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**WOUND REPAIR
AND
REGENERATION**

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Key Words:	volatile organic compound, skin, infection, cutaneous wounds

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**Volatile organic compound detection as a potential means of
diagnosing cutaneous wound infections**

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Abstract

Chronic cutaneous wound infections and surgical site infections (SSIs) present a huge burden on the healthcare system and can lead to increased morbidity and mortality. Current diagnostic methods of identifying and confirming infection involve culture-based and molecular methods. Both techniques are time consuming and delays commonly lead to untargeted empirical treatment. An ideal diagnostic method would be non-invasive and highly sensitive and detect pathogenic organisms with a high degree of accuracy in order to allow targeted treatment. Volatile organic compounds (VOCs) are a diverse group of carbon-based molecules produced and released by humans and microorganisms. VOC detection has the potential in aiding cutaneous wound infection diagnostics using non-invasive and time-efficient methods. This review provides a comprehensive update on VOCs produced and emitted by bacteria commonly associated with chronic wounds and SSIs. VOC sampling has the advantage of being painless, time-efficient, non-invasive and reproducible. VOCs emitted by these organisms are diverse. *In vitro* studies have identified potential signature volatile profiles, which can be used in detecting these microorganisms. Combining these profiles with volatile profiles emitted from acute, chronic and surgical wounds *in vivo* could potentially allow identification of bacterial-specific VOCs. VOC detection has the potential for a relatively inexpensive, portable, non-invasive and reliable clinical diagnostic tool, which could be used in detecting cutaneous wound infections and guiding their optimal management.

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Introduction

Chronic cutaneous wounds occur as a result of deficiencies in wound healing processes ^{1, 2}. Chronic wounds have a major impact on patient’s quality of life and present a huge burden on the healthcare system ^{3, 4}. Persistent cutaneous wound infection is a major contributor to delayed wound healing ^{5, 6}. Cutaneous infection following post-surgical intervention is also of clinical concern. Surgical site infections (SSIs) are common complications after surgery and can lead to increased morbidity and mortality ^{7, 8}. Common bacterial pathogens associated with chronic as well as superficial and deep SSIs include *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Streptococcus pyogenes* and *Enterococcus faecalis* ⁹⁻¹². Current diagnostic methods of identifying and confirming cutaneous infections involve culture-based and molecular methods. Both techniques are time consuming, and culture-based methods have limited sensitivity and are susceptible to over-estimation of skin commensals leading to incorrect causative bacterial identification ¹³⁻¹⁵. Delays in diagnostics often result in the use of untargeted empirical treatment with a risk for sub-optimal choice of antibiotics, the development of antibiotic resistance and increase in mortality ¹⁶. An ideal diagnostic method would be non-invasive and highly sensitive and detect pathogenic organisms with a high degree of accuracy in order to allow targeted treatment.

Volatile organic compounds (VOCs) are a diverse group of carbon based molecules, including alcohols, isocyanates, ketones, aldehydes, hydrocarbons and sulphides, which are volatile at ambient temperatures ^{17, 18}. VOC sampling has the advantage of being painless, non-invasive and reproducible. A compendium of VOCs emanating from the human body has been compiled with 1840 VOCs assigned from breath (872), saliva (359), blood (154),

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7 milk (256), skin secretions (532) urine (279), and faeces (381) ¹⁹. There is increasing
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9 evidence that VOCs or combinations of VOCs are unique to various disease states and their
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11 early detection could represent a useful means of diagnosis. VOCs have been identified as
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13 potential biomarkers in malignancies of the lung ²⁰, stomach ²¹, head and neck ²², breast ²³,
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15 liver ^{24, 25} colon and prostate ²⁶. They have also been detected as markers of asthma ²⁷⁻²⁹,
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17 chronic obstructive pulmonary disease ^{30, 31}, inflammatory bowel disease ^{32, 33} and diabetes ³⁴,
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19 ³⁵. Microorganisms produce and release VOCs and currently volatile detection via breath
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21 testing has been at the forefront in the potential of this technology to diagnose infection ³⁶.
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23 The ability to identify VOCs in cutaneous wound infections could lead to a non-invasive and
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25 time efficient method of diagnosis.
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30 The aim of this review is to provide a detailed update on VOCs produced and emitted by
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32 bacterial species commonly associated with cutaneous wound infections. It then describes the
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34 volatile profile of skin in health and disease. Finally, the role of volatile detection from
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36 cutaneous wounds is discussed. An extensive literature search was conducted on PubMed for
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38 relevant articles published from 1960 onwards. MeSH terms used included a variety of
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40 combinations including: SSI, surgical wound infection, VOCs, biofilm, *staphylococcus*,
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42 *Escherichia coli*, *streptococcus*, *enterococcus*, *Pseudomonas aeruginosa*, gram positive
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44 bacteria, gram negative bacteria, wound infection, post-operative wound infection, wound
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Chronic wounds and SSIs

Chronic wounds represent a major burden on patients with the National Health Service expenditure in managing these wounds in excess of £1 billion annually³. Biofilms are present in more than half of chronic wounds and present a major obstacle in healing³⁷. Over 50 million in-patient surgical procedures are performed in the USA annually with approximately 1-5% developing SSIs with a high associated mortality^{38, 39}. SSIs pose a significant burden on the healthcare system leading to increase postoperative in-patient stay, increased expenses and readmissions. The preliminary management of SSIs involves preventative measures which include but are not limited to peri-operative antibiotics and meticulous skin preparation prior to the procedure. However, despite these measures SSIs represent the most common hospital acquired infections in surgical patients⁴⁰. Risk factors associated with SSIs include patient and operative factors. Patient factors include diabetes, obesity, smoking and pre-operative active infection⁴¹⁻⁴³. Operative factors include wound characteristics which can be classified into four categories, namely, clean, clean-contaminated, contaminated and dirty-infected⁴⁴. An escalating risk of developing SSIs is linked to the level of wound characterisation ranging from 1.3% for clean wounds to 40% for dirty-infected wounds^{45, 46}. Other operative factors include preoperative showering and hair removal, hand-washing and surgical attire, and intra-operative skin preparation⁴⁷.

Planktonic bacteria undergo a four phase cycle of growth consisting of a lag phase, log phase, stationary phase and death phase. Biofilms are physiologically distinct from their planktonic counterparts and undergo a different developmental cycle, which involves initial attachment to the surface, irreversible attachment, maturation and dispersion⁴⁸. Biofilms are more prevalent than expected in surgical wounds with up to 80% of SSIs involving the presence of

biofilm⁴⁹. They are difficult to diagnose with culture methods proving obsolete in their detection⁵⁰. Biofilms have been found on closure materials of healed surgical wounds⁵¹ and on the surface of implanted surgical devices⁵². Biofilms are significantly more difficult to eradicate leading to a chronic inflammatory state which further negatively impacts wound healing⁵³ and persistence of infection⁵⁴. Management of SSI associated biofilms requires invasive management involving surgical debridement of devitalised tissue, removal of infected devices and parenteral broad-spectrum antimicrobials⁵⁵⁻⁵⁷. This further highlights the need for rapid and accurate diagnostic tools in order to avoid a delay in optimum management.

SSIs are classified into three categories: superficial incisional where the infection occurs around the area of the skin where the incision was made; deep incisional where the infection occurs deep to the incision effecting the underlying fascia and muscle; and organ/space where the infection effects the organs or body cavities⁴⁴. We propose the potential use of volatile detection as a means of diagnosing SSIs would be limited to superficial SSI and potentially deep SSI as these would allow the non-invasive capture of VOCs. The most typical causative micro-organisms of superficial and deep SSIs are pathogens native to the patient's skin which include *staphylococci*, *streptococci*, *enterococci* and gram negative *bacilli*⁴⁷.

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Microorganisms and VOCs

A portion of normal microbial metabolites produced are VOCs⁵⁸. They are thought to evolve as products or by-products of metabolic pathways⁵⁹. Zoller and Clark were the first to report laboratory evidence of bacterial production of volatiles⁶⁰. Many classification systems exist for microbial volatiles with one potential system categorising them ~~Bacterial volatiles can be classified~~ into fatty acid derivatives, aromatic compounds, nitrogen-containing compounds, sulphur compounds, terpenoids, halogenated selenium, tellurium and other metalloid compounds⁶¹. Their production is dependent on the bacterial species, bacterial strains, and its bacterial growth phase, co-cultures, pH, humidity, nutrients ~~and~~, temperature and other environmental factors^{62, 63}. The ability to rapidly sample and analyse VOCs, potentially allowing identification of bacteria, has massive implications in the management of infection in clinical settings.

VOC sampling and analytical techniques~~VOCs produced by microorganisms commonly associated with wound infection~~

There are numerous sampling ~~(figure 1)~~ and analytical ~~(figure 2)~~ techniques that have been utilised in order to collect and identify VOCs, the advantages and limitations of which are outlined in Figure 1s ~~produced and emitted by microorganisms. These include VOC collection onto adsorbents, such as Tenax or solid-phase micro-extraction (SPME) ⁶⁴, either directly or through an intermediary such as gauze or cotton pads ⁶⁵, collection in sealed containers ⁶⁶, direct or indirect solvent extraction ⁶⁷, or combinations of the above. The above methods do not often identify all VOCs present due to selective adsorption of the trapping material and these sampling techniques limit accurate quantification of VOCs and therefore direct sampling are utilised ⁶⁸.~~

~~This section details the VOCs identified from the common causative bacteria of wounds based on the different sampling and analytical techniques available.~~

~~Studies have~~ There are a ~~used a~~ variety of ~~methodological and~~ analytical chemical techniques used in VOC detection (Figure 2). Gas chromatography mass spectrometry (GCMS) is a combined analytical system and is one of the most widely used and powerful analytical chemical methods. It detects and quantifies VOCs over a range from parts per million to part per billion or less. Samples for GCMS must be in the gaseous phase where they are injected into the chromatograph where an inert carrier gas transports it through a tubular column. The length of the columns used varies with longer columns providing better chromatographic resolution and maximum separation of analytes. Each compound is emitted from the column at different times with the mass spectrometer used to detect eluting compounds through compound ionisation followed by measurement of the mass to charge ratio of each ion

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generating a unique mass spectrum for the compound ⁶⁹. The major advantages of GCMS are increased sensitivity and compound identification is greatly facilitated by the availability of extensive and easily searchable databases ⁷⁰. However, it is not typically strong at identifying unknown compounds. Other limitations of GCMS are increased processing and analysis times and similarity of molecular fragmentation patterns from structural isomers can make compound identification difficult ⁷¹. Also, due to high detection limits, sample pre-concentration using techniques described above are typically required, making quantification of VOCs very difficult. A time of flight (TOF) mass spectrometer serves a very similar purpose to GCMS with the exception of providing a combination of high mass accuracy and extended range of detectable metabolites ⁷².

Ion mobility spectrometry (IMS) separates gas-phase ions based on their size and shape ⁷³. This technology has long been used in the detection of explosives and illegal substances ⁷⁴. It can be coupled to a pre-separating multi capillary column (MCC) unit allowing two-dimensional volatile separation, significantly increasing the resolution of metabolites ⁷⁵. MCC allows minimisation or avoidance of analyte interactions in the ionisation region of the IMS, thus reducing the complexity of the measurement signal ⁷⁶. IMS is advantageous over GCMS as ambient air can be used as the carrier gas, eliminating the need for a pure speciality inert gas or a vacuum. This has allowed IMS to be portable and provide on-site measurements. IMS is highly sensitive with very low detection limits between the parts per billion to parts per trillion ranges, offers excellent low detection limits and gives relatively rapid results ⁷⁷. IMS is however not suitable for identification of unknown compounds.

Direct injection mass spectrometry methods include ion molecule reaction mass spectrometry (IMR-MS), secondary electrospray ionisation mass spectrometry (SESI-MS), selected ion flow tube mass spectrometry (SIFT-MS) and proton transfer reaction mass spectrometry (PTR-MS). A drawback of conventional mass spectrometry instrumentation is the high fragmentation of molecules in a complex gas mixture secondary to high electron ionisation leading to complex mass spectra with overlapping intensities, thus making quantification difficult or impossible. IMR-MS offers a very soft form of ionisation leading to less fragmentation. SESI-MS utilises electrospray ionisation technique in order to produce analytes suitable for mass analysis. In PTR-MS, compounds are ionised via proton transfer prior to analysis. This involves the production of H_3O^+ ions from high purity distilled water through a cathode ion source. SIFT-MS is similar to PTR-MS but is able to ionise a wider range of analytes as it uses a greater number of precursor ions (H_3O^+ , NO^+ or O_2^+) for chemical ionisation⁶⁹. The major advantage of direct injection methods is quantification. These direct methods also have lower detection limits than GCMS at parts per trillion ranges. They also provide real-time detection with no sample preparation or separation requirements. They are however limited in the range of VOCs detected compared to GCMS and PTR-MS is limited to the identification of compounds with a higher proton affinity than water.

