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14. ABSTRACT
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PAPER

Storage stability of exhaled breath on Tenax TA

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Abstract

Exhaled breath is coming to the forefront of non-invasive biomarker discovery efforts. Concentration of exhaled breath volatile organic compounds (VOCs) on thermal desorption (TD) tubes with subsequent analysis by gas chromatography-mass spectrometry (GC-MS) has dominated this field. As discovery experimentation increases in frequency, the need to evaluate the long-term storage stability of exhaled breath VOCs on thermal desorption adsorbent material is critical. To address this gap, exhaled breath was loaded on Tenax TA thermal desorption tubes and stored at various temperature conditions. 74 VOCs, 56 of which have been previously uncharacterized, were monitored using GC-MS over a period of 31 d. The results suggest that storage of exhaled breath at cold temperatures (4 °C) provides the most consistent retention of exhaled breath VOCs temporally. Samples were determined to be stable up to 14 d across storage conditions prior to gaining or losing 1–2 standard deviations in abundance. Through gene set enrichment analysis (GSEA), certain chemical classes were found to be positively (acids) or negatively (sulfur-containing) enriched temporally. By means of field sample collections, the effect of storage and shipping was found to be similar to those studies preformed in the laboratory at 4 °C. Collectively this study not only provides recommendations for proper storage conditions and storage length, but also illustrates the use of GSEA to exhaled breath based GC-MS data.

Introduction

Exhaled breath has become a highly researched sampling medium for non-invasive volatile organic compound (VOC) biomarker discovery. However due to the dilute nature of VOCs in breath, volatiles must be concentrated prior to offline analysis [1]. Several methods exist for VOC concentration, including solid-phase microextraction (SPME) and thermal desorption (TD) tubes. In general, these methods require binding of VOCs to a bed of adsorbent, commonly either a single or mixture of carbon or porous polymer, through passive or active sampling [2]. Subsequent desorption of volatiles from the media, via heat, allows for the introduction into the gas chromatograph-mass spectrometer (GC-MS) for separation and detection [2–4].

The need to store exhaled VOCs on adsorbent media, due to offsite sampling or instrumentation throughput, for prolonged periods of time will become an integral part of breath testing [5]. Historically, Tenax TA has been a popular adsorbent for exhaled breath trace volatiles analysis due to its hydrophobic nature and the wide range of compound retention [6–8]. As such, it is necessary to understand how exhaled breath compounds are retained on Tenax TA across varying storage durations, temperatures and field conditions.

The storage of volatiles within sampling bags has been extensively studied [9–19]. However, the storage duration of volatiles on Tenax adsorbents has not received the same sizable attention [5, 20–22]. For example, recently van der Schee *et al* showed that 10 exhaled breath compounds can be transported and

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stored for up to 14 d, on Tenax GR, at refrigerated conditions, while still allowing for discrimination between healthy and lung cancer patients [5]. However, the results did not demonstrate whether or not a mixture of samples stored at varying durations would impact results. High-throughput studies are likely to involve a mixture of samples stored at short and long durations, respectively. This difference in storage time may result in compound degradation, retention and potentially chemical reactivity. Effects that are attributed to storage duration may preclude identification of human-state VOC correlates, especially if matched pairs (i.e. pre versus post stimuli comparison) are not employed. Although collectively these studies have evaluated the storage characteristics of several VOCs on Tenax sorbent materials, a long term, highly sampled common source, time series of exhaled breath VOCs on Tenax TA has not been performed.

The purpose of this work is to provide an assessment of a large group of exhaled breath compounds and their storage stability across both laboratory and field sampling scenarios. In this manuscript, we show the overall storage stability of 74 known exhaled breath compounds, as referenced by de Lacy Costello *et al*, on Tenax TA evaluated over a period of 31 d across three distinct temperature conditions [23]. Next, we identify specific exhaled breath compounds and compound classifications as increasing or decreasing over the 31 d time course. Finally, we illustrate, via field sampling, that storage and transportation introduces variability of VOCs at similar rates to controlled laboratory experiments over 36 d of storage.

Experimental

Human subject recruitment and information

The human subject for laboratory testing was a non-smoking male volunteer in our laboratory. The volunteer was informed of the experimental parameters prior to experiment initiation.

All human subjects for field-testing were active duty military. Each test subject was a qualified aviator of variable age, rank and educational level stationed at an Air Force base in the Eastern United States. This research was deemed Not Human Use Research per the Air Force Research Laboratories Institutional Review Board (FWR20140142N). Sampling for this study was part of a larger command-directive aviator occupational health assessment. Subjects were informed, and free to opt out without penalty, but written consent was not required.

Experimental setup and exhaled breath collection

All test subjects were verbally instructed on the exhaled breath collection protocol as described in Harshman *et al* [24]. Briefly, participants were instructed to take a breath, exhale excess inspired air and the lung tidal volume (until their abdomen was tight) and fill the bag with the remaining air from the lungs (the functional residual capacity). This procedure was repeated until the bag was full (approximately 1–3 times for 1 l bags and >25 times for 25 l bags). All exhaled breath VOCs were concentrated on preconditioned stainless steel Tenax TA thermal desorption tubes, selected due to its hydrophobic properties, fitted with brass caps and polytetrafluoroethylene (PTFE) ferrules as recommended in the US EPA TO-17 method (TD, Markes International, South Wales, UK) [25]. Briefly, 550 ml of exhaled breath (total volume) was pulled through an inline Tenax TA TD tube using a calibrated MultiRae Pro pump (RAE Systems Inc., San Jose, CA) [24]. The maximum observed percent CO_2 was recorded via the MultiRae Pro pump real-time sensor, to confirm end-tidal portion of the exhaled breath was collected, for each bag sample [26, 27]. The CO_2 (%) data is provided in supplemental data 1 (stacks.iop.org/JBR/10/046008/mmedia). Samples with % CO_2 less than 3.5% were removed from the analysis. All sample TD tubes were capped (brass caps with PTFE ferrules) and stored at 4 °C (unless otherwise noted) until analysis (Markes International).

In the laboratory setting, the end tidal portion of exhaled breath from a single individual was collected in brand new 25 l ALTEF polypropylene bags in order to minimize the effect of bag related contaminants (three bags total, Jensen Inert Products, Coral Springs, FL). Additionally, to reduce potential changes in VOC composition, 30 individual Tenax TA thermal desorption tubes were immediately loaded from the bag after filling (approximately 2 h per bag and 6 h for 90 total TD tubes from the 3 separate bags) [19]. 10 tubes from each bag were placed at each cold (4 °C), ambient (21 °C) and hot (37 °C) temperatures (30 total tubes in each condition). Storage temperature was monitored daily during the workweek for the duration of experiment and the results are provided in supplemental data 2. Three tubes from each bag and condition, 9 total tubes, were analyzed immediately as baseline samples. Subsequently at each time point, three tubes were removed, 9 total tubes: one from each bag and each storage condition, twice a week (Monday and Thursday) for 5 total weeks (31 total days). An illustration depicting the experimental setup, a plot of the fill order versus analysis time and an illustration of GC-MS data analysis can be found in supplemental data 3(A)–(C). TD tubes were analyzed by TD-GC-MS as described below.

For field sampling, exhaled breath was collected from 12 volunteer pilots (79 total samples) over a 53 d period. The final portion of each test subject's exhaled breath was collected in 1 l ALTEF polypropylene bags as described previously, in the locker room adjacent to the flight line immediately prior to the 'step' for a training sortie, roughly 30 min prior to takeoff. TD tubes were loaded with the exhaled breath via MultiRae Pro, shipped and stored for variable lengths of time under refrigerated conditions (4 °C, supplemental data 1). An illustration depicting the sampling setup can be found

in supplemental data 3(D). TD tubes were analyzed by TD-GC-MS as described below.

