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Original Article

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Sampling the Body Odor of Primates: Cotton Swabs Sample Semivolatiles Rather Than Volatiles

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Abstract

We assessed the suitability of a frequently used sampling method employing cotton swabs for collecting animal body odor for gas chromatography–mass spectrometry (GC-MS) analysis of volatile organic compounds (VOCs). Our method validation showed that both sampling material and sampling protocols affect the outcome of the analyses. Thus, among the tested protocols swabs of pure viscose baked before use and extracted with hexane had the least blank interferences in GC-MS analysis. Most critical for the recovery of VOCs was the handling time: the significant recovery losses of volatiles experienced with this sampling procedure suggest that a rapid processing of such samples is required. In a second part, we used swab sampling to sample the body odor of rhesus macaques (*Macaca mulatta*), which lack scent glands. First results after GC-MS analysis of the samples collected from these nonhuman primates emphasize that proper analytical performance is an indispensable prerequisite for successful automated data evaluation of the complex GC-MS profiles. Moreover, the retention times and the nature of the identified chemical compounds in our samples suggest that the use of swabs is generally more appropriate for collecting semivolatile rather than VOCs.

Key words: body odor, GC-MS profiling, rhesus macaques, semivolatile organic compounds (sVOCs), validation of swab sampling, volatile organic compounds (VOCs)

Introduction

In recent years, it has become increasingly evident that nonhuman primates widely use the olfactory sense, relying on the reception and recognition of chemical signals, to assess their social and ecological environment (e.g., Palagi et al. 2003; Matsumoto-Oda et al. 2007). Primates use olfactory cues, for instance, to distinguish individuals (Palagi and Dapporto 2006; Scordato and Drea 2007), sex (Drea 2015) and females' fertility (Crawford and Drea 2015) as well as group membership (Henkel et al. 2015). Hence, it is not surprising that research on olfactory cues in primates increasingly pursues the presence and function of such signals.

Chemical signals range from volatile to nonvolatile compounds transported via different media, for example, urine, feces, sweat, or glandular secretions (Drea et al. 2013). A precondition for olfactory signaling is that compounds are volatile under the environmental conditions present during signaling between conspecifics. For the identification of volatile organic compounds (VOCs) emitted from primates, for example, as body odors, gas chromatography of vaporized compounds coupled to mass spectrometry (GC-MS) is the method of choice (Drea et al. 2013).

GC-MS analysis has been successfully applied in studies investigating if odor compounds emitted from mammals contain information, for example, on individual identity, group membership, health state or genetic relatedness within a social group (for example, Bechstein's bats [*Myotis bechsteinii*], Safi and Kerth 2003; Giant pandas [*Ailuropoda melanoleuca*], Zhang et al. 2008; Antarctic fur seals [*Arctocephalus gazella*], Stoffel et al. 2015). In primates, GC-MS studies primarily have been conducted in species with scent glands such as ring-tailed lemurs (*Lemur catta*, Scordato et al. 2007) and mandrills (*Mandrillus sphinx*, Setchell et al. 2011), but also in humans (*Homo sapiens*), who do not possess distinct scent glands (Chen and Haviland-Jones 1999; Ackerl et al. 2002; Lenochova et al. 2009; Curran et al. 2010). Body odors were typically collected with untreated cotton swabs or swabs precleaned with diethyl ether or supercritical fluid extraction by methanol modified carbon dioxide. After GC-MS analysis of these samples, 3 volatile substances were identified, namely decanal (Zhang et al. 2008) and 2 farnesenes (Scordato et al. 2007); most of the other compounds were nonvolatile higher organic acids and their esters.

Therefore, here we systematically assessed the suitability of swab sampling for collecting VOCs and subsequently analyzed body odor samples from rhesus macaques, a nonhuman primate without scent glands, as *proof of concept*. In order to establish an efficient method for data evaluation of the complex GC-MS data obtained, we compared the advantages and disadvantages of 2 different approaches, the targeted and nontargeted screening approach (Hernández et al. 2005), to analyze the GC-MS profiles of rhesus macaque samples.

Materials and methods

We examined various swab materials and sample preparation methods for possible contaminations and explored the recovery of volatiles from swabs under different sampling conditions. Swab sampling was applied for the first time to collect body odor from rhesus macaques.

Materials and chemicals

The following cosmetic fibers were used: 1) 100% viscose (*vi*), ebelin cosmetics & more, dm; 2) 50% viscose and 50% cotton wool (*vicw*), Rewe; 3) 100% cotton wool (*cw*) Lilibe Cosmetics, Rossmann; 4) 60% cotton wool, 25% microfiber from polyester and 15% polyester (*mix*) Lilibe, Rossmann; 5) 100% organic cotton (*bio*) from Lilibe Cosmetics, Rossmann; and 6) 100% cotton, Jean Carol Beauty Cosmetic Naturelle, W. Pelz; ordered via Roth.

Methanol, 2-propanol (IP), *n*-pentane (C5), dichloromethane (DCM), ethyl acetate (EA), and diethyl ether (DE) were obtained from VWR, Merck Darmstadt, Germany. Orange oil was purchased from Bioherba Naturprodukte, Heimertingen, Germany. Limonene and *n*-hexane (C6) were obtained from Sigma–Aldrich.

Contaminations from swab material, precleaning, and extraction procedures

Variation in the quality of the swab material, chemicals used for cleaning the swabs before sampling or those for finally extracting the target compounds from the swabs all can introduce contaminations potentially interfering with GC-MS analysis of such samples. We therefore tested 5 different commercially available swab materials (*vi*, *vicw*, *cw*, *mix*, and *bio)*, mainly cotton and viscose mixes, for potential contaminations. All swabs were cut to pieces of ~2 cm diameter held with metal tweezers that were washed in 99 % ethanol and air-dried before use.

In addition, we tested 7 different analytical grade solvents for "washing" the swabs before use as a precleaning procedure, namely water, methanol, isopropanol, ethyl acetate, diethyl ether, dichloromethane, and *n*-pentane, or baked the swabs at 130 °C for 30 min in a dry cabinet. The cleaning solvents were selected to dissolve a wide range of contaminants of different chemical nature. Each swab was washed with >10 mL of 1 of the 7 solvents and left to dry overnight under the fume hood.

For GC analysis of volatile compounds using liquid injection, the extraction solvent is not only required to dissolve the targeted substances but also needs to have a boiling point sufficiently lower than the target compounds to ensure a good separation by GC. We therefore tested diethyl ether and *n*-hexane as very volatile extraction solvents. Each swab was soaked for 10 min in the corresponding solvent (1.2 mL) and the extract was sequentially transferred to a GC vial with a 200 µL insert and was reduced to 60 µL under a gentle stream of air (Sample Concentrator). Each possible combination of swab type (N = 5), precleaning (N = 8), and extraction procedure (N = 2) was analyzed by GC-MS with 2 replicates per combination, respectively. The final experimental design is summarized in Supplementary Table 1.

In addition, we assessed volatile contaminations from cotton swabs *cw* and swabs of the type *mix* after precleaning by baking, whereby the swabs were 1) baked and stored for 1.5 years in a closed glass jar (not airtight), 2) baked and stored for 1.5 years in a closed glass jar (not airtight) and baked again before processing, 3) baked before processing, or 4) washed with 3×4 mL of methanol:DCM (1:1) and dried under the fume hood. After each treatment, swabs were analyzed without solvent extraction by means of headspace sampling for GC-MS. Each combination of treatment and extraction procedure was analyzed thrice (4 precleaning procedures × 3 replicates).

