Novel noninvasive identification of biomarkers by analytical profiling of chronic wounds using volatile organic compounds

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Manuscript received: March 22, 2009
Accepted in final form: March 26, 2010

DOI:10.1111/j.1524-475X.2010.00592.x

ABSTRACT

A complex profile of volatile organic compounds (''VOC''s) emanates from human skin, which is altered by changes in the body's metabolic or hormonal state, the external environment, and the bacterial species colonizing the skin surface. The aim of this study was to compare VOC profiles sampled from chronic leg wounds with those from asymptomatic skin. Five participants with chronic arterial leg ulcers were selected. VOC samples were obtained using polydimethylsilicone membranes (''skin-patch method'') and analyzed by gas chromatography-ion trap mass spectrometry. Resultant data were analyzed using multivariate analysis and mass spectral matches were compared against the National Institute of Standards and Technology database. Principal component analysis showed differences in profiles obtained from healthy skin and boundary areas and between profiles from healthy skin and lesion samples ($p < 0.05$). Partial least squares for discriminant analysis gave an average prediction accuracy of 73.3% ($p < 0.05$). Mass spectral matching (verified against microbial swab results) identified unique VOCs associated with each sample area, wound bacterial colonization, and ingested medications. This study showcases a reproducible, robust, noninvasive methodology that is applicable in a clinical setting and may offer a new, hitherto unexplored, class of biochemical markers underpinning the metabolism of chronic wounds.

Chronic wounds affect circa 200,000 people in the United Kingdom at any one time and of these wounds, leg ulcers are highly prevalent affecting up to 2% of the adult population.[1,2] The estimated cost of treating leg ulceration (as defined as a loss of skin that takes more than 6 weeks to heal[3]) to the UK's National Health Service amounts to £400 million–£600 million per year,[4,5] with the overall financial burden of all chronic wounds being estimated at more than £1 billion per annum.[6] Furthermore, patients with leg ulcers have reduced quality of life when compared with age-matched controls due to pain, odor, and decreased mobility.[3]

Identification of the causation of such wounds is problematic, as is ascertaining the most appropriate treatment method. Currently, patients with leg ulcers are assessed via the history, appearance of the lesion, and a vascular assessment (palpation of the pedal pulses, ankle brachial pressure index and duplex ultrasound scanning). The ability to delineate both the underlying cause and the bacterial colonization of such lesions via a noninvasive technique would be helpful in understanding the disease process, aiding appropriate treatment selection (possibly mitigating against the emergence of multiresistant organisms via correct antibiotic choice). The noninvasive technique used in this study is based on the collection and analysis of volatile organic compound (''VOC'') signatures emitted from leg ulcers.

VOC is a generic term used to classify a wide range of molecules with a boiling point of $\leq 300\,^{\circ}C$, for example, alcohols, aldehydes, ketones, isocyanates, sulfides, and hydrocarbons.[7] The human body is known to contain and emit a large number of these substances as essential nutrients and intermediates, waste products of endogenous processes, from the absorption of environmental contaminants, and also via exogenous bacterial metabolism. To date, these compounds have been detected from skin (sebum, sweat, skin emanations, and hair), breath, serum, urine, saliva, cerebrospinal fluid, feces, breast milk, semen, amniotic fluid, and tissue homogenates.[7,8] The VOCs emitted change with the body's metabolic or

NA	Nonadhesive
PC	Principal component
PCA	Principal component analysis
PLS-DA	Partial least square for discriminant analysis
VOC	Volatile organic compound

hormonal state[9] (for example, the metabolic changes associated with diabetes cause the release of acetone on breath[10]), with ingested dietary compounds,[11] by variations in the external environment (VOC emissions from the skin alter diurnally and seasonally[12]), and by alterations in the bacterial species colonizing the skin surface.[12–14]

Aside from the underlying causative factors that result in the formation of ulcers, another important aspect in their chronicity is concurrent bacterial colonization or infection of the ulcerated area.[15] Wounds are usually colonized by the commensal skin flora but pathogenic bacterial species are also commonly implicated.[16,17] Colonization of a wound is not itself a barrier to wound healing[15]; however, repeated infections have been shown to cause increased proinflammatory cytokines and matrix metallopeptidases, decreased tissue inhibition of these peptidases and decreased levels of growth factors—these changes have been hypothesized to be the underlying causes of wound chronicity.[18]

Historically, the degree and/or speed of wound healing were thought to be related to the bacterial count. A study of pressure ulcers showed that significant healing only occurred when the bacterial count was $< 10^6\,mL^{-1}$ bacteria.[19] More recent research indicates that while bacterial density at the wound surface is independently predictive of nonhealing, this is overly simplistic as factors such as bacterial diversity, microbial synergistic interactions, and the underlying host response are contributory.[16,18,20]

Current clinical methods of wound infection analysis are surface swabbing and wound exudate culture, wound tissue biopsy, and the clinician's judgment of the "classic" signs of infection (pain, erythema, edema, heat, and purulence). None of these methods is ideal. Surface swabbing is difficult to undertake reproducibly and reliably[21] and causes trauma to the granulating tissue.[22] Trauma is of greater concern with tissue biopsy, still deemed the "gold standard" method for quantitative wound infection analysis, with some authors finding that the accuracy of culture results obtained is comparable with that of surface swabbing.[20,21,23] Clinicians cannot provide reproducible, consistent, and accurate assessments: purely subjective observations result in a large variation in the sensitivity of results with little interobserver reproducibility.[24,25]

Development of an accurate, noninvasive method for the analysis of chronic wound etiology, infection, and healing would be of clinical use. In this study, we propose to sample the VOC profiles of vascular leg ulcers to demonstrate a novel technique for the future analysis of not only vascular ulcers but also other types of chronic wounds.

