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Review Article

Working Up a Good Sweat – The Challenges of Standardising Sweat Collection for Metabolomics Analysis

*Joy N Hussain,1 Nitin Mantri,2 Marc M Cohen

1School of Health and Biomedical Sciences, RMIT University, Bundoora, Vic. 3083; 2Health Innovations Research Institute, School of Applied Sciences, RMIT University, Bundoora, Vic. 3083, Australia.

*For correspondence: Dr Joy Hussain, joyhussain9@gmail.com

Abstract

Introduction

Human sweat is a complex biofluid of interest to diverse scientific fields. Metabolomics analysis of sweat promises to improve screening, diagnosis and self-monitoring of numerous conditions through new applications and greater personalisation of medical interventions. Before these applications can be fully developed, existing methods for the collection, handling, processing and storage of human sweat need to be revised. This review presents a cross-disciplinary overview of the origins, composition, physical characteristics and functional roles of human sweat, and explores the factors involved in standardising sweat collection for metabolomics analysis.

Methods

A literature review of human sweat analysis over the past 10 years (2006–2016) was performed to identify studies with metabolomics or similarly applicable ‘omics’ analysis. These studies were reviewed with attention to sweat induction and sampling techniques, timing of sweat collection, sweat storage conditions, laboratory derivation, processing and analytical platforms.

Results

Comparative analysis of 20 studies revealed numerous factors that can significantly impact the validity, reliability and reproducibility of sweat analysis including: anatomical site of sweat sampling, skin integrity and preparation; temperature and humidity at the sweat collection sites; timing and nature of sweat collection; metabolic quenching; transport and storage; qualitative and quantitative measurements of the skin microbiota at sweat collection sites; and individual variables such as diet, emotional state, metabolic conditions, pharmaceutical, recreational drug and supplement use.

Conclusion

Further development of standard operating protocols for human sweat collection can open the way for sweat metabolomics to significantly add to our understanding of human physiology in health and disease.

Introduction

Human sweat is a biological fluid (biofluid) that is generating increasing interest across a diverse set of fields including dermatology, paediatrics, toxicology, analytical chemistry, forensic pathology, psychiatry, illicit drug testing and infectious diseases. Currently sweat is primarily used in clinical medicine for chloride sweat testing which is used in the diagnosis of cystic fibrosis (CF). Additionally, some centres around the world use a sweat patch for monitoring drugs of abuse, while others have developed an indicator test (Neuropad) to detect peripheral neuropathy in the foot sweat of diabetics.1-3 Aside from these applications, the use of sweat in medical practice is limited in part due to challenges involved with sweat collection and the range and reproducibility of testing. This is likely to change as advances in analytical technology methods within metabolomics and other related ‘omics fields allow more complex physiological information to be derived from smaller amounts of sweat with less arduous processing. This is leading to a greater understanding of the physiology of human sweating and the skin’s excretory pathways in relation to metabolites, pathogens, and xenobiotics.4 Incorporation of Bluetooth capabilities with some of the newer wearable sweat electrolyte and metabolite detecting systems reflects even wider trends in applications to enhance personalised analysis.5-7
Each type of human biofluid or tissue sample has its own signature metabolome, but most of what is known about the human metabolome is based upon findings in the ‘serum metabolome’ and the ‘urine metabolome’. Further study and standardized procedures are now required to characterise the ‘sweat metabolome’ and how it fits into the bigger picture of the human metabolome, and whether the case exists for wider application of sweat metabolomic testing.

When applying a metabolomics approach to analysing human sweat, a number of variables need to be examined within the context of the origins, composition, physical characteristics and functional roles of sweat. These variables include: sweat induction and sampling techniques, timing of sweat collections, sweat storage conditions, and laboratory aspects such as metabolite quenching, extraction, concentration, fractionation, separation and other processing methods applicable to sweat. Exploring the variables within the framework of newer laboratory analytical platforms that optimise qualitative and quantitative detection of sweat metabolites will pave the way forward to make more rigorous and meaningful comparisons of sweat metabolomics studies.

Standardising the collection, handling, processing and storage of sweat for further metabolomics analysis is vital to this endeavour and working out the further steps necessary to achieve this standardisation is the focus of this review.

Background – Metabolomics

Metabolomics is the multidisciplinary science involving the measurement and analysis of low molecular weight metabolites such as electrolytes, sugars, lipids and other compounds that exist in a selected biofluid, cell, tissue or organ under a given set of physiological conditions. Its history and roots in the works of many biochemists who pioneered the discovery and detection of various vitamins in the 1940s and progressed the concepts of ‘metabolic variance’ and ‘biochemical individuality’.11-14

The exact number of unique metabolites in the human metabolome has yet to be firmly established, but it is generally thought that there is a lower number of metabolites in the human metabolome compared with the total number of genes (>30,000 in genome), RNA transcripts >30,000 in transcriptome) and proteins (>100,000 in proteome).15,16 Small changes in the transcriptome may translate into marked changes in metabolites.17,18 With presumed fewer total metabolites to analyse and a potentially more amplified signal to be detected, the power and potential of metabolomics to pick up minute but significant health-related changes holds promise.

As with all newly emerging fields, within metabolomics there is multiplicity and various expansions of terminology, although metabolomics and metabonomics are often used interchangeably in the literature, metabonomics technically refers to the study of the interactions of metabolites over a timeframe in a complex system.19 Fluxomics refers to an extension of metabolomics, in which metabolomics is applied at various experimental time points generating kinetic data which can then be used to study metabolic pathway fluxes.20 Exposomics, another extension of metabolomics, refers to identifying metabolites linked to environmental risk factors for disease.21-23 Metabolites can be classified into two categories: endogenous metabolites (synthesised and utilised within a biological system) and xenobiotic metabolites (imported from outside the biological system into the cell, such as drugs, xenobiotics and nutrients).3,9,10 The Human Metabolome Project (HMP) led by Dr David Wishart of the University of Alberta in Canada published a first draft of the human metabolome in 2007 which consisted of 2180 metabolites, 1200 protein and 3500 food compounds. A list of findings additional to the HMP is being compiled and verified on the Human Metabolome Database – a freely accessible and continually updated web resource (http://www.hmdb.ca).10,24,25 Not all known human metabolites can be found in any given biofluid because different biofluids serve different functions and play different metabolic roles. As of November 2016, the HMP had identified and/or quantified over 3848 metabolites: 440 metabolites in cerebrospinal fluid, 1233 metabolites in saliva, 2287 metabolites in blood, 1746 metabolites in urine, 695 metabolites in sweat and 62 metabolites in other tissues and biofluids including sweat.14

