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METHODS & TECHNIQUES

New methods for field collection of human skin volatiles and perspectives for their application in the chemical ecology of human–pathogen–vector interactions

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SUMMARY

Odours emitted by human skin are of great interest to biologists in many fields, with practical applications in forensics, health diagnostic tools and the ecology of blood-sucking insect vectors of human disease. Convenient methods are required for sampling human skin volatiles under field conditions. We experimentally compared four modern methods for sampling skin odours: solvent extraction, headspace solid-phase micro-extraction (SPME), and two new techniques not previously used for the study of mammal volatiles, contact SPME and dynamic headspace with a chromatoprobe design. These methods were tested and compared both on European subjects under laboratory conditions and on young African subjects under field conditions. All four methods permitted effective trapping of skin odours, including the major known human skin volatile compounds. In both laboratory and field experiments, contact SPME, in which the time of collection was restricted to 3 min, provided results very similar to those obtained with classical headspace SPME, a method that requires 45 min of collection. Chromatoprobe sampling also proved to be very sensitive, rapid and convenient for the collection of human-produced volatiles in natural settings. Both contact SPME and chromatoprobe design may considerably facilitate the study of human skin volatiles under field conditions, opening new possibilities for examining the olfactory cues mediating the host-seeking behaviour of mosquito vectors implicated in the transmission of major diseases.

Key words: chromatoprobe, human, sampling method, skin odour, SPME.

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INTRODUCTION

Numerous studies have investigated the chemical composition of human skin volatiles emitted by axillae (Zeng et al., 1996; Curran et al., 2005; Natsch et al., 2006), forearms and hands (Bernier et al., 2000; Zhang et al., 2005; Gallagher et al., 2008; Syed and Leal, 2009; DeGreeff and Furton, 2011), feet (Qiu et al., 2004; Ara et al., 2006; Kanda et al., 1990; Caroprese et al., 2009), or even the whole body (Logan et al., 2008; Harraca et al., 2012). Deciphering human body odours may provide tools for diagnosing human diseases or infections (Prugnolle et al., 2009), and has proved to have various applications in forensic studies (Prada and Furton, 2008). In addition, several studies of human body odours have aimed at understanding how blood-sucking insects locate and choose their vertebrate hosts for blood meals, with particular attention on anthropophilic mosquitoes that transmit pathogens to humans (Meijerink et al., 2000; Logan et al., 2008; Syed and Leal, 2009).

Various sampling methods have been used to analyse human skin volatiles (for review, see Dormont et al., 2013), such as solvent extraction of sweat samples or headspace collection of airborne skin volatiles onto adsorbent traps, usually following preliminary absorption on gauze or cotton pads, or sometimes on clothes that have been worn. Solid-phase micro-extraction (SPME) is a simple, sensitive, solvent-free technique originally developed for the monitoring of air pollutants and later extended to the sampling of volatiles from living organisms (Musteata and Pawliszyn, 2007). SPME is now widely applied for the collection of human odours, but has so far most frequently been used following preliminary collection of skin volatiles on an intermediate medium (sorbent material such as gauze or cotton), leading to the potential isolation of many exogenous compounds (Prada et al., 2011; DeGreeff et al., 2011).

In contrast to plant volatiles, where direct simultaneous comparisons of odour sampling methods have been often conducted (Agelopoulos and Pickett, 1998; Tholl et al., 2006), the results of different methods for sampling volatiles from mammals have never been rigourously compared. The purpose of the present work on skin volatiles was to compare and evaluate the efficiency of four different sampling methods, including two new techniques not previously used for the study of mammalian volatiles. The possible effects of pretreatments applied to the skin before sampling of volatiles were also tested in order to identify optimal conditions for sampling. Because we are interested in how the host-seeking behaviour of vector insects is affected by human-derived volatiles, we aimed at developing methods that can be easily used in outdoor conditions, in countries where vector-borne diseases are of major concern, and on the populations that are most exposed to the pathogens. Our comparative experiments were thus conducted both with French volunteers under laboratory conditions and with young child volunteers under real field conditions in Burkina Faso. We focused our work on odours emitted by human feet, as they have

been proved to strongly influence the behaviour of several blood-sucking mosquitoes (Lacey and Cardé, 2011), and because feet are the preferred biting sites for the main human malaria vectors in Africa, *Anopheles gambiae* and *Anopheles arabiensis* (De Jong and Knols, 1995; Dekker et al., 1998).