Recovery of volatiles from swabs using limonene as test standard

We tested swabs (*vi* and *vicw*) prepared by baking for their ability of adsorbing volatiles and for the effects of storage of samples. Orange oil was used as a model mixture with limonene as the most abundant VOC (>90%), a common volatile cyclic terpene present in many flavors. Its recovery was determined within each step of the sampling protocol, namely after concentrating the sample as well as after sampling from different surfaces after different time intervals.

Recovery losses by the extract evaporation were determined comparing 1:10 000 diluted orange oil with an extract of 600 µL of a 1:100 000 dilution concentrated to 60 µL. To assess limonene recovery from different surfaces, 60 µL of diluted orange oil (1:1,000) was applied onto 1) a Petri dish, 2) a defined area on the human forearm, or 3) directly onto a swab. After intervals of 0, 1, 5, and 30 min, for 1) and 2) a swab was used to absorb the applied sample by 10 s of wiping. The swabs were extracted using 600 µL hexane and were analyzed by GC-MS. Each combination of swab type, sampling surface and time interval was analyzed thrice. The corresponding dilution of orange oil in hexane served as a control.

Cotton swab odor samples are usually stored in gas-tight vessels at −80 °C (Lenochova et al. 2009) to avoid recovery losses

during their transport to the GC-MS laboratories. Therefore, we assessed the recovery of limonene after different storage times at −80 °C: swabs were spiked with 10 µL of a 1:1000 limonene dilution in hexane and immediately deposited in a 4 mL screw top vial or, alternatively, in a 4 mL glass ampoule sealed by heat. After a storage time of 1, 2, 7, 28, 56, and 74 days, respectively, swabs were extracted with 1.2 mL hexane as described above and analyzed by GC-MS. Each combination of vessel and storage time was analyzed thrice. In addition, swabs spiked and extracted immediately and a 1:10 000 dilution of orange oil in hexane served as controls.

Rhesus macaque samples

Odor samples were collected from the rhesus macaque population living on the island Cayo Santiago, Puerto Rico (USA), managed by the Caribbean Primate Research Center (CPRC). This semifree colony was established in 1938 and currently consists of about 1200 individuals living in several naturally formed groups (for more details see Widdig et al. 2016). Although the macaques forage up to 50% on natural vegetation (for example foliage, fruits, insects, and soil, see Marriott et al. 1989), they are daily provisioned with commercial monkey chow (0.23 kg/monkey/day) (Teklad NIB Primate Diet 8773, Teklad Harlan), limiting the potential influence of nutrition on variation in individual body odors (Henkel et al. 2015). From January to March 2011 we trapped and sampled 65 adult individuals (2 males and 63 females) between 6 and 21 years of age living in 6 different social groups (R, HH, S, KK, F, V). A total of 144 GC-MS samples were obtained.

Odor samples were collected from the armpit region of the narcotized macaques (ketamine, 10 mg/kg) using cotton swabs (Jean Carol) precleaned with methanol and pentane and dried overnight under a hood (Henkel et al. 2015). Swabs were rubbed for approximately 20 s against the armpit of the sampling subject using ethanol-cleaned forceps and were subsequently stored in precleaned glass vials at −80 °C after return to the laboratory of the CPRC on the mainland of Puerto Rico. Odor sample collection was performed within 30 min of anesthetization to minimize the possible influence of ketamine and stress on the body odor. Six blank swabs were taken, stored, and analyzed as controls.

All research procedures followed the institutional and national guidelines for the care and use of animals approved by the CPRC and the Institutional Animal Care and Use Committee (IACUC) of the University of Puerto Rico (protocol number 4060105).

GC-MS analysis

For blank assessment, storage time and rhesus samples, GC-MS analyses were carried out on an HP6890 Series GC System coupled to the Mass Selective Detector HP5973, MSD (Agilent) with electron-impact ionization (EI) at 70 eV and 250 °C, the scan range was set to *m/z* 50–550. The instrument was equipped with a J&W Fisher DB35-MS capillary column (30 m, 0.25 mm id, 0.25 µm film, Agilent) and a 5 m deactivated fused silica guard column. In general, 4 µL of the sample was injected at a temperature of 250 °C in splitless mode with helium as the carrier gas (flow rate 1.7 mL/min). The oven temperature program started at 35 °C held for 2 min, followed by a ramp of 10 °C/min up to 320 °C, held for 10 min. The GC conditions for the experiment on the storage time were optimized for limonene: 70 °C for 1 min; 20 °C/min up to 120 °C, then 7 °C/min up to 190 °C, held for 1 min.

GC-MS analysis of the blank samples after headspace sampling instead of solvent extraction was carried out on a Shimadzu QP-2010 ultra GC-EI-quadrupole-MS with the ion source at 200 °C and 70 eV and the analyser scanning from *m/z* 29–500. The instrument was equipped with a J&W Fisher DB5-MS column (30 m, 0.25 mm id, 0.25 µm film, Agilent) connected to an HS-20 headspace injector (Shimadzu). Headspace injection was done in loop mode (0.25 min load time and 1 min injection time) with a split of 10:1 at a helium flow of 0.95 mL/min; the oven was set to 200 °C, sample and transfer line to 300 °C with a pressurizing gas pressure of 95 kPa and 1 min pressurizing time. The GC program started at 35 °C for 1 min, ramped with 10°/min to 250 °C held for another 5 min.

For assessment of limonene recovery from swabs, 1 µL sample was injected in splitless mode at 230 °C onto a MAT 95 XP double focusing sector field MS coupled to a Trace GC Ultra (ThermoFinnigan) with a Trx 5-MS column (30 m, 0.25 mm id, 0.25 µm film, Thermo Scientific) and a deactivated 5 m guard column using helium as carrier gas at 1 mL/min. The initial oven temperature was set to 40 °C for 1 min, then heated with 10 °C/ min to a final temperature of 330 °C held for another 5 min. The EI source (70 eV) operated at 250 °C, the analyser with a scan range of *m/z* 50–800 and a scan rate of 0.5 s/d.

Data evaluation

Blanks

Full scan signal intensities were calculated as the sum of all signal intensities represented by the total ion current (TIC) per scan obtained from the chromatograms TIC text file. Peak detection was accomplished using the deconvolution algorithm of AMDIS 2.71 (Automated Mass-spectral Deconvolution and Identification System, Stein 1999) with one adjacent peak subtraction, medium resolution and sensitivity, and low shape requirements. The best 10 hits of a NIST08 library search (National Institute of Standards and Technologies) in conjunction with the retention time (Rt) were used to establish tentative identifications of blank contaminations for comparison with the rhesus samples (Supplementary Table 2).

Limonene recovery

The area of the *m/z* 68 peak (base peak) was used for quantitation (Agilent Chemstation software B.01.00 or XCalibur 1.4, respectively). Peak areas were divided through the corresponding area obtained from the designated control sample and multiplied by 100% to obtain the relative recovery values.