The methodology of metabolomics can be divided into different conceptual approaches such as targeted analysis, global metabolomics and metabolomics. Analytical methods used in metabolomics utilise complementary analytical methodologies such as liquid chromatography-mass spectrometry (LC-MS/MS), gas chromatography-mass spectrometry (GC-MS), and nuclear magnetic resonance (NMR) spectroscopy in a coordinated attempt at global metabolomic profiling.26 Metabolic fingerprinting refers to the metabolic ‘signature’ or metabolic fingerprinting/ metabolic footprinting.27 A targeted metabolomics approach involves a targeted search and quantitative analysis of a set of known metabolites or substances that play a particular role in a laboratory test. Global metabolomic profiling is untargeted and comprises an analysis of all measured metabolites or substances, including those known and unknown, which make up a metabolic profile of the total complement of metabolites in the human metabolome. Metabolomic profiling utilises complementary analytical methodologies such as liquid chromatography-dual mass spectrometry (LC-MS/MS), gas chromatography-mass spectrometry (GC-MS), and nuclear magnetic resonance (NMR) spectroscopy in a coordinated attempt at global metabolomic profiling.28 Metabolic fingerprinting refers to the metabolic ‘signature’ or mass profile of the biofluid or tissue sample of interest which is then compared in a large sample population to screen for differences between control samples. Two signals present in deep dermis layers: (i) upper dermal portion with straight and coiled parts; and (ii) intra-epidermal part often referred to as the acrosyringium. The dermal portion, or dermal duct, has epithelial cells connected at numerous sites by desmosomes and intercellular junctions that are believed to constitute a barrier between the luminal and extracellular compartments. The inner luminal cells contain various tonofilaments while the outer basal cells are surrounded by collagenous and fibrocyte-rich sheaths.22,23

Sweat Collection for Metabolomics Analysis

Whole body sweat is a complex mixture of cumulative secretions from millions (1.6-5 million) of eccrine, apocrine, sebaceous glands and apocrine sweat ducts from the skin surface in environmental studies, especially older studies, is often referred to as ‘eccrine’ sweat because eccrine sweat glands are the most numerous and ubiquitous glands in the skin, however many sweat samples also contain potentially trace amounts of apocrine, apocrine and sebaceous gland secretions (called sebum), depending upon the body site of sampling. This imprecision of nomenclature is the case in tissues toxicology literature dealing with sweat patch testing of illicit drugs and toxicology literature studying electrolyte changes in exercise. However, in the dermatology and facial cosmetic literature, ‘sebum’ can figure in addition to ‘sweat’ with more emphasis on the underlying structures within the skin. The converse can also be true with studies focused on collection of sebum. The term ‘residual skin surface components’ (RSSC) is another term for potential sweat glandular secretions and cellular debris (from stratum corneum – outermost epidermal skin layer).29-31

Mindful of the semantics surrounding sweat, it is useful to revisit the anatomy, histology and secretions of the four known gland types that contribute to sweat. This sets the stage for better understanding and targeting of future studies to fully characterise the sweat metabolome.

Eccrine Sweat Glands and Secretions

Eccrine sweat glands exist at birth and can be located all over the body’s skin except on lips, on the nail bed and on some fields of the genitalia (e.g. glans penis). They can average 100-200 µm body surface area, with higher densities (600-7000 µm2) on palms and soles, and at luminal diameters of 20-60 µm at skin openings.23,30 Eccrine glands consist of single tubules ranging 4-8 mm in length that are generally divided into two main sections: (i) deep coil portion in deep dermis layers; (ii) upper dermal portion with straight and coiled parts; and (iii) epidermal part often referred to as the acrosyringium. The dermal portion, or dermal duct, has epithelial cells connected at numerous sites by desmosomes and intercellular junctions that are believed to constitute a barrier between the luminal and extracellular compartments. The inner luminal cells contain various tonofilaments while the outer basal cells are surrounded by collagenous and fibrocyte-rich sheaths.22,23

Apocrine and Apocrine Sweat Glands and Secretions

Apocrine sweat glands also exist at birth but do not become active until the androgenic stimulation of puberty.34 They are composed of a single cell derived from the sebaceous gland and are typically found in the axilla, peri-umbilicus, perineal and genital areas since they open and secrete into adjacent hair ducts (e.g. apoliposebaceous ducts) before secretions reach the skin surface. They are generally larger than eccrine sweat glands with apocrine coil diameters of ~800 µm compared to eccrine coil diameters of ~500 µm, both located in the dermis and hypodermis.35 Apocrine ducts are relatively short and found in close proximity to hair follicles. The density of apocrine glands is highly variable within and between individuals. Apocrine glands are generally found in the axilla.35,36 Two different types of cells are visualised in apocrine glands: columnar secretory cells and myoepithelial cells. The secretory cells are generally noted to be full of mitochondria and different granules with convoluted cell membranes and microvilli presenting towards the lumen.37 Apocrine sweat glands are a mixed type gland as the name suggests and were first described in 1878 by Sato et al.38 They are composed of a cell derived from the sebaceous gland and are located near to bodily hair by areas. As many as 50% of all axillary sweat glands are thought to be apocrine. Component cells of

