

**REPORT DOCUMENTATION PAGE**Form Approved  
OMB No. 0704-0188

The public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

<b>1. REPORT DATE (DD-MM-YY)</b> 10-03-20		<b>2. REPORT TYPE</b> Journal Article		<b>3. DATES COVERED (From - To)</b>	
<b>4. TITLE AND SUBTITLE</b> Evaluation of a standardized collection device for exhaled breath sampling onto thermal desorption tubes				<b>5a. CONTRACT NUMBER</b> FA8650-17-F-6831	
				<b>5b. GRANT NUMBER</b>	
				<b>5c. PROGRAM ELEMENT NUMBER</b>	
<b>6. AUTHOR(S)</b> Sean W. Harshman <sup>1*</sup> , Rhonda L. Pitsch <sup>2</sup> , Christina N. Davidson <sup>3</sup> , Erica M. Lee <sup>4</sup> , Alexander M. Scott <sup>3</sup> , Elizabeth M. Hill <sup>1</sup> , Paras Mainali <sup>1</sup> , Zachary E. Brooks <sup>1</sup> , Kraig E. Strayer <sup>1</sup> , Nicole M. Schaeublin <sup>1</sup> , Taylor L. Wiens <sup>3</sup> , Michael C. Brothers <sup>1</sup> , Leslie A. Drummond <sup>4</sup> , Dirk P. Yamamoto <sup>6</sup> , Jennifer A. Martin <sup>3</sup>				<b>5d. PROJECT NUMBER</b>	
				<b>5e. TASK NUMBER</b>	
				<b>5f. WORK UNIT NUMBER</b> HOV3	
<b>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</b> 1 UES Inc., Air Force Research Laboratory, 711th Human Performance Wing/RHBB, 2510 Fifth Street, Area B, Building 840, Wright-Patterson Air Force Base, OH 45433 2 The Henry M. Jackson Foundation for the Advancement of Military Medicine, Air Force Research Laboratory, 711 HPW Wing/RHBB, Wright-Patterson Air Force Base, OH 45433 4 United States Air Force Academy, 2304 Cadet Drive, U S A F Academy, CO 80840 5 Naval Medical Research Unit-Dayton, Biomedical Sciences, 2624 Q Street, Area B, Building 851, Wright-Patterson Air Force Base, OH 45433 6 Air Force Research Laboratory, 711th Human Performance Wing/RHM, 2510 Fifth Street, Area B, Building 840, Wright-Patterson Air Force Base, OH 45433, USA				<b>8. PERFORMING ORGANIZATION REPORT NUMBER</b>	
<b>9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)</b> 3 Air Force Materiel Command Air Force Research Laboratory 711 <sup>th</sup> Human Performance Wing Airman Systems Directorate Airman Bioengineering Division Applied Biotechnology Branch Wright-Patterson AFB, OH 45433				<b>10. SPONSORING/MONITORING AGENCY ACRONYM(S)</b> 711 HPW/RHBB	
				<b>11. SPONSORING/MONITORING AGENCY REPORT NUMBER(S)</b> AFRL-RH-WP-JA-2020-0004	
<b>12. DISTRIBUTION/AVAILABILITY STATEMENT</b> Distribution A: Approved for public release. 88ABW-2019-6025, cleared 18 December 2019					
<b>13. SUPPLEMENTARY NOTES</b> Journal of Breath Research <a href="https://iopscience.iop.org/article/10.1088/1752-7163/ab7e3b">https://iopscience.iop.org/article/10.1088/1752-7163/ab7e3b</a>					
<b>14. ABSTRACT</b> The Respiration Collector for In Vitro Analysis (ReCIVA) sampler, marketed by Owlstone Medical, provides a step forward in exhaled breath sampling through active sampling directly onto thermal desorption (TD) tubes. Although an improvement to the issues surrounding breath bag sampling, the ReCIVA device, first released in 2015, is a relatively new research and clinical tool that requires further exploration. Here, data are presented comparing two distinct ReCIVA devices. The results, comparing ReCIVA serial numbers #33 and #65, demonstrate that overall statistically insignificant results are obtained via targeted isoprene quantitation ( $p > 0.05$ ). However, when the data are parsed by the TD tube type used to capture breath volatiles, either Tenax TA or the dual bed Tenax/Carbograph 5TD (5TD), a statistical difference ( $p < 0.05$ ) among the two different TD tubes was present. These data, comparing the two ReCIVA devices with both Tenax TA and 5TD tubes, are further supported by a global metabolomics analysis yielding 85% of z-scores, comparing ReCIVA devices, below the limit for significance. Experiments to determine the effect of breathing rate on ReCIVA function, using guided breathing for low (7.5 breaths min <sup>-1</sup> ) and high (15 breaths min <sup>-1</sup> ) breathing rates, demonstrate the ReCIVA device shows no statistical difference among breathing rates for quantitated isoprene ( $p > 0.05$ ). Global metabolomics analysis of the guided breathing rate data shows more than 87% of the z-scores, comparing high and low breathing rates using both the Tenax and the 5TD tubes, are below the level for significance. Finally, data are provided from a single participant who displayed background levels of isoprene while illustrating levels of acetone consistent with the remaining participants. Collectively, these data support the use of multiple ReCIVA devices for exhaled breath collection and provide evidence for an instance where exhaled isoprene is consistent with background levels.					
<b>15. SUBJECT TERMS</b> ReCIVA, breath collection, thermal desorption (TD) tubes, isoprene					
<b>16. SECURITY CLASSIFICATION OF:</b>			<b>17. LIMITATION OF ABSTRACT:</b> SAR	<b>18. NUMBER OF PAGES</b> 39	<b>19a. NAME OF RESPONSIBLE PERSON (Monitor)</b> Jennifer Martin <b>19b. TELEPHONE NUMBER (Include Area Code)</b>
<b>a. REPORT</b> Unclassified	<b>b. ABSTRACT</b> Unclassified	<b>c. THIS PAGE</b> Unclassified			

ACCEPTED MANUSCRIPT • OPEN ACCESS

## Evaluation of a standardized collection device for exhaled breath sampling onto thermal desorption tubes

To cite this article before publication: Sean William Harshman *et al* 2020 *J. Breath Res.* in press <https://doi.org/10.1088/1752-7163/ab7e3b>

### Manuscript version: Accepted Manuscript

Accepted Manuscript is “the version of the article accepted for publication including all changes made as a result of the peer review process, and which may also include the addition to the article by IOP Publishing of a header, an article ID, a cover sheet and/or an ‘Accepted Manuscript’ watermark, but excluding any other editing, typesetting or other changes made by IOP Publishing and/or its licensors”

This Accepted Manuscript is **Not subject to copyright in the USA. Contribution of UES Inc., Air Force Research Laboratory.**

As the Version of Record of this article is going to be / has been published on a gold open access basis under a CC BY 3.0 licence, this Accepted Manuscript is available for reuse under a CC BY 3.0 licence immediately.

Everyone is permitted to use all or part of the original content in this article, provided that they adhere to all the terms of the licence <https://creativecommons.org/licenses/by/3.0>

Although reasonable endeavours have been taken to obtain all necessary permissions from third parties to include their copyrighted content within this article, their full citation and copyright line may not be present in this Accepted Manuscript version. Before using any content from this article, please refer to the Version of Record on IOPscience once published for full citation and copyright details, as permissions may be required. All third party content is fully copyright protected and is not published on a gold open access basis under a CC BY licence, unless that is specifically stated in the figure caption in the Version of Record.

View the [article online](#) for updates and enhancements.

# Evaluation of a Standardized Collection Device for Exhaled Breath Sampling onto Thermal Desorption Tubes

Sean W. Harshman<sup>1\*</sup>, Rhonda L. Pitsch<sup>2</sup>, Christina N. Davidson<sup>3</sup>, Erica M. Lee<sup>4</sup>, Alexander M. Scott<sup>3</sup>, Elizabeth M. Hill<sup>1</sup>, Paras Mainali<sup>1</sup>, Zachary E. Brooks<sup>1</sup>, Kraig E. Strayer<sup>1</sup>, Nicole M. Schaeublin<sup>1</sup>, Taylor L. Wiens<sup>3</sup>, Michael C. Brothers<sup>1</sup>, Leslie A. Drummond<sup>4</sup>, Dirk P. Yamamoto<sup>6</sup>, Jennifer A. Martin<sup>3</sup>

1 UES Inc., Air Force Research Laboratory, 711th Human Performance Wing/RHBB, 2510 Fifth Street, Area B, Building 840, Wright-Patterson Air Force Base, OH 45433, USA

2 The Henry M. Jackson Foundation for the Advancement of Military Medicine, Air Force Research Laboratory, 711th Human Performance Wing/RHBB, Wright-Patterson Air Force Base, OH 45433, USA

3 Air Force Research Laboratory, 711th Human Performance Wing/RHBB, 2510 Fifth Street, Area B, Building 840, Wright-Patterson Air Force Base, OH 45433, USA

4 United States Air Force Academy, 2304 Cadet Drive, United States Air Force Academy, CO 80840, USA

5 Naval Medical Research Unit-Dayton, Biomedical Sciences, 2624 Q Street, Area B, Building 851, Wright-Patterson Air Force Base, OH 45433, USA

6 Air Force Research Laboratory, 711th Human Performance Wing/RHM, 2510 Fifth Street, Area B, Building 840, Wright-Patterson Air Force Base, OH 45433, USA

\* Address reprint requests and correspondence to Dr. Sean W. Harshman, Air Force Research Laboratory, 711<sup>th</sup> Human Performance Wing, Human Biosignatures Branch, 2510 Fifth Street, Area B, Building. 840, Wright-Patterson Air Force Base, Ohio 45433, USA. Phone (937) 938-3766, Fax (937) 656-6898, email sean.harshman.ctr@us.af.mil

## ABSTRACT

The Respiration Collector for In Vitro Analysis (ReCIVA) sampler, marketed by Owlstone Medical, provides a step forward in exhaled breath sampling through active sampling directly onto thermal desorption (TD) tubes. Although an improvement to the issues surrounding breath bag sampling, the ReCIVA device, first released in 2015, is a relatively new research and clinical tool that requires further exploration. Here, data are presented comparing two distinct ReCIVA devices. The results, comparing ReCIVA serial numbers #33 and #65, demonstrate that overall statistically insignificant results are obtained via targeted isoprene quantitation ( $p>0.05$ ). However, when the data are parsed by the TD tube type used to capture breath volatiles, either Tenax TA or the dual bed Tenax/Carbograph 5TD (5TD), a statistical difference ( $p<0.05$ ) among the two different TD tubes was present. These data, comparing the two ReCIVA devices with both Tenax TA and 5TD tubes, are further supported by a global metabolomics analysis yielding 85% of z-scores, comparing ReCIVA devices, below the limit for significance. Experiments to determine the effect of breathing rate on ReCIVA function, using guided breathing for low ( $7.5 \text{ breaths min}^{-1}$ ) and high ( $15 \text{ breaths min}^{-1}$ ) breathing rates, demonstrate the ReCIVA device shows no statistical difference among breathing rates for quantitated isoprene ( $p>0.05$ ). Global metabolomics analysis of the guided breathing rate data shows more than 87% of the z-scores, comparing high and low breathing rates using both the Tenax and the 5TD tubes, are below the level for significance. Finally, data are provided from a single participant who displayed background levels of isoprene while illustrating levels of acetone consistent with the remaining participants. Collectively, these data support the use of multiple ReCIVA devices for exhaled breath collection and provide evidence for an instance where exhaled isoprene is consistent with background levels.

