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PAPER

Standardization of the collection of exhaled breath condensate and exhaled breath aerosol using a feedback regulated sampling device

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Abstract

Exhaled breath condensate (EBC) and associated exhaled breath aerosols (EBA) are valuable non-invasive biological media used for the quantification of biomarkers. EBC contains exhaled water vapor, soluble gas-phase (polar) organic compounds, ionic species, plus other species including semi- and non-volatile organic compounds, proteins, cell fragments, DNA, dissolved inorganic compounds, ions, and microbiota (bacteria and viruses) dissolved in the co-collected EBA. EBC is collected from subjects who breathe 'normally' through a chilled tube assembly for approximately 10 min and is then harvested into small vials for analysis. Aerosol filters without the chilled tube assembly are also used to separately collect EBA. Unlike typical gas-phase breath samples used for environmental and clinical applications, the constituents of EBC and EBA are not easily characterized by total volume or carbon dioxide (CO_2) concentration, because the gas-phase is vented. Furthermore, EBC and associated EBA are greatly affected by breathing protocol, more specifically, depth of inhalation and expelled breath velocity. We have tested a new instrument developed by Loccioni Gruppa Humancare (Ancona, Italy) for implementation of EBC collection from human subjects to assess EBC collection parameters. The instrument is the first EBC collection device that provides instantaneous visual feedback to the subjects to control breathing patterns. In this report we describe the operation of the instrument, and present an overview of performance and analytical applications.

1. Introduction

There has been significant interest in the ability of breath analysis to identify target compounds and trends in the constituents of exhaled breath as a tool for diagnosing and monitoring medical conditions, and for discovering a growing number of biomarkers that might indicate recent environmental exposures or preclinical disease state [1–3]. In fact, there is ongoing research regarding the mixtures of exogenous, endogenous, and microbiome contributions to breath samples, and for volatiles emissions in other biological media, for deducing specific retrospective or prospective effects [4–7].

Exhaled breath contains measurable levels of volatile organic compounds (VOCs), semi-volatile organic compounds (SVOCs), proteins, lipids, DNA and microbiota such as bacteria and viruses. The central goal of exhaled breath analysis is to non-invasively gain insight into previous occupational or environmental exposures and/or the presence or status of a medical condition or infectious state [6, 8, 9]. Exhaled breath analysis is less invasive, more easily repeatable and less expensive than alternative invasive tests [10]. There are various methods for collecting and analyzing exhaled breath, depending on the constituent of interest. If the target of interest is a protein, lipid, SVOC, or microbiota, collection of exhaled breath

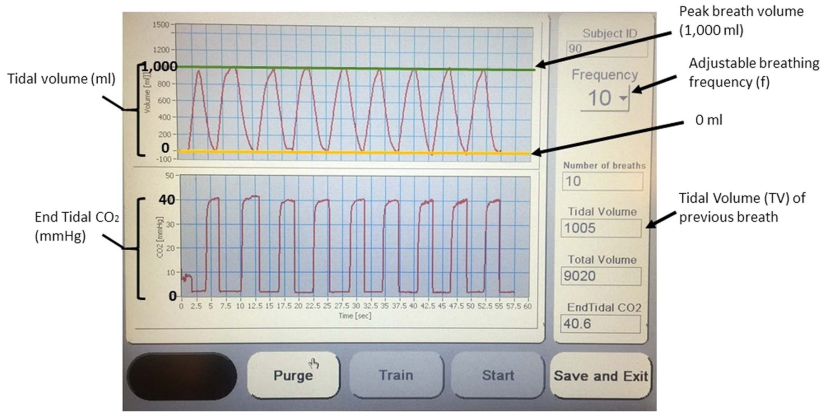

Figure 1. Loccioni instrument interactive screen. The interactive screen guides the subject's breathing pattern with instantaneous tidal volume measurements and an auditory metronome. The interactive screen allows the researcher to adjust breathing frequency and also provides the researcher with an instantaneous endtidal CO_2 measurement and total volume exhaled.

