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TITLE:

Capturing Actively Produced Microbial Volatile Organic Compounds From Human-associated Samples With Vacuum-assisted Sorbent Extraction

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KEYWORDS:

Volatile organic compounds, GC-MS, vacuum assisted sorbent extraction, headspace extraction, clinical samples, stable isotope probing

SUMMARY:

This protocol describes the extraction of volatile organic compounds from a biological sample with the vacuum-assisted sorbent extraction method, gas chromatography coupled with mass spectrometry using the Entech Sample Preparation Rail, and data analysis. It also describes culture of biological samples and stable isotope probing.

ABSTRACT:

Volatile organic compounds (VOCs) from biological samples have unknown origins. VOCs may originate from the host or different organisms from within the host's microbial community. To disentangle the origin of microbial VOCs, volatile headspace analysis of bacterial mono- and co-cultures of *Staphylococcus aureus, Pseudomonas aeruginosa,* and *Acinetobacter baumannii*, and stable isotope probing in biological samples of feces, saliva, sewage, and sputum were performed. Mono- and co-cultures were used to identify volatile production from individual bacterial species or in combination with stable isotope probing to identify the active metabolism of microbes from the biological samples.

Vacuum-assisted sorbent extraction (VASE) was employed to extract the VOCs. VASE is an easy-to-use, commercialized, solvent-free headspace extraction method for semi-volatile and volatile

compounds. The lack of solvents and the near-vacuum conditions used during extraction make developing a method relatively easy and fast when compared to other extraction options such as *tert*-butylation and solid phase microextraction. The workflow described here was used to identify specific volatile signatures from mono- and co-cultures. Furthermore, analysis of the stable isotope probing of human-derived biological samples identified VOCs that were either commonly or uniquely produced. This paper presents the general workflow and experimental considerations of VASE in conjunction with stable isotope probing of live microbial cultures.

INTRODUCTION:

Volatile organic compounds (VOCs) have great promise for bacterial detection and identification because they are emitted from all organisms, and different microbes have unique VOC signatures. Volatile molecules have been utilized as a non-invasive measurement for detecting various respiratory infections including chronic obstructive pulmonary disease[1], tuberculosis[2] in urine[3], and ventilator-associated pneumonia[4], in addition to distinguishing subjects with cystic fibrosis (CF) from healthy control subjects[5,6]. Volatile signatures have even been used to distinguish specific pathogen infections in CF (*Staphylococcus aureus*[7], *Pseudomonas aeruginosa*[8,9], and *S. aureus* vs. *P. aeruginosa*[10]). However, with the complexity of such biological samples, it is often difficult to pinpoint the source of specific VOCs.

One strategy for disentangling the volatile profiles from multiple infecting microbes is to perform headspace analysis of microorganisms in both mono- and co-culture[11]. Headspace analysis examines the analytes emitted into the "headspace" above a sample rather than those embedded in the sample itself. Microbial metabolites have often been characterized in mono-cultures because of the difficulty in determining the origin of microbial metabolites in complex clinical samples. By profiling volatiles from bacterial mono-cultures, the types of volatiles a microbe produces *in vitro* may represent a baseline of its volatile repertoire. Combining bacterial cultures, *e.g.*, creating co-cultures, and profiling the volatile molecules produced may reveal the interactions or cross-feeding between the bacteria[12].

Another strategy for identifying the microbial origin of volatile molecules is to provide a nutrient source that is labeled with a stable isotope. Stable isotopes are naturally occurring, non-radioactive forms of atoms with a different number of neutrons. In a strategy that has been utilized since the early 1930s to trace active metabolism in animals[13], the microorganism feeds off of the labeled nutrient source and incorporates the stable isotope into its metabolic pathways. More recently, a stable isotope in the form of heavy water (D_2O) has been used to identify metabolically active *S. aureus* in a clinical CF sputum sample[14]. In another example, ^{13}C-labeled glucose has been used to demonstrate the cross-feeding of metabolites between CF clinical isolates of *P. aeruginosa* and *Rothia mucilaginosa*[12] .

With the advancement of mass spectrometry techniques, methods of detecting volatile cues have moved from qualitative observations to more quantitative measurements. By using gas chromatography mass spectrometry (GC-MS), processing of biological samples has become within reach for most laboratory or clinical settings. Many methods to survey volatile molecules have been used to profile samples such as food, bacterial cultures, and other biological samples,

and air and water to detect contamination. However, several common methods of volatile sampling with high-throughput require solvent and are not performed with the advantages provided by vacuum extraction. In addition, larger volumes or quantities (greater than 0.5 mL) of sampled materials are often required for analysis[15–19], although this is substrate-specific and requires optimization for each sample type and method.

Here, vacuum-assisted sorbent extraction (VASE) followed by thermal desorption on a GC-MS was employed to survey the volatile profiles of bacterial mono- and co-cultures and identify actively produced volatiles with stable isotope probing from human feces, saliva, sewage, and sputum samples (**Figure 1**). With limited sample quantities, VOCs were extracted from as little as 15 µL of sputum. Isotope probing experiments with human samples required adding a stable isotope source, such as ^{13}C glucose, and media to cultivate the growth of the microbial community. The active production of volatiles was identified as a heavier molecule by GC-MS. Extraction of volatile molecules under a static vacuum enabled the detection of volatile molecules with increased sensitivity[20–22].

PROTOCOL:

1. Headspace sorbent pen (HSP) and sample analysis considerations

NOTE: The HSP containing the sorbent Tenax TA was selected to capture a broad range of volatiles. Tenax has a lower affinity for water compared to other sorbents, which enables it to trap more VOCs from higher-moisture samples. Tenax also has a low level of impurities and can be conditioned for re-use. Sorbent selection was also made in consideration with the column installed in the GC-MS (see the **Table of Materials**).

1.1. Generate negative controls by extracting media and/or sample blanks with the same conditions used for sample extraction.

1.2. Analyze a blank HSP (previously confirmed to be clean and free of significant background) on the GC-MS before analyzing extracted samples. Run blanks between sample types (*e.g.*, three replicates of bacteria mono-culture, blank, three replicates of bacteria co-culture, blank, etc.).

1.3. Limit use of fragrant personal care items or consumption of smelly foods prior to sample extraction and analysis. Ideally, prepare samples in a biosafety hood that has not been cleansed by alcohol or other volatile cleaners for at least 30 min. Turn on airflow in the biosafety hood for 30–60 min prior to sample preparation.

1.4. Keep samples on ice to limit volatile release during sample preparation.

2. Mono- and co-culture preparation

2.1. In the biosafety hood, inoculate cultures of *A. baumannii, S. aureus,* and *P. aeruginosa* in Todd Hewitt growth media. Incubate overnight at 37 °C with 200 rpm agitation.

2.2. After the overnight incubation, perform culture handling in the biosafety hood. Dilute each culture to optical density 0.05 at 500 nm.

2.3. Mix co-cultures in equal parts, and pipette 200 µL of control media, mono-, or co-culture into each well of a 96-well plate, and place in 37 °C incubator for 24 h. Prepare a second plate for a 48-h incubation.

2.4. At the end of the incubation period, prepare samples for extraction in section 4. Pipette liquid cultures into microcentrifuge tubes and store at -80 ℃.

NOTE: At this point, samples can be stored at -80 °C to extract later if needed.

3. Stable isotope probing in biological samples preparation

NOTE: The feces and saliva samples were donated from anonymous donors with approval from the University of California Irvine Institutional Review Board (HS# 2017-3867). The sewage came from San Diego, CA. The sputum samples were collected from subjects with cystic fibrosis as part of a larger study approved by the University of Michigan Medical School Institutional Review Board (HUM00037056).

3.1. Perform all biological sample preparations in the biosafety hood.

3.1.1. To prepare fecal samples, add 1 mL of deionized water to 100 mg of feces in a 1.5 mL microcentrifuge tube and vortex for 3 min. Place on ice when not in use.

3.1.1.1. To 15 µL of fecal and water mixture, add 485 µL of Brain Heart Infusion (BHI) medium with 20 mM ^{13}C glucose, or BHI with 30% deuterium (D_2O). Ensure that the final volume of the sample is 500 µL. Prepare samples in technical triplicates.

3.1.2. To prepare sewage samples, add 500 µL of sewage to 500 µL of BHI and 20 mM ^{13}C glucose or BHI with 30% D_2O for a total volume of 1 mL. Prepare samples in triplicate. Place on ice when not in use.

3.1.3. To prepare saliva samples, add 50 µL of saliva to 500 µL of BHI and 20 mM ^{13}C glucose or BHI with 30% D_2O for a total volume of 550 µL. Prepare samples in triplicate. Place on ice when not in use.

3.1.4. To prepare sputum samples to compare the volatiles present in the sample prior to and after culturing, perform a first extraction with 15 µL of sputum. Prepare samples in triplicate. Place on ice when not in use. Proceed to section 4 for sample extraction, and extract for 18 h at 37 °C with 200 rpm agitation.

3.1.4.1. After the completion of the first extraction of the uncultured sputum samples, save the

vials with sputum. Add 500 µL of BHI with 20 mM ^{13}C glucose to the vials with sputum from 3.5.1. Place on ice when not in use.

3.2. Proceed to section 4 for sample extraction.

4. Sample extraction

4.1. Place empty volatile organic analysis (VOA) vials (20 mL) on the cold plate, and place the cold plate on ice in the biosafety hood.