The electronic nose devices constitute a non-invasive technique capable of detecting and differentiating VOC patterns based on its ability to detect odour. The Cyranose 320 and E50835 electronic nose devices have been used in bacterial identification. These devices allow imprinting of an odour on its sensor chip micro-array which is composed of complex materials. When the sensors are exposed to a gas, the polymer absorbs the gas and swells, during which the distance between the conductive carbon particles increases and thus also increases the resistance of the sensor material⁷⁸. This change in resistance is transmitted to a

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computer with the pattern of change in the sensor array being used to detect the gas. Electronic nose devices are powerful at differentiating between non-identical samples, provide rapid results with on-site sampling. They are however limited due to temperature and humidity sensitivity, only detect patterns of VOCs programmed on their database and are not suitable for screening of unknown compounds.

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~~, therefore using very specific search criteria were abandoned as this would have led to exclusion of a large proportion of studies. With regards to VOC quantities, studies varied with some presenting relative abundances whereas others identified absolute concentrations of compounds and therefore quantitative analysis was not possible. The majority of studies have provided VOC abundances relative to other compounds identified (79-84). Studies where absolute abundances of VOCs are shown range from the parts per million volume to parts per trillion volume (85-87). Parts per million volume concentrations equate to µg/l sampled, parts per billion volume concentrations equate to ng/l sampled and parts per trillion volume concentrations equate to pg/l sampled. Studies in which more than 5 VOCs were identified for a specific bacterial species, only the top 5 compounds based on abundance (either relative or absolute) are presented (table 1). Studies where VOC production could not be attributed to an isolated bacterial species or strain were excluded.~~

~~Informatics approaches also varied between the studies evaluated. Four main methods were used to confirm identification of VOCs. Some studies compared mass spectra to reference libraries and databases, such as the National Institute of Standards and Technology (NIST) (83, 88), Wiley (89) and Massbank (90) libraries and pre-determined reference databases (91). Other studies compared mass spectra and peak retention times with those obtained from pure standard compounds (82, 85, 92). The majority of studies combined the use of reference~~

libraries and pure standard compounds for identification of VOCs (79, 81, 86, 87, 89, 93-97). A third technique used was the manual comparison of mass spectra with those available in the literature (94). Lastly, due to the limitations of certain analytical techniques in compound identification, concurrent techniques were utilised to confirm VOC identity (76, 98-101).

VOCs produced by microorganisms commonly associated with wound infection

This section details the VOCs identified from the common causative bacteria of wounds based on the different analytical techniques available. With regards to VOC quantities, based on analytical technique used studies varied with some presenting relative abundances whereas others identified absolute concentrations of compounds. The majority of studies have provided VOC abundances relative to other compounds identified⁷⁹⁻⁸⁴. Studies where absolute abundances of VOCs are shown range from the parts per million volume to parts per trillion volume⁸⁵⁻⁸⁷. Parts per million volume concentrations equate to µg/l sampled, parts per billion volume concentrations equate to ng/l sampled and parts per trillion volume concentrations equate to pg/l sampled. Studies in which more than 5 VOCs were identified for a specific bacterial species, only the top 5 compounds based on abundance (either relative or absolute) are presented (table 1). Studies where VOC production could not be attributed to an isolated bacterial species or strain were excluded.

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~~Gas chromatography-mass spectrometry (GCMS)~~

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~~GCMS has been the main analytical method utilised to identify VOCs.~~ Neerincx et al
inoculated *P. aeruginosa* in brain heart infusion broth and sampled the headspace using ~~glass~~
~~tubes filled with~~ Tenax TA at 16, 24 and 48 hours⁹³. The top 5 VOCs emitted from *P.*
aeruginosa cultures were methyl thiolacetate, 2,3-dimethyl-5-isopentylpyrazine, 2-methyl-3-
(2-propenyl)-pyrazine, 3-methyl-1 H-pyrrole and 6-tridecane. Scholler et al sampled volatile
metabolites emitted from *P. aeruginosa* ~~cultured in Autoinducer Bioassay medium~~ by
diffusive sampling ~~again using using stainless steel analytical thermal desorption tubes~~
~~packed with mesh~~ Tenax⁸⁹. The major headspace VOC identified was dimethyl disulphide
~~with isoprene, dimethyl trisulphide and 1-undecene also detected.~~ Bean et al were the first to
report the application of two-dimensional GC-TOF spectrometry to identify volatiles of *P.*
aeruginosa grown for 24 hours in lysogeny broth in sealed GC headspace vials via SPME
passive sampling⁸⁸. This method facilitated the identification of 56 VOCs emitted by *P.*
aeruginosa of which 28 were new including alcohols, heteroaromatics, ketones, benzenes and
aldehydes⁸⁸. Scott-Thomas et al used similar SPME sampling of the headspace of *P.*
aeruginosa and found high concentrations of 2-aminoacetophenone⁸⁴.

Saranya et al identified 2-[3-acetoxy-4,4,14-trimethylandroster-8-en-17-yl] propanoic acid was specific to *Staphylococcus* ~~when cultured in tryptone soya broth both in the culture and headspace (silica-coated discs)~~⁸⁰ and ~~Five VOCs namely~~ 3-methyl-1-butanol, 2-methylbutanal, 3-methyl-1-butanol, 3-methylbutanoic acid and 2-methylbutanoic acid have been found to be significantly expressed in the headspace of *S. epidermidis* ~~cultured in blood agar compared to controls~~ using the purge and trap technique ~~to collect the volatiles in glass cartridges containing Tenax TA~~⁷⁹.

Indole is produced by both pathogenic and non-pathogenic strains of *E. coli* cultured on a mixture of trypticase soy agar and brain heart infusion broth⁸⁶. ~~This was demonstrated by analysing the headspace of these cultures using a Super-Q porous polymer trapping method (86).~~ Bianchi et al inoculated commercial tins of peeled tomatoes with *E. coli* and found and collected headspace gas via a glass tube trap filled with Tenax TA at day 2 and 7 (81). ~~They found~~ the most abundant compounds detected were dimethylsulfide, 6-methyl-5-hepten-2-one, ethanol, ethyl acetate and 3-methyl furan⁸¹. Umber et al identified differing volatile signatures of *E. coli* dependant on the environment. Headspace analysis revealed *E. coli* inoculated whole blood released a different set of VOCs compared to *E. coli* cultured in Luria Bertani (LB) broth⁸⁵. VOCs specific to *E. coli*-infected whole blood were dimethyl sulfide, carbon disulphide, ethanol, acetaldehyde and methyl butanoate. Whereas dimethyl disulfide, dimethyl trisulfide, methyl propanoate, 1-propanol and methylcyclohexane were isolated only from *E. coli* cultured in LB broth⁸⁵.

Filipiak et al cultured *S. aureus* and *P. aeruginosa* in tryptic soy broth medium and collected ~~the~~ headspace samples on multi-bed sorption tubes ~~at differing time intervals for up to 28~~

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~~hours~~⁸⁷. *S. aureus* released 32 VOCs and *P. aeruginosa* 37 of diverse chemical classes ~~at different concentrations~~ comprising aldehydes, alcohols, ketones, acids, sulphur containing compounds, esters, hydrocarbons and nitrogen containing compounds. They found distinct differences in the bacteria-specific VOC profiles, especially with regard to aldehydes ~~(2-methylpropanal, acetaldehyde, 3-methylbutanal, (Z)-2-methyl-2-butenal)~~, which were ~~released-observed~~ by *S. aureus* with no release observed from *P. aeruginosa*⁸⁷. Preti et al sampled the headspace of *P. aeruginosa* and *S. aureus* cultured in blood agar medium in petri culture dishes and identified compounds specific to each species⁹⁴.

Zscheppank ~~(82)~~ et al utilised a novel needle trap technique consisting of a sorbent packed needle ~~novel technique of needle trap~~ to sample ~~the~~ headspace of *E. coli* and *P. aeruginosa* cultured in liquid media ~~over a 48 hour period. Needle trap is an extraction device that contains a sorbent packed inside of a needle~~¹⁰² and found. They ~~found~~ both organisms produced isoprene. ~~Dimethyl sulphide, 1-undecene and 2-nonanone were specific to *P. aeruginosa* and *E. coli* produced carbon disulfide, butanal and indole~~⁸².

Elgaali et al utilised purge and trap and passive SPME sampling of the headspace to analyse *E. coli* and *S. aureus*⁹⁵. Both sampling techniques identified indole as the principle component over the headspace of *E. coli* and the purge and trap technique identified long chain alcohols, 2-methylbutanol and 3-methylbutanol in abundance, whereas, passive SPME sampling of the headspace identified 2-tridecenone and dimethyldisulfide over the headspace of *S. aureus*⁹⁵.

~~Gas chromatography time-of-flight mass spectrometry (GC-TOF-MS)~~

~~A time-of-flight mass spectrometry measures the mass dependent time it takes ions of different masses to move from the ion source to the detector. It allows rapid detection and analysis of a wide range of smaller molecules (103).~~ Boots et al sampled the bacterial headspace, using desorption tubes packed with carbograph 1TD/Carbopack X, of *S. aureus*, methicillin-resistant *S. aureus* (MRSA), *P. aeruginosa* and *E. coli* cultured in a variety of agars and broths⁸³. Samples were analysed by GC-TOF-MS. They identified 25 VOCs which could be used to discriminate between the bacterial strains. They also found 1,1,2,2-tetrachloroethane, 2-heptanone and 1,4-dichlorobenzene are differentially excreted in the headspace of *S. aureus* and MRSA, with the latter two compounds significantly more abundant over the headspace of MRSA⁸³.

~~SPME-GCMS~~

~~SPME is a simple and efficient solvent-free sample preparation method that integrates sampling, extraction, concentration and sample introduction of VOCs into a single step (104). Bean et al were the first to report the application of two-dimensional gas chromatography (GC×GC) time-of-flight mass spectrometry to identify volatiles of *P. aeruginosa* grown for 24 hours in lysogeny broth utilising solid-phase microextraction (SPME) headspace sampling (88). GC×GC are commonly made up of two columns which separate sample compounds related to their relative volatility and polarity (105). The additional chromatographic~~

dimension improves spectral purity improving the detection of low abundance volatiles. This method facilitated the identification of 56 VOCs emitted by *P. aeruginosa* of which 28 were new including alcohols, heteroaromatics, ketones, benzenes and aldehydes (88). Scott Thomas et al found specific to *P. aeruginosa* cultured in sheep blood agar, high concentrations of 2-aminoacetophenone in the headspace sampled using SPME at 24 hours (84). Preti et al sampled the headspace of *P. aeruginosa* and *S. aureus* cultured in blood agar medium in petri culture dishes (94). Characteristic compounds of *S. aureus* in order of abundance were isovaleric acid, 2-methylbutyric acid, isobutyric acid, 1-hydroxy-2-propanone, 3-hydroxy-2-butanone, butyric acid, 4-methylhexanoic acid and 2-phenylethyl alcohol. For *P. aeruginosa*, signature VOCs detected were 2-Amino-acetophenone, dimethyldisulfide, undecene, dimethylpyrazine and dimethylsulfide (94). Elgaali et al utilised two collection methods (porous polymer Super Q and SPME) to analyse the headspace of *E. coli* and *S. aureus* cultured in tryptic soy agar and broth (95). Both sampling techniques identified indole as the principle component over the headspace of *E. coli* and the porous polymer trapping technique identified long-chain alcohols, 2-methylbutanol and 3-methylbutanol in abundance. SPME sampling identified 2-tridecenone and dimethyldisulfide over the headspace of *S. aureus* (95). Two studies have assessed the VOCs emitted in the headspace of sputum samples using SPME-GCMS (106, 107). These however did not isolate the bacterial strains and are therefore not discussed further.

