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Comparison of breath sampling methods: a *post hoc* analysis from observational cohort studies†

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Abstract

In this report, we present a post hoc analysis from two observational cohorts, comparing the global breath volatile profile captured when using polymer sampling bags (mixed breath) versus Bio-VOC™ (alveolar breath). The cohorts were originally designed to characterize the breath volatile profiles of Malawian children with and without uncomplicated falciparum malaria. Children aged 3–15 years were recruited from ambulatory pediatric centers in Lilongwe, Malawi. Breath sampling was carried out two months apart (one study using a Bio-VOC™ and the second using sampling bags), and all samples were analyzed by gas chromatography/mass spectrometry. The efficacy of breath collection was assessed by quantifying levels of two high prevalence breath compounds, acetone and isoprene, as well as determining the overall number of breath compounds collected and their abundance. We found that the mean number of volatiles detected using sampling bags was substantially higher than when using the Bio-VOC™ (137 vs. 47). Breath collection by Bio-VOC™ also yielded reduced levels of endogenous breath volatiles, isoprene and acetone, even after breath volume correction. This suggests that the Bio-VOC™ dilutes the volatiles and introduces dead air or ambient air. Our results suggest that sampling bags are better suited for biomarker discovery and untargeted search of volatiles in pediatric populations, as evidenced by superior breath volatile detection.

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Conflicts of interest

The authors report their interest as co-inventors on U.S. Provisional Application Filed 62/550283 (AOJ, CLS, and IT). In addition, AOJ currently serves on the Scientific Advisory Board of Pluton Biosciences, LLC.

Introduction

Analysis of exhaled air demonstrates great potential for medical diagnosis and therapeutic monitoring.¹ It offers a non-invasive strategy for monitoring potential biomarkers originating from normal biochemical processes, as well as from pathological disorders. A typical exhaled human breath contains hundreds of volatile organic compounds (VOCs)² and an expanding number of pathologic states have been linked to unique exhaled breath VOC profiles.^{3–5}

During breath sampling, investigators must determine which portion of the breath to collect for analysis. Most studies in breath analysis aim to collect either “mixed expiratory” breath or the “alveolar” portion. Mixed expiratory breath is considered the simplest breath portion to obtain and encompasses the total exhaled breath plus “dead space air” (ambient, mouth, and potentially nasal air). This portion of breath is unlikely to represent the optimum sample, due to greater abundance of non-endogenous contaminants. In contrast, alveolar breath sampling aims to minimize the contamination from dead space, thus enriching for samples with a greater relative contribution of endogenous VOCs that are produced within the host. Endogenous VOCs have the potential to provide a metabolic snapshot that correlates to the physiological state of an individual.

Once a sample has been collected, temporary storage is required prior to pre-concentration. Polymer bags comprise the majority of breath collection containers and are typically used to collect mixed expiratory breath samples. Sampling bags can collect a large volume of breath (>1 L). However, such bags are costly and challenging to use. In addition, while bags can be reused, significant time and effort are required to ensure efficient cleaning.^{6,7} Each specific bag type also requires empirically determined, standardized procedures for quality control, reusability, and recovery. The second most common option for the collection of breath is the Bio-VOC™ (Markes International, UK), originally developed to monitor environmental exposure to workplace hazards. Bio-VOC™ is among the simplest and least expensive options for breath collection.⁸ This breath collection apparatus boasts an easy, straightforward collection scheme and a reusable design. In addition, the Bio-VOC™ is intended to enrich for capture of alveolar air and is therefore of particular interest for blood-borne biomarker analysis.⁹

In this work, we present a *post hoc* analysis of results from two observational cohorts, in order to compare the global volatile profile captured when using polymer bags (mixed expiratory air) and Bio-VOC™ (alveolar breath). The two studies were originally designed to characterize the breath volatile profiles of Malawian children with and without uncomplicated falciparum malaria. Because the two cohorts were similar except for the methods employed for breath collection, they provide an additional opportunity for comparative studies that may inform which breath collection method is most suitable for pediatric biomarker discovery in field settings. Moreover, we were interested in whether the ease-of-use and breath quality obtained with Bio-VOC™ (alveolar breath) outperforms sampling bags (mixed air) for biomarker discovery.

