

Technical Note

Non-occlusive Sweat Collection Combined with Chemical Isotope Labeling LC-MS for Human Sweat Metabolomics and Mapping the Sweat Metabolomes at Different Skin Locations

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**Non-occlusive Sweat Collection Combined with Chemical Isotope Labeling
LC-MS for Human Sweat Metabolomics and Mapping the Sweat
Metabolomes at Different Skin Locations**

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Abstract

Human sweat is an excellent biofluid candidate for metabolomics due to its non-invasive sample collection and relatively simple matrix. We report a simple and inexpensive method for sweat collection over a defined period (e.g., 24 hr) based on the use of a non-occlusive style sweat patch adhered to a skin. This method was combined with differential chemical isotope labeling (CIL) LC-MS for mapping the metabolome profiles of sweat samples collected from skins of left forearm, lower back, and neck of 20 healthy volunteers. Three 24-hr sweat samples were collected at three different days from each subject for examining day-to-day metabolome variations. A total of 342 LC-MS runs including replicate analyses were carried out (two runs were discarded due to instrumental issue), resulting in the detection and relative quantification of 3140 sweat metabolites with 84 metabolites identified and 2716 metabolites mass-matched to metabolome databases. Multivariate and univariate analyses of the metabolome data revealed a location-dependence characteristic of the sweat metabolome, offering a possibility of mapping the sweat metabolic differences according to skin locations. Significant differences in male and female sweat metabolomes could be detected, demonstrating the possibility of using sweat metabolome to reveal biological variations among different comparative groups. Thus the combination of non-invasive sweat collection and CIL LC-MS is a robust analytical tool for sweat metabolomics with potential applications including daily monitoring of the sweat metabolome as health indicators, discovering sweat-based disease biomarkers, and metabolomic mapping of sweat collected from different areas of skin with and without injuries or diseases.

Introduction

Sweat is an excellent candidate for metabolomics applications since a large portion of sweat solutes are small molecules (<1000 Da).¹⁻⁵ Compared to other biofluids, sweat collection is non-invasiveness and can be conveniently done on a regular basis for continuous monitoring (e.g., hourly or daily).^{6, 7} In addition, sweat can serve as a media for detecting metabolic differences from skins with and without epidermal injuries or diseases (e.g., soft tissue injury).^{8, 9} While detecting low concentrations of sweat metabolites was a major challenge, recent advances in analytical techniques such as high-performance chemical isotope labeling LC-MS have made high-coverage metabolomic profiling possible.⁴ However, to enable sweat metabolomics for various applications including discovering disease biomarkers and mapping metabolites of sweat from different skin locations of a subject, the use of a proper sweat collection method that can be conveniently administrated plays a crucial role.

Pilocarpine iontophoresis is a sweat collection technique used for the diagnosis of cystic fibrosis in newborns.¹⁰ This method has also been used in untargeted sweat metabolomics studies.¹¹⁻¹³ However, it requires special equipment and caution to avoid any metabolite changes that may be induced by the applied chemical and the use of the device. Other sweat collection methods include the use of occlusive and non-occlusive patches. Occlusive sweat patches are ideal for collecting pure liquid sweat over a short time interval (minutes).^{4, 14, 15} This style of sweat collection uses exercise or heat to stimulate sweating, which is then collected into an absorbent material fixed to the skin by an impermeable barrier such as Teflon. This method is only suitable for short-term collection, as the skin is completely covered and unable to breathe during the collection time.

Non-occlusive style sweat patches are designed to be worn for longer periods of time (hours and days). For this style of collection, a semi-permeable adhesive (e.g., Tegaderm) is used to fix an absorbent material to the skin which collects passive sweat over the collection period.¹⁵ ¹⁶ Tegaderm allows the skin to breathe due to the semi-permeable nature of the adhesive. Water vapour, oxygen, and carbon-dioxide to pass through while larger molecular weight, non-volatile contaminants are prevented. Specially designed non-occlusive sweat patches are currently used for continuous monitoring of drug exposure (e.g., those from Pharmchek).^{13, 17, 18} However, these patches are relatively expensive and have not been used for untargeted metabolomics studies where special care must be considered to avoid environmental contamination during sweat collection and sample loss during sweat extraction for downstream analysis. In this work, we report the development of a simple and inexpensive non-occlusive patch to collect passive sweat from different skin locations of a body and demonstrate its application and performance, in combination with chemical isotope labeling (CIL) LC-MS,^{19, 20} for quantitative sweat metabolomics with high coverage.