Multi-capillary column-coupled ion mobility spectrometry (MCC-IMS)

IMS is a portable, sensitive and time-efficient instrumental analytical technique. IMS provides characterisation of organic and inorganic compounds based upon ion-mobility or size-to-charge ratio rather than mass-to-charge ratio (73). It can detect very low

concentrations of compounds without any pre-concentration; however it is not as powerful as GCMS in separating and identifying metabolites (108). Maddula et al utilised MCC-IMS to identify three VOCs namely, ethanol, heptan-2-one, and nonan-2-one emitted from the headspace of *E. coli* cultures which were cross validated with SPME-GCMS⁷⁶. Guaman et al evaluated IMS and SPME-GCMS in the detection of VOCs in the breath of rats injected intraperitoneally with *E. coli* or regular saline after 24 hours and found (98). They identified IMS had a higher sensitivity and specificity than SPME-GCMS in distinguishing the two groups based on volatile analysis⁹⁸. Kunze et al analysed using MCC-IMS the headspace of *E. coli* and *P. aeruginosa* cultured in Lysogeny Broth at four time points (91) and found - They found six VOCs (Decan-1-ol, Ethanol, Indole, Octan-1-ol [monomer and dimer] and P_755_105) exclusively over the headspace of *E. coli* cultures and 7 VOCs (2-Propanone, Azane [dimer], Dodecane, 2-Ethylhexan-1-ol, P_603_25, P_648_36 and P_778_4) emitted exclusively by *P. aeruginosa* cultures, with their concentrations differing across the time points analysed⁹¹.

Direct injection mass spectrometry Ion molecule reaction mass spectrometry (IMR-MS)

IMR-MS provides a highly sensitive method for online and off-line sampling of VOCs (109). It uses a non fragmenting chemical ionisation mode (110) and is extremely time efficient.

Only a single study to date has utilised IMR-MS to analyse the headspace over *E. faecalis*, *S. aureus* and *S. epidermidis* cultured in blood agar and brain heart infusion broth⁹². They were able to differentiate between the species based on the mass spectra generated from the VOCs.

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Secondary electrospray ionisation mass spectrometry (SESI-MS)

~~SESI-MS has the ability to directly sample ambient gas and provide real-time detection and analysis of VOCs (68).~~ Zhu et al were the first to report the application of SESI-MS to the detection and characterisation of VOCs produced by *P. aeruginosa*, *S. aureus* and *E. coli* cultured in tryptic soy broth ⁹⁰. They identified *P. aeruginosa* emitted ethanol and 4-Methylphenol in high abundance; *S. aureus* emitted high concentrations of butanol, acetone and acetic acid; *E. coli* principle emitted volatile was indole. A follow on study by the same group were able to distinguish 11 strains of *E. coli* from *S. aureus* based on VOCs ¹⁰³. In addition they identified 6 VOC biomarkers that were common in all *E. coli* strains.

Selected ion flow tube mass spectrometry (SIFT-MS)

~~SIFT-MS is rapid, has a high sensitivity for volatile detection and can detect small molecules not readily detectable by GCMS (108). It also provides quantification of VOCs and can monitor varying compound levels in real-time (112).~~ Carroll et al cultured different strains of *P. aeruginosa* on blood agar and pseudomonas-selective media and analysed the headspace using SIFT-MS ¹⁰⁴. They identified the majority of strains emitted high quantities of ammonia and hydrogen cyanide compared to controls. Shestivska et al corroborated the latter findings using both ~~SPME~~-GCMS and SIFT-MS ⁹⁶. They also identified a second compound, methyl thiocyanate, is commonly emitted from the majority of *P. aeruginosa* strains and most probably both compounds are biochemically associated. A follow on study by the same group identified differing production rates of VOC by genotypically different strains of *P.*

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7 *aeruginosa*⁹⁷, which may allow differentiation between not only bacterial species but also
8 strains of the same organism. Gilchrist et al identified elevated concentrations of hydrogen
9 cyanide in the breath of patients with chronic *P. aeruginosa* infections compared to controls
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12¹⁰⁵. However, it must be noted that the patients and controls may have been colonised by
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14 other bacteria.
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20 Allardyce et al inoculated blood culture bottles, supplemented with tryptic soy broth, with
21 isolated strains of *P. aeruginosa*, *S. aureus* and *E. coli* and analysed emitted VOCs using
22 SIFT-MS at 6 hours¹⁰⁶. Nine VOCs in total were assessed to differentiate between the
23 bacterial strains. *P. aeruginosa* cultures had relatively high absolute concentrations of acetic
24 acid and acetone; *E. coli* ethanol and acetaldehyde; and *S. aureus* ethanol and acetone¹⁰⁶.
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26 Storer et al used SIFT-MS to measure volatiles emitted from the headspace of urine samples
27 inoculated with *P. aeruginosa*, *S. aureus*, *S. epidermidis*, *E. coli* and *E. faecalis* at 6 hours¹⁰⁷.
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29 The principle VOC emitted compared to non-inoculated samples was formaldehyde for *E.*
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The principle VOC emitted compared to non-inoculated samples was formaldehyde for *E.*
coli, ethanol for *P. aeruginosa* and ammonia for *S. epidermidis*, *S. aureus* and *E. faecalis*. In
contrast, Sovova et al found the principle volatile emitted from the headspace of *E. coli*
cultured in nutrient broth enriched with glucose was ethanol¹⁰⁸. This could be explained by
the use of different media, strains and growth conditions between the studies. This
assumption is confirmed by Chippendale et al who analysed the VOC from the headspace of
E. coli cultured in two different media¹⁰⁹. *E. coli* cultured in Dulbecco's modified Eagle's
medium resulted in large amounts of ethanol, acetaldehyde and hydrogen sulphide
production, whereas *E. coli* cultured in lysogeny broth, ammonia was the major volatile
compound¹⁰⁹.

Proton transfer reaction mass spectrometry (PTR-MS)

~~PTR-MS uses protonated water as a chemical ionisation reagent to measure volatiles. It is a fast technique providing real time analysis (119). However, it is not as powerful as GCMS in separation and identification of volatiles and not all molecules are detectable.~~ Lechner et al ~~described the use of~~ PTR-MS to analyse the headspace ~~of of bacterial cultures. They cultured~~ *E. coli*, *P. aeruginosa* and *S. aureus* ~~cultured~~ in either MacConkey agar or Mannitol-salt agar ~~24 hours and analysed the headspace for signature volatiles~~⁹⁹. They identified patterns which were specific to the bacterial species however did not expand on the identity of the VOCs. Using PTR-MS, O'Hara and Mayhew analysed the headspace of *S. aureus* cultured in three different broths (nutrient, dextrose and brain heart bovine) and found although the VOCs emitted were specific to *S. aureus*, their concentrations differed dependant on the type of media they were grown in¹⁰⁰. Both Luchner et al¹⁰¹ and Bunge et al¹¹⁰ have utilised PTR-MS to identify volatiles in the headspace of *E. coli*.

Electronic nose

~~The electronic nose constitutes a non-invasive technique capable of detecting and differentiating VOC patterns based on its ability to detect odour. The Cyranose 320 and E50835 electronic nose devices have been used in bacterial identification. These devices allow imprinting of an odour on its sensor chip array which is composed of complex materials. When the sensors are exposed to a gas, the polymer absorbs the gas and swells, during which the distance between the conductive carbon particles increases and thus also increases the resistance of the sensor material (78). This change in resistance is transmitted to~~

~~a computer with the pattern of change in the sensor array being used to detect the gas.~~ The
Cyranose 320 and E50835 electronic nose devices have been utilised to differentiate
between *P. aeruginosa*, *S. aureus* and *E. coli* isolates based on VOC patterns from the
headspace^{81, 111-113}. However, it does not provide details regarding the individual volatile
compounds emitted by the bacteria.

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Skin and VOCs

Skin is the largest human organ ¹¹⁴ and forms an essential barrier between the body and the environment, protects from injury and provides vital homeostatic mechanisms including control of temperature, maintenance of fluid balance, and detection of sensations such as pain ¹¹⁵. A compilation of 1840 VOCs emitted from healthy human individuals has recently been published with skin containing 532 different compounds ¹⁹. The origins of VOCs emanating from skin are either from eccrine, sebaceous, and apocrine glandular secretions or metabolism of the skin microbiota ^{67, 116-119} (Figure 3). VOCs released from skin are diverse including ketones, aldehydes, heterocyclic compounds, hydrocarbons, terpenes, esters, volatile sulphur compounds and alcohols ^{66, 67, 120-122}. VOCs emitted from skin are affected by environmental factors such as diet and use of fragranced products such as soaps and perfumes ¹²³. This makes study design difficult with studies to date varying on the protocol with regards to skin preparation prior to volatile extraction. Some have asked subjects to make no changes ¹²⁴, others have stipulated the avoidance of fragranced products ⁶⁷, whilst others have implemented dietary restrictions ^{125, 126}. Studies examining the skin volatilome have employed different sampling techniques. These include solvent extraction, dynamic headspace absorption indirectly or directly onto absorbent traps, trapping tubes allowing direct insertion into the GC and SPME ^{68, 119, 127-130}. Each has their advantages and limitations including isolation of unexpected VOCs, exogenous contamination and loss of low molecular weight volatiles (Figure 1) ⁶⁴.

The top 5 VOCs identified from human skin are 6- methyl-5-hepten-2-one, nonanal, decanal, geranylacetone and (E)-2-nonenal ⁶⁴. Harraca et al analysed the VOCs of the whole human body by collection through customised heat sealed oven bags which participants wore from

the neck down and found the main compounds were heptanal, octanal, nonanal, decanal, 6-methyl-5-hepten-2-one and geranylacetone ¹²⁶. The array of VOCs emitted from human skin also varies according to the body site sampled (Figure 4). Axillary sampling has revealed high concentrations of alkanes, C6–C11 carboxylic acids, 3-methyl-2-hexenoic acid and 3-hydroxy-3-methylhexanoic acid ^{119, 127, 131}. However, different sampling techniques have led to a different array of volatiles identified ¹²⁸. VOCs emitted from the hands often consist of aldehydes and ketones ⁶⁴ and in addition forearm volatiles also comprise of alkanes and carboxylic acids ⁶⁷. The main volatiles released from feet are carboxylic acids ^{132, 133}. Ageing also seems to have an effect on VOC profiles of skin with four potential markers of ageing namely (E)-2-nonenal, dimethylsulphone, benzothiazole, and nonanal identified ^{67, 134}.

VOCs emitted from the skin have been studied as potential markers of disease. VOC patterns from skin melanoma have been identified using electronic nose devices ¹³⁵ and Kwak et al employed headspace SPME-GCMS to identify specific volatiles that could differentiate between melanoma and normal melanocyte cells cultured *in vitro* ¹³⁶. They found melanoma cells emit dimethyl disulfide and dimethyl trisulfide along with higher concentrations of isoamyl alcohol compared to normal melanocytes. Abaffy et al reported 3 studies utilising headspace SPME-GCMS for the differentiation of melanoma *in vivo* ¹³⁷⁻¹³⁹. Firstly they identified 4-methyl decane, dodecane and undecane were preferentially expressed in melanoma fresh and frozen tissue samples compared to control skin ¹³⁷. In a follow on case study they found 32 VOCs of which 23 were only detected from melanoma lesions compared to normal skin in the same individual ¹³⁸. They later followed this on with a pilot study in which they recruited 5 patients with melanoma and 5 patients with benign skin lesions and found increased levels of the fatty acids lauric acid and palmitic acid in melanoma ¹³⁹. The detection of VOCs from skin has also been applied experimentally to detect heart failure and

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diabetes^{140, 141}. Voss et al utilised an electronic nose device to detect emitted VOCs from skin and were able to discriminate between heart failure patients and controls with an accuracy of 87%¹⁴⁰. Turner et al used SIFT-MS to carry out a pilot study on five volunteers to determine VOC changes from skin before and after the ingestion of glucose in the fasting state¹⁴¹. VOCs were collected in a collection bag surrounding part of the arm and changes in acetone were noted post ingestion of glucose compared to the fasting state¹⁴¹.

