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

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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 breaths min^{-1}) and high (15 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

have shown exhaled breath bags are prone to leaking and volatile loss due to breath condensation [7-12]. Additionally, those providing breath must follow exhalation protocols to obtain consistent samples among individuals. To mitigate the issues observed with exhaled breath bags, the Respiration Collector for In Vitro Analysis (ReCIVA) was developed and marketed by Owlstone Medical. This sampler uses real-time exhaled CO_2 measurements to estimate the portion (lower airway, upper airway, etc.) of breath entering the device. Breath is sampled at the appropriate CO_2 levels via active pumps directly onto adsorbent tubes placed into the device. The ReCIVA device is a large step toward standardized exhaled breath sampling. However, due to the relative novelty, thorough experimentation surrounding the sources of variability associated with the ReCIVA sampler have yet to be adequately explored.

Previous work by Doran et. al. evaluated parameters for breath collection, such as sample volume and sampling flow rates, while noting contaminants associated with the breath sampler itself [13]. Further evaluation of the ReCIVA sampler by our research group established the importance of the ReCIVA software version in commanding flow rates applied by the device [14]. Additionally, the data illustrated that manually calibrated flow rates allow for comparable results among ReCIVA banks, i.e. duplicate samples, and statistically similar results among ReCIVA samples and exhaled breath bags [14]. While these studies establish several critical attributes surrounding the ReCIVA sampler, additional work must be performed to ensure proper performance for large clinical investigations.

In this manuscript, data are presented for further evaluation of the ReCIVA device using two routinely utilized thermal desorption adsorbent tubes for exhaled breath sampling, Tenax TA and Tenax/Carbograph 5TD (5TD). The results provided support the use of multiple ReCIVA devices. The data also demonstrate the limited impact breathing rate has on exhaled breath collected by the ReCIVA device. These data add additional evidence for the use of the ReCIVA device for more consistent, standardized, exhaled breath collection.

EXPERIMENTAL

PARTICIPANTS

The participants (n=20 max per experiment, 27 total participants) were volunteer, non-smoking, males at our research facility. The research described here was determined to be Not-Human Research by the United States Air Forces Research Laboratory's Institutional Review Board, (FWR2017161N) as the research is designed to interrogate exhaled breath sampling platforms. As a result, all participants were verbally informed of all experimental parameters and free to discontinue participation at any time. However, written consent was not provided.

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^{-1} 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^{-1} over a 1420 mL (approximately 100 measurements) collection volume using the software's "Always On" feature (Owlstone Medical,

Cambridge, UK). Flow rate was measured by connecting the open end of the TD tube to a DryCal Bios Defender 510 (Mesa Labs, Lakewood, CO, USA) through a hole cut into a ReCIVA mask. The measurements were taken one at a time using both TD tube types and individual ReCIVA banks to evaluate the flow at the sampling positions distal to the participants mouth (n=5 per tube type per bank). The remaining three sampling ports within the ReCIVA that were not being tested were blocked with clean 3.5" x 0.25" solid stainless-steel rods. ReCIVA-applied-flow was monitored and recorded via the DryCal Pro Software (v. 1.3, Mesa Labs). Additionally, using the same setup, the calibrated flow rate was determined, for ReCIVA #65, by manually adjusting the flow rate within the ReCIVA software until approximately 200 mL min^{-1} was measured on the DryCal Pro Software, as described above [14]. The determined calibrated flow rates for ReCIVA #65 were as follows: Tenax Bank A: 152 mL min^{-1}, Tenax Bank B: 150 mL min^{-1}, 5TD Bank A: 174.5 mL min^{-1}, and 5TD Bank B: 178.5 mL min^{-1}. The calibrated flow rates used for ReCIVA #33 were determined previously [14].

Experimental Design & Exhaled Breath Collection: Test 1 & 2

All exhaled breath samples for Tests 1 & 2 were collected using two different ReCIVA devices, serial numbers 33 (#33) or #65, and brand-new masks (Owlstone Medical). The mask assembly manufacture dates for Tests 1 & 2 were March 2018 and May 2019, respectively. For all experiments, the filter was removed, using gloves, from the mask assembly and the silicon portion of the mask was baked at 180 °C overnight. Prior to breath collection, the mask was cooled and, with gloves, the filter was reinserted. Test 1

was performed using the ReCIVA control software v. 1.46 and the calibrated flow rates with the calculated collection volumes for ReCIVA device #33 and device #65 (**Supplemental Data 1A**). Test 2 was performed using the ReCIVA control software v. 1.46 and the uncalibrated flow rates on each ReCIVA device (200 mL min^{-1}, and 550 mL collection volume, **Supplemental Data 1B**). All remaining ReCIVA settings were held constant and represented in **Supplemental Data 1C**.