Body odor samples from rhesus macaques

A common approach to analyze unknown multi-component mixtures is to identify all signals present in a set of samples before further processing of the data ("targeted approach," e.g., Hutschenreuther et al. 2012, 2013; Milkovska-Stamenova et al. 2015), which involves a substantial workload. To reduce this workload, knowledge about sample characteristics can be used to find signals of interest and identify only those ("nontargeted approach"). In this approach, signals are quantified with respect to their selective mass traces at a particular retention time (*mass spectral tag*, used as entry in our automated peak evaluation) without previous identification of the compounds. Data assessment then is accomplished unsupervised, which means finding a structure in the data by grouping similar samples, or follows a hypothesis-driven approach and sample knowledge is used to identify interesting target compounds (Figure 1). For example, one particular signal that is always relatively smaller in males compared with females could be regarded as an indicator for sex. Subsequently,

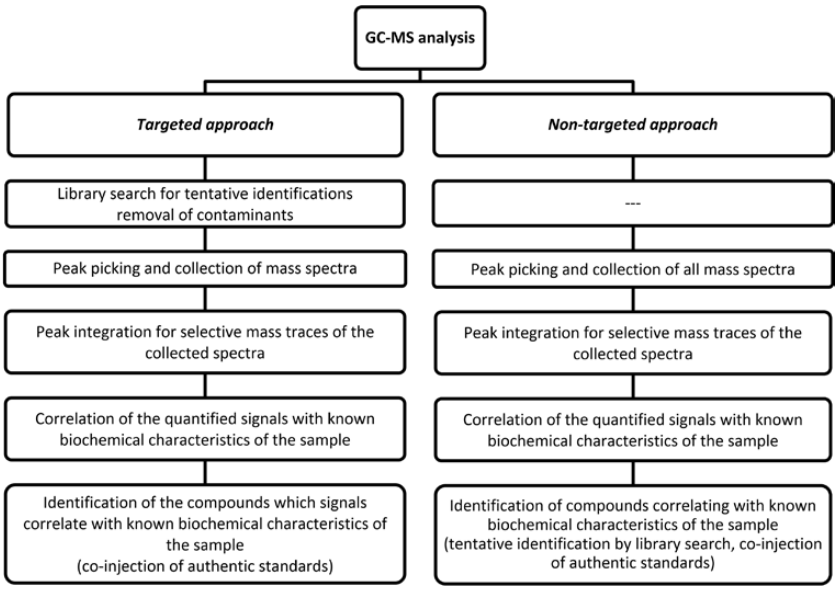

Figure 1. Workflow of the 2 different data evaluation strategies, denoted as targeted and nontargeted approach.

this compound may be tentatively identified by library search, its identity confirmed by spiking with an authentic standard, and finally can be subjected to bioassays to confirm the presumed relationship. The main difference between the 2 applied data evaluation procedures is that in the targeted approach only *manually selected* signals are quantified, whereas in the nontargeted approach *all automatically detected* signals are quantified.

AMDIS 2.71 was used for peak picking by signal deconvolution. The targeted approach started with the manual confirmation of the detected peaks and identification after library search against NIST08, manually deselecting multiple identifications and known contaminants such as siloxane peaks und adding the peaks unrecognized by the software. Selective mass traces of these manually confirmed signals were used to create an automated quantitation method in XCalibur 2.0.7 based on peak area integration. Correct integration from this procedure was manually confirmed or established where needed. The time-consuming, manually performed steps for confirmation of the obtained results were unavoidable particularly for correct integration of coeluting compounds with similar fragmentation patterns.

In the nontargeted approach, all deconvoluted signals by AMDIS 2.71 obtained at an area threshold >2000 and a retention time deviation among the chromatograms of ≤0.1 min for the same compound were collected in a compound library and 2 selective *m/z*, the most abundant below and the highest *m/z* with a relative abundance in the spectrum ≥10%, were used to create an automated quantitation method in XCalibur based on peak area integration. Correct integration from this procedure was manually confirmed or established where needed.

Data from both approaches were median-normalized by division through the median signal intensity within the corresponding chromatogram (Hutschenreuther et al. 2012).

Signal intensities in the obtained data sets were subjected to an unpaired *t*-test comparison between male and female samples to test the feasibility of both approaches to obtain similar results. However, given the very different sample size of male and female samples and apparent pseudoreplication (replicates of the same animal individual) of the male samples (8 samples from 2 individuals), this *t*-test is regarded useful only to illustrate the course of action for the nontargeted approach and to provide evidence if both approaches are capable to extract the same sample differences in a given data set; a biologically meaningful conclusion could not be confirmed with respect to the available data.

Results

Contaminations from swab material, precleaning, and extraction procedures

The different combinations of swab types, precleaning methods and extraction solvents exhibited contaminations of various intensities with the lowest noises in off-white (print version) or green (online version) and the most contaminated blanks in black (print) or red (online) color (Figure 2). Sums and averages of the obtained signals over each chromatogram had highly similar patterns, indicating that the differences were not just determined by single peaks of high abundance. Exceptions were the combinations of C5 and water precleaning for most swab types and baking of *mix* swabs, both with C6 extraction (Figure 2).

The purity of the blanks depended mostly on the swab material and extraction solvent used. The swab material had the highest impact, with cotton-containing materials exhibiting the most intense contaminations, while swabs with a higher viscose content were less contaminated. Swabs from pure viscose virtually exhibited no contaminations (Supplementary Figure 1).

Signal intensities of the contaminations were usually less intense by orders of magnitude if samples were extracted with hexane compared with diethyl ether (Figure 2); because most of the detected impurities of the diethyl ether samples were already detected in the solvent blank, these contaminations are expected to originate from the solvent itself. However, although diethyl ether introduced mainly volatile contaminations, it still may provide a better extraction efficiency for semivolatile compounds such as the sterols, carboxylic acids, and alcohols considering the higher polarity of diethyl ether as a solvent compared with hexane.

Different precleaning protocols produced different contaminations. For example, although pentane and baking effectively