For Peer Review

Wounds, SSIs and VOCs

There are no studies published to date investigating the use of VOC detection in the diagnosis of SSIs. The evidence of VOCs emitted from cutaneous wounds is sparse with only two *in vivo* studies to date. Parry et al utilised an electronic nose device which was able to differentiate between uninfected venous leg wounds and those infected with beta-Haemolytic *streptococci*¹⁴². Thomas et al obtained VOC samples from 5 patients with chronic lower limb wounds using a polydimethylsilicone membrane and analysed by gas chromatography ion trap mass spectrometry¹⁴³. They sampled from the wound, boundary areas around the wound and normal skin. They identified an array of VOCs which were unique to each of the sampled sites. They found significant differences in the VOC profile between normal skin and boundary skin and between normal skin and wounded skin but showed no difference between boundary skin and wound profiles. They recognised 6 compounds which may be responsible for this difference: 1-(1-methyethoxy) 2-propanol; dimethyl disulfide; 3-carene; 2-ethyl-1-hexanol; 3,5-bis(1,1-dimethylethyl)-phenol; and butylated hydroxytoluene. These compounds are often associated with preservatives found in creams and gels. However, they do elucidate that their protocol restricted the use of such creams and gels and that none of the patients reported using such products during the study period. Although study numbers were small, they provided a basis on which to develop further studies to identify the role of VOCs in wound healing. Dini et al captured the volatiles emitted from compressed and non-compressed body regions using GCMS and an electronic nose device¹⁴⁴. Their main aim was to identify if the pattern of volatiles emitted differed dependant on skin pressure which would allow the potential identification of patients at risk of developing decubitus wounds. They found emissions from compressed tissue differed from those of non-compressed tissue allowing the potential to be able to non-invasively detect those at risk of developing wounds

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Conclusions and future perspectives

VOCs emitted by microorganisms commonly associated with cutaneous wound infections are diverse. Studies isolating these pathogens have allowed the identification of potential signature combinations of volatiles which ~~would~~may allow detection of their presence. Although there are many VOCs that are shared between microorganisms, there are those identified to be unique to particular bacteria (table 2). This knowledge is imperative if ~~this~~ technology VOC detection is to be developed not only to identify if a wound is infected or not but differentiate between causative microorganisms. Although it must be considered that the majority of studies have only investigated the VOC profile of a limited number of species and strains and this may not hold true when extrapolating this to significantly more species and strains.

~~However,~~ Studies looking at the same organism have found different patterns of volatiles detected most probably explained by the use of different sampling methods and analytical techniques. Identifying and using the best suited sampling and analytical techniques will be critical. Although there is a wide spectrum of VOCs attributed to the presence of bacteria such as *P. aeruginosa* and *E. coli*, there is paucity in information available with regards to others such as *MRSA*, *E. faecalis* and *S. pyogenes*. Also of note is that the methodology of the studies thus far employed the use of various media and broths which are not ideal models for skin and cutaneous wounds. Therefore, one must be wary of inferring which volatiles will be emitted by organisms in skin and wounds by extrapolating the findings from these studies. Also, the *in vitro* studies discussed above have all identified VOCs specific to bacterial species in the planktonic phase. Extrapolating this to the identification of the bio-burden in a biofilm state must be approached with caution as the presence of other biofilm components

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such as the extracellular polymeric substance may alter the VOC profile. Therefore, future studies must compare the VOC profiles of bacterial species in both their planktonic and biofilm states.

In vivo studies assessing VOCs released from skin have identified a vast array of volatiles in both health and disease with potential markers of cutaneous malignancy and other common diseases identified. However, studies again have varied with regards to sampling methods which have proved much more difficult compared to *in vitro* experimental sampling. Also, controlling for external factors have proven difficult in human subjects leading to identification of exogenous and contaminant VOCs despite robust attempts to minimise this. Information with regards to VOCs emitted from cutaneous wounds is very limited; however the couple of studies to date have provided a direction for future work.

Current laboratory-based techniques - culture and non-culture based techniques - are time-consuming and culture over-estimates rapidly dividing non-fastidious bacteria and under-estimates more fastidious anaerobes⁵⁰. Molecular methods provide a detailed breakdown of the poly-microbial nature of wounds, however, are deficient in providing information regarding the dominant strain or strains driving the infection. Therefore, the use of untargeted empirical antimicrobial treatment is common based on the limited strain information available, which causes delay in optimal wound management as well as risk for development of antimicrobial resistance. The addition of VOC profiling could provide a more detailed outlook on the development state of a biofilm and on the metabolic processes microorganisms are relying on to thrive in the wound, thus allowing treatment to be specifically tailored.

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With regards to utilising VOC detection techniques in the diagnosis of chronic wound and SSIs, several limitations must be considered. As described above surgical patients undergo risk reducing measures such as peri- and post-operative antimicrobial administration and surgical skin preparation which will alter the wound microbiome. This in turn will alter the microbial metabolites produced and emitted from the wound surface. Also the use of cosmetic and sanitary products which themselves will release VOCs have the potential to interfere and mis-lead. Other significant limitations are the spectrum of surgical wounds ranging from clean to dirty-infected and the phase of bacterial growth will provide a spectrum of microorganisms and therefore a substantially variable array of metabolites produced. Also most chronic wounds are poly-microbial in nature with the presence of different strains of the same species and the presence of more than one active microorganism will produce a different volatile signature compared to its isolated state. Therefore it is vital that any ~~technology introduced~~technique used has the capability to detect a multitude of VOCs and the ability to identify poly-microbial infections in order to aid specificity in diagnoses of SSIs.

There are currently no approved tests based on VOC detection in the diagnosis of SSI or cutaneous wound infections. However, there are FDA approved devices available in the diagnosis of asthma ¹⁴⁵, *Helicobacter pylori* infection ¹⁴⁶ and heart transplant rejection ¹⁴⁷, which are all based on VOC detection ~~technology~~. There is currently a huge interest and a developing body of work in the use of VOC detection in the diagnosis and monitoring of various diseases ranging from infection to malignancy ^{148, 149}. Volatile detection via breath

testing has been at the forefront in the potential of this concept to diagnose infection. It is controversial whether an organism can be identified by its VOC profile as these are influenced by environmental factors, co-cultures and species strains⁶². However, recent studies on mono- and co-cultures of species in different growth conditions associated with infections in cystic fibrosis patients have yielded patterns of VOCs that allowed species identification^{93, 150, 151}. Also, Kunze et al found no difference between VOC patterns of different strains of a single species and concluded that VOC patterns of a bacterial strain can be transferred to other strains of the same species⁹¹. VOCs also provide information about host-infection interactions and the types of metabolic processes occurring^{120, 152}. Rather than just species identification, VOC profiling could be used to delineate the stage of infection based on the activation of certain metabolic processes¹⁵³, thus not only allowing identification of infection but allowing the tailoring of treatment of chronic wounds and monitoring treatment response.

~~The principles of this technology have the potential to be used in diagnosis of cutaneous infections.~~

We propose the need for further *in vitro* experimental work and robust clinical studies in order to identify signature volatiles of the common organisms attributed to the aetiology of chronic wounds and SSIs. Culturing these bacteria on skin explants and combining these with substrates such as Matrigel¹⁵⁴ to allow representation of cutaneous wounds *in vitro* would allow a more accurate determination of VOCs produced and emitted. Taking this information with volatiles emitted from acute, chronic and surgical wounds *in vivo* would potentially allow identification of VOCs which could be attributed to infected wounds and normal and abnormal wound healing. The ultimate goal would be to develop a clinical diagnostic tool

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which is inexpensive, portable, non-invasive, and reliable and offers time efficient results in detecting cutaneous wound infections in order to expedite accurate management. VOC detection has the potential to offer this.

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Conflicts of interest

None.

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Table 1. Common volatile organic compounds produced by bacteria associated with cutaneous wound and surgical site infections

Bacteria	VOC
<i>P. aeruginosa</i>	<ul style="list-style-type: none">- Isoprene ^{82, 83, 89}- 1-undecene ^{82, 83, 89, 94}- Dimethyl sulfide ^{82, 88, 94}- 2-butanone ^{83, 97}- Acetic acid ^{88, 106}- 2-aminoacetophenone ^{84, 94, 96}- Dimethyl disulphide ^{89, 94}- Ethanol ^{90, 106, 107}- Ammonia ^{104, 106}- Hydrogen cyanide ^{96, 97, 104, 105}- Methyl thiocyanate ^{96, 97}- Methyl thiolacetate, 2; 3-dimethyl-5-isopentylpyrazine; 2-methyl-3-(2-propenyl)-pyrazine; 3-methyl-1 H-pyrrole; 6-tridecane ⁹³- Dimethyl trisulphide ⁸⁹- 2-methylbutyl 2-methylbutyrate; amyl isovalerate; 2-methoxy-5-methylthiophene; 2-methylbutyl isobutyrate; 3-(ethylthio)-propanal ⁸⁷- 2-nonanone ⁸²- 2-pentene; 2-heptanone ⁸³- Thiocyanic acid methyl ester; 2-butanol; 2-Pentanol ⁸⁸- Dimethyl pyrazine ⁹⁴- 2-propanone; azane [dimer]; dodecane; 2-ethylhexan-1-ol; P_603_25 ⁹¹- 4-methylphenol ⁹⁰- Methyl mercaptan ¹⁰⁴- Acetophenone, ⁹⁷- Acetone; acetaldehyde ¹⁰⁶
<i>E. coli</i>	<ul style="list-style-type: none">- Indole ^{82, 83, 86, 90, 91, 95}- Dimethyl sulfide ^{81, 85, 106}- Ethanol ^{76, 81, 85, 91, 106, 108, 109}- Acetaldehyde ^{85, 106, 108, 109}- Propanol ^{83, 85, 108}- Isoprene ^{82, 83}- Acetic acid ^{83, 106, 108}- Acetone ^{76, 98, 106}- Hydrogen sulfide ^{107, 109}- Benzaldehyde; 2,5-dimethylpyrazine; 2,5-dimethyltetrahydrofuran; 2-nonanone ⁸⁶- 6-methyl-5-hepten-2-one; ethyl acetate; 3-methyl furan ⁸¹- Carbon disulphide; methyl butanoate; dimethyl disulfide, dimethyl trisulfide, methyl propanoate ⁸⁵- Carbon disulfide; butanal ⁸²- N-propylacetate ⁸³

	- 2-methylbutanol; 3-methylbutanol ⁹⁵
	- Heptan-2-one; nonan-2-one ⁷⁶
	- Methyl cyclohexane; carbon dioxide; pentafluoropropionamide; Dimethylether ⁹⁸
	- Decan-1-ol; Octan-1-ol ⁹¹
	- Formaldehyde; methyl mercaptan ¹⁰⁷
	- ammonia ¹⁰⁹
<i>S. aureus</i>	- Ethanol ^{87, 106}
	- Acetic acid ^{87, 90}
	- Acetaldehyde ^{87, 106}
	- Acetone ^{83, 90, 106}
	- Dimethyl disulfide ^{83, 95}
	- Ammonia ^{106, 107}
	- 3-methylbutanal; 2-methylpropanal ⁸⁷
	- 1,1,2,2-tetrachloroethane; dimethyl trisulfide ⁸³
	- Isovaleric acid; 2-methylbutyric acid; isobutyric acid; 1-hydroxy-2-propanone; 3-hydroxy-2-butanone ⁹⁴
	- 2-tridecenone ⁹⁵
	- Butanol ⁹⁰
	- Dimethyl sulfide ¹⁰⁶
<i>MRSA</i>	- 2-heptanone; 1,4-dichlorobenzene ⁸³
<i>S. pyogenes</i>	-
<i>E. faecalis</i>	- Ammonia ¹⁰⁷
<i>S. epidermidis</i>	- 3-methyl-1-butanol; 2-methylbutanal; 3-methylbutanoic acid ; 2-methylbutanoic acid ⁸⁰
	- Ammonia; acetone ¹⁰⁷

VOC – volatile organic compound; *P. aeruginosa* – *Pseudomonas aeruginosa*; *E. coli* – *Escherichia coli*; *S. aureus* – *Staphylococcus aureus*; *MRSA* – methicillin resistant *staphylococcus aureus*; *S. pyogenes* - *Streptococcus pyogenes*; *E. faecalis* - *Enterococcus faecalis*; *S. epidermidis* – *Staphylococcus epidermidis*.