MATERIALS AND METHODS

Human subjects

In the first experiment, conducted in Montpellier, southern France, 10 volunteers, five males (age 27–45 years) and five females (age 4–35 years), were recruited. A second experiment was conducted under field conditions in villages around Bobo Dioulasso, Burkina Faso, where people are exposed to malaria infection. Because children are more affected by malaria than adults, we recruited volunteers among young subjects, with the approval of their parents. A total of 16 young volunteers participated in the experiment, nine males (age 5–11 years) and seven females (age 5–11 years).

Trapping of human foot volatiles

Solvent extraction

Sweat volatiles were collected from each volunteer by lightly scraping the foot around the ankle and malleolus regions with a single-use steel razor blade, held perpendicular to the surface, over a period of 20 s. The blade was then placed for 2 min in a small glass vial containing 3 ml of diethyl ether or dichloromethane (a different blade was used for each foot and each solvent).

Headspace SPME

Sampling by SPME was performed using two types of SPME fibre: 65 μm PDMS-DVB Stableflex fibres and 50/30 μm PDMS-DVB/CAR Stableflex fibres (Supelco, Sigma-Aldrich, Bellefonte, PA, USA). The two types of fibre were used simultaneously on the same sampled foot, for each volunteer. For each subject, one foot was enclosed in a non-reactive plastic bag made of polyethylene terephthalate (Nalophan; Kalle Nalo GmbH, Wursthüllen, Germany) and allowed to equilibrate for 15 min. A fibre was then introduced with a manual holder into the Nalophan bag containing the foot. The fibre was left in close proximity (2 cm) to the skin for 45 min. An SPME fibre was also inserted into an empty Nalophan bag (control).

Contact SPME

To examine whether the experimental procedure for sampling human skin volatiles of young children in the field could be simplified, we tested a novel way to use SPME fibres: instead of being exposed to the air surrounding the foot (headspace method as described above), fibres were handled by the operator and lightly stroked over the skin of the foot, around the ankle and malleolus regions, for a period of 3 min. During these 3 min, the tip of the fibre (with the adsorbent polymer-coated phase) was continuously stroked all over the skin so that some part of the adsorbant phase was always in direct contact with the skin. As for headspace SPME, the two types of SPME fibre were used simultaneously on each subject.

Chromatoprobe dynamic headspace

The principle of this solvent-free method is that volatiles are collected into miniaturized trapping tubes filled with a porous polymer, and trapping tubes are then loaded into a chromatoprobe device for direct thermal desorption in the gas chromatography (GC) injector (Dötterl et al., 2005). Adsorbent tubes consisted of ChromatoProbe quartz microvials (length 15 mm, inner diameter 2 mm; Varian, Palo Alto, CA, USA), cut at the closed end, and filled with 1.5 mg Carbotrap B (mesh 20–40, Supelco, Bellefonte, PA, USA) and 1.5 mg Tenax-TA (mesh 60–80; Supelco). The second foot of each subject was enclosed in a Nalophan bag. The air inside the bag was then withdrawn (flow rate 300 ml min^{-1}) over the trapping tube. Collection occurred over 5 and 20 min. A control bag was simultaneously sampled by withdrawing air from an empty Nalophan bag through the trapping tubes.

Pretreatment procedure

A first collection of foot volatiles was performed before any treatment occurred, using solvent extraction and contact SPME. Immediately after odour collection, both feet of the subject were washed with tap water and left to air-dry for 10 min. Foot volatiles were then sampled a second time with the same two methods. After this second sampling, each foot was enclosed in a separate Nalophan bag, as described above. Room air temperature was high (36°C), so that the foot skin became sweaty in a few minutes. After a 15 min equilibration time (as above), an SPME fibre was inserted into one of the bags and exposed for 45 min (headspace SPME collection). Volatiles were simultaneously sampled from the other foot using the chromatoprobe device. One hour after enclosing the feet, we removed Nalophan bags, and foot volatiles from sweaty skin were immediately sampled again using both contact SPME and solvent extraction techniques.