condensate (EBC) is considered the ideal approach [11–14]. While there is significant interest in the collection and analysis of biomarkers in EBC for disease detection and monitoring, there are currently substantial issues with the collection and analysis of breath, in general, and in EBC specifically with respect to inter-subject and intra-subject variability and a lack of standardization [15, 16] until very recent recommendations [28]. Concentration of biomarkers as well as total sample volume in EBC can vary significantly depending on subject's health, profession, diet, genotype, age, gender, and recent exposure to exogenous chemicals [10]. In addition, a subject's respiratory rate, exhaled breath velocity, and volume of each exhaled breath has been shown to significantly affect EBC volume [17–19]. Currently there is minimal standardization of methods or techniques to collect EBC, resulting in substantial difficulty comparing results within and across studies [15]. Given that the applications of examining EBC biomarkers are extremely broad, the complete standardization of EBC collection protocols is unrealistic [12, 15]. However, an instrument that assists in the control of a subject's breathing rate and exhalation volume may be useful in reducing inter-subject and intra-subject variability within a specific study and for certain biomarkers.

Herein, we report the efficacy of one such device, the Loccioni instrument, which was adapted from a prototype clinical instrument used previously for gas-phase health studies [20]. This instrument provides real-time feedback and breath cues to the subject to regulate breathing frequency and tidal volume. In this study, we compare consistency of EBC volume and pH of samples collected by controlled 'paced' breathing using a new instrument with verbal and audio prompts to samples collected by uncontrolled free breathing. 'Paced' breathing, with the goal of producing a fairly reproducible tidal volume and frequency, typically involves audio, visual, and/or vibratory prompts for the subject; we use this interchangeable with controlled breathing for the purposes of this report. This work is in two parts; the first is based on a collection of various samples drawn from exploratory sample collections from research into environmental exposures, and the second part is a detailed longitudinal comparison of specific parameters between groups. The primary goal is to assess if the controlled breathing technique presents any advantage to EBC sample standardization, such as decreased variability. We utilized two collection durations—a short (6 min) collection when a minimal amount of time to provide a relatively small EBC volume is required, and a longer (10 min) one for potential studies needing a larger amount of EBC volume.

2. Methods

2.1. Instrument

The instrument, provided by Loccioni Gruppa HumanCare (Ancona Italy), instantaneously measures CO_2 concentration, volume of each breath and total volume exhaled during sampling. While CO_2 concentration was not directly recorded for our research, it may be useful for determining where in the lung the exhaled breath originated or to confirm complete exhalation [21, 22]. In addition, the Loccioni instrument also has an interactive screen that visually guides the subject's volume of air inhaled and exhaled and also acts as a audio metronome in order to prompt a subject when to reverse direction of breathing (figure 1). The respiratory rate for controlled breathing can be programmed from 6 to 16 breaths min^{-1}, and individual breath volume programmed from 100 to 1500 ml/breath, which may allow for targeting of air from a specific region within the lung or breathing pattern. Total exhaled volume is also provided. There is no valve to allow collection of specific exhaled breath fractions.

