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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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## Author Questionnaire

**1. Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**

**2. Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes, all done**

**3. Interview statements:** Considering the COVID-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

Interviewees wear masks until videographer steps away (≥6 ft/2 m) and begins filming, then the interviewee removes the mask for line delivery only. When take is captured, the interviewee puts the mask back on. Statements can be filmed outside if weather permits.

**4. Filming location:** Will the filming need to take place in multiple locations?  **No**

**Current Protocol Length**

Number of Steps: 21

Number of Shots: 49

# Introduction

1. **Introductory Interview Statements**

**REQUIRED:**

* 1. Joann Phan**:** This protocol allows us to easily concentrate and identify volatile metabolites and actively produced volatiles from microbial organisms in a variety of biological samples **[1]**.
     1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: Figure 1.*
  2. Joseph Kapcia III**:** VASE is a more user-friendly way of concentrating low abundance volatiles. All you need is a sample under near vacuum, and let physics do the rest **[1].**
     1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 3.5.3.*

**OPTIONAL:**

* 1. Katrine Whiteson**:** The implications of this technique include the possibility of identifying metabolic biomarkers that may have importance in different diseases or phenotypes of interest. The pathogen driving an airway infection or successful antibacterial treatment could be detected from sputum, saliva, or breath **[1]**.
     1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 6.7.*

**Ethics Title Card**

* 1. The feces and saliva samples were donated from anonymous donors with approval from the University of California Irvine Institutional Review Board. 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.

