Analytical Methods

MINIREVIEW

View Article Online
View Journal | View Issue

Cite this: *Anal. Methods*, 2015, 7, 818

The *in vitro* identification and quantification of volatile biomarkers released by cystic fibrosis pathogens

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Received 15th December 2014
Accepted 6th January 2015

DOI: 10.1039/c4ay02981j

www.rsc.org/methods

There is interest in the development of exhaled breath tests for the detection of lower airway infection in children with cystic fibrosis. The first stage of this process is the identification of volatile organic compounds (VOCs) released into the gas phase by CF pathogens that can be used as breath test biomarkers. Selected ion flow tube mass spectrometry (SIFT-MS) is ideally suited to these *in vitro* studies as it allows simultaneous quantification of multiple VOCs in real time. We review a decade of *in vitro* experiments using SIFT-MS to analyse the VOCs released by respiratory pathogens. This includes identification and quantification of VOCs and the investigation of the *in vitro* factors that affect their production. We also report on how our culture methodology has been refined over the years to better account for variations in bacterial mass. Finally, we discuss how these *in vitro* findings have been translated into clinical trials and assess possible future applications.

Introduction

CF is the commonest inherited life-limiting condition in the Caucasian population affecting over 10 000 patients in the United Kingdom.[1] It is characterised by recurrent pulmonary infections leading to chronic inflammation, respiratory failure and death. The early and accurate detection of respiratory infections is a vital component of good CF care and is associated with reduced morbidity and mortality.[2] This is difficult in children who are often unable to expectorate sputum for microbiological culture. There is an urgent need for a non-invasive, child-friendly method to detect pulmonary infections in such children. One exciting possibility is the use of exhaled breath tests.[3] The concept of an exhaled breath test to detect a lower airway infection in children with CF was considered by our team after a number of patients were noted to have halitosis around the time they acquired a respiratory infection with one of the most important CF pathogens, *Pseudomonas aeruginosa* (PA). We believed that if a characteristic smell was present, then it should be possible to quantify this. The non-invasive and child-friendly nature of exhaled breath tests makes this a very appealing way of diagnosing lower airway infection in children with CF who cannot expectorate sputum. The potential for significant clinical value has driven our programme of research.

The first stage in the development of an exhaled breath test for the detection of a respiratory pathogen is the *in vitro* detection of volatile organic compounds (VOCs) that can potentially be used as biomarkers.[4,5] Identified VOCs must then be quantified and factors affecting their production established.[6–8] The second stage involves the analysis of exhaled breath samples taken from patients known to have pulmonary infection with the relevant pathogen.[9,10] This investigates if the VOCs identified *in vitro* can also be identified *in vivo* and attempts to assess the effect of host factors such as co-infection, dietary intake and diurnal variation.[11,12] If confirmed, further studies may be undertaken to investigate if the VOCs are detectable in exhaled breath early enough after new infection to allow them to be used as a screening test. This review will focus on the first stage of this process, specifically the methodologies used for the *in vitro* identification and quantification of the biomarkers of CF pathogens. The second stage is clearly of equal importance but is beyond the scope of this review. Our team has over a decades experience in the analysis of VOCs released into the gas phase by cultures of CF pathogens.[13] Our work initially focussed on PA as this is the most important CF pathogen but more recently we have investigated *Staphylococcus aureus* (SA), *Streptococcus pneumoniae* (SP), *Haemophilus influenzae* and *Aspergillus fumigatus* (AF). See Fig. 1.

Methods of VOC analysis

There are a number of different analytical techniques that have been used to identify the VOCs released into the gas phase by *in vitro* cultures. These include but are not limited to selected ion flow tube mass spectrometry (SIFT-MS),[6] gas chromatography