Thermal desorption gas chromatography mass spectrometry (TD-GC-MS)

Thermal desorption and GC-MS analysis were conducted as described in Harshman *et al* [24]. Briefly, thermal desorption of all sorbent tubes was carried out on a Markes International TD-100 thermal desorber. Prior to primary desorption, 2 ppm TO-14A internal standard (bromocholoromethane, 4-bromofluorobenzene, chlorobenzene-d5, and 1,4-difluorobenzene) were automatically applied by the TD-100 (Linde Gas North America, Stewartsville, NJ). Primary thermal desorption was performed at 310 °C over 10 min. The cold trapping was performed on an Air Toxics trap, a dual bed of Carbograph 1 and Carbosieve S-III, operated at a flow rate 50 ml min^{-1}, flow path temperature 160 °C, trap purge time 1 min, low temperature of 25 °C, high trap temperature 315 °C, trap heating rate 40 °C s^{-1} and a post trap split 3.5:1 (Markes International).

GC-MS analysis was performed on a Thermo Scientific Trace Ultra-ISQ gas chromatograph in line with a single quadraple mass spectrometer (Waltham, MA). Desorbed compounds were separated on a Restek Rxi-624Sil capillary column (60 m $\times$ 0.32 mm ID $\times$ 1.80 μm df), with 2 ml min^{-1} constant flow of helium carrier gas (99.999%, Bellefonte, PA). Separations were performed from 40 °C to 240 °C with temperature increasing at a rate of 10 °C min^{-1}. Temperature was held at 240 °C for 20 min. 70 eV electron ionization was conducted with an ion source of 275 °C. Spectral scans of 35–300 m/z were acquired every 0.154 s. Data was acquired using the Thermo Scientific Trace Finder EFS software package (v. 3.0). RAW to CDF file conversion was performed using the file conversion tool of the Xcalibur Software package (v. 3.0.63, Thermo Scientific, Waltham, MA).

Compound identification & computational peak registration

Exhaled breath GC-MS data was registered and aligned using the metabolite differentiation and discovery lab (MeDDL) software with the settings provided in supplemental data 4 [28, 29]. The registered data set was down selected to include only individual abundant ions that were unique to a particular compound via manual inspection. All compounds were tentatively identified based on spectral match to the NIST 11 Mass Spectral Library, as implemented in Thermo XCalibur Qual Browser Software package (v. 2.0, National Institute of Standards and Technology, Gaithersburg, MD, v 3.0.63, Thermo Scientific, Waltham, MA). The most abundant registered/aligned masses were used in down stream analyses. In total, 110 compounds were found in the laboratory data set. The compounds were further reduced to 74 compounds, as being previously found in exhaled breath based on de Lacy Costello *et al* [23]. The corresponding peak abundance values from these 74 compounds were used for further analyses, both laboratory and field (as found) samples.

Statistical analyses

All statistical analyses were performed within Matlab© software environment (v. R2013a, MathWorks, Natick, MA). The R statistical software environment and ggplot2 (v2.0) package was used to graph discovered trends [30]. Registered/aligned total peak abundance distributions were observed to be variable across samples, likely due to collection or instrument variation, and hence, quantile normalization was applied [31, 32]. When applying quantile normalization, it is assumed that all samples follow a common distribution function and that individual samples are randomly warped in scale [33]. It was hypothesized that the main driver of total VOC variation in this study was tube-to-tube loading differences and run-to-run instrument variation, respectively. After quantile normalization, all statistical comparisons are relative to sample abundance ranks rather than absolute abundance [33]. Unfortunately, isoprene, a highly abundant breath VOC, was invariant after normalization i.e. any chemical feature (i.e. m/z abundance) that has an invariant rank will show abundance invariance after quantile normalization. A $\log_2$ transformation was applied to all peak abundances to force Gaussian distribution behavior. VOC abundances were standardized by their respective mean and standard deviation to remove any location or scale bias between VOCs. Field GC-MS samples were registered/aligned separately due to differences in instrument tune and chromatography but were otherwise identically processed. Isoprene, a major VOC found in exhaled breath, was used as a proxy quality control metric for field samples. A sample was removed from downstream analyses if un-normalized Isoprene ($m/z = 67$, RT $= 5.3$ min) was not among the top 10 registered/aligned peak abundances. 8/87 samples were excluded with a median Isoprene rank order of 225 given 1094 registered/aligned m/z features.

VOC variation was assessed by projecting all ions, with a tentative chemical identification, onto a lower dimensional space via principal component analysis (PCA). Spearman rank correlation (ρ), a measure to assess monotonic relationships, was measured between identified chemical markers (e.g. m/z 59 at 5.4 min or Acetone) and storage duration (days). VOC's were ordinated by storage duration spearman rank correlation for each condition separately. Those VOC's that met an arbitrarily set effect size (ρ: ± 0.6, $p < 0.01$) were graphed for trend assessment. In order to assess VOC loss or gain, standardized z-scores were calculated by subtracting the average compound abundance from individual compound abundance, then dividing each value by their standard deviation. These values were calculated separately for each compound and plotted in box-whisker plots. Standardization was performed due to differences in VOC concentrations across samples.

By removing the mean and standard deviation we focus on the temporal variation, making other extraneous sources of variation equal. Additionally, a locally weighted scatterplot smoothing (LOESS) was applied between storage duration and resultant abundance via ggplot2 implementation [30].

Gene set enrichment analysis (GSEA), a common bioinformatics technique applied to gene-expression phenotypic data to assess biological pathway activity that may vary across studies but share a common biological purpose or origin, i.e. to assess how random a subset of features, was dispersed across the ordinated feature list, was applied [34, 35]. The enrichment score (ES) indicates the non-randomness associated with the subset list placement, the hits, within the overall list, the rank ordered data set, and hence, enrichment [34]. The scores obtained are relative to a modified random walk model, i.e. increasing or decreasing a running sum statistic based on a feature's presence, indicating how different a subset of features is from a uniform placement [34]. Enrichment analysis is visualized by plotting a running enrichment score, as shown in figure 3(A), where the ES is the value represented by maximum distance from zero from walking the list [34]. An ideal result is to have a queried subset of features only at the top (positive enrichment) or bottom (negative enrichment) of the ordinated list. The arrival of features at the bottom or top of an ordinated list is assumed to be non-random, indicating a group or enrichment effect, such as a correlation with storage duration. The normalized enrichment score (NES) removes differences attributed to size of feature sets allowing for comparison of results across feature sets [34].

The GSEA was applied to our ordinated VOC markers to assess the overall chemical properties and storage stability to help generalize observed effects. All tentatively identified chemicals were annotated as Sulfur containing (7), Aldehyde (12), Ether (10), Alcohol (9), Ketone (15), Acids (7), Phenyl Hydrocarbon (6) or Alkene (6), based on De Lacy Costello *et al* [23]. The resultant annotations or feature subsets were queried against the VOC storage correlation spectrum to assess whether or not a specific chemical class tended to decrease (negatively enriched) or increase (positively enriched) with storage duration. Enrichment scores and normalized enrichment scores were reported for each chemical classification.

Field sample storage duration was calculated as the days between sample collection and instrument analysis (supplemental data 1). The correlation between peak abundance and storage duration was assessed for each subject from the field sampling ($n = 12$) separately, and averaged, or overall. A conservative effect size (ρ: ± 0.6, $p < 0.01$) was applied to mimic the laboratory test criteria. Storage stability was assessed per subject to account for the random effects known to occur between individuals due to, for example, dietary differences. We found that storage duration VOC correlates were fairly consistent across subjects with the exception of two, but whose dissimilarity is likely explained by poor storage range; the shortest duration for both was 17 d while 6.5 was the groups median. A rank product analysis, which in our case assesses correlation consistency, was applied across subjects to discover a robust set of less stable chemicals [36]. As a result, the two dissimilar examples previously mentioned were excluded.