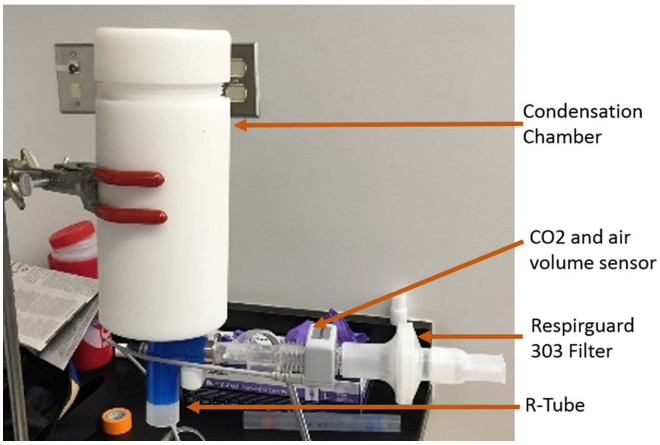

Figure 2. EBC collection system. The principal components of the collection system include the R-tube, the condensation chamber, the Respirguard 303 filter and the CO_2 and air volume sensor. The condensation chamber is filled with dry ice, which cools the R-tube to −78 °C. A Respirguard 303 filter is in place to prevent accumulation of microbes within the Loccioni instrument. The CO_2 and air volume sensor are located between the R-tube and the Respirguard 303 filter.

2.2. Human subjects

EBC biological specimens were collected from four adult volunteers during the methods development phase of studies conducted under the Institutional Review Board (IRB) auspices of IRB Study #: 09-1344 and IRB Study# 99-283, University of North Carolina, Chapel Hill NC. Additional longitudinal evaluations were conducted ad hoc under an exemption to the common rule for biological specimens.

2.3. EBC collection method

EBC samples were collected using R-tubes (Respiratory Research Inc. Austin, TX, USA). The R-tube was placed inside an aluminum sleeve, which was surrounded by dry ice within an 8 cm diameter co-axial container ('condensation chamber'). A Respirguard 303 filter (Vital Signs, San Diego, CA) was placed at the intake of the R-tube in order to prevent the introduction of microbes into the Loccioni Instrument (figure 2). A plastic fitting containing the CO_2 and air volume sensor was installed between the Respirguard 303 filter and the R-tube (figure 2). The sensor was installed on both the spontaneous and controlled sampling methods for consistency and to measure the final total air volume exhaled. The metronome and individual breath volume feedback mechanisms were not used for the spontaneous breathing samples.

Subjects remained seated while breathing through the filter without nose clips. Upon completion of sample collection, the R-tube was capped and condensate thawed at room temperature. Once thawed, the condensate was consolidated using the internal O-ring squeegee in collaboration with the R-tube plunger. The volume of each sample was measured and samples transferred to a 2 ml polypropylene centrifuge tube for storage in −20 °C freezer until pH analysis.

For this initial study, we collected 114 samples. 102 of the 114 samples were collected in sets of three. Between samples within the same set, an approximately 7 min gap was necessary to prepare for the following sample. Of the 114 samples, 57 samples had a 6 min collection time, while the remaining 57 samples were collected over a 10 min sample time. Sixty-three of the 114 samples were collected using the Loccioni instrument to control breathing rate at 10 breaths per minute and a breath volume of 1000 ml of air per breath. This was defined as controlled breathing. Of the 63 controlled samples, 30 were collected for 6 min, while the remaining 33 samples were collected over a 10 min sampling period. The remaining 51 samples did not use the Loccioni instrument to control breathing rate and volume. Instead, the subject was instructed to breathe at a regular frequency and tidal volume. Of the 51 spontaneous samples, 27 were collected for 6 min, while the remaining 24 were collected over a 10 min sampling period.

2.4. pH analysis

pH was measured for all EBC samples with sufficient volume. A minimum of approximately 1.3 ml of EBC was required to accurately cover the pH meter electrode bulb. Few individual EBC samples had a volume of 1.3 ml or more, so EBC samples from each triplicate set (collected within 45 min) were blended together to provide sufficient volume for a single pH measurement. pH was analyzed using a Thermo Scientific Ross Ultra pH Electrode, connected to Thermo Scientific Orion Star A211 pH meter (Waltham, MA). Thermo Scientific Orion Pure Water Low Ionic Strength pH buffers were selected for pH meter calibration. Argon was bubbled into each pH sample for 10 min, then the pH electrode was immediately placed into the sample. pH was recorded once the value stabilized.

2.5. Statistical analysis

Statistical analyses and tests were performed using Prism 7 software (GraphPad Software, Sn Diego CA).