Experimental Section

Non-occlusive Sweat Patch. Figure 1A shows a schematic diagram of the patch consisting of a Tegaderm tape and two layers of Whatman filter paper. To apply the sweat patch to a skin (Figure 1B), the sampling site was first cleansed by wiping the collection area with a Kimwipe soaked in 70% isopropyl alcohol in water. The area was left to **air dry**. While waiting, the protective backing was removed from the Tegaderm (6 cm × 7 cm) which was then placed adhesive side up on a flat surface. Two clean 1.5-cm-diameter filter papers were adhered to the Tegaderm approximately 4 cm apart from one another. Two clean 1.27-cm-diameter filter papers

were then carefully rested onto the collection area approximately 4 cm apart. The Tegaderm was secured overtop of the 1.27-cm filter papers on the collection area, making sure to line up the filter papers on the adhesive and the skin. The subject was asked to wear the patch for 24 hours.

Metabolite Extraction. Non-occlusive sweat collection resulted in a dried sweat in the filter paper. Water, 1:1 (v/v) acetonitrile:water, and acetonitrile (ACN), all compatible to dansylation labeling solvent (1:1 ACN:H₂O), were tested as sweat extraction solvents. To examine the relative extraction efficiency, 40 μ L of pure sweat was spiked in two cycles (20 μ L each with a drying step in between to avoid saturation) onto a 1.27-cm filter paper. Pure sweat was collected from the proximal forearm of a single volunteer using a gauze sponge sweat patch method previously described.⁴ After the final drying step, the filter paper was placed into a 1.5 mL microcentrifuge tube containing an insert with a fritted bottom. Sweat was then extracted by pipetting 40 μ L of either water, 1:1 ACN:water, or ACN into the insert, incubating for 5 min at ambient temperature, and centrifugation at 20000 g for 5 min. An aliquot (25 μ L) of the extract was labeled with ¹²C-dansyl chloride (DnsCl),¹⁹ and 2 μ L of the labeled extract was injected into LC-UV to measure the peak area of labeled metabolites at 338 nm.²¹ Extraction efficiency was calculated by comparing the total peak area of labeled sweat metabolites from the extraction to the total peak area after labeling 25 μ L of pure sweat without filter paper spiking and extraction.

Environmental Contamination Testing. The sweat patch was worn over a period of time and thus environmental contamination may arise from the air, objects that may come in contact with the patch, bathing, etc. To study the sweat patch's ability to resist contamination, a modified sweat patch (Figure 1C) was constructed and worn on the left and right forearms of a volunteer for 24 hr. The design of this sweat patch is similar to the normal patch; however, another layer of Tegaderm is placed between the filter paper and the skin on one of the two

sampling sites. If the non-occlusive patch resists environmental contamination, then the labeled extract from the control filter paper worn between the 2 layers of Tegaderm should be identical to an extract from a new filter paper. Detection of a large amount of unique metabolites present in this control sample compared to a new filter paper would signify a large amount of contamination from the environment or the patch itself. For LC-MS analysis, 10 nmol of labeled sweat was injected and an equivalent volume to 10 nmol injection was used for analyzing the control and new filter paper extracts. The peak pairs detected exclusively in filter paper in contact with the skin represents true sweat metabolites.

Study Design and Sweat Collection. Ten males and ten females were recruited to donate sweat for this study with Ethics Approval from the University of Alberta. For each recruit, sweat patches were placed on 3 sampling locations (left forearm, lower back, and back of neck) for 24 hr. This procedure was repeated twice more for each recruit on separate days in three weeks. Each sweat patch contained 2 sampling sites for biological duplicates for a total of 360 sweat samples collected (20 recruits $\times$ 3 days $\times$ 3 locations $\times$ 2 replicates). After 24 hr, patches were removed and sweat metabolites were extracted from the filter paper. Samples were stored at -80°C until analysis.

