In vitro volatile organic compound profiling using GC×GC-TOFMS to differentiate bacteria associated with lung infections: A proof-of-concept study

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Abstract. Chronic pulmonary infections are the principal cause of morbidity and mortality in individuals with cystic fibrosis (CF). Due to the polymicrobial nature of these infections, the identification of the particular bacterial species responsible is an essential step in diagnosis and treatment. Current diagnostic procedures are time-consuming, and can also be expensive, invasive and unpleasant in the absence of spontaneously expectorated sputum. The development of a rapid, non-invasive methodology capable of diagnosing and monitoring early bacterial infection is desired. Future visions of *real-time*, *in situ* diagnosis *via* exhaled breath testing rely on the differentiation of bacteria based on their volatile metabolites. The objective of this proof-of-concept study was to investigate whether a range of CF-associated bacterial species (*i.e. Pseudomonas aeruginosa*, *Burkholderia cenocepacia*, *Haemophilus influenzae*, *Stenotrophomonas maltophilia*, *Streptococcus pneumoniae* and *Streptococcus milleri*) could be differentiated based on their *in vitro* volatile metabolomic profiles. Headspace samples were collected using solid phase microextraction (SPME), analyzed using comprehensive two-dimensional gas chromatography – time-of-flight mass spectrometry (GC×GC-TOFMS) and evaluated using principal component analysis (PCA) in order to assess the multivariate structure of the data. Although it was not possible to effectively differentiate all six bacteria using this method, the results revealed that the presence of a particular pattern of VOCs (rather than a single VOC biomarker) is necessary for bacterial species identification. The particular pattern of VOCs was found to be dependent upon the bacterial growth phase (*e.g.* logarithmic *vs.* stationary) and sample storage conditions (*e.g.* short-term *vs.* long-term storage at -18 °C). Future studies of CF-associated bacteria and exhaled breath condensate will benefit from the approaches presented in this study and further facilitate the production of diagnostic tools for the early detection of bacterial lung infections.

1. Introduction

Cystic fibrosis (CF) is the most common lethal inherited disorder in the Caucasian population [1]. It is characterized by the accumulation of excessively thick, sticky mucus that blocks the airways and is associated with the increased prevalence of certain pathogenic bacteria. Persistent bacterial colonization in the lungs precedes recurrent pulmonary infections causing inflammation and irreversible lung damage, which often leads to respiratory failure and death. Some studies have suggested that early, aggressive antibiotic therapy may increase the chances of preventing or delaying chronic bacterial colonization [2–5]. Early detection of established colonization with pathogenic bacteria (such as *Pseudomonas aeruginosa*) is critical for CF patients because chronic respiratory infections are difficult to eradicate from CF-infected airways once acquired.

The detection of CF lung infections generally relies on culturing of pathogenic microbiological organisms from lower airway secretions [6]. As expectorated samples can be difficult to obtain in patients with negligible sputum production, particularly in children, alternative methods such as sputum induction [7] or bronchoalveolar lavage [8] are often required. These procedures are time-consuming, expensive, invasive and unpleasant, especially for young children and infants.

CF lungs can be colonized and infected by many different bacterial species (*e.g. Haemophilus influenzae*, *Burkholderia cepacia* complex, *Pseudomonas aeruginosa*, etc.) [1]. Identification of the specific pathogen allows for targeted antibiotic therapy, which reduces the likelihood for the development of antibiotic resistance, and improves bacterial eradication and clinical response [6]. The correct culture conditions are vital for bacterial identification [2], and may require several different selective growth media and several days for identification and antibiotic susceptibility testing.

For these reasons, a rapid non-invasive diagnostic technique to monitor bacterial infection in CF patients is highly desirable. Current focus in the literature is aimed at developing techniques for exhaled breath analysis [9–13]. Future visions involve *real-time* analysis of exhaled breath (*e.g.* bedside diagnosis using an electronic nose) [14–16], which could provide clinicians with immediate information, facilitating rapid diagnosis and treatment, in addition to therapeutic monitoring. The non-invasive nature of exhaled breath testing is also appealing for pediatric patients that may have difficulty producing sputum samples for microbiological culturing. However, before these objectives can be met, the first step in the development of an exhaled breath test for the diagnosis of respiratory infections is the identification of volatile organic compounds (VOCs) that: 1) can be used as suitable pathogen-specific biomarkers [17,18]; and 2) may reflect alterations in growth characteristics in important bacterial populations.

A number of different analytical techniques have been reported throughout the literature for the detection and identification of VOCs produced by *in vitro* cultures of CF pathogens including: selected ion flow tube mass spectrometry (SIFT-MS) [17,19], proton transfer reaction mass spectrometry (PTR-MS) [20,21], ion mobility spectrometry (IMS) [22,23], gas chromatography-mass

spectrometry (GC-MS) [18,24,25] and more recently comprehensive two-dimensional gas chromatography – time-of-flight mass spectrometry (GC×GC-TOFMS) [26,27]. Although GC-MS is typically considered the gold standard amongst analytical methods in this field [18], it often suffers from insufficient peak capacity, limited sensitivity and restricted selectivity; this makes co-eluting peaks, chromatographic artefacts and dynamic range difficult to manage [28]. Multidimensional techniques, such as GC×GC-TOFMS, offer distinct advantages when faced with complex matrices that exhibit these issues. The benefits of GC×GC have been highlighted in many fields including: petroleum products [29,30], food and flavour [31,32], environmental studies [33], forensic science [28,34,35] and metabolomics [36,37]. In the first reported application of GC×GC-TOFMS for *in vitro* bacterial headspace characterization, Bean et al. identified 28 new VOCs that had not been previously reported for *Pseudomonas aeruginosa* [26]. This nearly doubled the list of previously published volatile metabolites for one of the most prevalent bacterial species associated with CF lung infections, demonstrating the wealth of information that can be gained by using GC×GC-TOFMS.