Experimental

Patient enrolment

This study is a *post hoc* analysis of samples collected within two observational cohort studies conducted in Malawi. For both cohorts, patients were enrolled at Kamuzu Central Hospital and Bwaila Health Centre in Lilongwe, Malawi. Human studies approval from both the University of Malawi College of Medicine Research and Ethics Committee (# P. 05/14/1572) and the Institutional Review Board of Washington University School of Medicine (#201504128) were obtained for this study. Informed consent was obtained for any experimentation with human subjects. Inclusion and exclusion criteria are described in ESI.[†]

Breath collection

Using Bio-VOC™ (exhaled alveolar breath).—For the first study (November 2015), the Bio-VOC™ breath sampler was used. It is a device that collects ~100 mL of exhaled, alveolar breath.¹⁰ The Bio-VOC™ can be described as a chemically inert syringe open at both ends, pictured in Fig. 1a. For breath collection, a mouthpiece was attached to the upper end of the Bio-VOC™. Participants were instructed to take a normal breath in and then to exhale fully into the Bio-VOC™. Since the volume of the syringe is smaller than the volume of a normal breath, the early portion of the breath is forced out by the later portion, with the goal of semi-selectively retaining the alveolar portion of the breath. After each exhalation, the mouthpiece is removed and a plunger is rapidly attached to the upper end of the Bio-VOC™ and a sorbent tube to the other end. The retained breath is expelled through the sorbent tube by manually pushing down the plunger slowly. Three breath samples from each participant were collected and stored in a single sorbent tube (ESI Table 1[†]). Because of the design of the Bio-VOC™ sampler, flow rate and volume are not controlled. We estimated this method to have sampled between 264 and 387 mL total (per three breaths, based on 88–129 mL per breath). A schematic of the breath collection method is shown in Fig. 1a and b and shown in ESI Movies 1 and 2.[†]

Using sampling bags (mixed exhaled breath).—For the second study (February 2016), breath was collected as previously described.¹¹ In brief, patients were asked to put the cardboard tube between the lips (Fig. 1c) and exhale as much as they comfortably could. As soon as the patient finished breathing out, the red valve was closed. Neither a nose clip nor VOC filter were used in our studies, as nose clips can be uncomfortable or frightening for sick children. This process was repeated multiple times until ~1 L of exhaled breath was collected in a 3 L SamplePro Flexfilm sample bag (SKC Inc., Pennsylvania). Using a set flow pump (ACTI-VOC, Markes International), exactly 1 L of breath was pumped through a sorbent tube using a flow of 100 mL min⁻¹. This method is pictured and diagrammed in Fig. 1c and d, and demonstrated in ESI Movie 3.[†]

Samples of room air were collected using both methods to assess possible environmental contaminants ($n = 5$ for Bio-VOC™ and $n = 2$ for sampling bags). In order to minimize the potential effect of background contamination, all breath samples were collected in the same

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physical location and the same time of the year, and enrolment occurred over a short period of time (2 weeks) in both cohorts.

The two studies were otherwise nearly identical in downstream sample processing (ESI Table 1†), as breath samples were transferred to sorbent tubes for storage and transport within minutes of collection. Samples were analyzed by GC/MS one month after initial collection (ESI Table 1†). Additional machine settings are provided in ESI.†

Chromatographic analyses

Chromatographic analysis of all breath samples (from both cohorts) were performed with a TurboMatrix 650 ATD (PerkinElmer, Massachusetts) connected to a Pegasus 4D GCxGC-TOFMS system (LECO, Michigan). Before analysis, sorbent tubes were brought to room temperature and purged for 5 min with BiP N₂ (Airgas) at 60 mL min⁻¹. A gaseous standard mixture (20.1 ng fluorobenzene, 18.6 ng toluene-D₈, 21.7 ng bromofluorobenzene, 20.3 ng 1,2-dichlorobenzene-D₄) was added to each tube by the TurboMatrix 650 immediately prior to analysis. Dry purge of 4 minutes was applied to our samples to reduce water peak and tubes were then desorbed at 270 °C, 40 mL min⁻¹ He flow, with recollection on a secondary Tenax cold trap at 10 °C. Analytes were released from the secondary trap by heating to 295 °C with 20% transferred to the GC/MS. The GC had a 30 m length × 0.25 mm ID × 0.25 µm film thickness DB-5 column (Agilent). The GC oven was programmed to hold at 40 °C for 3 min, ramp 5 °C min⁻¹ to 200 °C, then ramp 10 °C min⁻¹ to 250 °C, final ramp 25 °C min⁻¹ to 300 °C, then hold at 300 °C for 3 min. The TOFMS had a sampling frequency of 50 Hz and a mass recording range of 34–400 amu.