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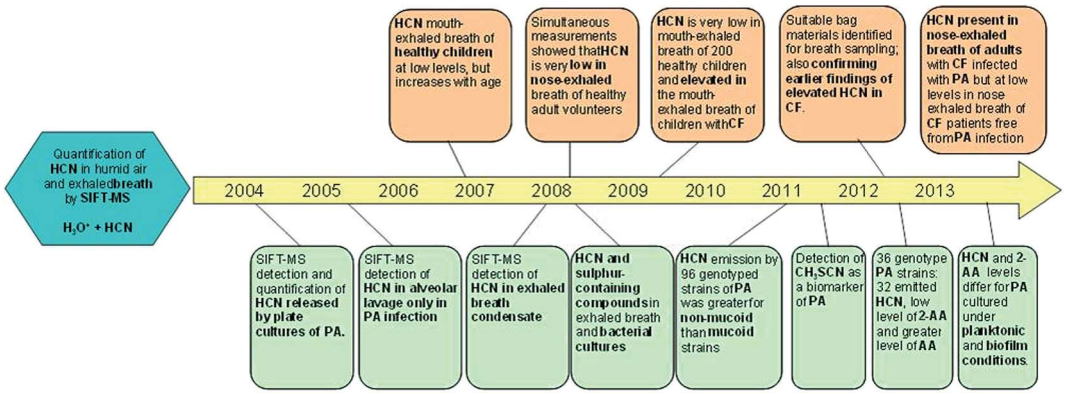

Fig. 1 The timeline of the research programme directed towards the study of gaseous HCN as a biomarker of PA infection in CF. The upper part of the scheme traces the *in vivo* studies with human subjects and the lower part traces the related parallel *in vitro* studies. Reproduced with permission from Smith *et al.*[13]

mass spectrometry (GCMS),[14] proton transfer reaction mass spectrometry (PTR-MS),[15] ion mobility spectrometry (IMS).[16] A detailed review of each of these techniques is beyond the scope of this review but each has relative pro and cons.[17] The technique most frequently used by our team is SIFT-MS and this will therefore be discussed in more detail.

Selected ion flow tube mass spectrometry

SIFT-MS combines fast flow tube reactors and quantitative mass spectrometry to allow accurate real-time quantification of VOCs down to a concentration of close to one part per billion by volume (ppbv).[18,19] See Fig. 2. The ability to give absolute, real time quantification of a number of compounds simultaneously makes SIFT-MS ideally suited to non-invasive and direct analysis of culture headspace. A mixture of precursor (reagent) ions is generated in a discharge ion source. The appropriate ion species (H_3O^+, NO^+ or O_2^+) is mass selected by a quadrupole mass filter, according to the compounds to be quantified, and then injected as selected ionic species into fast-flowing helium carrier gas in a flow tube. The gas to be analysed (in this case the culture headspace) is sampled directly into the carrier gas/precursor ion swarm at a known rate *via* a calibrated capillary. The reagent ions react with the trace gases in the sample producing ions that are characteristic of the trace gas molecules. These characteristic product ions, and the precursor ions and their hydrates are detected and counted by a downstream quadrupole mass spectrometer/ion detection system. An on-line