4.2. Turn on the 5600 sorbent pen extraction unit (SPEU), and adjust to the desired temperature as required for each method.

NOTE: For stable isotope probing experiments at 37 °C, reaching the setpoint can take up to 15 min. For mono- and co-culture experiments at 70 °C, reaching the setpoint can take up to 60 min.

4.3. Collect clean HSPs that are equal to the number of samples prepared, including HSPs for media or sample controls.

4.4. Label 20 mL VOA vials according to samples, replicates, and HSP IDs as needed. Use a marker that resists water in case condensation forms on the outside of the vial while on ice.

4.5. Inside the biosafety hood, unscrew the white cap on the vial, quickly pipet sample into the vial, and assemble the black cap, lid liner, and HSP.

NOTE: Samples should not come into contact with the HSP, and sample volume will depend on sample type.

4.6. Place the vial containing the sample and HSP back on the cold plate.

4.7. Repeat steps 4.5 and 4.6 for each sample. Perform these steps per sample instead of all at once to prevent sample warming and thus, premature volatile release.

4.8. Once all samples have been prepared in the glass vials, perform the following steps outside the biosafety hood on the bench. Turn on the vacuum pump, place the vials under vacuum to 30 mmHg, and remove the vacuum source.

NOTE: The vials do not need to be on the cold tray after vacuum application has been completed.

4.9. Double-check the pressure after placing all samples under vacuum using the pressure gauge. If a vial has a leak, ensure that the cap is screwed on tightly, and that the white O-rings of the HSP and lid liners are properly in place.

NOTE: A compromised seal can result in decreased volatile detection compared to a vial under vacuum.

4.10. Place vials in the SPEU for the optimized time and temperature with agitation at 200 rpm. Extract cultures for 1 h at 70 °C, and extract stable isotope probing experiments with fecal, sewage, saliva, and sputum samples for 18 h at 37 °C.

4.11. Place the cold plate at -80 °C for use after the extraction period is complete.

4.12. When extraction is complete, place samples on the cold plate for 15 min to draw out water vapor from the HSP and vial headspace.

4.13. Transfer the HSPs to their sleeves.

NOTE: The experiment can be paused here for up to ~1 week at room temperature before losing volatiles from the HSPs.

5. Analyze samples on the gas chromatography–mass spectrometer (GC-MS)

5.1. Use the following GC-MS (see the **Table of Materials**) settings: 35 °C with a 5 min hold, 10 °C/min ramp to 170 °C, and a 15 °C/min ramp to 230 °C with a total runtime of 38 min.

5.2. Set the desorption method as follows: 2 min, 70 °C preheat; 2 min 260 °C desorption; 34 min, 260 °C bakeout; and 2 min, 70 °C post bake.

5.3. Set up the sequence of samples, and start the run according to instrumentation.

5.3.1. To set up a sequence on the Entech Software, open the program. In the options to the right of the instrument dropdown menu, select **5800 | Sequence**.

5.3.2. Observe the sequence table in the Entech software similar to that in the GC-MS software. Name the **Sample ID** column according to **Current date_vial number**. Keep in mind that **Name** is analogous to **Name** in the GC-MS sequence table, and **5800 Method** determines the rate of temperature ramp, holding times, etc. (opens a menu to select the method generated in step 5.2).

5.3.3. Keep in mind that the **Tray** and **Position** columns determine where the Sample Preparation Rail (SPR) will go to pick up the HSPs.

5.3.3.1. Observe the two trays with 30 spots each to the immediate left, laid out as six columns with five spots each; the spot furthest and leftmost on each tray is spot 1, while the closest and rightmost spot is spot 30.

5.3.3.2. Note that these trays are **HSP A or B**, where HSP B is the tray closer to the SPR (innermost

tray), and directly behind HSP B is HSP Blank. Place the extracted samples into the trays, and select the spot on the sequence accordingly.

5.3.4. Save the sequence table, select **Run** on the left-hand side, then **Start with blank in desorber** if the blank HSP is in the desorber (denoted by a HSP marked by yellow label).

5.4. Note that HSPs will be handled by the SPR for each sample in the sequence. Let the SPR warm up, then a message will appear at the top of the screen to confirm if the blank is in the desorber. Click on **Skip** to confirm that the pen is there. Allow the SPR to run all samples automatically, and the sequence on the GC-MS side will automatically record the data in separate files.

6. Data analysis

6.1.1. Quality-filter data on GC-MS software (**Table of Materials**).

6.1.2. Review each peak on the chromatogram, and annotate peaks that match the National Institute of Standards & Technology (NIST) library (or with another available library).

6.1.3. Add annotated chromatogram peaks to the processing method. Set the criteria for selecting peaks to include compounds with a greater than 75% probability, and ensure that the alignment of each identifying ion of the compound lies within the center of the peak.

6.1.3.1. To add a peak to the processing method, select **Calibrate | Edit Compound | Name | insert compound** under **External Standard Compound**. Add the name of the compound, retention time, Quant Signal **Target Ion**. Add the three largest peaks. To save, select **ok | Method | Save**.

6.1.4. Once the process method is set up, proceed to **Quantitate | Calculate**, and **View | QEdit Quant Result**.

6.1.5. Inspect each compound to ensure that the peaks align with their expected retention times and are above background noise.

6.1.6. Once QEdit has been completed, select **Exit | Yes** to save the QEdits and return to the main chromatogram. Export the area integrations by opening the file on the left-hand side. Select **Quantitate | Generate Report**.

6.1.7. To export files for use in DExSI, select **File | Export Data** to **AIA format | Create New Directory**, and select a location for the file or **Use Existing Directory**.

6.1.8. Observe a new window opening up to select files for export. Move the files to the right side of the window and click on **Process**. Wait for a few seconds to a few minutes depending on the number of files being converted.

6.2. Correct for isotope abundance in DExSI according to instructions for the DExSI software (https://github.com/DExSI/DExSI), and perform analysis with a favorite software or program (*e.g.*, R).

REPRESENTATIVE RESULTS:

Mono- and co-cultures of *S. aureus*, *P. aeruginosa*, and *A. baumannii*

The mono- and co-cultures consisted of the bacterial species *S. aureus, P. aeruginosa,* and *A. baumannii*. These are common opportunistic pathogens found in human wounds and chronic infections. To identify the volatile molecules present in the mono- and co-cultures, a short 1-h extraction was performed at 70 °C with 200 rpm agitation. From the mono- and co-cultures at 24- and 48-h timepoints, 43 annotated volatile molecules were detected (**Figure 2**) among which were aldehydes, ketones, alcohols, sulfuric compounds, hydrocarbons, carboxylic acids or esters, and aromatics. There were a small number of volatile molecules that were only detected in certain mono- or co-cultures at certain timepoints. For example, acetoin and 3-hydroxy-2-butanone acetate were only detected in the *S. aureus* cultures at the 48-h timepoint (**Figure 2**).

Volatile 1-propanol 2-methyl was detected only in the *P. aeruginosa* and *A. baumannii* co-culture at 48 h (**Figure 2**). Ethyl acetate was present in *A. baumannii* co-cultures with either *S. aureus* or *P. aeruginosa* at 48 h (**Figure 2**). The metabolites heptane, 2,3-dimethyl and pentane, 2-methyl were only detected in the *A. baumannii* culture at 24 h (**Figure 2**). Acetaldehyde and ethanol had higher relative abundances in the *A. baumannii* and *S. aureus* co-culture at the 24-h timepoint compared to 48 h and either of the strains in culture alone (**Figure 2**). Some of the volatiles were more abundant in cultures at either the 24- or 48-h timepoint. Short-chain fatty acids, including acetic acid, butanoic acid, and propanoic acid, were at high relative abundances in cultures at 48 h, but were not detected in the 24-h cultures (**Figure 2**). Hexane was more abundant in the TH control at 24 h compared to 48 h (**Figure 2**).

Stable isotope labeling of fecal, sewage, and saliva samples

To identify active production of volatile molecules from a biological sample, a labeled nutrient source, ^{13}C glucose or D_2O, and media were added to support the growth of the microbial community. One unique sample was analyzed from each of the different sample types of fecal, sewage, and saliva samples in triplicate. There was more incorporation of the ^{13}C into fully labeled volatile molecules (**Figure 3A–D**) compared to incorporation with deuterium (**Figure 3E**). The ^{13}C was incorporated into 2-butanone, 3-hydroxy; 2,3-butanedione; acetic acid; and phenol for all fecal, sewage, and saliva samples (**Figure 3A**).

The other labeled volatiles were detected in either two or one sample types. For example, acetone, butanoic acid, and propanoic acid were detected as labeled in saliva and sewage (**Figure 3B**). The labeled volatiles, dimethyl trisulfide and disulfide dimethyl, were enriched in both fecal and saliva samples (**Figure 3C**). Volatiles, 1-propanol, 2-butanone, benzophenone, ethanol, and methyl thiolacetate, were enriched only in sewage (**Figure 3D**). The labeled volatile, 2,3-pentanedoine, was enriched in saliva (**Figure 3D**). Deuterium was incorporated into the volatiles, acetic acid; benzaldehyde, 4-methyl; dimethyl trisulfide; and phenol, from either saliva or sewage

samples (**Figure 3E**). In addition to the isotope-enriched volatiles, there were volatiles detected that did not contain incorporated stable isotopes. For example, pyrazine compounds, except for pyrazine, 2,5-dimethyl, were detected in fecal, sewage, and saliva samples, but were not fully enriched with ^{13}C (**Supplemental Figure S1**).