METHODS

Participant selection

Participants were selected from a cohort of inpatients under the care of the vascular surgery department at University Hospital of South Manchester NHS Foundation Trust, Wythenshawe, Manchester, UK. The inclusion criteria were that a participant was male, of Caucasian descent, between the ages of 55 and 95 and have a leg ulcer of predominantly arterial etiology (proven by the evidence of significant arterial disease on duplex ultrasound scanning). This was necessary to minimize confounding factors associated with comparing ulcers of variable etiology. A participant was deemed to be of Caucasian descent if his parents and all grandparents were stated as Caucasian—this was relevant because ethnicity and sex are known to alter skin VOC profiles.[26–30] There were no specific exclusion criteria; however, the selected cohort was necessarily limited to five participants due to the prospective nature of the study and the time required to process and analyze the samples—this provided 50 samples, allowing the appropriate use of the chosen statistical tests. For each participant, a comprehensive history was elicited including that of the lesion to be sampled, ingested medication, and the treatment applied to the lesion both historically and before the sampling period. Also noted were any toiletries used and whether specific consumables that affect the emitted VOC profile, e.g., spicy food, coffee had been ingested during 48 hours before sampling.

Equipment preparation

The sampling methodology has been described recently and evaluated.[11] Briefly, polydimethylsilicone skin-sampling patches measuring 20 mm × 15 mm × 0.45 mm (Goodfellow Cambridge Ltd., Huntingdon, UK), were prepared by washing and conditioning at 180 °C in a vacuum oven before storage in inert thermal desorption tubes (Markes International Ltd., Rhondda Cynon Taff, UK). Before utilization, the patches were thermally desorbed to verify that they were free from contamination.

Ethics and participant preparation

This study was conducted in accordance with the ethical principles of Good Clinical Practice and the Declaration of Helsinki. The Local Research Ethics Committee (Manchester, UK) approved the protocol before commencement of the study and all subjects gave written, informed consent. Twenty-four hours before sampling, the dressing covering the participant's lesion was removed, the area was irrigated with distilled water to remove any remnants of prior dressing materials, and a sterile nonadhesive (NA) dressing (Johnson & Johnson Medical Ltd., Ascot, UK) was applied using an aseptic technique. A secondary dressing layer comprising Softform gauze and crepe bandaging was applied over the NA dressing. The same procedure was used to apply a second sterile NA dressing on a more proximal, lesion-free region of the ipsilateral leg, at least 10 cm away from the ulcer edge. This was used to collect the VOC profile of normal skin. The participant was advised to keep the dressings dry and not to use any toiletries until post-sampling 24 hours later.

Sampling procedure

Twenty-four hours after applying the dressings, the prepared thermal desorption tubes were removed from refrigeration and transported to the participant. The participant's local environment was assessed for signs of significant exogenous VOC contamination (cleaning, cooking, or other medical interventions) and other clinical staff and patients were excluded from the sampling locality to reduce exogenous sample contamination.

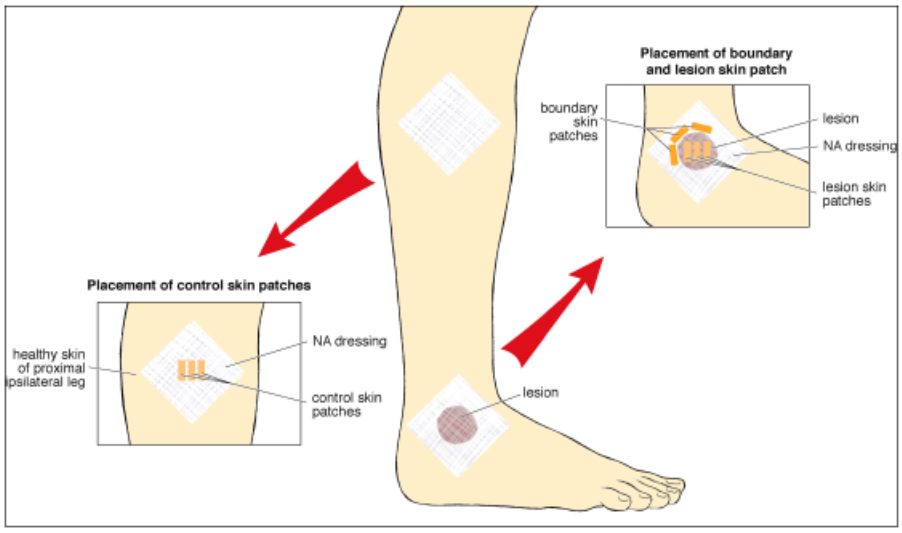

Figure 1. A diagram showing the positioning of the skin patches on a participants' foot. Patches were applied in triplicate for 30 minutes to the lesion, boundary, and control areas above the nonadhesive dressings applied previously.