For analysis of the overall VOC profile, GC/MS data files were first exported in the “.cdf” format. GC/MS data files were then converted to “.D” *via* GC/MS Translator Pro (ChemSW, Inc.), and modified to work with MassHunter *via* GC/MS ChemStation (Agilent Technologies, Inc.). Deconvoluted compound lists were imported into Mass Profiler Professional (Agilent) for alignment. Peaks were normalized to the 1,2-dichlorobenzene-D₄ internal standard (*m/z* 150@11.7 min). Compounds were given annotations using the “IDBrowser Identification” feature using the NIST v11 reference library. *M/z* expansion was set to –0.3 amu/+0.7 amu. Retention time (RT) matching was not employed. Minimum match score was set to 40. Compounds were first filtered out if present in only one sample. Then, compounds present only at a raw abundance of less than 20 000 counts or identified as a silicon contaminant were filtered out. One patient from the cohort using the Bio-VOC™ was removed from the malaria negative cohort for both terpenes using Grubbs’ test for outliers ($\alpha = 0.0001$).

For GC/MS data analysis of individual compounds (isoprene, acetone, α -pinene, 3-carene, and the 1,2-dichlorobenzene-D₄ internal standard), OpenChrom was applied and the “ion remover filter” function was used to remove all ions except the *m/z* of interest.¹² Then, the peak at the proper retention time (determined by comparison to a true standard) was isolated by the “first derivative peak detector” function, with threshold set to “medium”, the “selected” box unticked, minimum S/N set to 0, and “moving average window size” set to 3. Finally, the base ion peak area was calculated with the “peak integrator trapezoid” function on default settings (no selected ions, no minimums, peak background not included) and

normalized to the base ion peak area of 1,2-dichlorobenzene-D₄. For each compound, a Mann–Whitney *U*-test was performed to detect significant differences between samples collected with bags and Bio-VOC™. Raw data and normalized data can be found in ESI † and ref. 11. The identities of individual compounds were verified using chemical standards.

As quality control for instrument performance, the mean base ion peak area of each internal standard was calculated over the period of GC/MS analysis. Levels of the internal standards for the Bio-VOC™ were up to 2 fold higher compared to levels obtained with bag sampling (ESI Fig. 1†).

Results and discussion

To evaluate the performance characteristics of distinct breath collection methods, we performed a *post hoc* analysis of breath volatile data from two distinct studies, designed and performed for the purpose of malaria breath biomarker discovery. Both were conducted in Lilongwe, Malawi during the wet season and reached their enrolment targets within two weeks (ESI Table 1†). Enrolled subjects were distinct (no individuals participated in both studies), but the two cohorts were demographically similar (ESI Table 2†). For both methods (bags and Bio-VOC™), a similar number of individuals with and without malaria were enrolled; moreover individuals were enrolled without knowledge of their malaria testing status. For sampling bags, 49% of children were positive with malaria, while for Bio-VOC™ studies 55% were positive for malaria (ESI Table 2†). Malaria positive participant numbers were not significantly different between the groups.

Because fever may influence VOC profile, inclusion criteria for both studies included fever as a presenting complaint. As expected based on this criteria, we found similar proportions of objectively measured fever at the time of emergency room evaluation in both studies (ESI Table 2†). For the Bio-VOC™ study, 91% of all participants had fever, while when using the sampling bags 89% had fever at the time of breath collection.