Exhaled breath collections, for both Test 1 and Test 2, were performed using a two TD tube setup by occluding the two ReCIVA sampling ports proximal to the mouth with clean solid stainless-steel rods (3.5" x 0.25"). In the remaining ReCIVA sampling ports, distal to the mouth, one of each type of TD tube, Tenax TA or 5TD, were randomly inserted. Additionally, participants (n=20 for Test 1 and n=18 for Test 2) were randomly assigned to a specific ReCIVA device, #33 or #65. All randomizations, for both TD tube placement and ReCIVA device, were performed using the RANDBETWEEN function of Microsoft Excel (Redmond, WA, USA). Please refer to **Supplemental Data 1D** for an illustration of the Test 1 & 2 experimental design.

Prior to performing an exhaled breath collection, all participants abstained from food or drink, except for water, for at least 1 hour. Immediately prior to collection, all participants thoroughly rinsed their mouths with filtered water and sat in a relaxed upright position for greater than five minutes [37]. Using an Alicat Scientific MCP-100 SLPM mass flow controller (MFC) and the FlowVIsion SC software (v. 1.3.28.0), 40 L min^{-1} of medical

grade breathing air (21% O_2 with N_2 balance) was provided to the ReCIVA device via Tygon tubing (Indiana Oxygen, Indianapolis, IN, USA; Alicat Scientific, Tuscon, AZ, USA). Participants donned the ReCIVA device affixed with the mask TD tube assembly. The head straps were adjusted until comfortable and no leaking was observed. Please refer to **Supplemental Data 1E** for a picture of the laboratory setup for exhaled breath collection and **Supplemental Data 2** for a summary of the participants and samples collected. Participants were instructed to breathe normal, slow breaths through their mouths only. No other direction or intervention, such as nose plugs, were provided for exhaled breath collection. Upon completion of the collection, all TD tubes were capped and stored at ambient temperature. Thermal desorption gas chromatography-mass spectrometry (TD-GC-MS) analysis was initiated on the same day [34].

Experimental Design & Exhaled Breath Collection: Test 3 & 4

The ReCIVA devices for Tests 3 and 4 were set up exactly as described above for Test 1 i.e., new, prebaked ReCIVA masks (manufacture date May 2019), with random placement of one Tenax TA and one 5TD tube in each ReCIVA bank distal to the mouth, manually calibrated ReCIVA flow rates, adjusted collection volumes, and ReCIVA control software v. 1.46. However, Test 3 (n=20) was performed only with ReCIVA #65 and Test 4 (n=18) only used ReCIVA #33 (**Supplemental Data 3A & 3B**). For both Tests 3 & 4 participants were randomly assigned to a breathing rate, high (15 breaths min^{-1} or 1 s inhale and 3 s exhale) or low (7 breaths min^{-1} or 2 s inhale and 6 s exhale). Breathing rate was controlled by having participants follow along with the Breathe+ iPhone app, set to

the breathing rate parameters assigned, projected on a large television (Dynamic App Design LLC, **Supplemental Data 3C & 3D**). Participants were instructed to inhale, as the bar of the app rose, and the inverse as the bar lowered. Please refer to the **Supplemental Video 1 & 2** for examples of the app function and rates and **Supplemental Data 4** for a summary of the participants and samples collected.