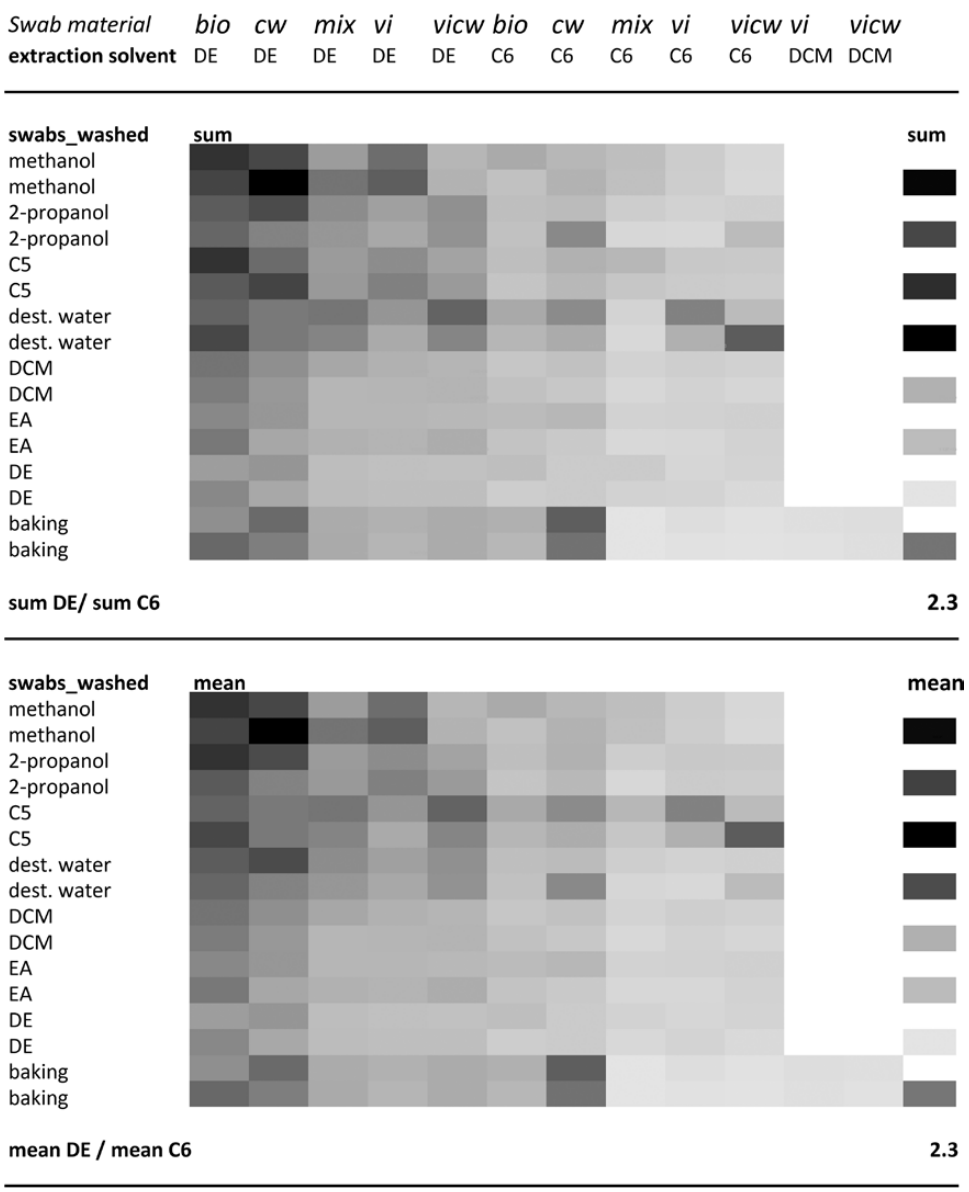

Figure 2. Heat map illustrating the signal intensity of contaminations of the blanks using the grey-scale (printed version)/traffic light color-coding (online version): off-white (print)/green (online) as the least contamination (minimum intensity across the data set), black (print)/red (online) as the highest contamination (maximum intensity across the data set) and grey (print)/yellow (online) as the intermediate levels. Sum (top) and averages (bottom) over all detected signals are illustrated. Ratios of chromatogram intensities after diethyl ether extractions by intensities after hexane extractions are given below each figure. All abbreviations are given in the experimental section. $n = 2$ each.

removed volatiles potentially adsorbed during storage by cotton material, methanol rather removed the late eluting contaminations (Supplementary Figure 1).

The sampling method we used to collect the body odor of rhesus macaques was adopted from earlier studies on primates, where cotton precleaned with methanol and pentane was used (Scordato and Drea 2007; Scordato et al. 2007). We therefore evaluated these blanks in detail. Pentane-washed swabs exhibited higher abundances of a series of higher alkane residues (C_{18}–C_{30}). From methanol-washed swabs, on the other hand, early eluting short-chain ether structures such as ethyl acetate and trimethyl dioxalane were extracted whereas long chain alkylic structures, hexadecanol, oleyl alcohol, C_{16} and C_{18} carboxylic acids, and phytosterols such as sitosterol were found among the less abundant contaminations with both precleaning solvents (Supplementary Figure 1).

Baking proved to be effective for precleaning the swabs, restricted to the viscose-type materials though. Analysis of the dry but not air-tight stored cotton swabs showed accumulation of multiple volatile compounds that could be removed again by baking (Figure 3A). Thus, for the swabs that were stored for 1.5 years at room temperature after baking, we detected high abundances of ketones and short chain carboxylic acid esters (tentatively identified by NIST search) that were not detected when the swabs were freshly baked before analysis.

However, baking did not remove several other contaminations from cotton swabs. We detected peaks with tentative identifications of, for instance, nonanal (9.79 min), decanal (11.36 min), and nonanoic acid (12.17 min) as contaminants (Figure 3B). These peaks were present with a higher abundance in the DCM precleaned blanks as well, thus suggesting that these contaminations may indeed originate

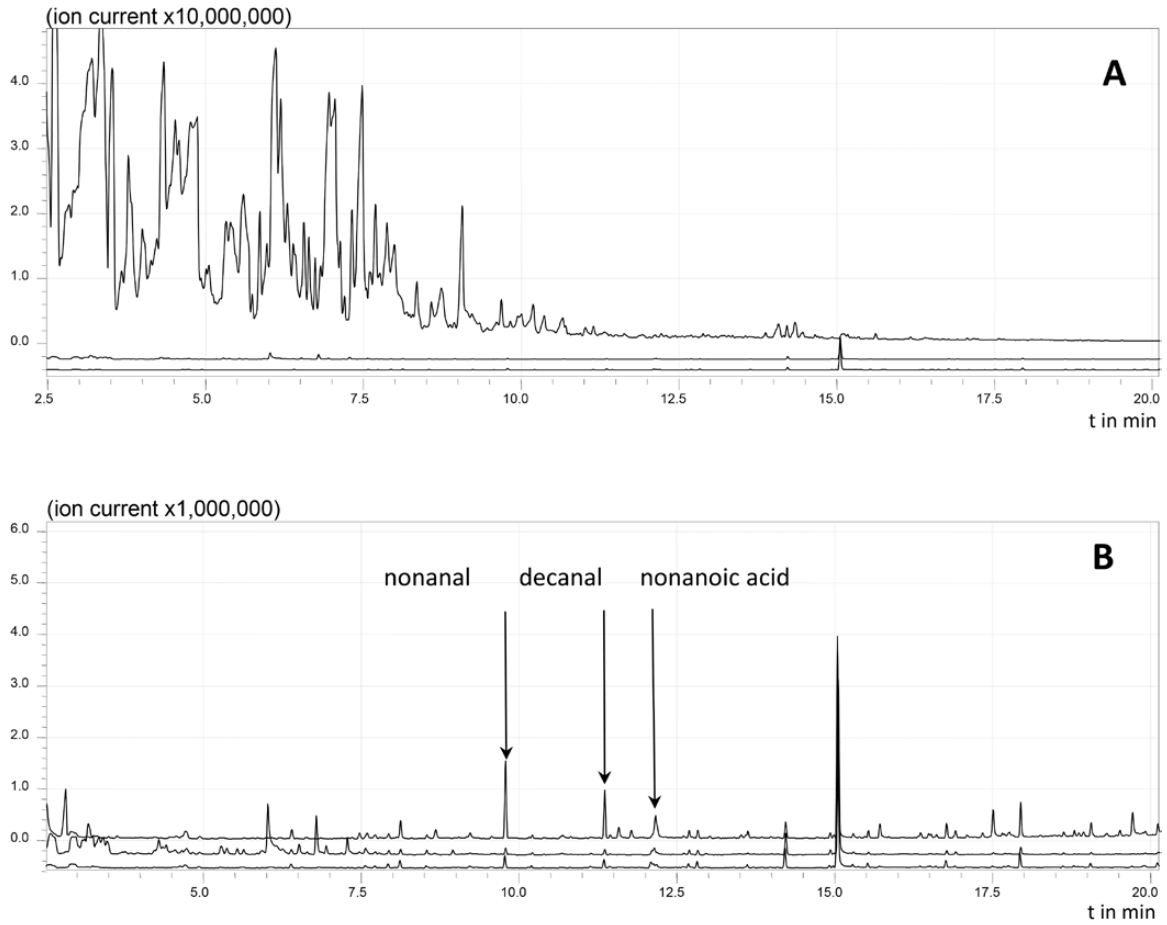

Figure 3. Headspace GC-MS analysis of the thermodesorbed sampling swabs (no solvent extraction), example chromatograms. (**A**)Three overlaid chromatograms with an offset; upper chromatogram: 1.5 years stored after baking; first offset, centered chromatogram: 1.5 years stored after baking, rebaked before usage; bottom chromatogram: fresh swab baked before use. (**B**)Three overlaid chromatograms with an offset; upper chromatogram: DCM cleaned; first offset, centered chromatogram: 1.5 years stored after baking, rebaked before usage; bottom chromatogram: fresh swab baked before use. $n = 3$ each.

from the swab material with the solvent aiding its subsequent evaporation.