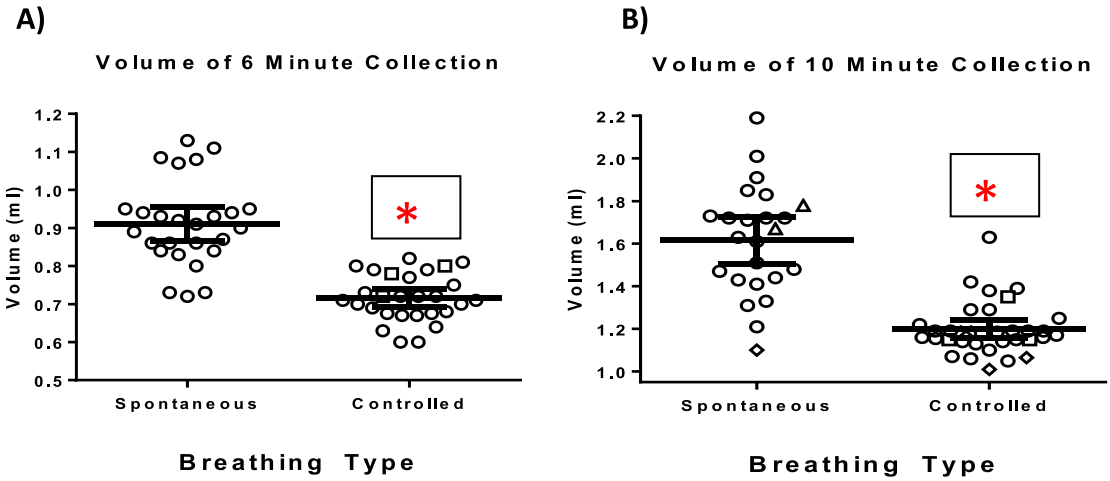

Figure 3. Comparison of EBC volume by collection time and method with mean and 95% confidence interval displayed. A reduction in variability between the spontaneous and controlled sampling methods is evident. (A) A comparison of volume of 6 min spontaneous ($N = 26$) samples collected from a single subject, and controlled ($N = 29$) samples collected from two subjects. (B) A comparison of volume of 10 min spontaneous ($N = 24$) samples collected from three subjects, and controlled ($N = 33$) samples collected from four subjects. (Each shape reflects a data point from a specific subject.) *There was a statistically significant difference between controlled and spontaneous breathing patterns at each collection time; $p < 0.01$ (one way ANOVA with Sidak Multiple Comparison Test).

3. Results

3.1. EBC volume

Reducing variability in EBC sample volume and biomarker concentration is a key goal for the standardization of breath analysis. To determine the efficacy of the Loccioni instrument in reducing variability, the EBC sample volume collected over 6 and 10 min using the Loccioni instrument was compared to the 6 and 10 min samples that were collected through spontaneous breathing. The variability of EBC volume using the Loccioni instrument was notably reduced in comparison to the spontaneous breathing EBC samples, regardless of collection time (figure 3). For samples collected for 6 min using the spontaneous breathing method, the mean value was 0.91 ml, with a range of 0.72–1.13 ml and a coefficient of variation of 0.124. In contrast, for samples collected using the controlled breathing method, the mean value was 0.71 ml, with a range of 0.52–0.82 ml and a coefficient of variation of 0.100. For samples collected for 10 min using the spontaneous breathing method, the mean value was 1.62 ml, with a range of 1.10–2.19 ml, and a coefficient of variation of 0.159, compared to the mean value being 1.20 ml, with a range of 1.01–1.63 ml, and a coefficient of variation of 0.103 using the controlled breathing method. The larger volume with the spontaneous breathing pattern likely derives from larger and more frequent breaths especially hyperventilation at the initiation of collection.