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Table 2. Unique and shared VOCs between bacteria

VOC	PA	EC	SA	MRSA	EF	SE
Acetophenone; 4-methylphenol; 2-ethylhexan-1-ol; Dodecane; Dimethyl pyrazine; 2-Pentanol; 2-butanol; 2-pentene; 3-(ethylthio)propanal; 2-methylbutyl isobutyrate; 2-methoxy-5 methylthiophene; amyl isovalerate; 2-methylbutyl 2-methylbutyrate; 6-tridecane; 3-methyl-1 H-pyrrole; 2-methyl-3-(2-propenyl)-pyrazine; 2; 3-dimethyl-5-isopentylpyrazine; Methyl thiolacetate; Methyl thiocyanate; Hydrogen cyanide; 2 aminoacetophenone; 2-butanone; 1-undecene;	*					
Formaldehyde; Octan-1-ol; Decan-1-ol; Dimethylether; Pentafluoropropionamide; carbon dioxide; Methyl cyclohexane; 2-methylbutanol; N-propylacetate; Butanal; Carbon disulfide; methyl propanoate; methyl butanoate; 3-methyl furan; ethyl acetate; 6-methyl-5-hepten-2-one; 2,5-dimethyltetrahydrofuran; 2,5-dimethylpyrazine; Benzaldehyde; Hydrogen sulfide; Propanol; Indole		*				
2-methylpropanal; 3-methylbutanal; 1,1,2,2-tetrachloroethane; Butanol; 2-tridecenone; 3-hydroxy-2-butanone; 1-hydroxy-2-propanone; Isobutyric acid;			*			
1,4-dichlorobenzene				*		
2-methylbutanoic acid;			*			*
methyl mercaptan; 2-nonanone; Isoprene	*	*				
2-methylbutanal; 3-methyl-1-butanol		*				*
3-methylbutanoic acid			*			*
2-heptanone	*	*		*		
Dimethyl sulfide; Dimethyl trisulfide; Dimethyl disulfide; Acetaldehyde; Acetic acid; Ethanol	*	*	*			

Ammonia	*		*		*	*
Acetone	*	*	*			*

VOC – volatile organic compound; PA – *Pseudomonas aeruginosa*; EC – *Escherichia coli*;
SA – *Staphylococcus aureus*; MRSA – *methicillin resistant staphylococcus aureus*; SP -
Streptococcus pyogenes; EF - *Enterococcus faecalis*; SE – *Staphylococcus epidermidis*.

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Figure Legend

Figure 1. Volatile organic compound sampling techniques. Spider diagram outlining the common volatile organic compound sampling techniques including their advantages and limitations. *VOC – volatile organic compound; GC- gas chromatography; SPME – solid phase micro-extraction; TD – thermal desorption.*

Figure 2. Volatile organic compound analytical techniques. Spider diagram outlining the common volatile organic compound analytical techniques including their advantages and limitations. *ToF – time of flight; GC- gas chromatography; MS – mass spectrometry; VOC – volatile organic compound; SIFT-MS - Selected ion flow tube-mass spectrometry; IMR-MS - Ion molecule reaction mass spectrometry; SESI-MS - Secondary electrospray ionisation-mass spectrometry; PTR-MS - Proton transfer reaction-mass spectrometry; IMS – ion mobility spectrometry; MCC – multi capillary columns.*

Figure 3. Common volatile organic compounds produced and emitted from skin. Volatile organic compounds in skin are produced by interactions between the secretions from sebaceous, apocrine and eccrine glands and bacteria. Volatile organic compounds detected can be affected by environmental factors. *VOC – volatile organic compound.*

Figure 4. Volatile organic compounds emitted bodily sites. Volatile organic compounds emitted from the human body vary dependent on site.

Volatile organic compound detection as a potential means of diagnosing cutaneous wound infections

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Running title: VOC detection and wound infection diagnosis

Keywords: Cutaneous wounds; volatile organic compound; skin; infection

Abstract

Chronic cutaneous wound infections and surgical site infections (SSIs) present a huge burden on the healthcare system and can lead to increased morbidity and mortality. Current diagnostic methods of identifying and confirming infection involve culture-based and molecular methods. Both techniques are time consuming and delays commonly lead to untargeted empirical treatment. An ideal diagnostic method would be non-invasive and highly sensitive and detect pathogenic organisms with a high degree of accuracy in order to allow targeted treatment. Volatile organic compounds (VOCs) are a diverse group of carbon-based molecules produced and released by humans and microorganisms. VOC detection has the potential in aiding cutaneous wound infection diagnostics using non-invasive and time-efficient methods. This review provides a comprehensive update on VOCs produced and emitted by bacteria commonly associated with chronic wounds and SSIs. VOC sampling has the advantage of being painless, time-efficient, non-invasive and reproducible. VOCs emitted by these organisms are diverse. *In vitro* studies have identified potential signature volatile profiles, which can be used in detecting these microorganisms. Combining these profiles with volatile profiles emitted from acute, chronic and surgical wounds *in vivo* could potentially allow identification of bacterial-specific VOCs. VOC detection has the potential for a relatively inexpensive, portable, non-invasive and reliable clinical diagnostic tool, which could be used in detecting cutaneous wound infections and guiding their optimal management.

Introduction

Chronic cutaneous wounds occur as a result of deficiencies in wound healing processes^{1, 2}. Chronic wounds have a major impact on patient's quality of life and present a huge burden on the healthcare system^{3, 4}. Persistent cutaneous wound infection is a major contributor to delayed wound healing^{5, 6}. Cutaneous infection following post-surgical intervention is also of clinical concern. Surgical site infections (SSIs) are common complications after surgery and can lead to increased morbidity and mortality^{7, 8}. Common bacterial pathogens associated with chronic as well as superficial and deep SSIs include *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Streptococcus pyogenes* and *Enterococcus faecalis*⁹⁻¹². Current diagnostic methods of identifying and confirming cutaneous infections involve culture-based and molecular methods. Both techniques are time consuming, and culture-based methods have limited sensitivity and are susceptible to over-estimation of skin commensals leading to incorrect causative bacterial identification¹³⁻¹⁵. Delays in diagnostics often result in the use of untargeted empirical treatment with a risk for sub-optimal choice of antibiotics, the development of antibiotic resistance and increase in mortality¹⁶. An ideal diagnostic method would be non-invasive and highly sensitive and detect pathogenic organisms with a high degree of accuracy in order to allow targeted treatment.

Volatile organic compounds (VOCs) are a diverse group of carbon based molecules, including alcohols, isocyanates, ketones, aldehydes, hydrocarbons and sulphides, which are volatile at ambient temperatures^{17, 18}. VOC sampling has the advantage of being painless, non-invasive and reproducible. A compendium of VOCs emanating from the human body has been compiled with 1840 VOCs assigned from breath (872), saliva (359), blood (154),

milk (256), skin secretions (532) urine (279), and faeces (381) ¹⁹. There is increasing evidence that VOCs or combinations of VOCs are unique to various disease states and their early detection could represent a useful means of diagnosis. VOCs have been identified as potential biomarkers in malignancies of the lung ²⁰, stomach ²¹, head and neck ²², breast ²³, liver ^{24, 25} colon and prostate ²⁶. They have also been detected as markers of asthma ²⁷⁻²⁹, chronic obstructive pulmonary disease ^{30, 31}, inflammatory bowel disease ^{32, 33} and diabetes ^{34, 35}. Microorganisms produce and release VOCs and currently volatile detection via breath testing has been at the forefront in the potential of this technology to diagnose infection ³⁶. The ability to identify VOCs in cutaneous wound infections could lead to a non-invasive and time efficient method of diagnosis.

The aim of this review is to provide a detailed update on VOCs produced and emitted by bacterial species commonly associated with cutaneous wound infections. It then describes the volatile profile of skin in health and disease. Finally, the role of volatile detection from cutaneous wounds is discussed. An extensive literature search was conducted on PubMed for relevant articles published from 1960 onwards. MeSH terms used included a variety of combinations including: SSI, surgical wound infection, VOCs, biofilm, *staphylococcus*, *Escherichia coli*, *streptococcus*, *enterococcus*, *Pseudomonas aeruginosa*, gram positive bacteria, gram negative bacteria, wound infection, post-operative wound infection, wound healing and skin.

Chronic wounds and SSIs

Chronic wounds represent a major burden on patients with the National Health Service expenditure in managing these wounds in excess of £1 billion annually³. Biofilms are present in more than half of chronic wounds and present a major obstacle in healing³⁷. Over 50 million in-patient surgical procedures are performed in the USA annually with approximately 1-5% developing SSIs with a high associated mortality^{38, 39}. SSIs pose a significant burden on the healthcare system leading to increase postoperative in-patient stay, increased expenses and readmissions. The preliminary management of SSIs involves preventative measures which include but are not limited to peri-operative antibiotics and meticulous skin preparation prior to the procedure. However, despite these measures SSIs represent the most common hospital acquired infections in surgical patients⁴⁰. Risk factors associated with SSIs include patient and operative factors. Patient factors include diabetes, obesity, smoking and pre-operative active infection⁴¹⁻⁴³. Operative factors include wound characteristics which can be classified into four categories, namely, clean, clean-contaminated, contaminated and dirty-infected⁴⁴. An escalating risk of developing SSIs is linked to the level of wound characterisation ranging from 1.3% for clean wounds to 40% for dirty-infected wounds^{45, 46}. Other operative factors include preoperative showering and hair removal, hand-washing and surgical attire, and intra-operative skin preparation⁴⁷.

Planktonic bacteria undergo a four phase cycle of growth consisting of a lag phase, log phase, stationary phase and death phase. Biofilms are physiologically distinct from their planktonic counterparts and undergo a different developmental cycle, which involves initial attachment to the surface, irreversible attachment, maturation and dispersion⁴⁸. Biofilms are more prevalent than expected in surgical wounds with up to 80% of SSIs involving the presence of

biofilm⁴⁹. They are difficult to diagnose with culture methods proving obsolete in their detection⁵⁰. Biofilms have been found on closure materials of healed surgical wounds⁵¹ and on the surface of implanted surgical devices⁵². Biofilms are significantly more difficult to eradicate leading to a chronic inflammatory state which further negatively impacts wound healing⁵³ and persistence of infection⁵⁴. Management of SSI associated biofilms requires invasive management involving surgical debridement of devitalised tissue, removal of infected devices and parenteral broad-spectrum antimicrobials⁵⁵⁻⁵⁷. This further highlights the need for rapid and accurate diagnostic tools in order to avoid a delay in optimum management.

SSIs are classified into three categories: superficial incisional where the infection occurs around the area of the skin where the incision was made; deep incisional where the infection occurs deep to the incision effecting the underlying fascia and muscle; and organ/space where the infection effects the organs or body cavities⁴⁴. We propose the potential use of volatile detection as a means of diagnosing SSIs would be limited to superficial SSI and potentially deep SSI as these would allow the non-invasive capture of VOCs. The most typical causative micro-organisms of superficial and deep SSIs are pathogens native to the patient's skin which include *staphylococci*, *streptococci*, *enterococci* and gram negative *bacilli*⁴⁷.

Microorganisms and VOCs

A portion of normal microbial metabolites produced are VOCs⁵⁸. They are thought to evolve as products or by-products of metabolic pathways⁵⁹. Zoller and Clark were the first to report laboratory evidence of bacterial production of volatiles⁶⁰. Many classification systems exist for microbial volatiles with one potential system categorising them into fatty acid derivatives, aromatic compounds, nitrogen-containing compounds, sulphur compounds, terpenoids, halogenated selenium, tellurium and other metalloid compounds⁶¹. Their production is dependent on the bacterial species, bacterial strains, bacterial growth phase, co-cultures, pH, humidity, nutrients, temperature and other environmental factors^{62, 63}. The ability to rapidly sample and analyse VOCs, potentially allowing identification of bacteria, has massive implications in the management of infection in clinical settings.

VOC sampling and analytical techniques

There are numerous sampling techniques that have been utilised in order to collect and identify VOCs, the advantages and limitations of which are outlined in Figure 1. These include VOC collection onto adsorbents, such as Tenax or solid-phase micro-extraction (SPME) ⁶⁴, either directly or through an intermediary such as gauze or cotton pads ⁶⁵; collection in sealed containers ⁶⁶; direct or indirect solvent extraction ⁶⁷; or combinations of the above. The above methods do not often identify all VOCs present due to selective adsorption of the trapping material and these sampling techniques limit accurate quantification of VOCs and therefore direct sampling are utilised ⁶⁸.