Many of the microbial species that can produce pulmonary infections in CF patients experience similar metabolic pathways; therefore, the challenge is determining whether CF-associated bacterial species can, in fact, be discriminated by their volatile metabolites [18]. Previous studies have attempted to identify species-specific volatile biomarkers for CF-associated pathogens; however, the number of bacterial species studied is often limited (*e.g.* n = 2) [24,25]. The principal objective of this proof-of-concept study was to investigate whether a range of bacterial species (n = 6) could be differentiated based on their volatile metabolomic profiles collected using headspace solid phase microextraction (HS-SPME) and analyzed by GC×GC-TOFMS. GC×GC-TOFMS was employed in order to expand the VOC profile available for characterization. The bacterial species investigated were chosen based on their association with CF lung infections [1,38–40]. The species examined included: *Pseudomonas aeruginosa*, *Burkholderia cenocepacia*, *Haemophilus influenzae*, *Stenotrophomonas maltophilia*, *Streptococcus pneumoniae* and *Streptococcus milleri*. Because access to analytical instrumentation may be limited and samples often need to be stored prior to analysis, a secondary aim of this study was to investigate whether the volatile metabolomic profiles of the bacteria changed over time. To do this bacterial headspace samples were collected and analyzed after both short-term (*i.e.* 2 – 5 days) and long-term (*i.e.* 48 – 50 days) storage at -18 °C. Each bacterial species was also cultured under two distinct growth phase conditions (*i.e.* logarithmic and stationary) in order to examine the volatile metabolomic profiles produced during clearly contrasting metabolic states.

2. Materials and Methods

2.1. Bacterial Culturing

The bacterial isolates were stored at -80 °C in Luria broth, Lennox (LB-Lennox; 10 g tryptone, 5 g yeast extract, 5 g NaCl) with 10 – 15% glycerol. *H. influenzae* was subcultured onto chocolate agar plates while all other isolates were subcultured onto blood agar (Oxoid Blood Agar base No. 2, Basingstoke, Hampshire, England) containing 5% defibrinated horse blood. The agar plates were incubated aerobically at 37 °C overnight. *S. pneumoniae* and *H. influenzae* plates were incubated overnight at 37 °C with 5% CO_2. Single colonies were taken from fresh culture plates grown overnight and inoculated into 5 mL of LB-Lennox or Brain Heart Infusion (BHI) broth inside sterilized 20 mL headspace vials, which were sealed airtight with a screw cap containing a 1.3 mm thick polytetrafluoroethylene/silicone septum (Sigma-Aldrich, Castle Hill, NSW, Australia). *S. pneumoniae* and *H. influenzae* were cultured in BHI broth while all other bacteria were grown in LB-Lennox. All strains were cultured at 37 °C with vigorous shaking (200 rpm) for 16 h in order to advance the development of samples of each bacterial species to the stationary phase of growth. After 16 h of growth, bacterial cultures were cooled on ice. To obtain samples of each bacterial species in the logarithmic phase of growth, freshly grown cultures (prepared overnight as described above) were inoculated as 1:100 dilutions in 5 mL of LB-Lennox or BHI broth inside sterilized 20 mL headspace vials sealed airtight. All strains were then cultured at 37 °C for 5 h with vigorous shaking (approximately 220 rpm) before being cooled on ice. Following bacterial culturing, which was performed off-site, the samples were transported on ice to the laboratory where they were stored at -18°C prior to analysis.

Each bacterial species was prepared in quadruplicate during both logarithmic and stationary phases of growth. Two of the bacteria samples were stored for 2 – 5 days at -18 °C prior to HS-SPME VOC collection and GC×GC-TOFMS analysis (*i.e.* short-term storage), while the other two samples were stored for 48 – 50 days at -18 °C (*i.e.* long-term storage) prior to analysis, therefore resulting in duplicate bacteria samples per overall treatment. Duplicate samples of LB-Lennox and BHI broth controls (*i.e.* broth only) were also prepared under both logarithmic and stationary growth phase conditions for the purpose of determining background VOCs associated with the growth media. Similar to the bacteria samples, half of the LB-Lennox and BHI broth control samples were stored under short-term storage conditions while the other half were stored under long-term storage conditions; this resulted in one replicate LB-Lennox control and one replicate BHI broth control per overall treatment.

2.2. HS-SPME VOC Collection

VOC collection was carried out *via* a previously optimized headspace sampling method (adapted from Bean et al. [26]) using a 50/30 μm divinylbenzene/carboxen/polydimethylsiloxane

(DVB/CAR/PDMS) 24 Ga Stableflex SPME fibre and manual fibre holder (Supelco, Bellefonte, PA, USA). The fibre was initially conditioned for 60 min at 270 °C before first use, according to the manufacturer's recommendations. Fibre reconditioning (5 min at 250 °C) was performed as necessary. A fibre blank was completed before sampling and after every 4 sample injections using the GC×GC-TOFMS method described below.

Prior to VOC collection, the SPME fibre was pre-loaded with an internal standard. This was performed by exposing the SPME fibre to the headspace of 200 µL of a 100 ppm solution of bromobenzene (GC grade, Sigma-Aldrich), prepared in methanol (HPLC grade, Sigma-Aldrich) inside a sealed headspace vial, for 15 s at room temperature. The pre-loading of an internal standard onto a SPME fibre has previously been demonstrated to exhibit good reproducibility for volatile and semivolatile internal standard analytes [41]. Using this approach the internal standard vial may be reused for numerous analyses without exhibiting significant loss [41]. This technique has been previously optimized in our laboratory to provide a relative standard deviation of <15% in the internal standard peak area. In this study, the intraday precision ranged from 4.2 – 14.7%, with an interday precision of 12.2% for the overall trial.

The HS-SPME sampling method (adapted from Bean et al. [26]) included sample thawing, sample incubation and sample headspace extraction. Sample thawing was performed at 4 °C overnight. Prior to headspace extraction, the sample was incubated for 10 min at 50 °C in a dry bath heating block (Thermoline Scientific, Wetherill Park, NSW, Australia). Sample extraction was achieved by exposing the SPME fibre to the sample headspace for 10 min while the sample was maintained at 50 °C. The SPME fibre was thermally desorbed in the GC×GC inlet for 5 min at 250 °C.