# Protocol

1. **Stable Isotope Probing in Biological Samples Preparation**
   1. To prepare fecal samples, add 1 milliliter of deionized water to 100 milligrams of feces in a 1.5-milliliter microcentrifuge tube **[1]** and vortex for 3 minutes. Keep the samples on ice when not in use **[2]**.
      1. Talent adding deionized water to the feces sample in a microcentrifuge tube.
      2. Talent vortexing the tube.
   2. Add 485 microliters of BHI *(B-H-I)* medium with 20 millimolar 13C *(13-C)* glucose or BHI with 30% deuterium to 15 microliters of fecal and water mixture, ensuring that the final volume of the sample is 500 microliters **[1-TXT]**.Prepare samples in technical triplicates **[2]**.
      1. Talent adding BHI medium with 13C glucose or 30% deuterium to the sample. **TEXT: BHI: Brain Heart Infusion**
      2. Shot of prepared samples in triplicates.
   3. To prepare sewage samples, add 500 microliters of sewage to 500 microliters of BHI medium with 13C glucose or 30% deuterium for a total volume of 1 milliliter **[1]**. Prepare samples in triplicates and keep them on ice **[2]**.
      1. Talent adding BHI to sewage sample.
      2. Shot of prepared samples on ice.
   4. To prepare saliva samples, add 50 microliters of saliva to 500 microliters of BHI medium with 13C glucose or 30% deuterium for a total volume of 550 microliters **[1]**. Prepare samples in triplicates and keep them on ice **[2]**.
      1. Talent adding BHI medium to saliva sample.
      2. Shot of prepared samples on ice.
   5. To prepare sputum samples, add 15 microliters of sputum into a vial **[1]**. Prepare samples in triplicates and keep them on ice **[2]**.
      1. Talent adding sputum sample to vial.
      2. Shot of prepared samples on ice.
2. **Sample Extraction**
   1. Place empty VOA *(V-O-A)* vials on the cold plate **[1-TXT]**, and place the cold plate on ice in the biosafety hood **[2]**
      1. Talent placing the empty VOA vials on the cold plate. **TEXT: VOA: Volatile organic analysis**
      2. Talent placing the cold plate on ice in biosafety hood.
   2. Turn on the 5600 SPEU *(S-P-E-U)* **[1-TXT]** and adjust it to the required temperature **[2].**
      1. Talent turning on the SPEU. **TEXT: SPEU:** S**orbent pen extraction unit**
      2. Talent adjusting the temperature.
   3. Label 20-milliliter VOA vials according to samples, replicates, and HSP IDs *(H-S-P-eye-dees)* using a water-resistant marker that resists water in case condensation forms on the outside of the vial while on ice **[1-TXT].**
      1. Talent labeling the VOA vials. **TEXT: HSP: Headspace sorbent pen**
   4. Inside the biosafety hood, unscrew the white cap on the vial **[1]**, quickly pipette sample into the vial **[2]**, and assemble the lid liner **[3]**, black cap **[4]**, and HSP **[5].**
      1. Talent unscrewing the white cap of the vial.
      2. Talent pipetting the sample to the vial.
      3. Talent assembling the lid liner.
      4. Talent assembling the black cap.
      5. Talent assembling the HSP.
   5. Place the vial containing the sample and HSP back on the cold plate **[1].** Once all samples have been prepared in the glass vials, turn on the vacuum pump **[2]**,place the vials under vacuum **[3-TXT]**,and remove the vacuum source **[4].** *Videographer: This step is important!*
      1. Talent placing the vial containing sample on the cold plate.
      2. Talent turning on the vacuum pump.
      3. Talent placing the vials under vacuum. **TEXT:** **30 mmHg**
      4. Talent removing the vacuum source.
   6. Double-check the pressure after placing all samples under vacuum using the pressure gauge **[1]**. If a vial is leaking, ensure that the cap is screwed on tightly **[2]** and that the white O-rings of the HSP and lid liners are correctly in place **[3].** *Videographer: This step is important!*
      1. Talent checking the pressure of all samples using a pressure gauge.
      2. Talent screwing the cap tightly.
      3. Talent placing the HSP and lid liners correctly.
   7. Place vials in the SPEU for the optimized time and temperature with agitation at 200 rpm **[1]**. Extract cultures for 1 hour at 70 degrees Celsius, and extract stable isotope probing experiments with fecal, sewage, saliva, and sputum samples for 18 hours at 37 degrees Celsius **[2]**.
      1. Talent placing the vials in the SPEU with agitation for extracting the culture and samples.
      2. Talent setting the time and temperature.
   8. Place the cold plate at minus 80 degrees Celsius for use after the extraction period is complete **[1].** 
      1. Talent placing the cold plate at minus 80 degrees.
   9. When extraction is complete, place samples on the cold plate for 15 minutes to draw out water vapor from the HSP and vial headspace **[1]**, thentransfer the HSPs *(H-S-Pees)* to their sleeves **[2].** *Videographer: This step is important!*
      1. Talent placing the samples on cold plate.
      2. Talent transferring the HSPs to their sleeves.
3. **Analyze Samples on the Gas Chromatography–Mass Spectrometer (GC-MS)**
   1. Set up the sequence of samples on the Entech Software. Open the program **[1]** and select **5800** and **Sequence** in the options to the right of the instrument dropdown menu **[2].**
      1. SCREEN: 4.1.1 edited.mov. 00:00-00:03. *Videographer: Obtain a few shots of talent clicking the mouse and typing on the keyboard to use as b-roll throughout the video.*
      2. SCREEN: 4.1.2 edited.mov. 00:00-00:28. *Video Editor: Speedup and play.*
   2. Save the sequence table **[1]**, select **Run** on the left-hand side, then **Start with blank in desorber** if the blank HSP is in the desorber **[2].**
      1. SCREEN: 4.2.1 edited.mov. 00:00-00:15.
      2. SCREEN: 4.2.2 edited.mov. 00:00-00:03.
   3. Note that HSPs will be handled by the SPR for each sample in the sequence. Let the SPR warm up **[1-TXT]**.
      1. SCREEN: 4.3.1 and 4.4.2 edited.mov. 00:00-00:08 **TEXT: SPR: Sample Preparation Rail.**
   4. Allow the SPR to run all samples automatically. The sequence on the GC-MS *(G-C-M-S)* side will automatically record the data in separate files **[1].** *Videographer: This step is important!*
      1. SCREEN: 4.3.1 and 4.4.2 edited.mov. 00:08-00:18
4. **Data Analysis**
   1. Add a peak to the processing method by selecting **Calibrate,** followed by **Edit Compound, Name,** and **insert compound** under **External Standard Compound**. Add the name of the compounds, retention time, and Quant Signal **Target Ion** **[1]**.
      1. SCREEN: 5.1.1 edited.mov 00:00-00:31.
   2. Add the three largest peaks, which include compounds with a greater than 75% probability, ensuring that the alignment of each identifying ion of the compound lies within the center of the peak **[1].** Save it by selecting **ok** followed by **Method** and **Save** **[2].**
      1. SCREEN: 5.2.1 edited.mov 00:00-00:34. *Video Editor: Speedup and play.*
      2. SCREEN: 5.2.2 edited.mov. 00:00-00:18.
   3. Once the process method is set up, proceed to **Quantitate** and **Calculate**, then **View** and **QEdit Quant Result** to quantitate the data **[1]**.*Videographer: This step is important!*
      1. SCREEN: 5.3.1 edited.mov. 00:00-00:25.