Dansylation LC-UV and LC-MS analysis. Supplemental Note S1 describes the experimental details on preparing the control and blank samples, dansylation labeling, LC-UV for measuring the total concentration of labeled metabolites, LC-QTOF-MS setup, data processing,²² statistical analysis and metabolite identification.²³

Results and Discussion

Sweat extraction. Non-occlusive sample collection results in a dried sweat due to the breathable nature of the patch. The sweat metabolites must be extracted from the absorbent collection material using a proper solvent and extraction technique in order to maximize metabolite detection sensitivity. To optimize the extraction process, three solvents, water, 1:1 water:ACN, and ACN, were investigated. They were chosen because the dansyl-labeling reaction takes place in 1:1 water:ACN and the use of one of these solvents can avoid a dry-down step.

To examine the extraction efficiency, a 1.27-cm filter paper spiked with pure sweat and left to dry was subjected to each solvent extraction, followed by dansyl labeling and LC-UV measurement of the labeled metabolites. Figure 2A shows the measured UV peak area for pure sweat, different extracts and their controls (i.e., with water applied to the filter paper, instead of sweat), while Figure 2B shows the number of peak pairs detected in LC-MS for the corresponding samples. Similar results were observed for water and water:ACN with extraction efficiencies of >90% and good reproducibility, while ACN gave poor extraction. The peak number distribution shown in Figure 2C indicates that most metabolites were common between the water and water:ACN extracts. Water extraction produced a slightly lower number of background peak pairs (see Figure 2B for controls). These results are not surprising as sweat is approximately 99% H₂O which suggests a mainly polar metabolome. Multiple extractions with smaller volumes were also attempted and produced similar results (Figure 2A-B, right side). Based on these results it was concluded that a single extraction with pure H₂O was optimal.

Resisting environmental contamination. In a real world, one would expect an individual wearing the sweat patch to undergo a variety of activities including sleeping and bathing. We tested the patch's ability to resist environmental contamination by comparing extracts of a clean filter paper with extracts from a filter paper worn for 24-hr but not in contact

with the skin (control) (Figure 1C). This experiment was performed in duplicate by placing the patch on the right and left forearm on a single volunteer. The volunteer was instructed to undergo their daily routine including bathing and sleeping as if the patches were not being worn. After extraction and dansyl-labeling, the UV peak areas of the control samples were almost identical to the blank filter paper samples (Supplemental Figure S1A). After injecting the same volume for LC-MS analysis (10 μ L), total peak pair numbers were almost identical (Supplemental Figure S1B). The Venn diagrams of the peak pairs detected from left and right forearm experiments are shown in Supplemental Figures S1C and S1D, respectively. In both arms, only 11 unique metabolites were detected in the control sample. These 11 metabolites can be attributed to the method. Over 700 unique metabolites could be attributed to sweat secretion. Overall, these results demonstrated the selective nature of the patch for not allowing contaminants to pass, assuring that environmental contamination is not an issue during sample collection.

Sweat submetabolome. A total of 365 peak pairs were detected in six method blank runs with an average of 310 ± 12 peak pairs per run ($N=6$). A total of 201 peak pairs were shared between sweat samples and the method blank. Shared peak pairs were excluded from the sweat analysis if they were present in at least 50% of the method blank runs. While it is possible that these shared features are naturally occurring in sweat, we conservatively excluded them from the data analysis. After the background pairs were removed, a total of 3140 peak pairs were detected in all the sample runs. Dansyl library search of these metabolites resulted in 84 definitive identifications (Supplemental Table S1). Metabolites not identified were subjected to HMDB and EML searches based on accurate mass.²³ These searches resulted in 947 and 1769 putative IDs, respectively (Supplemental Tables S2 and S3).