There are a variety of analytical chemical techniques used in VOC detection (Figure 2). Gas chromatography mass spectrometry (GCMS) is a combined analytical system and is one of the most widely used and powerful analytical chemical methods. It detects and quantifies VOCs over a range from parts per million to part per billion or less. Samples for GCMS must be in the gaseous phase where they are injected into the chromatograph where an inert carrier gas transports it through a tubular column. The length of the columns used varies with longer columns providing better chromatographic resolution and maximum separation of analytes. Each compound is emitted from the column at different times with the mass spectrometer used to detect eluting compounds through compound ionisation followed by measurement of the mass to charge ratio of each ion generating a unique mass spectrum for the compound ⁶⁹. The major advantages of GCMS are increased sensitivity and compound identification is greatly facilitated by the availability of extensive and easily searchable databases ⁷⁰. However, it is not typically strong at identifying unknown compounds. Other limitations of GCMS are increased processing and analysis times and similarity of molecular fragmentation

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3 patterns from structural isomers can make compound identification difficult ⁷¹. Also, due to
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5 high detection limits, sample pre-concentration using techniques described above are
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7 typically required, making quantification of VOCs very difficult. A time of flight (TOF) mass
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9 spectrometer serves a very similar purpose to GCMS with the exception of providing a
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11 combination of high mass accuracy and extended range of detectable metabolites ⁷².
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18 Ion mobility spectrometry (IMS) separates gas-phase ions based on their size and shape ⁷³.
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20 This technology has long been used in the detection of explosives and illegal substances ⁷⁴. It
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22 can be coupled to a pre-separating multi capillary column (MCC) unit allowing two-
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24 dimensional volatile separation, significantly increasing the resolution of metabolites ⁷⁵.
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26 MCC allows minimisation or avoidance of analyte interactions in the ionisation region of the
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28 IMS, thus reducing the complexity of the measurement signal ⁷⁶. IMS is advantageous over
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30 GCMS as ambient air can be used as the carrier gas, eliminating the need for a pure speciality
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32 inert gas or a vacuum. This has allowed IMS to be portable and provide on-site
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34 measurements. IMS is highly sensitive with very low detection limits between the parts per
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36 billion to parts per trillion ranges, offers excellent low detection limits and gives relatively
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38 rapid results ⁷⁷. IMS is however not suitable for identification of unknown compounds.
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46 Direct injection mass spectrometry methods include ion molecule reaction mass spectrometry
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48 (IMR-MS), secondary electrospray ionisation mass spectrometry (SESI-MS), selected ion
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50 flow tube mass spectrometry (SIFT-MS) and proton transfer reaction mass spectrometry
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52 (PTR-MS). A drawback of conventional mass spectrometry instrumentation is the high
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54 fragmentation of molecules in a complex gas mixture secondary to high electron ionisation
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56 leading to complex mass spectra with overlapping intensities, thus making quantification
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difficult or impossible. IMR-MS offers a very soft form of ionisation leading to less fragmentation. SESI-MS utilises electrospray ionisation technique in order to produce analytes suitable for mass analysis. In PTR-MS, compounds are ionised via proton transfer prior to analysis. This involves the production of H_3O^+ ions from high purity distilled water through a cathode ion source. SIFT-MS is similar to PTR-MS but is able to ionise a wider range of analytes as it uses a greater number of precursor ions (H_3O^+ , NO^+ or O_2^+) for chemical ionisation⁶⁹. The major advantage of direct injection methods is quantification. These direct methods also have lower detection limits than GCMS at parts per trillion ranges. They also provide real-time detection with no sample preparation or separation requirements. They are however limited in the range of VOCs detected compared to GCMS and PTR-MS is limited to the identification of compounds with a higher proton affinity than water.

The electronic nose devices constitute a non-invasive technique capable of detecting and differentiating VOC patterns based on its ability to detect odour. The Cyranose 320 and E50835 electronic nose devices have been used in bacterial identification. These devices allow imprinting of an odour on its sensor chip micro-array which is composed of complex materials. When the sensors are exposed to a gas, the polymer absorbs the gas and swells, during which the distance between the conductive carbon particles increases and thus also increases the resistance of the sensor material⁷⁸. This change in resistance is transmitted to a computer with the pattern of change in the sensor array being used to detect the gas. Electronic nose devices are powerful at differentiating between non-identical samples, provide rapid results with on-site sampling. They are however limited due to temperature and humidity sensitivity, only detect patterns of VOCs programmed on their database and are not suitable for screening of unknown compounds.

VOCs produced by microorganisms commonly associated with wound infection

This section details the VOCs identified from the common causative bacteria of wounds based on the different analytical techniques available. With regards to VOC quantities, based on analytical technique used studies varied with some presenting relative abundances whereas others identified absolute concentrations of compounds. The majority of studies have provided VOC abundances relative to other compounds identified⁷⁹⁻⁸⁴. Studies where absolute abundances of VOCs are shown range from the parts per million volume to parts per trillion volume⁸⁵⁻⁸⁷. Parts per million volume concentrations equate to µg/l sampled, parts per billion volume concentrations equate to ng/l sampled and parts per trillion volume concentrations equate to pg/l sampled. Studies in which more than 5 VOCs were identified for a specific bacterial species, only the top 5 compounds based on abundance (either relative or absolute) are presented (table 1). Studies where VOC production could not be attributed to an isolated bacterial species or strain were excluded.

Informatics approaches also varied between the studies evaluated. Four main methods were used to confirm identification of VOCs. Some studies compared mass spectra to reference libraries and databases, such as the National Institute of Standards and Technology (NIST)^{83, 88}, Wiley⁸⁹ and Massbank⁹⁰ libraries and pre-determined reference databases⁹¹. Other studies compared mass spectra and peak retention times with those obtained from pure standard compounds^{82, 85, 92}. The majority of studies combined the use of reference libraries and pure standard compounds for identification of VOCs^{79, 81, 86, 87, 89, 93-97}. A third technique used was the manual comparison of mass spectra with those available in the literature⁹⁴. Lastly, due to the limitations of certain analytical techniques in compound identification, concurrent techniques were utilised to confirm VOC identity^{76, 98-101}.

GCMS

Neerincx et al inoculated *P. aeruginosa* in brain heart infusion broth and sampled the headspace using Tenax TA at 16, 24 and 48 hours⁹³. The top 5 VOCs emitted from *P. aeruginosa* cultures were methyl thiolacetate, 2,3-dimethyl-5-isopentylpyrazine, 2-methyl-3-(2-propenyl)-pyrazine, 3-methyl-1 H-pyrrole and 6-tridecane. Scholler et al sampled volatile metabolites emitted from *P. aeruginosa* by diffusive sampling again using Tenax⁸⁹. The major headspace VOC identified was dimethyl disulphide. Bean et al were the first to report the application of two-dimensional GC-TOF spectrometry to identify volatiles of *P. aeruginosa* grown for 24 hours in lysogeny broth in sealed GC headspace vials via SPME passive sampling⁸⁸. This method facilitated the identification of 56 VOCs emitted by *P. aeruginosa* of which 28 were new including alcohols, heteroaromatics, ketones, benzenes and aldehydes⁸⁸. Scott-Thomas et al used similar SPME sampling of the headspace of *P. aeruginosa* and found high concentrations of 2-aminoacetophenone⁸⁴.

Saranya et al identified 2-[3-acetoxy-4,4,14-trimethylandro-8-en-17-yl] propanoic acid was specific to *Staphylococcus*⁸⁰ and 3-methyl-1-butanol, 2-methylbutanal, 3-methyl-1-butanol, 3-methylbutanoic acid and 2-methylbutanoic acid have been found to be significantly expressed in the headspace of *S. epidermidis* using the purge and trap technique⁷⁹.

Indole is produced by both pathogenic and non-pathogenic strains of *E. coli* cultured on a mixture of trypticase soy agar and brain heart infusion broth⁸⁶. Bianchi et al inoculated commercial tins of peeled tomatoes with *E. coli* and found the most abundant compounds detected were dimethylsulfide, 6-methyl-5-hepten-2-one, ethanol, ethyl acetate and 3-methyl

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3 furan ⁸¹. Umber et al identified differing volatile signatures of *E. coli* dependant on the
4 environment. Headspace analysis revealed *E. coli* inoculated whole blood released a different
5 set of VOCs compared to *E. coli* cultured in Luria Bertani (LB) broth ⁸⁵. VOCs specific to *E.*
6 *coli*-infected whole blood were dimethyl sulfide, carbon disulphide, ethanol, acetaldehyde
7 and methyl butanoate. Whereas dimethyl disulfide, dimethyl trisulfide, methyl propanoate, 1-
8 propanol and methylcyclohexane were isolated only from *E.coli* cultured in LB broth ⁸⁵.
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20 Filipiak et al cultured *S. aureus* and *P. aeruginosa* in tryptic soy broth medium and collected
21 headspace samples on multi-bed sorption tubes ⁸⁷. *S. aureus* released 32 VOCs and *P.*
22 *aeruginosa* 37 of diverse chemical classes comprising aldehydes, alcohols, ketones, acids,
23 sulphur containing compounds, esters, hydrocarbons and nitrogen containing compounds.
24 They found distinct differences in the bacteria-specific VOC profiles, especially with regard
25 to aldehydes which were observed by *S. aureus* with no release observed from *P. aeruginosa*
26 ⁸⁷. Preti et al sampled the headspace of *P. aeruginosa* and *S. aureus* cultured in blood agar
27 medium in petri culture dishes and identified compounds specific to each species ⁹⁴.
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42 Zscheppank et al utilised a novel needle trap technique consisting of a sorbent packed needle
43 to sample headspace of *E. coli* and *P. aeruginosa* cultured in liquid media ¹⁰² and found both
44 organisms produced isoprene ⁸².
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52 Elgaali et al utilised purge and trap and passive SPME sampling of the headspace to analyse
53 *E. coli* and *S. aureus* ⁹⁵. Both sampling techniques identified indole as the principle
54 component over the headspace of *E. coli* and the purge and trap technique identified long
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chain alcohols, 2-methylbutanol and 3-methylbutanol in abundance, whereas, passive SPME sampling of the headspace identified 2-tridecenone and dimethyldisulfide over the headspace of *S. aureus*⁹⁵.

Boots et al sampled the bacterial headspace, using desorption tubes packed with carbograph 1TD/Carbopack X, of *S. aureus*, methicillin-resistant *S. aureus* (*MRSA*), *P. aeruginosa* and *E. coli* cultured in a variety of agars and broths⁸³. Samples were analysed by GC-TOF-MS. They identified 25 VOCs which could be used to discriminate between the bacterial strains. They also found 1,1,2,2-tetrachloroethane, 2-heptanone and 1,4-dichlorobenzene are differentially excreted in the headspace of *S. aureus* and *MRSA*, with the latter two compounds significantly more abundant over the headspace of *MRSA*⁸³.

IMS

Maddula et al utilised MCC-IMS to identify ethanol, heptan-2-one, and nonan-2-one emitted from the headspace of *E. coli* cultures⁷⁶. Guaman et al evaluated IMS and GCMS in the detection of VOCs in the breath of rats injected intraperitoneally with *E. coli* or regular saline after 24 hours and found IMS had a higher sensitivity and specificity than GCMS in distinguishing the two groups⁹⁸. Kunze et al analysed using MCC-IMS the headspace of *E. coli* and *P. aeruginosa* cultured in Lysogeny Broth and found six VOCs (Decan-1-ol, Ethanol, Indole, Octan-1-ol [monomer and dimer] and P_755_105) exclusively over the headspace of *E. coli* cultures and 7 VOCs (2-Propanone, Azane [dimer], Dodecane, 2-Ethylhexan-1-ol, P_603_25, P_648_36 and P_778_4) emitted exclusively by *P. aeruginosa* cultures⁹¹.

Direct injection mass spectrometry

Only a single study to date has utilised IMR-MS to analyse the headspace over *E. faecalis*, *S. aureus* and *S. epidermidis* cultured in blood agar and brain heart infusion broth⁹². They were able to differentiate between the species based on the mass spectra generated from the VOCs.