All Venn diagrams were generated by the web application BioVenn [37].

Results

Overall exhaled breath compound stability

Compound stability on Tenax TA has been investigated, among many different compounds, conditions and sources, with generally mixed or conflicting results [5, 20–22]. To establish the optimum storage conditions in a laboratory setting, for consistent retention of exhaled breath compounds on Tenax TA over time, exhaled breath from a single individual filling three separate bags was analyzed regularly by GC-MS over a period of 31 d. Seventy-four known exhaled breath VOCs were monitored across this time period for changes in abundance in relation to the baseline samples [23]. A graphical depiction of the experimental setup and VOC information is provided in supplemental data 3 & 5. Figure 1 shows a plot of the first principal component (PC1) by days in storage. The PC1 explains 28% of the variation in the laboratory data set. The locally weighted scatterplot smoothing regression, or LOESS fit, applied to each storage condition separately suggests the cold (4 °C) storage temperature has less variation of overall exhaled breath VOCs over time, when compared to the ambient (21 °C) and hot (37 °C) storage temperatures. These data indicate that refrigerated conditions are preferred for the most stable exhaled breath VOC detection on Tenax TA thermal desorption tubes.

To assess additional sources of variation in the data set, such as from the experimental design, the second principal component (PC2) was calculated and plotted against storage length (supplemental data 6(A)). The 2nd principal component explains 11% of the variation in the laboratory data set. A distinct separation was noted and largely explained by sample-bag membership with samples from bag 1 being distinct from those samples from bag 2 and bag 3 (supplemental data 6(A)). Individual compounds related to this effect were identified from PC2 latent coefficients and Random Forest modeling input sensitivity (supplemental data 6(B)) [38]. The decision space for selecting the predictive set was determined to be >0.2 permuted variable delta error and >0.1 or <-0.1 PC2 latent coefficients. From this analysis, 12 compounds were identified as related to the PC2 effect (supplemental data 6(C)). Upon review of the identified compounds, a majority of the compounds can be attributed to exogenous sources, such as diet/flavorings, cleaning reagents/solvents and petroleum/combustion emissions (supplemental data 6(D)) [39–48]. Additionally, compounds associated with endogenous metabolism were identified (2,3-butan-

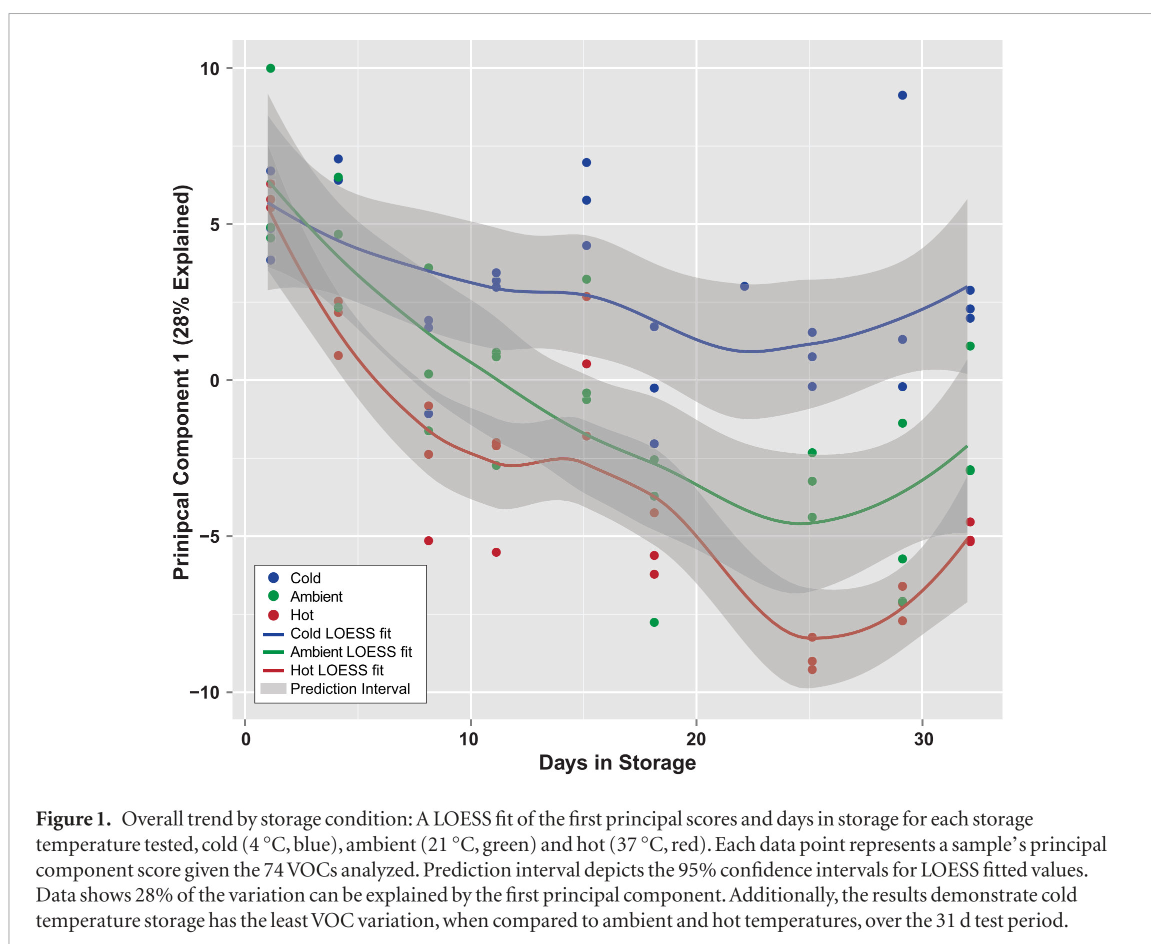


Figure 1. Overall trend by storage condition: A LOESS fit of the first principal scores and days in storage for each storage temperature tested, cold (4 °C, blue), ambient (21 °C, green) and hot (37 °C, red). Each data point represents a sample's principal component score given the 74 VOCs analyzed. Prediction interval depicts the 95% confidence intervals for LOESS fitted values. Data shows 28% of the variation can be explained by the first principal component. Additionally, the results demonstrate cold temperature storage has the least VOC variation, when compared to ambient and hot temperatures, over the 31 d test period.

edione and 2-pentanone) [42, 47, 48]. However, these compounds have been attributed to diet and food flavorings as well [39–41]. Collectively, these results suggest that although variation in the data set can be attributed to the sample-bag (PC2), the compounds associated with this trend are primarily of exogenous sources. As a result, the primary source of VOC variation in the laboratory data set is principal component 1 (figure 1).

Trends in exhaled breath compound stability

The data presented in figure 1 suggest a temporal trend in exhaled breath VOCs across all storage temperatures. To determine temporal changes of individual compounds across storage conditions, a Spearman rank correlation (ρ) was measured between identified chemical markers and storage duration (table 1, ρ: $\pm 0.6, p < 0.01$). These data suggest 24 compounds from the hot temperature (37 °C), 16 compounds from the ambient temperature (21 °C) and 5 compounds from the cold temperature (4 °C) have significant changes in abundance over the 31 d test period. Furthermore, the relative average abundance ratio (mean abundance of time point divided by median abundance of baseline ($t = 0$) time point) was calculated and included in table 1 to illustrate the fold change relative to the baseline measurement for these compounds. Collectively, these results illustrate the cold storage condition has the least number of compounds significantly changing temporally with minimal relative change to the baseline measurement over the entire test period. As a result, cold storage is recommended for prolonged storage of exhaled breath VOCs on Tenax TA TD tubes.

