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Characterization of Standardized Breath Sampling for Off-Line Field Use

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ABSTRACT

Due to several sources of potential variability associated with exhaled breath bag sampling procedures for off-line analysis, the Respiration Collector for In Vitro Analysis (ReCIVA) sampler was developed. Although designed to improve upon several pitfalls of sampling with exhaled breath bags, the ReCIVA remains a minimally studied research tool. In this manuscript, several attributes of the ReCIVA sampler are investigated among three individual tests, such as background contamination, control software version, performance of different adsorbent tubes, duplicate sample production, and comparison to exhaled breath bags. The data shows greater than a 58% reduction in background siloxanes can be achieved with submersion of ReCIVA masks in ethyl alcohol or baking the masks at a high temperature (200°C). The results illustrate the ReCIVA control software version plays a key role in the flow rates applied to thermal desorption (TD) tubes. Using exhaled isoprene as a representative analyte, the data suggest duplicate samples among ReCIVA pump banks can be achieved using two different thermal desorption tubes, Tenax TA and Tenax/Carbograph 5TD, when using an updated control software and manually calibrating the ReCIVA pumps to uniform flow rates (Tenax $p=0.3869$, 5TD $p=0.3131$). Additionally, using the updated control software and manual ReCIVA flow calibration, the data suggest the ReCIVA can produce statistically similar results among TD tube types ($p=0.3824$) and compared to standard exhaled breath bags ($p=0.1534$). Collectively, these results establish a method for manually calibrating the flow of the ReCIVA device to allow for the most consistent results. These data support further experimentation into the use of the ReCIVA sampler for exhaled breath research.

INTRODUCTION

Analysis of exhaled breath is traditionally performed via two mechanisms. On-line analysis via Proton-Transfer-Reaction Mass Spectrometry (PTR-MS) or Selected Ion Flow Tube Mass Spectrometry (SIFT-MS) which provides a real-time volatiles analysis without preconcentration. However, these methods are difficult for field or clinical applications as they require specialized equipment at the collection site. Conversely, off-line analysis, collection of exhaled breath in a container, such as a bag, and subsequent transfer of volatiles to an adsorbent tube for concentration and storage is more amendable to field or off-site sample collection with later transfer to the analytical lab.

Exhaled breath bags, such as ALTEF or Tedlar, continue to dominate the off-line exhaled breath research field. However, bags represent several sources of variability [1-7]. For example, exhaled breath bags rely on a subject's adherence to an exhalation protocol for collection of end-tidal breath [8]. Furthermore, breath bags have been shown to be "leaky" and require an additional pump to transfer volatiles from a bag to an adsorbent tube [7]. As a result, exhaled breath bags can provide irregular exhaled breath samples if care is not taken. To mitigate sources of variability observed with bag exhaled breath samples, Respiration Collector for In Vitro Analysis (ReCIVA) was developed for off-line exhaled breath collection.

The ReCIVA was designed for consistent exhaled breath collection directly onto thermal desorption (TD) tubes for later off-line gas chromatography-mass spectrometry (GG-MS)

analysis. Using a silicone facemask, exhaled CO_2 is monitored in real-time for sample pump activation and dynamic exhaled breath sampling. Real-time monitoring allows for versatile sampling of different portions of exhaled breath, such as lower-airways, upper-airways, whole breath, etc. Furthermore, two separate pump banks within the ReCIVA allow for capture of up to four redundant airway samples, e.g. all lower, different airway, or lower and upper airway, samples. The features associated with the ReCIVA sampler are intended to mitigate the variability associated with exhaled breath samples while allowing a researcher optimum versatility.

Although the ReCIVA represents a significant step toward consistent exhaled breath sampling, the monetary requirement, as compared to exhaled breath bags, for the ReCIVA is large. For instance, there is a significant investment in the unit itself and in the associated consumables (single use masks) presumably keeping it from widespread use in many laboratories. As a result, many questions surrounding the ReCIVA sampler remain.

The initial work utilizing the ReCIVA, by Doran et al., has provided evidence for recommended sampling parameters such as breath fraction, sample volume and collection flow rate, and the observation of potential background contaminants [9]. This study provides a starting point for further investigations surrounding the ReCIVA sampler for research use, such as reduction of background, performance of different adsorbent

tubes, reuse of ReCIVA masks, comparison of results to exhaled breath bags and the ability to generate duplicate tubes among ReCIVA banks.

In this manuscript, several of the additional questions surrounding the ReCIVA will be addressed. Using exhaled isoprene (2-methyl-1,3-butadiene) as a representative breath analyte, Tenax TA and Tenax/Carbograph 5TD (5TD) tubes were evaluated for consistent sampling using the ReCIVA device and exhaled breath bags. The data presented here support additional research into the use of the ReCIVA sampler for off-line exhaled breath studies.

EXPERIMENTAL

PARTICIPANTS

All of the volunteer participants (n=12 per experiment, 19 total participants) for the experiments were healthy, male, non-smokers within our research facility. While ideal to use the exact same 12 participants for all of the experiments, due to limited availability 19 total participants had to be used. As the goal of this research was to evaluate sampling media platforms, not relate volatile profiles to physiological parameters, the United States Air Force Research Laboratory's Institutional Review Board deemed this research Non-Human Use (FWR20170161N). Therefore, participants were informed of the procedure and free to quit at any time, but written consent was not required.

All volunteers had refrained from food or drink, except for water, for greater than one hour prior to taking part in the experiments. Before entering the laboratory, all participants rinsed their mouths briefly with filtered water (Brita, Oakland, CA, USA). The volunteers sat upright in a relaxed position for more than five minutes prior to exhaled breath collection. The participants remained in the relaxed upright sitting position for all breath collections [10].

THERMAL DESORPTION TUBES

All samples were collected on preconditioned stainless steel Tenax TA (35/60 mesh) or Tenax/Carbograph 5TD thermal desorption tubes (Markes International, South Wales, UK). Preconditioning was performed on a Markes International TC-20 with 85 mL min^{-1} (99.999%) nitrogen flow at 320ºC for 1 hour, as recommended by the manufacturer. All tubes were capped with brass caps fitted with a polytetrafluoroethylene ferrule and stored at ambient temperature until use.