For preconditioning by baking, we tested the extraction of swabs with dichloromethane as a third alternative solvent for GC-MS analysis of volatile compounds, which produced blanks of similar quality as with hexane; however, the DCM extracts of the polyester-containing material *mix* had a glassy appearance and led to destructive jamming of the GC injection syringe so that this combination of extraction solvent and swab material should be avoided.

Recovery of volatiles from swabs using limonene as test standard

For the optimal swab material viscose, the recovery of limonene as a test volatile compound was investigated after evaporation and adsorption from different surfaces (Figure 4).

The evaporation of the extraction solvent only slightly decreased the recovery of the volatile target compound to ~90% (Figure 4A), while sampling from different surfaces provoked serious recovery losses (Figure 4B) even though we tried to avoid time losses between spiking and sampling. Notably, recoveries of samples from (human) skin, the anticipated surface for future odor sampling, were below 10%.

Although we obtained better recoveries with viscose, we investigated the cotton-containing material in more detail because the results were expected to better resemble the rhesus sampling that was done using cotton swabs. According to the results with the cotton-containing material, handling time was even more critical for the recovery of VOCs (Figure 4C) compared with the surface; when extraction was performed 1 min after spiking, 40% of the compound was still recovered, whereas after 10 min the signal was below the quantitation limit.

In addition to this low recovery, we found a high variance in the obtained values. Although this protocol featured an approximate relative standard deviation (RSD) of 50% (Figure 4), it rose above 100% for operators using this protocol for the first time, introducing an inacceptable variance to the obtained data.

When testing the storage of the swab samples at −80 °C in screw top vials, recoveries of limonene decreased from ~80% within the first 2 days to ~30% after the first week, and remained fairly constant thereafter (Figure 5).

The initial recovery after storage in the ampoules was already at ~40% and thus quite low from the start, but appeared to remain constant thereafter. From 1 week of storage on, recoveries were similar for both types of vessels.

Rhesus macaque samples

Initial analyses of 12 samples from rhesus macaques with headspace sampling suggested that VOCs could not be recovered from the

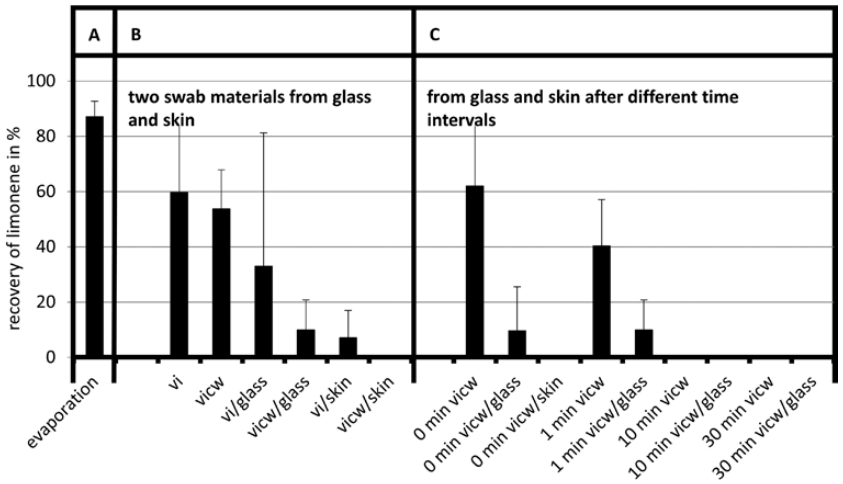

Figure 4. Influence of sampling steps on the recovery of the volatile compound limonene: recovery of limonene (**A**) after evaporation of solvent to obtain a more concentrated target solution, (**B**) after being spiked directly to the swab or applied to glass or human skin and taken off by a swab, (**C**) after different time intervals when applied directly to the swab or taken off with a swab from glass. *n* = 3 each. *vi*, viscose; *vicw*, viscose/cotton 1:1.

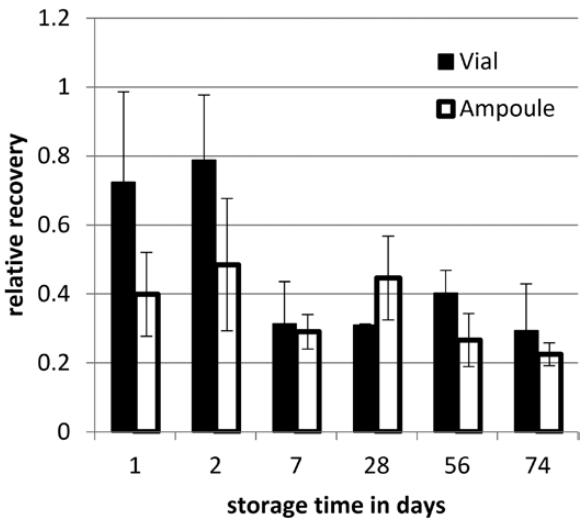

Figure 5. Mean relative recovery of limonene by storage time over various periods of days (d) and type of vessel (vial versus ampoule). *n* = 3 each.

cotton swabs (not shown). Therefore, liquid injection after solvent extraction of the swabs was used instead to include also the semi-volatile compounds sampled from the animals (compounds with boiling points between 240–400 °C). A set of ~410 entries (unique combinations of retention time and *m/z*, 2–4 entries on average per substance) corresponding to 140 chromatographic peak signals was quantified over the whole data set. The number of entries detected in a single chromatogram ranged from 143 to 382, whereby >300 entries (corresponding to ~90 substances) could be quantified in more than half of the 144 chromatograms.

Problems occurred in quantitation due to bad chromatographic performance of free carboxylic acids, steroids, and other compounds featuring polar chemical substituents. These unsuitable peak shapes often failed to be recognized as a signal by automated integration procedures and needed to be manually corrected. Indeed, often we failed at identifying selective *m/z* for coeluting similar compounds such as for instance the wax esters, so that several peaks could not be separately integrated by automated methods in a reproducible manner. Moreover, the chromatogram intensities exhibited a very high variation considering that the overall signal intensities (sums of peak areas) differed between the chromatograms by a factor as high as ~130. The highest RSDs (>100% for all selective mass traces) were observed for the peak areas of nonanal, decanal, and several carboxylic acids (C_{16}, C_{16} methyl and propyl, C_{18} methyl, 9-C_{18}, 9-C_{18} methyl). Deviations <50% for at least one selective mass trace were obtained for many wax esters, nonacosane, cholestenone, cholestadienol, octacosanol, b-amyrin, ursadienone, and lanostenone.

In the rhesus samples, we were able to tentatively identify a total of 68 compounds by spectral comparison with the NIST08 library (match > 900) and manual confirmation of the assignments by spectral similarities to library hits and among the observed fragmentation patterns (Supplementary Table 2). Apart from steroid and alkylic structures including alcohols, aldehydes and carboxylic acids that are ubiquitously found in biological samples as structural units of triglycerides (Charpentier et al. 2012), a pronounced series of wax esters was detected, most probably valeric and isovaleric, and butyric and isobutyric acid esters with saturated, long-chain alcohols. Wax esters are typical for plants and their fruits; they were strongly coeluting and had highly similar mass spectra, and therefore, could not all be accurately integrated for relative quantitation. Moreover, many of the tentatively identified substances such as nonanal, decanal, and long chain alcohols such as hexadecanol, octadecenol, and octadecanol were already found in our blanks. Hence, these substances rather originated from the sampling material than from the animal, which stresses the need of including an appropriate number of blanks into biological sampling. Figure 6 illustrates the comparison of typical chromatograms obtained from the samples.