Forceps used to handle the patches were sterilized with an isopropyl alcohol wipe and air-dried. A cotton-wool pad was located 1–2 m from the participant onto which a sampling patch was placed. This was exposed for 30 minutes, providing a baseline of the environmental VOCs present. Concurrently, the secondary dressings were removed and three skin patches were applied to each sampling region (Figure 1), covered with a cotton-wool pad and left in situ for 30 minutes. At the end of the sampling time, each patch was removed from the sampling site and resealed into its thermal desorption tube. The sampled area was photographed and swabbed for microbiological analysis before being redressed. The samples were placed in storage at 4 °C to await analysis within 24 hours of sample collection.

Sample analysis

Sampled VOCs were recovered from the skin patches via a two-stage thermal desorption procedure (Markes International Ltd.), separated along 60 m of a 0.25 mm diameter capillary column with a 0.25 µm thick 5% phenyl, 95% methyl polysiloxane stationary phase (DB-5MS; Agilent, West Lothian, UK) before passing into the Varian 2200 (Varian Ltd., Oxford, UK) ion trap mass spectrometer, the operating parameters of which are summarized in supporting information Table S1.

Mass spectral searching and data visualization

All chromatographic data were evaluated and checked to ensure reproducibility. The chromatograms were assessed on a peak-by-peak basis and five mass spectra were averaged from consecutive scans for all resolved peaks $> 5\%$ of the maximum peak intensity. The resultant list of mass spectra was searched against the National Institute of Standards and Technology library and provisional assignments were made to the isolated compounds. The assignments were then reviewed and those with incompatible physical chemical characteristics were removed from the list of candidate compounds and labeled as unknown. The compounds were then examined by sample type to assess the variability of the samples obtained from the different sites.

Data preparation

The gas chromatography-mass spectrometric (GC-MS) data files were converted into netCDF format using a conversion program (Palisade MASSTransit, Scientific Instrument Services, Ringoes, NJ, USA) enabling the data to be exported into the multivariate statistical processing software (Matlab, Mathworks, Natick, MA, USA) (Figure 2). Within Matlab, linear interpolation algorithms with resampling were applied to ensure that all data sets were the same size—the accumulation of small variations in the instrument control unit results in GC-MS data surfaces of different dimensions over a range of time off-sets. The resampled data were then aligned using correlated optimized warping and baseline corrected by applying asymmetric least squares. The final process was to remove those parts of the data set that did not contain chemical information—the gaps between chromatographic peaks. This was achieved by studying the chromatogram visually and identifying the level of the base line, along with the standard deviation for the baseline. A standard deviation filter was subsequently applied to the data sets and all data that were within three standard deviations of the base line were removed. The resultant chromatogram was normalized such that the total integrated peak areas of the peaks were equal to unity.[31]

Chemometric analysis

The chromatograms were analyzed for natural trends or outliers using principal component analysis (PCA) and the result of this unsupervised learning analysis was visualized by plotting the first three principal components scores (PC 1, PC 2, and PC 3).[9,32,33] Hotelling's T^2-statistics[33] were applied to the PCA scores to determine the statistical significance of the observed intersite differences.

Partial least squares for discriminant analysis (PLS-DA)[9,31–33] was then used to model the VOC data for its ability to discriminate between different sites (lesion,

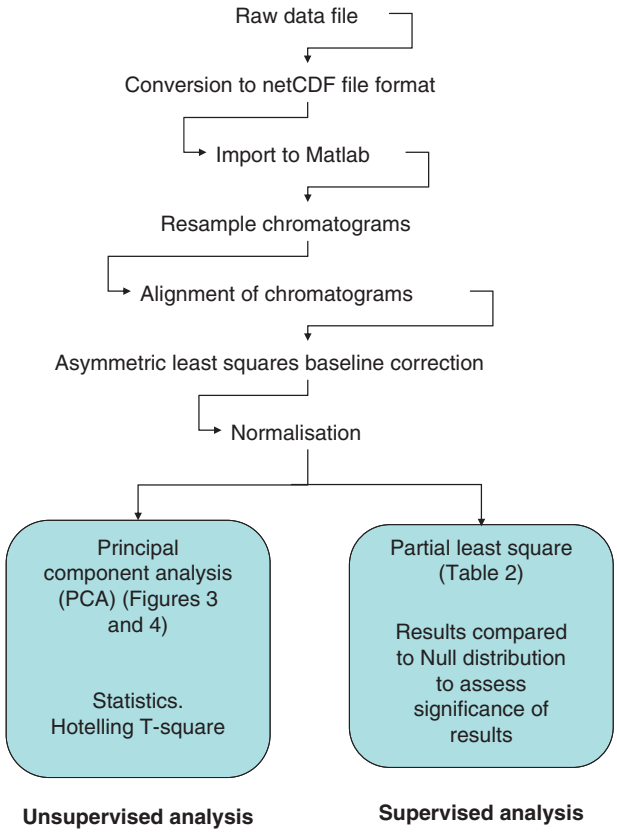

Figure 2. Data handling flow-chart.

control and boundary). The data were randomly divided into two sets: 80% was used to build a training model and the remaining 20% was used to test the accuracy of the model. In order to avoid biasing the analysis (vide infra), the selection of the training set and test set was based on participants rather than samples. That is, if the data from a participant were selected for use as part of the training set, all the other data from this participant were also used in the training set. Consequently, four out of five of the participants' data were used for training and the remaining participant's data were used for testing. This procedure was repeated five times, each time using one different individual. This minimized the influence of the characteristic individual VOC signatures that might have resulted in overoptimistic results if the training and test data contained profiles from the same participant. Because of the limited sample size, the classification results obtained were averaged and further evaluated using a permutation test.[34–36] In this test, 2,500 Monte-Carlo simulations were carried out and in each simulation, the order of labels was shuffled randomly—the same PLS-DA procedure was applied to the data set post-shuffling. The results formed the null distribution and the averaged classification result obtained as described above was compared against it to assess the significance level of the result.