Metabolome profiles at different skin locations. Unlike other homogenous biofluids such as urine, sweat can be collected at different skin locations and thus we examined whether collection location has any effect on the metabolome profiles. Concentrations of labeled metabolites in sweat extracts after dansyl labeling were determined by LC-UV, based on the total area of the LC peak produced from the elution of the labeled metabolites in high organic solvent of a step-gradient and a standard calibration curve of a labeled amino-acid-mixture.²¹ Figure 1D shows the average concentrations of the labeled sweat samples with error bars depicting the standard deviation of each group. Lower back samples yielded the highest concentration of labeled metabolites on average. The large error bars for each of collection areas show just how variable the resulting concentrations were for each collection area. This was an expected result as concentration of an extract should correlate with how much an individual sweated through the collection period. Each individual performs different activities through the day and are expected to sweat different amounts depending on their level of activity.

Within a subject, the concentrations of individual metabolites in sweat samples collected from different locations can be different. This can be visualized using a heat map for the metabolome data set collected from lower back, forearm and neck areas of an individual. Figure 3 shows two examples (see Supplemental Figure S2 for the heat maps of other individuals). In these heat maps, only the 100 top-ranked metabolites with significant variations in three locations are shown. In Figure 3, for most metabolites shown, concentration variations in the 24-hr sweat samples collected from the same location on three different days are small. However, the concentrations of many of the metabolites are very different from one location to another. This location dependence characteristic is interesting from the perspective of mapping the metabolic differences in sweat samples collected from different location to reveal metabolite

distributions in different areas of skins. However, this location dependence also suggests that, to reveal biological variations among different groups, we need to take the collection site into consideration when comparing the metabolomes of different subjects (see below).

Metabolomic Comparison of Male and Female. Figure 4 shows the PLS-DA plots for comparing the male and female sweat samples collected from all areas or different locations and Supplemental Figures S3-S6 show the corresponding permutations test results for model validation. Univariate analysis was performed to identify metabolites with significantly differing changes in abundance between sexes (Supplemental Figure S7). Taking sex comparison from all sweat samples as an example (Figure 4A and Supplemental Figure S7A), multiple-testing correction was performed on the p -values from the t-test to calculate q -values. Based off the table of q -values, in order to maintain a false discovery rate of $\leq 5\%$, a $p \leq 0.02$ must be used as a cut-off. Therefore for this analysis, metabolite level changes were considered significantly different if the ratio had an $FC \geq 1.5$ or ≤ 0.67 , and a $p \leq 0.02$. This criterion revealed 78 metabolites with significantly differing levels between sexes. Dansyl library search resulted in definitive identification of seven of these metabolites (Supplemental Table S4). HMDB and EML library searches based on accurate mass resulted in 22 and 42 matches, respectively (Supplemental Tables S5 and S6). We note that we did not detect hormone/pheromone compounds in sweat in this work; they either could not be labeled by dansylation chemistry or, if labeled, had concentrations below the detection limit of the method. Future work of using other labeling chemistries in combination with concentrating the sweat samples may afford the detection of these compounds.

Figure 4B-D show the PLS-DA plots of the metabolome data from samples collected in three separate locations. Supplemental Note S2 describes the comparison results and significant

metabolites detected to separate sexes from each comparison. Combining common dansyl library matches from each of the 3 collection areas, there were a total of 10 different metabolites with significantly differing levels between sexes, 9 of which are endogenous metabolites (see Supplemental Note S2 for the discussion of these metabolites). Overall, a greater separation could be observed by using the metabolome data of samples collected from the same location.

Conclusions

We have developed a method to profile the sweat metabolome using non-occlusive sample collection and CIL LC-MS. Our results revealed the dynamic nature of the sweat metabolome and how important it is to consider sweat collection area when conducting metabolomic studies. This location dependence characteristic may also be explored further. For example, we are in the process of mapping the metabolomic profile differences in normal and affected areas of lymphedema patients for discovering potential metabolite biomarkers with diagnosis and prognosis values including measuring the severity of an affected area and guiding the management regimens (e.g., massaging areas and frequencies).

Acknowledgements

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Disclosure

The authors declare no competing financial interest.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website:

- Supplemental Note S1 for experimental, Note S2 for results, Figures S1-S7 for comparison results, and Tables S1-S6 listing metabolite identification results.