The chromatograms are depicted divided into 3 regions by the retention times of the 3 main compound groups, namely fatty acids and derivatives, wax esters, and steroids. In the chromatograms' general appearance, the most prominent peaks were hexadecanol, octadecanol, octadecenol, cholesterol, and sitosterol coeluting with an unknown contaminant; the least abundant signals were found to be a few less abundant wax esters and other compounds such as a few higher alkanes, C_{18} and C_{16} acids and their methyl esters, nonanal, decanal, decanol and octacosanol, ursadienone, and tocopherol acetate. Nonanal, decanal and decanol, as well as long chain alkanes, alkanols and carboxylic acids, sitosterol, cholestadienol,

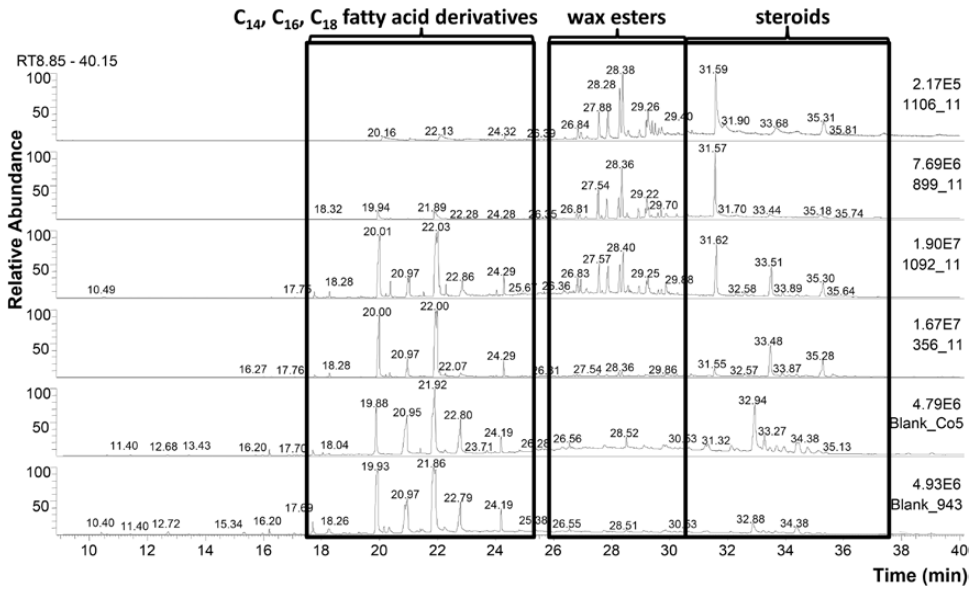

Figure 6. Example chromatograms from 4 female individuals (top) and 2 blank samples (bottom) from swabs precleaned with methanol and pentane. The 3 main compound groups in the chromatogram, namely fatty acid derivatives ≤C18, wax esters and steroids, are highlighted by black frames. The first 2 rhesus samples show a similar peak pattern differing in intensity by a factor of ~20; the 2 most abundant peaks (at 20.0 and 22.0 min) of the blank are here of only minor intensity. The wax esters are not detected with the blanks and exhibit a similar pattern in all rhesus samples with a different intensity while the pattern of the 3 main steroids (cholesterol, sitosterol, stigmasta-3,5-dien-7-one) was different among the chromatograms. The following identifications are suggested for the largest peaks: Rt 20.0 min hexadecanol; Rt 20.9 min hexadecanoic acid; Rt 21.9/22.0 min octadecanol/octadecenol; Rt 22.8 min octadecenol; Rt 24.3 min tributyl acetylcitrate; Rt 31.6/33.5/35.3 min cholesterol/sitosterol/coelution of stigmasta-3,5-dien-7-one with an unknown.

cholestadiene and cholesterol (steroids), squalene, ursadienone and amyrin (terpenoids) but also many wax esters were present in >140 chromatograms whereas octacosanol and 9,12-C_{18} methyl ester were the only substances detected in <50 chromatograms. Notably, already the visual inspection indicates a very different appearance of peak patterns in the different chromatograms.

From the 6 largest peaks, only the wax esters and cholesterol can be unambiguously accepted to originate from the skin of the sampled animals and not from the swab material. Nonanal, decanal, and decanol were indeed the only volatiles identified in the whole set of chromatograms, considering a boiling point below 250 °C (standard conditions); all remaining compounds were semivolatile organic compounds (sVOCs) with higher boiling points, most of them present in the blanks as well. The majority of other signals (for example the wax esters and most of the sterols) seemed to originate, if not from the cotton blank, from other plants, possibly from the food of the macaques or physical interaction with their environment. Apart from the wax esters, just 7 out of 140 compounds (5%, all unknown except a steroid and a steroid ester) were found only in the animal samples, only 2 of which had a relative intensity larger than 0.1% of overall chromatogram intensity, namely an unknown compound and the tentative steroid ester (Supplementary Table 2). However, other compounds may still originate from the animal skin or fur, such as cholesterol and (3β,5α)-cholest-7-en-3-ol considering that those were consistently more abundant in the animal samples compared with the blanks.

Targeted and nontargeted data evaluation of rhesus macaques' samples

Applying the nontargeted approach, automated peak deconvolution extracted a total of 653 unique peak signals for our data set compared with the targeted approach with 140 signals. The reason for this very different number in signals is not only the fact that in the targeted approach obvious contaminations from the system were excluded. Within this context, a critical parameter in automated peak detection is also its success in recognizing the same compound in different chromatograms. However, the automatized nontargeted approach decreased the workload of signal recognition dramatically.

When comparing the normalized signal intensities of samples from 63 females and 2 males in the targeted approach, octadecenol, (3β,5α)-cholest-7-en-3-ol, cholesta-3,5-diene, cholesterol, cholest-4-en-3-one, a cholesteryl ester, and hexadecanoic acid were different by a factor of 2 between the sexes. Table 1 gives an overview of the average signal ratios of the tentative steroid compounds between female and male samples and the *t*-test *P*-values.

The results of the *t*-test between female and male samples for the nontargeted data set are similar to those obtained for the targeted approach above, suggesting the peaks corresponding to cholesterol, cholestenol, cholestenone, and the 2 detected cholesterol esters as significantly different between the 2 sex groups (not shown).

Possibly as a consequence of the very different sample size, all steroid compounds except cholestadienone appeared to be more abundant in the female samples. Moreover, given the apparent pseudoreplication, this *t*-test is used here only to provide an example for the data processing procedure with the nontargeted approach and to show that both approaches are feasible to extract the same sample differences.