Background Sample Collection

For each test event (Tests 1-4), prior to the first participant's exhaled breath collection and following the last participant's exhaled breath collection, a room blank, medical grade air blank, and mask blank sample were collected on each adsorbent tube type as described previously [14]. Briefly, 550 mL of room air was pulled through each TD tube type (room blank), Tenax TA and 5TD, using a GilAir Plus pump operated at 200 mL min^{-1}. On each TD tube type, 550 mL of medical grade air was sampled at 200 mL min^{-1}, as described above, from a 1 L ALTEF bag filled with the air. Finally, ReCIVA mask blanks (550 mL at 200 mL min^{-1}) 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^{-1} 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-diflurobenzene, 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^{-1}) 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^{-1} trap purge was performed using a flow path temperature of 180 °C followed by a 5 min 315 °C trap desorption (40 °C s^{-1}). 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^{-1} with a constant 2 mL min^{-1} 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 quadrapole 0.154 scans s^{-1} 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 µg 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 mL^{-1} 99.999% nitrogen backflush (**Supplemental Data 5**, Hamilton, Reno, NV, USA) [38,39]. The zero point of calibration curves were 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 Tracefineder 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, Gaitherburg, 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_2 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

the sampling conditions are similar between the two ReCIVA tests and do not significantly influence the exhaled breath results.

Examination of the quantitated exhaled isoprene values from Tests 1 and 2 show no statistical difference ($p>0.05$ one-way ANCOVA) in isoprene amount among the two ReCIVA devices, independent of ReCIVA flow rate calibration (**Figure 1A**). To further evaluate the exhaled volatiles from the samples beyond isoprene alone, the internal standard normalized response ratios of ten abundant features were calculated and principal component analysis (PCA) was performed. **Figure 1B** illustrates that a relatively small amount of the overall variation (40.8%) is captured by the first two principal components (PC1 26.8%, PC2 14.0%). Additionally, the 95% confidence ellipses overlap in space, suggesting no statistical difference among the samples based on ReCIVA unit (comparing among ellipses colors) or calibration (comparing ellipses within the same color) (**Figure 1B**). To further inspect differences among feature abundances, z-scores of the ten IS normalized response ratios between ReCIVA #65 and #33 were calculated and parsed by ReCIVA flow calibration (**Supplemental Data 8A**). Overall the data suggest greater than 85% (17/20) of the comparison are within the $\pm$ 1.96 significance value. Within the global data set, the feature 5.35, 67.1 (RT, m/z) corresponds to isoprene (**Supplemental Data 9**). The data presented in **Supplemental Data 8A** show isoprene abundance is not significantly different between ReCIVA devices supporting the quantitated isoprene results by a global metabolomics approach. Overall these data indicate similar results are obtained by both quantitated values and relative abundances, among two separate ReCIVA devices independent of ReCIVA flow rate.

Previous data suggest variability among ReCIVA banks can occur, likely due to separate active pumps in each bank, and manual calibration of ReCIVA flow rates can remedy the differences among ReCIVA banks [14]. To determine if there are significant differences between banks among the two ReCIVA devices, the isoprene quantities were parsed for each bank (A and B) and Test (1: calibrated ReCIVA flow rate, 2: uncalibrated ReCIVA flow rate, **Figure 1C**). The data suggest there is no statistically significant difference among banks of the ReCIVA independent of the ReCIVA device or flow rate ($p>0.05$ one-way ANCOVA, **Figure 1C**). Additionally, a recalculation of the 95% confidence ellipses of the PCA, based on ReCIVA bank and ReCIVA device, illustrate a high amount of overlap between ReCIVA devices (comparing among ellipses colors) and banks within the same device (comparing ellipses within the same color, **Figure 1D**). To identify divergence among selected features across samples, z-scores of the ten IS normalized response ratios between ReCIVA banks (B - A) were calculated and parsed by ReCIVA device and ReCIVA flow calibration (**Supplemental Data 8B**). The z-scores show greater than 97% (39/40) comparisons fall within significance limits (**Supplemental Data 8B**). Similar to previous results, the feature corresponding to isoprene (5.35 min, 67.1 m/z) displays an insignificant difference among banks, providing further support for the quantitated data for isoprene shown in **Figure 1C**. These data demonstrate that there are minimal differences between banks of the ReCIVA devices independent of ReCIVA flow rate for sampling by both quantitated and relative approaches.