General breath volatiles profile

Using untargeted metabolomics to determine the compounds present in the breath of each subject, we found that the mean number of volatiles using sampling bags was substantially higher than the number detected using the Bio-VOC™ (116 vs. 28, $p < 0.0001$) (Fig. 2a), even though instrument analytical response was somewhat higher at the time the Bio-VOC™ samples were analyzed (ESI Fig. 1†). On average, nearly four times fewer volatiles were detected in the Bio-VOC™ samples compared to those obtained using sampling bags. This calculation excluded experimentally determined contaminants arising from sampling devices ($n = 18$ for Bio-VOC™ and $n = 21$ for sampling bags). The most dominant chemical classes of the contaminants were aromatic hydrocarbons, hydrocarbons, and alcohols (ESI Table 3†).

The differences in sample abundance between Bio-VOC™ and sampling bags are illustrated by comparing total ion chromatograms (TICs) from representative breath samples collected (Fig. 2b). The retention time range (3.5 to 5.5 minutes) corresponds to low boiling point compounds (non-polar molecules) that are characteristic in breath samples.² Peak heights

are higher and a greater number of peaks can be visualized from analysis of samples obtained using the sampling bags (Fig. 2b). The Bio-VOC™ aims to collect predominantly the alveolar portion of the breath and therefore endogenous compounds, while sampling bags collect mixed exhaled air samples comprised of endogenous and exogenous compounds. This may in part explain the lower number of VOCs observed in Bio-VOC™, or may also reflect the reduced volume of breath sampled.

To minimize the impact of patient-to-patient variability, we focused our analysis on compounds present in the breath of at least 50% of all subjects from either study. We thus determined that nearly 10 times as many compounds were detected with the sampling bag method compared to the Bio-VOC™ (Fig. 3a). Only 7 VOCs were common between the two breath collection methods and populations. The distribution patterns (e.g. “breathprints”) of these 7 VOCs were distinct between the two cohorts; moreover, lower signals were detected by using the Bio-VOC™ (Fig. 3b).

Relative abundance of the most common endogenous volatiles

We determined the levels of two well-characterized endogenous breath VOCs, isoprene and acetone, as detected using the Bio-VOC™. These compounds are typically found at higher concentrations in alveolar air than in dead space air.¹⁴ We also compared the levels of isoprene and acetone in Bio-VOC™ samples to those from sampling bags, which reflect mixed exhaled air. We found that both acetone and isoprene (Fig. 4a) were reliably detected in breath samples collected by either method (Bio-VOC™ or sampling bags), with average abundance levels roughly 10-fold greater than levels found in room air controls (ESI Fig. 2†). However, when breath was collected by Bio-VOC™, levels of acetone and isoprene were both significantly lower than in breath samples collected *via* sampling bags (Fig. 4b, $p < 0.0001$).

The concentration of isoprene in exhaled breath increases significantly during physical exertion,² while acetone typically increases in levels during fasting, due to fatty acid breakdown.¹³ Due to the nature of patient recruitment as they presented for care in a resource-limited emergency room setting, we were unable to control for time of last meal or physical exertion. Notably, physical exertion is expected to be modest for the febrile children enrolled, although these acutely ill subjects may have had variable flow rates during exhalation.

The markedly lower levels of isoprene and acetone captured by Bio-VOC™ compared to sampling bags are due in part to the different volumes of breath collected. Due to the need to expedite clinical care for enrolled patients, Bio-VOC™ collection was limited to three breaths (approximately 300 mL), compared to 1 L for sampling bags. To correct for this difference, we calculated the ratio of peak areas of acetone and isoprene from different sampling methods ($\text{peak area}_{\text{alv}}/\text{peak area}_{\text{mixed}}$) and multiplied the peak area of VOCs from Bio-VOC™ (alveolar air) by 3.3, to determine the concentration of the VOC in about 1 L of breath (assuming 300 mL of breath were collected by the Bio-VOC™). We found that the ratios of alveolar and mixed air for isoprene and acetone were 0.3 and 0.4, respectively. Thus, even when corrected for volume, the estimated concentrations of endogenous volatiles collected by Bio-VOC™ did not reach the levels expected for alveolar air samples (*i.e.*,

ratios of 1.75 for acetone and 1.9 for isoprene¹⁴). The above results suggest that other breath compounds, estimated to be present at yet lower concentrations, may fail to be detected using this method.