RESULTS

The researchers observed no discomfort to the participants and no participant reported any discomfort arising from the procedure.

All sampled lesions were of mixed etiology: the major component was limb ischemia due to arterial occlusion, but elements of neurogenic injury and superimposed wound colonization were also present. There was heterogeneity in the character of each lesion, varying between dry and necrotic to wet and exudative. The clinical description correlated with bacterial colonization of each lesion (see Table 1 for a summary of the microbiological swab results). The organisms cultured were mainly Gram-negative aerobic bacilli, coagulase-negative staphylococci or Proteus species and coliforms. Methicillin-resistant *Staphylococcus aureus* was cultured in one patient.

Data characterization and visualization

Examples of GC-MS data obtained from lesion samples are represented in Figure 3. The complexity of the chromatogram is striking: the range of intensities of responses spans more than three orders of magnitude, with more than 300 clearly resolved signatures. There were many other nonresolved chromatographic peaks with peak shapes suggesting the presence of significant numbers of

Table 1. Microbiology swab results taken from the center of the lesion, boundary area of the lesion and control (reference) skin site

Participant	Control (reference site)	Boundary area of the lesion	Lesion
1	Mixed coagulase-negative staphylococci (mixed skin-type flora)	Methicillin-resistant *Staphylococcus aureus*	Methicillin-resistant *Staphylococci aureus*
2	No growth	No growth	Mixed coliforms and *Proteus* species
3	Mixed coagulase-negative staphylococci (mixed skin-type flora)	Mixed coagulase-negative staphylococci (mixed skin-type flora)	Mixed coagulase-negative staphylococci (mixed skin-type flora)
4	No growth	Mixed coagulase-negative staphylococci (mixed skin-type flora)	Mixed coagulase-negative staphylococci (mixed skin-type flora)
5	No growth	Mixed coagulase-negative staphylococci and enterococci	Mixed coagulase-negative staphylococci and enterococci

Rotational spot swabbing was utilized due to the limited surface area available on some sites. Unsurprisingly, all lesions were associated with bacterial growth whereas the majority of healthy control skin demonstrated no bacterial growth.