EXPERIMENTAL DESIGN & EXHALED BREATH COLLECTION: TEST 1

Test 1 was performed using the ReCIVA control software v. 1.30 and brand new ReCIVA masks (masks manufactured June 2017, Owlstone Medical, Cambridge, UK). Please refer to **Supplemental Data 1** for a summary of the experimental design. Twelve participants were randomly assigned an adsorbent tube type, 6 participants Tenax TA and 6 participants 5TD, for exhaled breath collection. For participants assigned to the

Tenax TA adsorbent tube group, exhaled breath was collected by two separate mechanisms, exhaled breath bags and the ReCIVA sampler, while those assigned to the 5TD tube group only performed a ReCIVA device collection. For the Tenax TA group, immediately prior to and following use of the ReCIVA device, 1L ALTEF breath bags, affixed with a mouth piece, were filled with end-tidal breath using our established exhalation protocol [11-13]. Briefly, volunteers were instructed to take a breath, exhale to the point where they would normally take another breath and their abdomen was tight (lung tidal volume) and fill the bag with the remaining breath in the lungs (functional residual capacity). Exhaled breath (550 mL) was immediately adsorbed onto thermal desorption tubes at 200 mL min^{-1}, the same flow rate used on the ReCIVA device, using a Gilian GilAir Plus pump (Sensidyne LP, St. Petersburg, FL, USA). The flow rate pulled by the GilAIR pump was verified prior to transferring each volunteer's breath from the bag using an unanalyzed Tenax TA TD tube and a DryCal Bios Defender 510 flow rate monitor, as historically the pump drifts from set point over time, (Mesa Labs, Lakewood, CO, USA). Please refer to **Supplemental Data 2** for a figure of the setup for verifying the GilAir Plus' flow rate, the measured flow rates of the GilAir pump, and the calculated sampling time for transferring 550 mL of exhaled breath on to the adsorbent tube via the GilAir pump. The percent CO_2 for each breath bag was determined via a MultiRAE Lite pump and percent exhaled CO_2 measured from each exhaled breath bag is provided in **Supplemental Data 2B** (RAE Systems, San Jose, CA, USA).

Between breath bags, duplicate breath samples, one on ReCIVA Bank A and one on ReCIVA Bank B, were collected using a brand new ReCIVA device (Owlstone Medical).

The ReCIVA was set up for two tube collection by placing two clean 3.5" x 0.25" solid stainless-steel rods in the positions proximal to the participant's mouth and thermal desorption tubes in the distal positions, as recommended by the manufacturer. Please refer to **Supplemental Data 3A** for a diagram of the sample collection setup. Medical grade breathing air was supplied to the mask at 40 L min^{-1} using an Alicat Scientific MCP-100 SLPM mass flow controller and manually monitored throughout the experiment using the FlowVision SC software v. 1.3.28.0 (AirGas, Radnor, PA, USA, Alicat Scientific, Tuscon AZ, USA). Please refer to **Supplemental Data 3B** for a figure of the overall ReCIVA setup. The mask was affixed to the participant using the head straps supplied with the ReCIVA and adjusted until comfortable. The ReCIVA control software (v. 1.30, Owlstone Medical) was set up to collect 550 mL of lower airway breath onto two TD tubes at 200 mL min^{-1} each. Remaining ReCIVA settings were as recommended by the device manufacturer and are provided in **Supplemental Data 4A**. Participants were instructed prior to ReCIVA exhaled breath collections to breathe through their mouth only, taking normal slow breaths. Participants randomly assigned to the 5TD adsorbent tube group only participated in the ReCIVA exhaled breath collections, as described above, using duplicate 5TD tubes, one tube in each bank positioned distal to the mouth, in the ReCIVA device (**Supplemental Data 3A**). Please refer to **Supplemental Data 5** for a summary of the participant groupings and the number of exhaled breath samples acquired from each participant. All TD tubes were stored at 4 °C until analysis and run on the TD-GC-MS instrumentation on the same day [12].

All ReCIVA masks used during Test 1 exhaled breath collections were retained (Day 0) for background siloxane contamination removal with ethyl alcohol, as described below. Mask siloxane background and residual ethanol was evaluated using a glass head on Day 4, as described in a following section. Please refer to **Supplemental Data 1** for a summary of the experimental design of Test 1.

CONTROL AND BACKGROUND SAMPLE COLLECTION

Room blank control samples were collected by passing 550 mL of laboratory room air through TD tubes with a GilAir Plus pump (200 mL min^{-1}, **Supplemental Data 2C**). Background samples were collected from the medical grade breathing air by filling 1L ALTEF bags with the air and loading, via GilAir pump, 550 mL onto TD tubes as described above (**Supplemental Data 2C**). ReCIVA mask background samples were collected using a brand-new mask affixed securely to a clean glass head supplied with 40 L min^{-1} of medical grade air. See **Supplemental Data 4B** for a figure illustrating the mask background collection setup. Air within the mask (550 mL) was collected from Bank A using “Always On” feature of the ReCIVA software, as described by Doran et al. (MVAP Medical Supplies, Newbury Park, CA, USA, **Supplemental Data 4C**) [9]. All control and background thermal desorption tubes were analyzed by TD-GC-MS on the same day as collection [12].

ReCIVA Flow Rate Measurements

The flow rate for each adsorbent tube type (Tenax TA and 5TD) and bank (A & B distal to the mouth) was measured individually by placing clean solid stainless-steel rods in three of the four sampling ports of the ReCIVA (two proximal and one distal to the glass head). In the remaining port, a clean reconditioned adsorbent tube, either Tenax TA or 5TD, was added and flow was monitored from the open end of the TD tube connected to a DryCal Defender through a hole cut in a ReCIVA mask. Please refer to **Supplemental Data 4D** for a figure depicting the setup to measure the ReCIVA flow rate. Each bank was tested individually (n=5 per bank per TD tube type) at 200 mL min^{-1} using the "Always On" ReCIVA settings provided in **Supplemental Data 4C** over a collection of 1420 mL (approximately 100 measurements). The measured flow rates were collected continuously using the DryCal Pro Software (v. 1.3, Mesa Labs).