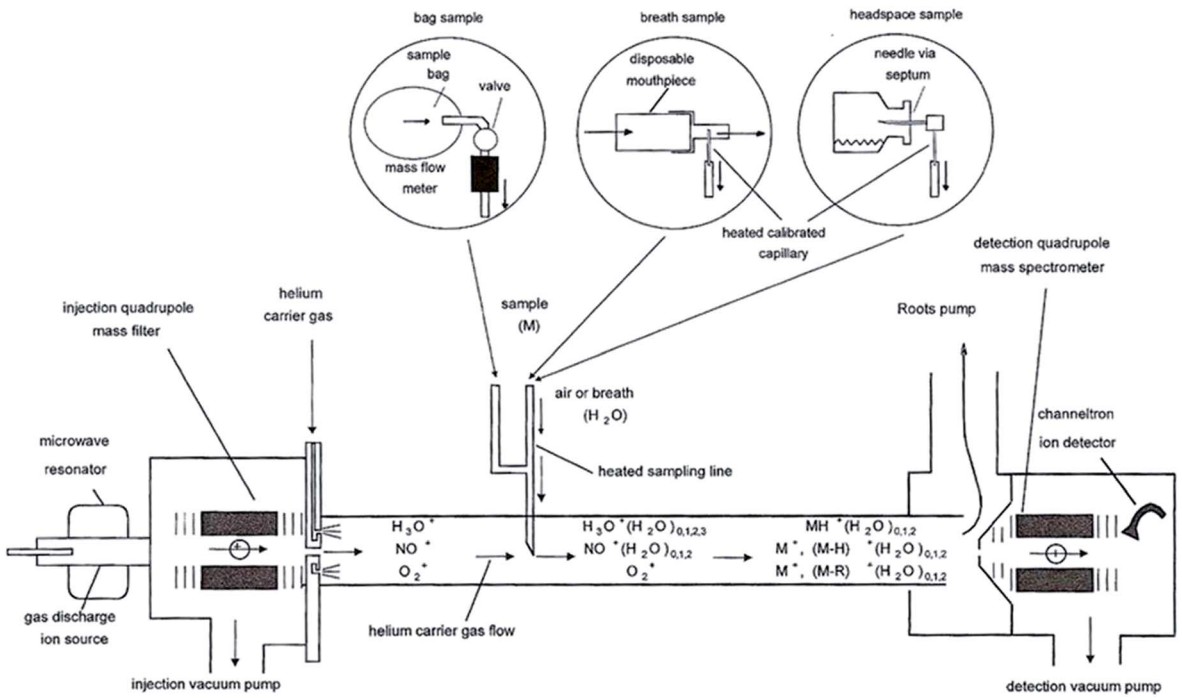

Fig. 2 The SIFT-MS Instrument. Used with permission from Španěl *et al.*[21]

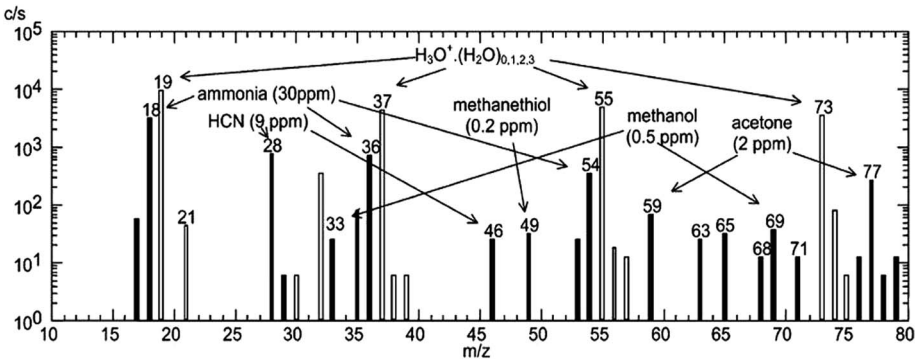

Fig. 3 SIFT-MS spectra of *Pseudomonas aeruginosa* culture headspace using H_3O^+ precursor ions. Expected compounds in culture headspace included ammonia, methanethiol, methanol and acetone. Compounds have multiple peaks due to the addition of water molecules. The peaks at *m*/*z* 28 and 46 were unexpected and identified as HCN. Used with permission from Španěl *et al.*[31]

computer calculates the partial pressures of the trace gases in the air sample from the precursor ion and characteristic product ion count rates and the sample gas flow rate as established by the calibrated capillary.[20]

SIFT-MS instruments can be operated in ether the full scan (FS) or the multiple ion monitoring (MIM) mode. Using the full-scan (FS) mode a complete mass spectrum is obtained by sweeping the detection quadrupole over a chosen mass–charge ratio (*m*/*z*) range. This is done for a selected time whilst a sample of air or breath is introduced into the carrier gas at a steady flow rate. The count rates of the ions are then calculated from the numbers of counts and the total sampling time for each ion. The mass spectra are interpreted by relating the product ion peaks to the trace gases present in the sample from a detailed knowledge of the ion chemistry and the in-built database. An example is shown in Fig. 3. This mode is used in the initial experiments to identify the VOCs released by particular organism. In the MIM mode, only the count rates of the precursor ions and those of selected product ions are monitored. This is achieved by rapidly switching the downstream mass spectrometer between the masses of all the primary ions and the selected product ions and dwelling on each of these masses for a predetermined short time interval, which effectively increases the analysis time and hence the precision of measurement. This real-time monitoring is possible because of the fast time response of SIFT-MS, which is about 20 milliseconds and is largely determined by the fast flow rates of the carrier gas along the flow tube and the sample gas along the inlet tube. There is no fundamental limit to the number of different ion masses that can be recorded simultaneously using this technique, but fewer ions result in greater analytical precision within a given analytical time frame.[21] The MIM mode is used once the VOCs of interest have been identified and the aim of the experiment changes from identification to quantification.