## INTRODUCTION

Since the observation by Pauling in 1971 that several hundred volatile organic compounds (VOCs) were detectable in exhaled breath, research utilizing this biosource for biomarker discovery has been substantial [1-4]. However, due to the low abundance of analytes, large amount of background, and lack of standardized sampling among off-line studies (sampling exhaled breath onto adsorbent tubes remotely and transfer to centralized lab for analysis) researchers have found reproducibility of exhaled breath data difficult [5,6]. Ultimately, these parameters have seriously restricted the clinical utility of exhaled breath as a source for biomarker discovery.

While real-time analysis of exhaled breath where participants exhale directly into analytical instrumentation using techniques such as selected ion flow tube mass spectrometry (SIFT-MS) and proton-transfer-reaction mass spectrometry (PTR-MS) have addressed many of the limitations associated with off-line analysis, real-time instrumentation is expensive and not easily portable for use among multiple sampling sites as required for large clinical studies. Therefore, off-line analysis still remains the primary tool for large multi-site exhaled breath studies.

As a stated previously, a limitation of off-line analysis is the lack of standardized sampling among laboratories and studies. Off-line exhaled breath is routinely sampled via exhaled breath bags, made of several materials such as Tedlar and fluoropolymer film (ALTEF), and transferred onto adsorbent tubes via an external pump. However, several studies

1  
2  
3 have shown exhaled breath bags are prone to leaking and volatile loss due to breath  
4 condensation [7-12]. Additionally, those providing breath must follow exhalation protocols  
5 to obtain consistent samples among individuals. To mitigate the issues observed with  
6 exhaled breath bags, the Respiration Collector for In Vitro Analysis (ReCIVA) was  
7 developed and marketed by Owlstone Medical. This sampler uses real-time exhaled CO<sub>2</sub>  
8 measurements to estimate the portion (lower airway, upper airway, etc.) of breath  
9 entering the device. Breath is sampled at the appropriate CO<sub>2</sub> levels via active pumps  
10 directly onto adsorbent tubes placed into the device. The ReCIVA device is a large step  
11 toward standardized exhaled breath sampling. However, due to the relative novelty,  
12 thorough experimentation surrounding the sources of variability associated with the  
13 ReCIVA sampler have yet to be adequately explored.  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31

32  
33 Previous work by Doran et. al. evaluated parameters for breath collection, such as sample  
34 volume and sampling flow rates, while noting contaminants associated with the breath  
35 sampler itself [13]. Further evaluation of the ReCIVA sampler by our research group  
36 established the importance of the ReCIVA software version in commanding flow rates  
37 applied by the device [14]. Additionally, the data illustrated that manually calibrated flow  
38 rates allow for comparable results among ReCIVA banks, i.e. duplicate samples, and  
39 statistically similar results among ReCIVA samples and exhaled breath bags [14]. While  
40 these studies establish several critical attributes surrounding the ReCIVA sampler,  
41 additional work must be performed to ensure proper performance for large clinical  
42 investigations.  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

1  
2  
3  
4  
5  
6  
7 In this manuscript, data are presented for further evaluation of the ReCIVA device using  
8 two routinely utilized thermal desorption adsorbent tubes for exhaled breath sampling,  
9 Tenax TA and Tenax/Carbograph 5TD (5TD). The results provided support the use of  
10 multiple ReCIVA devices. The data also demonstrate the limited impact breathing rate  
11 has on exhaled breath collected by the ReCIVA device. These data add additional  
12 evidence for the use of the ReCIVA device for more consistent, standardized, exhaled  
13 breath collection.  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26

## 27 **EXPERIMENTAL**

### 28 **PARTICIPANTS**

29  
30  
31 The participants (n=20 max per experiment, 27 total participants) were volunteer, non-  
32 smoking, males at our research facility. The research described here was determined to  
33 be Not-Human Research by the United States Air Forces Research Laboratory's  
34 Institutional Review Board, (FWR2017161N) as the research is designed to interrogate  
35 exhaled breath sampling platforms. As a result, all participants were verbally informed of  
36 all experimental parameters and free to discontinue participation at any time. However,  
37 written consent was not provided.  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

## THERMAL DESORPTION TUBES

Exhaled breath was concentrated simultaneously on stainless steel reconditioned Tenax TA (35/65 mesh, PN: C1-AXXX-5003) and Tenax/Carbograph 5TD (5TD, PN: C2-AXXX-5149), thermal desorption (TD) tubes (Markes International, South Wales, UK). The Tenax TA adsorbent tubes were selected due to their frequent use among exhaled breath researchers [14-36]. Tenax/Carbograph 5TD tubes were selected based on the recommendation of Owlstone Medical, the ReCIVA manufacturer. To minimize TD tube batch variability, the same forty tubes, twenty of each adsorbent material, were used randomly for all exhaled breath collections. Control and background samples were collected on each TD tube adsorbent type, Tenax TA or 5TD, using serial numbers independent of those used for exhaled breath collections. Reconditioning, following each use, was performed at 320 °C for 1 hour with 85 mL min<sup>-1</sup> of 99.999% nitrogen backflush on a Markes International TC-20. All thermal desorption tubes were stored at ambient temperature with brass caps and polytetrafluoroethylene ferrules affixed to each end until experimental use.

## RECIVA MANUAL FLOW RATE MEASUREMENT & CALIBRATION

The uncalibrated flow rate pulled by the ReCIVA device across thermal desorption tubes was determined using a clean glass head as described previously [14]. Briefly, the flow rate applied by ReCIVA serial number 65 (#65) was evaluated using ReCIVA control software v. 1.46 set at 200 mL min<sup>-1</sup> over a 1420 mL (approximately 100 measurements) collection volume using the software's "Always On" feature (Owlstone Medical,

1  
2  
3 Cambridge, UK). Flow rate was measured by connecting the open end of the TD tube to  
4 a DryCal Bios Defender 510 (Mesa Labs, Lakewood, CO, USA) through a hole cut into a  
5 ReCIVA mask. The measurements were taken one at a time using both TD tube types  
6 and individual ReCIVA banks to evaluate the flow at the sampling positions distal to the  
7 participants mouth (n=5 per tube type per bank). The remaining three sampling ports  
8 within the ReCIVA that were not being tested were blocked with clean 3.5" x 0.25" solid  
9 stainless-steel rods. ReCIVA-applied-flow was monitored and recorded via the DryCal  
10 Pro Software (v. 1.3, Mesa Labs). Additionally, using the same setup, the calibrated flow  
11 rate was determined, for ReCIVA #65, by manually adjusting the flow rate within the  
12 ReCIVA software until approximately 200 mL min<sup>-1</sup> was measured on the DryCal Pro  
13 Software, as described above [14]. The determined calibrated flow rates for ReCIVA #65  
14 were as follows: Tenax Bank A: 152 mL min<sup>-1</sup>, Tenax Bank B: 150 mL min<sup>-1</sup>, 5TD Bank  
15 A: 174.5 mL min<sup>-1</sup>, and 5TD Bank B: 178.5 mL min<sup>-1</sup>. The calibrated flow rates used for  
16 ReCIVA #33 were determined previously [14].  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39

## 40 EXPERIMENTAL DESIGN & EXHALED BREATH COLLECTION: TEST 1 & 2

41  
42 All exhaled breath samples for Tests 1 & 2 were collected using two different ReCIVA  
43 devices, serial numbers 33 (#33) or #65, and brand-new masks (Owlstone Medical). The  
44 mask assembly manufacture dates for Tests 1 & 2 were March 2018 and May 2019,  
45 respectively. For all experiments, the filter was removed, using gloves, from the mask  
46 assembly and the silicon portion of the mask was baked at 180 °C overnight. Prior to  
47 breath collection, the mask was cooled and, with gloves, the filter was reinserted. Test 1  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

1  
2  
3 was performed using the ReCIVA control software v. 1.46 and the calibrated flow rates  
4 with the calculated collection volumes for ReCIVA device #33 and device #65  
5  
6 **(Supplemental Data 1A)**. Test 2 was performed using the ReCIVA control software v.  
7  
8 1.46 and the uncalibrated flow rates on each ReCIVA device (200 mL min<sup>-1</sup>, and 550 mL  
9  
10 collection volume, **Supplemental Data 1B**). All remaining ReCIVA settings were held  
11  
12 constant and represented in **Supplemental Data 1C**.  
13  
14  
15  
16  
17  
18  
19  
20

21 Exhaled breath collections, for both Test 1 and Test 2, were performed using a two TD  
22  
23 tube setup by occluding the two ReCIVA sampling ports proximal to the mouth with clean  
24  
25 solid stainless-steel rods (3.5" x 0.25"). In the remaining ReCIVA sampling ports, distal to  
26  
27 the mouth, one of each type of TD tube, Tenax TA or 5TD, were randomly inserted.  
28  
29 Additionally, participants (n=20 for Test 1 and n=18 for Test 2) were randomly assigned  
30  
31 to a specific ReCIVA device, #33 or #65. All randomizations, for both TD tube placement  
32  
33 and ReCIVA device, were performed using the RANDBETWEEN function of Microsoft  
34  
35 Excel (Redmond, WA, USA). Please refer to **Supplemental Data 1D** for an illustration of  
36  
37 the Test 1 & 2 experimental design.  
38  
39  
40  
41  
42  
43  
44  
45

46 Prior to performing an exhaled breath collection, all participants abstained from food or  
47  
48 drink, except for water, for at least 1 hour. Immediately prior to collection, all participants  
49  
50 thoroughly rinsed their mouths with filtered water and sat in a relaxed upright position for  
51  
52 greater than five minutes [37]. Using an Alicat Scientific MCP-100 SLPM mass flow  
53  
54 controller (MFC) and the FlowVision SC software (v. 1.3.28.0), 40 L min<sup>-1</sup> of medical  
55  
56  
57  
58  
59  
60