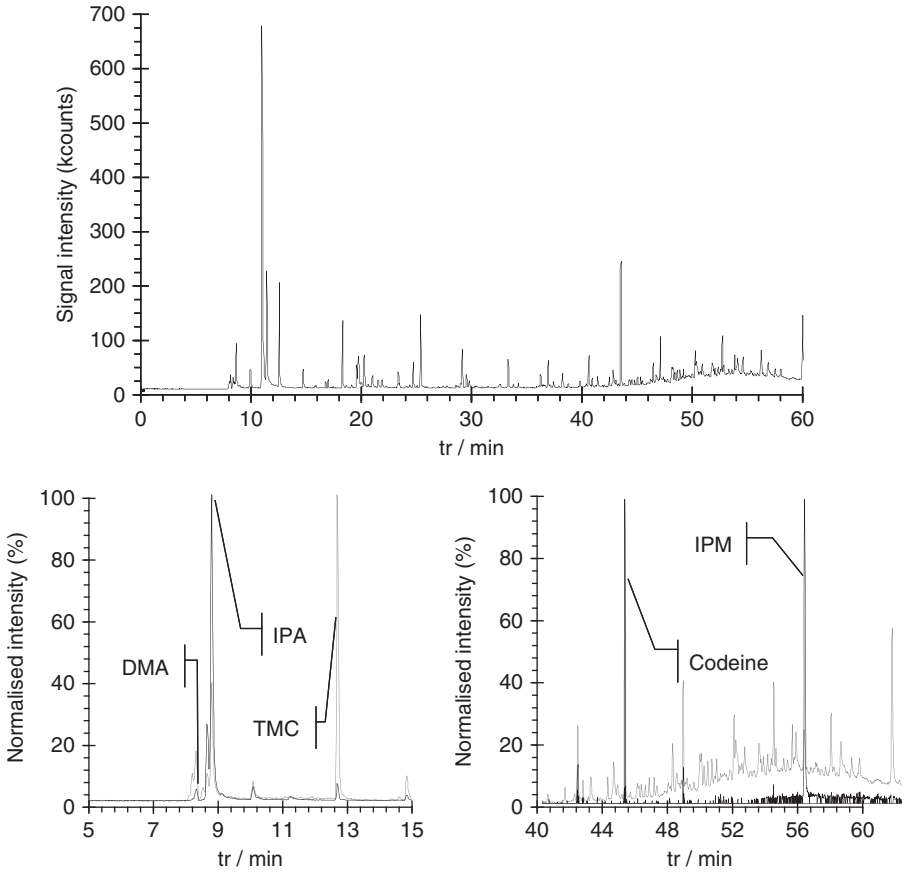

Figure 3. Examples of the gas chromatography-mass spectrometric data obtained from lesion samples. The top trace shows the total ion chromatogram while the two bottom traces show normalized plots of selected ion chromatograms (shown in black), superimposed on the total ion trace (gray lines). The selected ions were *m/z* 45 for the bottom left trace and *m/z* 299, and 228 for the bottom right trace. The complexity of the data observed within the total ion chromatogram in the top trace derives from a variety of sources (examples as follows): Endogenous metabolites—trimethylcarbazole (TMC, at a retention time [T_r] of ca. 12.7 minutes) bottom left; exogenous volatile organic compounds—isopropylalcohol from the sampling protocol (IPA, T_r=ca. 9 minutes) bottom left, isopropylmyristate from personal care products (IPM, T_r=ca. 56.3 minutes) bottom right and Codeine (T_r=ca. 44.8 minutes) bottom right; and from the bacterial activity within the lesion-dimethylamine (DMA, T_r=ca. 8.3 minutes) bottom left.

close and coeluting components. Indeed, a preliminary assessment of the data with deconvolution software (ACD/Labs IntelliXtract, Toronto, Canada) indicates the presence of many hundreds of hitherto undocumented VOCs in the skin samples. Such chemical diversity has been discussed previously[11] and arises from the underlying metabolism of the participant (affected by phenotype, diet and environment); exogenous inputs (medication, environmental contamination); compounds specifically related to the metabolism of bacteria in the lesion; and the associated pathology of tissue damage.

Compounds of varying chemical groups were recovered, including esters, alcohols, thiols, hydrocarbons, carboxylic acids, amines, amides, ketones and siloxanes. Visualization of the data resulted in the list of compounds in Table 2—this summarizes the tentative assignments of compounds that were associated exclusively with each individual sampling site. Although site-unique compounds may be discerned, it is helpful to note that the distribution of compounds across the different sites arises from a variety of mechanisms: exogenous materials may be washed out by wound exudates; VOCs generated within the lesion may diffuse into the blood stream to be released from the skin at the control site; and VOCs released from the affected area into the surrounding air may be present in the environmental control samples. Certainly, a strong odor associated with infected, necrotic tissue was frequently encountered during sampling. Thus, while the compound list in Table 2 is encouraging, the information associated with peak intensities is also important, i.e., compound abundances, and as such, chemometric approaches are required to prospect these data to identify the chemical differentiators.

Chemometric analysis

Chemometric analysis was utilized as this allows the discrimination of statistically significant differences in the VOC profiles acquired from each sample site. Figure 4 shows the results of PCA of the complete data set (comprising 50 samples). There was no separation observed between the lesion and boundary areas ($p > 0.05$). However, significant differences between the control and lesion areas and between the control and boundary areas were observed (both $p < 0.05$). In addition, it was possible to identify which chemical compounds might be responsible for such separation via comparison of the scoring plot and the corresponding loading plot (see Figure 5). Because chromatograms were used for the data analysis, each chromatographic peak (correlating to a specific chemical compound) was represented by a series of adjacent variables. From the loadings plot, the variable clusters on the extremes were mainly responsible for the separation exhibited in the scores plot whereas those close to the origin had little or no contribution to such separation. Examination of the loading plot revealed six peaks with the highest

Table 2. Tentative identification of the extracted compounds that were unique to each of the sampled sites—this list was derived from mass spectral matches against the NIST data base and is subject to further confirmation