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apoecrine glands include eccrine secretory cells, apocrine secretory cells and myoepithelial cells. Identification of these morphologically distinct glands can be made with specific protein markers (i.e. phalloidin, S-100, CD15). Apocrine and apoecrine glands are both classed as apocrine glands. With apocrine glands, secretion occurs via pinching-off of the cell’s plasma membrane producing membrane-bound vesicles, which helps to account for comparatively more viscous secretions. Sato et al. determined that Na+ and K+ concentrations obtained from the isolated ducts of the apocrine glands are curiously more similar to that of eccrine sweat compared to apocrine sweat. While the composition of apocrine sweat has not yet been fully elucidated, apocrine sweat has been demonstrated to contain carrier proteins for volatile odor molecules like volatile organic compounds (VOCs) and pheromones with amino acid conjugates produced by bacterial enzymes. Apocrine bromhidrosis (more commonly known as BO or body odour) is thought to be linked to large amounts (i.e. over 10^9 bacteria/cm^3) of resident microflora such as aerobic cocci, *diphtheroid species*, *Corynebacterium species* and *Staphylococcus epidermidis*. Sebaceous Glands and Sebum Sebaceous glands are located in the skin of all surface areas except for the palms of hands and soles of feet. They are particularly numerous on the forehead, scalp, midline back, chest, perineum and surrounding the orifices of the human body. Densities of up to 400–900 glands/cm^2 occur on the face, especially in the T-zone area of the face which starts from the midpoint and sides of the forehead, and extends downward toward the middle of the nose, including the sides of the nose and the midline part of chin. Sebaceous gland density decreases towards the extremities of the body. Sebaceous glands can be divided into two types: pilosebaceous glands, when associated with hair follicles, and free sebaceous glands seen mostly at transitional zones between skin and mucous membranes. A well-known example of a free sebaceous gland is the Meibomian gland of the eyelid. All sebaceous glands consist of single or multiple lobules, or acini, with ducts emptying into a main sebaceous duct. Secretory lobules contain sebocytes and keratinocytes, with studies identifying differences based upon cholesterol and squalene conversion enzymes.

Collected Sweat Setting aside the above distinctions in gland origins, the vast majority of sweat studies in the literature have analysed a collective form of sweat with eccrine gland secretions predominating. Depending upon location of sweat sampling and various cleaning and collection strategies used during sampling or processing, trace amounts of sebum and/or apocrine and apoecrine gland secretions, cellular matter from the epidermis and associated ~10^5 skin microbes, as well as other metabolites like xenobiotics may feature in sweat samples. The systems-based approach of analysing sweat with metabolomics offers the prospect of uniting all these different subcomponents of sweat. With such a metabolomics approach, studies of ‘normal’ sweat obtained from ‘healthy’ people have detected highly variable metabolite compositions with large numbers of different small molecules, of both microbial and human origin, in a primarily water-based (~99%), relatively acidic (mean pH 6.3) solution (Figure 2). This rich complexity of sweat content hints at its functions both at the level of the skin and at the level of the organism as a whole.

Functions of Sweat Temperature and Fluid Homeostasis Sweat is integral to the regulation of body core temperature by water evaporative heat dissipation. Blood flow regulation and vasodilation of superficial blood vessels largely contribute to this homeothermic control and the finding that eccrine sweat production is under the control of cholinergic and, to a lesser extent, adrenergic innervation is consistent with this hypothesis. Various stimuli of this system include temperature, emotions, intellectual stimulation and gustatory stimulation. Sweat volumes vary widely as a result. Global insensible fluid losses can be approximately 1000 ml daily for the whole human body, including more than half of fluid losses through the skin via perspiration with the remaining losses being through the lungs. However, there are reports of individuals perspiring up to several litres per hour, 12 L per day under certain extreme physiological conditions.

Eccrine sweat activity appears intermittent over a large portion of the body; cycles of periodic discharges alternating with pauses occurring from <1 to 12 geyser-like emissions per hour with single sweat gland emissions recorded every 3.3 min in one recent study. This activity differs among
individuals, environmental circumstances and body sites with approximately 50% of total body volume of sweat being thought to be produced by the trunk, 25% by the legs and 25% by the head and upper extremities.46 Even in cases of profuse sweating, it is thought that only approximately 50% of sweat pores release sweat at any given time, except for the palmoplantar regions where the sweat gland activity is largely synchronised.47 How these findings fit within sweat’s overall functions in the human body is still unclear.

In contrast to eccrine glands, apocrine gland activity is reported to be more continuous in its fluid secretions, while still receiving predominantly cholinergic and some adrenergic innervation.48,49 Apocrine gland stimulation by physiological and pharmacological stimuli appears to be distinct from those controlling eccrine or apocrine glands. Apocrine glands respond quickly to psychological stress and are thought to more significantly contribute to the abundant sweat produced in the axilla.50,51

Sweat Electrolyte Regulation

Another important component of sweat that impacts human fluid balances is sodium. The concentrations of Na+ in sweat can be highly variable, ~20–100 mmol/L, and some individuals can lose an estimated 4–6 g of Na+ per day, equivalent to 12–15 g of NaCl daily through sweating, especially if working in moderately hot conditions.47 Eccrine gland duct cells reabsorb several ions, including Na+ and Cl−, via a number of known anion exchangers such as Na+/K+-ATPases (on basolateral membranes), cystic fibrosis transmembrane conductance regulators (CFTRs, mutations of which provide the basis for Cystic Fibrosis Sweat Chloride testing), carbonic anhydrases II, and vacuolar proton pumps (V-H+ATPase).52 Sweat Na+ and Cl− concentrations have been documented to increase with age from 12–19 years then stabilise thereafter.51,56 Sweat Na+, Cl−, and K+ concentrations also have reported body regional variations.57

Skin Protection

Sweat also provides lubricating, water-proofing, antimicrobial and skin barrier-promoting properties that support skin in the first line of defence against many environmental insults. In extreme hot conditions, the lipid-rich secretions of apocrine and sebaceous glands can emulsify sweat produced by eccrine glands to create a hydrolipid film that is not as readily evaporated. This is thought to be of importance in delaying dehydration. In colder conditions, the lipid nature of sweat becomes more solid and, in coating the hair and skin, sources of unwanted moisture like rain or snow can theoretically be more effectively repelled.58 Palmar hydration, which is directly linked to eccrine sweat production, increases the skin friction coefficient which therefore improves the adherence of hands to objects and contributes to a heightened sense of touch.59 Sweat also contains antimicrobial peptides (AMPs) like dermicidin, lactoferrin, and LL-37, an AMP of the cathelicidin family, which serve to control certain pathogenic bacterial counts on the skin surface.44,60 However, the precise qualitative and quantitative content of skin microbiota and associated microbe-microbe and microbe-host dynamics via sweat are areas of active research with early findings hinting at rich metabolic inter-relationships with impacts on skin integrity, especially in skin inflammatory states.61