Intra-subject variability in EBC volume and biomarker concentration is inherent in EBC samples collected on different days. Therefore, to compare intra-subject variability between spontaneous and controlled breathing in samples collected in rapid succession, the majority of EBC samples were collected in sets of three using an identical sampling method and collection time. To determine whether the Loccioni instrument reduced same-day variability, EBC volume from each sample was graphed by sample set. The variability between 10 min samples collected in rapid succession using the Loccioni instrument was notably smaller in comparison to the 10 min samples collected in rapid succession using the spontaneous breathing method (figure 4). The largest volume difference between samples collected in rapid succession from the same subject for spontaneous breathing and controlled breathing is approximately 0.6 ml and 0.2 ml, respectively. In addition, variability in sample sets collected on different days was also markedly reduced in samples collected using the controlled breathing method.

3.2. pH analysis

pH of EBC samples is an easily measured biomarker to monitor respiratory acidification, which is associated with numerous respiratory illnesses [23]. Most of the EBC samples (31 of 37) had a pH value considered within the range of normal healthy individuals (7.08 ± 0.69) [24] (figures 5(A) and (B)). For the 10 min EBC samples, one sample from a subject was slightly below the pH range found in nominally healthy individuals ($pH = 6.12$), and two samples collected from the same subject, approximately 20 min apart, had pH values of 4–5, far below the range of healthy individuals. No asthma-related symptoms were reported on the day of sample collection but the subject reported a *Staphylococcus aureus* infection and had just begun treatment using antibiotics the day before. Previous studies have linked bacterial infections with increased levels of nitric oxide, which may

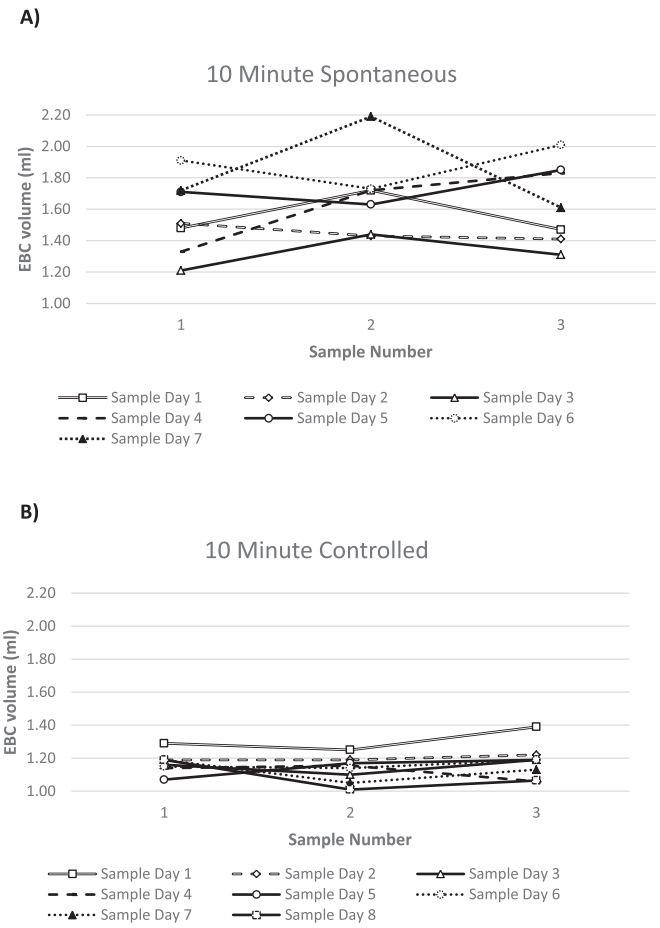

Figure 4. Variability of EBC volume by sample set. Variability within sample sets using controlled EBC collection method is significantly reduced in comparison to spontaneous breathing method. (A) EBC volume collected using spontaneous breathing method for 10 min. Minimal horizontal linearity is indicative of significant variability in intra-day samples ($N = 7$), collected from a single subject over approximately 2 months. (B) EBC volume collected using controlled breathing method for 10 min. Relative horizontal linearity of each set indicates reduced variability using Loccioni instrument ($N = 8$), collected from two subjects over approximately 2 months.