For all experimental tests, one of each TD tube type, Tenax and 5TD, was inserted randomly within the ReCIVA device in the positions distal to the mouth. To ascertain how different TD tubes, contribute to the variability among ReCIVA devices, quantitated isoprene values were parsed by TD tube type (Tenax and 5TD) and calibrated (Test 1) and uncalibrated (Test 2) ReCIVA flow rates (**Figure 1E**). One-way ANCOVA of the isoprene results provided an overall significant ($p=0.0005$) difference among the samples. To determine which groups differed significantly, Tukey's multiple comparisons test correction was used. The data demonstrate significant p-values ($p<0.05$) for all comparisons between the Tenax and 5TD results, within each Test, except for the test using the calibrated flow rate on ReCIVA #65 (**Figure 1E**). Again, the 95% confidence ellipses of the PCA were recalculated based on TD tube type and ReCIVA device (**Figure 1F**). The data show overlap between each TD tube type (comparing among ellipses colors of the same line style) independent of ReCIVA device while showing greater separation between TD tube types within the same device (comparing ellipses of the same color, **Figure 1F**). Evaluation of the calculated z-scores from the ten IS normalized response ratios (Tenax – 5TD) parsed by ReCIVA flow calibration and ReCIVA device, show more than 52% (21/40) of the global metabolomic features are within the ± 1.96 range of significance based on tube type (**Supplemental Data 8C**). Inspection of the tentative identifications associated with the features, outside the significant limits, suggest propene and methoxy-phenyl-oxime are significantly retained on the 5TD tubes (**Supplemental Data 9**). However, the data illustrate that the Tenax tubes retain a greater amount of isoprene (feature 5.35 min, 67.1 m/z) than the 5TD tubes, which is in-line with the isoprene quantified results. Collectively, the data suggest the differences among TD tubes

provide the greatest source of variability among the two ReCIVA devices independent of ReCIVA flow rate or ReCIVA bank.

Effect of Breathing Rate on ReCIVA Performance

The ReCIVA device is designed to monitor CO_2 in real time to allow for versatile airway collection, e.g., lower airway, upper airway, or whole breath, via an active sampling pump. As breathing rate and depth of breathing could contribute to variability in exhaled breath results sampled with the ReCIVA device due the frequency and duration of active pumping, two guided breathing experiments, Test 3 (n=20) using ReCIVA #65 and Test 4 (n=18) using ReCIVA #33, were performed by asking participants to inhale and exhale along with an iPhone app (Breath+) projected on a television (**Supplemental Data 3C, 3D, 4, and Supplemental Videos 1 & 2**). Representative plots of the measured CO_2 from the low (7.5 breaths min^{-1}) and high (15 breaths min^{-1}) breathing rate groups are provided in **Supplemental Data 10A & 10B**. Inspection of the attributes associated with sampling, such as collection time, exhalation rate, total number of exhalations, and mean max CO_2, shows statistically significant differences ($p<0.05$) between high and low breathing rate groups for all attributes independent of ReCIVA device (**Supplemental Data 10C - 10F**). As expected, the low breathing rate groups had shorter collection times attributed to fewer overall exhalations and higher mean max CO_2 (longer exhalations) while the opposite is true of the high breathing rate group. All aspects of sampling, except for mean max CO_2 between ReCIVA #65 (Test 3) and #33 (Test 4) low breathing rate samples, are not significantly different ($p>0.05$) between breathing rate groups no matter the ReCIVA

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

To assess if breathing rate significantly affects exhaled breath results between ReCIVA devices, isoprene was quantitated from the exhaled breath from each Test and plotted by breathing rate and ReCIVA device (**Figure 2A**). The data show no significant difference among all the isoprene values regardless of ReCIVA device or guided breathing rate ($p>0.05$ by one-way ANCOVA). A PCA of the 38 samples corresponding to 11 features (IS normalized response ratios) extracted from the exhaled breath GC-MS data show a small amount of the overall variation within the data (42.4%) is accounted for by the first two principal components (PC1 25.1% and PC2 17.3%, **Figure 2B**). Overlay of the 95% confidence ellipses by ReCIVA device and breathing rate shows a high amount of overlap among not only the breathing rates, (high and low, comparing ellipses of the same color) but also between ReCIVA devices (comparing among ellipse colors, **Figure 2B**). To further explore the global analysis, z-scores were determined between ReCIVA devices (65 – 33) and parsed by breathing rate (**Supplemental Data 11A**). The data show that more than 87% (29/33) of the comparisons are below the threshold for significance ($\pm$ 1.96) suggesting most features are not observed at significantly different levels between ReCIVA devices. The results also show that isoprene, feature 5.35 min, 67.1 m/z, is not significantly different between devices, suggesting that the global isoprene data correspond to that of the quantitated isoprene values. Furthermore, z-scores were