Discussion

Sources of contamination in blanks

The extraction procedure and the swab material had the most obvious effects on the intensity of contaminations in blanks, whereby the best results were achieved when viscose swabs were extracted by *n*-hexane. Diethyl ether extraction introduced several early eluting compounds that may lead to a bias in odor analysis

Table 1. *t*-Test and ratio of means female/male for steroid compounds in the data set obtained with the targeted approach (female/male, min. number of replicates > 6, 2-sided, unequal variance)

Tentative identification	Ratio (female/male)	*P* value
Cholesterol	1:2.2	<0.001
(3β,5α)-Cholest-7-en-3-ol	3.1	<0.001
Cholesta-3,5-diene	2.1	<0.001
Cholesta-3,5-dien-7-one	0.7	0.05
Cholest-4-en-3-one	7.1	<0.001
Steroid ester 1	1.8	<0.01
Steroid ester 2	2.7	<0.001

and should therefore be avoided. Extraction with DCM was similarly suitable as hexane, however, DCM reacted unfavorably with polyester-containing swabs.

Swabs from cotton introduced several contaminations of non-industrial origin from the material itself that may also be present on the skin or fur to be sampled and are therefore indistinguishable from those. Consequently, we recommend avoiding the use of cotton for the sampling of odor compounds.

Optimal precleaning procedures depended on the combination with the swab material. Baking proved effective in removing any blank contaminations of lower boiling points (Figure 3), making it suitable for materials with negligible high-boiling contaminations such as viscose. Eventually, a combination of baking with methanol pre-cleaning removing the late eluting compounds may prove particularly useful when working with other than viscose-type materials. Because baking enables a much easier handling combined with an environment-friendly work up and the stability of all swab materials including cotton during baking was satisfactory, we therefore recommend the use of this particular preconditioning.

In conclusion, selecting a viscose-type batting material, baking as sample preparation and extraction of the swabs after sampling with hexane appeared to be the best combination for swab-based sampling.

Notably, the overall quantitative reproducibility of blank contamination analysis of the commercially available swabs was rather poor (Supplementary Figures 1 and 2). Therefore, normalization of sample signals to blank signal intensities seems not feasible; if doubtful, any signals detected in blank samples should be excluded from further analysis of target samples. Moreover, studies using swab sampling should include a representative number of blank replicates to enable a reliable assessment of any blank contaminations.

Recovery of volatiles from swab samples

The recovery of a volatile model compound, limonene, sampled with swabs was generally poor and depended mainly on the time between the application and sampling, but also on the surface from which it was sampled and on the storage time of the sampled swabs. Therefore, if swabs are used for volatile sampling, the samples should be processed as quickly as possible to avoid any unwanted recovery losses.

The recovery of limonene from human skin was poorer than from glass, possibly because 1) skin has a higher temperature compared with glass (~37 °C in mammals compared with ~22 °C room temperature), leading to better evaporation (and therefore higher loss) of VOCs, or 2) skin may be the better adsorber compared with glass. Consequently, it can be assumed that when sampling animals with swabs, only a very small proportion of the produced volatiles will actually be sampled. In contrast, the fact that blank swabs adsorbed volatile compounds during storage indicates that swab material generally should be able to adsorb compounds. However, considering the low recovery of volatiles, the adsorption capabilities of swabs may be either compound-specific or require a long interaction time. Another possibility would be that we observed volatile break-down products from the swab material after storage rather than volatiles adsorbed from the air.

Recovery losses of limonene during storage suggest that swab samples are best analyzed within the first 2 days after sampling. However, thereafter the recovery did not change noticeably, so that after this time samples might be stored for weeks or even months without further loss. This is in line with data from ring-tailed lemurs, where cold storage of samples for several years did not negatively affect the chemical richness of the samples (Scordato et al. 2007). Notably, storage of VOCs by sealing a glass ampoule did not perform better than storage of VOCs in closed screw-top vials stored at −80 °C. Rather, the heat applied during sealing of the ampoule already seemed to cause fast initial losses that resulted in lower recoveries from the ampoules compared with the vials shortly after sampling. Consequently, as screw top vials are easier to handle, they can be recommended to replace the ampoules.

Swab-based sampling of rhesus macaques' body odor

From 140 compounds detected within the rhesus samples, only 3 had a boiling point below 200 °C whereas all other compounds can be considered sVOCs with a limited potential of being used in olfactory communication. The most abundant substances were hexadecanol, octadecanol, cholesterol, and sitosterol; however, ~60% of the identified compounds were present in the blanks as well, whereas only ~5% of the detected compounds (mainly unknowns and 2 steroid compounds) were unique to the animal samples. However, a few of the identified compounds such as cholesterol and (3β,5α) -cholest-7-en-3-ol may originate from swab material *and* animal skin or fur, considering that those were consistently higher in the animal samples compared with the blanks.

According to Charpentier et al. (2012), saturated and mono- and polyunsaturated fatty acids are ubiquitous in living organisms; several sterols and terpenoids may be of mammalian origin as well. Many studies on GC-MS analysis of scents presented similar results exhibiting fatty acid derivatives as the most prominent representatives in such samples (Thom and Hurst 2004; Smith 2006; Scordato and Drea 2007; Scordato et al. 2007; Burgener et al. 2009; Karlsson et al. 2010). However, given our results on blank contaminations, the use of swabs made of plant material (cotton) might be an unfavorable choice. Indeed, many of the detected sterols in the samples were typical phytosterols such as sitosterol, stigmasteroids or campesterol, or plant terpenoids such as the ursanoids, originating from the swab material rather than the animal itself. This stresses the need of a careful consideration and validation of the sampling protocol prior to sampling.

The rhesus GC-MS profiles illustrated a considerable variation among the samples which can possibly be related to variances in the sampled amounts or otherwise introduced by the sampling procedure and material. The median RSD of the samples over all analytes describing the median variability of an analyte within the data set was ~120%; within the normalized data set, it decreased to ~70%. However, for 3 animals 5 replicates were analyzed; with those we obtained a mean RSD of ~25%.

The large variation introduced by the sampling procedure and material may also be one reason that highly abundant contaminations in some chromatograms were not detected in all chromatograms, thus exhibiting irreproducible responses. Consequently, a small RSD of a particular signal was unfortunately not useful for defining a compound as contaminant. Cotton is a biological material and, as such, of course not standardized for the amount of extractable compounds. Within this context, it needs to be emphasized that for cotton swab samples an automatable strategy to exclude compounds as contaminants from data analysis without earlier, proper identification of the compound was not feasible.

Targeted and nontargeted data evaluation

Although the nontargeted approach produces a higher noise in the data, it is easier to automatize and decreases the workload of library search and subsequent manual inspection of the obtained identifications. Furthermore, the nontargeted approach is able to extract compounds that are hardly found by manual inspection (e.g., consistently very low abundant and/or coeluting compounds, respectively) but still may be important. However, it needs to be emphasized that an optimized chromatographic performance preventing from inappropriate peak shapes and coelution of compounds should be established to reduce the workload for subsequent manual correction of automated peak recognition and integration. Although the anticipated proper integration in case of our data set still required experienced personnel for manual corrections and failed to reduce the work load of manual assessment, we still expect the automated approach to be highly efficient in conjunction with a reasonable chromatographic separation and overall performance of analysis.

Implications for sampling body odors with swabs

Our results are generally in line with those of Drea et al. (2013), who emphasized the importance of appropriate precleaning procedures, choice of suitable extraction solvents and the use of blanks to help separating contaminants from potentially biologically meaningful substances. In addition to Drea's methodological considerations, we identified baking as a precleaning method suitable to remove volatile contaminations and found that the swab material should be chosen carefully to reduce contaminations. Importantly, the expected composition of an odor ultimately determines the appropriate manner of scent investigation including the analytical procedures.