Experimental Design & Exhaled Breath Collection: Test 2

Test 2 was performed exactly as described above for Test 1. However, Test 2 was acquired with updated ReCIVA control software (v. 1.46) and brand new ReCIVA masks (masks manufactured March 2018, Owlstone Medical). Please refer to **Supplemental Data 1** for a summary of the experimental design of Test 2. All masks used during ReCIVA exhaled breath collections for Test 2 were retained for background siloxane contamination removal by baking, as described below. Masks were evaluated for siloxane removal using a glass head as described previously for ReCIVA mask background samples. **Supplemental Data 5** provides a summary of the groupings and the samples

acquired from each participant from Test 2. All thermal desorption tubes were run on the TD-GC-MS instrumentation on the same day as collection [12].

Background Contaminant Removal and Cleaning Evaluation

Removal of background siloxane contamination was performed by two separate methods. First, masks retained from Test 1 using the ReCIVA device (n=12) were immediately subjected to the ethanol wash cleaning procedure (**Supplemental Data 6**). Briefly, masks and filters were completely submerged twice (60 minutes and 45 minutes) in 100% ethyl alcohol, changing the solvent for each wash (Decon Laboratories, Prussia, PA, USA) [14,15]. Bulk solvent was removed with house compressed air. The remaining solvent was removed by placing under vacuum for approximately 3 days (Labconco, Kansas City, MO, USA). Masks were re-evaluated for removal of background siloxanes and residual solvent, as described above, for the ReCIVA mask background samples 4 days following the initial collection (**Supplemental Data 1, 4B, & 4C**). Cleaning evaluation samples were analyzed by TD-GC-MS, as described in a following section, on the same day as collection [12].

Second, masks retained from Test 2 using the ReCIVA device (n=12) were subjected to the mask bake protocol (**Supplemental Data 6**). Filters were removed from the retained masks following ReCIVA exhaled breath collections and the silicon portion of masks were baked at 200 °C for approximately 68 hours. Masks were cooled to room temperature and the filters were reinserted. The reassembled masks were evaluated for removal of

siloxane background contamination, as described above for ReCIVA mask background samples (**Supplemental Data 1, 4B, & 4C**). TD tubes were placed at 4 °C until TD-GC-MS analysis was initiated on the same day as collection.

ReCIVA Manual Flow Rate Calibration

The ReCIVA flow rates were calibrated for each TD tube type and ReCIVA bank by adjusting the flow rate within the ReCIVA software (v. 1.46) until 200 mL min^{-1} was measured on the DryCal Defender. The calibrated flow rates were verified (n=5 per ReCIVA bank per TD tube type) over 1420 mL collections (approximately 100 individual measurements) as described above for ReCIVA flow rate measurements. For exhaled breath collections using the calibrated flow rates, an adjustment to the overall collection volume per tube must be made to obtain 550 mL volume on each TD tube. The calculation and adjusted volumes are shown in **Supplemental Data 7**.

Experimental Design & Exhaled Breath Collection: Test 3

Test 3 was performed as described above for Test 1 and Test 2. However, Test 3 was performed using the determined calibrated flow rates, for each TD tube type and ReCIVA bank, with reduced collection volumes as shown in **Supplemental Data 7**, ReCIVA control software v. 1.46, and new ReCIVA masks (masks manufactured March 2018, Owlstone Medical). Please refer to **Supplemental Data 1** for a summary of the experimental design of Test 3 and **Supplemental Data 4E & 4F** for the specific Test 3

ReCIVA settings. All ReCIVA masks used were baked as described above to remove siloxane contamination prior to exhaled breath collection. Participant groupings and the exhaled breath samples collected from each participant are provided in **Supplemental Data 5**. All breath and control samples were run on TD-GC-MS instrumentation as described below on the day of collection [12].

TD-GC-MS Analysis

All thermal desorption was carried out on a Markes International TD-100xr affixed to a Trace Ultra-ISQ GC-MS system (Thermo Scientific, Waltham, MA, USA). TD tubes were purged with nitrogen (99.999%) for 1 minute at 20 mL min^{-1} prior to initial desorption performed at 310°C for 10 minutes onto a Markes International Air Toxics cold trap. Trap desorption was carried out at 315°C with a 40°C s^{-1} heating rate for 5 minutes. Trap was purged for 1 minute at 50 mL min^{-1} with a flow path temperature of 180°C. The trap outlet split was 5 mL min^{-1} (3.64:1 overall split ratio). 25 ppm of 1,4-diflurobenzene internal standard was added to each thermal desorption tube prior to initial desorption by the TD-100xr (Restek, Bellefonte, PA, USA). Chromatographic separations were performed at a constant 2 mL min^{-1} helium flow (99.999%) on an Rxi-624Sil 60 m x 0.32 mmID x1.80 µm df GC column (Restek). The GC gradient was carried out with a 40 °C hold for one minute then a linear increase at 10 °C min^{-1} to 240 °C over 20 minutes. Temperature was held at 240 °C for an additional 20 minutes. Ions were generated via 70 eV electron impact ionization at a temperature of 275 °C. Single quadrapole detection was carried out over 35-300 m/z range with 0.154 scans s^{-1}. All data was acquired in a random order with

Tracefinder EFS software (v. 3.2, Thermo Scientific). Unknown siloxanes were tentatively identified by comparison of spectra to the NIST 11 Mass Spectral Library (v. 2.0, National Institute of Standards and Technology, Gaithersburg, MD). GC-MS data was visualized and manually inspected using the XCalibur software package (v. 3.0.63, Thermo Scientific).

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

Calibration curves were generated for isoprene for each test experiment from a custom 0.99 ppm isoprene compressed gaseous cylinder for Tests 1 & 2 and 1.10 ppm for Test 3 (Linde Gas North America LLC, Alpha, NJ, USA, Scott Medical Products, Plumsteadville, PA, USA). Briefly, the curve for Test 1 ranged from 15.314 to 918.824 ng isoprene for Tenax TA and 15.314 to 459.412 ng for 5TD tubes. For Test 2, the calibration curve ranged from 0 ng to 918.824 ng isoprene for Tenax TA and 0 ng to 1.071 µg for 5TD tubes. For Test 3, the calibration spanned 0 ng to 1.071 µg for Tenax TA and 0 ng to 918.824 ng for 5TD tubes. Calibration curves were adjusted to encompass both the linear range and the isoprene values based on preliminary testing. Each standard was individually spiked onto separate thermal desorption tubes using a gas-tight syringe (Hamilton, Reno, NV, USA) and a Markes International Standard Loading Rig supplemented with 60 mL^{-1} nitrogen (99.999%) flow [16,17]. Tubes were run as described above. The peak areas for isoprene (Q-ion m/z 67) and 1,4-difluorobenzene (internal standard, Q-ion m/z 114) were tabulated using the Tracefineder EFS software. An internal standard normalized response ratio (isoprene area/internal standard area) was calculated

and plotted against theoretical isoprene concentration. Please refer to **Supplemental Data 8** for a summary of the calibration curves from each test calibration. The amount of isoprene on each sample tube was calculated by inputting the unknown isoprene internal standard normalized abundance into the linear fit line equation for each calibration curve. Instrument performance was evaluated with standard prior to each GC-MS analysis based on % difference to calibration as outlined in the EPA TO-15/17 method [18,19].