Methodology of culture headspace analysis

The aim analysing culture headspace using SIFT-MS is to achieve high quality sampling with little or no disturbance of the culture.[7] To achieve this, the cultures are prepared, sealed inside individual low-density polyethylene (LDPE) bags and then incubated. At the time of analysis, the bag is punctured with a hypodermic needle connected directly to the sample input port of the SIFT-MS instrument. The needle is carefully placed so that it is in the headspace but does not come into direct contact with the culture (see Fig. 2). The gas/vapour developed above the cultures is introduced at a set flow rate (*via* a heated calibrated capillary) into the carrier gas of the SIFT-MS instrument. The sample can be analysed using either the FS or MIM mode of SIFT-MS. When the MIM is used, the sample is analysed for 100 seconds and the mean concentrations of the relevant compounds are recorded over this time. Following analysis, the hypodermic needle is removed whilst keeping the bag sealed, the lid replaced and the puncture hole left by the needle covered with tape. This methodology allows the culture headspace of a single culture to be analysed at multiple durations of incubation with minimal disruption to the culture itself. Whenever cultures are analysed in this way it is also necessary to analyse control cultures. These are samples of sterile culture medium (agar or liquid) that are sealed in LDPE bags and incubated using the same methodology as the cultures. Comparison of the VOCs released into the gas phase by the cultures and control cultures confirms which are released by the bacteria and which are released by the culture medium or LDPE bags.

Culture preparation

Agar plate cultures

Our initial studies used simple agar plate cultures.[4,6,7] Clinical isolates were plated out onto the appropriate agar plate, covered with a lid, sealed in an individual LDPE bag (127 mm × 203 mm) and incubated. When the headspace was sampled the lid was lifted to allow the needle attached to the sample input port of the SIFT-MS to be sited in the headspace whilst keeping the bag sealed. After analysis, the needle was removed, the lid replaced and the puncture hole sealed with tape. The main disadvantage of using agar plate cultures is that it is not possible to account for the effect of bacterial mass which will potentially affect the quantity of VOCs produced by a culture.

Liquid cultures

In an attempt to standardise the bacterial mass, liquid cultures were used in our later studies.[7,22–24] The bacteria sample was initially cultured overnight on agar, the colonies that formed then used to produce saline suspensions. The turbidity of the suspensions was adjusted to approximately 0.5 optical density units (assessed using a spectrophotometer at 600 nm), which approximates to 10^8 colony forming units (CFUs) per mL. 150 mL glass bottles containing 10 mL of the appropriate liquid media were then inoculated with 0.5 mL of the saline suspension. The bottles were immediately sealed with lids containing a rubber septum and incubated without agitation. The quantity of cells in each inoculum was additionally assessed by plating 10^5 fold dilutions of the saline suspensions onto agar plates, which were incubated for 24 h after which the colonies were counted.

The headspace of the liquid cultures was sampled by piercing the rubber septum with a hypodermic needle connected directly to the sample inlet port of the SIFT-MS instrument. The headspace volume over the 10 mL liquid culture was typically 140 mL. A sample flow rate of 30 mL min^{-1} was used and the pressure in the bottle was maintained at atmospheric pressure by balancing the loss of headspace gas/vapour by a flexible bag containing dry sterile cylinder air. During the sampling period of 100 seconds, a temporary dilution of the headspace occurred resulting in a small reduction in the VOC concentrations. Headspace/liquid phase equilibrium in the VOC concentrations was rapidly re-established.[22,23]

Planktonic and biofilm cultures

A number of CF pathogens, including *Pseudomonas aeruginosa* (PA) and *Burkholderia cepacia complex* (BCC), are able to form biofilms.[8] During early pulmonary infection, these organisms attach to the respiratory epithelium and multiply whilst in a planktonic (free-floating) form. Once a certain bacterial density is reached, growth slows and biofilm production is signalled. This signalling occurs by quorum sensing in which extracellular molecules are used to regulate phenotype in response to population density.[25] Biofilms are communities of non-motile organisms embedded in an exopolysaccharide matrix attached to a solid surface.[26] When growing in this form, these organisms are more difficult to remove by mucociliary clearance and have increased antibiotic resistance.[27] A mucoid phenotype is a marker of biofilm formation.[28]

As a number of quorum sensing molecules are known to be volatile, it was important to be able to produce *in vitro* biofilm cultures to assess their effect on the pattern of headspace VOCs.[7,24] Initially a liquid culture was produced, as detailed above; 10 mL of this liquid culture was pipetted into a sterile Petri dish containing 4 mm diameter sterile glass beads (sufficient to evenly cover the bottom of the Petri dish). A lid was then placed on the Petri dishes and the dish sealed in an individual LDPE bag and incubated. The liquid media was changed daily by pipetting off approximately 10 mL of spent media and replacing with 10 mL of fresh media.[7,24] Planktonic cultures were created by pipetting the liquid culture in to a Petri dish that did not contain glass beads; all other aspects of the preparation and analysis was identical to the biofilm cultures. This technique was adapted from previously published papers.[29] The biofilm forms around the glass beads during the next 48–72 hours (see Fig. 4).