Many of the previous studies detected very few volatiles but mostly semivolatiles (Thom and Hurst 2004; Smith 2006; Scordato and Drea 2007; Scordato et al. 2007; Burgener et al. 2009; Karlsson et al. 2010). Based on our study this may be due to the fact that volatiles are largely intangible when using swab-based sampling and many (potentially relevant) compounds are not sampled at all. We argue that if swab-based or related sampling is the method of choice, its limitations such as a careful assessment of sampling material, pre- and post-sampling procedures and the appropriate number of blanks to accurately account for the variation of contaminations should be carefully considered *before* starting the experiments.

Conclusions

Swab-based sampling of body odor exhibited considerable contaminations introduced by swab material, cleaning, and extraction. If swab-based sampling shall be used for volatile analysis at all, we recommend using viscose-type swabs cleaned by baking and extracted in hexane, whereby samples collected that way should be analyzed as soon as possible within the first 2 days after storage at −80 °C in airtight vessels. In addition, we found swab-based sampling for GC-MS analysis after liquid injection of hexane extracts appropriate for analysis of semivolatiles rather than volatiles. Consequently, we recommend the well-established sampling of volatile compounds with thermodesorption tubes instead. Researchers should carefully consider to select sampling methods suiting the anticipated type of compounds. Finally, according to our experiences, chromatographic performance should be thoroughly optimized before performing an experiment to keep the required manual assessment to a minimum.

Supplementary material

Supplementary material can be found at http://www.chemse.oxfordjournals.org/

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References

Ackerl K, Atzmueller M, Grammer K. 2002. The scent of fear. *Neuro Endocrinol Lett*. 23(2):79–84.

Burgener N, Dehnhard M, Hofer H, East ML. 2009. Does anal gland scent signal identity in the spotted hyaena?. *Anim Behav*. 77:707–715.

Charpentier MJE, Barthes N, Proffit M, Bessière J-M, Grison C. 2012. Critical thinking in the chemical ecology of mammalian communication: roadmap for future studies. *Funct Ecol*. 26:769–774.

Chen D, Haviland-Jones J. 1999. Rapid mood change and human odors. *Physiol Behav*. 68:241–250.

Crawford JC, Drea CM. 2015. Baby on board: olfactory cues indicate pregnancy and fetal sex in a non-human primate. *Biol Lett*. 11(2):20140831.

Curran AM, Prada PA, Furton KG. 2010. The differentiation of the volatile organic signatures of individuals through SPME-GC/MS of characteristic human scent compounds. *J Forensic Sci*. 55(1):50–57.

Drea CM. 2015. D'scent of man: a comparative survey of primate chemosignaling in relation to sex. *Horm Behav*. 68:117–133.

Drea CM, Boulet M, Delbarco-Trillo J, Greene LK, Sacha CR, Goodwin TE, Dubay GR. 2013. The "secret" in secretions: methodological considerations in deciphering primate olfactory communication: methods in deciphering olfactory communication. *Am J Primatol*. 75:621–642.

Henkel S, Lambides AR, Berger A, Thomsen R, Widdig A. 2015. Rhesus macaques (*Macaca mulatta*) recognize group membership via olfactory cues alone. *Behav Ecol Sociobiol*. 69:2019–2034.

Hernández F, Pozo OJ, Sancho JV, López FJ, Marín JM, Ibáñez M. 2005. Strategies for quantification and confirmation of multi-class polar pesticides and transformation products in water by LC–MS2 using triple quadrupole and hybrid quadrupole time-of-flight analyzers. *TrAC-Trend Anal Chem*. 24:596–612.

Hutschenreuther A, Birkenmeier G, Bigl M, Krohn K, Birkemeyer C. 2013. Glycerophosphoglycerol, beta-alanine, and pantothenic acid as metabolic companions of glycolytic activity and cell migration in breast cancer cell lines. *Metabolites*. 3(4):1084–1101.

Hutschenreuther A, Kiontke A, Birkenmeier G, Birkemeyer C. 2012. Comparison of extraction conditions and normalization approaches for cellular metabolomics of adherent growing cells with GCMS. *Anal Method*. 4(7):1953–1963.

Karlsson AC, Jensen P, Elgland M, Laur K, Fyrner T, Konradsson P, Laska M. 2010. Red junglefowl have individual body odors. *J Exp Biol*. 213(Pt 10):1619–1624.

Lenochova P, Roberts SC, Havlicek J. 2009. Methods of human body odor sampling: the effect of freezing. *Chem Senses*. 34(2):127–138.

Marriott BM, Roemer J, Sultana C. 1989. An overview of the food intake patterns of the Cayo Santiago rhesus monkeys (*Macaca mulatta*): report of a pilot study. *P R Health Sci J*. 8(1):87–94.

Matsumoto-Oda A, Kutsukake N, Hosaka K, Matsusaka T. 2007. Sniffing behaviors in Mahale chimpanzees. *Primates*. 48(1):81–85.

Milkovska-Stamenova S, Schmidt R, Frolov A, Birkemeyer C. 2015. GC-MS method for the quantitation of carbohydrate intermediates in glycation systems. *J Agric Food Chem*. 63(25):5911–5919.

Palagi E, Dapporto L. 2006. Beyond odor discrimination: demonstrating individual recognition by scent in *Lemur catta*. *Chem Senses*. 31(5):437–443.

Palagi E, Telara S, Borgognini Tarli SM. 2003. Sniffing behavior in *Lemur catta*: seasonality, sex and rank. *Int J Primatol*. 24:335–350.

Safi K, Kerth G. 2003. Secretions of the interaural gland contain information about individuality and colony membership in the Bechstein's bat. *Anim Behav*. 65:363–369.

Scordato ES, Drea CM. 2007. Scents and sensibility: information content of olfactory signals in the ringtailed lemur (*Lemur catta*). *Anim Behav*. 73:301–314.

Scordato ES, Dubay G, Drea CM. 2007. Chemical composition of scent marks in the ringtailed lemur (*Lemur catta*): glandular differences, seasonal variation, and individual signatures. *Chem Senses*. 32(5):493–504.

Setchell JM, Vaglio S, Abbott KM, Moggi-Cecchi J, Boscaro F, Pieraccini G, Knapp LA. 2011. Odour signals major histocompatibility complex genotype in an Old World monkey. *Proc R Soc B Biol Sci*. 278:274–280.

Smith T. 2006. Individual olfactory signatures in common marmosets (*Callithrix jacchus*). *Am J Primatol*. 68(6):585–604.

Stein SE. 1999. An integrated method for spectrum extraction and compound identification from GC/MS data. *J Am Soc Mass Spectrom*. 10:770–781.

Stoffel MA, Caspers BA, Forcada J, Giannakara A, Baier M, Eberhart-Phillips L, Müller C, Hoffman JI. 2015. Chemical fingerprints encode mother-offspring similarity, colony membership, relatedness, and genetic quality in fur seals. *Proc Natl Acad Sci U S A*. 112(36):E5005–E5012.

Thom MD, Hurst JL. 2004. Individual recognition by scent. *Ann Zool Fennici*. 41:765–787.

Widdig A, Kessler MJ, Bercovitch FB, Berard JD, Duggleby C, Nürnberg P, Rawlins RG, Sauermann U, Wang Q, Krawczak M, *et al*. 2016. Genetic studies on the Cayo Santiago rhesus macaques: a review of 40 years of research. *Am J Primatol*. 78(1):44–62.

Zhang JX, Liu D, Sun L, Wei R, Zhang G, Wu H, Zhang H, Zhao C. 2008. Potential chemosignals in the anogenital gland secretion of giant pandas, *Ailuropoda melanoleuca*, associated with sex and individual identity. *J Chem Ecol*. 34(3):398–407.