GC–mass spectrometry of skin volatiles

GC–mass spectrometry (GC–MS) analyses of the solvent and SPME extracts were performed using electronic impact ionization mode on a Varian Saturn 2000 ion trap spectrometer, interfaced with a Varian GC CP-3800 apparatus. The Varian CP-3800 was equipped with a 1079 split-splitless injector (260°C) and a 30 m×0.25 mm×0.25 μm film thickness ID WCOT CPSil-8CB fused silica capillary column (Chrompack, Bergen op Zoom, The Netherlands), with helium as the carrier gas (1 ml min^{-1}), and programmed to run 2 min isothermal at 50°C, then 50°C to 250°C at 5°C min^{-1}. For chromatoprobe extracts, an 1079PTV injector was used (Programmed Temperature Vaporizator) with the chromatoprobe equipment (Varian). Mass spectra were recorded in electronic impact (EI) at 70 eV, and identified by matching the mass spectra with data of the NIST 98 MS software library, with previously published data (Adams, 2007) and with data from our personal laboratory bank. Compound identification was confirmed by comparison of retention index (RI) with data from libraries and published data (Adams, 2007), and also by comparison with the retention time of external standards. Peaks were quantified using Star Chromatography Software (Varian). All the peaks of each chromatogram were considered, including compounds present at very low and trace amounts. Non-natural compounds, such as industrial chemicals (e.g. 2-ethyl hexanol, naphthalene) and/or compounds not naturally produced by living organisms (Charpentier et al., 2012), were considered as contaminants and were not included.

RESULTS AND DISCUSSION

Chemical composition of foot volatiles

A total of 44 volatile compounds were detected and identified in human foot volatiles (Table 1). In most samples, the volatile profile was dominated by the presence of two aldehydes, nonanal (mean 34% of the total volatile compounds) and decanal (18%), followed by a few other important compounds, mostly methylheptenone (7%), octanal (6%), undecanal (2%) and geranylacetone (5%). These six compounds have been regularly cited as major compounds of human

Table 1. Volatile compounds emitted by human feet, and collected with different sampling methods: solvent extraction, solid-phase microextraction (SPME) (contact and headspace) and chromatoprobe dynamic headspace

RT	RI	*N*	Compound	Solvent extraction	Contact SPME	Headspace SPME	Dynamic headspace
5.02	628	1	Ethyl acetate	*			
5.13	803	2	Hexanal[1]	*	*		*
6.39	900	3	Nonane	*	*	*	*
7.37	936	4	α-Pinene	*	*	*	
7.72	940	5	Diethylsulfide[2]	*			*
8.60	957	6	(E)-2-Heptenal		*	*	*
8.82	962	7	Benzaldehyde	*	*	*	*
9.30	987	8	6-Methyl-5-hepten-2-one	**	**	**	**
9.58	1000	9	Decane	*	*	*	*
9.83	1006	10	Octanal	**	**	**	**
9.98	1033	11	Limonene	*	*	*	*
11.38	1052	12	(E)-2-Octenal				*
12.50	1100	13	Undecane	*	*	*	*
12.58	1101	14	Linalool	*	*	*	*
12.60	1104	15	Nonanal	***	***	***	***
14.27	1152	16	(E)-2-Nonenal[1]	*			*
14.75	1179	17	Octanoic acid				*
15.44	1200	18	Dodecane	*	*	*	*
15.58	1205	19	Decanal	**	***	***	***
15.87	1214	20	(E,E)-2,4-Nonadienal				*
16.20	1240	21	Benzothiazole				*
17.15	1253	22	(E)-2-Decenal				*
17.50	1275	23	Nonanoic acid	*			*
18.21	1300	24	Tridecane	*	*	*	*
18.38	1306	25	Undecanal	**	**	**	**
19.79	1359	26	γ-Nonalactone				*
19.88	1362	27	(E)-2-Undecenol				*
20.08	1373	28	Decanoic acid				*
20.61	1398	29	Tetradecene	*	*	*	*
20.84	1400	30	Tetradecane	*	*	*	*
21.06	1406	31	Dodecanal	*	**	*	*
22.02	1448	32	Geranylacetone	**	**	**	**
22.50	1471	33	Dodecanol				*
23.14	1498	34	Pentadecene	*			*
23.45	1500	35	Pentadecane	*	*	*	*
23.60	1506	36	Tridecanal	*	*	*	*
23.98	1522	37	Lilial[3]				*
25.14	1576	38	Hexadecene				*
25.52	1600	39	Hexadecane	*	*	*	*
25.72	1700	40	Heptadecane	*	*	*	*
26.63	1926	41	Methyl hexadecanoate[2]	*			
26.05	2125	42	Methyl octadecanoate[2]	*			
30.37	2208	43	Octadecyl acetate[2]	*			
37.12	2847	44	Squalene[2]	*			