Stable isotope labeling of sputum samples

The stable isotope labeling strategy was implemented for identifying actively produced volatiles with sputum samples from seven human subjects with cystic fibrosis. The volatiles in the sample were compared with those that emerged from samples cultured with a stable isotope label. Each volatile component of each sample was analyzed twice: before and after stable isotope probing with ^{13}C glucose and media. The samples collected from the subjects spanned three different timepoints or clinical states: baseline, exacerbation, and treatment[23]. The volatiles detected as labeled in the cultured sputum samples had different relative abundances compared to the unlabeled volatiles from the uncultured sputum samples. Culturing conditions in the stable isotope probing experiments with sputum may favor the growth of certain microbes, leading to differences in relative abundances of volatiles compared to the uncultured sputum samples.

For example, acetic acid, dimethyl trisulfide, acetone, and propanal, 2-methyl were more abundant in the cultured sputum samples compared to the uncultured sputum samples (**Figure 4**). Detecting ^{13}C-labeled ethanol, which can be present in variable amounts in the background room air, provides evidence that the ethanol was actively produced by microbial metabolism from ^{13}C glucose. The amount of variation was explained by subject as assessed by Permutational Multivariate Analysis of Variance (PERMANOVA) and was also different for the two different volatile datasets (**Table 1** and **Supplemental Figure S2**). For the ^{13}C-labeled cultured sputum, 51% of the variation was explained by the subject, while 33% of the variation was explained by subject from the volatiles in the uncultured sputum samples (**Table 1**). The microbiome community composition as determined by 16S rRNA amplicon sequencing from the seven subjects was unique to each subject (**Supplemental Figure S3**), and the individual signatures were also reflected in both the cultured and uncultured sputum volatile molecules detected.

In cultured sputum, 23 volatiles were detected that were fully labeled with ^{13}carbon. The isotope-enriched (active) volatiles detected from the sputum samples were different for each subject. The volatiles with isotope enrichment detected in the sputum samples from all seven subjects were 2,3-butanedione; acetic acid; acetone; dimethyl trisulfide; disulfide, dimethyl; and pyrazine, 2,5-dimethyl (**Figure 5**). Although those volatiles were detected in all subjects, the isotope enrichment for each subject varied. Samples from subject 7 had higher isotope enrichment of disulfide dimethyl compared to the other six subjects (**Figure 5B**). Acetone was higher in subjects 4 and 6 (**Figure 5**). Some volatiles were enriched with ^{13}C only in certain subjects. For example, 1-butanol, 3-methyl and propanoic acid, 2-methyl were only enriched in a subset of samples from subject 2 (**Figure 5**). In addition to the isotope-enriched volatiles, there were volatiles also detected as unlabeled from the same cultured sputum (**Supplemental Figure S4**). Volatiles 2-piperidinone; benzaldehyde, 4-methyl; benzothiazole; butanoic acid, 3-methyl; hexanal; hexane; isopropyl alcohol; phenol; propanoic acid, 2-methyl; and pyrrolo 1,2-apyrazine-1,4-dione, hexahydro were detected in the sputum samples, but were not isotope-enriched (**Supplemental**

Figure S4).

FIGURE AND TABLE LEGENDS:

Figure 1: Protocol schematic. A biological sample is placed into a glass vial and assembled with the lid liner and headspace sorbent pen. A vacuum is applied to the vial until a pressure of approximately 30 mmHg is reached. The vacuum source is removed, and the vials are placed in the sorbent pen extraction unit where a static extraction is performed with the aid of heat, agitation, and time. After extraction, vials are placed on a cold metal block to remove water from the headspace and HSP. The HSPs are collected and run via thermal desorption on the GC-MS. The data are analyzed with ChemStation, DExSI, and R. Abbreviations: HSP = headspace sorbent pen; GC-MS = gas chromatography–mass spectrometry.

Figure 2: Heatmap of mono- and co-cultures. VOCs detected from mono- and co-cultures at 24- and 48-h timepoints. The co-cultures are the combinations of the letters representing each strain. All samples were extracted for 1 h at 70 °C with 200 rpm agitation. Heatmap intensity values are column Z-scores, normalized by metabolite. The Z-score was calculated by the difference of value from the mean of values, divided by the standard deviation of values. The dendrogram was generated with the **cluster_cols** option in the **pheatmap** function of R. The dendrogram represents hierarchical clustering in which metabolites that cluster together have more similar Z-scores across samples. Abbreviations: A = *A. baumanii*; P = *P. aeruginosa*; S = *S. aureus;* TH = Todd Hewitt media (control).

Figure 3: Percent conversion of ^{13}C into volatile molecule mass in fecal, saliva, and sewage samples during 18 h of simultaneous incubation and extraction. The % conversion was calculated for fully labeled compounds by taking the mass of the fully labeled compound (M+N) and dividing it by (M+N) + the mass of the unlabeled volatile mass (M), where N is the maximum number of possible carbons (in **A–D**) or hydrogens (in **E**) that can be labeled in each volatile molecule. Compounds are considered to be fully labeled when all carbons of the volatile are replaced by ^{13}C. Where data are missing, the volatile was not detected. For example, in (**D**), 1-propanol was not detected in fecal or saliva samples. Number of replicates per sample = 3. (**A**) The ^{13}C-labeled volatiles detected in all sample types (feces, saliva, and sewage). (**B**) The ^{13}C-labeled volatiles only detected in saliva and sewage samples. (**C**) The ^{13}C-labeled volatiles detected in feces and saliva samples. (**D**) The ^{13}C-labeled volatiles detected in one of the three different sample types. (**E**) The deuterium-labeled volatile molecules.

Figure 4: Heatmap of ^{13}C-labeled volatiles from cultured sputum and volatile molecules detected from uncultured sputum. The labeled volatiles come from the stable isotope probing experiments where ^{13}C glucose and Brain Heart Infusion medium were added to sputum during the extraction step to cultivate microbial growth and capture active volatile production. The unlabeled volatile molecules were detected directly from sputum samples. The heatmap intensities are Z-scores as described in the caption of **Figure 2**. However, the Z-scores were calculated within each experiment for the cultured and uncultured sputum experiments. The dendrogram was generated as described in **Figure 2**.

Figure 5: Percent conversion of ^{13}C into volatile molecule mass in sputum samples from seven subjects with cystic fibrosis during 18 h of simultaneous incubation and extraction. The % conversion was calculated as described in the caption of **Figure 3**. Volatiles not detected in samples are indicated by the absence of data. N = 1–3. (**A**) The ^{13}C-labeled volatiles detected at a higher percent conversion in the majority of sputum samples. (**B**) The ^{13}C-labeled volatiles detected at a lower percent conversion in the majority of sputum samples. (**C**) The ^{13}C-labeled volatiles detected at a lower percent conversion in a minority of the sputum samples. Abbreviations: B = baseline; E = exacerbation; T = treatment.

Table 1: Permutated multivariate analysis of variance (PERMANOVA) of sputum samples. The PERMANOVA was generated using the **adonis** function from the **vegan** package in R.

Supplemental Figure S1: The relative abundances of labeled (M+N(max)) and unlabeled (M+0) volatiles across fecal, saliva, and sewage samples.

Supplemental Figure S2: Non-metric multidimensional scaling of cultured sputum with stable isotope probing and uncultured sputum. (**A**) The NMDS of cultured sputum with ^{13}C glucose and media was generated with k = 3 dimensions. The stress value was 0.07. (**B**) The NMDS of uncultured sputum was generated with k = 3 dimensions. The stress value was 0.13. Abbreviations: NMDS = non-metric multidimensional scaling; B = baseline; E = exacerbation; T = treatment.

Supplemental Figure S3: Microbial community composition of sputum samples from subjects with cystic fibrosis. Assessed by 16S rRNA amplicon sequencing as part of a larger study, further information about approach found in Carmody et al. 2020[19]. from subjects with cystic fibrosis. Each stacked bar is a different timepoint. Abbreviations: B = baseline, E = exacerbation, T = treatment.

Supplemental Figure S4: The relative abundances of labeled (M+N(max)) and unlabeled (M+0) volatiles across sputum samples from seven subjects with cystic fibrosis.

DISCUSSION:

To identify volatile production in *in vitro* cultures and human-associated samples, volatile analysis of mono- and co-cultures of *P. aeruginosa, S. aureus,* and *A. baumanii* and stable isotope probing of different biological samples were performed. In the analysis for the mono- and co-cultures, volatiles were detected by performing a short extraction for 1 h at 70 °C. The volatile analysis of mono- and co-cultures allowed the survey of the compounds produced both by individual species and during their interactions with other species. There were differences in relative abundances across the different culture types and time points. In the stable isotope probing experiments, the biological samples included feces and saliva from healthy subjects, sewage, and sputum from subjects with cystic fibrosis. Stable isotope profiling enabled the identification of actively produced volatile molecules by extracting for 18 h at 37 °C. The long extraction time with a lower temperature enabled growth and metabolism of microbes present in the biological samples.

Comparing the ^{13}C glucose and D_2O enrichments showed that there was more extensive isotope enrichment with labeled ^{13}C.