1  
2  
3 grade breathing air (21% O<sub>2</sub> with N<sub>2</sub> balance) was provided to the ReCIVA device via  
4 Tygon tubing (Indiana Oxygen, Indianapolis, IN, USA; Alicat Scientific, Tuscon, AZ, USA).  
5  
6 Participants donned the ReCIVA device affixed with the mask TD tube assembly. The  
7  
8 head straps were adjusted until comfortable and no leaking was observed. Please refer  
9  
10 to **Supplemental Data 1E** for a picture of the laboratory setup for exhaled breath  
11  
12 collection and **Supplemental Data 2** for a summary of the participants and samples  
13  
14 collected. Participants were instructed to breathe normal, slow breaths through their  
15  
16 mouths only. No other direction or intervention, such as nose plugs, were provided for  
17  
18 exhaled breath collection. Upon completion of the collection, all TD tubes were capped  
19  
20 and stored at ambient temperature. Thermal desorption gas chromatography-mass  
21  
22 spectrometry (TD-GC-MS) analysis was initiated on the same day [34].  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32

### 33 EXPERIMENTAL DESIGN & EXHALED BREATH COLLECTION: TEST 3 & 4

34  
35 The ReCIVA devices for Tests 3 and 4 were set up exactly as described above for Test  
36  
37 1 i.e., new, prebaked ReCIVA masks (manufacture date May 2019), with random  
38  
39 placement of one Tenax TA and one 5TD tube in each ReCIVA bank distal to the mouth,  
40  
41 manually calibrated ReCIVA flow rates, adjusted collection volumes, and ReCIVA control  
42  
43 software v. 1.46. However, Test 3 (n=20) was performed only with ReCIVA #65 and Test  
44  
45 4 (n=18) only used ReCIVA #33 (**Supplemental Data 3A & 3B**). For both Tests 3 & 4  
46  
47 participants were randomly assigned to a breathing rate, high (15 breaths min<sup>-1</sup> or 1 s  
48  
49 inhale and 3 s exhale) or low (7 breaths min<sup>-1</sup> or 2 s inhale and 6 s exhale). Breathing rate  
50  
51 was controlled by having participants follow along with the Breathe+ iPhone app, set to  
52  
53  
54  
55  
56  
57  
58  
59  
60

1  
2  
3 the breathing rate parameters assigned, projected on a large television (Dynamic App  
4 Design LLC, **Supplemental Data 3C & 3D**). Participants were instructed to inhale, as the  
5 bar of the app rose, and the inverse as the bar lowered. Please refer to the **Supplemental**  
6  
7  
8 **Video 1 & 2** for examples of the app function and rates and **Supplemental Data 4** for a  
9  
10 summary of the participants and samples collected.  
11  
12  
13  
14  
15  
16  
17  
18

## 19 BACKGROUND SAMPLE COLLECTION

20  
21 For each test event (Tests 1-4), prior to the first participant's exhaled breath collection  
22 and following the last participant's exhaled breath collection, a room blank, medical grade  
23 air blank, and mask blank sample were collected on each adsorbent tube type as  
24 described previously [14]. Briefly, 550 mL of room air was pulled through each TD tube  
25 type (room blank), Tenax TA and 5TD, using a GilAir Plus pump operated at 200 mL min<sup>-1</sup>.  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

1. On each TD tube type, 550 mL of medical grade air was sampled at 200 mL min<sup>-1</sup>, as described above, from a 1 L ALTEF bag filled with the air. Finally, ReCIVA mask blanks (550 mL at 200 mL min<sup>-1</sup>) were obtained, as described by Doran et al., using the "Always On" feature of the ReCIVA device software and new prebaked masks attached to a clean glass head that was provided 40 L min<sup>-1</sup> of medical grade air [13]. All background samples were stored at room temperature and analyzed by GC-MS initiated on the same day as collection [34].

## TD-GC-MS ANALYSIS

Internal standard (1,4-difluorobenzene, 25ppm) was automatically added by the Markes TD-100xr to each thermal desorption tube. Thermal desorption of adsorbed volatiles from the TD tubes was performed using a Markes TD-100xr in line with a Thermo Scientific Trace Ultra-ISQ GC-MS system (Waltham, MA, USA). A 1 min (20 mL min<sup>-1</sup>) predesorption dry purge was conducted with 99.999% nitrogen followed by a 10 min 310 °C primary desorption onto an Air Toxics cold trap (Markes International). A 1 min 50 mL min<sup>-1</sup> trap purge was performed using a flow path temperature of 180 °C followed by a 5 min 315 °C trap desorption (40 °C s<sup>-1</sup>). A 3.64:1 split ratio was applied to desorbed volatiles via the trap outlet prior to introduction onto a Rxi-624Sil 60 m x 0.32 mmID x1.80 µm df GC column (Restek, Bellefonte, PA, USA). Volatiles were chromatographically separated following a 40 °C hold for 1 min using a linear gradient to 24 °C over 20 min at a rate of 10 °C min<sup>-1</sup> with a constant 2 mL min<sup>-1</sup> helium carrier flow (99.999%). The column was held at 240 °C for 20 min. 70 eV electron impact ionization was applied to the column eluent at a temperature of 275 °C. Ion detection was performed on a single quadrupole 0.154 scans s<sup>-1</sup> over a 35-300 m/z range. TD tubes were analyzed in a random order using Tracefinder EFS software (v. 3.2, Thermo Scientific). All GC-MS data were manually inspected utilizing the Thermo Scientific XCalibur software package (v. 3.0.63) to ensure no coelution of compounds about peaks of interest.

## CALIBRATION AND QUANTITATION OF ISOPRENE (2-METHYL-1,3-BUTADIENE)

Single point isoprene calibration curves were created using each TD tube type, from a custom 1.10 ppm pressurized canister (Linde Gas North America LLC, Alpha, NJ, USA). Briefly, using a Hamilton gas-tight syringe, different volumes of gaseous standards, corresponding to 0 - 1.071  $\mu\text{g}$  for Tenax TA and 0 - 918.8 ng for 5TD, were individually spiked onto separate thermal desorption tubes using a Markes Standard Loading Rig supplied with 60  $\text{mL}^{-1}$  99.999% nitrogen backflush (**Supplemental Data 5**, Hamilton, Reno, NV, USA) [38,39]. The zero point of calibration curves were  $\text{N}_2$  backflush loaded tubes. All calibration curve TD tubes were analyzed as described in the previous section. GC-MS instrument performance was evaluated prior to each data acquisition using the isoprene standard. For each analysis, the isoprene % difference to calibration met the requirements set forth in the EPA TO-15/17 methods [40,41].

The single ion peak areas for isoprene (Q-ion 67 m/z) and the internal standard (1,4-difluorobenzene, Q-ion 114 m/z) were determined using the Tracefinder EFS software. The theoretical isoprene concentration was plotted against the internal standard normalized response ratio and fitted with a linear regression line using Microsoft Excel. The calibration curves for each TD tube type are provided in **Supplemental Data 5**. The internal standard normalized response was generated for each sample and background TD tube. The amount of unknown isoprene was determined using the linear fit equation and the internal standard normalized response for each sample.

## GLOBAL FEATURE EXTRACTION

For each test, retention time alignment and feature extraction were performed in the Metabolite Differentiation and Discovery Lab (MeDDL, v. 1.22) as described previously using the settings provided in **Supplemental Data 6** [35,42]. Background samples from each test were aligned and extracted separately using the same MeDDL settings used for the samples. Following sample extraction, data reduction was performed within the MeDDL software by time binning each sample at 0.1 minutes and 1E6 abundance. The feature list was further reduced by removing features with missing values among any sample. A list of the mean abundance of the features that were found in both the reduced samples and background samples was tabulated. Features found in both background and samples were removed from the sample data set. Following removal of compounds found in the background samples, the internal standard (IS) normalized response ratio was generated from the resulting feature list for each test. These data were used for further analysis. Tentative identifications were manually performed using the NIST 11 Mass Spectral Library (v. 2.0, Gaithersburg, MD).

## ACETONE AREA DETERMINATION

Acetone (Q-ion 58 m/z) peak areas were determined using the Tracefinder EFS software and normalized to the 1,4-difluorobenzene internal standard areas (internal standard normalized response ratios) as described for the isoprene quantitation.

## STATISTICAL ANALYSIS

Prism GraphPad Software suite (v. 8.3.0(328)) was used for basic statistical analysis such as t-tests and one-way Analysis of Variance (ANOVA, Graphpad Software Inc., LaJolla, CA, USA). One-way Analysis of Covariance (ANCOVA) and principal component analysis was conducted in the R software suite (v. 3.5.0) utilizing the prcomp, ggbiplot, and ggplot packages [43-45]. Tukey's multiple comparisons test was used to correct for the ANCOVA multiple comparisons, and to illustrate what groups, if any, showed statistical significance.