Malaria breath biomarker comparison

To test this prediction, we qualitatively and quantitatively compared the performance of each collection method for a number of malaria volatile biomarkers. We have previously reported two potential mosquito attractant compounds, the terpenes α -pinene and 3-carene, which were present at higher levels in the breath of malaria-infected patients, when breath was collected using sampling bags.¹¹ Using the Bio-VOCTM breath collection method, α -pinene and 3-carene were successfully detected. However, as was found for the canonical breath compounds acetone and isoprene, detection of breath terpenes was also reduced with Bio-VOCTM collection compared to sampling bags, with a 5-fold reduction in 3-carene and an 11-fold reduction in α -pinene levels across all patients between collection methods ($p < 0.0001$, Mann–Whitney *U*-test). On average, individuals with malaria had breath levels of α -pinene and 3-carene that were 1.6-fold and 2.1-fold higher, respectively, than the breath levels of these compounds in uninfected individuals; however, this finding did not reach statistical significance (ESI Fig. 3;† $p = 0.08$ and $p = 0.19$).

In addition, using the sampling bags, we have also reported a suite of 6 compounds that classified malaria infection status with 83% accuracy. When the Bio-VOCTM was used for breath collection, 5 of the 6 classifying volatiles were not detected in more than half of patients. The undetectable volatiles were: methyl undecane, dimethyl decane, trimethyl hexane, nonanal and tridecane. The remaining biomarker, isoprene, was readily detected using Bio-VOCTM collection, but did not differ in abundance based on infection status (see ESI†) in this cohort.

To the best of our knowledge, this is the first reported comparison of the two most widely used breath collection techniques in a field setting. While a single prior study did report the inferior performance of Bio-VOCTM for breath composition of an individual child (analyzed once using sampling bags and once using Bio-VOCTM),¹⁵ comprehensive comparisons of breath collection methods with consistent downstream analyses have been lacking.

Direct study-to-study comparisons have so far proven extremely challenging, due to the large number of additional variables involved in experimental designs, including differences in the amount of breath collected, data pre-processing methodologies, and the type of detector employed. Collecting breath *via* sampling bags is expected to result in a more mixed breath sample (oral plus alveolar breath) that includes an increased number of contaminants compared to the end-expiratory (alveolar) breath that is harvested by Bio-VOCTM. Moreover, endogenous compounds like isoprene and acetone are normally present at higher concentrations in alveolar air than in mixed exhaled air.⁹ In contrast, our current work indicates that breath collection by Bio-VOCTM yields reduced levels of typical breath volatiles, (*i.e.* acetone and isoprene), and fewer total VOCs present compared to sampling bags (Fig. 2). The lower-than-expected levels of acetone and isoprene, even after breath volume correction, suggest that the Bio-VOCTM dilutes the volatiles and introduces dead air or ambient air. With the Bio-VOCTM, the investigator must retrieve the device from the

patient and transfer breath contents over a sorbent tube (Fig. 1a and b), potentially permitting some VOC diffusion out of the open ends of the device prior to capture. The degree of potential leaks due to the open ends of the device is unknown; unfortunately, this is an inherent feature of the device that cannot be controlled by the operator. Another possible explanation for the apparently poorer-than-expected Bio-VOC™ performance is that sampling bags may be more airtight in design. Once the requisite liter of breath is collected, there is negligible potential for leaking or diffusion with sampling bags.

Other significant VOCs in breath, previously associated with malaria, α -pinene and 3-carene, failed to reach significance with the Bio-VOC™ based on infection status. Our data suggest that this failure may be a result of greater variability in the levels of these compounds when sampled by Bio-VOC™. The coefficients of variation were 104% and 130% for α -pinene and 3-carene for the Bio-VOC™ study, *versus* 28% and 30% for the terpenes for the sampling bags study. Therefore, our results suggest that the increased variability is caused by measuring near the limit-of-detection (and therefore outside the dynamic range) in samples collected *via* Bio-VOC™, *versus* well above it for samples collected *via* sampling bags. An additional source of variability may be differences in flow rates introduced by manually plunging air in the sorbent tube when using the Bio-VOC™ sampler. Regardless, the Bio-VOC™ cohort provides modest additional support for the hypothesis that malaria infection increases breath levels of mosquito-attractive terpene compounds.