The free amino acid composition of sweat is curiously different from other biofluids. Data from a recent study suggest the amino acid content of sweat is remarkably similar to the amino acid content of an epidermal protein, profilaggrin. Since profilaggrin is thought to be the key contributor of free amino acids making up the natural moisturising factor within the stratum corneum, it is postulated that sweat plays a role via interactions with profilaggrin in maintaining the barrier integrity of human skin.62

Immune System

Sweat has links to many immune-mediated mechanisms. Skin epithelial cells interact with various external stimuli to produce cytokines, and sweat directly activates epidermal keratinocytes to produce various cytokines using in vitro models with cultured human keratinocytes from surgically discarded neonatal skin samples.63 It is postulated that sweat may play both beneficial and pathological roles in immune-mediated communications. For example, sweat is well-recognised in exacerbating atopic dermatitis (AD) lesions and is associated with increased itching (pruritus) which has associations with enhanced expression of IL-31 (newer member of IL-6 family of cytokines) in tissue samples of exacerbated AD lesions.62 Sweat also contains cystatin A, a proteinase inhibitor of bacterial cysteine proteases. Given these exogenous proteases are known to break down the epidermal barrier, cystatin A in sweat may serve both immune

Figure 2. Metabolomic sweat content.
and skin protective roles.\textsuperscript{13} Quantitative levels of IL-1\textalpha, IL-1\textbeta, IL-6, TNF-\textalpha, IL-8 and TGF-\beta have been measured in human sweat although the precise cellular origin of these cytokines is still unknown and could be derived from sweat, blood or epidermal cells.\textsuperscript{14}

**Excretion Functions and Drug Delivery Mechanisms**

While the excretory function of sweat has previously been considered negligible compared to the kidney, recent studies challenge this notion. There is evidence that several toxic elements and xenobiotics may be preferentially excreted through human sweat.\textsuperscript{15,16} Some studies report arsenic, cadmium, lead and mercury being excreted in appreciable quantities via sweat, with the rates of excretion matching or exceeding urinary excretion.\textsuperscript{17} Furthermore, while excess dietary nicotinamide cannot be eliminated through urine because of its reabsorption by the renal tubules, it can be effectively excreted by sweating.\textsuperscript{18} Many therapeutic pharmaceutical drugs are also excreted via sweat and the role of sweat patch technology in monitoring illicit drug use is based on dozens of studies examining the pharmacodynamics and pharmacokinetics of amphetamines, cocaine, cannabis, opiates and associated metabolites excreted in sweat.\textsuperscript{19} Drug binding to various skin fractions and reabsorption of drugs from pooled sweat on skin has also been observed. The relative concentration of unmetabolised drugs is reported to be occasionally higher in sweat than in blood, urine or saliva.\textsuperscript{20,21}

The above findings suggest that molecules of drugs/metabolites/xenobiotics may reach the skin surface from blood by various proposed routes: via sweat or sebum by active or passive inter- and/or transcellular mechanisms; and by transcutaneous diffusion or lipid bilayer penetration of sweat glands.\textsuperscript{22} A number of endogenous metabolites/xenobiotics may reach the skin surface from blood and are associated with hot flashes linked to increased sweat production.\textsuperscript{23}

Night sweats can indicate serious systemic infections (e.g. tuberculosis) and malignancy, while local hyperhidrosis around a bite site can indicate toxic envenomations such as occurs with Australian redback spider bites.\textsuperscript{24} Hypoglycaemia, hyperthermia, hypercapnia and vagus nerve stimulation can all lead to stimulation of eccrine sweat production and alterations in effective sweating may arise directly from certain skin conditions.\textsuperscript{25} For example, some hyperkeratotic disorders such as pityriasis versicolor and psoriasis interfere with the excretion of sweat and are associated with decreased sweat output as visualised with skin capacitance imaging of lesions.\textsuperscript{26} Abnormalities in the transport of sweat onto the skin’s surface may also cause a severe prickly sensation and skin inflammation resulting in the intra-epidermal retention of sweat, such as occurs with miliaria rubra which has been linked to elevated levels of IL-1 and IL-31 detectable in sweat.\textsuperscript{27}

**Therapeutic and Wellness Functions of Sweat**

It is hypothesised that sweat produced by different activities may differ in composition. For example, IL-1 concentrations are increased in sweat induced by both exercise and sauna bathing\textsuperscript{28} and exercise is linked to increases in the generation of several end-metabolites like reactive oxygenated species that are in turn linked to oxidative stresses. This is thought not to be the case with sauna-induced sweat although this remains to be validated by further studies.\textsuperscript{29}

**Lipid Homeostasis**

Sebum production changes have been linked to diet. Caloric deprivation in the setting of obesity decreases sebum production,\textsuperscript{30} while exercise has been shown to increase it.\textsuperscript{31} Increases in energy intake have been associated with increased excretion of triglycerides, cholesterol and associated esters in sebum.\textsuperscript{32} As newer studies in sweat and skin surface lipidoemetabolites are being done, more definitive information regarding these links and potential mechanisms of action are likely to emerge.\textsuperscript{33}

**Methods**

Puhbmed, Medline, Google Scholar, Embase, Science Direct, Scopus, Ovid, Web.ofScience, Proquest, Toxline and UpToDate databases were initially searched with keywords ‘sweat’ and ‘metabolomics’ with restrictions of English language and of dates 2006-2016. These records were then supplemented with searches for other research by key authors, searches of citations and reference lists of key papers, and additional searches with expanded keywords relating to sweat including perspiration, sauna, exercise, secretion and/or excretion from human skin and residual skin surface components as well as expanded keywords relating to metabolomics including exosomes, xenometabolomics, toxicometabolomics and fluxomics. Older studies of sweat (before 2006) have been used in compiling background information, but not for the detailed analysis of sweat collection methods.

Of the 1320 records identified for review as of 1 June 2016, all 17 studies presenting quantitative human data utilising a sweat metabolomics methodology of analysis between the dates 2006-2016 of experimental design. An additional three sweat proteomics-based studies were identified that utilised similar laboratory platforms relevant to metabolomics and were also included in the comparison analysis.