Evaluation of Adsorbent Tube Purge Time and Humidity Effects

The adsorbent tube purge time was evaluated by two separate methods. First, ten tubes of each adsorbent type (Tenax TA or 5TD) were spiked with 20 mL of isoprene standard (61.25 ng), as described above for calibration curve generation. The TD tubes were subjected to either 15 minutes (n=5) or 1-minute (n=5) of nitrogen purge at 20 mL min^{-1}, on the TD-100xr, prior to primary desorption followed by TD-GC-MS analysis as described above. Second, six participants performed a ReCIVA collection of 550 mL of lower airway breath at 200 mL min^{-1} (unadjusted flow rate) twice in a single day as described for Test 2. Each collection was carried out with either Tenax TA or 5TD tubes placed in both banks, A and B, in the positions distal to the mouth as described previously and depicted in **Supplemental Data 3A**. Each set of tubes was run with either 1-minute nitrogen purge (bank B samples) or 15-minute nitrogen purge (bank A samples) at 20 mL min^{-1} prior to primary desorption in the thermal desorption system. TD-GC-MS analysis followed as described above. The peak areas, retention times, and the full width at half

max (FWHM) of isoprene and 1,4-difluorobenzene peaks were tabulated using the Tracefinder EFS and XCalibur software packages.

Relative Quantitation

Relative quantitation among contaminants was calculated by comparing internal standard normalized contaminant areas (internal standard: 1,4-difluorobenzene, m/z 114, ethyl alcohol (m/z 46), siloxane contaminants: RT 7.13 m/z 75, RT 8.12 m/z 147, RT 10.82 m/z 207, RT 11.91 m/z 221, RT 13.89 m/z 281, RT 15.17 m/z 207, and RT 16.65 m/z 73). All peak areas were determined using the Tracefinder EFS software package.

Statistical Analysis

Basic statistical analysis was performed using the Prism GraphPad Software package (v. 8.1.1(224), Graphpad Software Inc., LaJolla, CA, USA). Linear mixed modeling was conducted in the R software suite (v. 3.4) utilizing the LME package [20]. Linear mixed modeling was utilized to account for correlation among repeated individuals.

RESULTS

Siloxane Contamination via ReCIVA Collection Apparatus: Test 1 & 2

Although the goal of this study was to characterize the attributes of the ReCIVA breath sampler as it applies to exhaled breath results, manual inspection of the raw data, from ReCIVA Test 1 exhaled breath collections (ReCIVA control software v. 1.30, masks

manufactured June 2017) on both adsorbent tube types, found significantly greater areas ($p<0.0001$), except for the contamination at RT 16.65 on 5TD tubes $p=0.7085$, of background siloxanes in the samples collected on the ReCIVA device compared to control samples (**Figure 1A & 1B**). Similarly, ReCIVA Test 2 exhaled breath samples collected with ReCIVA software v. 1.46 on ReCIVA masks manufactured March 2018 contained significantly greater peak areas of contamination at RT 7.13 and RT 8.12 compared to the control samples on both TD tube types tested ($p<0.0001$, **Figure 1C & 1D**). As these were the two main siloxane peaks detected in the masks from Test 2, p-values were only generated for the contamination at RT 7.13 and RT 8.12. These data suggest a reduction in the overall number of siloxanes background peaks as the mask manufacturing process becomes refined over time. However, siloxane background is still present above control sample levels.

To attempt to mitigate the observed siloxane contamination, masks were retained following each participant's exhaled breath collection via the ReCIVA device and cleaned by two distinct methods, ethyl alcohol wash (Test 1 masks) and baking (Test 2 masks) as outlined in **Supplemental Data 6**. The evaluation of the background following each cleaning protocol was performed using a glass head as shown in **Supplemental Data 4B**. **Figure 1** illustrates cleaning can reduce background siloxane peak area 78.4% to 99.9% by ethanol wash and 58.8% to 100.0% by baking (%=1-(Cleaning Evaluation Normalized Area/Test Exhaled Breath Normalized Area). While a significant reduction in the siloxane background was obtained by the ethyl alcohol wash protocol, a greater than 85% increase, compared to exhaled breath samples, in residual ethanol persisted in the

cleaning evaluation samples suggesting additional measures must be considered to utilize this method for cleaning (**Supplemental Data 9**). Collectively, these results suggest cleaning protocols can reduce the siloxane contamination derived from the ReCIVA masks. However, cleaning the masks by baking is preferred to ethyl alcohol washing due to the persistence of ethanol following the cleaning protocol.

COMPARISON OF EXHALED ISOPRENE VIA RECIVA BANKS

A benefit of the ReCIVA sampler is the ability to collect multiple samples at the same time from a single participant. To test if duplicate lower airway samples can be acquired across ReCIVA pump banks (A & B) distal to the participants mouth, isoprene was quantitated from all ReCIVA samples across both adsorbent tube types tested, Tenax TA and 5TD, and the three separate test collections. **Figure 2A** shows a box-and-whisker plot of the quantitated isoprene values from each test. The data show statistically similar isoprene quantities are present, bank A compared to bank B, from Test 1 collected with ReCIVA control software v. 1.30 (**Figure 2A Left**). However, a statistically significant difference was found between the TD tube types tested, Tenax compared to 5TD tubes, using the v. 1.30 ReCIVA control software. It was hypothesized that the cause of the observed difference between TD tube types found during Test 1 may be a result of incorrect flow rates applied to the TD tubes by the ReCIVA device software (v. 1.30). To test this hypothesis, the flow rates were determined over approximately 100 measurements 1420 mL). **Figure 2B Left** illustrates that the overall mean flow rate, independent of the TD tube type, applied by the ReCIVA device during Test 1 (software v.1.30) has a statistically