## RESULTS

### COMPARISON OF TWO SEPARATE RECIVA DEVICES

As use of the ReCIVA device becomes more prominent within the breath community, the use of multiple ReCIVA devices within a single study, potentially at several remote sampling locations, will become more frequent. To determine if similar exhaled breath results are obtained from multiple ReCIVA devices, two tests were performed utilizing two separate ReCIVA devices (#33 and #65) with calibrated ReCIVA flow rates (Test 1, n=20) and uncalibrated ReCIVA flow rates (Test 2, n=18). Since minimal direction was provided to participants for exhaled breath collection, the parameters surrounding the collections were investigated. **Supplemental Data 7** illustrate no significant differences in collection times, exhalation rates, total exhalations, and mean max CO<sub>2</sub> values (estimation of depth of breath, p>0.05 one-way ANOVA) were observed among tests using calibrated ReCIVA flow rates (Tests 1) and uncalibrated ReCIVA flow rates (Test 2). These data suggest that

1  
2  
3 the sampling conditions are similar between the two ReCIVA tests and do not significantly  
4 influence the exhaled breath results.  
5  
6  
7  
8  
9

10  
11  
12 Examination of the quantitated exhaled isoprene values from Tests 1 and 2 show no  
13 statistical difference ( $p > 0.05$  one-way ANCOVA) in isoprene amount among the two  
14 ReCIVA devices, independent of ReCIVA flow rate calibration (**Figure 1A**). To further  
15 evaluate the exhaled volatiles from the samples beyond isoprene alone, the internal  
16 standard normalized response ratios of ten abundant features were calculated and  
17 principal component analysis (PCA) was performed. **Figure 1B** illustrates that a relatively  
18 small amount of the overall variation (40.8%) is captured by the first two principal  
19 components (PC1 26.8%, PC2 14.0%). Additionally, the 95% confidence ellipses overlap  
20 in space, suggesting no statistical difference among the samples based on ReCIVA unit  
21 (comparing among ellipses colors) or calibration (comparing ellipses within the same  
22 color) (**Figure 1B**). To further inspect differences among feature abundances, z-scores  
23 of the ten IS normalized response ratios between ReCIVA #65 and #33 were calculated  
24 and parsed by ReCIVA flow calibration (**Supplemental Data 8A**). Overall the data  
25 suggest greater than 85% (17/20) of the comparison are within the  $\pm 1.96$  significance  
26 value. Within the global data set, the feature 5.35, 67.1 (RT, m/z) corresponds to isoprene  
27 (**Supplemental Data 9**). The data presented in **Supplemental Data 8A** show isoprene  
28 abundance is not significantly different between ReCIVA devices supporting the  
29 quantitated isoprene results by a global metabolomics approach. Overall these data  
30 indicate similar results are obtained by both quantitated values and relative abundances,  
31 among two separate ReCIVA devices independent of ReCIVA flow rate.  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

1  
2  
3  
4  
5  
6  
7 Previous data suggest variability among ReCIVA banks can occur, likely due to separate  
8 active pumps in each bank, and manual calibration of ReCIVA flow rates can remedy the  
9 differences among ReCIVA banks [14]. To determine if there are significant differences  
10 between banks among the two ReCIVA devices, the isoprene quantities were parsed for  
11 each bank (A and B) and Test (1: calibrated ReCIVA flow rate, 2: uncalibrated ReCIVA  
12 flow rate, **Figure 1C**). The data suggest there is no statistically significant difference  
13 among banks of the ReCIVA independent of the ReCIVA device or flow rate ( $p > 0.05$  one-  
14 way ANCOVA, **Figure 1C**). Additionally, a recalculation of the 95% confidence ellipses of  
15 the PCA, based on ReCIVA bank and ReCIVA device, illustrate a high amount of overlap  
16 between ReCIVA devices (comparing among ellipses colors) and banks within the same  
17 device (comparing ellipses within the same color, **Figure 1D**). To identify divergence  
18 among selected features across samples, z-scores of the ten IS normalized response  
19 ratios between ReCIVA banks (B - A) were calculated and parsed by ReCIVA device and  
20 ReCIVA flow calibration (**Supplemental Data 8B**). The z-scores show greater than 97%  
21 (39/40) comparisons fall within significance limits (**Supplemental Data 8B**). Similar to  
22 previous results, the feature corresponding to isoprene (5.35 min, 67.1 m/z) displays an  
23 insignificant difference among banks, providing further support for the quantitated data  
24 for isoprene shown in **Figure 1C**. These data demonstrate that there are minimal  
25 differences between banks of the ReCIVA devices independent of ReCIVA flow rate for  
26 sampling by both quantitated and relative approaches.  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

1  
2  
3 For all experimental tests, one of each TD tube type, Tenax and 5TD, was inserted  
4 randomly within the ReCIVA device in the positions distal to the mouth. To ascertain how  
5 different TD tubes, contribute to the variability among ReCIVA devices, quantitated  
6 isoprene values were parsed by TD tube type (Tenax and 5TD) and calibrated (Test 1)  
7 and uncalibrated (Test 2) ReCIVA flow rates (**Figure 1E**). One-way ANCOVA of the  
8 isoprene results provided an overall significant ( $p=0.0005$ ) difference among the samples.  
9  
10 To determine which groups differed significantly, Tukey's multiple comparisons test  
11 correction was used. The data demonstrate significant  $p$ -values ( $p<0.05$ ) for all  
12 comparisons between the Tenax and 5TD results, within each Test, except for the test  
13 using the calibrated flow rate on ReCIVA #65 (**Figure 1E**). Again, the 95% confidence  
14 ellipses of the PCA were recalculated based on TD tube type and ReCIVA device (**Figure**  
15 **1F**). The data show overlap between each TD tube type (comparing among ellipses colors  
16 of the same line style) independent of ReCIVA device while showing greater separation  
17 between TD tube types within the same device (comparing ellipses of the same color,  
18 **Figure 1F**). Evaluation of the calculated  $z$ -scores from the ten IS normalized response  
19 ratios (Tenax – 5TD) parsed by ReCIVA flow calibration and ReCIVA device, show more  
20 than 52% (21/40) of the global metabolomic features are within the  $\pm 1.96$  range of  
21 significance based on tube type (**Supplemental Data 8C**). Inspection of the tentative  
22 identifications associated with the features, outside the significant limits, suggest propene  
23 and methoxy-phenyl-oxime are significantly retained on the 5TD tubes (**Supplemental**  
24 **Data 9**). However, the data illustrate that the Tenax tubes retain a greater amount of  
25 isoprene (feature 5.35 min, 67.1  $m/z$ ) than the 5TD tubes, which is in-line with the  
26 isoprene quantified results. Collectively, the data suggest the differences among TD tubes  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

1  
2  
3 provide the greatest source of variability among the two ReCIVA devices independent of  
4  
5 ReCIVA flow rate or ReCIVA bank.  
6  
7  
8  
9  
10

## 11 EFFECT OF BREATHING RATE ON RECIVA PERFORMANCE

12  
13  
14 The ReCIVA device is designed to monitor CO<sub>2</sub> in real time to allow for versatile airway  
15 collection, e.g., lower airway, upper airway, or whole breath, via an active sampling pump.  
16  
17 As breathing rate and depth of breathing could contribute to variability in exhaled breath  
18 results sampled with the ReCIVA device due the frequency and duration of active  
19 pumping, two guided breathing experiments, Test 3 (n=20) using ReCIVA #65 and Test  
20 4 (n=18) using ReCIVA #33, were performed by asking participants to inhale and exhale  
21 along with an iPhone app (Breath+) projected on a television (**Supplemental Data 3C,**  
22 **3D, 4, and Supplemental Videos 1 & 2**). Representative plots of the measured CO<sub>2</sub> from  
23 the low (7.5 breaths min<sup>-1</sup>) and high (15 breaths min<sup>-1</sup>) breathing rate groups are provided  
24 in **Supplemental Data 10A & 10B**. Inspection of the attributes associated with sampling,  
25 such as collection time, exhalation rate, total number of exhalations, and mean max CO<sub>2</sub>,  
26 shows statistically significant differences (p<0.05) between high and low breathing rate  
27 groups for all attributes independent of ReCIVA device (**Supplemental Data 10C - 10F**).  
28  
29 As expected, the low breathing rate groups had shorter collection times attributed to fewer  
30 overall exhalations and higher mean max CO<sub>2</sub> (longer exhalations) while the opposite is  
31 true of the high breathing rate group. All aspects of sampling, except for mean max CO<sub>2</sub>  
32 between ReCIVA #65 (Test 3) and #33 (Test 4) low breathing rate samples, are not  
33 significantly different (p>0.05) between breathing rate groups no matter the ReCIVA  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

1  
2  
3 device used to sample (**Supplemental Data 10C - 10F**). Collectively, these data indicate,  
4  
5 regardless of the ReCIVA used for sampling, differences are primarily associated with the  
6  
7 breathing rate group (high or low). These results support the ability to compare exhaled  
8  
9 breath results among breathing rate groups and ReCIVA devices.  
10  
11

12  
13  
14  
15  
16  
17 To assess if breathing rate significantly affects exhaled breath results between ReCIVA  
18  
19 devices, isoprene was quantitated from the exhaled breath from each Test and plotted by  
20  
21 breathing rate and ReCIVA device (**Figure 2A**). The data show no significant difference  
22  
23 among all the isoprene values regardless of ReCIVA device or guided breathing rate  
24  
25 ( $p > 0.05$  by one-way ANCOVA). A PCA of the 38 samples corresponding to 11 features  
26  
27 (IS normalized response ratios) extracted from the exhaled breath GC-MS data show a  
28  
29 small amount of the overall variation within the data (42.4%) is accounted for by the first  
30  
31 two principal components (PC1 25.1% and PC2 17.3%, **Figure 2B**). Overlay of the 95%  
32  
33 confidence ellipses by ReCIVA device and breathing rate shows a high amount of overlap  
34  
35 among not only the breathing rates, (high and low, comparing ellipses of the same color)  
36  
37 but also between ReCIVA devices (comparing among ellipse colors, **Figure 2B**). To  
38  
39 further explore the global analysis, z-scores were determined between ReCIVA devices  
40  
41 (65 – 33) and parsed by breathing rate (**Supplemental Data 11A**). The data show that  
42  
43 more than 87% (29/33) of the comparisons are below the threshold for significance ( $\pm$   
44  
45 1.96) suggesting most features are not observed at significantly different levels between  
46  
47 ReCIVA devices. The results also show that isoprene, feature 5.35 min, 67.1 m/z, is not  
48  
49 significantly different between devices, suggesting that the global isoprene data  
50  
51 correspond to that of the quantitated isoprene values. Furthermore, z-scores were  
52  
53  
54  
55  
56  
57  
58  
59  
60

1  
2  
3 calculated based on breathing rate (high – low) and separated by the ReCIVA device  
4 used for collection (**Supplemental Data 11B**). The data show that 29 of 33 (>87%)  
5  
6 comparisons are within the  $\pm 1.96$  significance level. Interestingly, the feature  
7  
8 corresponding to isoprene (5.35 min, 67.1 m/z) shows that the low breathing rate IS  
9  
10 normalized abundance from ReCIVA #33 is significantly higher than the high breathing  
11  
12 rate abundance within the same ReCIVA device (illustrated by negative z-scores,  
13  
14 **Supplemental Data 11B**). This result is an inverse of the quantitated isoprene values  
15  
16 and could be a result of the substantial variability associated with the ReCIVA #33 low  
17  
18 breathing rate samples (**Figure 2A**). Overall, the data suggest that neither breathing rate  
19  
20 nor ReCIVA device plays a significant role in disrupting the consistency of the exhaled  
21  
22 breath results obtained from a ReCIVA sampler.  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32