Compounds	Molecular weight	Formula
Lesion		
2-Indazol-2-ylphenylamine	209	$C_{13}H_{11}N_3$
1,4-Methanoazulen-3-ol decahydro-1,5,5,8a-tetramethyl-, [1s-(1.α.,3.β.,3a.β.,4.α.,8a.β.)]-	222	$C_{15}H_{26}O$
Z,Z-2,5-Pentadecadien-1-ol	224	$C_{15}H_{28}O$
E-2-Methyl-3-tetradecen-1-ol acetate	268	$C_{17}H_{32}O_2$
Pentanoic acid, 2,2,4-trimethyl-3-carboxyisopropyl, isobutyl ester	286	$C_{16}H_{30}O_4$
Octadecane, 1-chloro-	288	$C_{18}H_{37}Cl$
2,6-Nonadienoic acid, 7-ethyl-9-(3-ethyl-3-methyloxiranyl)-3-methyl-, methyl ester, [2R-[2.α.(2E,6E),3.α.]-	294	$C_{18}H_{30}O_3$
Z-3-Octadecen-1-ol acetate	310	$C_{20}H_{38}O_2$
.α.-Ethylether of 11-epi-dihydroartemisinin	312	$C_{17}H_{28}O_5$
4-Trifluoroacetoxypentadecane	324	$C_{17}H_{31}F_3O_2$
1,2-Benzenedicarboxylic acid, butyl 2-ethylhexyl ester	334	$C_{20}H_{30}O_4$
Boundary area		
2-Propanol, 1-(2-methoxy-1-methylethoxy)-	148	$C_7H_{16}O_3$
2-t-Butyl-5-propyl-[1,3]dioxolan-4-one	186	$C_{10}H_{18}O_3$
3-Decen-1-ol, (E)-	156	$C_{10}H_{20}O$
E-2-Tetradecen-1-ol	212	$C_{14}H_{28}O$
1-Dodecanol, 3,7,11-trimethyl-	228	$C_{15}H_{32}O$
Myristic acid, 9-hexadecenyl ester, (Z)-	450	$C_{30}H_{58}O_2$
Reference skin site (nonlesion)		
(S)-(+)-1,2-Propanediol	76	$C_3H_8O_2$
1,3-Pentanediol, 2,2,4-trimethyl-	146	$C_8H_{18}O_2$
Cyclodecanol	156	$C_{10}H_{20}O$
5,9-Undecadien-2-one, 6,10-dimethyl-, (E)-	194	$C_{13}H_{22}O$
Tetradecanal	212	$C_{14}H_{28}O$
1-Decanol, 2-hexyl-	242	$C_{16}H_{34}O$
1-Nonadecene	266	$C_{19}H_{38}$
1-Eicosanol	298	$C_{20}H_{42}O$
4-Trifluoroacetoxytetradecane	310	$C_{16}H_{29}F_3O_2$
Octadecanoic acid, 4-hydroxy-, methyl ester	314	$C_{19}H_{38}O_3$
Cyclopropaneoctanoic acid, 2-[(2-pentylcyclopropyl)methyl]-, methyl ester, trans,trans-	322	$C_{21}H_{38}O_2$
2-Trifluoroacetoxypentadecane	324	$C_{17}H_{31}F_3O_2$
Dodecane, 1,2-dibromo-	326	$C_{12}H_{24}Br_2$
Background compounds (field blanks)		
Nonanal	142	$C_9H_{18}O$
Cyclodecanol	156	$C_{10}H_{20}O$
7-Tetradecene	196	$C_{14}H_{28}$
1-Propyl-3,6-diazahomoadamantan-9-ol	210	$C_{12}H_{22}N_2O$
2-Methyl-Z-4-tetradecene	210	$C_{15}H_{30}$
Propanoic acid, 2-methyl-, 3-hydroxy-2,4,4-trimethylpentyl ester	216	$C_{12}H_{24}O_3$
Butylated hydroxytoluene	220	$C_{15}H_{24}O$
2-Hexadecanol	242	$C_{16}H_{34}O$
3-tert-butyl-5-chloro-2-hydroxybenzophenone	288	$C_{17}H_{17}ClO_2$
3-Benzoylmethyl-3-hydroxy-5-nitro-2-indolinone	326	$C_{17}H_{14}N_2O_5$
1,3,5-Tris(trimethylsiloxy)benzene	342	$C_{15}H_{30}O_3Si_3$
3-Isopropyl-6a,10b-dimethyl-8-(2-oxo-2-phenyl-ethyl)-dodecahydro-benzo[f]chromen-7-one	396	$C_{26}H_{36}O_3$

NIST, National Institute of Standards and Technology.

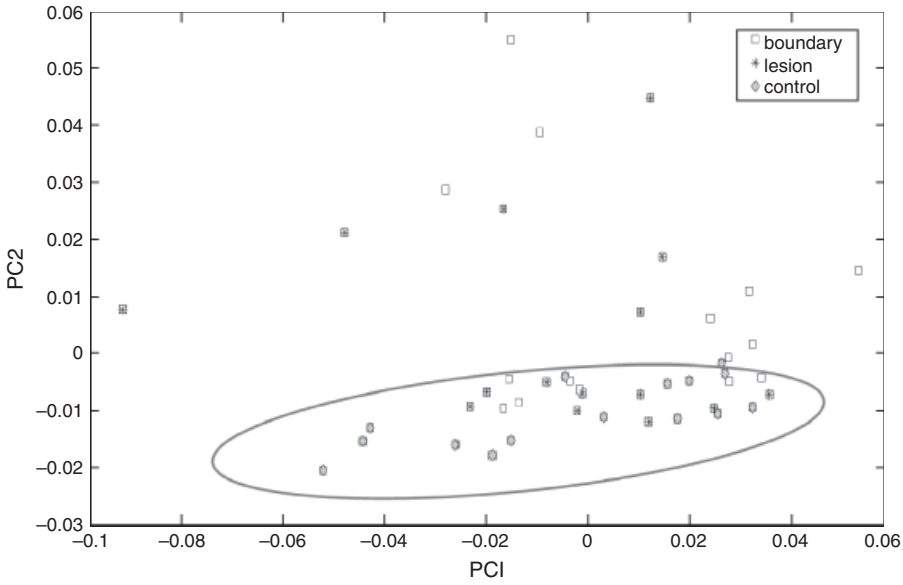

Figure 4. Principal component analysis (PCA) scores biplot of gas chromatography mass spectrometric data from lesion (denoted by asterisk symbol), boundary (denoted by square symbol), and control (denoted by diamond symbol) areas where principal component (PC) 1 and PC 2 exhibit 62.08 and 21.29% of the total explained variance (TEV), respectively. The first two PC scores in PCA were plotted against each other in order to look for underlying trends within the data set, where no obvious patterns were observed for the boundary and lesion areas. However, the reference (control) areas were observed to be localized as a broad cluster at the lower left hand side of the PCA plot (encircled in gray).

variability (see Table 3 for the tentative assignments of these peaks)—the compounds identified in this pilot study are often associated with preservatives typically used in the formulation of creams and gels. However, as the experimental protocol restricted the use of such creams and gels during the experiment and none of the patients reported using such products, the source of these compounds is unclear.