lower rate ($p<0.0001$) than the set point of 200 mL min^{-1}. Furthermore, there is a statistical difference between the measured mean flow rates between banks A and B, with bank B providing a higher flow rate than bank A regardless of the TD tube type tested during Test 1 (**Figure 2B Left**). The difference in flow rates among banks is further highlighted during Test 1, on Tenax TA TD tubes specifically, by plotting the delta isoprene values among banks (bank B-A). **Figure 2C Left** illustrates there is a significant difference in the isoprene detected among banks (delta absolute value) with more isoprene detected on tubes loaded by bank B versus bank A (raw delta) for Tenax tubes. Whereas 5TD tubes do not show the same trend by linear mixed modeling. Taken together, these results demonstrate that deviations in flow rate provided by the ReCIVA device using control software v. 1.30, may account for the variability of measured isoprene during Test 1.

During examination of the Test 1 data, the ReCIVA manufacturer, Owlstone Medical, was contacted about the results. It was proposed that a software update, from v. 1.30 to v. 1.46, would alleviate the flow rate differences observed. To test the updated software, a second test, Test 2, was performed with ReCIVA software v. 1.46. **Figure 2B Center** shows the flow rate with the updated software is significantly different from the 200 mL min^{-1} set point for both banks with Tenax TA tubes (bank A: $p=0.0107$, bank B: $p=0.0002$) while only bank A with 5TD tubes is significantly different from the set point (bank A: $p=0.0148$, bank B: $p=0.0754$). Additionally, bank B, with ReCIVA software v. 1.46, had a significantly higher measured flow rate than that found on bank A independent of TD tube type tested, similar to the results observed during Test 1 (**Figure 2B Left & Center**). The quantitated isoprene values from exhaled breath from Test 2 show Tenax TA TD tubes

have a significant difference in measured isoprene between ReCIVA banks (p=0.0091) while the 5TD do not (p=0.8015, **Figure 2A Center**). The data suggest bias for greater isoprene on bank B, shown by calculated delta isoprene values bank B-A, may be evident on the 5TD tubes (p=0.0026, **Figure 2C Center**). A comparison between the two TD tube types from Test 2 show an insignificant difference (p=0.2321) in the overall measured isoprene values (**Figure 2A Center**). Collectively, these data illustrate the update in ReCIVA control software, from v. 1.30 to v. 1.46, improved the consistency between the two different TD tube types. However, the measured flow rates remain statistically inconsistent with the set point. Additionally, the quantitated exhaled isoprene values do not provide similar results among ReCIVA banks for Tenax TA TD tubes.

While the updated ReCIVA control software (v. 1.46) improved several attributes in flow rate and isoprene quantities, variability and flow rate differences among ReCIVA banks persisted. As flow rate appeared to play a role in the observed differences among banks, it was hypothesized that manual calibration of the flow rate, by adjusting the flow rate within the settings of the ReCIVA control software (v. 1.46) to a measured flow rate of 200 mL min^{-1}, would alleviate the differences among both the TD tube types and ReCIVA banks. To test this hypothesis, the flow rates within the ReCIVA control software (v. 1.46) were manually adjusted to a measured flow rate of 200 mL min^{-1} resulting in calibrated flow rates for each tube type and bank (Tenax bank A: 180 mL min^{-1}, Tenax bank B: 182 mL min^{-1}, 5TD bank A: 197 mL min^{-1}, and 5TD bank B: 200 mL min^{-1}, **Figure 2B Right**). Exhaled breath collected using these ReCIVA pump flow rates (Test 3) and corresponding reduced collection volumes (Tenax A: 495 mL, Tenax B: 501 mL, 5TD A:

542 mL, and 5TD B: 550 mL, **Supplemental Data 7**), resulted in statistically similar isoprene quantities between both ReCIVA banks independent of TD tube type (Tenax: p=0.3869, 5TD: p=0.3131, **Figure 2A Right**) with no significant delta isoprene values between banks (p>0.05, **Figure 2C Right**). Furthermore, there was not a significant difference in the measured exhaled isoprene between the two different adsorbent tubes tested (p=0.3824, **Figure 2A Right**). Overall, the data presented in **Figure 2** illustrate the ReCIVA control software version plays a significant role in exhaled breath collection flow rates. Additionally, updated software with manual calibration of the ReCIVA flow rates for each ReCIVA bank and TD tube type allows for the most consistent results from the ReCIVA device.

Comparison of Isoprene via ReCIVA and ALTEF Bag Samples

Exhaled breath for off-line analysis has most commonly been collected in bags, such as Tedlar, ALTEF, etc., for research purposes. To determine if the ReCIVA produces similar quantities of isoprene to this standard collection method, exhaled breath samples were collected via ALTEF polypropylene bags prior to and following the ReCIVA collections for each test using Tenax TA TD tubes. **Figure 3 Left and Center** show a significant decrease in the isoprene quantities from the ReCIVA sampler compared to the matched exhaled breath bags from Test 1 (ReCIVA Software v. 1.30) and Test 2 (ReCIVA Software v. 1.46). However, the data collected using the calibrated flow rates on the ReCIVA (Test 3 ReCIVA software v. 1.46) are not statistically significant from the ALTEF bag samples (p=0.1534, **Figure 3 Right**). These data further highlight the improvement in the data

collected via the ReCIVA device with calibrated flow rates. Furthermore, these results suggest samples collected by a ReCIVA device can give comparable quantities of isoprene as exhaled breath bags.

Evaluation of TD Tube Purge Time on Exhaled Breath Results

While the Tenax/Carbograph 5TD tubes may give wider compound coverage (C_4-C_{30}) compared to other adsorbent tubes, such as Tenax TA (C_6-C_{30}), the carbograph adsorbent contained in the 5TD tubes may cause effects corresponding to water or humidity acquired during sampling. A common method to mitigate water effects on TD adsorbent material is an extended dry purging, prior to primary desorption, with high purity gas, such as nitrogen or helium, for an extended period of time. To provide preliminary results surrounding the use of extended purge time to potentially reduce humidity effects, two experiments were performed.