When extracting different sample types, there were initial optimizing steps taken prior to starting a full run. First, test different volumes of a sample as a trial run. For some sample types, sputum, for example, only small sample volumes were available. It is recommended to start with a lower volume or smaller amount of sample first, depending on sample type and available sample quantities. Do not extract a large volume or too much of the sample because it could overwhelm the column and contaminate the HSP. Column overload can be evident when peaks in the chromatogram are saturated or appear in subsequent runs. HSP contamination has occurred if carryover is present when the HSP is re-run on the GC-MS. In the mono- and co-culture experiments, 200 µL of culture was sufficient to detect a variety of volatiles. In the stable isotope probing experiments, depending on the sample type, the volume for each experiment ranged from 500 µL to 1 mL. Second, depending on the sample type and compounds of interest, the extraction time and temperature as well as the GC-MS and thermal desorption methods will need to be adjusted to optimize volatile detection. These methods were determined to be appropriate for the analytes of interest and column type.

After optimizing the method, the critical steps in the protocol pertained to the steps prior to and following extraction. During sample preparation, samples were placed on ice so that the volatiles present in the sample did not escape. It was also important to make sure the vacuum seal was tight, and the lid was securely closed. Otherwise, there would be an inefficient extraction and decreased detection of the volatiles from the sample. Leaks can arise from the O-rings around the lid liner or the HSP. To ensure that the vial was under vacuum, a gauge was used prior to extraction to make sure the O-rings were still functional. In addition, after the extraction, samples were placed on the ice block so that water was drawn out of the headspace for a determined period. Water in the column could lead to changes in retention times and abundances of the volatiles.

There are both limitations and advantages to these methods with respect to alternative methods. Evaluating the mono- and co-culture experiments, there were volatile signatures detected that were specific to a particular microbe or co-culture. There were also changes in volatile abundances across time. As for the stable isotope probing experiments in the different types of biological samples, deuterium labeling did not result in as many isotope-enriched volatiles as ^{13}C glucose. The metabolism required to produce isotope-enriched volatiles with deuterium may be more limited. In addition, media was added to the biological samples to enhance microbial growth, which may lead to changes in the microbial community composition. Culture conditions of stable isotope probing experiments may be selecting for the favored growth of certain microbes in a biological sample. This was opposed to the short extraction for one hour at 70 °C designed to detect the volatiles in the community sample before one or few of the microbes begin taking over the community. The complexity of the microbial and chemical compositions of the fecal, sewage, saliva, and sputum sample types make it difficult in assigning specific chemical signatures to any given microbial or human origin without additional analyses such as sequencing. This method provided a high-throughput, solvent-free vacuum-extraction that led to

more sensitive detection of low volume samples. The volumes used for these experiments ranged from 200 µL to 1 mL of cultured biological samples. In other cases (data not shown), biological samples (*e.g*, sputum and fecal samples) as low as 15 µL or 10 µg were extracted.

For future applications of the method, a wide range of sample types could be analyzed with small or limited volumes. Dozens of samples could be extracted simultaneously, and the run time would depend on the parameters of the specific GC-MS instrument. In the case of stable isotope probing, when coupled with metagenomic sequencing, there is the possibility of identifying the microbes responsible for the production of the volatile molecules. The metagenome of the biological sample could be sequenced prior to and after extraction with stable isotope probing to identify changes in microbial community composition. Metagenomic sequencing would allow identification of genes responsible for the production of the isotope enriched volatile molecules. Highlighted here are a few examples of the sample types and approaches that could be used as input for the presented protocol, which has already been established in different industries. Because volatile molecules are important diagnostic indicators, the use of this protocol could be expanded to biological laboratories and clinical healthcare settings.

ACKNOWLEDGMENTS:

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DISCLOSURES:

V. L. V and S. J. B. D. were former employees of Entech Instruments Inc., and K. W. is a member of Entech's University Program. J. P., J. K., and C. I. R. have no conflicts of interest to declare.

REFERENCES:

1. Van Berkel, J. J. B.N. et al. A profile of volatile organic compounds in breath discriminates COPD patients from controls. *Respiratory Medicine*. **104** (4), 557–563 (2010).
2. Nakhleh, M. K. et al. Detecting active pulmonary tuberculosis with a breath test using nanomaterial-based sensors. *European Respiratory Journal*. **43** (5), 1522–1525 (2014).
3. Lim, S. H. et al. Rapid diagnosis of tuberculosis from analysis of urine volatile organic compounds. *ACS Sensors*. **1** (7), 852–856 (2016).
4. Schnabel, R. et al. Analysis of volatile organic compounds in exhaled breath to diagnose ventilator-associated pneumonia. *Scientific Reports*. **5**, 17179 (2015).
5. Paff, T. et al. Exhaled molecular profiles in the assessment of cystic fibrosis and primary ciliary dyskinesia. *Journal of Cystic Fibrosis*. **12** (5), 454–460 (2013).
6. Robroeks, C. M. H. H. T. et al. Metabolomics of volatile organic compounds in cystic fibrosis patients and controls. *Pediatric Research*. **68** (1), 75–80 (2010).
7. Neerincx, A. H. et al. Hydrogen cyanide emission in the lung by *Staphylococcus aureus*. *European Respiratory Journal*. **48** (2), 577–579 (2016).
8. Goeminne, P. C. et al. Detection of *Pseudomonas aeruginosa* in sputum headspace through volatile organic compound analysis. *Respiratory Research*. **13**, 87 (2012).
9. Joensen, O. et al. Exhaled breath analysis using Electronic Nose in cystic fibrosis and primary ciliary dyskinesia patients with chronic pulmonary infections. *PLOS ONE*. **9** (12), e115584

(2014).
10. Nasir, M. et al. Volatile molecules from bronchoalveolar lavage fluid can 'rule-in' *Pseudomonas aeruginosa* and 'rule-out' *Staphylococcus aureus* infections in cystic fibrosis patients. *Scientific Reports*. **8** (1), 826 (2018).
11. Tyc, O., Zweers, H., de Boer, W., Garbeva, P. Volatiles in inter-specific bacterial interactions. *Frontiers in Microbiology*. **6**, 1412 (2015).
12. Gao, B. et al. Tracking polymicrobial metabolism in cystic fibrosis airways: *Pseudomonas aeruginosa* metabolism and physiology are influenced by *Rothia mucilaginosa*-derived metabolites. *mSphere*. **3** (2), e00151-18 (2018).
13. Schoenheimer, R., Rittenberg, D. Deuterium as an indicator in the study of intermediary metabolism. *Science*. **82** (2120), 156–157 (1935).
14. Neubauer, C. et al. Refining the application of microbial lipids as tracers of *Staphylococcus aureus* growth rates in cystic fibrosis sputum. *Journal of Bacteriology*. **200** (24), e00365-18 (2018).
15. Cordell, R. L., Pandya, H., Hubbard, M., Turner, M. A., Monks, P. S. GC-MS analysis of ethanol and other volatile compounds in micro-volume blood samples—quantifying neonatal exposure. *Analytical and Bioanalytical Chemistry*. **405** (12), 4139–4147 (2013).
16. Mayor A, R. S. Optimisation of sample preparation for direct SPME-GC-MS analysis of murine and human faecal volatile organic compounds for metabolomic studies. *Journal of Analytical & Bioanalytical Techniques*. **5** (2), 184 (2014).
17. Camarasu, C. C. Headspace SPME method development for the analysis of volatile polar residual solvents by GC-MS. *Journal of Pharmaceutical and Biomedical Analysis*. **23** (1), 197–210 (2000).
18. Charry-Parra, G., DeJesus-Echevarria, M., Perez, F. J. Beer volatile analysis: optimization of HS/SPME coupled to GC/MS/FID. *Journal of Food Science*. **76** (2), C205–C211 (2011).
19. Bicchi, C., Cordero, C., Liberto, E., Rubiolo, P., Sgorbini, B. Automated headspace solid-phase dynamic extraction to analyse the volatile fraction of food matrices. *Journal of Chromatography A*. **1024** (1), 217–226 (2004).
20. Trujillo-Rodríguez, M. J., Anderson, J. L., Dunham, S. J. B., Noad, V. L., Cardin, D. B. Vacuum-assisted sorbent extraction: An analytical methodology for the determination of ultraviolet filters in environmental samples. *Talanta*. **208**, 120390 (2020).
21. Mollamohammada, S., Hassan, A. A., Dahab, M. Immobilized algae-based treatment of herbicide-contaminated groundwater. *Water Environment Research*. **93** (2), 263–273 (2021).
22. Psillakis, E. The effect of vacuum: an emerging experimental parameter to consider during headspace microextraction sampling. *Analytical and Bioanalytical Chemistry*. **412** (24), 5989–5997 (2020).
23. Carmody, L. A. et al. The daily dynamics of cystic fibrosis airway microbiota during clinical stability and at exacerbation. *Microbiome*. **3**, 12 (2015).
24. Carmody, L. A. et al. Fluctuations in airway bacterial communities associated with clinical states and disease stages in cystic fibrosis. *PLOS ONE*. **13** (3), e0194060 (2018).
25. Mahboubi, M. A. et al. Culture-based and culture-independent bacteriologic analysis of cystic fibrosis respiratory specimens. *Journal of Clinical Microbiology*. **54** (3), 613–619 (2016).