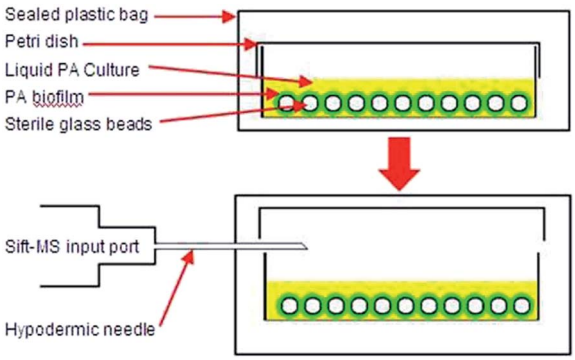

Fig. 4 Headspace analysis of a biofilm culture of *Pseudomonas aeruginosa* using SIFT-MS. Used with permission of Gilchrist *et al.*[7]

Biofilm formation was assessed visually on a daily basis and then quantified using spectrophotometry after 96 hours (after headspace analysis had been completed). To do this, individual glass beads were removed from the culture and washed in crystal violet that penetrates the bacteria cell wall and membrane. Each bead was then submerged in a defined volume of industrial methylated spirits (IMS) to solubilise the biofilm. The IMS (containing the solubilised biofilm) was analysed using the spectrophotometer set at 600 nm. As the number of bacteria in the biofilm increases, the aqueous concentration of crystal violet increases which causes increased absorbance. This method of assessing biofilm formation is well established,[29] a possible criticism of this method is that it is actually assessing bead adherence and not biofilm formation. True biofilm formation can be assessed by the detection of non-volatile quorum sensing signalling molecules, but we do not have this facility in our laboratory.[30]

VOCs released by cultures of PA

Detection of hydrogen cyanide

The VOCs released by *in vitro* cultures of PA were the first that our team analysed.[4] Using the H_3O^+ precursor ions, the SIFT-MS FS spectrum reveals the presence of several VOCs. Unusually, it consistently showed product ions with mass-to-charge-ratio (m/z) of 28 and 46 (see Fig. 3). These ions were not present at significant levels in the headspace of control cultures (see Fig. 5). The product ion with an m/z value of 28 was identified as protonated HCN (H_2CN^+). $C_2H_4^+$, N_2^+ and CO^+ were excluded as possibilities as they cannot be formed in the reaction of H_3O^+ with either C_2H_4, N_2 and CO (all with molecular mass of 28). It follows from the known ion chemistry of HCN, as investigated thoroughly in our extensive study, that the product ion at m/z 46 is $HCNH^+ \cdot H_2O$, the monohydrate of protonated HCN.[31] This thorough study of the ion chemistry of HCN allowed the required SIFT-MS kinetics database to be constructed, thereby allowing accurate quantification of gaseous HCN in moist air