be associated with decreased EBC pH, thus potentially explaining anomalous pH readings in the two sets of samples [25, 26]. A Grubbs' outlier test identified pH values of 4.9 and 6.12 as a significant outlier in the 6 and 10 min collections with spontaneous breathing, respectively. With the outlier removed, the mean pH of the samples using the spontaneous breathing method for 6 min was 8.29, with a range of 7.8–8.62. With the outlier removed, the mean pH of sample sets using the spontaneous breathing method for 10 min was 8.39, with a range of 7.93–8.66.

4. Discussion

We have illustrated the implementation of a novel method of controlled cued breathing using a Loccioni instrument for standardization of EBC. Results from this instrument evaluation indicated a reduction of both intra-day and inter-day variability of EBC volume with the instrument use. EBC pH results were fairly consistent across samples from same subjects, regardless of collection method and time, which is important for the application of the R-tube for assaying asthma status. Additionally, EBC recovery volume and variability over multiple time points were quantified and compared, allowing for determination of ideal collection time for subsequent EBC studies. To determine an optimal collection time, it is necessary to determine the ideal collection volume, which is partially dependent on the number and assay types chosen. If planning to measure cytokine concentrations and pH, while reserving a sufficient volume for replicate or follow-up assays, we estimated a sample volume of at least 0.8 ml would be necessary. The majority (27/29) samples collected for 6 min using the controlled method had a final volume of 0.8 ml or less but only 4 of 26 measured samples using the spontaneous method had a final volume of $\geqslant$0.8 ml. However, due to the reduced variability using the controlled method, the 6 min spontaneous sampling technique was considered inferior to both the 6 and 10 min controlled collection method if low volume reproducibility is the criterion or less volume is needed. These findings with controlled breathing were obtained without knowledge of the variability in TV and freq for each