2.3. GC×GC-TOFMS Analysis

Sample analysis was conducted on a Pegasus® 4D GC×GC-TOFMS system (LECO, Castle Hill, NSW, Australia) equipped with a liquid nitrogen cryogenic quad jet modulator. The column configuration consisted of a 30 m × 0.250 mm inner diameter (ID), 1.40 µm film thickness Rxi®-624Sil MS column (Restek Corporation, Bellefonte, PA, USA) in the first dimension (^{1}D) and a 2 m × 0.250 mm ID, 0.50 µm film thickness Stabilwax® column (Restek Corporation) in the second dimension (^{2}D). The ^{1}D and ^{2}D columns were connected by a SilTite™ µ-Union (SGE Analytical Science, Wetherill Park, NSW, Australia).

The speed optimized flow [42] and optimal heating rate [43] were calculated in order to obtain optimal resolution in the ^{1}D. High purity helium (BOC, Sydney, NSW, Australia) was used as the carrier gas with a constant flow rate of 2.0 mL/min. Sample introduction was performed using splitless injection with a 30 s purge time. The primary oven was programmed to begin at 40 °C (held for 0.20 min) and was increased to 230 °C (held for 0.80 min) at a rate of 10 °C/min. Relative to the

primary oven, the secondary oven was programmed to have a constant offset of +5 °C and the modulator a constant offset of +30 °C. A modulation period of 4 s was used with a 0.4 s hot pulse and 1.6 s cooling time between stages. Mass spectra were collected from *m/z* 25 – 500 at a rate of 200 Hz with an acquisition delay of 120 s. A 200 V offset above the optimized detector voltage was used, the electron ionization energy was set at 70 eV and the ion source and MS transfer line were maintained at 200 °C and 250 °C, respectively.

2.4. Data Processing

ChromaTOF® (version 4.51.6.0; LECO) was used for data processing. The baseline was automatically smoothed by the software with an 80% offset. The ^{1}D peak width was set at 20 s while the ^{2}D peak width was set at 0.1 s. The minimum signal-to-noise ratio (*S/N*) for the base peak and sub-peaks was set at 250 and 20, respectively. A minimum similarity match >800 to the 2011 National Institute of Standards and Technology (NIST) mass spectral library database was used for initial identification.

Peak identifications were supported with the use of ^{1}D retention indices and retention time matching with chemical standards when possible using a standard test mixture containing a range of compounds covering several different compound classes (complete list of standards documented in Perrault et al. [44]). The ^{1}D retention indices were calculated using the *n*-alkanes within the standard test mixture and a Retention Index Method created in ChromaTOF®. For analysis of the standards, the SPME fibre was exposed to 1 µL of a 100 ppm solution of the test mixture, prepared in carbon disulfide (≥99% anhydrous, Sigma-Aldrich) inside a sealed headspace vial, for 10 min at 50 °C in a dry bath heating block. The SPME fibre was then desorbed for analysis as previously described.

The Statistical Compare software feature in ChromaTOF® was used for peak alignment. Samples were input into Statistical Compare and two approaches were taken to facilitate visualization by multivariate analysis. In the first approach (denoted approach A), the samples were processed in two separate files based on growth phase; within each of these files seven classes were created that included the control samples (*i.e.* media) as a single class (n = 4 samples) and one class for each individual bacterial species (n = 6 classes with n = 4 samples within each class). For approach A, analytes were only retained if found in 75% of the samples within a class. This approach was used to investigate the primary objective of the paper (*i.e.* to determine whether the six bacterial species could be differentiated based on their volatile metabolomic profiles). In a second approach (denoted approach B), the samples were input into a single file and separated into two classes: control (n = 8 samples) and bacteria (n = 48 samples). For approach B, analytes were only retained if found in 24 samples out of the 56 total samples or if found in 50% of the samples within a class. This approach was applied to see if any further information could be extracted from the data with the use of a two-class model, conforming to the original design of Statistical Compare.

For both approaches, the following settings applied. A *S/N* of 20 was used to search for peaks not found during the initial peak finding step. A mass spectral match >600 was required for peaks to be identified as the same compound across chromatograms during alignment. When analytes did not meet this mass spectral match threshold during alignment they were removed from the final compound list. To allow for retention time deviations between samples the maximum retention time differences specified in the ^{1}D and ^{2}D were 4 s (*i.e.* 1 modulation period) and 0.6 s, respectively. After alignment, the analyte peak areas (calculated using unique mass) were normalized using the bromobenzene internal standard peak area. A Fisher ratio (*i.e.* the ratio of between-class variance to within-class variance) was also calculated for each analyte using the Statistical Compare software feature. In the case where an analyte was absent from a class or only detected in a single sample in a class, the within-class variance could not be calculated (or was equal to 0) and a value of undefined was given for the Fisher ratio. Analytes with higher Fisher ratio values (or those labelled as "undefined") indicated compounds that statistically differed in abundance between the defined classes.

Fisher ratio filtering was performed based on its success in previous applications for identifying class-distinguishing compounds [45–49]. Compounds with Fisher ratios above the critical value (F_{crit} = 2.57 and 4.02 for approaches A and B, respectively), which includes those labelled as "undefined", were exported as a *.csv file and imported into Microsoft Excel for the manual removal of chromatographic artefacts (*i.e.* column bleed and phthalates). The *F*-distribution was used to calculate F_{crit} for each aligned Statistical Compare compound list. The F_{crit} value is computed based on three approach-dependent criteria: the number of classes in the analysis, the degrees of freedom for each class and the significance level chosen (α = 0.05). Principal component analysis (PCA) was carried out in The Unscrambler® X (version 10.3; CAMO Software, Oslo, Norway). Data pre-processing steps performed in The Unscrambler® X prior to PCA included mean centring, variance scaling and unit vector normalization. These pre-treatment steps have been previously demonstrated for multivariate VOC analyses [50,51]. Following PCA, each dataset was evaluated for outlying samples by means of the Hotelling's T^2 95% confidence limit. All data were verified to contain no outliers.