33 The ReCIVA contains two separate active pumps, one in each bank of the device.  
34  
35 Controlling exhaled breath sampling may disrupt the consistency among banks as the  
36  
37 active pump functions more frequently (high breathing rate) or for longer periods of time  
38  
39 (low breathing rate). To investigate this hypothesis, quantitated isoprene values were  
40  
41 parsed by bank, breathing rate, and ReCIVA device (**Figure 2C**). The data show, by one-  
42  
43 way ANCOVA ( $p>0.05$ ), that there is not a significant difference in the isoprene among  
44  
45 any of these attributes. A recalculation of the 95% confidence ellipses of the 38 samples  
46  
47 from Tests 3 & 4, accounting for bank, breathing rate, and ReCIVA device, illustrate a  
48  
49 high amount of overlap among ellipses in not only banks of the same ReCIVA device  
50  
51 (comparing ellipses of the same color) but also between the different ReCIVA samplers  
52  
53 (comparing among ellipse colors and line styles) (**Figure 2D**). The calculated z-scores (B  
54  
55  
56  
57  
58  
59  
60

1  
2  
3 – A) from the IS normalized response ratios of the global extraction demonstrate a high  
4 percentage of comparisons are below the significance level (43/44, 87.5%) suggesting  
5 minimal difference by a relative global approach (**Supplemental Data 11C**). The feature  
6 corresponding to isoprene (5.35 min, 67.1 m/z) shows insignificant z-scores for all  
7 comparisons in line with the quantitated isoprene results (**Figure 2C & Supplemental**  
8 **Data 11C**). Altogether, these data indicate the ReCIVA bank does not add significant  
9 variability to the exhaled breath results independent of ReCIVA device or breathing rate.  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22

23 The data shown in **Figure 1E** suggest that TD tube type plays a significant role in the  
24 variability associated with the ReCIVA sampler, as expected due to the different  
25 adsorbent packing material of the Tenax and 5TD tubes. To evaluate the impact of TD  
26 tube variability along with breathing rate and ReCIVA device, the quantitated isoprene  
27 data were further parsed by TD tube type. **Figure 2E** shows no statistically significant  
28 difference ( $p=0.1672$  one-way ANCOVA) among the isoprene measurements indicating  
29 that within and among a breathing rate groups the two different TD tubes perform  
30 similarly. Recalculation of the 95% confidence ellipses of the PCA analysis, to consider  
31 TD tube type and ReCIVA device, show a large amount of overlap between TD tube types  
32 within a ReCIVA device (comparing ellipses of the same color) and among TD tubes of  
33 the same type (comparing ellipses the same line style among the two colors, **Figure 2F**).  
34 Inspection of the calculated z-scores (Tenax – 5TD), show similar results to those from  
35 Test 1 & 2 comparing TD tube types, in that the 5TD samples have greater propene and  
36 methoxy-phenyl-oxime while the Tenax samples tend to retain greater amounts of  
37 isoprene (feature 5.35 min, 67.1 m/z, **Supplemental Data 9 & 11D**). Similar trends for  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

1  
2  
3 isoprene are observed in the quantitated data (**Supplemental Data 11D, Figure 2E**).  
4  
5 Collectively, these data indicate breathing rate does not play a significant role in the  
6  
7 functionality of the ReCIVA device.  
8  
9  
10  
11  
12  
13

#### 14 IDENTIFICATION OF PARTICIPANT WITH LOW ISOPRENE

15  
16  
17 Exhaled breath is most commonly characterized by the presence of two highly abundant  
18  
19 compounds, acetone (2-propanone) and isoprene. A single participant, Participant #28,  
20  
21 performed exhaled breath collections for both Tests 2 & 4. While tabulating the isoprene  
22  
23 peak areas for quantitation, it was observed that this participant had levels of breath  
24  
25 isoprene ( $\log_2$  IS normalized response ratio) similar to background, among the two  
26  
27 sampling events, no matter the TD tube type used for sampling (**Figure 3A**). To verify the  
28  
29 exhaled breath was otherwise similar to the remaining participants, the acetone peak  
30  
31 areas were tabulated for all participants including Participant #28 and  $\log_2$  IS normalized  
32  
33 response ratios were calculated and plotted (**Figure 3B**). The data show that while the  
34  
35 isoprene values were similar to background, the acetone levels were dispersed among  
36  
37 the other participants. These data suggest the exhaled breath was overall similar for  
38  
39 Participant #28, except for the background levels of isoprene. As these data would have  
40  
41 skewed the results, Participant #28's samples were removed from the overall analysis.  
42  
43 However, the lack of isoprene in a participant's exhaled breath is quite rare and worth  
44  
45 noting when observed [46].  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

## DISCUSSION

Currently, only exhaled nitric oxide (FeNO) has been adopted into clinical practice due to extensive research, among both the clinic and the lab, into the utility of this compound for diagnosis and monitoring therapeutic response in asthmatics [47]. For additional exhaled breath compounds to be adapted clinically, large-scale, multi-site exhaled breath studies will be required to support laboratory research. To this end, it is imperative that exhaled breath sample collection be consistent among sampling sites. Data provided in **Figure 1** illustrates that two distinct ReCIVA devices yield similar results, using both calibrated and uncalibrated ReCIVA flow rates. The flow rate data contradict previous results suggesting manual flow rate calibration allowed for comparable isoprene values between Tenax and 5TD tubes, although these two studies have different experimental designs [14]. As a result, further experimentation is required to determine the ultimate utility of manual flow rate calibration. However, in this study, all exhaled breath samples were collected using the same 40 TD tubes, 20 Tenax and 20 5TD, used randomly. It is hypothesized that much of the variability associated with the previous study can be attributed to the batch-to-batch variability among the TD tubes rather than the ReCIVA sampler. Preliminary data with flow rate measurements among new and old TD tubes, within our lab, support these results. However, controlled experiments utilizing calibrated flow rates for each individual TD tube and ReCIVA bank must be performed to truly validate this hypothesis.

As expected, when utilizing two different TD tube types, Tenax and 5TD, within the ReCIVA device simultaneously, comparable exhaled breath results are not obtained (**Figure 1E**). Although there is inherent variability of exhaled isoprene among individuals,

1  
2  
3 it is interesting that the 5TD tubes, which are a dual bed of Tenax and carbograph  
4 adsorbents intended for compounds ranging from C<sub>4</sub>-C<sub>30</sub>, retained less isoprene among  
5 both experiments although designed for lower molecular weight species than the single  
6 bed Tenax tubes (C<sub>6</sub>-C<sub>30</sub>, **Figure 1E & 2E**) [48-53]. Initially, the data were evaluated to  
7 determine if background or an interfering ion was responsible for the observed increase  
8 in isoprene on the Tenax TA TD tubes. However, the isoprene in the background samples  
9 did not show any statistical difference between TD tube types among all of the tests  
10 (p=0.2653 one-way ANOVA). Furthermore, manual inspection of the spectra about the  
11 isoprene peak suggests no interfering ions are present. To confirm the peak was truly  
12 isoprene, a secondary ion (53 m/z, S-ion) peak area was monitored and compared to the  
13 quantitative ion (Q-ion) for isoprene (67 m/z) for all injections. The ratio of S-ion/Q-ion for  
14 sample injections was  $\pm 30\%$  from the S-ion/Q-ion of the standard isoprene injections.  
15 Collectively, these data suggest that the peak in question is in fact isoprene and  
16 background or interfering ions are not responsible for the observed increase in isoprene  
17 on the Tenax TA tubes. Another probable cause may be breakthrough of isoprene with  
18 5TD tubes. However, preliminary experimentation of breakthrough on 5TD tubes, using  
19 isoprene standards and methods previously established, shows little or no isoprene found  
20 on the second tube (data not shown) [39]. Next, it is speculated that the difference in the  
21 observed isoprene retention may be attributed to a pump initiation delay caused by  
22 resistance, i.e. backpressure, of the carbograph particles. As Tenax is much larger in  
23 size, flow can initiate easier with each pump activation. A delay in pump activation, caused  
24 by backpressure, repeated over approximately 75 exhalations could account for the  
25 differences observed. However, this hypothesis is only minimally supported by the  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

1  
2  
3 breathing rate data (**Figure 2E**) and off-line backpressure experiments (data not shown).  
4  
5 Finally, data were recently presented suggesting Tenax/Carbograph 5TD tubes retain a  
6  
7 greater amount of water than Tenax adsorbent tubes [54]. Therefore, it is hypothesized,  
8  
9 due to the high solubility of isoprene in water, that the isoprene is removed from the 5TD  
10  
11 tubes during the dry purge of the thermal desorption cycle, with the water [54]. As Tenax  
12  
13 TD tubes retain less water, less isoprene would be removed during the dry purge, yielding  
14  
15 the observed higher isoprene values compared to the 5TD tubes, even though Tenax has  
16  
17 illustrated potential breakthrough of isoprene [54]. Additionally, it is also plausible that  
18  
19 humidity accumulation within the 5TD tubes blocks binding sites within the adsorbent,  
20  
21 occluding isoprene from binding and ultimately being retained [54]. However, as these  
22  
23 data have yet to be peer reviewed, additional experimentation may be required to confirm  
24  
25 this hypothesis. Regardless of the cause for the differences between the two adsorbent  
26  
27 tube types, the intent of the experiments was to illustrate that sampling can be performed  
28  
29 with two different TD tube types within the ReCIVA sampler, one in each ReCIVA bank.  
30  
31 However, the resulting exhaled breath data are only comparable among the same TD  
32  
33 tube types.  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43

44 Inspection of the z-scores for the global metabolomics comparisons, again, highlighted  
45  
46 the differences in TD tube types rather than substantial differences among ReCIVA  
47  
48 devices, breathing rates, or device banks (**Supplemental Data 8 & 11**). The data suggest  
49  
50 for both experiments, that 5TD tubes have greater amounts of propene (3.81 min, 43.1  
51  
52 m/z) and an unknown compound methoxy-phenyl-oxime (14.08 min, 133.1 m/z) while  
53  
54 Tenax tubes retain compounds more routinely found in breath such as isoprene (feature  
55  
56  
57  
58  
59  
60

1  
2  
3 5.35 min, 67.1 m/z) and acetone (5.57 min, 43.1 m/z, **Supplemental Data 9**). While  
4  
5 propene has been identified in breath previously and methoxy-phenyl-oxime was included  
6  
7 in the analysis as it was an unknown, it is hypothesized this is a result of greater  
8  
9 background from the 5TD tubes themselves even though both TD tube types were  
10  
11 reconditioned as recommended by the manufacturer prior to each test [34]. Therefore,  
12  
13 these data indicate careful consideration must be utilized when selecting TD tube  
14  
15 adsorbent materials and adequate control samples must be utilized for exhaled breath  
16  
17 collection while using the ReCIVA device.  
18  
19  
20  
21  
22  
23  
24  
25