Figure 1

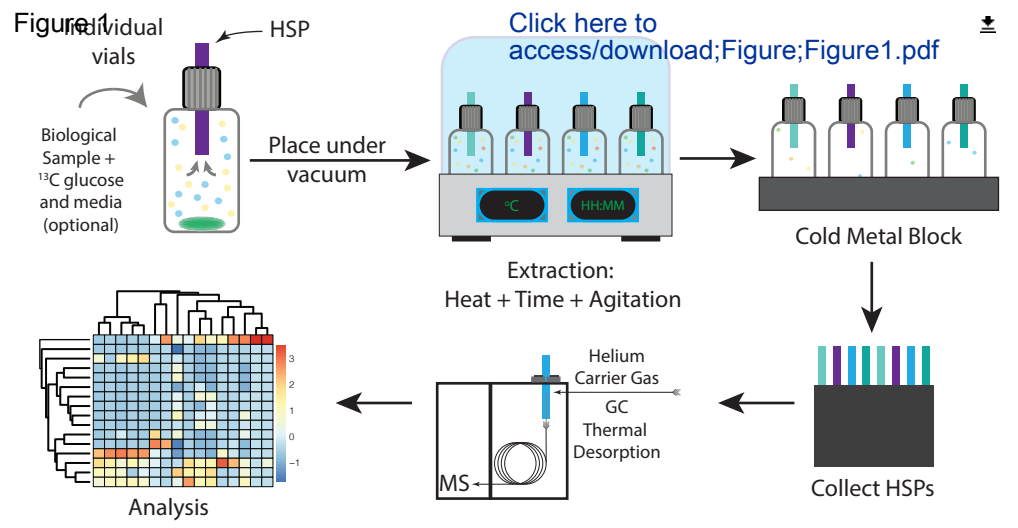


Figure 2

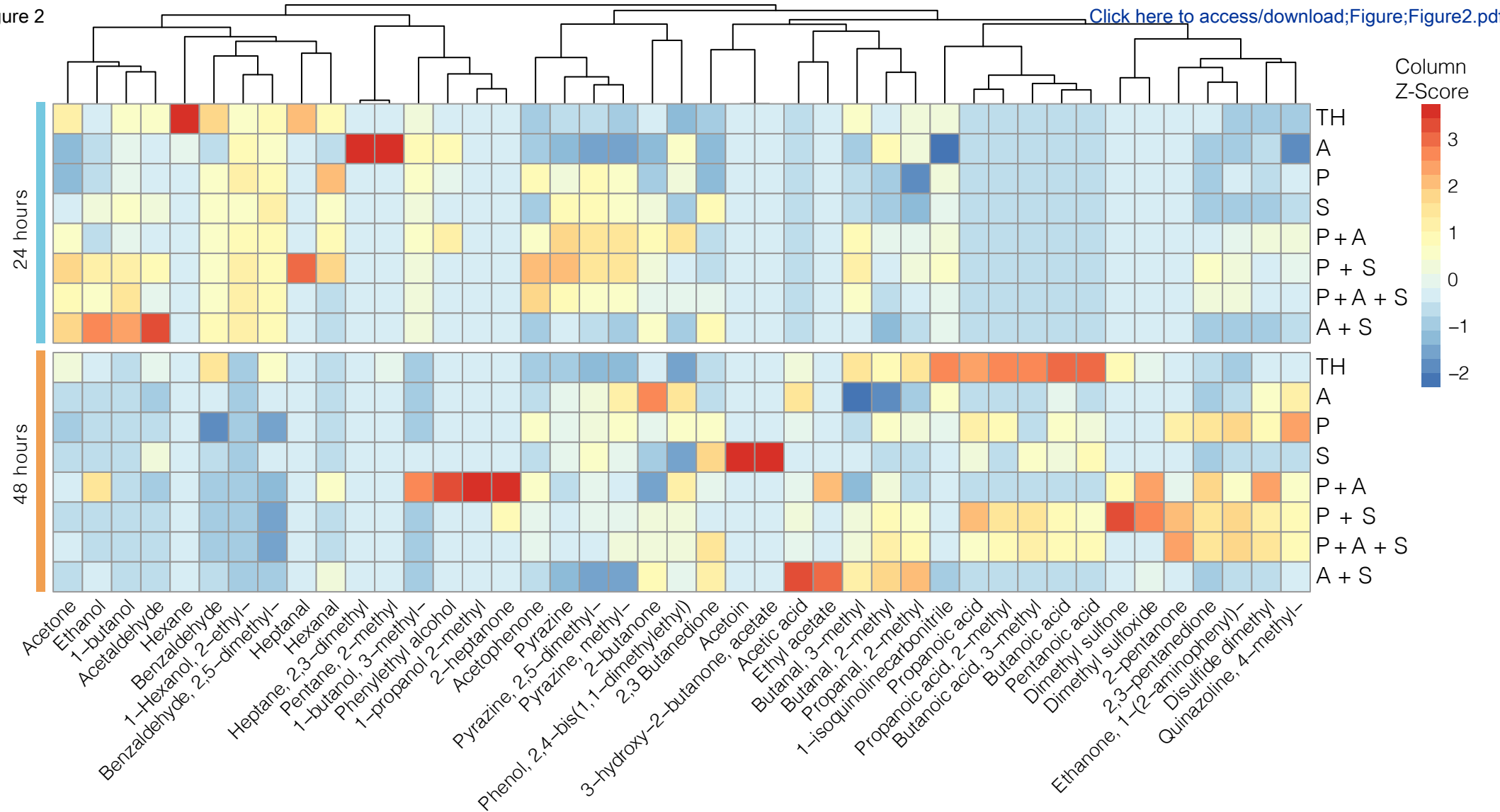


Figure 3

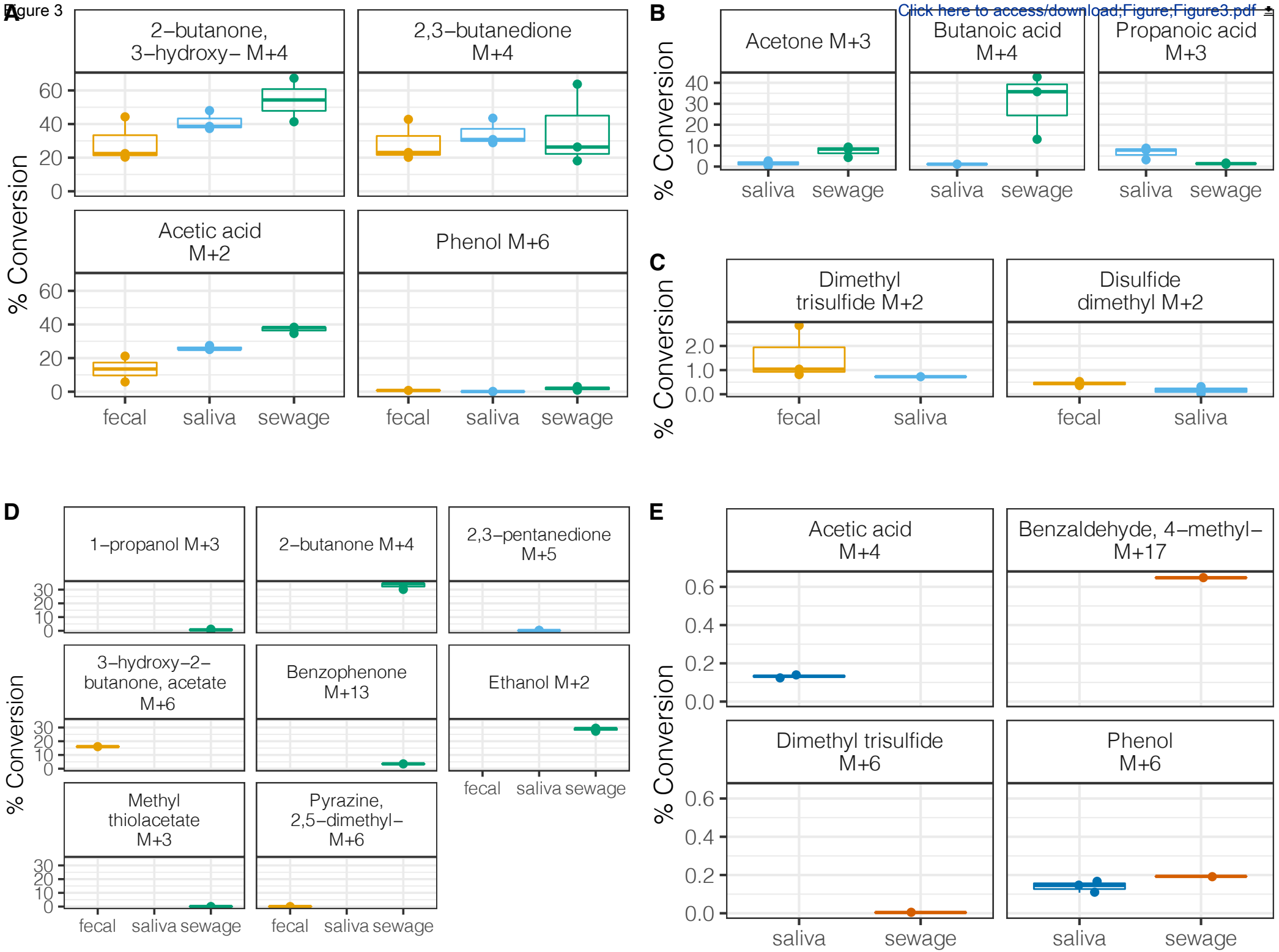


Figure 4

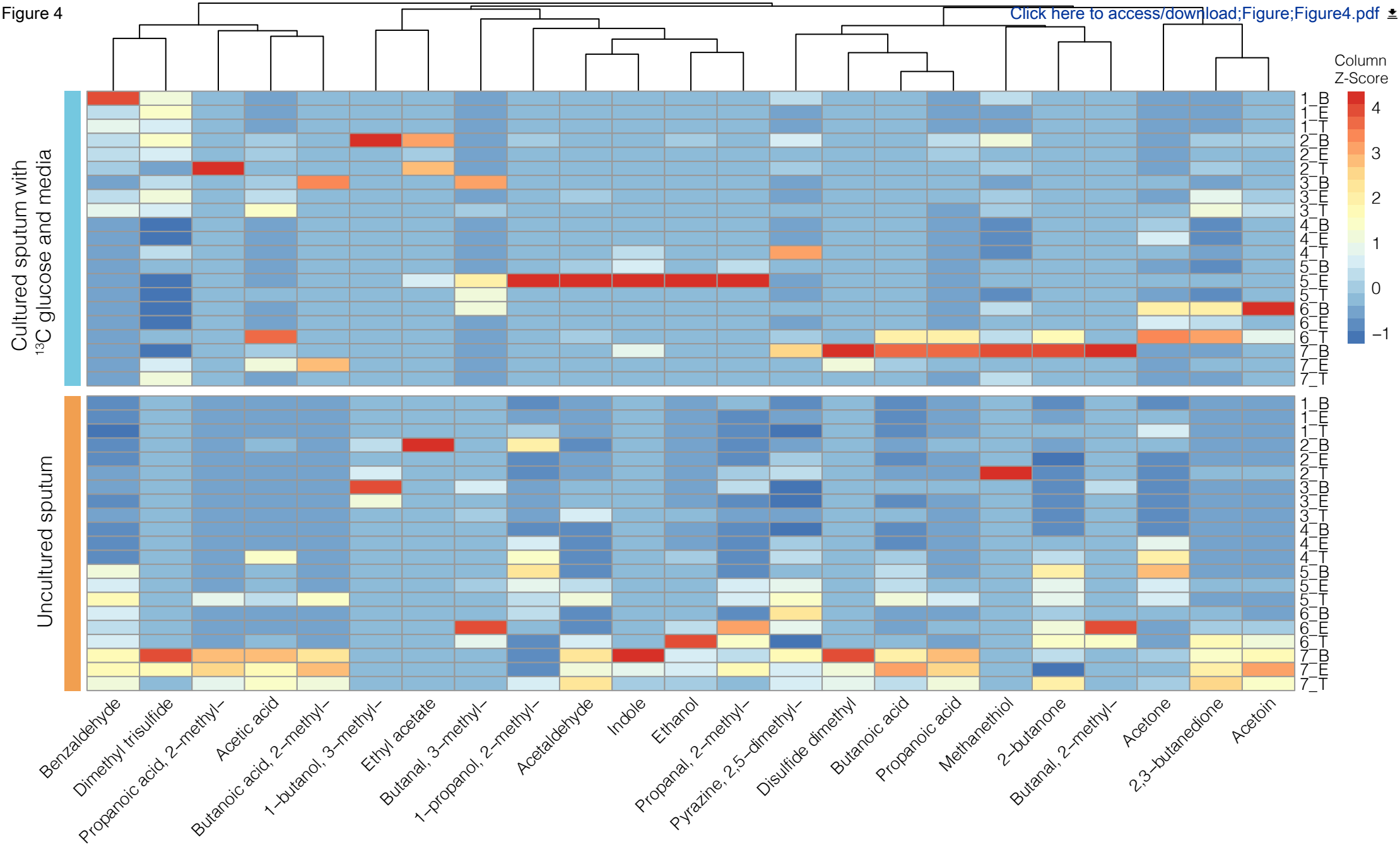


Figure 5

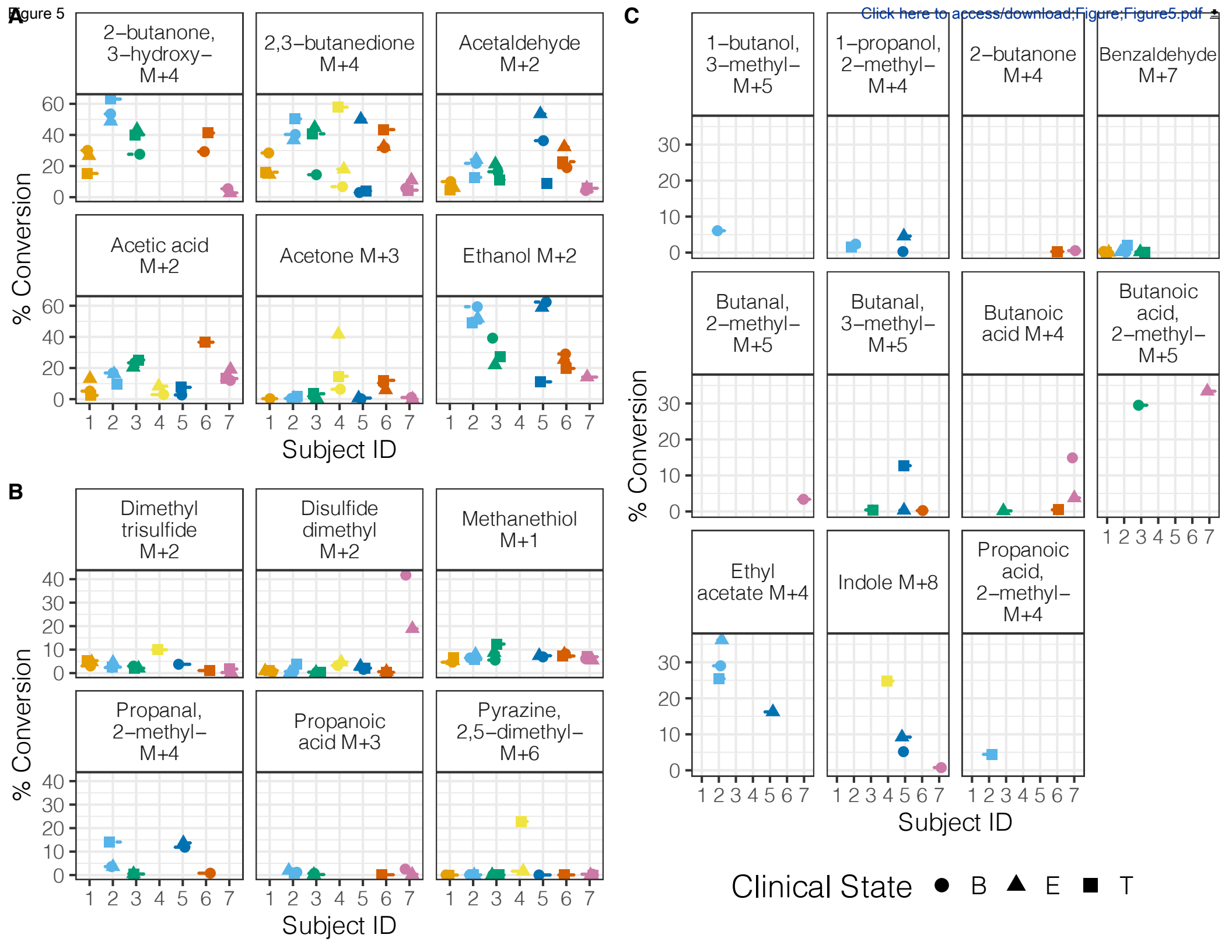

A
2–butanone, 3–hydroxy– M+4
2,3–butanedione M+4
Acetaldehyde M+2
Acetic acid M+2
Acetone M+3
Ethanol M+2
% Conversion
Subject ID
B
Dimethyl trisulfide M+2
Disulfide dimethyl M+2
Methanethiol M+1
Propanal, 2–methyl– M+4
Propanoic acid M+3
Pyrazine, 2,5–dimethyl– M+6
% Conversion
Subject ID
C
Click here to access/download;Figure;Figure5.pdf
1–butanol, 3–methyl– M+5
1–propanol, 2–methyl– M+4
2–butanone M+4
Benzaldehyde M+7
Butanal, 2–methyl– M+5
Butanal, 3–methyl– M+5
Butanoic acid M+4
Butanoic acid, 2–methyl– M+5
Ethyl acetate M+4
Indole M+8
Propanoic acid, 2–methyl– M+4
% Conversion
Subject ID
Clinical State B E T

Table 1

		Degrees of Freedom	R^2
Uncultured sputum	Subject	6	0.33
	Clinical State	2	0.01
	Subject: clinical State	12	0.12
Cultured sputum with ^{13}C glucose and media	Subject	6	0.51
	Clinical State	2	0.02
	Subject: clinical state	12	0.11