3. Results and Discussion

3.1. HS-SPME-GC×GC-TOFMS Results

During this study, headspace samples were collected from six different CF-associated bacterial species and two different growth media (*i.e.* controls) using HS-SPME and analyzed using GC×GC-TOFMS. Figure 1 displays GC×GC-TOFMS total ion current (TIC) contour plots of the BHI control broth and the two bacterial species cultured in BHI broth: *H. influenzae* and *S. pneumoniae*. GC×GC-TOFMS TIC contour plots of the LB-Lennox control broth and the bacterial species cultured

in LB-Lennox (*i.e. P. aeruginosa*, *B. cenocepacia*, *S. maltophilia* and *S. milleri*) are displayed in figure 2. A scale of 0 – 40% of the normalized signal intensity was used in figure 1 and figure 2 in order to assist with chromatographic visualization of trace components, as a result of the wide dynamic range detected. The contour plots displayed in figure 1 and figure 2 illustrate that chromatographic differences can be observed between: 1) the individual bacterial species; and 2) between the bacterial samples and the respective control samples.

With a TIC *S/N* greater than 250, an average of 397 peaks were detected in the control samples compared to an average of 472 peaks detected in the bacterial samples. This represents an order-of-magnitude increase in the number of VOCs detected using GC×GC-TOFMS when compared to the volatile profiles of CF-associated bacterial species obtained *in vitro* using traditional one-dimensional GC-MS [18,24,25]. This is a direct result of the increased peak capacity, sensitivity and selectivity afforded by GC×GC-TOFMS. These benefits allowed co-eluting peaks, chromatographic artefacts and dynamic range to be more easily managed in this study, leading to an overall increase in peak detectability. This resulted in a more comprehensive volatile metabolomic profile of each sample, with each individual VOC having a higher likelihood of being detected and quantified accurately. Compounds detected before filtering included acids, alcohols, aldehydes, aliphatic hydrocarbons (*i.e.* alkanes, alkenes and alkynes), aromatic hydrocarbons, esters, ethers, functionalized benzenes (*i.e.* benzene ring with various N, O, or S heteroatomic functional groups), heteroaromatics (*i.e.* aromatic ring containing a N, O, or S heteroatom), ketones, sulfur-containing compounds, nitrogen-containing compounds, chromatographic artefacts (*e.g.* siloxanes, silanols, silanes and phthalates) and "other" compounds containing multiple functional groups. A similar range of chemical classes has previously been reported in the *in vitro* headspace analysis of *P. aeruginosa* by HS-SPME-GC×GC-TOFMS [26]. Overall, the use of GC×GC-TOFMS in this study highlights the wealth of additional information that can be gained from this technique for volatile metabolomic profiling and the prospects for new advancements in future applications of disease monitoring.

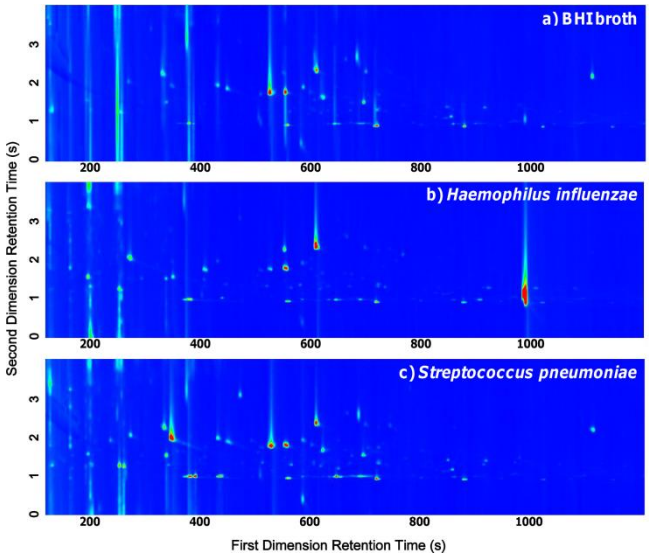

Figure 1. GC×GC-TOFMS TIC contour plots of a) BHI control broth and those bacterial species cultured in BHI broth under stationary growth phase conditions and analyzed after short-term storage: b) *H. influenzae* and c) *S. pneumoniae*.

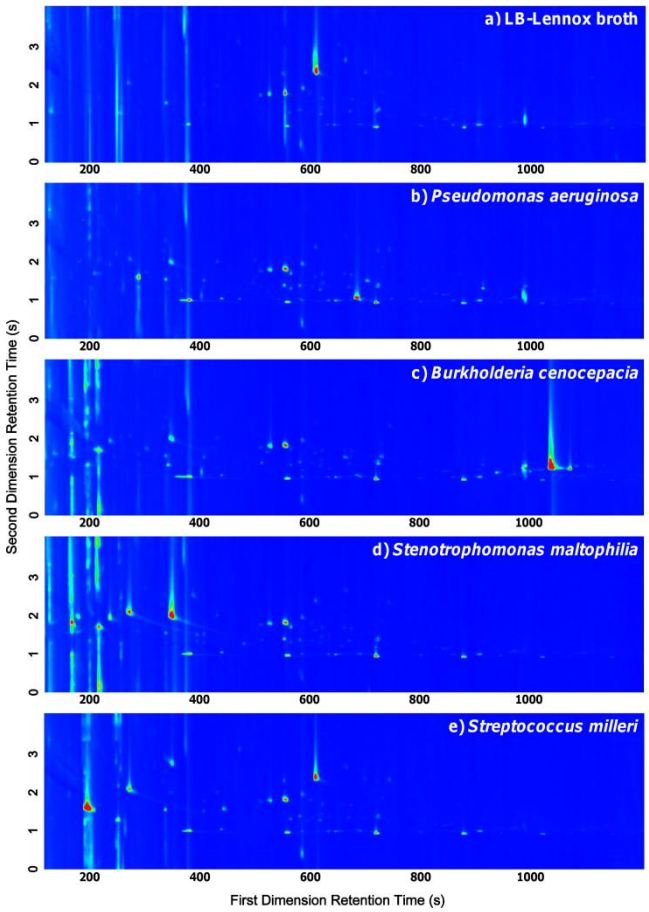

Figure 2. GC×GC-TOFMS TIC contour plots of a) LB-Lennox control broth and those bacterial species cultured in LB-Lennox under stationary growth phase conditions and analyzed after short-term storage: b) *P. aeruginosa*, c) *B. cenocepacia*, d) *S. maltophilia* and e) *S. milleri*.