Zhu et al were the first to report the application of SESI-MS to the detection and characterisation of VOCs produced by *P. aeruginosa*, *S. aureus* and *E. coli* cultured in tryptic soy broth⁹⁰. They identified *P. aeruginosa* emitted ethanol and 4-Methylphenol in high abundance; *S. aureus* emitted high concentrations of butanol, acetone and acetic acid; *E. coli* principle emitted volatile was indole. A follow on study by the same group were able to distinguish 11 strains of *E. coli* from *S. aureus* based on VOCs¹⁰³. In addition they identified 6 VOC biomarkers that were common in all *E. coli* strains.

Carroll et al cultured different strains of *P. aeruginosa* on blood agar and pseudomonas-selective media and analysed the headspace using SIFT-MS¹⁰⁴. They identified the majority of strains emitted high quantities of ammonia and hydrogen cyanide compared to controls. Shestivska et al corroborated the latter findings using both GCMS and SIFT-MS⁹⁶. They also identified a second compound, methyl thiocyanate, is commonly emitted from the majority of *P. aeruginosa* strains and most probably both compounds are biochemically associated. A follow on study by the same group identified differing production rates of VOC by genotypically different strains of *P. aeruginosa*⁹⁷, which may allow differentiation between not only bacterial species but also strains of the same organism. Gilchrist et al identified elevated concentrations of hydrogen cyanide in the breath of patients with chronic *P.*

aeruginosa infections compared to controls ¹⁰⁵. However, it must be noted that the patients and controls may have been colonised by other bacteria. Allardyce et al inoculated blood culture bottles, supplemented with tryptic soy broth, with isolated strains of *P. aeruginosa*, *S. aureus* and *E. coli* and analysed emitted VOCs using SIFT-MS at 6 hours ¹⁰⁶. Nine VOCs in total were assessed to differentiate between the bacterial strains. *P. aeruginosa* cultures had relatively high absolute concentrations of acetic acid and acetone; *E. coli* ethanol and acetaldehyde; and *S. aureus* ethanol and acetone ¹⁰⁶. Storer et al used SIFT-MS to measure volatiles emitted from the headspace of urine samples inoculated with *P. aeruginosa*, *S. aureus*, *S. epidermidis*, *E. coli* and *E. faecalis* at 6 hours ¹⁰⁷. The principle VOC emitted compared to non-inoculated samples was formaldehyde for *E. coli*, ethanol for *P. aeruginosa* and ammonia for *S. epidermidis*, *S. aureus* and *E. faecalis*. In contrast, Sovova et al found the principle volatile emitted from the headspace of *E. coli* cultured in nutrient broth enriched with glucose was ethanol ¹⁰⁸. This could be explained by the use of different media, strains and growth conditions between the studies. This assumption is confirmed by Chippendale et al who analysed the VOC from the headspace of *E. coli* cultured in two different media ¹⁰⁹. *E. coli* cultured in Dulbecco's modified Eagle's medium resulted in large amounts of ethanol, acetaldehyde and hydrogen sulphide production, whereas *E. coli* cultured in lysogeny broth, ammonia was the major volatile compound ¹⁰⁹.

Lechner et al used PTR-MS to analyse the headspace of *E. coli*, *P. aeruginosa* and *S. aureus* cultured in either MacConkey agar or Mannitol-salt agar ⁹⁹. They identified patterns which were specific to the bacterial species however did not expand on the identity of the VOCs. Using PTR-MS, O'Hara and Mayhew analysed the headspace of *S. aureus* cultured in three different broths (nutrient, dextrose and brain heart bovine) and found although the VOCs emitted were specific to *S. aureus*, their concentrations differed dependant on the type of

media they were grown in ¹⁰⁰. Both Luchner et al ¹⁰¹ and Bunge et al ¹¹⁰ have utilised PTR-MS to identify volatiles in the headspace of *E. coli*.

Electronic nose

The Cyranose 320 and E50835 electronic nose devices have been utilised to differentiate between *P. aeruginosa*, *S. aureus* and *E. coli* isolates based on VOC patterns from the headspace ^{81, 111-113}. However, it does not provide details regarding the individual volatile compounds emitted by the bacteria.

Skin and VOCs

Skin is the largest human organ ¹¹⁴ and forms an essential barrier between the body and the environment, protects from injury and provides vital homeostatic mechanisms including control of temperature, maintenance of fluid balance, and detection of sensations such as pain ¹¹⁵. A compilation of 1840 VOCs emitted from healthy human individuals has recently been published with skin containing 532 different compounds ¹⁹. The origins of VOCs emanating from skin are either from eccrine, sebaceous, and apocrine glandular secretions or metabolism of the skin microbiota ^{67, 116-119} (Figure 3). VOCs released from skin are diverse including ketones, aldehydes, heterocyclic compounds, hydrocarbons, terpenes, esters, volatile sulphur compounds and alcohols ^{66, 67, 120-122}. VOCs emitted from skin are affected by environmental factors such as diet and use of fragranced products such as soaps and perfumes ¹²³. This makes study design difficult with studies to date varying on the protocol with regards to skin preparation prior to volatile extraction. Some have asked subjects to make no changes ¹²⁴, others have stipulated the avoidance of fragranced products ⁶⁷, whilst others have implemented dietary restrictions ^{125, 126}. Studies examining the skin volatilome have employed different sampling techniques. These include solvent extraction, dynamic headspace absorption indirectly or directly onto absorbent traps, trapping tubes allowing direct insertion into the GC and SPME ^{68, 119, 127-130}. Each has their advantages and limitations including isolation of unexpected VOCs, exogenous contamination and loss of low molecular weight volatiles (Figure 1) ⁶⁴.

The top 5 VOCs identified from human skin are 6- methyl-5-hepten-2-one, nonanal, decanal, geranylacetone and (E)-2-nonenal ⁶⁴. Harraca et al analysed the VOCs of the whole human body by collection through customised heat sealed oven bags which participants wore from

the neck down and found the main compounds were heptanal, octanal, nonanal, decanal, 6-methyl-5-hepten-2-one and geranylacetone¹²⁶. The array of VOCs emitted from human skin also varies according to the body site sampled (Figure 4). Axillary sampling has revealed high concentrations of alkanes, C6–C11 carboxylic acids, 3-methyl-2-hexenoic acid and 3-hydroxy-3-methylhexanoic acid^{119, 127, 131}. However, different sampling techniques have led to a different array of volatiles identified¹²⁸. VOCs emitted from the hands often consist of aldehydes and ketones⁶⁴ and in addition forearm volatiles also comprise of alkanes and carboxylic acids⁶⁷. The main volatiles released from feet are carboxylic acids^{132, 133}. Ageing also seems to have an effect on VOC profiles of skin with four potential markers of ageing namely (E)-2-nonenal, dimethylsulphone, benzothiazole, and nonanal identified^{67, 134}.

VOCs emitted from the skin have been studied as potential markers of disease. VOC patterns from skin melanoma have been identified using electronic nose devices¹³⁵ and Kwak et al employed headspace SPME-GCMS to identify specific volatiles that could differentiate between melanoma and normal melanocyte cells cultured *in vitro*¹³⁶. They found melanoma cells emit dimethyl disulfide and dimethyl trisulfide along with higher concentrations of isoamyl alcohol compared to normal melanocytes. Abaffy et al reported 3 studies utilising headspace SPME-GCMS for the differentiation of melanoma *in vivo*¹³⁷⁻¹³⁹. Firstly they identified 4-methyl decane, dodecane and undecane were preferentially expressed in melanoma fresh and frozen tissue samples compared to control skin¹³⁷. In a follow on case study they found 32 VOCs of which 23 were only detected from melanoma lesions compared to normal skin in the same individual¹³⁸. They later followed this on with a pilot study in which they recruited 5 patients with melanoma and 5 patients with benign skin lesions and found increased levels of the fatty acids lauric acid and palmitic acid in melanoma¹³⁹. The detection of VOCs from skin has also been applied experimentally to detect heart failure and

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3 diabetes ^{140, 141}. Voss et al utilised an electronic nose device to detect emitted VOCs from skin
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5 and were able to discriminate between heart failure patients and controls with an accuracy of
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7 87% ¹⁴⁰. Turner et al used SIFT-MS to carry out a pilot study on five volunteers to determine
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9 VOC changes from skin before and after the ingestion of glucose in the fasting state ¹⁴¹.
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11 VOCs were collected in a collection bag surrounding part of the arm and changes in acetone
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13 were noted post ingestion of glucose compared to the fasting state ¹⁴¹.
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For Peer Review

Wounds, SSIs and VOCs

There are no studies published to date investigating the use of VOC detection in the diagnosis of SSIs. The evidence of VOCs emitted from cutaneous wounds is sparse with only two *in vivo* studies to date. Parry et al utilised an electronic nose device which was able to differentiate between uninfected venous leg wounds and those infected with beta-Haemolytic *streptococci*¹⁴². Thomas et al obtained VOC samples from 5 patients with chronic lower limb wounds using a polydimethylsilicone membrane and analysed by gas chromatography ion trap mass spectrometry¹⁴³. They sampled from the wound, boundary areas around the wound and normal skin. They identified an array of VOCs which were unique to each of the sampled sites. They found significant differences in the VOC profile between normal skin and boundary skin and between normal skin and wounded skin but showed no difference between boundary skin and wound profiles. They recognised 6 compounds which may be responsible for this difference: 1-(1-methoxy) 2-propanol; dimethyl disulfide; 3-carene; 2-ethyl-1-hexanol; 3,5-bis(1,1-dimethylethyl)-phenol; and butylated hydroxytoluene. These compounds are often associated with preservatives found in creams and gels. However, they do elucidate that their protocol restricted the use of such creams and gels and that none of the patients reported using such products during the study period. Although study numbers were small, they provided a basis on which to develop further studies to identify the role of VOCs in wound healing. Dini et al captured the volatiles emitted from compressed and non-compressed body regions using GCMS and an electronic nose device¹⁴⁴. Their main aim was to identify if the pattern of volatiles emitted differed dependant on skin pressure which would allow the potential identification of patients at risk of developing decubitus wounds. They found emissions from compressed tissue differed from those of non-compressed tissue allowing the potential to be able to non-invasively detect those at risk of developing wounds

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Conclusions and future perspectives

VOCs emitted by microorganisms commonly associated with cutaneous wound infections are diverse. Studies isolating these pathogens have allowed the identification of potential combinations of volatiles which may allow detection of their presence. Although there are many VOCs that are shared between microorganisms, there are those identified to be unique to particular bacteria (table 2). This knowledge is imperative if VOC detection is to be developed not only to identify if a wound is infected or not but differentiate between causative microorganisms. Although it must be considered that the majority of studies have only investigated the VOC profile of a limited number of species and strains and this may not hold true when extrapolating this to significantly more species and strains.

Studies looking at the same organism have found different patterns of volatiles detected most probably explained by the use of different sampling methods and analytical techniques. Identifying and using the best suited sampling and analytical techniques will be critical. Although there is a wide spectrum of VOCs attributed to the presence of bacteria such as *P. aeruginosa* and *E. coli*, there is paucity in information available with regards to others such as *MRSA*, *E. faecalis* and *S. pyogenes*. Also of note is that the methodology of the studies thus far employed the use of various media and broths which are not ideal models for skin and cutaneous wounds. Therefore, one must be wary of inferring which volatiles will be emitted by organisms in skin and wounds by extrapolating the findings from these studies. Also, the *in vitro* studies discussed above have all identified VOCs specific to bacterial species in the planktonic phase. Extrapolating this to the identification of the bio-burden in a biofilm state must be approached with caution as the presence of other biofilm components such as the extracellular polymeric substance may alter the VOC profile. Therefore, future

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3 studies must compare the VOC profiles of bacterial species in both their planktonic and
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5 biofilm states.
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11 *In vivo* studies assessing VOCs released from skin have identified a vast array of volatiles in
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13 both health and disease with potential markers of cutaneous malignancy and other common
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15 diseases identified. However, studies again have varied with regards to sampling methods
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17 which have proved much more difficult compared to *in vitro* experimental sampling. Also,
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19 controlling for external factors have proven difficult in human subjects leading to
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21 identification of exogenous and contaminant VOCs despite robust attempts to minimise this.
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23 Information with regards to VOCs emitted from cutaneous wounds is very limited; however
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25 the couple of studies to date have provided a direction for future work.
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33 Current laboratory-based techniques - culture and non-culture based techniques - are time-
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35 consuming and culture over-estimates rapidly dividing non-fastidious bacteria and under-
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37 estimates more fastidious anaerobes⁵⁰. Molecular methods provide a detailed breakdown of
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39 the poly-microbial nature of wounds, however, are deficient in providing information
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41 regarding the dominant strain or strains driving the infection. Therefore, the use of untargeted
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43 empirical antimicrobial treatment is common based on the limited strain information
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45 available, which causes delay in optimal wound management as well as risk for development
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47 of antimicrobial resistance. The addition of VOC profiling could provide a more detailed
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49 outlook on the development state of a biofilm and on the metabolic processes
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51 microorganisms are relying on to thrive in the wound, thus allowing treatment to be
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53 specifically tailored.
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With regards to utilising VOC detection techniques in the diagnosis of chronic wound and SSIs, several limitations must be considered. As described above surgical patients undergo risk reducing measures such as peri- and post-operative antimicrobial administration and surgical skin preparation which will alter the wound microbiome. This in turn will alter the microbial metabolites produced and emitted from the wound surface. Also the use of cosmetic and sanitary products which themselves will release VOCs have the potential to interfere and mis-lead. Other significant limitations are the spectrum of surgical wounds ranging from clean to dirty-infected and the phase of bacterial growth will provide a spectrum of microorganisms and therefore a substantially variable array of metabolites produced. Also most chronic wounds are poly-microbial in nature with the presence of different strains of the same species and the presence of more than one active microorganism will produce a different volatile signature compared to its isolated state. Therefore it is vital that any technique used has the capability to detect a multitude of VOCs and the ability to identify poly-microbial infections in order to aid specificity in diagnoses of SSIs.