**Results**

The Table presents a summary of the pertinent information regarding sweat induction and collection methods extracted from the 20 identified studies for comparison.\textsuperscript{34}

**Discussion**

**Sweat Induction Protocols**

Induction of perspiration represents a phenomenon involving a complex chain of metabolic reactions, with many possible triggers, as already discussed. Exercise, stress, psychological state, relative humidity, hormonal and sympathetic/parasympathetic nervous system parameters, diet, skin colonisation factors, xenobiotics exposure – both pharmacological and non-pharmacological – can influence sweat volumes and content.\textsuperscript{35} Refer to the fourth column in the Table describing sweat induction modes utilised in the reviewed studies. A number of important factors are apparent when obtaining sweat for metabolomics analysis: (i) ensuring adequate amount of sweat is available to complete the analysis, including enough volume for controls and potential further analysis; (ii) ensuring the mode of sweat induction does not interfere with the utility of the results; and (iii) ensuring that skin composition and sweat collection happen in a timely manner that optimises metabolic quenching and metabolite stability.\textsuperscript{36,37}

**Pilocarpine Iontophoresis**

Several active research groups rely on a chemical pilocarpine iontophoresis method of inducing sweat.\textsuperscript{29,30,31,32} This method takes advantage of the bioelectric properties of skin which allow the application of low intensity electrical current (i.e. 1.5 mA) for 5 min. The resulting opposition offered by skin to this electrical current, called bio-impedance, is present in intra- and extracellular fluids and the capacitive reactance of cell membranes. For a typically applied chemical such as pilocarpine (0.5\% pilocarpine nitrate solution), a drug with cholinergic parasympathomimetic activity which aims to stimulate primarily the eccrine sweat glands, to be absorbed through human skin, the electrical current must overcome the bio-impedance imposed on its flow to reach the target tissue of sweat glands with sufficient intensity. This bio-impedance can be influenced by a range of factors, some of which are electricity source-dependent such as the distance between electrodes positioning, pulsed direct current vs constant direct current source, and size and content of iontophoresis electrodes (typically containing 70\% copper, 30\% zinc with diameter of 30 mm).

Some of the important host-dependent factors involved with this mode of sweat induction include the amounts of keratin and the variable thickness of stratum corneum (SC) at different body sites, fluctuating amounts of fluid in skin layers with overall hydration status, ambient temperature increasing or decreasing hydration of keratin, adipose tissue thickness (especially with some sweat glands residing in deep dermis/ subcutaneous fat) and individual pain/tolerance to the electric current. All of these factors can alter biological responses, thereby potentially confounding metabolic results. Therefore, the argument can be made that using pilocarpine with iontophoresis induces production of a particular type of primarily eccrine sweat but whether the detailed metabolomic composition of eccrine/systemic sweat is the same as physiologic sweat and/or thermally-induced sweat and/or exercise-induced sweat remains unknown.

After all, the original method of cholinergic stimulation with pilocarpine iontophoresis on the skin to facilitate sweat production dates back to the 1959 Gibson and Cooke publication describing implementation and standardisation of the ‘classic sweat test’ targeting sweat chloride levels for the purposes of diagnosing cystic fibrosis. The original method involved the Webster Sweat Inducer system coupled with a patented Macrodust Sweat Collector used more recently to study sweat metabolomics studies originates from a further enhancement of the pilocarpine method, again designed to specifically improve the classic sweat test for CE.\textsuperscript{38} The quantitative pilocarpine iontophoresis test (QPIIT) remains the gold standard for sweat induction in terms of CF-related testing and now has over 50 years of progressive standardisation.\textsuperscript{39} Despite better uniformity in collecting sweat specimens from different individuals, based on age, defined rates of sweating and the volume of sweat to be...
### Study