3.2. Bacterial Differentiation

In order to determine whether the six bacterial species could be differentiated based on their volatile metabolomic profiles, the chromatographic results were evaluated using principal component analysis (PCA). This allowed visualization of the multivariate structure of the data based on the volatile metabolomic profile as opposed to a univariate approach based on individual compounds. In order to examine the structure of the data related to bacterial differentiation, two separate PCA analyses were conducted whereby samples prepared under logarithmic and stationary growth phase conditions were evaluated as two separate datasets. Within each dataset the samples were separated into seven classes (*i.e.* six individual bacterial classes and one control class) for alignment and Fisher ratio filtering.

Using this method (designated as Statistical Compare approach A), analytes were only retained if found in 75% of the samples within a class, where the number of samples in each class was equal to four (*i.e.* two short-term storage samples and two long-term storage samples). This filtering method allowed for the identification of analytes that were detected in both short-term and long-term storage samples for a particular bacteria or control, which is important for laboratories that may need to store samples prior to analysis while waiting for analytical instrumentation to become available. Using Statistical Compare approach A, 324 compounds were retained after alignment for the samples cultured under logarithmic growth phase conditions. Of the 324 compounds, 38 analytes were identified that met all post-processing criteria (*i.e.* found to have a Fisher ratio above the F_{crit} threshold of 2.57, sufficient spectral matching and not related to chromatographic artefacts). Hereafter the term "detected compounds" refers to the compounds that met these criteria and were used for PCA analysis. Similarly, for samples cultured under stationary growth phase conditions, 334 compounds were initially retained after alignment using Statistical Compare approach A. After post-processing procedures, 76 detected compounds remained that were used for PCA analysis.

The additional information gained through the use of GC×GC-TOFMS (compared to traditional GC-MS) in combination with complementary statistical software features allowed for the above described approach to be applied in order to select compounds of interest rather than relying on all compounds identified. The compounds of interest selected in this post-processing list should provide optimal discrimination when using PCA.

Figure 3 and figure 4 display the PCA scores and loadings plots produced for the detected compounds in both the logarithmic (figure 3(a) and figure 3(b), respectively) and stationary (figure 4(a) and figure 4(b), respectively) growth phase analyses. Compound identifications corresponding to the numbers in the loadings plots can be found in Table S-1 (samples cultured under logarithmic growth phase conditions) and Table S-2 (samples cultured under stationary growth phase conditions) in the Supplementary Data. The explained variance observed for the principal component axes in figure 3 and figure 4 (*i.e.* 43% and 38%, respectively) is considered to be moderate. These values

were originally higher before data transformations (*i.e.* mean centring, variance scaling and unit vector normalization) were performed; however, data transformations and filtering reduced the amount of noise introduced into the overall dataset and therefore by performing these steps the resulting axis loadings were reduced. This is not a negative point but rather increases the ability to differentiate the samples based on the structure of the data. In addition, the more principal components considered, the larger the amount of variation that is taken into account. For this reason PC-3 was also investigated but was not found to provide any further differentiation between the bacterial species under logarithmic or stationary growth phase conditions.

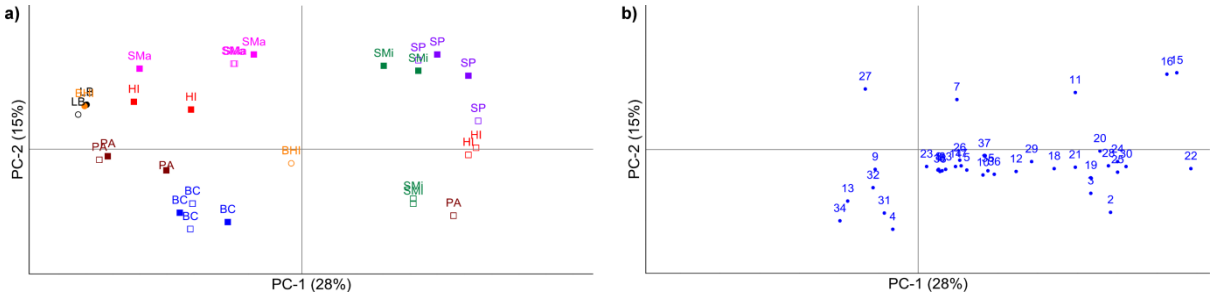

Figure 3. Principal component analysis using pre-processed GC×GC-TOFMS peak area data for compounds detected in logarithmic growth phase samples with Statistical Compare approach A: a) scores plot; b) loadings plot (list of compounds available in Table S-1 in the Supplementary Data). Closed symbols represent samples analyzed after short-term storage and open symbols represent samples analyzed after long-term storage. PA = *P. aeruginosa*; BC = *B. cenocepacia*; HI = *H. influenzae*; SMa = *S. maltophilia*; SP = *S. pneumoniae*; SMi = *S. milleri*; LB = Luria broth, Lennox; and BHI = Brain Heart Infusion broth.