To estimate a threshold of allowable days in storage prior to excessive gain or loss in VOC concentration, a box-whisker plot was constructed by plotting z-scores (standard deviation from the mean) across the storage duration for those compounds meeting the significance criteria (ρ: $\pm 0.6, p < 0.01$) for each storage temperature (figure 2). These data show that the day 14-time point is the longest temporal point where variation appears to be stable at the average value (z-score $= 0$) for most conditions. However, there are examples where stability is affected prior to 14 d. For example, the compounds increasing under ambient conditions show a 10 d stability. Notwithstanding, the variation present after deviation from the average value ($z = 0$) is more subdued and may be attributed to a significant reduction or saturation of compound. Taken together, these results suggest, in general, analysis by day 14 is recommended to avoid significant gain or loss of VOCs attributed to prolonged storage.

A high amount of compound overlap across storage conditions was observed with regards to the highlighted trends (table 1). To illustrate this point, a Venn diagram was constructed of the exhaled breath compound IDs from table 1 (supplemental data 7). These results show, of the 25 total exhaled breath compounds identified to change temporally, only one compound tentatively identified as acetaldehyde, is distinct to a

Table 1. Summary of the compounds identified to change temporally across laboratory storage conditions.

CAS	Compound	Storage condition	Spearman rank correlation	Overall trend	Relative day 3	Relative day 14	Relative day 31
98-86-2	Acetophenone	Hot	0.779 31	Increase	1.4096	1.3481	2.1872
79-31-2	2-methylpropanoic acid	Hot	0.893 61	Increase	1.2297	1.5581	2.08
79-09-4	Propanoic acid	Hot	0.712 01	Increase	1.2909	1.1977	1.7724
67-68-5	Dimethyl sulfoxide	Hot	0.926 28	Increase	7.5568	14.7421	19.872
60-35-5	Acetamide	Hot	0.611 52	Increase	4.2927	4.9969	6.5224
142-62-1	Hexanoic acid	Hot	0.643 13	Increase	1.5787	1.5994	3.5095
111-14-8	Heptanoic acid	Hot	0.659 81	Increase	1.2989	1.588	2.8382
107-92-6	Butanoic acid	Hot	0.746 78	Increase	1.1371	1.2197	1.889
107-02-8	2-propenal	Hot	0.714 62	Increase	1.2717	1.7039	1.9552
100-52-7	Benzaldehyde	Hot	0.766 93	Increase	0.9963	1.0303	1.5097
96-17-3	3-methylbutanal	Hot	−0.727 42	Decrease	0.9216	0.9086	0.6186
75-21-8	Ethylene oxide	Hot	−0.742 87	Decrease	0.9771	0.8869	0.7673
75-18-3	Dimethylsulfide (thiopropane)	Hot	−0.9311	Decrease	0.7227	0.3839	0.1856
75-09-2	Methylene chloride (dichloromethane)	Hot	−0.644 11	Decrease	0.8127	0.9956	0.6174
67-64-1	2-propanone (acetone)	Hot	−0.787 36	Decrease	0.8475	0.7955	0.5477
66-25-1	Hexanal	Hot	−0.665 55	Decrease	0.7325	0.6325	0.5399
513-86-0	3-hydroxy-2-butanone	Hot	−0.950 87	Decrease	0.5229	0.1301	0.049
463-58-1	Carbonyl sulphide	Hot	−0.613 46	Decrease	0.8851	0.7797	0.6924
42848-06-6	1-(methylthio)-1-propene, (1E)-	Hot	−0.911 95	Decrease	0.9262	0.7764	0.433
3877-15-4	1-(methylthio)-propane	Hot	−0.827 15	Decrease	0.875	0.7616	0.5055
2216-34-4	4-methyloctane	Hot	−0.606 27	Decrease	0.9008	0.9203	0.7132
115-07-1	Propene	Hot	−0.899 82	Decrease	0.8806	0.726	0.5512
108-10-1	4-methyl-2-pentanone	Hot	−0.845 82	Decrease	0.9803	0.8838	0.6832
10152-76-8	3-(methylthio)-1-propene (allyl methylsulfide)	Hot	−0.949 95	Decrease	0.7817	0.4413	0.1857
79-31-2	2-methylpropanoic acid	Ambient	0.895 31	Increase	1.2281	1.736	2.1844
79-09-4	Propanoic acid	Ambient	0.645 17	Increase	0.9711	1.4078	1.383
67-68-5	Dimethyl sulfoxide	Ambient	0.905 08	Increase	3.1386	11.3056	14.7823
60-35-5	Acetamide	Ambient	0.704 65	Increase	2.6019	5.132	5.7111
107-92-6	Butanoic acid	Ambient	0.684 59	Increase	1.3555	1.9907	1.9411
96-17-3	3-methylbutanal	Ambient	−0.627 18	Decrease	1.1419	0.9118	0.8175
75-21-8	Ethylene oxide	Ambient	−0.624 16	Decrease	1.0722	0.9663	0.8446
75-18-3	Dimethylsulfide (thiopropane)	Ambient	−0.827 53	Decrease	0.9358	0.6862	0.457
75-09-2	Methylene chloride (dichloromethane)	Ambient	−0.6757	Decrease	0.8746	0.7915	0.6544
67-64-1	2-propanone (acetone)	Ambient	−0.773 98	Decrease	1.0192	0.6604	0.4896
513-86-0	3-hydroxy-2-butanone	Ambient	−0.600 65	Decrease	0.4482	0.5371	0.1625
42848-06-6	1-(methylthio)-1-propene, (1E)-	Ambient	−0.733 04	Decrease	1.1043	0.9164	0.6731
3877-15-4	1-(methylthio)-propane	Ambient	−0.615 39	Decrease	1.0638	0.9183	0.747
115-07-1	Propene	Ambient	−0.756 12	Decrease	1.0212	0.7875	0.6524
108-10-1	4-methyl-2-pentanone	Ambient	−0.613 55	Decrease	1.0824	0.8976	0.7712
10152-76-8	3-(methylthio)-1-propene (allyl methylsulfide)	Ambient	−0.915 78	Decrease	1.0489	0.7358	0.5044
75-07-0	Acetaldehyde	Cold	0.665 66	Increase	1.01	1.306	2.0663
67-68-5	Dimethyl sulfoxide	Cold	0.704 55	Increase	0.8923	1.9972	2.6303
67-64-1	2-propanone (acetone)	Cold	−0.648 15	Decrease	0.9668	0.9547	0.5107
513-86-0	3-hydroxy-2-butanone	Cold	−0.634 19	Decrease	0.6766	0.3187	0.1407
75-18-3	Dimethylsulfide (thiopropane)	Cold	−0.650 05	Decrease	1.2631	0.9997	0.7675

Note. Relative average abundance ratio: mean abundance of time point/median abundance of baseline ($t = 0$) time point. Data shows cold storage has the least number of compounds significantly change with minimal relative change with regards to the baseline measurement.