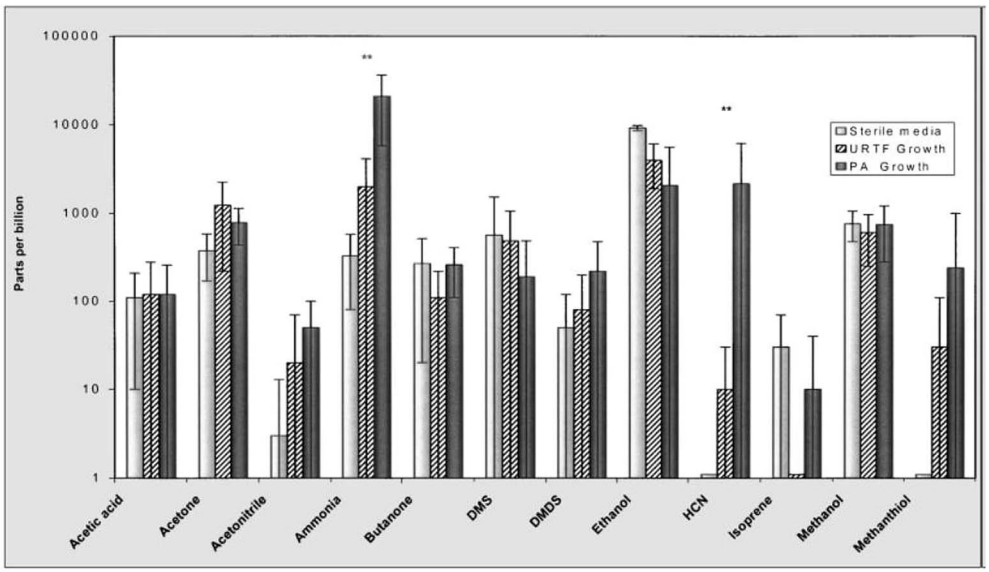

Fig. 5 Volatile compounds detected in the headspace of *Pseudomonas aeruginosa*, upper respiratory tract flora and blank cultures. Mean concentration of volatiles in parts per billion (ppb). ** indicate statistical significance of the difference ($p < 0.05$). Used with permission Carroll *et al.*[4]

samples. SIFT-MS studies then confirmed the presence of HCN in the headspace of PA cultures. Although it had been known for a number of years that PA can produce cyanide ions (CN^-), this SIFT-MS study was the first study to detect and quantify HCN released into the gas phase by PA cultures, revealing it as a possible biomarker in exhaled breath of PA infection of the airways.

In total, 22 PA cultures and 13 control cultures (6 sterile plates and 7 cultures of mixed upper respiratory tract flora [URTF]) were analysed after 48 hours incubation.[4] HCN was detected in the headspace of 15/22 PA and 1/7 URTF cultures. The mean concentration of the 15 positive PA cultures (2170 ppbv) was significantly higher than the concentration of the single positive URTF culture (60 ppbv) (see Fig. 5). Using a headspace HCN of > 100 ppbv for the identification of PA gave a sensitivity of 68% and a specificity of 100%. The other VOCs identified, were not specific to PA.

Subsequently, to confirm that PA was the only CF pathogen to produce HCN we analysed the culture headspace of multiple samples of *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Moraxella catarrhalis*, *Haemophilus influenzae* and *Aspergillus fumigatus*. For all these organisms, the headspace HCN concentration remained very low (<10 ppb) at all incubation time points. The headspace HCN concentration in the control cultures (sterile agar plates) also remained <10 ppbv at all durations of incubation.[6]

The effect of genotype, phenotype and biofilm formation

As part of a previous study assessing cross infection between CF patients, 96 PA isolates obtained were genotyped and then stored on cryogenic beads (Pro-Lab Diagnostics. Microbank™ Product code PL 172) at −70 degrees Celsius. After gaining ethical approval, we analysed the HCN released into the headspace after 24, 48, 72 and 96 hours of incubation.[6] All 96 PA cultures had elevated HCN concentrations in the culture headspace, but the quantity varied according to the PA genotype.

Increased HCN concentrations were found in the headspace of non-mucoid PA samples compared to mucoid samples. This was in contrast to previous studies that identified elevated concentrations of the non-volatile ion cyanide (CN^-) in mucoid PA cultures.[32,33] One possible explanation for this is that mucoid PA cultures do produce cyanide, but the layer of alginate that covers mucoid cultures prevents this from being released as HCN into the gas phase. It is again important to emphasize that the SIFT-MS method of detecting HCN in the culture headspace does not disrupt the culture in contrast to the detection of cyanide using a cyanide ion-selective electrode. Since any *in vivo* breath test for PA detection would be based on the analysis of VOCs, we focus on the factors that affect gaseous HCN (volatile) rather than cyanide ions (non-volatile).

As HCN is a quorum sensing molecule, we expected the formation of biofilms to increase the production of HCN by PA as seen with BCC.[29] However, a subsequent study using a small number of the same samples did not reveal any difference in the headspace HCN concentration for biofilm and planktonic PA cultures.[7] The likely explanation for this is related to our explanation for the difference in the HCN production by non-mucoid and mucoid PA cultures. This is, PA biofilm cultures do produce cyanide ions, but any gaseous HCN remains trapped within the biofilm and is therefore not released into the gas phase. Our team and our collaborators have thoroughly investigated the other VOCs released into the gas phase by cultures of PA, but HCN remains the most reliable biomarker.[34,35]