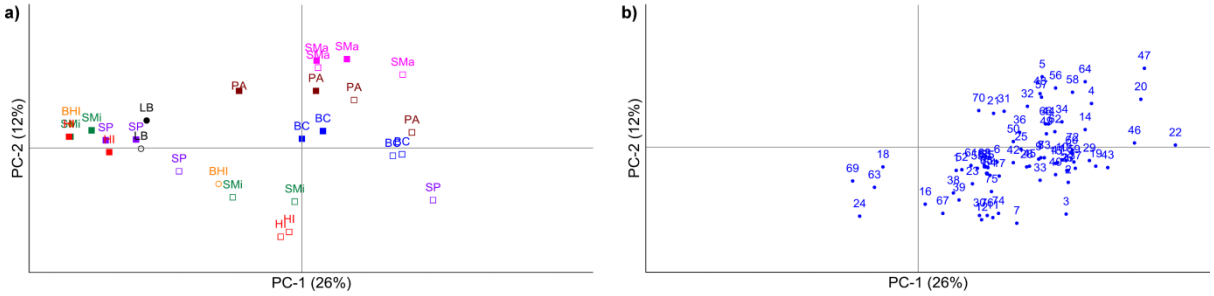

Figure 4. Principal component analysis using pre-processed GC×GC-TOFMS peak area data for compounds detected in stationary growth phase samples with Statistical Compare approach A: a) scores plot; b) loadings plot (list of compounds available in Table S-2 in the Supplementary Data). Closed symbols represent samples analyzed after short-term storage and open symbols represent samples analyzed after long-term storage. PA = *P. aeruginosa*; BC = *B. cenocepacia*; HI = *H. influenzae*; SMa = *S. maltophilia*; SP = *S. pneumoniae*; SMi = *S. milleri*; LB = Luria broth, Lennox; and BHI = Brain Heart Infusion broth.

Similar to the chromatographic profiles of the bacteria samples in figure 1 and figure 2, PCA allowed the visualization of varying degrees of differentiation and similarity between the volatile metabolomic profiles of the bacteria investigated. *P. aeruginosa* and *B. cenocepacia* produced similar volatile metabolomic profiles when prepared under both logarithmic and stationary growth phase

conditions. Under logarithmic growth phase conditions (figure 3(a) and figure 3(b)), *P. aeruginosa* and *B. cenocepacia* exhibited comparable concentrations of 2,4-dimethyl-heptane (13), 2-nonanone (34), 2-methyl-3-isopropylpyrazine (31), 2-methyl-furan (4), 1-decene (32) and mercaptoacetone (9) based on the available compound identification information (bracketed numbers following the compound names refer to the compound identification numbers in the corresponding loadings plot). However, under stationary growth phase conditions (figure 4(a) and figure 4(b)), the similarities in these bacterial species was attributed to a broader spectrum of compounds. *S. pneumoniae* and *S. milleri* also produced similar metabolic by-products to each other during both growth phases investigated. Under logarithmic conditions (figure 3(a) and figure 3(b)), *S. pneumoniae* and *S. milleri* exhibited comparable concentrations of acetic acid butyl ester (16) and 3-methyl-3-heptanol (22). Under stationary growth phase conditions (figure 4(a) and figure 4(b)), the similarities between *S. pneumoniae* and *S. milleri* were mainly attributed to the following VOCs: 1-ethylpropyl-benzene (67), 3-methyl-butanal (16), 2-methyl-butanal (18), hexyl-benzene (69), 2,3-pentanedione (24) and α-methyl-benzeneacetaldehyde (63). During the logarithmic growth phase (figure 3(a) and figure 3(b)), *S. maltophilia* generated a comparable VOC profile to two of the *H. influenzae* replicates with a similar concentration of L-leucine methyl ester (27); however, under stationary growth phase conditions (figure 4(a) and figure 4(b)), *S. maltophilia* produced similar VOCs (*e.g.* L-leucine methyl ester (57), 1-propanol (5), methyl thiolacetate (20), butyl 2-methylbutanoate (56), etc.) to *P. aeruginosa* and *B. cenocepacia*. *H. influenzae* produced a more variable VOC profile than the other bacteria under all conditions tested.

Under logarithmic growth phase conditions (figure 3(a)) the bacteria samples exhibited a higher degree of discrimination from the control samples in comparison with stationary growth phase conditions (figure 4(a)). There was also reduced variation between replicates for *B. cenocepacia*, *S. pneumoniae* and *S. maltophilia* under logarithmic growth phase conditions, which resulted in an increased overall discrimination between species.

Further investigation demonstrated that the variation observed within a single bacterial species (*e.g. H. influenzae* and *S. maltophilia*) was a result of the different storage conditions used (*i.e.* short-term *vs.* long-term storage). For example, the two *H. influenzae* replicates grouped on the left-side of the scores plot in figure 3(a) (closed symbols) were analyzed after short-term storage, while the two replicates grouped on the right-side of the plot (open symbols) were analyzed after long-term storage. Similar results were also found for the *H. influenzae* samples cultured under stationary growth phase conditions (figure 4(a)).

The two different growth media used herein (*i.e.* LB-Lennox and BHI broth) were selected for the optimal *in vitro* growth of the bacterial species investigated (*i.e.* LB-Lennox is poor for culturing *S. pneumoniae* and *H. influenzae*). It is of course expected that the use of different growth media may result in different volatile metabolomic profiles; unfortunately this is an unavoidable part of *in vitro*

analysis. Nevertheless, the use of two different growth media can be considered reflective of future *in vivo* samples (*e.g.* exhaled breath) where there is no media involved but there is a higher probability of biological variability (*i.e.* growth conditions) in the lung environment between patients. Despite the use of two different growth media, *S. milleri* (cultured using LB-Lennox) and *S. pneumoniae* (cultured using BHI broth), two bacteria belonging to the same genus, were still found to produce similar volatile metabolomic profiles as previously described.

When comparing all six of the CF-associated bacterial species it is apparent that the presence of a particular pattern of VOCs (rather than a single VOC biomarker) is necessary for bacterial species differentiation and identification. The particular pattern of VOCs also appears to be dependent upon the bacterial growth phase and sample storage conditions. Previous studies have claimed to identify species-specific biomarkers for CF-associated pathogens; however, the number of bacterial species studied is often limited (*e.g.* n = 2) [24,25]. Although it may be possible to effectively differentiate two or three bacterial species based on their respective VOC profiles (*e.g. B. cenocepacia, S. maltophilia* and *S. pneumoniae* in figure 3(a)), the results of this study highlight the importance of studying several different bacterial species with similar metabolic pathways when the objective is to identify species-specific biomarkers/VOC patterns. Increasing the number of replicates in the future and maintaining sample storage conditions, as discussed in the next section, may yield even greater differentiation between bacterial species.