Foot volatiles were collected from 10 volunteers in France (five males and five females), and from 16 young volunteers in Burkina Faso (nine males and seven females). The relative content of each compound is expressed with respect to the total content of volatile compounds: *<1%; **1–10%; ***>10%.

RT, retention time; RI, retention index; *N*, peak number.

[1]Compound found only in foot volatiles of French volunteers. [2]Compounds found only in diethylether extracts (not with dichloromethane). [3]Compound found only in foot volatiles of Burkina Faso volunteers, together with other terpenes found as traces: sabinene, β-pinene, *p*-cymene.

skin volatiles in the literature (Logan et al., 2008; Prada and Furton, 2008; Syed and Leal, 2009; DeGreeff et al., 2011). Interestingly, we also isolated the unsaturated C9 aldehyde (E)-2-nonenal in many headspace extracts of foot volatiles, including those of several young participants. This compound has been proposed to be a key component of body odour associated with ageing (>40 years) (Haze et al., 2001; Yamazaki et al., 2010), but recent findings have not supported this assumption (Curran et al., 2005; Gallagher et al., 2008).

More than 300 compounds have been reported from hand skin (Bernier et al., 2000), but that list included many compounds, isolated by solvent extraction, that are not volatile at naturally occurring body temperature, as well as many non-natural and industrial compounds. In results reported by most other studies, skin odour was usually composed of many fewer compounds, e.g. 36–54 compounds found in axilla emissions (Zeng et al., 1996; Curran et al., 2005; Mebazaa et al., 2011), 35–98 compounds in emissions from hands and forearms (Zhang et al., 2005; Gallagher et al., 2008; Curran et al., 2010), or 9–23 compounds in foot volatiles (Qiu et al., 2004; Ara et al., 2006; Caroprese et al., 2009).

In this study, we isolated only a few carboxylic acids in foot volatiles, namely octanoic, nonanoic and decanoic acids. Carboxylic acids have sometimes been cited as main components of skin odours, e.g. in emissions from axillae (Natsch et al., 2006), hands (Prada et al., 2011) or the forehead region (Cork and Park, 1996), and have been shown to be attractive to *Anopheles* mosquitoes (Knols and De Jong, 1996; Meijerink and van Loon, 1999). In contrast, several other authors, and particularly those

who processed headspace collection of skin volatiles, found no or very few carboxylic acids in the skin volatiles of axillae (Mebazaa et al., 2011), hands (Zhang et al., 2005; Kusano et al., 2013) or whole body (Logan et al., 2008). Two studies (Ara et al., 2006; Caroprese et al., 2009) reported nine short-chain fatty acids in solvent extracts of foot sweat and in the interdigital area of the foot, respectively. However, no carboxylic acids were detected in the headspace extracts of foot volatiles (Qiu et al., 2004). Isolation of short-chain fatty acids from skin emanations is thus likely dependent on both the sampling method used and the precise area examined. Moreover, the production of these types of compound may be substantially modified through microbial action (Ara et al., 2006; Natsch et al., 2006) or inhibited by various fragrant agents (Caroprese et al., 2009). In our case, future experiments should test whether a longer duration of volatile collection (for both SPME and chromatoprobe sampling), a change of adsorbent polymer type or even changes during the pretreatment procedure before volatile sampling result in the isolation of more carboxylic acids.