calculated based on breathing rate (high – low) and separated by the ReCIVA device used for collection (**Supplemental Data 11B**). The data show that 29 of 33 (>87%) comparisons are within the ± 1.96 significance level. Interestingly, the feature corresponding to isoprene (5.35 min, 67.1 m/z) shows that the low breathing rate IS normalized abundance from ReCIVA #33 is significantly higher than the high breathing rate abundance within the same ReCIVA device (illustrated by negative z-scores, **Supplemental Data 11B**). This result is an inverse of the quantitated isoprene values and could be a result of the substantial variability associated with the ReCIVA #33 low breathing rate samples (**Figure 2A**). Overall, the data suggest that neither breathing rate nor ReCIVA device plays a significant role in disrupting the consistency of the exhaled breath results obtained from a ReCIVA sampler.

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

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

The data shown in **Figure 1E** suggest that TD tube type plays a significant role in the variability associated with the ReCIVA sampler, as expected due to the different adsorbent packing material of the Tenax and 5TD tubes. To evaluate the impact of TD tube variability along with breathing rate and ReCIVA device, the quantitated isoprene data were further parsed by TD tube type. **Figure 2E** shows no statistically significant difference (p=0.1672 one-way ANCOVA) among the isoprene measurements indicating that within and among a breathing rate groups the two different TD tubes perform similarly. Recalculation of the 95% confidence ellipses of the PCA analysis, to consider TD tube type and ReCIVA device, show a large amount of overlap between TD tube types within a ReCIVA device (comparing ellipses of the same color) and among TD tubes of the same type (comparing ellipses the same line style among the two colors, **Figure 2F**). Inspection of the calculated z-scores (Tenax – 5TD), show similar results to those from Test 1 & 2 comparing TD tube types, in that the 5TD samples have greater propene and methoxy-phenyl-oxime while the Tenax samples tend to retain greater amounts of isoprene (feature 5.35 min, 67.1 m/z, **Supplemental Data 9 & 11D**). Similar trends for

isoprene are observed in the quantitated data (**Supplemental Data 11D, Figure 2E**). Collectively, these data indicate breathing rate does not play a significant role in the functionality of the ReCIVA device.

IDENTIFICATION OF PARTICIPANT WITH LOW ISOPRENE

Exhaled breath is most commonly characterized by the presence of two highly abundant compounds, acetone (2-propanone) and isoprene. A single participant, Participant #28, performed exhaled breath collections for both Tests 2 & 4. While tabulating the isoprene peak areas for quantitation, it was observed that this participant had levels of breath isoprene (log_2 IS normalized response ratio) similar to background, among the two sampling events, no matter the TD tube type used for sampling (**Figure 3A**). To verify the exhaled breath was otherwise similar to the remaining participants, the acetone peak areas were tabulated for all participants including Participant #28 and log_2 IS normalized response ratios were calculated and plotted (**Figure 3B**). The data show that while the isoprene values were similar to background, the acetone levels were dispersed among the other participants. These data suggest the exhaled breath was overall similar for Participant #28, except for the background levels of isoprene. As these data would have skewed the results, Participant #28's samples were removed from the overall analysis. However, the lack of isoprene in a participant's exhaled breath is quite rare and worth noting when observed [46].