3.3. Storage Conditions

Statistical Compare was initially designed by LECO as an optional feature within ChromaTOF® to help manage large sets of metabolomics data allowing for analyte alignment and the calculation of descriptive statistics. For this reason, Statistical Compare appears to be well-suited for two-class models (*e.g.* cancerous *vs.* non-cancerous samples or diabetic *vs.* non-diabetic samples). In an attempt to conform to the design of Statistical Compare, the data was further analyzed using a two-class model (*i.e.* bacteria *vs.* control), which is referred to throughout as Statistical Compare approach B. With this approach, more stringent filtering rules were applied during alignment in an effort to better characterize the difference in the volatile metabolomic profiles collected after short-term (*i.e.* 2 – 5 days) *vs.* long-term (*i.e.* 48 – 50 days) storage at -18 °C.

The volatile metabolomic profiles acquired from the samples analyzed after long-term storage were clearly more complex as noted in both the GC×GC-TOFMS TIC contour plots (figure 5) and in the number of VOCs detected (*e.g.* an average increase of approximately 200 VOCs compared to samples analyzed after short-term storage). Similar results have also been reported for the analysis of volatiles from blood samples analyzed periodically over 5 weeks after storage at room temperature (25 °C), under refrigeration (4.5 °C) and in a freezer (-18 °C), with VOC profiles becoming more complex with increasing storage length [52]. The variation observed in the volatile metabolomic

profiles as a result of the different storage periods could be due to contamination, sample degradation, residual bacterial activity (which may not be halted even when samples are frozen at -18 °C [53]), or a combination of such events.

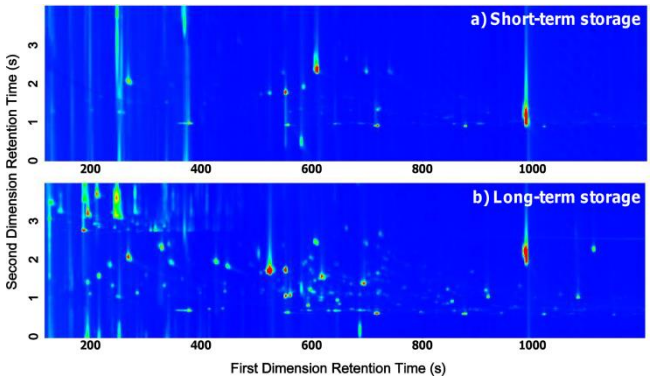

Figure 5. GC×GC-TOFMS TIC contour plots of *H. influenzae* cultured under logarithmic growth phase conditions and analyzed after a) short-term and b) long-term storage.

Overall, Statistical Compare approach B generated a list of 320 aligned peaks. Of these 320 peaks, 25 detected compounds met the previously described post-processing criteria and were subsequently used for PCA analysis. Discrimination was observed between the samples analyzed after short-term storage compared with long-term storage using PCA as shown in the scores plots in figure 6(a). This discrimination can be attributed to the compounds identified in the loadings plot (figure 6(b)) which are listed in Table S-3 in the Supplementary Data. Overall, the first principal component (PC-1) accounted for 33% of the variation in the dataset. Discrimination occurred along this axis between the short-term and long-term storage conditions. The second principal component (PC-2) accounted for 16% of the variation in the dataset, and discrimination between the bacteria and controls was observed along this axis.

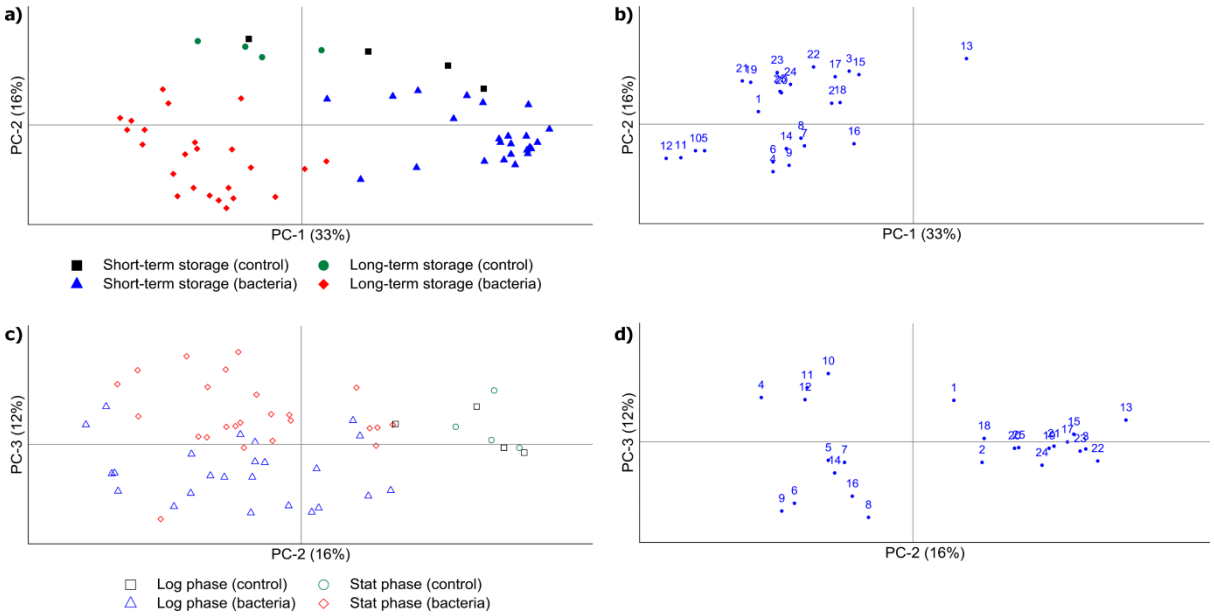

Figure 6. Principal component analysis using pre-processed GC×GC-TOFMS peak area data for compounds detected in analyzed samples using Statistical Compare approach B: a) scores plot of PC-1 and PC-2 distinguishing storage length; b) loadings plot of PC-1 and PC-2; c) scores plot of PC-2 and PC-3 distinguishing growth phase; d) loadings plot of PC-2 and PC-3. Compound numbers in loadings plots correspond to the list of compounds in Table S-3 in the Supplementary Data. Log = logarithmic growth phase and stat = stationary growth phase.