First, isoprene standard was spiked onto each TD tube type, Tenax and 5TD, and subjected to dry purges of 1 minute or 15 minutes, at 20 mL min^{-1} prior to primary desorption. The data shows, for both TD tube types, a statistically significant reduction in isoprene (5TD $p=0.0079$, Tenax $p<0.0001$) and the 1,4-difluorobenzene internal standard (5TD $p=0.0079$, Tenax $p<0.0001$) raw peak areas with the 15-minute purge time (**Supplemental Data 10A**). Similarly, the second experiment, utilizing exhaled breath collected on the ReCIVA device with Tenax and 5TD tubes run with either 1 minute or 15-minute dry purge times, shows a significant reduction in both isoprene (5TD $p=0.0062$,

Tenax p<0.0029) and 1,4-difluorobenzene (5TD p<0.0001, Tenax p=0.0002) raw peak areas (**Supplemental Data 5 & 10B**). Collectively, these results suggest the extended dry purge time of 15 minutes may cause breakthrough of volatiles from the TD tube.

Evidence of water or humidity in gas chromatographic separations can appear as peak broadening and retention time shifts of compounds within the raw data [21]. To determine if evidence of water or humidity effects were present in the exhaled breath samples acquired to evaluate purge time, the full width at half max (FWHM) and peak retention times for isoprene were determined for each sample. The results show that the 1-minute dry purge significantly reduces the FWHM on both tube types (5TD p<0.0001, Tenax p=0.0126, **Supplemental Data 10C**) while no significant change is observed in retention times of isoprene among the purge times for each TD tube type (5TD p=0.1017, Tenax p=0.3409, **Supplemental Data 10D**). Furthermore, the retention times of isoprene among the injections showed an overall ≤0.4% RSD for any set of parameters tested. However due to the relatively short period of time for these experiments, impact on the overall instrument maintenance, potentially caused by any excess water collected during sampling, could not be assessed. Collectively, these data suggest that extended dry purge time may not be suitable to mitigate humidity on tubes due to breakthrough. However, initial results using exhaled breath collected via the ReCIVA device suggest there may be little or no effect attributed to humidity or water on chromatography, independent of TD tube type, as illustrated by consistent peak FWHM and retention times among the breath samples.

DISCUSSION

Initial examination of Test 1 exhaled breath raw data from samples collected via the ReCIVA device showed a large abundance of siloxane contamination emanating from the silicon of the ReCIVA masks. However, this is not the first study to detect background siloxanes [9]. Doran et al. observed a significant increase in several siloxanes, such as hexamethyl cyclotrisiloxane and hexamethyl disiloxane, compared to room air controls [9]. Mitigation of the siloxane background is necessary to identify potential co-eluting compounds at these retention times. Literature suggested organic solvent could remove the siloxane contamination observed [14,15]. Soaking in ethyl alcohol was shown to reduce siloxane abundance by more than 78% (**Figure 1**) [14,15]. However, removal procedures for the alcohol solvent were insufficient. These results lead to a greater than 85% increase in ethyl alcohol following the alcohol wash procedure (**Supplemental Data 9**). Alternatively, masks retained from exhaled breath collection following Test 2 were subjected to a baking protocol. A greater than 94% reduction in the most dominant siloxane background contamination was achieved using the baking protocol without the addition of solvent to the masks (**Figure 1**). While both cleaning methods reduce the siloxane background associated with the ReCIVA masks, additional removal strategies, such as mask baking and/or autoclaving, must be explored to remove all of the residual solvent from the ReCIVA masks when the ethanol wash method is utilized for cleaning and/or sterilization. Therefore, the mask baking procedure is recommended to remove siloxane contamination from new masks, as illustrated by Test 3 baking the new masks prior to exhaled breath collection, while a combination of the two cleaning methods may

be appropriate for repeated use of the masks. However, data supporting multiple uses of masks following a sterilization protocol is still needed.

While significant siloxane background attributed to the ReCIVA masks has been observed in not only this study but others, the data illustrate a reduction in the overall background contamination based on the ReCIVA mask's manufacture date [9]. For instance, the masks used in Test 1 and Test 2 were manufactured approximately nine months apart, but the masks used in Test 2 have significantly less overall number siloxane background peaks (**Figures 1**). These data suggest the manufacturer of the mask is aware of the background contamination and seems to be working to mitigate the issue to a point where cleaning procedures may be ultimately unnecessary.

The results show that the flow rate applied by the ReCIVA device to TD tubes directly impacts the consistency of exhaled breath results, estimated via isoprene quantities, obtained by this collection method. While it is not surprising that the flow rates differ from Tenax TA to the 5TD tubes as flow rate is in part controlled by the TD tube adsorbent material flow resistance, the effect the ReCIVA control software version plays in regulating the flow rate is surprising. Furthermore, the data illustrate that the updated ReCIVA control software (v. 1.46) did not provide the set flow rate of 200 mL min^{-1} for either Tenax TA TD tubes or the recommended 5TD tubes for which the ReCIVA device is calibrated. The results show an additional "in-house" flow calibration should be considered for each TD tube type and each ReCIVA bank used for collection, and the

most up-to-date ReCIVA control software should be installed prior to initiating new studies utilizing the ReCIVA device for collection. It should be noted that the data in this manuscript were generated using a single ReCIVA unit among several TD tube batches with the date of manufacturer flow rate calibration unknown. Therefore, additional experimentation among multiple ReCIVA units, controlling for potential TD tube batch effects, will be required to fully characterize the variability associated with ReCIVA breath sampler results and flow rates. It is plausible that other ReCIVA units may have different flow rates compared to the one tested, depending on adsorbent tube type/batch and ReCIVA bank, making comparisons of results among different ReCIVA units difficult without “in-house” flow calibration prior to collection on each ReCIVA to be compared. However, evidence supporting this hypothesis still needs to be generated.