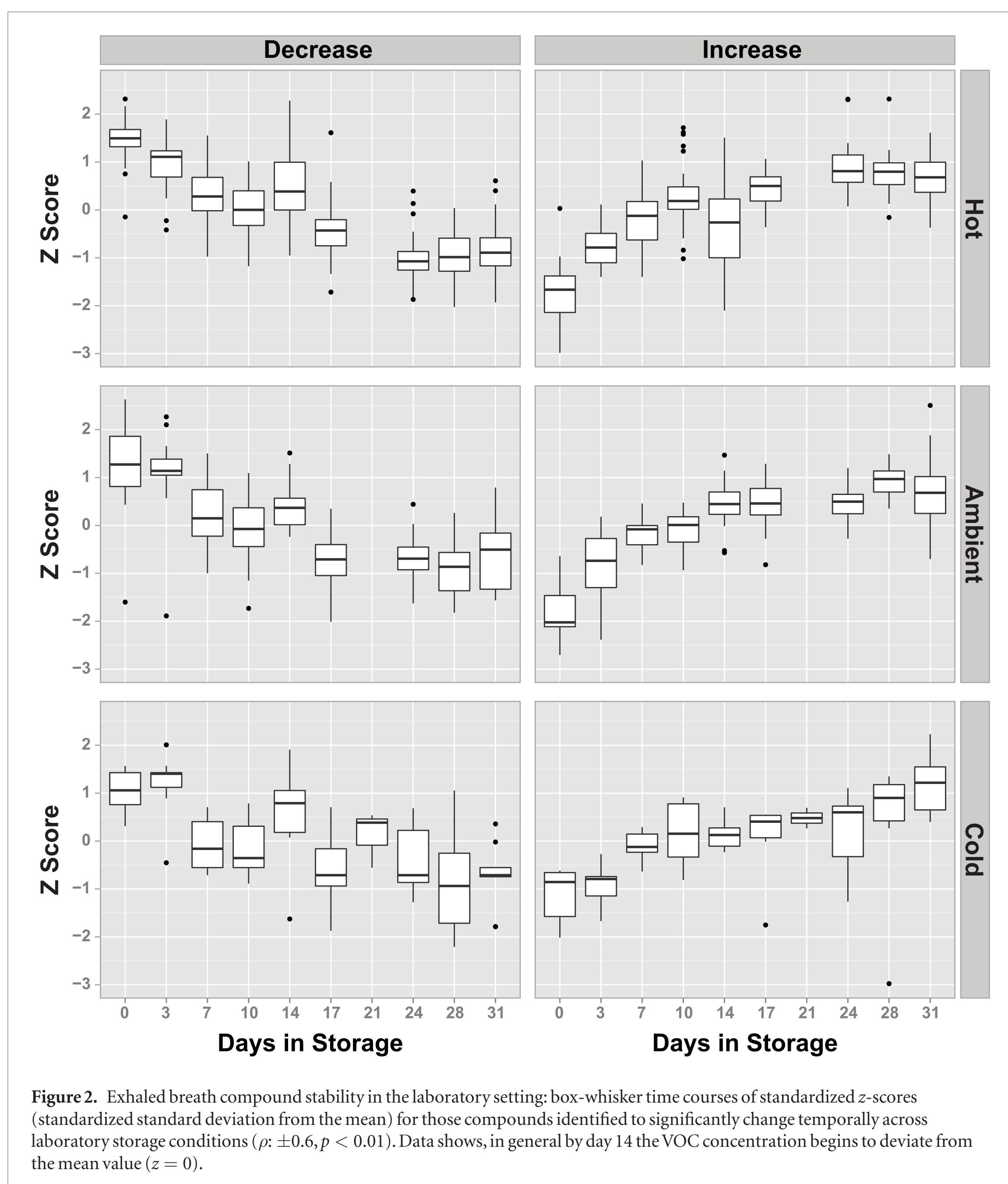
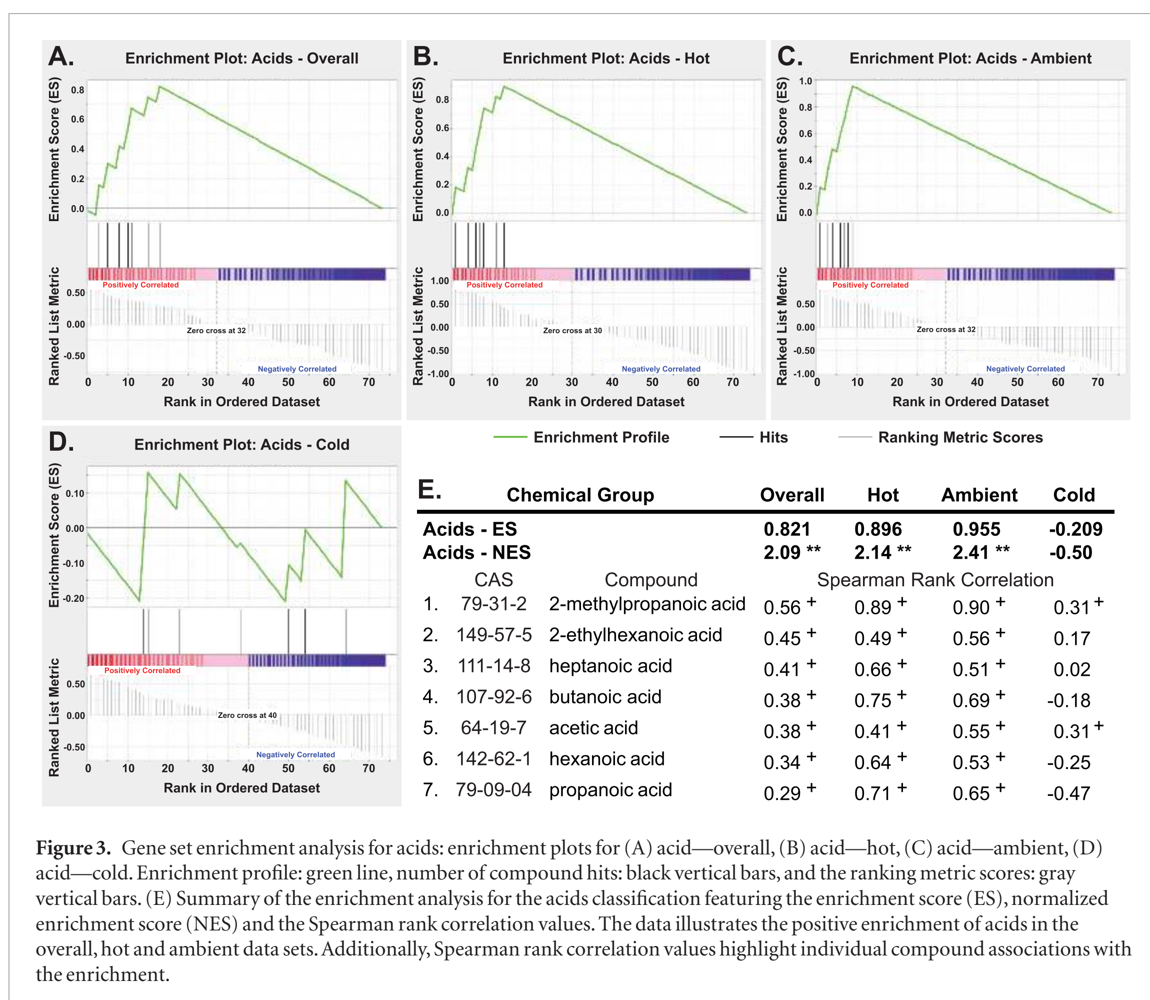


Figure 2. Exhaled breath compound stability in the laboratory setting: box-whisker time courses of standardized *z*-scores (standardized standard deviation from the mean) for those compounds identified to significantly change temporally across laboratory storage conditions (ρ: ± 0.6, $p < 0.01$). Data shows, in general by day 14 the VOC concentration begins to deviate from the mean value ($z = 0$).

specific storage temperature (cold, supplemental data 7). The remaining 24 compounds are proper subsets of the proceeding higher temperature tested. Furthermore, Venn diagrams, constructed from parsed exhaled breath compounds previously determined as increasing or decreasing temporally, show a strong overlap in identifications across each group (supplemental data 8(A) and (B)). Collectively, these data suggest exhaled breath compounds have a high amount of overlap based on storage temperature.