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<td>Develop and validate a method for metabolic analysis of human sweat using GC-TOF/MS</td>
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<td>Webster Sweat Inducer – Pilogel® iontophoretic discs; 1.5 mA electric current x 5 min</td>
<td>Macroduct® Sweat Collector – part of Macroduct® Sweat Analysis System – covers forearm skin x 15 min; sample transferred into micro-Eppendorf tube</td>
<td>5 min induction + 15 min collection</td>
<td>&gt;70 µL each participant – pooled into one sample</td>
<td>Frozen at -80°C</td>
<td>GC-TOF/MS, full scan mode, untargeted</td>
</tr>
<tr>
<td>Calderon-Santiago et al., 2015&lt;sup&gt;14&lt;/sup&gt;</td>
<td>Identify metabolic markers of lung cancer in sweat to develop screening tool for diagnosis of lung cancer</td>
<td>96</td>
<td>Webster Sweat Inducer – Pilogel® iontophoretic discs; 1.5 mA electric current x 5 min</td>
<td>Macroduct® Sweat Collector – part of Macroduct® Sweat Analysis System – covers forearm skin x 15 min; sample transferred into micro-Eppendorf tube</td>
<td>5 min induction + 15 min collection</td>
<td>&gt;10 µL</td>
<td>Frozen at -80°C until analysed</td>
<td>LC-QTOF MS/MS, untargeted</td>
</tr>
<tr>
<td>Porecznik et al., 2015&lt;sup&gt;15&lt;/sup&gt;</td>
<td>Targeted detection of BPA in sweat in comparison to urine for biomonitoring</td>
<td>50</td>
<td>Passive sampling – no artificial modes of sweat induction</td>
<td>Sweat patches (PharmChek®) applied after skin cleansed with alcohol wipes, to either upper-outter arm or front/back midriff</td>
<td>7 days</td>
<td>Not specified</td>
<td>Sweat patches extracted with methanol; evaporated in Turboprep®; reconstituted with ammonium bicarbonate: ACN (mobile phase)</td>
<td>UHPLC-MS-MS, targeted; using methods initially designed for urine samples</td>
</tr>
</tbody>
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*Table. Sweat induction and collection methods for metabolomics.*
<table>
<thead>
<tr>
<th>Study</th>
<th>Aims</th>
<th>n</th>
<th>Sweat Induction Mode</th>
<th>Methods</th>
<th>Sweat Collection Timing</th>
<th>Amount</th>
<th>Storage</th>
<th>Sweat Preparation Protocols</th>
<th>Analytical Chemistry Platforms</th>
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<td>Dukiewicz et al., 2014&lt;sup&gt;18&lt;/sup&gt;</td>
<td>Untargeted metabolomics profiling of human sweat to evaluate hydrogel micropatch collection linked with direct mass spectrometry</td>
<td>9</td>
<td>Passive sampling – in room temperature, ~25°C, 45% relative humidity</td>
<td>Skin pre-wiped with cellulose tissue soaked with isopropanol: H&lt;sub&gt;2&lt;/sub&gt;O; fabricated agarose hydrogel micropatch embedded with PTFE probe attached to forehead area with adhesive bandage tape</td>
<td>1 min - 1 h</td>
<td>'single droplet' – unable to estimate volume of sweat sample accurately</td>
<td>Hydrogel micropatch probe covered with glass slide, stored at 4°C</td>
<td>Direct coupling of hydrogel micropatch probe to nanospray desorption electrospray ionisation mass spectrometer</td>
<td>ESI + IT + FT-ICR-MS</td>
</tr>
<tr>
<td>Calderon-Santiago et al., 2014&lt;sup&gt;37&lt;/sup&gt;</td>
<td>Untargeted global metabolomics profiling of human sweat to optimise laboratory methods and chemometrics</td>
<td>96</td>
<td>Passive sampling at room temperature: 18–25°C, 50-60% relative humidity</td>
<td>Forehead pre-wiped with cotton soaked in diethyl ether, allowed to dry. Cigarette paper applied, held in place with elastic headband, in duplicate x 1 h, fresh cigarette paper replaced every hour for total 3 h</td>
<td>3 h</td>
<td>Totals not specified; peak amounts 0.11–0.12 +/-, 0.06–0.07 mg/cm&lt;sup&gt;2&lt;/sup&gt; of RSSC collected in first hour</td>
<td>Frozen at -80°C</td>
<td>Pooled samples diluted with formic acid:H&lt;sub&gt;2&lt;/sub&gt;O with additional protocols: (i) hydrolysis with 0.1M NaOH or HCL in H&lt;sub&gt;2&lt;/sub&gt;O; vortexed, evaporated to dryness, reconstituted in chromatographic mobile phase A; (ii) solid phase extraction using C18 and hydrophilic centrifugal Micro SpinColumn™</td>
<td>LC-QTOF MS/MS, untargeted</td>
</tr>
<tr>
<td>Shetage et al., 2014&lt;sup&gt;21&lt;/sup&gt;</td>
<td>Identify collection methods for RSSC and evaluate effects of ethnicity, gender and age on amount and composition</td>
<td>315</td>
<td>Passive sampling at room temperature: 18–25°C, 50-60% relative humidity</td>
<td>Forehead pre-wiped with cotton soaked in diethyl ether, allowed to dry. Cigarette paper applied, held in place with elastic headband, in duplicate x 1 h, fresh cigarette paper replaced every hour for total 3 h</td>
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<td>LC-QTOF MS/MS, untargeted</td>
</tr>
<tr>
<td>Mark et al., 2013&lt;sup&gt;31&lt;/sup&gt;</td>
<td>Detailed amino acid analysis of sweat to better understand key biological mechanisms governing its composition</td>
<td>12</td>
<td>Hot room; 40°C; 60% relative humidity x 15–40 min</td>
<td>Sweat droplets removed from axilla with positive displacement pipette using polypropylene tips; sample transferred directly into 'low binding' Eppendorf tube kept at 4°C</td>
<td>~20 min</td>
<td>&gt;500 µL</td>
<td>Frozen at -70°C</td>
<td>Two methods: (i) ninhydrin derivatisation for amino acid automated analyser + GC-TOF/MS (ii) oxidation and trimethyl-silylation for GC-TOF/MS</td>
<td>Targeted amino acid analysis; automated amino acid analyser + GC-TOF/MS, targeted</td>
</tr>
<tr>
<td>Raiszadeh et al., 2012&lt;sup&gt;38&lt;/sup&gt;</td>
<td>Untargeted and targeted analysis of healthy control and schizophrenic patient sweat, to identify candidate biomarkers of disease</td>
<td>78</td>
<td>Passive sampling at room temperature: 18–25°C, 50-60% relative humidity</td>
<td>Forehead pre-wiped with cotton soaked in diethyl ether, allowed to dry. Cigarette paper applied, held in place with elastic headband, in duplicate x 1 h, fresh cigarette paper replaced every hour for total 3 h</td>
<td>30 min</td>
<td>50–60 µL</td>
<td>Stored on dry ice</td>
<td>Pooled samples; reduction (dithio-threitol/urea), alkylation (iodo-acetamide), overnight enzymatic digestion (trypsin/ammonium bicarbonate), quenching (glacial acetic acid, then angiotensin II), desalting (C-18 Zip Tips), drying in vacuum concentrator, reconstitution in 0.1% formic acid</td>
<td>LC-MS/MS, LC-MS/MS + spectral counting; MRM-MS verification</td>
</tr>
<tr>
<td>Gemuis et al., 2012&lt;sup&gt;35&lt;/sup&gt;</td>
<td>Targeted profiling of phthalate compounds in blood, sweat and urine</td>
<td>20</td>
<td>Passive sampling at room temperature: 18–25°C, 50-60% relative humidity</td>
<td>Direct collection from any body site into 500 mL glass jar using stainless steel spoutula; participant-delivered to commercial laboratory; transferred to 4 mL glass jars at laboratory</td>
<td>No time parameters around sweat collection except conditional within 1 week of blood collection (before/after)</td>
<td>100 mL</td>
<td>Stored at -20°C; shipped frozen on dry ice from Canada to Sweden for analysis</td>
<td>Not specified</td>
<td>HPLC/MS, targeted; GC/MS, targeted</td>
</tr>
<tr>
<td>Gemuis et al., 2012&lt;sup&gt;35&lt;/sup&gt;</td>
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<td>HPLC/MS, targeted; GC/MS, targeted</td>
</tr>
<tr>
<td>Gemuis et al., 2011&lt;sup&gt;30&lt;/sup&gt;</td>
<td>Targeted profiling of 120 compounds (toxins) in blood, sweat and urine</td>
<td>20</td>
<td>Passive sampling at room temperature: 18–25°C, 50-60% relative humidity</td>
<td>Direct collection from any body site into 500 mL glass jar using stainless steel spoutula; participant-delivered to commercial laboratory; transferred to 4 mL glass jars at laboratory</td>
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<td>HPLC/MS, targeted; GC/MS, targeted</td>
</tr>
</tbody>
</table>
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Stored at ~4°C

Timing

Passeive sampling

Sample storage not specified

77-79°F

Lipid-free absorbent papers placed on 6 areas

Axillary sweat sampled with devised twister

Natural environmental heat

Frozen at -80°C until analysis.