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Figure Captions

- Figure 1. Side view (A) and top view (B) of a non-occlusive sweat patch design. (C) Side view of a special patch design for investigating environmental contaminations from wearing the patch. (D) Average concentrations of labeled metabolites measured by LC-UV in sweat and blank samples (error bar = one standard deviation).
- Figure 2. (A) UV peak areas of labeled metabolites from LC-UV analyses of labeled samples. (B) Peak pair numbers detected from LC-MS analyses of labeled samples. Pure sweat control was generated by labeling an equal volume of H₂O. Control data for each solvent was generated by extracting a blank filter paper, labeling, and injecting the same volume as the experimental. Error bars represent standard deviation (n=3). (C) Venn diagram of peak pairs detected using each solvent combination.
- Figure 3. Heat maps of two individuals for the 100 top-ranked significant metabolites with varying concentrations in sweat samples collected from three locations. The bottom axis, Wx-y, refers to the sample collected from week x and analyzed as replicate y (e.g., W1-1 refers to the sample collected in week 1 and analyzed as the 1st replicate, while W1-2 refers to the same sample, but analyzed as the 2nd replicate).
- Figure 4. PLS-DA plots comparing sexes using sweat samples collected from (A) all areas ($R^2X=0.590$, $R^2Y=0.905$, $Q^2=0.770$), (B) lower back ($R^2X=0.582$, $R^2Y=0.965$, $Q^2=0.844$), (C) left forearm ($R^2X=0.683$, $R^2Y=0.983$, $Q^2=0.759$) and (D) neck ($R^2X=0.626$, $R^2Y=0.951$, $Q^2=0.740$).

Figure 1.

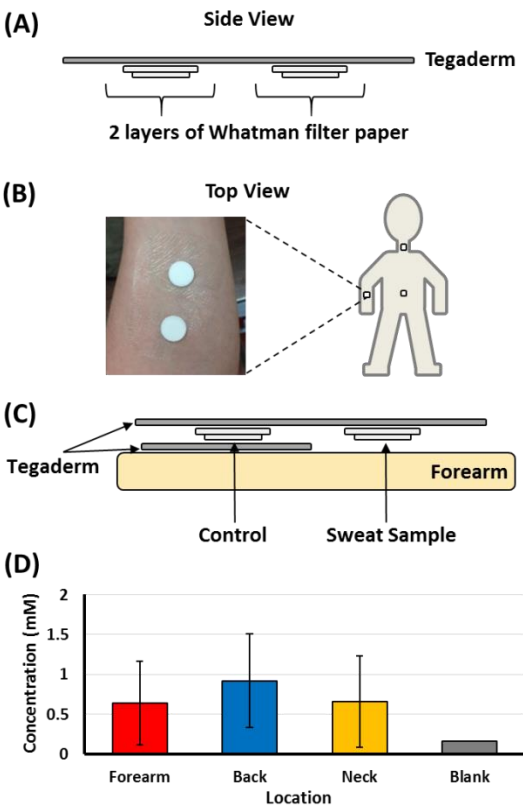


Figure 2.