Analysis clearly shows that participant-specific "fingerprints" were identified (see supporting information Table S1). Therefore in the supervised classification, the selected training and test sets had to be based on participants rather than samples in order not to create overoptimistic results. The data from the lesion and boundary samples were combined into a single class, termed the affected class, as the PCA scoring plot showed no clear separation between these samples. A predictive model based on PLS-DA supervised classification was formulated, which gave an average prediction accuracy of 73.3%. When this was compared with the null distribution that showed an average prediction accuracy of 51.1% and when only 122 out of 2,500 simulations ($\sim$4.9%) obtained a better prediction accuracy, it was concluded that our classification results were significant to a confidence level of 95% ($p < 0.05$)

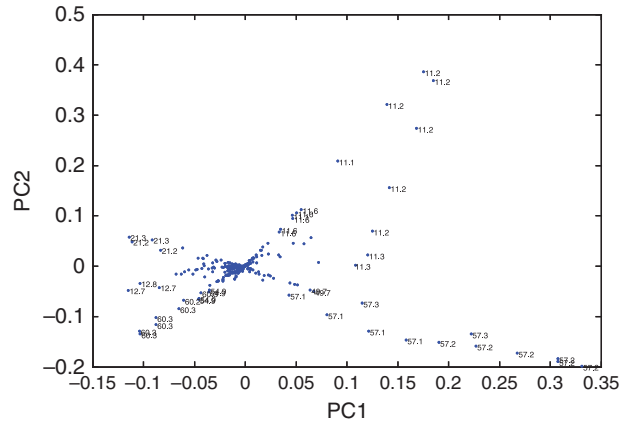

Figure 5. Principal component analysis loadings plot of the reduced chromatographic data. Data points that lay close to origin (zero), have little or no contributions toward separations shown in the scores plot, whereas points that lay further away from the origin (zero) have more significant contributions toward the separations. Because the separation between control and lesion/boundary most appeared in principal component (PC) 2, the variables that show a large diversity in PC 2 are more likely to be the chemicals that differentiate these classes. The numbers represent retention times of the variables, which correspond to one or multiple peaks observed in chromatograms.

Table 3. Tentative identification of unique compounds recovered from the loading plot of the reduced chromatographic data—these compounds were not unique to a single sample area but significantly discriminate between the areas

T_r/minutes	Compound	Molecular weight	Formula
11.20	2-propanol, 1-(1-methyethoxy),	118	$C_6H_{14}O_2$
11.63	Disulfide, dimethyl	94	$C_2H_6S_2$
20.9	3-Carene	136	$C_{10}H_{16}$
21.2	1-Hexanol, 2-ethyl-	130	$C_8H_{18}O$
43.1	Phenol,3,5-bis(1,1-dimethylethyl)-	206	$C_{14}H_{22}O$
43.2	Butylated hydroxytoluene	220	$C_{15}H_{24}O$
43.3	Straight chain hydrocarbon		

They may serve as a focus for future chronic wound VOC analysis.
VOC, volatile organic compound.

Table 4. Summary of partial least square for discriminate analysis (PLS-DA)

	Control vs. lesion/ boundary areas	Lesion vs. boundary areas
Average CCR (%)	73.3	56.67
Minimum CCR (%)	55.56	50
Maximum CCR (%)	88.89	83.33
Standard deviation (%)	14.91	14.91

(Table 4). This shows that the sampling technique generates reproducible, information-rich, complex VOC profiles which, when analyzed with chemometric methods, show statistically significant differences between the affected, i.e., lesion plus boundary areas and the control, i.e., healthy, normal skin areas. The most quantitatively prevalent of these differences were revealed by the loading plot, allowing identification of the key statistically significant differentiating VOC peaks from among the hundreds of compounds delineated through visual characterization alone.

DISCUSSION

The aim of this study was to show for the first time the utility of a novel, noninvasive sampling method for the analysis of chronic wounds and specifically chronic arterial leg ulcers. This was achieved by sampling from three separate areas (the lesion center, a control area of healthy skin and a boundary area between the two) on five individuals with chronic ulcers. VOC profile differentiation was achieved between the control and lesion areas and between the control and boundary areas (to $p < 0.05$) but not between the lesion and boundary areas ($p > 0.05$). VOCs unique to each sampling area were identified, with evidence of VOCs from ingested medication (e.g., Codeine) and secondary to the metabolic processes of colonizing bacteria (e.g., 2-indazole-2ylphenylamine). Although the VOC species cannot be defined as specific biomarkers for wound infection or healing, some may be attributed as the products of reactive oxidative stress while others may be due to preferential absorption of exogenous and ubiquitous environmental contaminants into damaged tissue. Species such as biogenic diamines and thiol compounds may also be related to bacterial degradation of the tissues. Conversely, it may be alterations in the overall VOC profile rather than quantitative analysis of specific VOC species that will give the greatest insight into chronic wound metabolic processes. Overall, it must be noted that it would be premature to definitively attribute the identified compounds to specific wound healing or degenerative processes due to the limited sample size—this would necessitate a larger, precisely described, phenotypically matched cohort of participants.