# Results

*Videographer: The authors want to use their own voice for the Results section. Please record the audio while the authors read the result statements below (6.1-6.9).*

1. **GC-MS, Heatmaps, and Data Analysis of the 13C-Labeled Volatiles in Fecal, Saliva, Sewage, Cultured and Uncultured Sputum Samples**
   1. Here, vacuum assisted sorbent extraction was followed by thermal desorption on a GC-MS 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.
      1. LAB MEDIA: Figure 1
   2. The mono- and co-cultures consisted of the bacterial species *Staphylococcus aureus, Pseudomonas aeruginosa,* and *Acinetobacter baumannii*. 43 annotated volatile molecules were detected from the mono- and co-cultures at 24- and 48-hour timepoints **[1].**
      1. LAB MEDIA: Figure 2.
   3. There was more incorporation of 13C into fully labeled volatile molecules **[1]** than the deuterium **[2].** 13C was incorporated into 2-butanone, 3-hydroxy; 2,3-butanedione; acetic acid; and phenol for all fecal, sewage, and saliva samples **[3].**
      1. LAB MEDIA: Figure 3A-3D.
      2. LAB MEDIA: Figure 3E.
      3. LAB MEDIA: Figure 3A.
   4. Acetone, butanoic acid, and propanoic acid were detected as labeled in saliva and sewage **[1]**,whereas dimethyl trisulfide and disulfide dimethyl were enriched in both fecal and saliva samples **[2].**
      1. LAB MEDIA: Figure 3B.
      2. LAB MEDIA: Figure 3C.
   5. Volatiles 1-propanol, 2-butanone, benzophenone, ethanol, and methyl thiolacetate, were enriched only in sewage **[1]** and 2,3-pentanedione in saliva **[2].**
      1. LAB MEDIA: Figure 3D. *Video editor: Focus on all the graphs with green points.*
      2. LAB MEDIA: Figure 3D. *Video editor: Focus on the 2,3-pentanedoine graph with blue points.*
   6. Deuterium was incorporated into the volatiles, acetic acid; benzaldehyde, 4-methyl; dimethyl trisulfide; and phenol, from either saliva or sewage samples **[1].**
      1. LAB MEDIA: Figure 3E.
   7. Acetic acid, dimethyl trisulfide, acetone, and propanal, 2-methyl were more abundant in the cultured sputum samples than uncultured sputum samples **[1].**
      1. LAB MEDIA: Figure 4.
   8. We conducted a statistical test to ask how much variance is explained by different factors describing our Cystic Fibrosis sputum samples. A permutated multivariate analysis of variance (a PERMANOVA) was performed on a bray-curtis distance matrix of the volatile abundances from the cystic fibrosis sputum samples. We found that the subject who donated the sample explains 51% of the variation in 13C-labeled cultured sputum and 33% of the variation in uncultured sputum **[1]**.

Variation: A permutated multivariate analysis of variance (a PERMANOVA) was performed to ask how much variance in volatile abundance patterns is explained by the subject who donated the cystic fibrosis sample or their clinical state. We found that the subject who donated the sample explains 51% of the variation in 13C-labeled cultured sputum and 33% of the variation in uncultured sputum

* + 1. LAB MEDIA: Table 1.
  1. Here, the success of stable isotope labeling with 13C glucose in volatiles from cultured sputum samples collected from 7 people with cystic fibrosis is shown. Volatiles that have higher 13C incorporation for the majority samples are shown in panel 5A, **[1]** those with lower percent 13C incorporation in the majority of sputum samples are in panel 5B, **[2]** and molecules with lower 13C percent conversion in a minority of the sputum samples are shown in panel 5C **[3]**.
     1. LAB MEDIA: Figure 5A.
     2. LAB MEDIA: Figure 5B.
     3. LAB MEDIA: Figure 5C.

# Conclusion

1. **Conclusion Interview Statements**
   1. Joseph Kapcia III**:** Always double check your vial pressures after about a minute. A broken vacuum defeats the sensitivity and speed of the VASE method **[1]**.
      1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 3.6.*
   2. **Joann Phan:** Following this procedure, if stable isotope probing was performed, DNA can be extracted from the remaining material to identify the microbial community or species that may have contributed to the production of the volatile molecules **[1]**.
      1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 6.1.*

* 1. **Joann Phan:** This method also applies to detecting volatiles from any sample type without isotope labeling. With the advantages of sensitivity and low sample volume, many applications can benefit from this technique **[1]**.
     1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.