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,

it is interesting that the 5TD tubes, which are a dual bed of Tenax and carbograph adsorbents intended for compounds ranging from C_4-C_{30}, retained less isoprene among both experiments although designed for lower molecular weight species than the single bed Tenax tubes (C_6-C_{30}, **Figure 1E & 2E**) [48-53]. Initially, the data were evaluated to determine if background or an interfering ion was responsible for the observed increase in isoprene on the Tenax TA TD tubes. However, the isoprene in the background samples did not show any statistical difference between TD tube types among all of the tests ($p=0.2653$ one-way ANOVA). Furthermore, manual inspection of the spectra about the isoprene peak suggests no interfering ions are present. To confirm the peak was truly isoprene, a secondary ion (53 m/z, S-ion) peak area was monitored and compared to the quantitative ion (Q-ion) for isoprene (67 m/z) for all injections. The ratio of S-ion/Q-ion for sample injections was ± 30% from the S-ion/Q-ion of the standard isoprene injections. Collectively, these data suggest that the peak in question is in fact isoprene and background or interfering ions are not responsible for the observed increase in isoprene on the Tenax TA tubes. Another probable cause may be breakthrough of isoprene with 5TD tubes. However, preliminary experimentation of breakthrough on 5TD tubes, using isoprene standards and methods previously established, shows little or no isoprene found on the second tube (data not shown) [39]. Next, it is speculated that the difference in the observed isoprene retention may be attributed to a pump initiation delay caused by resistance, i.e. backpressure, of the carbograph particles. As Tenax is much larger in size, flow can initiate easier with each pump activation. A delay in pump activation, caused by backpressure, repeated over approximately 75 exhalations could account for the differences observed. However, this hypothesis is only minimally supported by the

breathing rate data **(Figure 2E**) and off-line backpressure experiments (data not shown). Finally, data were recently presented suggesting Tenax/Carbograph 5TD tubes retain a greater amount of water than Tenax adsorbent tubes [54]. Therefore, it is hypothesized, due to the high solubility of isoprene in water, that the isoprene is removed from the 5TD tubes during the dry purge of the thermal desorption cycle, with the water [54]. As Tenax TD tubes retain less water, less isoprene would be removed during the dry purge, yielding the observed higher isoprene values compared to the 5TD tubes, even though Tenax has illustrated potential breakthrough of isoprene [54]. Additionally, it is also plausible that humidity accumulation within the 5TD tubes blocks binding sites within the adsorbent, occluding isoprene from binding and ultimately being retained [54]. However, as these data have yet to be peer reviewed, additional experimentation may be required to confirm this hypothesis. Regardless of the cause for the differences between the two adsorbent tube types, the intent of the experiments was to illustrate that sampling can be performed with two different TD tube types within the ReCIVA sampler, one in each ReCIVA bank. However, the resulting exhaled breath data are only comparable among the same TD tube types.

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

5.35 min, 67.1 m/z) and acetone (5.57 min, 43.1 m/z, **Supplemental Data 9**). While propene has been identified in breath previously and methoxy-phenyl-oxime was included in the analysis as it was an unknown, it is hypothesized this is a result of greater background from the 5TD tubes themselves even though both TD tube types were reconditioned as recommended by the manufacturer prior to each test [34]. Therefore, these data indicate careful consideration must be utilized when selecting TD tube adsorbent materials and adequate control samples must be utilized for exhaled breath collection while using the ReCIVA device.

It was hypothesized, due to the active pumping of the ReCIVA for exhaled breath sampling, that frequency of pumping (high number of exhalations) and length of pumping (length and depth of exhalations) would affect the performance of the device. As a result, guided breathing rate experiments were performed. The data illustrate that similar results are obtained independent of the breathing rate (**Figure 2**). However, substantial isoprene variability was observed in the low breathing rate test from ReCIVA #33 (**Figure 2A**). As the Mean Max CO_2 (an estimate of breath depth) for this test is also substantially variable, the data would suggest that consistent depth of breathing would lead to more consistent results as observed with the other three tests (**Supplemental Data 10, Figure 2A**). Therefore, to truly obtain the most consistent data, it is speculated that guided breathing, at a comfortable rate, should be applied for all exhaled breath collections using the ReCIVA device.

Data were presented demonstrating a participant (#28), sampled during Tests 2 and Test 4, had background levels of exhaled isoprene while showing acetone levels similar to the remaining participants (**Figure 3**). Spanel et. al. first reported a similar case of extremely low isoprene using SIFT-MS detection [46]. However, they were unable to determine a cause as blood cholesterol values of the subject in the Spanel et. al. study were similar to controls [46]. No additional cases have been reported in the literature. However, unpublished evidence of similar cases, with little or no isoprene, have been presented at exhaled breath conferences. Preliminary data suggest genetics may play a role in this phenomenon. While it would be extremely interesting to further explore the genetic hypothesis with Participant #28 from this study, due to ethical restraints, further experimentation is not possible at this time. Although the data presented here represent only a single participant, it is important to highlight such cases to determine not only the overall prevalence of the phenomenon but also, ultimately, the underlying cause.