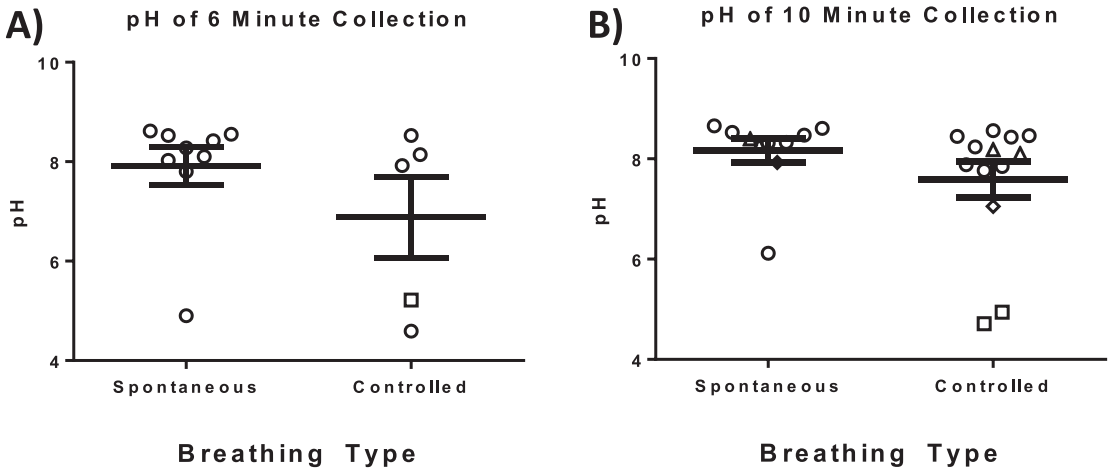

Figure 5. Comparison of pH of EBC by collection time and method with mean and standard error displayed. No variability in pH between sampling collection method and collection time was observed. (A) A comparison of pH of 6 min spontaneous ($N = 9$) samples collected from a single subject, and controlled ($N = 5$) samples collected from two subjects. All pH values are derived from triplicate pooled EBC samples. The pH of three samples are noticeably lower than the mean pH of their respective sample group. (B) A comparison of pH of 10 min spontaneous ($N = 10$) samples collected from three subjects and controlled breathing ($N = 13$) samples collected from four subjects. For the spontaneous values, seven were pooled from triplicate samples, and three were individual samples with an adequate volume to measure the pH; for the controlled breathing pH values, nine were pooled from triplicate samples, three were individual samples, and one was pooled from duplicates. The pH of two sets of samples from the same subject are noticeably lower than the mean of the pH of their respective sample collection method. (Each shape reflects a data point from a specific subject.) Two-way ANOVA performed comparing sampling method and collection duration.

collection, though the total volume was near the target (100 l), which could be improved with software reprogramming.

A potential benefit of the 10 min collection time is the decrease in variability in volume or biomarker levels relative to spontaneous breathing due to hyperventilation by the subject, which typically can occur when the subject first expires into the R-tube mouthpiece and Respirguard 303 filter. It is not clear what role hyperventilation plays in the recovery of EBC or concentration of cytokines. While hyperventilation reduces the pH of blood, previous studies have found no link between hyperventilation and changes in EBC pH [27].

Our results indicated that there is less range of EBC volume in the 6 min samples than in corresponding 10 min samples, regardless of method of collection, but that the variability expressed as COV were similar for both the 6 and 10 min collections. This is reasonable, since there is less time for the sample volume to diverge. However a smaller EBC volume is collected over the 6 min. While there was less variability of EBC volume when using the controlled method, this difference may be more pronounced if the sample subject size is expanded further. Additionally, early in the study, the subjects' spontaneous respiratory rate was markedly different than the programmed controlled respiratory rate of 10 breaths per minute. In later sample collections, the subjects' spontaneous respiratory rate was noted to be considerably closer to the controlled breathing rate of 10 breaths per minute, possibly attributable to acclimation to the controlled breathing rate. Further examination of what different breathing patterns, e.g. panting, breath holding, would have on the measured endpoints is needed. Additional subjects would allow testing of how generalizable the findings described herein are to the general population. Because the general population has a wider range of lung volumes, the utility of using controlled breathing patterns of similar frequency and volumes may not be optimal to minimize variability. Adjustment to a more appropriate volume based on, e.g. lung volume capacity, may prove to be a better method to collect samples with minimal variability. Within the whole population, consideration of the frequency and volume of the controlled breathing pattern should be given to individuals with lung disease as well, possibly to document changes towards normal values due to therapies. While not examined in this report, the total exhaled volume is reported on the Loccioni instrument so that volume of EBC can be compared with the breath volume, as per recent recommendations by a European Respiratory taskforce on exhaled breath collection [28].

5. Conclusions

A controlled breathing pattern using visual and audio clues to set inhalation and exhalation volumes and frequency was shown to have decreased variability in EBC volume compared to spontaneous breathing by an individual. This decrease in variability using the controlled breathing was observed with EBC collected for both 6 and 10 min.

With the controlled breathing pattern, a smaller EBC volume was collected at both collection durations

examined. The collection time may be critical for procuring adequate volume for subsequent analyses.

The use of the controlled breathing pattern provided EBC which did not have a difference in pH from the EBC produced by spontaneous breathing.

Further examination of the controlled breathing pattern with different frequencies and volumes within a wider population with different lung sizes are needed for further validation of decreased variability in the EBC endpoints.

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Disclaimer

The research described in this article has been reviewed by the US EPA National Health and Environmental Effects Research Laboratory and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the Agency, nor does the mention of trade names of commercial products constitute endorsement or recommendation for use.

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