While Bio-VOC™ has been successfully used for the analysis of breath volatiles to study a number of diseases and health status,^{16–20} the targeted exhaled breath volatiles were known *a priori*, therefore the suitability of the Bio-VOC™ to detect these compounds had been established prior to study. However, our pilot results suggest that sampling bags are better suited for biomarker discovery and untargeted search of volatiles in pediatric populations, due to the capacity for collection of a larger number of volatiles.

Our current work has several potential limitations. Most notably, our work represents an unplanned comparison of data from two distinct patient populations, rather than a prospective analysis of the same patients with two distinct methods. Although our two cohorts appear largely similar in terms of overall clinical characteristics, they may differ in some unaccounted-for manner that affects the number or diversity of VOCs detected, independent of collection methodology. We also note that the Bio-VOC™ was designed for use by adults, not the pediatric population (ages 3 to 12 years) under investigation in this work. However, the average forced vital capacity of preschool-aged (and older) children is greater than 1 L, suggesting that the poor performance of the Bio-VOC™ is not likely to be due to incomplete filling of the ~100 mL device.^{21–23}

Conclusions

Altogether, our results provide strong preliminary evidence that the use of sampling bags is ideal for breath biomarker discovery studies in pediatrics. While the Bio-VOC™ may demonstrate distinct performance characteristics with children *versus* adults, our studies indicate that the Bio-VOC™ sampler may be more suitable for the detection and analysis of

disease-specific endogenous biomarkers, in particular those found at high concentrations. Future prospective studies, in particular studies that examine repeated breath sampling from the same subjects, are needed to confirm these preliminary findings.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Comparison of Bio-VOC™ and sampling bag for breath collection. Pediatric breath collection via Bio-VOC™ (a) and sampling bag (c). For each method, field-site videos of breath collection are presented in Movies S1 and S2,[†] respectively. Schematic of breath collection methods for Bio-VOC™ (b) and sampling bag (d). Step 1, The empty collection device. Step 2, patient exhales into the collection device. Unique breath volatile organic compounds visualized as different color dots. Step 3, Sorbent tube is affixed to the collection device. Step 4, Breath volume driven through sorbent tube by depressing plunger or using an air pump. Breath metabolites are captured on the sorbent tube. Step 5, Breath metabolites are released from the sorbent tube by thermal desorption and measured by GC/MS. LOD: Limit of detection. Overall, the sampling bag (c, d) collects a larger volume of breath (Step 3) leading to a greater quantity of breath metabolites captured. This in turn is reflected as higher signals by GC/MS, including multiple breath metabolites that were undetectable via the Bio-VOC™ (Step 5).

Fig. 2.

Comparison of breath volatile organic compounds (VOCs) using two different collection methods (Bio-VOC™ and sampling bags). (a) Median detected number of volatiles in both breath collection methods, Bio-VOC™ and bag. Median and interquartile range are shown. P-Values, Mann Whitney *U*-tests. (b) Portion of representative total ion chromatogram from both studies. * = internal standard, ** = column contaminant, number = compound.

Compound annotations are as follows: 1 = 1,3,5-cycloheptatriene, 2 = acetic acid, 3 = 4-ethylbenzamide, 4 = hexanal, 5 = propyl-propanedioic acid, and 6 = 2,3,4-trimethyl-hexane.

Fig. 3. Common breath volatiles using Bio-VOC™ (alveolar air) and sampling bags (mixed air). (a) Comparison of total VOCs detected above the limit-of-detection in more than half of patients in one or both studies. (b) Heat maps showing intensities of the 7 compounds found in common to both breath collection methods studies (ND = compound not detected).

Fig. 4.

Comparison of endogenous volatiles isoprene and acetone using two different collection methods (Bio-VOC™ and sampling bags). (a) Structure and chemical information for the two most common and abundant compounds in breath. MW = molecular weight, RT = retention time. (b) Breath abundance of acetone and isoprene across the two studies. Median and interquartile range are shown. *P*-Values, Mann Whitney *U*-tests.