Variation in storage conditions can clearly impact the number of VOCs detected in bacteria samples and the VOC profile reported. Studies that remove cells by centrifugation prior to volatile analysis may reduce some of the variation described above. Storage at lower temperatures (*e.g.* -70 to -80 °C) could also further reduce or inhibit bacterial activity [53,54]. Regardless, as access to analytical instrumentation may be limited, future work should explicitly document sample storage conditions in order to prevent misrepresentation of the data and to allow for datasets within the literature to be appropriately compared. Standardizing the storage conditions of samples may also increase the ability to discriminate between bacterial species using multivariate statistics.

3.4. Growth Phase

In vitro bacterial growth can be modeled by plotting the natural logarithm of the number of bacteria cells *vs.* incubation time, resulting in a growth curve with 4 distinct phases: lag phase, logarithmic (or exponential) phase, stationary phase and death phase [55]. During the lag phase the bacteria adapt to their new environment (*e.g.* following inoculation into fresh growth medium) and the cells begin to grow. Shortly after the bacteria adjust to their new growth conditions they enter the logarithmic phase where they reach their maximum growth rate. Continued exponential growth begins to exhaust essential nutrients and the formation of metabolic waste alters the conditions of the growth medium producing the horizontal linear portion of the growth curve referred to as the stationary phase. Eventual depletion of essential nutrients and accumulation of waste material leads to cell death.

Since chronic respiratory infections are not easily eradicated from the CF lung once acquired, the ability to distinguish early-stage, acute infections from chronic infections may provide clinicians with additional information about the most appropriate treatment strategy. In this study, we compared logarithmic and stationary growth phases for each bacterial species as these provide clearly contrasting metabolic states.

The VOC profiles acquired of samples from stationary phase cultures were more complex than those samples from logarithmic phase cultures, with an average increase of approximately 40 VOCs. When the third principal component (PC-3) was taken into consideration for the detected compounds obtained from Statistical Compare approach B, the logarithmic and stationary phases of growth could be differentiated based on their VOC profiles (figure 6(c)). Overall, PC-3 accounted for 12% of the variation in the dataset, and discrimination between the growth phases was observed along this axis. This discrimination can be attributed to the compounds shown in the loadings plot (figure 6(d)) which are listed in Table S-3 in the Supplementary Data. In order to determine if the growth phases could be differentiated within an individual bacterial species, each species was analyzed independently by PCA using the 25 detected compounds isolated from Statistical Compare approach B. Using this approach, logarithmic and stationary phase metabolic profiles could be differentiated using PC-3 for *P. aeruginosa*, *S. milleri* and *S. pneumoniae* but not for *B. cenocepacia*, *H. influenzae* and *S. maltophilia*. Figure 7 demonstrates a representative PCA scores plot of PC-2 *vs.* PC-3 obtained for *P. aeruginosa*.

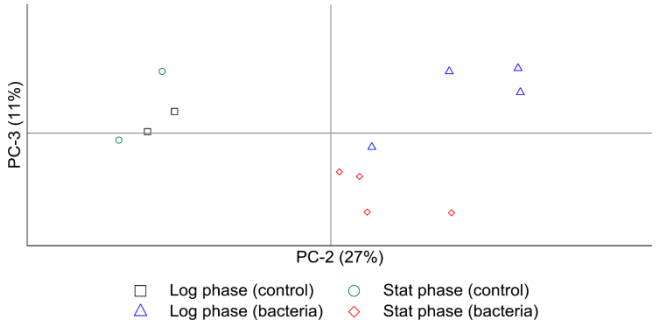

Figure 7. Principal component analysis using pre-processed GC×GC-TOFMS peak area data for *P. aeruginosa* and LB-Lennox control samples using compounds detected with Statistical Compare approach B. Log = logarithmic growth phase and stat = stationary growth phase.

4. Concluding Remarks

Previous studies considering only one or two bacterial species have claimed to identify species-specific biomarkers for CF-associated pathogens. The main objective of this study was to investigate whether six different CF-associated bacterial species could be differentiated based on their volatile metabolomic profiles collected and analyzed using HS-SPME-GC×GC-TOFMS. Although it was not possible to effectively differentiate all six bacteria using the methods outlined herein, this

study has demonstrated that a large subset of the bacterial volatome (*i.e.* VOC profile), rather than a single biomarker, is required for bacterial species identification.

Due to similarities in the metabolic pathways of CF-associated bacteria, it is important to investigate the broad range of bacteria that may be detected in the CF lung in order to accurately recognize and differentiate their volatomes. Expanding volatile metabolomic profiling to encompass additional bacterial species will aid in the development of *in situ* tools for diagnostic purposes which are capable of differentiating common bacterial species that could be present in the airways as a result of a variety of different lung infections worldwide such as tuberculosis. This study did not aim to identify species-specific biomarkers; however, the approaches used herein could be applied in future studies (with the use of high replicate datasets) for species-specific volatome discovery, including other more difficult situations/organisms (*e.g.* influenza virus).

Sample storage conditions are often not noted in published studies; however, this study has shown that storage length can alter the VOC profile and should be considered when reporting bacterial volatomes (or biomarkers). Although the growth phases could not be differentiated for all of the bacterial species investigated, the differentiation of all six bacteria was improved under logarithmic growth phase conditions compared with stationary growth phase conditions. This knowledge may be important when considering future diagnostic tools using exhaled breath analysis for the early detection and identification of acute pulmonary infections.

Acknowledgements

The authors wish to thank the University of Technology Sydney laboratory technical staff, Dr. David Bishop and Dr. Ronald Shimmon, for their ongoing support. SGE Analytical Science and Restek are recognized for donating research supplies. Financial support for this work was provided in part by the University of Technology Sydney and the Westmead Millennium Institute.

Conflict of Interest

The authors declare that they have no conflict of interest.

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