To determine if specific chemical classes, such as alkanes, aldehydes etc within each storage temperature tend to change temporally, a GSEA was applied to the laboratory data. For example, the GSEA suggests the exhaled breath compounds tentatively identified and classified as acids are positively enriched, or tend to increase with storage duration, in hot (37 °C) and ambient (21 °C) storage temperatures, but tend to be stable, generally, when samples are stored at cold temperature (4 °C) (figures 3(A)–(D)). These results would indicate a potential chemical reaction, such as oxidation of aldehydes, potentiated by increased storage temperature, resulting in the observed increase in acid abundance over time. Similarly, the GSEA analysis shows a marginal negative enrichment effect (*fdr* = 0.08, nominal *p*-value = 0.047) for sulfur containing compounds retained on Tenax TA over time at ambient temperatures. This effect could be attributed to the high reactivity of sulfur containing compounds [49]. Though more likely, as our data and others have suggested, several sulfur compounds are not retained well on Tenax TA at elevated temperatures [49, 50]. However, additional, more targeted and controlled experimentation would be required to confirm the hypotheses surrounding the GSEA result mechanisms e.g. if these results are a consequence of bag artifacts or



Chemical Group		Overall	Hot	Ambient	Cold
Acids - ES		**0.821**	**0.896**	**0.955**	**-0.209**
Acids - NES		**2.09 ****	**2.14 ****	**2.41 ****	**-0.50**
CAS	Compound	Spearman Rank Correlation			
1. 79-31-2	2-methylpropanoic acid	0.56 +	0.89 +	0.90 +	0.31 +
2. 149-57-5	2-ethylhexanoic acid	0.45 +	0.49 +	0.56 +	0.17
3. 111-14-8	heptanoic acid	0.41 +	0.66 +	0.51 +	0.02
4. 107-92-6	butanoic acid	0.38 +	0.75 +	0.69 +	-0.18
5. 64-19-7	acetic acid	0.38 +	0.41 +	0.55 +	0.31 +
6. 142-62-1	hexanoic acid	0.34 +	0.64 +	0.53 +	-0.25
7. 79-09-04	propanoic acid	0.29 +	0.71 +	0.65 +	-0.47

Figure 3. Gene set enrichment analysis for acids: enrichment plots for (A) acid—overall, (B) acid—hot, (C) acid—ambient, (D) acid—cold. Enrichment profile: green line, number of compound hits: black vertical bars, and the ranking metric scores: gray vertical bars. (E) Summary of the enrichment analysis for the acids classification featuring the enrichment score (ES), normalized enrichment score (NES) and the Spearman rank correlation values. The data illustrates the positive enrichment of acids in the overall, hot and ambient data sets. Additionally, Spearman rank correlation values highlight individual compound associations with the enrichment.

on tube reactions [19, 51, 52]. Interestingly, a majority of the chemical classifications, such as alcohols and alkenes, do not show a significant enrichment in any storage temperature (supplemental data 9). Supplemental data 9 shows the complete summarized results from the GSEA with classification normalized enrichment scores (NES) and individual compound Spearman rank correlations. In general, these results establish the use of enrichment analyses or GSEA as a means to look for concerted effects in exhaled breath given chemical properties associated with tentatively identified chemicals.

Exhaled breath compound stability under field scenarios

The data presented in figures 1 and 2 and table 1 are representative of well-controlled laboratory conditions. However, these ideal conditions are often not attainable in large multi-site sampling schemes [5]. To determine if field sampling, i.e. alternative environments, different exhaled breath compositions, travel and storage duration, affect compound stability, exhaled breath was collected from 12 volunteer pilots off site over a 53 d period, shipped cold (4 °C) to the laboratory, stored for variable lengths of time (5–36 d) at cold temperatures (4 °C) and analyzed by GC-MS (supplemental data 1). A subset of the original 74 exhaled breath compounds (54) used in the laboratory data analysis with matching ions and retention times found in the field sampling data set were monitored across this time period for changes in peak abundance over time (supplemental data 5). Figure 4(A) shows the overall trend in the data by plotting the first principal component by days in storage. The cold lab temperature principal component, derived separately, was included for graphical comparison. In the field data set, the first principal component accounts for 19% of the variability in the data set.

To discover exhaled breath compounds driving the overall trends from figure 4(A), the Spearman rank correlations were measured, as applied previously to the laboratory data. Figure 4(B) depicts the three exhaled breath compounds determined to significantly change temporally, octane increasing while 6-methyl-5-hepten-2-one and 2-pentanone decrease (ρ: ± 0.6, $p < 0.01$). Interestingly, none of these exhaled breath compounds, in the reduced field sampling data set, were found to change in any of the laboratory storage temperatures (table 1). This result maybe a consequence of the approximately 27% (54/74) reduction in the compounds analyzed for the field sampling data set (supplemental data 5). This is most likely a consequence of differences in the exhaled breath composition between the studies. For example, dimethyl sulfoxide was found to increase in all three storage temperatures tested. However, no matching ion and retention time pair was found in the field sampling data set, and hence not reported for the analy-