P value

0.001
0.46
0.092
0.001
0.095
0.194

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
^{13}C glucose	Sigma-Aldrich	389374-1G	
2-Stg Diaph Pump	Entech Instruments	01-10-20030	
20 mL VOA vials	Fisher Scientific	5719110	
24 mm Black Caps with hole, no septum	Entech Instruments	01-39-76044B	holds lid liner in place on vial
24 mm vial liner for sorbent pens	Entech Instruments	SP-L024S	allows pens to make a vacuum seal at top of via
5600 Sorbent pen extraction unit (SPEU)	Entech Instruments	5600-SPES	5600 Sorbent Pen Extraction Unit -120 VAC
96-well assay plates	Genesee	25-224	
Brain Heart Infusion (BHI) media	Sigma-Aldrich	53286-500G	
ChemStation Stofware	Agilent		
DB-624 column	Agilent	122-1364E	60 m, 0.25 mm ID, 1.40 micron film thickness, ir
Deuterium oxide	Sigma-Aldrich	151882-1L	
Dexsi sofware	Dexsi (open source)		
GC-MS (7890A GC and 5975C inert XL MSD with Triple-Axis Detector)	Agilent		7890A GC and 5975C inert XL MSD with triple-a
Headspace Bundle HS-B01, 120VA	Entech Instruments	SP-HS-B01	Items for running headspace extraction include
Headspace sorbent pen (HSP) - blank	Entech Instruments	SP-HS-0	
Headspace sorbent pen (HSP) Tenax TA (35/60 Mesh)	Entech Instruments	SP-HS-T3560	
Microcentrifuge tubes (2 mL)	VWR	53550-792	
O-rings	Entech Instruments	SP-OR-L024	
Sample Preparation Rail	Entech Instruments		
Sorbent pen thermal conditioner	Entech Instruments	3801-SPTC	
Todd Hewitt (TH) media	Sigma	T1438-500G	

l

ı GC-MS

xis detector
d in bundle

Editorial comments:
Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

Response: **We thank the editor and reviewers for their constructive feedback. Any comments and suggestions given are addressed below. Authors have thoroughly proofread the manuscript and all abbreviations are defined at first use.**

2. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials, e.g.,

Entech autosampler**; (named the Sample Preparation Rail (SPR))**
HSP with Tenax TA; **(named the Headspace sorbent pen (HSP) Tenax TA (35/60) Mesh)**
5600 sorbent pen extraction unit;
Agilent GC-MS (7890A GC and 5975C inert XL MSD with Triple-Axis Detector) with a DB-624 column;
ChemStation software etc.

Obviously, if you have optimized your protocol with certain instruments/reagents/software, you will need to mention them, but include just the bare minimum information (e.g., name of product) in the manuscript and come up with a generic term to refer to the product (include this in the comments column in the Table of Materials) after the first mention so that you don't keep repeating commercial terms throughout the paper.

Response: **Trademark symbols are not included in the manuscript. Company names have been removed before an instrument or reagent and generic names are used. Commercial products are referenced in the Table of Materials. We revised the Materials document to include the listed materials above.**

3. Please revise the text, especially in the protocol, to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Response: **The use of personal pronouns is not included in the protocol.**

4. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

Response: **Text in the protocol section is written in the imperative tense. Suggestive text is included as "Note." The safety procedures and use of hoods has been added to the protocol.**

5. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g., button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

Response: **Specific details for performing the protocol and software actions are included in the protocol.**

6. After including a one line space between each protocol step, highlight up to 3 pages of protocol text for inclusion in the protocol section of the video. This will clarify what needs to be filmed.

Response: **One line space has been added between each protocol step. The highlighted materials for filming have been updated.**

7. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article

Title. Source (italics). Volume (bold) (Issue), FirstPage–LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Please include volume and issue numbers for all references and do not abbreviate journal names.

Response: **The references are updated to the format above. The full journal names are listed.**

8. Please sort the Materials Table alphabetically by the name of the material.

Response: **The Materials table is alphabetically sorted by the name of the material.**

Reviewer #1:
Manuscript Summary:
The manuscript deals with the application of headspace vaccum assisted sorbent extraction (VASE) technique for determining the volatile organic compounds (VOCs) profile of biological samples using GC-MS. Specifically, it was first applied for the analysis of VOCs from different bacterial cultures to elucidate the origin of the compounds. Then, it was applied for the analysis of several type of human biological samples to identify the VOCs produced: the isotope labelling was studied and then used for the analysis of sputum samples from different individuals.
I think the study is very interesting considering the potential of the technique. Indeed, the advantages of the VASE technique are perfectly highlighted during the procedure description and the discussion of the results, while its limitations and precautions are also discussed. The experimental procedures for both samples preparation and analytical determination are described in detail and give the required information to perform the experiments. Therefore, I think the manuscript is suitable for publication.

Major Concerns:
No applicable.

Minor Concerns:
- I suggest adding some information regarding the selection of the extraction phase (composition) of the HSP. Is only the type of GC column the reason behind the selection of Tenax? What other considerations should be taken into account? Type of sample, target compounds? What about the reproducibility of the technique?

Response: **The GC column is not the only reason behind the selection of Tenax. Tenax is capable of capturing a broad range of volatile molecules and has a lower affinity for water compared to other sorbents, which enables it to trap VOCs from higher moisture samples. Tenax also has a low level of impurities and can be conditioned for re-use. As for the reproducibility of the technique, we have performed analyses in triplicate, but the data are not shown.**

- I also suggest adding the paramters considered for the identification of the peaks-compounds using the NIST library: minimum match of mass spectrum? Probability?

Response: **The parameter for the identification of the peaks using the NIST library was at least 75% probability. We also selected peaks based on the alignment of the identifying ions within the center of the peak during quality filtering in the QEdit step in section 6 of the protocol.**

- Just out of curiosity, the data demonstrated the volatile profile in the analysis of sputum is specific for each individual (according to the statistical analysis), but what about the volatile profile in the different clinical stages? Is there any difference for any of the individuals? I think this should be discussed in the text. Considering the wide variety of VOCs identified and the features of the technique, it may be a promising alternative as a prognosis, diagnosis and evaluation tool for some disorders (cystic fibrosis in this particular case).

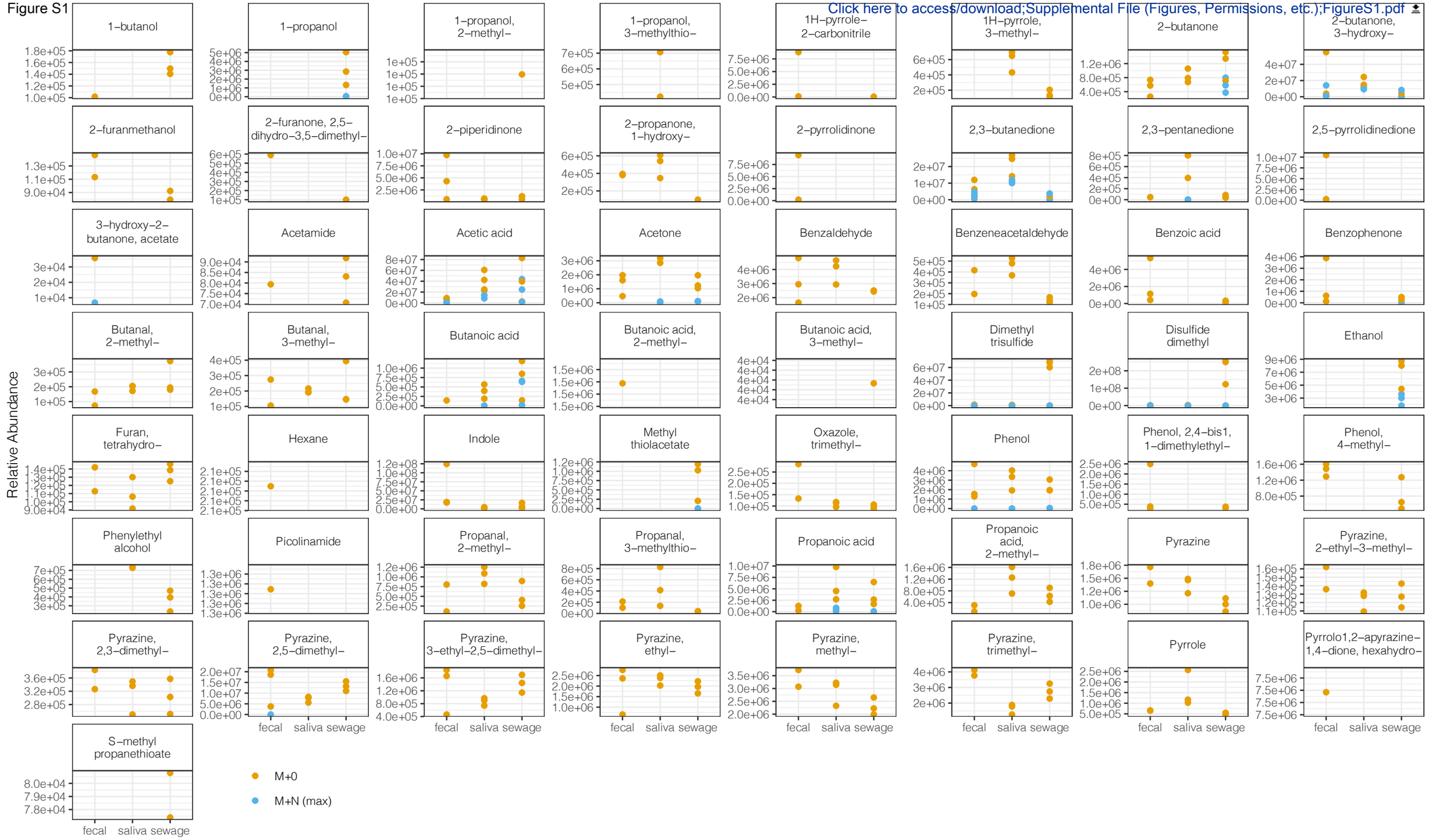
Response: **There were no significant differences in clinical states found in the overall cohort for the uncultured or cultured sputum samples. When clinical state was nested within the subject in a permutated multivariate analysis of variance (PERMANOVA) model, there was no significant amount of variation explained by clinical state (Subject: Clinical State, Table 1). There may be important signals of clinical state within an individual, but the analysis was performed on a limited number of samples (one sample per clinical state per person).**

Reviewer #2:
Manuscript Summary:
The manuscript describes analytical methods for the extraction of volatile and semi-volatile organic compounds in complex biological matrices using vacumm-assisted sorbent extraction. This approach is incipient, but the results clearly illustrated the potential of VASE for GC-MS methods.