The results illustrate that by using updated ReCIVA control software and an “in-house” flow calibration creates duplicate samples using a single adsorbent tube type and can be generated for a single ReCIVA breath collection. It is noteworthy that the results and conclusions generated here are based on isoprene quantity alone. Isoprene was selected as a representative compound, for this study, due to the relatively high abundance found in exhaled breath, the ability to acquire a commercially available neat gaseous standard for calibration, and the overall large body of work that has been performed on exhaled isoprene [22-31]. While the data illustrates flow rate plays a role in the determined isoprene results from the ReCIVA, a portion of the overall variability in the results could correspond to person-to-person differences in exhaled isoprene [27-32]. For example, the isoprene results from breath bags collected prior to the ReCIVA sampling show a wide

range of concentrations (Test 2: 236.6 – 543.2ng, Test 3: 66.2 – 279.3ng). To account for this potential factor, moving forward, if isoprene is to be used as a representative compound for these types of experiments, it is recommended to utilize an increased number of participants for sampling.

While others have shown, that through breath-to-breath analysis using PTR-MS, isoprene values can change with slight movements or breath maneuvers of an individual, such as change in posture or breath holding, these studies were conducted with relatively short collection windows (≤2 minutes) with real-time analysis [10,33]. As collections with the ReCIVA device for our test, have shown samples collected over seven minutes (425 s) and more than 85 exhalations on average. Therefore, breath-to-breath changes in exhaled isoprene from posture changes or breath holding will only have a small effect on the overall isoprene detected across the lengthier collection time (**Supplemental Data 11**). While it is hypothesized that other, less abundant compounds in exhaled breath will perform similarly to isoprene, these experiments must be performed for further discovery applications.

Finally, for these ReCIVA test collections participants were given simple directions, like breath normal slow breaths in and out through the mouth only. Therefore, breathing rate and breathing depth was not controlled beyond these simplistic instructions. It is currently unknown what effect these and other parameters, such as nose breathing, modified gas composition provided to the test subject, gender, etc., may have on the exhaled breath

results acquired with the ReCIVA sampler. However, these data provide additional evidence for further investigation surrounding the ReCIVA for exhaled breath collections. The results illustrate the utility of the ReCIVA breath collector for providing consistent exhaled breath onto adsorbent media, eliminating the pitfalls associated with collecting breath in bags, while gaining comparable results to samples collected by standard breath bags.

CONCLUSIONS

Studies surrounding the use of the Respiration Collector for In Vitro Analysis have been few. The data presented support the use of ethyl alcohol submersion and high temperature baking to reduce the siloxane background associated with the ReCIVA masks. Additionally, the results highlight some variabilities associated with using the ReCIVA sampler and approaches for mitigating the inconsistencies. The data illustrate that flow rates applied by the ReCIVA device should be checked and adjusted prior to sample acquisition to ensure the most consistent results among samples. Collectively, the results demonstrate that the ReCIVA sampler represents a significant step towards standardized exhaled breath sampling for off-line analysis and further research is warranted to improve overall exhaled breath sampling.

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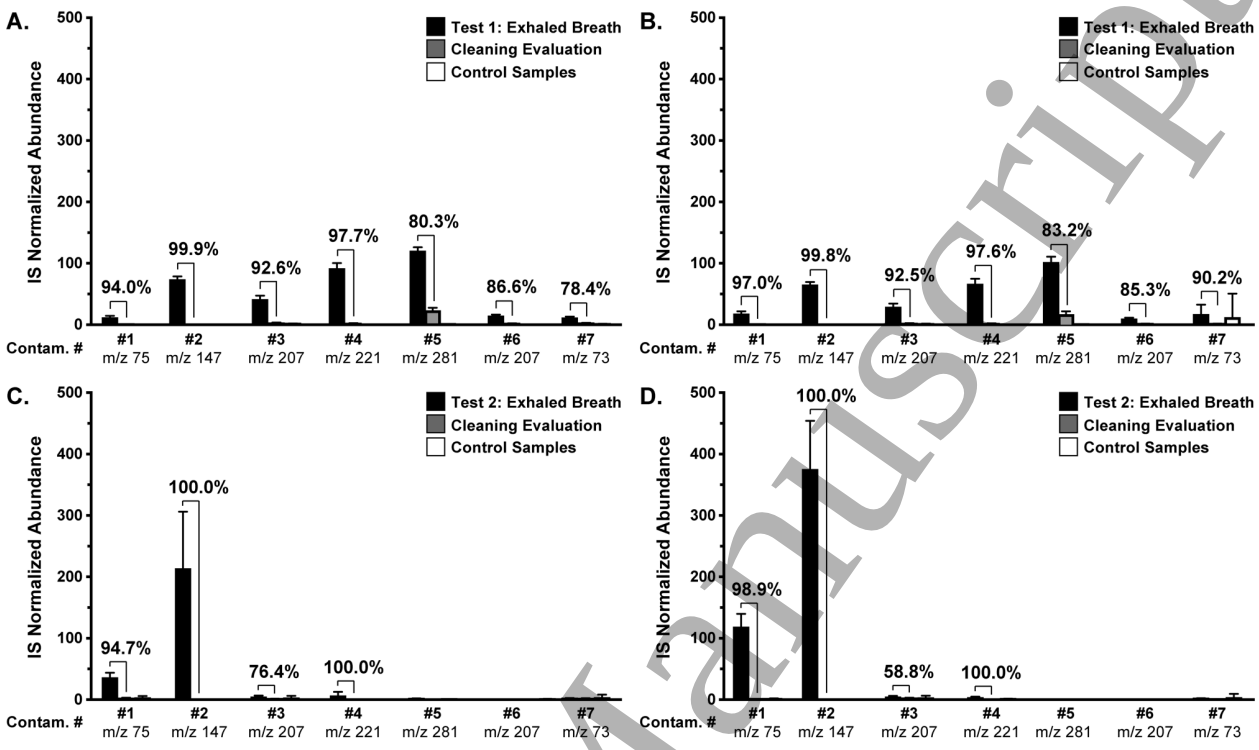
FIGURE CAPTIONS

Figure 1: A summary of the internal standard normalized abundance of background compounds collected from ReCIVA Test 1 (ReCIVA Software v.1.30, masks manufactured June 2017) **A)** Tenax TA (n=6 per session) and **B)** 5TD (n=6 per session) and ReCIVA Test 2 (ReCIVA Software v.1.46, masks manufactured March 2018) **C)** Tenax TA (n=6 per session) and **D)** 5TD (n=6 per session) thermal desorption tubes. Percentages signify the % reduction in the siloxane background following cleaning. Error bars represent the 95% confidence interval. All p-values between exhaled breath and cleaning evaluation samples are $p<0.05$ by Student's t-test. The data illustrate a reduction in siloxane background can be achieved with ethanol mask washes and heating masks at 200°C for approximately 68 hours.