CONCLUSIONS

As the ReCIVA becomes a more widely used tool, investigations into parameters affecting functionality and data variability must be explored. The data presented here support the use and comparison of results from two ReCIVA devices. Additionally, the results highlight that the ReCIVA performs consistently, independent of breathing rate while significant differences were noted between TD tubes of different types. Collectively, the results support the combination of individual TD tube flow rate calibration within the ReCIVA and guided breathing during exhaled breath collections to yield the most

consistent data. Overall, the results provide evidence that the ReCIVA sampler may be used among multiple sampling sites for exhaled breath collection.

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Conflicts of Interest

The authors have no conflicts of interest to report

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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

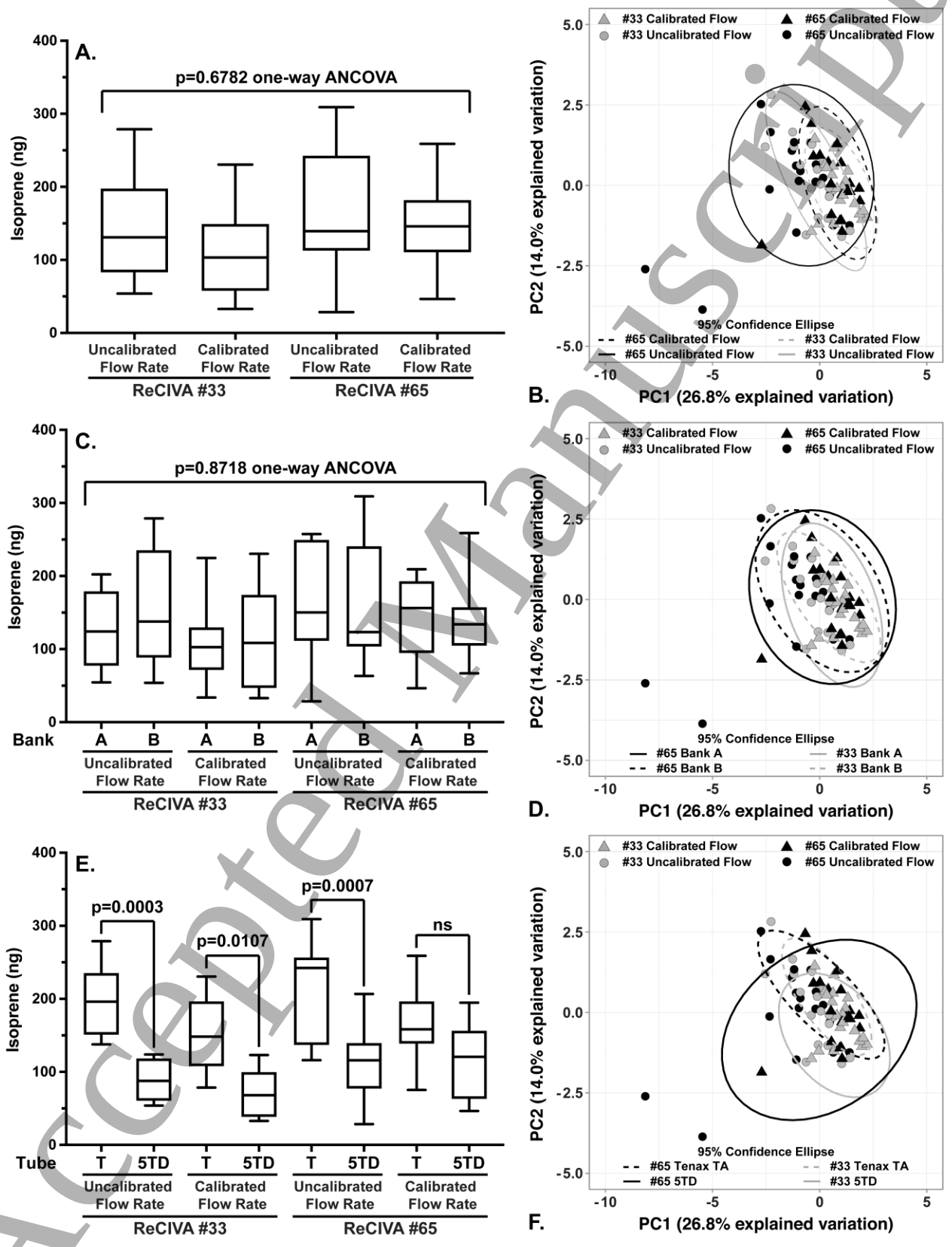

FIGURE 2

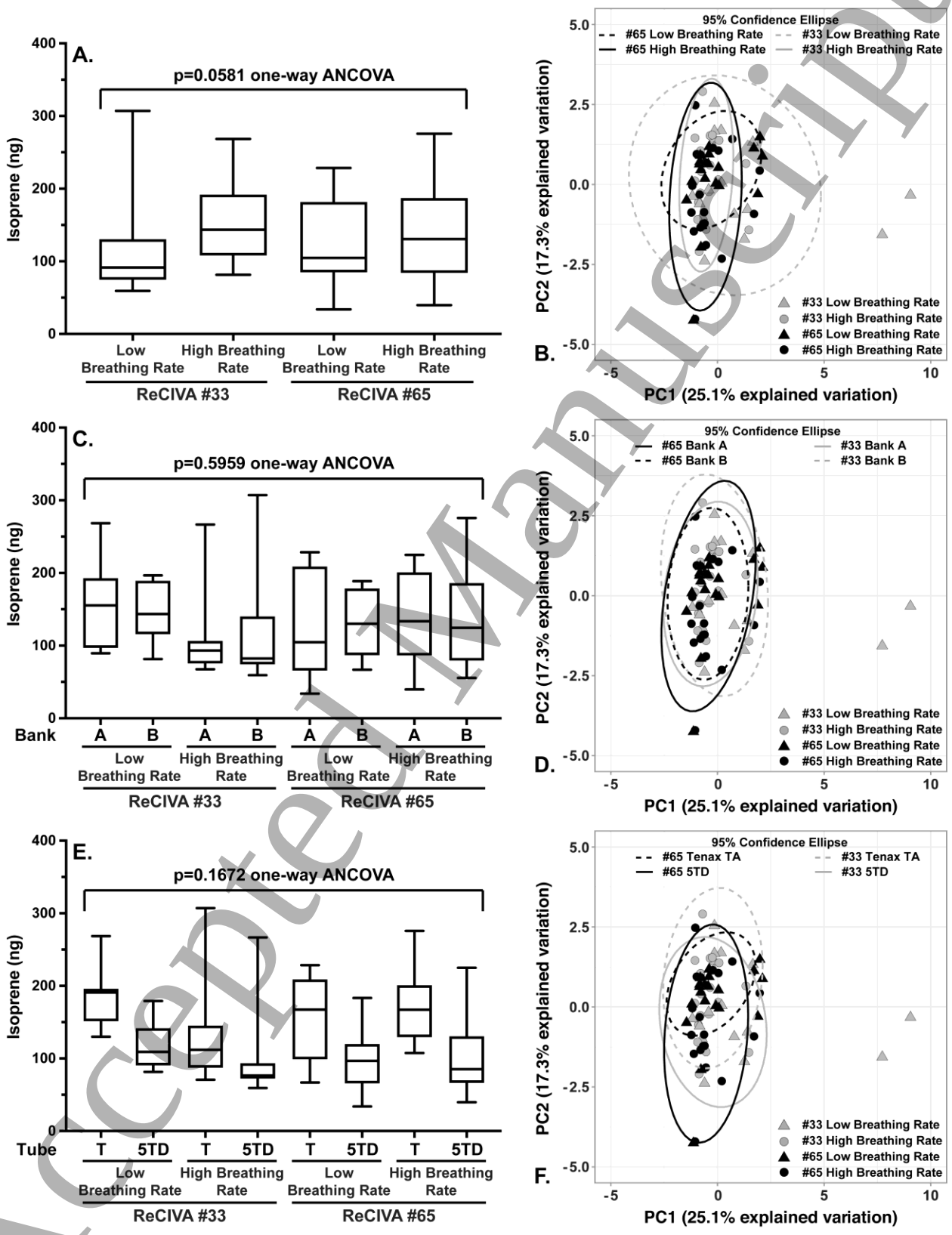

FIGURE 3