There are currently no approved tests based on VOC detection in the diagnosis of SSI or cutaneous wound infections. However, there are FDA approved devices available in the diagnosis of asthma ¹⁴⁵, *Helicobacter pylori* infection ¹⁴⁶ and heart transplant rejection ¹⁴⁷, which are all based on VOC detection. There is currently a huge interest and a developing body of work in the use of VOC detection in the diagnosis and monitoring of various diseases ranging from infection to malignancy ^{148, 149}. Volatile detection via breath testing has been at the forefront in the potential of this concept to diagnose infection. It is controversial whether an organism can be identified by its VOC profile as these are influenced by environmental factors, co-cultures and species strains ⁶². However, recent studies on mono- and co-cultures of species in different growth conditions associated with infections in cystic fibrosis patients

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3 have yielded patterns of VOCs that allowed species identification^{93, 150, 151}. Also, Kunze et al
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5 found no difference between VOC patterns of different strains of a single species and
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7 concluded that VOC patterns of a bacterial strain can be transferred to other strains of the
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9 same species⁹¹. VOCs also provide information about host-infection interactions and the
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11 types of metabolic processes occurring^{120, 152}. Rather than just species identification, VOC
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13 profiling could be used to delineate the stage of infection based on the activation of certain
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15 metabolic processes¹⁵³, thus not only allowing identification of infection but allowing the
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17 tailoring of treatment of chronic wounds and monitoring treatment response.
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21 We propose the need for further *in vitro* experimental work and robust clinical studies in
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23 order to identify signature volatiles of the common organisms attributed to the aetiology of
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25 chronic wounds and SSIs. Culturing these bacteria on skin explants and combining these with
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27 substrates such as Matrigel¹⁵⁴ to allow representation of cutaneous wounds *in vitro* would
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29 allow a more accurate determination of VOCs produced and emitted. Taking this information
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31 with volatiles emitted from acute, chronic and surgical wounds *in vivo* would potentially
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33 allow identification of VOCs which could be attributed to infected wounds and normal and
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35 abnormal wound healing. The ultimate goal would be to develop a clinical diagnostic tool
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37 which is inexpensive, portable, non-invasive, and reliable and offers time efficient results in
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39 detecting cutaneous wound infections in order to expedite accurate management. VOC
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41 detection has the potential to offer this.
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Conflicts of interest

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Table 1. Common volatile organic compounds produced by bacteria associated with cutaneous wound and surgical site infections

Bacteria	VOC
<i>P. aeruginosa</i>	<ul style="list-style-type: none"> - Isoprene^{82, 83, 89} - 1-undecene^{82, 83, 89, 94} - Dimethyl sulfide^{82, 88, 94} - 2-butanone^{83, 97} - Acetic acid^{88, 106} - 2-aminoacetophenone^{84, 94, 96} - Dimethyl disulphide^{89, 94} - Ethanol^{90, 106, 107} - Ammonia^{104, 106} - Hydrogen cyanide^{96, 97, 104, 105} - Methyl thiocyanate^{96, 97} - Methyl thiolacetate; 2, 3-dimethyl-5-isopentylpyrazine; 2-methyl-3-(2-propenyl)-pyrazine; 3-methyl-1 H-pyrrole; 6-tridecane⁹³ - Dimethyl trisulphide⁸⁹ - 2-methylbutyl 2-methylbutyrate; amyl isovalerate; 2-methoxy-5-methylthiophene; 2-methylbutyl isobutyrate; 3-(ethylthio)-propanal⁸⁷ - 2-nonanone⁸² - 2-pentene; 2-heptanone⁸³ - Thiocyanic acid methyl ester; 2-butanol; 2-Pentanol⁸⁸ - Dimethyl pyrazine⁹⁴ - 2-propanone; azane [dimer]; dodecane; 2-ethylhexan-1-ol; P_603_25⁹¹ - 4-methylphenol⁹⁰ - Methyl mercaptan¹⁰⁴ - Acetophenone,⁹⁷ - Acetone; acetaldehyde¹⁰⁶
<i>E. coli</i>	<ul style="list-style-type: none"> - Indole^{82, 83, 86, 90, 91, 95} - Dimethyl sulfide^{81, 85, 106} - Ethanol^{76, 81, 85, 91, 106, 108, 109} - Acetaldehyde^{85, 106, 108, 109} - Propanol^{83, 85, 108} - Isoprene^{82, 83} - Acetic acid^{83, 106, 108} - Acetone^{76, 98, 106} - Hydrogen sulfide^{107, 109} - Benzaldehyde; 2,5-dimethylpyrazine; 2,5-dimethyltetrahydrofuran; 2-nonanone⁸⁶ - 6-methyl-5-hepten-2-one; ethyl acetate; 3-methyl furan⁸¹ - Carbon disulphide; methyl butanoate; dimethyl disulfide, dimethyl trisulfide, methyl propanoate⁸⁵ - Carbon disulfide; butanal⁸² - N-propylacetate⁸³

	- 2-methylbutanol; 3-methylbutanol ⁹⁵
	- Heptan-2-one; nonan-2-one ⁷⁶
	- Methyl cyclohexane; carbon dioxide; pentafluoropropionamide; Dimethylether ⁹⁸
	- Decan-1-ol; Octan-1-ol ⁹¹
	- Formaldehyde; methyl mercaptan ¹⁰⁷
	- ammonia ¹⁰⁹
<i>S. aureus</i>	- Ethanol ^{87, 106}
	- Acetic acid ^{87, 90}
	- Acetaldehyde ^{87, 106}
	- Acetone ^{83, 90, 106}
	- Dimethyl disulfide ^{83, 95}
	- Ammonia ^{106, 107}
	- 3-methylbutanal; 2-methylpropanal ⁸⁷
	- 1,1,2,2-tetrachloroethane; dimethyl trisulfide ⁸³
	- Isovaleric acid; 2-methylbutyric acid; isobutyric acid; 1-hydroxy-2-propanone; 3-hydroxy-2-butanone ⁹⁴
	- 2-tridecenone ⁹⁵
	- Butanol ⁹⁰
	- Dimethyl sulfide ¹⁰⁶
<i>MRSA</i>	- 2-heptanone; 1,4-dichlorobenzene ⁸³
<i>S. pyogenes</i>	-
<i>E. faecalis</i>	- Ammonia ¹⁰⁷
<i>S. epidermidis</i>	- 3-methyl-1-butanol; 2-methylbutanal; 3-methylbutanoic acid ; 2-methylbutanoic acid ⁸⁰
	- Ammonia; acetone ¹⁰⁷

VOC – volatile organic compound; *P. aeruginosa* – *Pseudomonas aeruginosa*; *E. coli* – *Escherichia coli*; *S. aureus* – *Staphylococcus aureus*; *MRSA* – methicillin resistant *staphylococcus aureus*; *S. pyogenes* - *Streptococcus pyogenes*; *E. faecalis* - *Enterococcus faecalis*; *S. epidermidis* – *Staphylococcus epidermidis*.

Table 2. Unique and shared VOCs between bacteria

VOC	PA	EC	SA	MRSA	EF	SE
Acetophenone; 4-methylphenol; 2-ethylhexan-1-ol; Dodecane; Dimethyl pyrazine; 2-Pentanol; 2-butanol; 2-pentene; 3-(ethylthio)-propanal; 2-methylbutyl isobutyrate; 2-methoxy-5 methylthiophene; amyl isovalerate; 2-methylbutyl 2-methylbutyrate; 6-tridecane; 3-methyl-1 H-pyrrole; 2-methyl-3-(2-propenyl)-pyrazine; 2; 3-dimethyl-5-isopentylpyrazine; Methyl thiolacetate; Methyl thiocyanate; Hydrogen cyanide; 2 aminoacetophenone; 2-butanone; 1-undecene;	*					
Formaldehyde; Octan-1-ol; Decan-1-ol; Dimethylether; Pentafluoropropionamide; carbon dioxide; Methyl cyclohexane; 2-methylbutanol; N-propylacetate; Butanal; Carbon disulfide; methyl propanoate; methyl butanoate; 3-methyl furan; ethyl acetate; 6-methyl-5-hepten-2-one; 2,5-dimethyltetrahydrofuran; 2,5-dimethylpyrazine; Benzaldehyde; Hydrogen sulfide; Propanol; Indole		*				
2-methylpropanal; 3-methylbutanal; 1,1,2,2-tetrachloroethane; Butanol; 2-tridecenone; 3-hydroxy-2-butanone; 1-hydroxy-2-propanone; Isobutyric acid;			*			
1,4-dichlorobenzene				*		
2-methylbutanoic acid;			*			*
methyl mercaptan; 2-nonanone; Isoprene	*	*				
2-methylbutanal; 3-methyl-1-butanol		*				*
3-methylbutanoic acid			*			*
2-heptanone	*	*		*		
Dimethyl sulfide; Dimethyl trisulfide; Dimethyl disulfide; Acetaldehyde; Acetic acid; Ethanol	*	*	*			

Ammonia	*		*		*		*
Acetone	*		*		*		*

VOC – volatile organic compound; PA – *Pseudomonas aeruginosa*; EC – *Escherichia coli*;
SA – *Staphylococcus aureus*; MRSA – *methicillin resistant staphylococcus aureus*; SP -
Streptococcus pyogenes; EF - *Enterococcus faecalis*; SE – *Staphylococcus epidermidis*.

For Peer Review

Figure Legend

Figure 1. Volatile organic compound sampling techniques. Spider diagram outlining the common volatile organic compound sampling techniques including their advantages and limitations. *VOC* – volatile organic compound; *GC*- gas chromatography; *SPME* – solid phase micro-extraction; *TD* – thermal desorption.

Figure 2. Volatile organic compound analytical techniques. Spider diagram outlining the common volatile organic compound analytical techniques including their advantages and limitations. *ToF* – time of flight; *GC*- gas chromatography; *MS* – mass spectrometry; *VOC* – volatile organic compound; *SIFT-MS* - Selected ion flow tube-mass spectrometry; *IMR-MS* - Ion molecule reaction mass spectrometry; *SESI-MS* - Secondary electrospray ionisation-mass spectrometry; *PTR-MS* - Proton transfer reaction-mass spectrometry; *IMS* – ion mobility spectrometry; *MCC* – multi capillary columns.

Figure 3. Common volatile organic compounds produced and emitted from skin. Volatile organic compounds in skin are produced by interactions between the secretions from sebaceous, apocrine and eccrine glands and bacteria. Volatile organic compounds detected can be affected by environmental factors. *VOC* – volatile organic compound.

Figure 4. Volatile organic compounds emitted bodily sites. Volatile organic compounds emitted from the human body vary dependent on site.

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Figure 4. Volatile organic compounds emitted bodily sites. Volatile organic compounds emitted from the human body vary dependent on site.

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