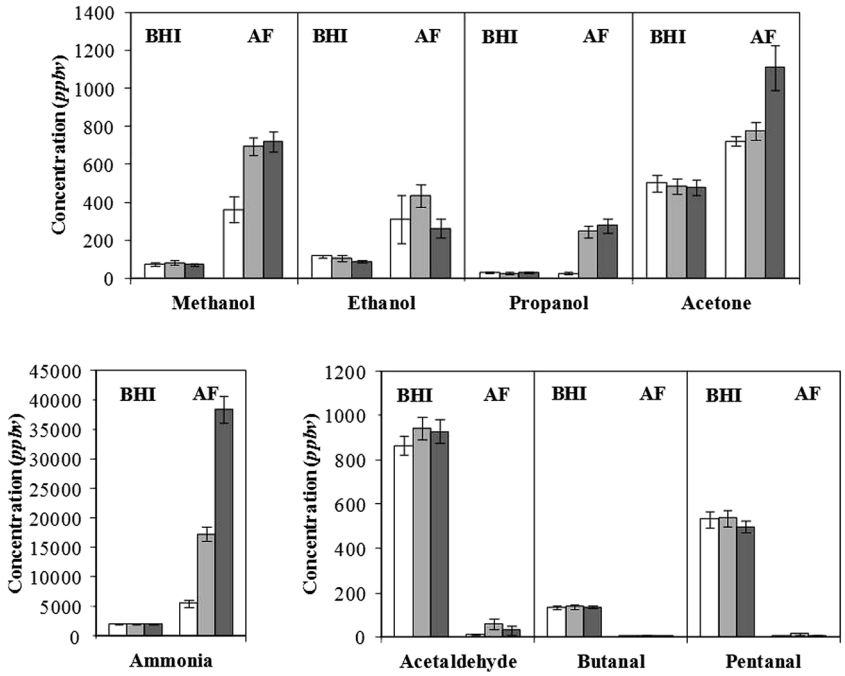

Fig. 6 Bar plots of the concentrations of several compounds present in the headspace of sterile culture media (BHI) and cultures of *Aspergillus fumigatus* (AF). Various durations of incubation: 24 h (open bars), 48 h (light grey bars) and 72 h (dark grey bars). Error bars indicate the standard errors. Note the abbreviations DMS (dimethyl sulphide) and DMDS (dimethyl disulphide). Used with permission from Chippendale.[23]

VOCs released by other CF pathogens

Staphylococcus aureus (SA)

The headspace of liquid SA cultures was analysed using SIFT-MS in FS mode at various period of incubation. Control cultures (sterile culture medium) were analysed using the same methodology. Six VOCs were present in higher concentration in the SA headspace compared to the control cultures. These were ammonia, methanol, acetaldehyde, ethanol, propanol and pentanal.[22]

Streptococcus pneumoniae (SP)

The VOCs released in the headspace above liquid SP cultures were analysed using SIFT-MS in FS mode over various durations of incubation. The medium used to culture SP was the same as that used to culture SA. The same control cultures (sterile culture medium) were therefore used. Eight VOCs were present in higher concentration in the SP headspace compared to the control cultures. These were methanol, acetaldehyde, ethanol, acetone, propanol, butanal, butanone and pentanal. Unlike the SA culture headspace, the concentrations of all of the identified compounds continuously increased during the incubation period. The ammonia concentration was lower in the SP headspace compared to the control culture headspace. This is likely related to the lower pH of the culture.[22]

Aspergillus fumigatus (AF)

Using a similar methodology to the SA and SP experiments, the headspace of liquid AF fungal cultures was compared to the headspace of control cultures (sterile culture medium). High concentrations of ammonia, methanethiol, dimethyl sulphide and dimethyl disulphide were identified in the AF culture headspace. The AF headspace had lower concentrations of acetaldehyde, butanal and pentanal (see Fig. 6). Interestingly, the VOCs emitted by co-cultures of AF with PA, SA and SP revealed that the biomarker HCN (for PA) is not compromised by the presence of AF, and the organosulphur compounds (for AF) are not compromised by the presence of SA or SP.[23]

Conclusions

The development of exhaled breath tests for the detection of lower airway infections in children with CF could revolutionise their care. The first stage in this process is the *in vitro* identification of a VOC or a pattern of VOCs that is specific to that organism. In the past decade our team has gained great experience in the microbiology and SIFT-MS techniques necessary to identify and quantify potential volatile biomarkers. Our work investigating HCN as marker of PA infection has demonstrated that VOCs identified in the headspace of bacterial cultures can be used as biomarkers in an exhaled breath test. We hope to develop a similar programme of research in relation to other respiratory pathogens including SA, SP and AF.

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