26 It was hypothesized, due to the active pumping of the ReCIVA for exhaled breath  
27  
28 sampling, that frequency of pumping (high number of exhalations) and length of pumping  
29  
30 (length and depth of exhalations) would affect the performance of the device. As a result,  
31  
32 guided breathing rate experiments were performed. The data illustrate that similar results  
33  
34 are obtained independent of the breathing rate (**Figure 2**). However, substantial isoprene  
35  
36 variability was observed in the low breathing rate test from ReCIVA #33 (**Figure 2A**). As  
37  
38 the Mean Max CO<sub>2</sub> (an estimate of breath depth) for this test is also substantially variable,  
39  
40 the data would suggest that consistent depth of breathing would lead to more consistent  
41  
42 results as observed with the other three tests (**Supplemental Data 10, Figure 2A**).  
43  
44 Therefore, to truly obtain the most consistent data, it is speculated that guided breathing,  
45  
46 at a comfortable rate, should be applied for all exhaled breath collections using the  
47  
48 ReCIVA device.  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

1  
2  
3 Data were presented demonstrating a participant (#28), sampled during Tests 2 and Test  
4  
5 4, had background levels of exhaled isoprene while showing acetone levels similar to the  
6  
7 remaining participants (**Figure 3**). Spanel et. al. first reported a similar case of extremely  
8  
9 low isoprene using SIFT-MS detection [46]. However, they were unable to determine a  
10  
11 cause as blood cholesterol values of the subject in the Spanel et. al. study were similar  
12  
13 to controls [46]. No additional cases have been reported in the literature. However,  
14  
15 unpublished evidence of similar cases, with little or no isoprene, have been presented at  
16  
17 exhaled breath conferences. Preliminary data suggest genetics may play a role in this  
18  
19 phenomenon. While it would be extremely interesting to further explore the genetic  
20  
21 hypothesis with Participant #28 from this study, due to ethical restraints, further  
22  
23 experimentation is not possible at this time. Although the data presented here represent  
24  
25 only a single participant, it is important to highlight such cases to determine not only the  
26  
27 overall prevalence of the phenomenon but also, ultimately, the underlying cause.  
28  
29  
30  
31  
32  
33  
34  
35  
36

## 37 **CONCLUSIONS**

38  
39 As the ReCIVA becomes a more widely used tool, investigations into parameters affecting  
40  
41 functionality and data variability must be explored. The data presented here support the  
42  
43 use and comparison of results from two ReCIVA devices. Additionally, the results  
44  
45 highlight that the ReCIVA performs consistently, independent of breathing rate while  
46  
47 significant differences were noted between TD tubes of different types. Collectively, the  
48  
49 results support the combination of individual TD tube flow rate calibration within the  
50  
51 ReCIVA and guided breathing during exhaled breath collections to yield the most  
52  
53  
54  
55  
56  
57  
58  
59  
60

1  
2  
3 consistent data. Overall, the results provide evidence that the ReCIVA sampler may be  
4  
5 used among multiple sampling sites for exhaled breath collection.  
6  
7  
8  
9  
10

## 11 **ACKNOWLEDGEMENTS**

12 UES Inc. was provided support from United States Air Force Defense Health Program  
13  
14 funds under subcontract (FA8650-17-6891). Opinions, interpretations, conclusions, and  
15  
16 recommendations are those of the authors and not necessarily endorsed by the United  
17  
18 States Government. The authors would like to thank Ms. Tenika Dearmond and Ms.  
19  
20 Heather Lyons for their support for this manuscript.  
21  
22  
23  
24  
25

## 26 **Conflicts of Interest**

27  
28  
29 The authors have no conflicts of interest to report  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

## REFERENCES

- [1] Pauling L, Robinson A B, Teranishi R and Cary P 1971 Quantitative Analysis of Urine Vapor and Breath by Gas-Liquid Partition Chromatography *Proc Natl Acad Sci USA* **68** 2374–6
- [2] van Oort P M, Povoia P, Schnabel R, Dark P, Artigas A, Bergmans D C J J, Felton T, Coelho L, Schultz M J, Fowler S J, Bos L Don behalf of the BreathDx Consortium 2018 The potential role of exhaled breath analysis in the diagnostic process of pneumonia—a systematic review *J Breath Res* **12** 024001–12
- [3] Davis M D, Fowler S J and Montpetit A J 2019 Exhaled breath testing - A tool for the clinician and researcher *Paediatric Respiratory Reviews* **29** 37–41
- [4] Zhou J, Huang Z-A, Kumar U and Chen D D Y 2017 Review of recent developments in determining volatile organic compounds in exhaled breath as biomarkers for lung cancer diagnosis *Anal Chim Acta* **996** 1–9
- [5] Risby T H 2008 Critical issues for breath analysis *J Breath Res* **2** 030302–4
- [6] Beauchamp J D and Pleil J D 2013 Simply breath-taking? Developing a strategy for consistent breath sampling *J Breath Res* **7** 042001–4
- [7] Beauchamp J, Herbig J, Gutmann R and Hansel A 2008 On the use of Tedlar bags for breath-gas sampling and analysis *J Breath Res* **2** 046001
- [8] Sulyok M, Haberhauer-Troyer C, Rosenberg E and Grasserbauer M 2001 Investigation of the storage stability of selected volatile sulfur compounds in different sampling containers *J Chromatogr A* **917** 367–74
- [9] Deng C, Zhang J, Yu X, Zhang W and Zhang X 2004 Determination of acetone in human breath by gas chromatography-mass spectrometry and solid-phase microextraction with on-fiber derivatization *J Chromatogr B* **810** 269–75
- [10] Groves W A and Zellers E T 1996 Investigation of organic vapor losses to condensed water vapor in Tedlar bags used for exhaled-breath sampling *Am Ind Hyg Assoc J* **57** 257–63
- [11] Mochalski P, King J, Unterkofler K and Amann A 2013 Stability of selected volatile breath constituents in Tedlar, Kynar and Flexfilm sampling bags *Analyst* **138** 1405–18
- [12] Steeghs M M, Cristescu S M and Harren F J 2007 The suitability of Tedlar bags for breath sampling in medical diagnostic research *Physiol Meas* **28** 73–84
- [13] Doran S L F, Romano A and Hanna G B 2017 Optimisation of sampling parameters for standardised exhaled breath sampling *J Breath Res* **12** 016007

- 1  
2  
3 [14] Harshman S W, Pitsch R L, Davidson C N, Scott A M, Hill E M, Smith Z K, Strayer  
4 K E, Schaeublin N M, Wiens T L, Brothers M C, Slusher G M, Steele M L, Geier  
5 B A, Fan M, Drummond L A and Martin J A 2019 Characterization of standardized  
6 breath sampling for off-line field use. *J Breath Res* **14** 016009  
7  
8  
9 [15] He J, Zou Z and Yang X 2019 Measuring whole-body volatile organic compound  
10 emission by humans: A pilot study using an air-tight environmental chamber *Build*  
11 *Environ* **153** 101–9  
12  
13 [16] Gashimova E M, Temerdashev A Z, Porkhanov V A, Polyakov I S, Perunov D V,  
14 Azaryan A A and Dmitrieva E V 2019 Evaluation of the Possibility of Volatile  
15 Organic Compounds Determination in Exhaled Air by Gas Chromatography for  
16 the Noninvasive Diagnostics of Lung Cancer *J Anal Chem* **74** 472–9  
17  
18 [17] Preti G, Labows J N, Kostelc J G, Aldinger S and Daniele R 1988 Analysis of lung  
19 air from patients with bronchogenic carcinoma and controls using gas  
20 chromatography-mass spectrometry. *J Chromatogr* **432** 1–11  
21  
22 [18] Prado C, Marín P and Periago J F 2003 Application of solid-phase microextraction  
23 and gas chromatography–mass spectrometry to the determination of volatile  
24 organic compounds in end-exhaled breath samples *J Chromatogr A* **1011** 125–  
25 34  
26  
27 [19] Salvo P, Ferrari C, Persia R, Ghimenti S, Lomonaco T, Bellagambi F and Di  
28 Francesco F 2015 A dual mode breath sampler for the collection of the end-tidal  
29 and dead space fractions *Med Eng Phys* **37** 539–44  
30  
31 [20] Grabowska-Polanowska B 2019 Breath analysis as promising indicator of  
32 hemodialysis efficiency *Clin Exp Nephrol* **23** 251–7  
33  
34 [21] van Oort P M P, Nijssen T, Weda H, Knobel H, Dark P, Felton T, Rattray N J W,  
35 Lawal O, Ahmed W, Portsmouth C, Sterk P J, Schultz M J, Zakharkina T, Artigas  
36 A, Pova P, Martin-Loeches I, Fowler S J and Bos L D J 2017 BreathDx –  
37 molecular analysis of exhaled breath as a diagnostic test for ventilator–  
38 associated pneumonia: protocol for a European multicentre observational study  
39 *BMC Pulm Med* **17** 1–8  
40  
41 [22] Gaida A, Holz O, Nell C, Schuchardt S, Lavae-Mokhtari B, Kruse L, Boas U,  
42 Langejuergen J, Allers M, Zimmermann S, Vogelmeier C, Koczulla A R and  
43 Hohlfeld J M 2016 A dual center study to compare breath volatile organic  
44 compounds from smokers and non-smokers with and without COPD *J Breath Res*  
45 **10** 026006–19  
46  
47 [23] He J, Sun X and Yang X 2019 Human respiratory system as sink for volatile  
48 organic compounds: Evidence from field measurements *Indoor Air* **29** 968–78  
49  
50 [24] Grabowska-Polanowska B, Skowron M, Miarka P, Pietrzycka A and Śliwka I 2017  
51 The application of chromatographic breath analysis in the search of volatile  
52  
53  
54  
55  
56  
57  
58  
59  
60