It is possible to differentiate visually between healthy control skin and diseased lesions but such a technique allows a greater understanding of the chronic wound microenvironment. Each VOC profile reflects the compounds carried to and from the skin within the blood stream, the metabolites of the underlying epidermal and dermal cellular layers, the superimposed metabolites from the normal skin bacterial flora and the environmental VOCs adsorbed at the skin surface. In the case of ulcerated regions, the profiles may be altered by the loss of normal skin cellular layers and their metabolic products and/or by the metabolites associated with the processes of necrosis, healing, and superimposed bacterial infection. Therefore, the technique of VOC profiling can provide a greater insight into the complex processes of skin infection and healing. It may, with further refinement and in conjunction with other existing modalities, become a useful method to assess the lifecycle of chronic wounds, the bioavailability of applied treatments, and in vivo bacterial antibiotic susceptibility. It will also allow for the identification of single VOCs or profiles that could act as biomarkers of specific bacterial wound infections. However, with specific reference to arterial ulceration, while this technique yields extensive chemical information regarding processes occurring at the local skin level that may be used in the assessment of infection and healing, its application in the planning of vascular interventional procedures is likely to remain adjunctive to imaging modalities.

The benefits of such a technique include its noninvasive, painless nature, its accuracy and reproducibility and the fact that it offers the possibility of rapid point-of-care testing at the bedside. At present, however, the laboratory equipment would need further optimization and miniaturization to allow this to occur. In addition, aspects of the sample processing are as yet not fully automated and thus it remains labor intensive. There are currently no VOC libraries available that are specific to chronic wounds with which to rapidly identify the sampled VOCs; certainly this will change with further development in this field of research.

This work has been successful in proving the applicability of the VOC sampling technique in a clinical setting with specific reference to the assessment of chronic wounds. The sample size investigated was adequate to allow chemometric analysis to be undertaken in order to show both the significance of the results in differentiating between VOC profiles and the reproducibility of the method. Despite this, future work would aim to extend the scope of sampling to a larger number of participants with a variety of chronic wounds in individuals of different ethnicities.

Research into the efficacy of VOC analysis in a medical setting has shown promise in a variety of conditions including lung carcinoma,[37] asthma,[38] aerodigestive tract carcinoma,[39] pulmonary tuberclosis,[40] hyperglycemia,[41] heart transplant rejection[42]), gastrointestinal disease (ulcerative colitis, *Clostridium difficile* and *Clostridium jejuni* infections),[43] bacteremia,[44] bacterial vaginosis,[45] and in the detection of an array of microbes. Few studies of wound infection have been undertaken, with those to date utilizing "electronic nose" machines[46,47] rather than GC-MS.

Chronic wounds have a major effect on both morbidity and mortality worldwide but currently are only assessed by dated, invasive methods (i.e., swabbing and biopsy). These methods do allow for an accurate identification of infecting organisms but take no account of the complex interdependent relationship between those cultured bacteria and an individual's genetic susceptibility or response to such an infection. Research has indicated that while bacterial density at the wound surface is independently predictive of wound nonhealing, this view is overly simplistic; bacterial

diversity, microbial synergistic interactions, and the underlying host response all play a role,[16,18,20] the effects of which could be assessed via VOC analysis.

The application of this technique to chronic wounds is still in its infancy but shows great promise. Future work will involve compiling a library of wound bacterial VOCs both in vitro and in vivo with the eventual identification of biomarkers or VOC fingerprints by which future noninvasive diagnosis of wound infection and etiology could be achieved. Another focus of substantial future work will be an investigation into the chronological VOC changes within healing and nonhealing chronic wounds and an analysis of the effect of ingested medications, e.g., antibiotics, on the VOC profiles of such wounds. The ultimate aim will be to minimize the equipment to allow hand-held bedside VOC analysis that would allow rapid, cheap, and painless analysis of chronic wounds and other skin conditions.

In conclusion, this preliminary study has shown how VOC profiles of chronic human skin lesions may be sampled and studied. The data processing methods applied to this study reliably differentiated control profiles from boundary profiles and control profiles from lesion profiles. The methodology has been shown to yield reproducible data from complex, previously intractable sampling environments. The development and extension of this approach may be appropriate for use in the future clinical evaluation of ulcers, wounds, and other skin lesions. An important next step in this area will be the creation of a phenotypically matched library of volatile skin metabolites.

ACKNOWLEDGMENTS

Our gratitude goes to the study participants and nursing staff of South Manchester University Hospitals NHS Trust. S.R. would like to thank Unilever Research and Development, Port Sunlight for their continued assistance throughout this research. Y.X. and R.G. are grateful to the EU project Biotracer for financial support. Final thanks must go to Ian Fleet and Eric Warburton of the Centre for Instrumentation and Analytical Science. The authors state no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Summary of instrumentation parameters.

Fig. S1. Distance heat map plot. The distance between each pair of samples are represented by a colour as indicated by the colour bar on right (red to blue). The higher the distance, the lower (bluer) the similarity between two samples. The 5 different subjects, each subject has 3 samples from each class, respectively, were labeled by numbers from 1–5 and samples from the sample class (e.g., Boundary, Control or Lesion) were placed together. Since each subject has 3 samples for each class, the 3×3 blocks in the diagonal of the picture represent the similarities between the samples from the same subject and also the same class which thereby show the reproducibility of the sampling methodology.

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