Figure 2: **A)** A plot of the quantitated isoprene (ng) parsed by ReCIVA pump bank, adsorbent tube type, and ReCIVA test (n=6 per plot for each test). Whiskers signify the min and max. **B)** A plot of the mean flow rate provided to individual TD tubes (n=5, per tube type per bank) by the ReCIVA sampler. Measurements for Test 1 & 2, the ReCIVA was set to 200 mL min^{-1}. The measurements for Test 3 represent the manual calibrated flow rates (Tenax A: 164 mL min^{-1}, Tenax B: 168 mL min^{-1}, 5TD A: 197 mL min^{-1} and 5TD 200 mL min^{-1}) to bring the ReCIVA flow to 200 mL min^{-1} overall. The dashed line indicates flow set/target of 200 mL min^{-1}, * indicates statistical difference from 200 mL min^{-1} set point/target ($p<0.05$), and error bars signify the 95% confidence interval. **C)** A plot of the delta between banks (Bank B-A, absolute value and raw delta) for isoprene among ReCIVA Tests (n=6 per plot for each test). Whiskers signify the min and max. Significance was determined by Student's t-test, paired t-test, and by linear mixed modeling as appropriate. Data show statistically similar isoprene values can be obtained among banks and TD tube types by using calibrated flow rates and updated ReCIVA control software. Additionally, calibrated flow rates reduce the bias of the ReCIVA between banks for both Tenax and 5TD tubes.

Figure 3: A plot of the quantitated isoprene values from Tenax tubes for all three tests collected by the ReCIVA sampler (n=12 per plot) and from ALTEF exhaled breath bags (n=12 per plot, 1 each pre and post n=6). Whiskers signify the min and max. p-values were determined by linear mixed modeling. The results illustrate that calibrating the flow rates on the ReCIVA device allows for statistically similar results to be obtained between the ReCIVA sampler and ALTEF exhaled breath bags. * Indicates estimated values, as quantities were above the calibration curve.

FIGURE 1



Contam. #	RT (min)	Tentative Compound ID	Ref.
1	7.13	silanol, trimethyl-	-
2	8.12	disiloxane, hexamethyl-	[9]
3	10.82	cyclotrisiloxane, hexamethyl-	[9]
4	11.91	trisiloxane, octamethyl-	[9]
5	13.89	cyclotetrasiloxane, octamethyl-	-
6	15.17	tetrasiloxane, decamethyl-	-
7	16.65	p-trimethylsilyloxyphenyl-bis(trimethylsilyloxy)ethane	-

FIGURE 2

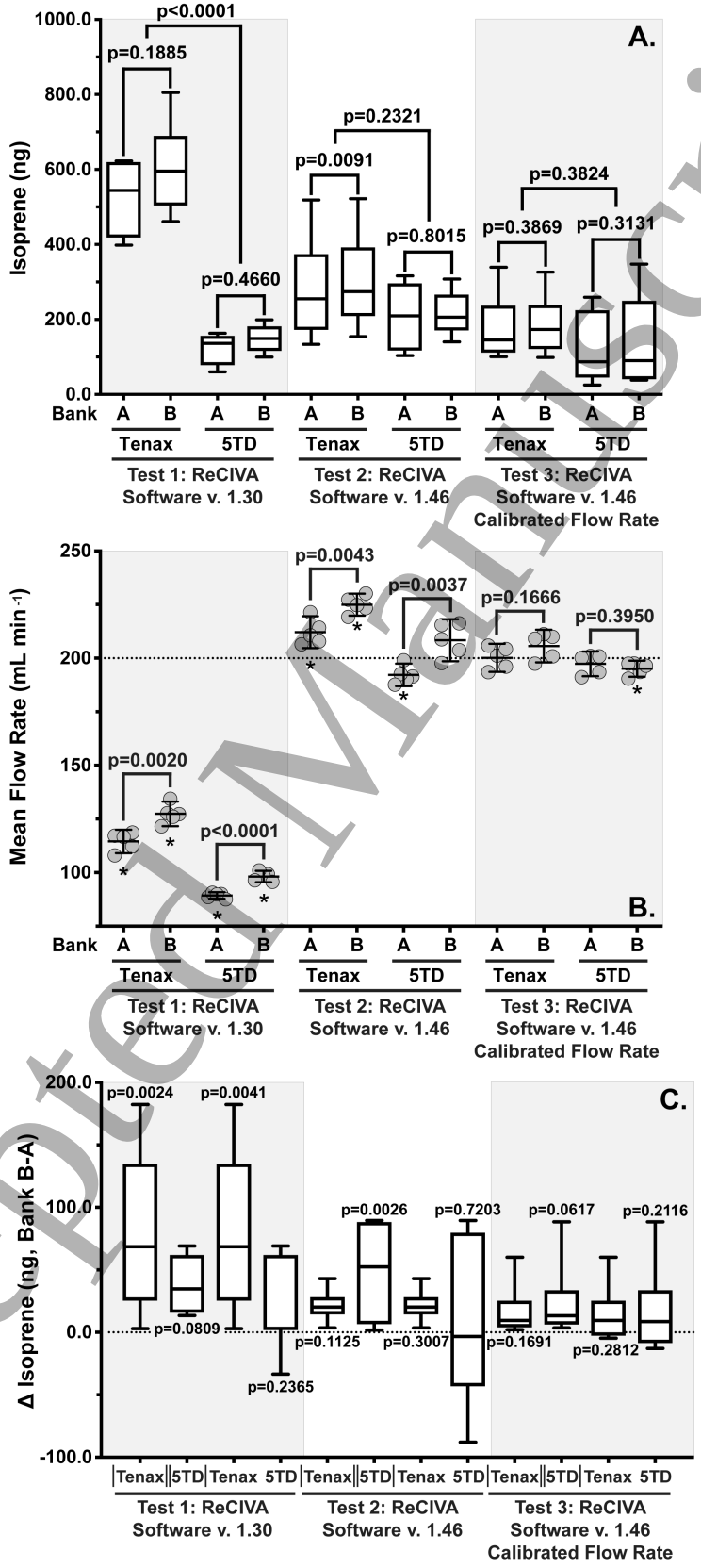


FIGURE 3