- 1  
2  
3 biomarkers of chronic kidney disease and coexisting type 2 diabetes mellitus *J*  
4 *Chromatogr B* **1060** 103–10  
5
- 6 [25] Rothbart N, Holz O, Koczulla R, Schmalz K and Hübers H-W 2019 Analysis of  
7 Human Breath by Millimeter-Wave/Terahertz Spectroscopy *Sensors* **19** 2719–12  
8  
9
- 10 [26] van der Schee M P, Fens N, Brinkman P, Bos L D J, Angelo M D, Nijsen T M E,  
11 Raabe R, Knobel H H, Vink T J and Sterk P J 2013 Effect of transportation and  
12 storage using sorbent tubes of exhaled breath samples on diagnostic accuracy of  
13 electronic nose analysis. *J Breath Res* **7** 016002  
14
- 15 [27] Tangerman A, Meuwese-Arends M T and van Tongeren J H 1983 A new sensitive  
16 assay for measuring volatile sulphur compounds in human breath by Tenax  
17 trapping and gas chromatography and its application in liver cirrhosis. *Clin Chim*  
18 *Acta* **130** 103–10  
19  
20
- 21 [28] Gordon S M, Szidon J P, Krotoszynski B K, Gibbons R D and O'Neill H J 1985  
22 Volatile organic compounds in exhaled air from patients with lung cancer. *Clin*  
23 *Chem* **31** 1278–82  
24
- 25 [29] Grabowska-Polanowska B, Faber J, Skowron M, Miarka P, Pietrzycka A, Śliwka I  
26 and Amann A 2013 Detection of potential chronic kidney disease markers in  
27 breath using gas chromatography with mass-spectral detection coupled with  
28 thermal desorption method *J Chromatogr A* **1301** 179–89  
29  
30
- 31 [30] Marco E and Grimalt J O 2015 A rapid method for the chromatographic analysis  
32 of volatile organic compounds in exhaled breath of tobacco cigarette and  
33 electronic cigarette smokers *J Chromatogr A* **1410** 51–9  
34
- 35 [31] Reynolds J C, Blackburn G J, Guallar-Hoyas C, Moll V H, Bocos-Bintintan V, Kaur-  
36 Atwal G, Howdle M D, Harry E L, Brown L J, Creaser C S and Thomas C L P 2010  
37 Detection of Volatile Organic Compounds in Breath Using Thermal Desorption  
38 Electropray Ionization-Ion Mobility-Mass Spectrometry *Anal Chem* **82** 2139–44  
39  
40
- 41 [32] Pennazza G, Santonico M, Incalzi R A, Scarlata S, Chiurco D, Vernile C and  
42 D'Amico A 2014 Measure chain for exhaled breath collection and analysis: A  
43 novel approach suitable for frail respiratory patients *Sensor Actuac B-Chem* **204**  
44 578–87  
45
- 46 [33] Harshman S W, Geier B A, Qualley A V, Drummond L A, Flory L E, Fan M, Pitsch  
47 R L, Grigsby C C, Phillips J B and Martin J A 2017 Exhaled isoprene for monitoring  
48 recovery from acute hypoxic stress. *J Breath Res* **11** 047111  
49  
50
- 51 [34] Harshman S W, Mani N, Geier B A, Kwak J, Shepard P, Fan M, Sudberry G L,  
52 Mayes R S, Ott D K, Martin J A and Grigsby C C 2016 Storage stability of exhaled  
53 breath on Tenax TA. *J Breath Res* **10** 046008  
54  
55  
56  
57  
58  
59  
60

- 1  
2  
3 [35] Harshman S W, Geier B A, Fan M, Rinehardt S, Watts B S, Drummond L A, Preti  
4 G, Phillips J B, Ott D K and Grigsby C C 2015 The identification of hypoxia  
5 biomarkers from exhaled breath under normobaric conditions. *J Breath Res* **9**  
6 047103  
7  
8  
9 [36] Kwak J, Fan M, Harshman S W, Garrison C E, Dershem V L, Phillips J B, Grigsby  
10 C C and Ott D K 2014 Evaluation of Bio-VOC Sampler for Analysis of Volatile  
11 Organic Compounds in Exhaled Breath *Metabolites* **4** 879–88  
12  
13 [37] Sukul P, Trefz P, Schubert J K and Miekisch W 2014 Immediate effects of breath  
14 holding maneuvers onto composition of exhaled breath *J Breath Res* **8** 037102  
15  
16 [38] Harshman S W, Rubenstein M H, Qualley A V, Fan M, Geier B A, Pitsch R L,  
17 Slusher G M, Hughes G T, Dershem V L, Grigsby C C, Ott D K and Martin J A  
18 2017 Evaluation of thermal desorption analysis on a portable GC–MS system *Int*  
19 *J Environ Anal Chem* **00** 1–17  
20  
21 [39] Harshman S W, Dershem V L, Fan M, Watts B S, Slusher G M, Flory L E, Grigsby  
22 C C and Ott D K 2015 The stability of Tenax TA thermal desorption tubes in  
23 simulated field conditions on the HAPSITE ER *Int J Environ Anal Chem* **95** 1014–  
24 29  
25  
26 [40] US Environmental Protection Agency 1999 *Compendium Method TO-15*  
27 *Determination of Volatile Organic Compounds (VOCs) in Air Collected in*  
28 *Specially-Prepared Canisters and Analyzed by Gas Chromatography/Mass*  
29 *Spectrometry (GC/MS)* **EPA/625/R-96/010b**  
30  
31 [41] US Environmental Protection Agency 1999 *Compendium Method TO-17*  
32 *Determination of Volatile Organic Compounds in Ambient Air Using Active*  
33 *Sampling onto Sorbent Tubes* **EPA/625/R-96/101b**  
34  
35 [42] Grigsby C C, Rizki M M, Tamburino L A, Pitsch R L, Shiyarov P A and Cool D R  
36 2010 Metabolite Differentiation and Discovery Lab (MeDDL): A New Tool for  
37 Biomarker Discovery and Mass Spectral Visualization *Anal Chem* **82** 4386–95  
38  
39 [43] Wickham H 2016 *ggplot2: elegant graphics for data analysis* (Springer-Verlag)  
40  
41 [44] Vu V Q 2011 ggbiplot: A ggplot2 based biplot. R package version 0.55.  
42  
43 [45] R Core Team 2018 R: A language and environment for statistical computing  
44  
45 [46] Španěl P, Davies S and Smith D 1999 Quantification of breath isoprene using the  
46 selected ion flow tube mass spectrometric analytical method. *Rapid Commun*  
47 *Mass Spectrom* **13** 1733–8  
48  
49 [47] Dweik R A, Boggs P B, Erzurum S C, Irvin C G, Leigh M W, Lundberg J O, Olin  
50 A-C, Plummer A L, Taylor D R American Thoracic Society Committee on  
51 Interpretation of Exhaled Nitric Oxide Levels (FENO) for Clinical Applications 2011  
52  
53  
54  
55  
56  
57  
58  
59  
60

- 1  
2  
3 An official ATS clinical practice guideline: interpretation of exhaled nitric oxide  
4 levels (FENO) for clinical applications. *Am J Respir Crit Care Med* **184** 602–15  
5  
6  
7 [48] Bajtarevic A, Ager C, Pienz M, Klieber M, Schwarz K, Ligor M, Ligor T, Filipiak W,  
8 Denz H, Fiegl M, Hilbe W, Weiss W, Lukas P, Jamnig H, Hackl M, Haidenberger  
9 A, Buszewski B, Miekisch W, Schubert J and Amann A 2009 Noninvasive  
10 detection of lung cancer by analysis of exhaled breath. *BMC Cancer* **9** 348  
11  
12 [49] King J, Kupferthaler A, Frauscher B, Hackner H, Unterkofler K, Teschl G,  
13 Hinterhuber H, Amann A and Högl B 2012 Measurement of endogenous acetone  
14 and isoprene in exhaled breath during sleep. *Physiol Meas* **33** 413–28  
15  
16 [50] King J, Mochalski P, Kupferthaler A, Unterkofler K, Koc H, Filipiak W, Teschl S,  
17 Hinterhuber H and Amann A 2010 Dynamic profiles of volatile organic compounds  
18 in exhaled breath as determined by a coupled PTR-MS/GC-MS study *Physiol*  
19 *Meas* **31** 1169–84  
20  
21  
22 [51] Kushch I, Arendacká B, Stolc S, Mochalski P, Filipiak W, Schwarz K, Schwentner  
23 L, Schmid A, Dzien A, Lechleitner M, Witkovský V, Miekisch W, Schubert J,  
24 Unterkofler K and Amann A 2008 Breath isoprene--aspects of normal physiology  
25 related to age, gender and cholesterol profile as determined in a proton transfer  
26 reaction mass spectrometry study. *Clin. Chem. Lab. Med.* **46** 1011–8  
27  
28  
29 [52] McGrath L T, Patrick R and Silke B 2001 Breath isoprene in patients with heart  
30 failure. *Eur. J. Heart Fail.* **3** 423–7  
31  
32 [53] Hornuss C, Zagler A, Dolch M E, Wiepcke D, Praun S, Boulesteix A-L, Weis F,  
33 Apfel C C and Schelling G 2012 Breath isoprene concentrations in persons  
34 undergoing general anesthesia and in healthy volunteers *J Breath Res* **6** 046004–  
35 8  
36  
37  
38 [54] Wilkinson M, White I R, Goodacre R, Nijssen T and Fowler S J 2019 Effects of high  
39 relative humidity and dry purging on VOCs obtained during breath sampling on  
40 common sorbent tubes Breath Biopsy Conference, Cambridge UK  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60