Not specified

~4°C

Frozen at -80°C until analysis.

Not specified

GC/MS and LC/MS/MS, untargeted

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1H NMR Spectroscopy – high resolution, both one dimensional and two dimensional, untargeted

HTGC-MS, with electron impact and chemical ionisation

SBSE with thermal desorption GC-MS

*See Appendix (online supplement) for an expanded version of this table including more detailed information of sweat preparation protocols, chemometrics, databases and key findings pertaining to studies.


collected at standardised sites as well as newer confirmatory CFTR-based testing, there are still complicating factors.27,28 Documented reports of false positive and false negative sweat chloride tests are in the literature, hypothesised to be due to such wide ranging factors as contaminating topical gels, interfering dermatological lesions (i.e. atopic dermatitis), autonomic nervous system dysfunction, prostaglandin use and other medication uses (e.g. topiramate), arsenic toxicity, malnutrition states, immunoglobulin deficiencies, autoimmune disorders such as systemic lupus erythematosus, and various abnormal endocrine states such as untreated hypothyroidism and Addison’s Disease.29,30

Exercise/Sauna/Hot Rooms

Other forms of sweat induction used in the studies presented in the Table include exercise and sauna activity or exposure to elevated temperatures with varying humidity levels. Older non-metabolomics studies have suggested distinct differences in metabolic content when sweat is obtained from exercise or sauna activity, especially in Ca2+ and Mg2+ concentrations.24

As this is an active area of ongoing research, it cannot be assumed that metabolomics studies using exercise and/or sauna-produced sweat have interchangeable results. A further potential confounder for sweat studies is the humidity level of sauna or hot rooms as this may contaminate sweat samples with condensation of airborne water droplets potentially containing bacteria, viruses, fungi, and/or xenobiotics. This is

*See Appendix (online supplement) for an expanded version of this table including more detailed information of sweat preparation protocols, chemometrics, databases and key findings pertaining to studies.

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describe another unique, specially-designed and Michael-Jubeli® issues of background (potentially due to variable body®,® include high and interactions with differing individuals’ skin microbiota alterations of skin pH, disruptions to skin barrier properties (i.e. glass pipettes and rollers, hydrogel micropatches) to direct collection of sweat off skin into microcentrifuge tubes for metabolomics studies. These techniques vary from simple, reduced or redundant. The need for complicated sweat induction methods will likely be done, as discussed more extensively in a recent review by de Giovanni and Fucci.1 With newer technological trends of sweat patch with commercial potential of PharmChek® simultaneously to collect larger amounts of sweat that are then pooled for analysis, but differing sweat rates at different collection sites, amplification of inconsistent dilutional effects and difficulty in attaching the Macroduct® to other body sites produces confounding results.5,9,38,45

Sweat Collection Protocols
A brief overview of the sweat collection methods in the Table reveals a diverse range of techniques employed to collect sweat for metabolomics studies. These techniques vary from simple, direct collection of sweat off skin into microcentrifuge tubes or elaborate specifically-designed implementations (i.e. glass pipettes and rollers, hydrogel micropatches) to more commercially-available products like the Macroduct® Sweat Collector or PharmChek® sweat patches. Large variations between individuals in the amounts and location of sweat produced create major difficulties for those attempting to design a universal sweat collection device. Skin irritation, alterations of skin pH, disruptions to skin barrier properties and interactions with differing individuals’ skin microbiota are just some of the difficulties to be encountered in designing an ideal sweat collecting apparatus.1

Commercial Sweat Collection Devices
The Macroduct® (ELITECH Wescor®, Inc., Logan, UT, USA) is a popular sweat collector that employs a plastic capillary-coil device of 29 mm diameter to wick the sweat off the skin surface, usually of the forearms.48 Since its introduction in 1986, it has been used in several sweat studies, including several addressing sweat metabolome optimisation.23,24 The Macroduct® is a component of the Macroduct® Sweat Analysis System, involving a commercial apparatus covering the skin after iontophoresis stimulation using pilocarpine23-31,37,72,73 that was developed to reduce the problems encountered with older filter pad- and tissue paper-based sweat chloride testing for CF.46 Macroduct® helps to overcome issues of background contamination, encapsulation (which increases local skin temperature and sweat gland secretion) and hidromicosis (the progressive decline in sweat rates that occurs when skin is thoroughly wetted and/or with higher humidity) encountered with the older methods.48 The Macroduct® has a capacity of ~0.1 mL of sweat collection per device session. Some researchers have considered placing more than one Macroduct® simultaneously, collecting the amounts of sweat that are then pooled for analysis, but differing sweat rates at different collection sites, amplification of inconsistent dilutional effects and difficulty in attaching the Macroduct® to other body sites produces confounding results.5,9,38,45

The same corporation (ELITECH Wescor®, Inc., Logan, UT, USA) developed a larger version of the Macroduct® called the Megaduct®. This is a round, plastic concave-based device with a larger collection area of 22.1 cm² and a central aperture through which sweat collects into a capped capillary tubing. While the Megaduct® has an increased sweat volume capacity of ~0.5 mL,48,49 its utility is limited by the duration of heat and/or exercise necessary to sweat long enough to fill the Megaduct® reservoir. For example, in one study, it required 65-75 min to collect the full 0.5 mL of sweat from 10 healthy men, with varying exercise intensities (VO2 = 0.5 – 2.0 L/min), temperatures (20–40 °C) in a controlled 50% humidity environment.50 Increasing sweat collection times to this range (~60 min) can potentially impact the power of metabolic findings, especially with the issues of metabolic quenching and time course of metabolic changes.51 For example, it is known that concentrations of sweat electrolytes and minerals such as zinc and iron change in relatively short periods of time.52 Furthermore, the Macroduct® and Megaduct® are designed primarily for forearm placement. As discussed already, the human body does not have a uniform sweat rate or composition over all skin locations. In fact, results of one study suggest forearm sweat rate is 30-60% less than that of the chest or back.53