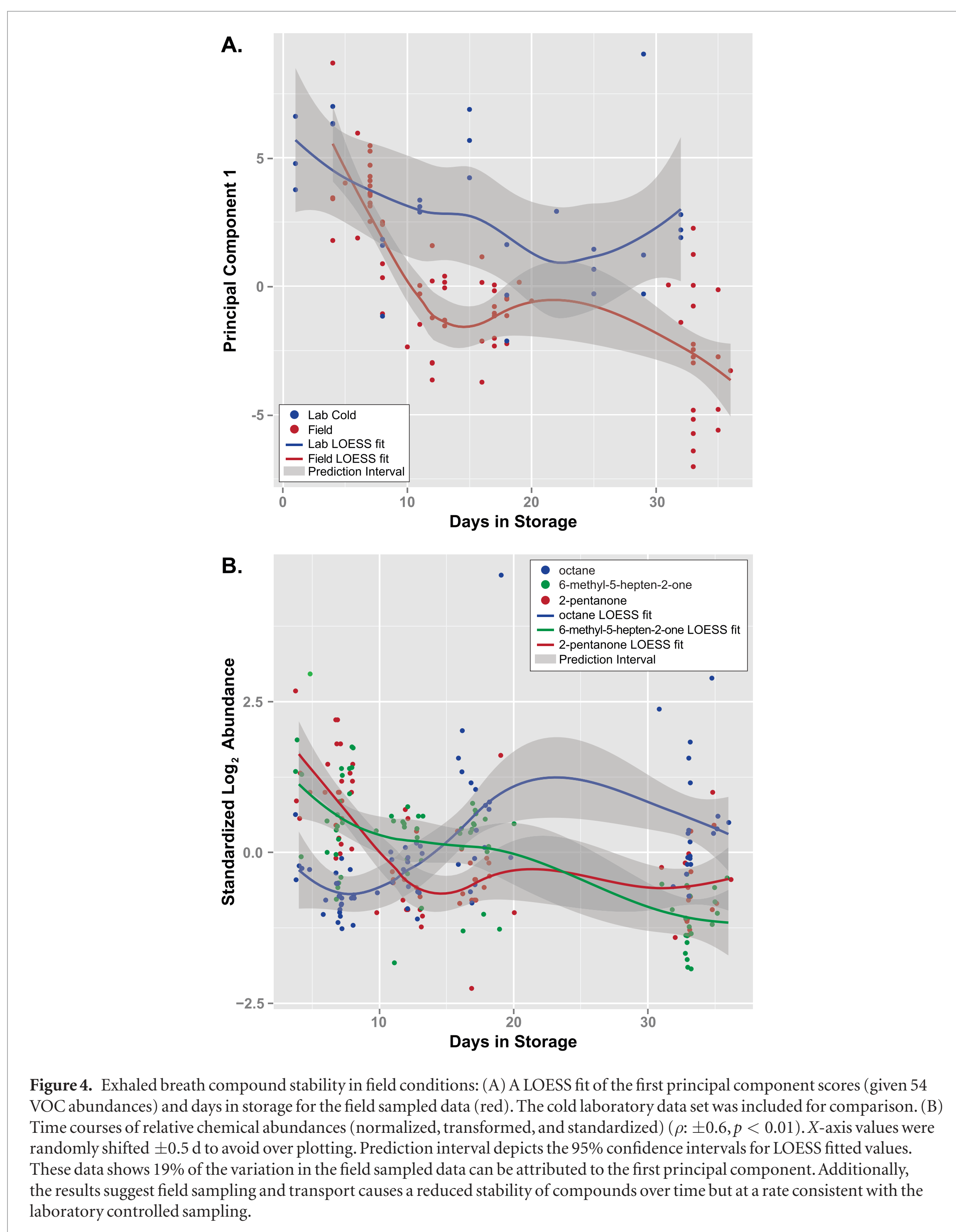


Figure 4. Exhaled breath compound stability in field conditions: (A) A LOESS fit of the first principal component scores (given 54 VOC abundances) and days in storage for the field sampled data (red). The cold laboratory data set was included for comparison. (B) Time courses of relative chemical abundances (normalized, transformed, and standardized) (ρ: $\pm0.6, p < 0.01$). X-axis values were randomly shifted ±0.5 d to avoid over plotting. Prediction interval depicts the 95% confidence intervals for LOESS fitted values. These data shows 19% of the variation in the field sampled data can be attributed to the first principal component. Additionally, the results suggest field sampling and transport causes a reduced stability of compounds over time but at a rate consistent with the laboratory controlled sampling.

sis. Similarly, ion and retention time matched pairs were not found in the field sampling data set for 5 additional compounds found to significantly change in the laboratory data set, an overall reduction of 24% potential candidates. Although significant at ρ: $\pm0.6, p < 0.01$, these results were generated with the conservative statistical cutoffs used for the laboratory data analysis. Supplemental data 10(A) and (B) depicts the traces and tentative IDs of the top 15 compounds, identified via rank product across subject Spearman correlation, that either increase or decrease, respectively. A Venn diagram was drawn to assess correlation overlap between field and laboratory study. Supplemental data 10(C) shows that only 7 of the compounds found in the expanded field results are found in any laboratory storage temperatures. Taken together, these results suggest that field sampling and transport, even at cold temperatures, of exhaled breath VOCs on Tenax TA thermal desorption tubes, can cause a reduced stability of compounds over time but at a rate consistent (3/51 in field versus 5/74 in lab cold storage; ρ: $\pm0.6, p < 0.01$) with laboratory controlled sampling.

Discussion

As the clinical utility of exhaled breath VOC analysis is embraced, the need to assess the storage stability of a wide number of VOCs on sampling media is

required for biomarker discovery applications. In this manuscript, 74 known exhaled breath compounds were monitored over a 31 d period [23]. As stated previously, others have tested the stability of volatiles on adsorbent media [5, 20–22, 53]. For example, Peters *et al* previously reported a 12 month stability of benzene, toluene and m-xylene on Tenax TA TD tubes [20]. Although it is only a single example, collectively the previous reports have assessed 29 total volatiles for stability on Tenax sampling media [5, 20–22]. We have further built upon these studies by monitoring an additional 56 exhaled breath compounds not previously characterized.

Eighteen of the 74 compounds found in this study were previously assessed for storage stability on Tenax sampling media [5, 20–22]. Our results are, in general, in line with the reported individual compound stabilities. For example, Brown *et al* have shown that under ambient temperatures, n-hexane, 4-methyl-2-pentanone and toluene are stable for 4 weeks on Tenax TA [21]. Furthermore, van der Schee *et al* have shown that under refrigerated temperatures isoprene, ethanol, limonene, toluene and N,N′-dimethylacetamide are stable on Tenax GR for 2 weeks [5]. These results were found to be consistent within this report.

Although a majority of the data is in agreement with previous reports, conflicts do exist. For instance, previous reports suggest both acetone and dimethyl sulfide to remain stable for up to 2 weeks under refrigerated conditions [5]. However, in the present study these compounds were identified to decrease over time, 31 d, under the same storage temperature. It is likely that the observed difference between the studies is due to the increased duration samples were stored. Similarly, hexanal has been reported to have substantially decreased recovery on Tenax TA, at 4 weeks, under ambient (20 °C) temperatures [21]. However, in the present study, hexanal was observed to decrease only at the hot (37 °C) temperature. This discrepancy may be attributed to differences in abundances of hexanal between the two studies. Although conflicts occur, these results highlight the consistencies of the current study with previous results, across an extended number of compounds, storage temperatures and durations, further validating these experiments.

Regarding chemical classes, the most striking enrichment result was found for carboxylic acids. We hypothesize that this effect was formed as a result of oxidation of structurally similar aldehydes. For example, a paired decrease in hexanal was observed with an increase in hexanoic acid under hot storage conditions. However, not all aldehydes showed this same trend suggesting some alternative reaction may be occurring. The hypothesis of on tube oxidation is supported by the identification of oxidative products of Tenax TA at high storage temperatures in our data set, leading to increased amounts of common degradation products acetophenone and benzaldehyde [54, 55]. Taken together, these results support the hypothesis that oxidation of VOCs on Tenax TA occurs in some quantity among all storage temperatures. Additionally, these data suggest the oxidative reactions can be lessened through storage at cold temperatures [21]. However as stated previously, further, more targeted and controlled experimentation will be required to adequately test this hypothesis as this study cannot discount a potential for sampling bag related artifacts contributing to the observed results [19, 51, 52].

The field sampling study was conducted to assess how alternative sampling environments, different exhaled breath compositions, transportation, and storage of samples may affect statistical interpretation. This is a direct result of the growing acceptance of exhaled breath as a diagnostic tool and the need to conduct large scale biomarker discovery experiments sampled at off-site locations [5]. While previously assessed, our results show that travel and storage, even at cold temperatures, have detrimental affects on the stability of VOCs on Tenax TA TD tubes. However, the effects are found to be present at a similar rate to controlled laboratory experiments [5]. It is interesting that no compounds were found to consistently change between the two studies, laboratory and field. This is to some extent a consequence of the reduced compound set used for the field sampling analysis. Regardless, these results establish the need for multi-site biomarker discovery experiments to analyze exhaled breath VOCs on Tenax TA as soon as possible to lessen extraneous variability.

This study does not come without limitations. The use of breath sampling bags remains controversial due to possible intrinsic reactivity, permeability and low-integrity of the bags resulting in possible introduction of artifacts to the analysis [19]. Additionally due to a lack of sample randomization, potential sources of variability in the data, such as contamination or artifacts brought on by the sampling bags, cannot be accounted for and have the potential to confound the analysis. This study is limited to the exhaled breath VOCs captured both by Tenax TA thermal desorption tubes and the dual bed Air Toxics cold trap. Therefore, these results may not be applicable to different TD tube and/or cold trap sorbent combinations.

The results presented in this manuscript stress the need for investigators to be critical of results obtained from breath studies with VOCs stored for prolonged periods of time. While these data provide experimental support for the storage of exhaled breath at refrigerated temperatures, further investigation is required to confirm the duration of storage of specific compounds. Such results are required to truly allow for large-scale biomarker discovery from exhaled breath across multiple sites.

Conclusion

In summary, we have characterized the storage stability of 74 exhaled breath compounds on Tenax TA across three storage conditions over a period of 31 d. The data indicates cold storage is the optimum storage condition

for exhaled breath on Tenax TA. Additionally, the results suggest analysis by day 14 in storage will minimize a potential 1–2 standard deviation gain or loss of VOC concentration. This initial study provides preliminary data to critically evaluate biomarker discovery efforts that require prolonged storage. However, these data represent a narrow range of VOC concentration derived from healthy individuals, which may be different in specific disease conditions. Therefore, further studies with more absolute quantitation, rather than relative quantitation, for the 74 exhaled breath compounds, among many different concentrations, is required to ultimately characterize the storage stability over time.