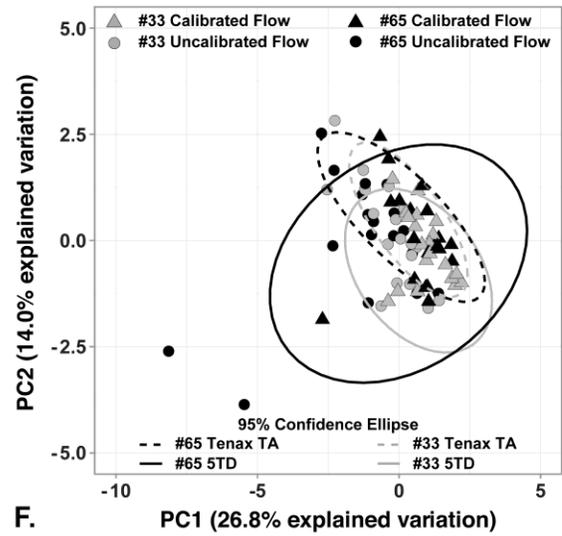
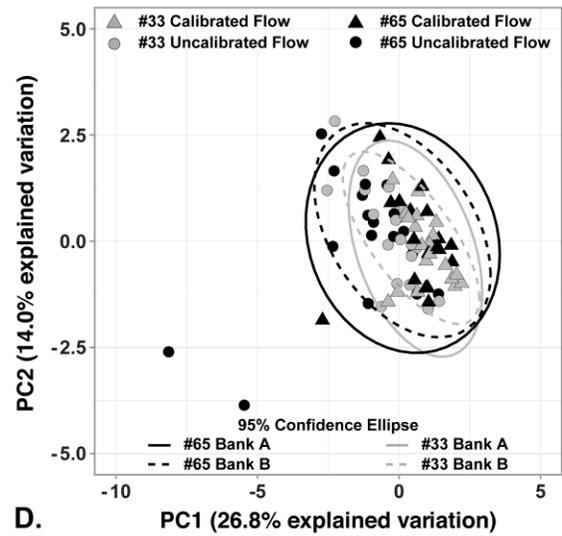
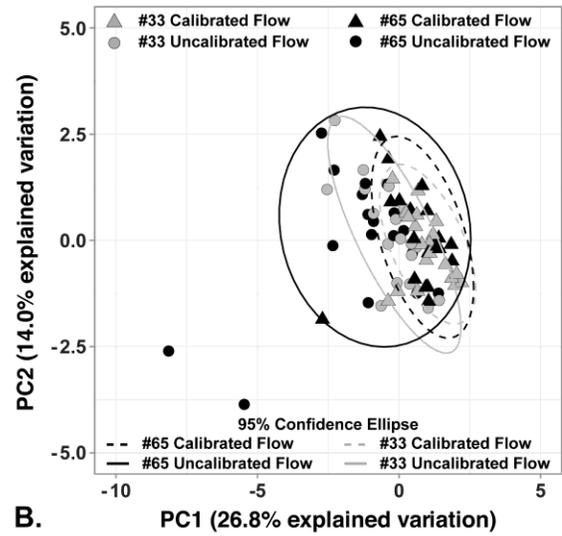
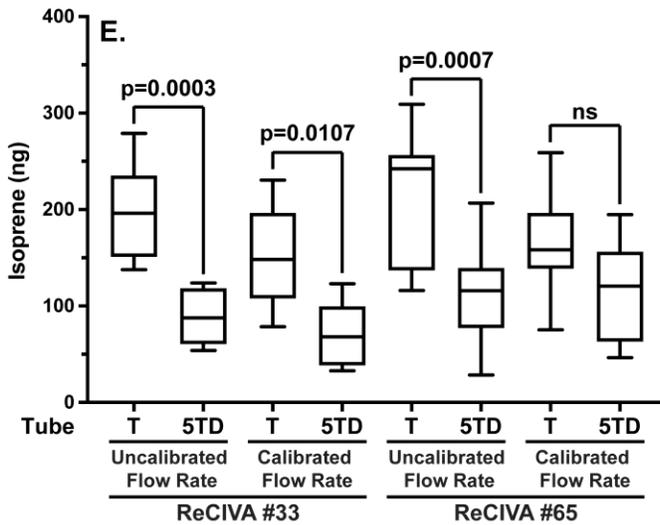
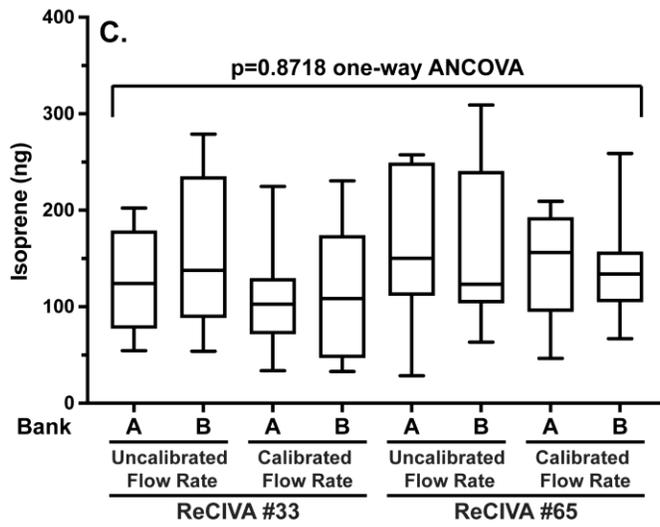
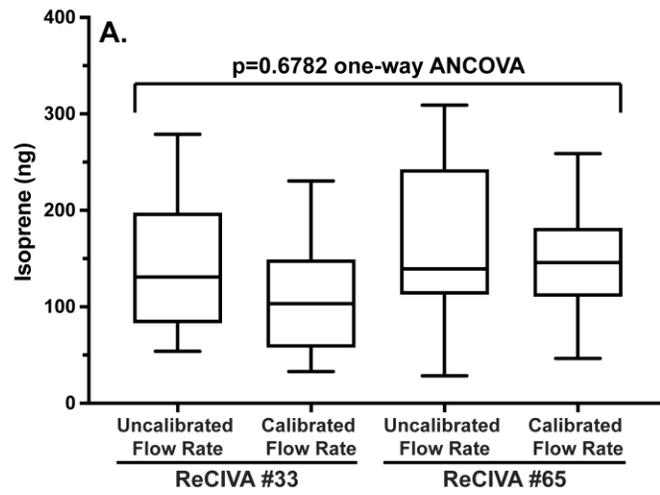
## FIGURE CAPTIONS

**Figure 1:** Box plots of the quantitated isoprene values obtained from participants using two distinct ReCIVA devices **A)** calibrated and uncalibrated ReCIVA flow rates, **C)** ReCIVA banks (A & B), and **E)** TD tube types (T=Tenax). A & C represent data from both TD tube types. The whiskers on the boxplots represent the highest and lowest observations. Principal component analysis (PCA) of ten high abundant features (internal standard normalized response ratios) extracted from samples obtained from participants using two distinct ReCIVA devices with the 95% confidence ellipses corresponding to **B)** calibrated and uncalibrated ReCIVA flow rates, **D)** ReCIVA banks, and **F)** TD tube type. The results show the primary source of variability in both isoprene and global metabolite abundance is the TD tube type.

**Figure 2:** Box plots of the quantitated isoprene values obtained from participants using two distinct ReCIVA devices with calibrated ReCIVA flow rates **A)** low and high breathing rates, **C)** ReCIVA banks, and **E)** TD tube types. A & C represent data from both TD tube types. The whiskers on the boxplots represent the highest and lowest observations. Principal component analysis (PCA) of 11 high abundant features (internal standard normalized response ratios) extracted from samples obtained from participants using two distinct ReCIVA devices with calibrated ReCIVA flow rates. The 95% confidence ellipses corresponding to **B)** low and high breathing rates, **D)** ReCIVA banks, and **F)** TD tube type. The data illustrate breathing rate does not have an effect on the performance of the ReCIVA device.

**Figure 3:** A scatter plot of the  $\log_2$  internal standard normalized response ratios of **A)** isoprene and **B)** acetone from Tests 2 and Test 4. Bars signify the median and 95% confidence interval, \* indicates  $p < 0.0001$  for exhaled breath isoprene to background isoprene by t-test. The data demonstrate participant #28 has isoprene abundance similar to the background levels while retaining acetone levels similar to the remaining participants from these tests.

**FIGURE 1**



**FIGURE 2**

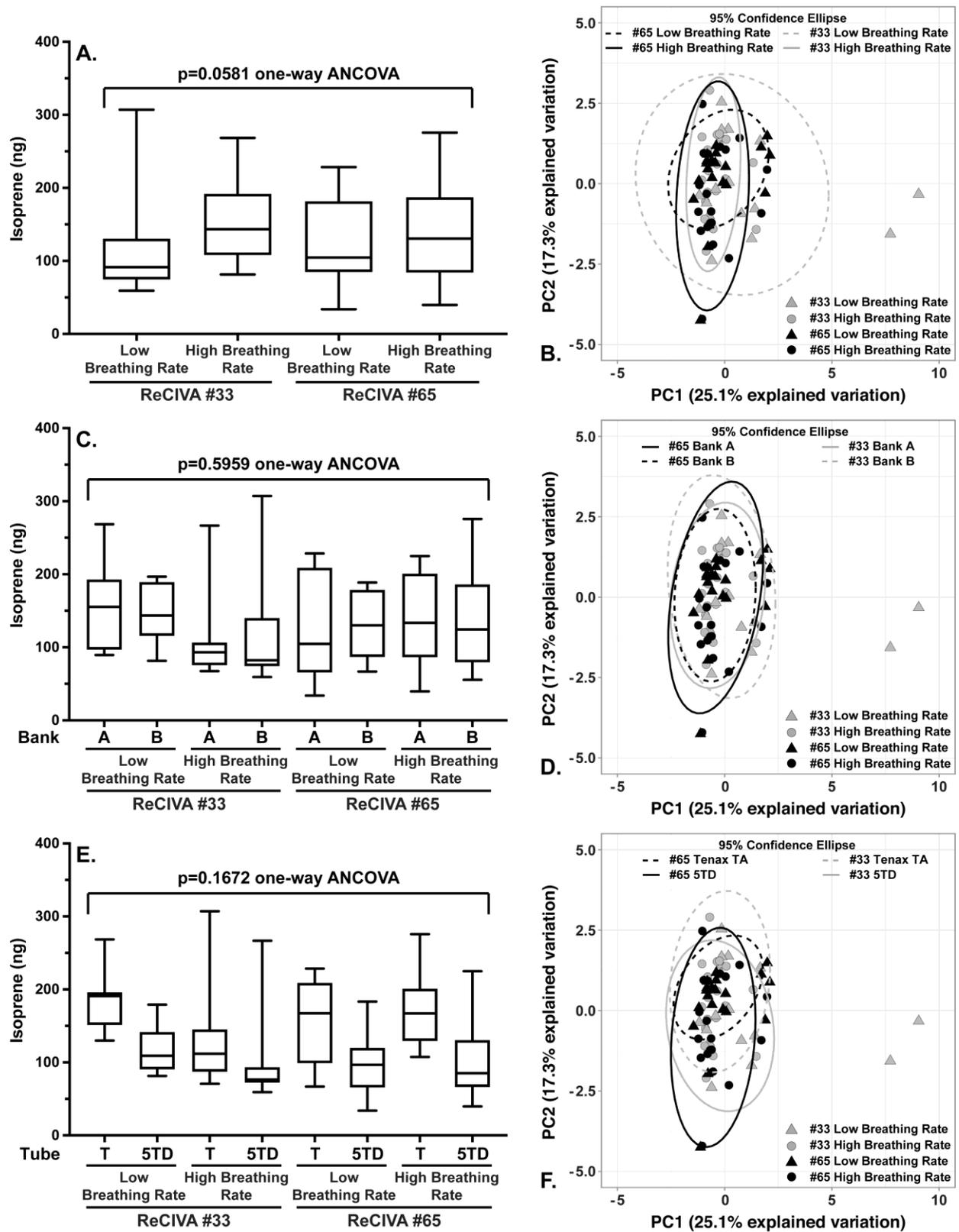


FIGURE 3