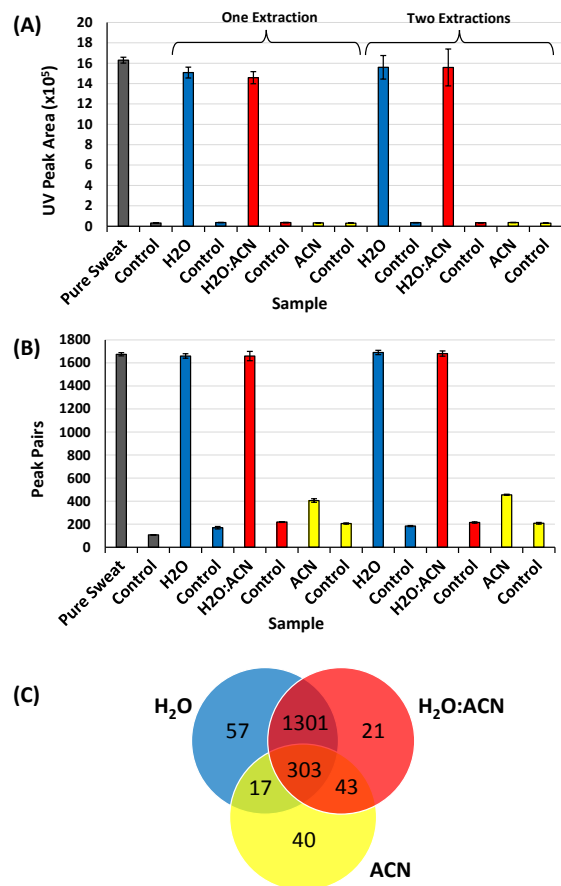


Figure 3.

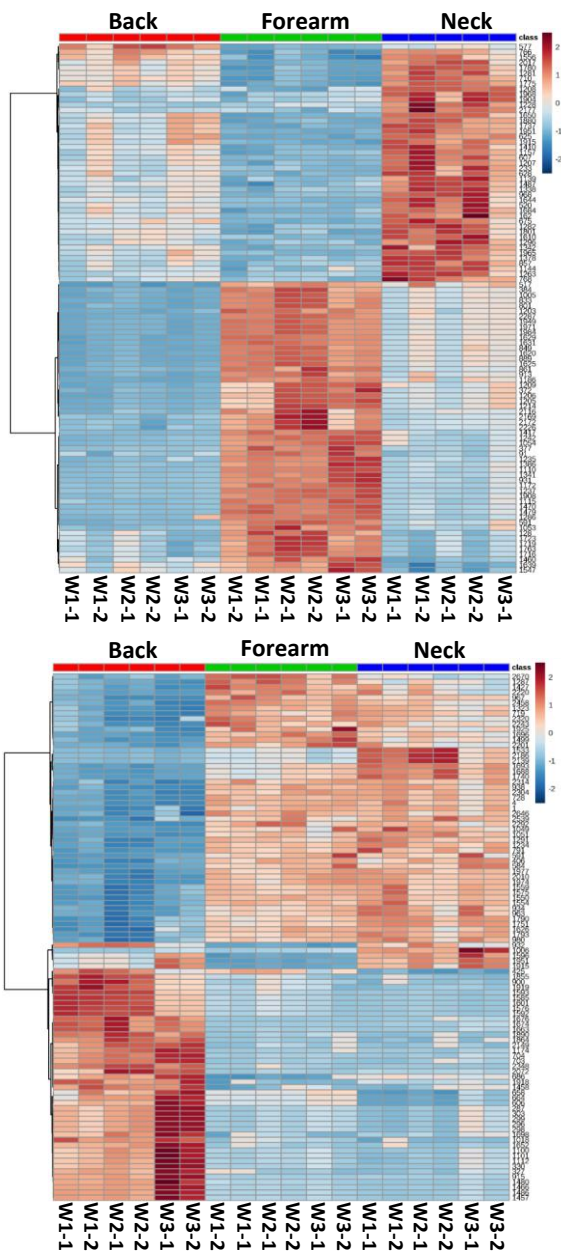
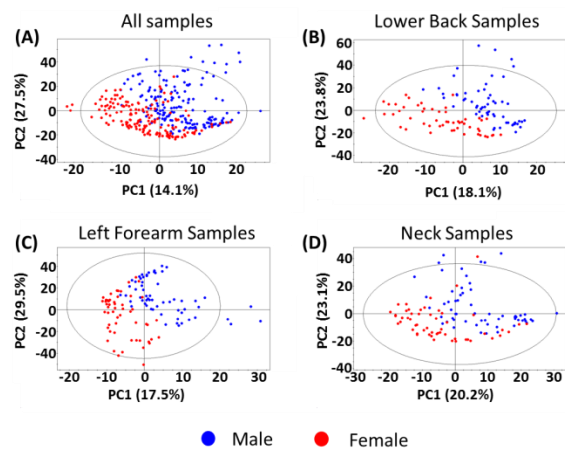


Figure 4.



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