The manuscript is well written and organized.Introduction: the background is clearly presented to the interested reader. The analytical challenges were not clearly stated. Material and methods: the authors provided a complete description of the methods, which should allow non-expert users to reproduce the experiments. The results are supported by the described methods. Results and discussion: the discussion is supported by the discussion. Major Concerns: none Minor Concerns: introduction could be improved.

Response: **Thank you for your concerns stated in your summary. We included the analytical challenges of other techniques that have been addressed with the methods presented in this paper. One of the analytical challenges include the complex sample types. In this protocol, we included fecal, sewage, saliva, and sputum samples. The complexity of the sample makes it difficult in assigning specific chemical signatures to any given microbial or human origin without additional analyses such as sequencing. The second analytical challenge is the difficulty in sampling volatiles for several reasons. The heterogeneous water content of sputum sometimes made it difficult to reproducibly extract the same sputum sample. In addition, volatile signatures may change upon sample culturing during the simultaneous extraction and incubation step for stable isotope probing. There may also be potential loss of very volatile molecules due to the strength of Tenax at room temperature. In the manuscript, we addressed these main comments and advised the user to optimize the sample preparation, extraction, and run conditions for analysis.**

Figure S1



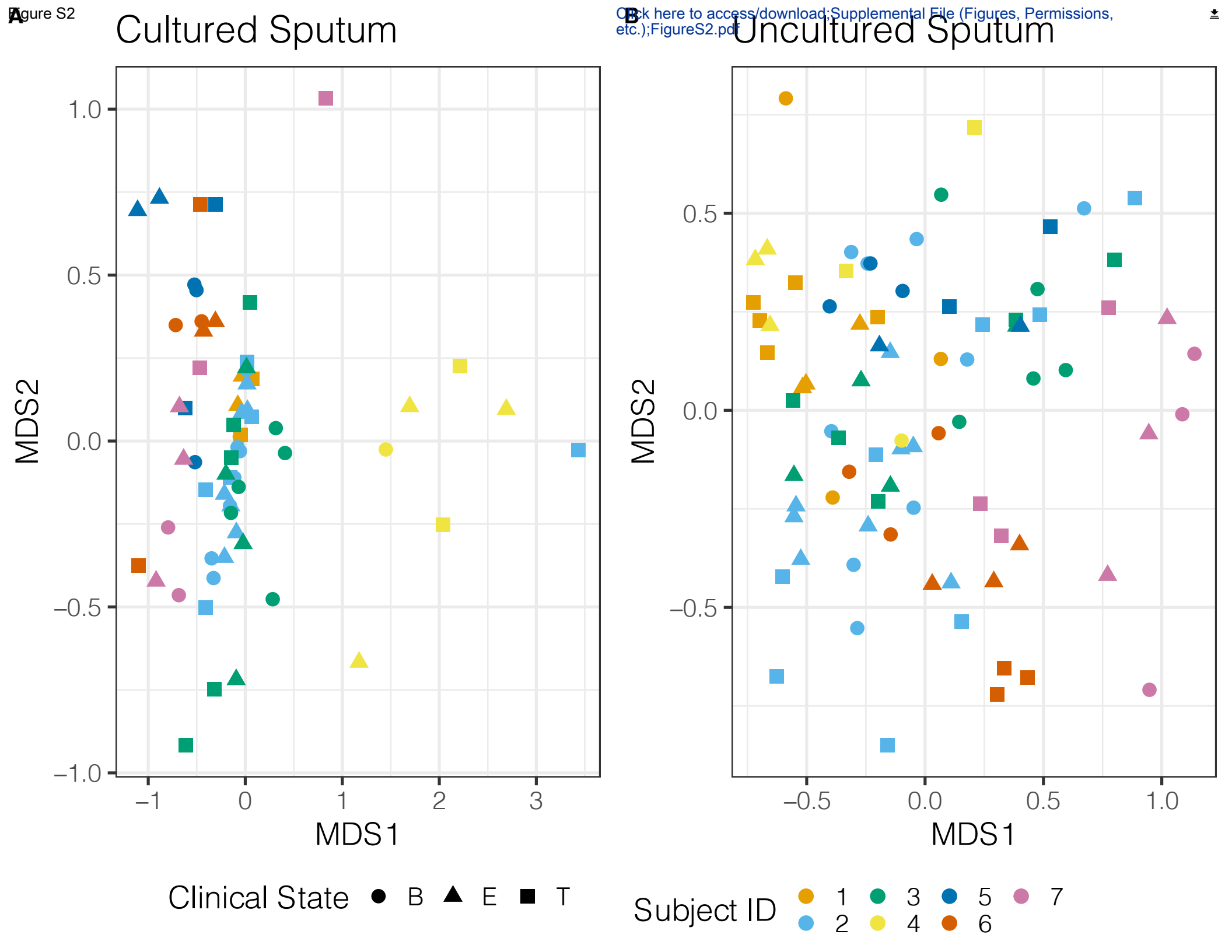


Figure S3

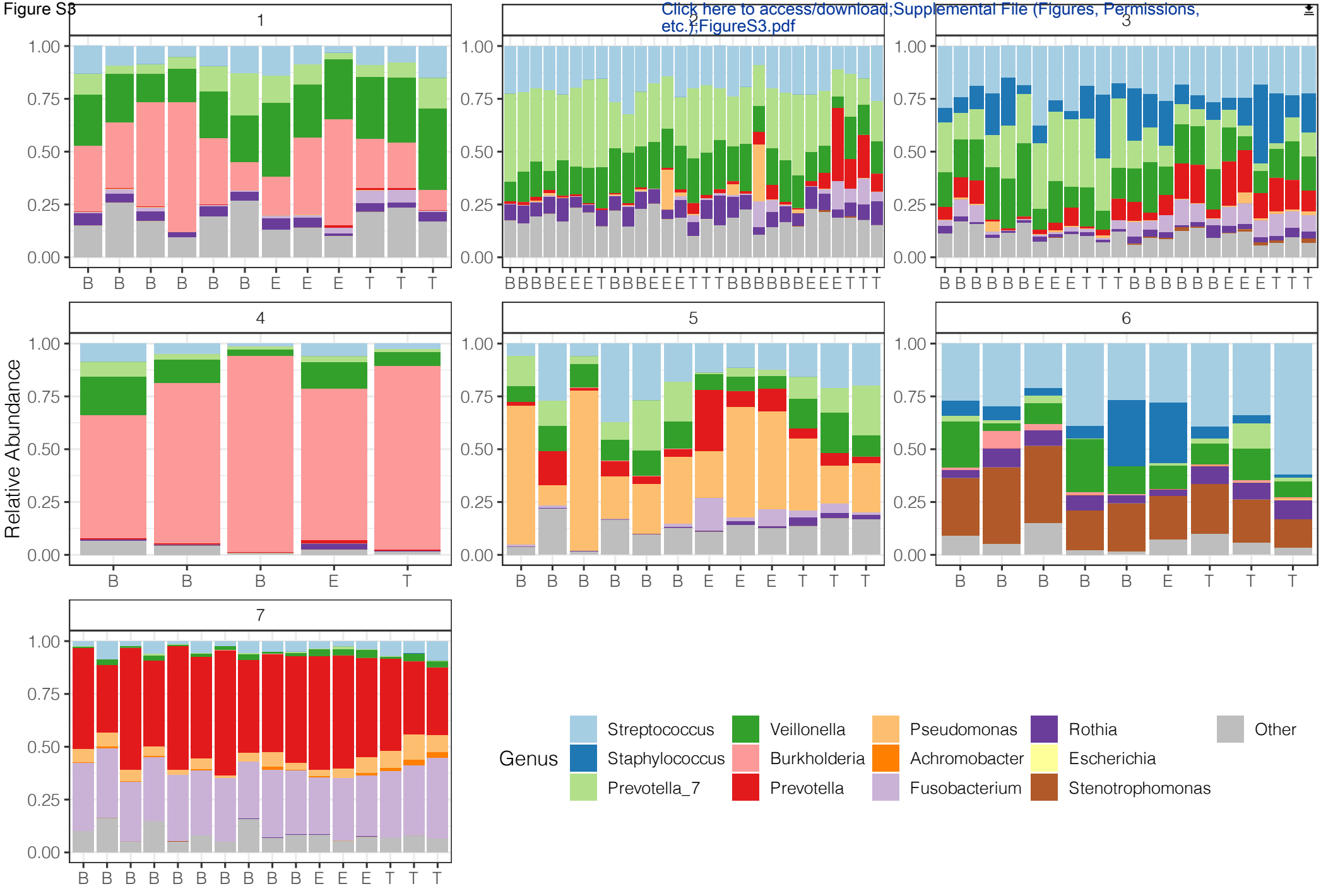


Figure S4