Qualitative differences in the composition of foot volatiles between young subjects sampled in France and young subjects sampled under field conditions in villages in Burkina Faso concerned minor compounds: more terpene products were found in the volatile profiles of African samples (sabinene, β-pinene, *p*-cymene, lilial, each accounting for less than 0.2% of the total compounds), whereas some aldehydes were isolated only in the foot volatiles of French subjects [hexanal, (E)-2-nonenal]. These few differences remain difficult to explain, given that monoterpenes are not naturally produced by humans (Charpentier et al., 2012). Both ethnic background and foot environment might be hypothesized to influence the production of such distinct volatiles: European subjects spend a large portion of their daily lives wearing shoes (in which a particular temperature and humidity are maintained), in contrast to African young volunteers in our study. No marked differences were found between males and females in our sampling, except a few quantitative differences for some components, e.g. nonanal, undecanal and geranylacetone.

Variation with pretreatment procedures

The pretreatment procedures affected odour composition only slightly. The washing pretreatment led to slight differences and a reduction in the quantities of exogenous terpene volatile compounds, such as α-pinene, camphene, *p*-cymene, terpinolene, α-copaene and longifolene. The amount of several alkanes (e.g. dodecane, tetradecane) was very low following washing but increased after sudation.

Variation with sampling methods

Our comparative experiments did not show strong effects attributable to the sample technique used. Qualitative variation among the four different sampling methods and their variants (type of fibres or nature of solvent) concerned only minor compounds of the volatile profile (Table 1, Fig. 1). The dynamic headspace method allowed the trapping of more foot volatiles than the two SPME procedures: 38 compounds were trapped with the chromatoprobe tubes, whereas 23 and 24 compounds were detected using headspace SPME and contact SPME, respectively. No difference was found between the results of tests using the two types of SPME fibre. Sampling with the chromatoprobe device allowed the isolation of compounds present at very low amounts (less than 0.05% of the total volatiles) that were not detected with either headspace or contact SPME but are known to be attractive for some blood-sucking insects, e.g. (E)-2-octenal, benzothiazole, nonanoic acid and decanoic acid (Cork and Park, 1996; Qiu et al., 2004; Siljander et al., 2008). Solvent extracts allowed the trapping of 32 compounds, including some compounds that were not collected with the three other methods, mostly molecules of relatively high weight that are probably poorly volatilized at naturally occurring body temperatures.