Another popular commercial sweat collection device is the PharmChek® or PharmChek® (PharmChem Inc., Fort Worth, TX, USA) paper-based sweat collector that was developed to reduce sweat contamination, encapsulation (which increases local skin temperature and sweat gland secretion) and hidromicosis already discussed as well as inter-subject variability and other exercise. It has a release liner that allows removal of the collection pad only once from the adhesive layer after use thereby preventing removal, reapplication or tampering with the patch. Underneath the polyurethane layer is a unique 9-digit number printed on the patch that is visible through a purpose-made window for legal (or research) applications. These features make this device useful in sweat testing for illicit drugs.1

Some of the disadvantages of PharmChek® include high inter-subject variability (potentially due to variable body site placement), high cost, possibility of environmental contamination either before patch application or after patch removal, risk of accidental removal before desired monitoring period and differing rates of drug/metabolite/ xenobiotic penetration through the membrane, depending upon charged or uncharged state. Molecules in an uncharged state have been recorded to migrate more rapidly than charged species in studies of PharmChek®.1,14

Non-Commercial Sweat Collection Techniques
A newer form of sweat patch with commercial potential described by Dukiewicz et al. is a specifically-designed agarose hydrogel micropatch with polytetrafluoroethylene (PTFE) support that has been developed for simplified collection of very small amounts of sweat that can be analysed directly within minutes using various MS platforms.40 This unique method of sweat collection shows promise, but still requires further validation and optimisation of signal sensitivity and performance at higher temperatures and at increased sweat rates.48 Other noncommercial techniques of sweat collection for metabolomics studies are also documented in the Table. Lee et al. describe a ‘sweat collection patch’ placed on the lower back with sweat collected at three time points (10-20 min, 30-50 min, and 60-90 min) of cycling ergometer.”6 Sweat was frozen on dry ice, and then stored at ~80 °C until prepared and analysed. Unfortunately, there is limited mention of skin preparation, the type of sweat collection patch used, how the sweat is frozen, either intact in patch or transferred to another collection tube, or how sweat is prepared for untargeted metabolomics analysis.48 Occlusive skin patches consisting of 2-3 layers of filter paper or gauze have been used in other sweat collection studies but limitations of excessive pH variations and skin irritation with some degree of presumed skin disruption have been significant detractors.49

Shetage et al. and Michael-Jubeli et al. both use passive sampling with ‘cigarette paper’ and ‘lipid-free absorbent paper’ to collect the desired RSCCs or surface skin lipids (SSLs), respectively. These collection methods have advantages of economics and simplicity but still have the disadvantages of encapsulation and hidromicosis already discussed as well as long collection times of 3 h and 30 min respectively.54,55

Kutynecenko et al. describe specially-designed glass rollers and glass pipettes for sweat collection. The rollers were used on lower sweat-producing regions (e.g. arms) moistened with a sterile distilled water spray gun beforehand whilst the glass pipettes were used on heavier sweat-producing areas, namely forehead, chest and back.56 Although the use of glass is compelling with its relative inertness and is certainly of benefit when metabolically targeting plastics-related xenobiotics, the confounders of varied locations of sweat harvesting, dilutional effects of adding sprayed distilled water and a lack of standardisation of temperature and humidity are likely to complicate the untargeted findings of this study.

Perrn et al. describe another unique, specially-designed method of collecting sweat with a polydimethylsiloxane-coated stir bar that is rolled directly onto skin. The fact that sweat samples can then directly go through the necessary extraction steps using a thermos with a cold compound and GC-MS for analysis is attractive. However, the fact that samples had to be shipped at 4 °C overseas to a special laboratory is a limitation and raises the issues of sample contamination and metabolite degradation during transportation.56

Unsupervised Sweat Collection Techniques
In studies by Genuss et al. and Sheng et al., participants were instructed to collect perspiration from any site on their body directly into a laboratory-provided PTFE-coated glass jar, acid- and water-rinsed 500 mL glass jar or by using a stainless steel spatula against their skin to transfer perspiration directly into the same laboratory glass jar.48,57,75 Sweat was collected within one week before or after specified blood collection and participants deposited the collected sweat sample themselves into a laboratory without any specified storage or transport

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detailed subjects

- To improve the sampling task as drawbacks. 11 The choice of harvesting sweat from the axillae, rich in apocrine and apoeccrine sweat as well as eccrine sweat, in both of these studies complicates the comparisons to be made with other metabolomics studies harvesting sweat from other specific areas of the body with minimal apocrine or apoeccrine contributions.

Some similar advantages and disadvantages are appreciated with the sweat collection methods of Jia et al. 9 A leg skin cleansing with alcohol pads followed by distilled water rinsing and drying precede the direct collection of sweat into microcentrifuge tubes which were placed immediately on dry ice to effect metabolic quenching. 10 The cleansing and rinsing beforehand, as well as the physical, direct contact with the microcentrifuge tube may however disrupt the skin surface and again potentially alter skin integrity with its possible effects on fluid migrations from plasma to skin surface.

Summary

A diverse range of sweat induction modes and sweat collection methods are presented in the Table, all with their own advantages and disadvantages. Issues of variable location, timing and amounts of sweat induction and sampling as well as inconsistent sample processing steps and storage conditions confound most comparisons between methods. Optimising these parameters and exploring newer identified sweat collection based upon updated information about sweat glands and the collective contents of their secretions will generate more meaningful results to build and improve our knowledge of the sweat metabolome. Standard operating protocols (SOPs) for collecting human biofluids like blood and sweat for metabolomics studies are crucial to help control for the wide variety of factors that can influence metabolite concentrations. The SOPs for human sweat collection require updating beyond cystic fibrosis and melanoma/metabolism streams, as well as defining standardised conditions and topical applications of antiperspirant and soap products, detailing dietary limitations and specifying shaving of the axillary hair. However, the wiping of underarms immediately before sweat collection introduces potential issues of altered skin integrity on a molecular level, which may impact the content of sweat analysis with ‘H NMR spectroscopy. As mentioned in the section discussing sweat functions, the skin integrity is thought to influence pathways of water and other molecules/metabolism streams, which may impact the composition of sweat and alter the composition of sweat. The cleansing and rinsing procedure is a critical step to furthering human sweat metabolomics. If this is achieved, it is anticipated that sweat may become a more utilised biofluid capable of delivering easily accessible, individualised information that can be used early in the diagnosis of peripheral neuropathy in type 2 diabetic patients.

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