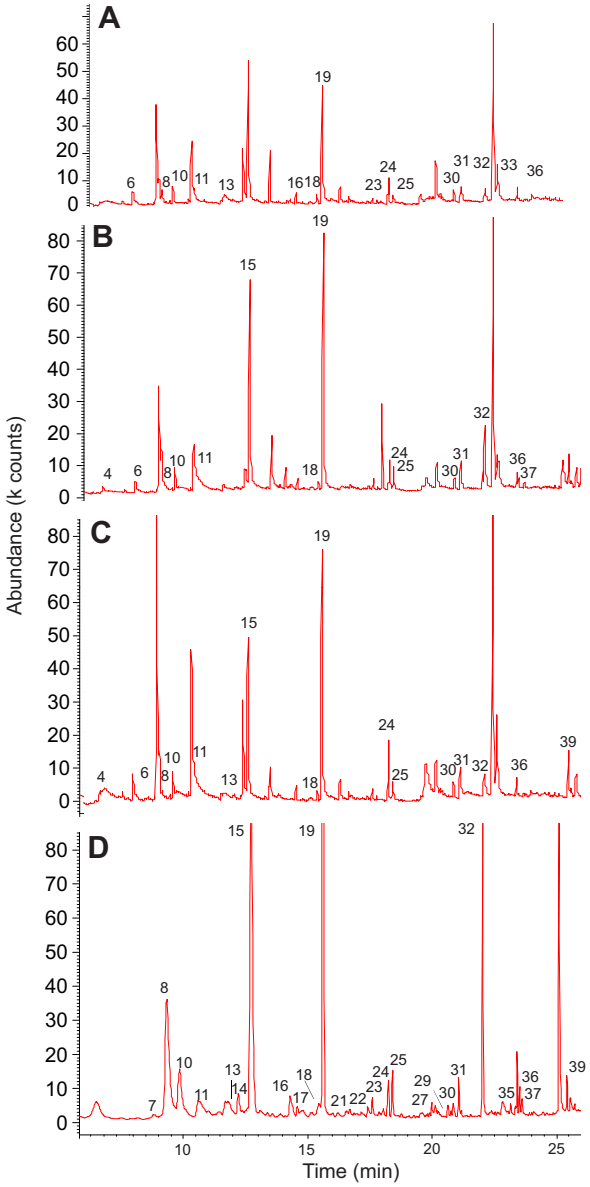

Fig. 1. Gas chromatograms of foot volatiles collected with four different sampling techniques. The four chromatograms show the volatile profile of the same foot successively sampled by the four following methods: (A) solvent extraction – a steel razor blade was used for scraping the foot skin and then put in diethyl ether or dichloromethane for 2 min; (B) headspace solid-phase micro-extraction (SPME) – the foot was enclosed inside a Nalophan bag and sampled by introducing a SPME fibre into the bag for 45 min; (C) contact SPME – a SPME fibre was lightly stroked over the skin of the foot for a period of 3 min; (D) chromatoprobe – the foot was enclosed within a Nalophan bag and sampled by the dynamic headspace method (20 min collection) using chromatoprobe trapping tubes filled with both Carbotrap and Tenax adsorbents. Peak designations are given in Table 1.

Solvent extracts obtained with diethylether permitted detection and identification of five more minor peaks than those obtained with dichloromethane.

An interesting result came from the new procedure we used to sample skin volatiles with SPME fibres: the chemical profiles obtained with the two SPME methods were very similar. To our knowledge, this is the first time SPME fibres have been utilized in this way (direct contact) for the collection of mammalian volatiles. This new procedure requires careful manipulation of the SPME fibre when it is stroked over the skin. Stableflex fibres are flexible but breakable, and adsorbent fibre coatings might also be eroded when used in this way. Both types of SPME fibre used in this study remained usable after more than 10 successive samplings, but how many individual 'contact' samplings can be processed with a single fibre remains to be evaluated. However, the enormous advantage of contact SPME is a considerable reduction in the time required for volatile collection (from 45 min to 3 min), making it easier to sample human skin volatiles under field conditions, especially for young subjects.

We conclude that contact SPME sampling is a faster and more convenient method for field experiments, while sampling with chromatoprobe appears more sensitive and is also easily usable under field conditions.

Whether the volatile profiles obtained with these sampling methods reflect effective olfactory cues perceived by flying blood-feeding insects remains under question. Further behavioural experiments and olfactory tests, e.g. GC–electroantennographic detection (EAD), should examine whether the volatile extracts resulting from headspace collection of human skin odours are actually attractive to mosquitoes. In fact, many of the foot volatile compounds isolated in our study have already been reported to be physiologically active to different mosquito species, e.g. nonanal, decanal, methylheptenone, octanal and geranylacetone, as well as other minor components of foot volatiles, including hexanal, linalool, E-nonen-2-al, octanoic acid, nonanoic acid, decanoic acid, pentadecane and hexadecane (Cork and Park, 1996; Meijerink et al., 2000; Costantini et al., 2001; Qiu et al., 2004; Logan et al., 2008; Syed and Leal, 2009). Preliminary electroantennogram (EAG) tests with adult *A. gambiae* showed that the mosquitoes also detected several other compounds isolated from foot volatiles in our study (L.D. and A.C., unpublished data). Such results may have direct practical applications by improving the efficiency of odour-baited traps, e.g. by adding natural attractants, in mosquito control programmes. We hope that the new methods tested in this work will facilitate the study of human skin volatiles under field conditions, and will allow elucidation of exciting mechanisms in human–vector–pathogen interactions.

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AUTHOR CONTRIBUTIONS

A.C. and L.D. conceived the study, conducted experiments in France, analysed the data, and wrote the paper. A.C. conducted field experiments in Burkina Faso. J.-M.B. performed chemical identifications. D.McK. helped with interpretation and revised the paper.

COMPETING INTERESTS

No competing interests declared.

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