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References

[1] Amann A, Costello Bde L, Miekisch W, Schubert J, Buszewski B, Pleil J, Ratcliffe N and Risby T 2014 The human volatilome: volatile organic compounds (VOCs) in exhaled breath, skin emanations, urine, feces and saliva *J. Breath Res.* **8** 034001

[2] Schmidt K and Podmore I 2015 Current challenges in volatile organic compounds analysis as potential biomarkers of cancer *J. Biomark.* **2015** 1–16

[3] Amann A and Smith D 2005 *Breath Analysis for Clinical Diagnosis and Therapeutic Monitoring* (Singapore: World Scientific)

[4] Pawliszyn J 2002 *Sampling and Sample Preparation in Field and Laboratory* (Amsterdam: Elsevier)

[5] van der Schee M P, Fens N, Brinkman P, Bos L D J, Angelo M D, Nijsen T M E, Raabe R, Knobel H H, Vink T J and Sterk P J 2013 Effect of transportation and storage using sorbent tubes of exhaled breath samples on diagnostic accuracy of electronic nose analysis *J. Breath Res.* **7** 016002

[6] McCaffrey C A, MacLachlan J and Brookes B I 1994 Adsorbent tube evaluation for the preconcentration of volatile organic compounds in air for analysis by gas chromatography-mass spectrometry *Analyst* **119** 897–902

[7] Baya M P and Siskos P A 1996 Evaluation of Anasorb CMS and comparison with Tenax TA for the sampling of volatile organic compounds in indoor and outdoor air by breakthrough measurements *Analyst* **121** 303–7

[8] Helmig D and Vierling L 1995 Water adsorption capacity of the solid adsorbents Tenax TA, Tenax GR, Carbotrap, Carbotrap C, Carbosieve SIII, and Carboxen 569 and water management techniques for the atmospheric sampling of volatile organic trace gases *Anal. Chem.* **67** 4380–6

[9] Nielsen A T and Jonsson S 2002 Quantification of volatile sulfur compounds in complex gaseous matrices by solid-phase microetraction *J. Chromatogr.* A **963** 57–64

[10] Sulyok M, Haberhauer-Troyer C, Rosenberg E and Grasserbauer M 2001 Investigation of the storage stability of selected volatile sulfur compounds in different sampling containers *J. Chromatogr.* A **917** 367–74

[11] Hyspler R, Crhova S, Gasparic J, Zadak Z, Cizkova M and Balasova V 2000 Determination of isoprene in human expired breath using solid-phase microextraction and gas chromatography-mass spectrometry *J. Chromatogr.* B **739** 183–90

[12] Deng C, Zhang J, Yu X, Zhang W and Zhang X 2004 Determination of acetone in human breath by gas chromatography-mass spectrometry and solid-phase microextraction with on-fiber derivatization *J. Chromatogr.* B **810** 269–75

[13] Groves W A and Zellers E T 1996 Investigation of organic vapor losses to condensed water vapor in Tedlar bags used for exhaled-breath sampling *Am. Ind. Hyg. Assoc. J.* **57** 257–63

[14] Mochalski P, King J, Unterkofler K and Amann A 2013 Stability of selected volatile breath constituents in Tedlar, Kynar and Flexfilm sampling bags *Analyst* **138** 1405–18

[15] van Harreveld A P T 2003 Odor concentration decay and stability in gas sampling bags *J. Air Waste Manag. Assoc.* **53** 51–60

[16] Petka J, Etievant P and Callement G 2000 Suitability of different plastic materials for head or nose spaces short term storage *Analusis* **28** 330–5

[17] Steeghs M M, Cristescu S M, Munnik P, Zanen P and Harren F J 2007 An off-line breath sampling and analysis method suitable for large screening studies *Physiol. Meas.* **28** 503–14

[18] Steeghs M M, Cristescu S M and Harren F J 2007 The suitability of Tedlar bags for breath sampling in medical diagnostic research *Physiol. Meas.* **28** 73–84

[19] Beauchamp J, Herbig J, Gutmann R and Hansel A 2008 On the use of Tedlar bags for breath-gas sampling and analysis *J. Breath Res.* **2** 046001

[20] Peters R J and Bakkeren H A 1994 Sorbents in sampling. Stability and breakthrough measurements *Analyst* **119** 71–4

[21] Brown V M, Crump D R, Plant N T and Pengelly I 2014 Evaluation of the stability of a mixture of volatile organic compounds on sorbents for the determination of emissions from indoor materials and products using thermal desorption/gas chromatography/mass spectrometry *J. Chromatogr.* A **1350** 1–9

[22] Patil S F and Lonkar S T 1994 Evaluation of Tenax TA for the determination of chlorobenzene and chloronitrobenzenes in air using capillary gas chromatography and thermal desorption *J. Chromatogr.* A **684** 133–42

[23] de Lacy Costello B, Amann A, Al-Kateb H, Flynn C, Filipiak W, Khalid T, Osborne D and Ratcliffe N M 2014 A review of the volatiles from the healthy human body *J. Breath Res.* **8** 014001

[24] Harshman S W, Geier B A, Fan M, Rinehardt S, Watts B S, Drummond L A, Preti G, Phillips J B, Ott D K and Grigsby C C 2015 The identification of hypoxia biomarkers from exhaled breath under normobaric conditions *J. Breath Res.* **9** 047103

[25] US Environmental Protection Agency 1999 Compendium method TO-17 determination of volatile organic compounds in ambient air using active sampling onto sorbent tubes EPA/625/R-96/101b (www3.epa.gov/ttnamti1/files/ambient/airtox/to-17r.pdf)

[26] Schubert J K, Spittler K-H, Braun G, Geiger K and Guttman J 2001 CO_2 controlled sampling of alveolar gas in mechanically ventilated patients *J. Appl. Physiol.* **90** 486–92

[27] Birken T, Schubert J, Miekisch W and Noldge-Schomburg G 2006 A novel visually CO_2 controlled alveolar breath sampling technique *Technol. Health Care* **14** 499–506

[28] Grigsby C C, Rizki M M, Tamburino L A, Pitsch R L, Shiyanov P A and Cool D R 2010 Metabolite differentiation and discovery lab (MeDDL): a new tool for biomarker discovery and mass spectral visualization *Anal. Chem.* **82** 4386–95

[29] Grigsby C C, Zmuda M A, Boone D W, Highlander T C, Kramer R M and Rizki M M 2012 Differential profiling of volatile organic compound biomarker signatures utilizing a logical statistical filter-set and novel hybrid evolutionary classifiers *Proc. SPIE* **8402** 84020K

[30] Wickham H and Chang W 2015 An implementation of the grammar of graphics (https://cran.r-project.org/web/packages/ggplot2/ggplot2.pdf)
[31] Lee J, Park J, Lim M, Seong S J, Seo J J, Park S M, Lee H W and Yoon Y 2012 Quantile normalization approach for liquid-chromatography-mass spectrometry-based metabolomics data from healthy human volunteers *Anal. Sci.* **28** 801–5
[32] Gallon S, Loubes J M and Maza E 2013 Statistical properties of the quantile normalization method for density curve alignment *Math. Biosci.* **242** 129–42
[33] Wu Y and Li L 2016 Sample normalization methods in quantitative metabolomics *J. Chromatogr.* A **1430** 80–95
[34] Subramanian A *et al* 2005 Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles *Proc. Natl Acad. Sci. USA* **102** 15545–50
[35] Bid H K *et al* 2013 Development, characterization, and reversal of acquired resistance to the MEK1 inhibitor Selumentinib (AZD6244) in an *in vivo* model of childhood astrocytoma *Clin. Cancer Res.* **19** 6716–29
[36] Breitling R, Armengaud P, Amtmann A and Herzyk P 2004 Rank products: a simple, yet powerful, new method to detect differentially regulated genes in replicated microarray experiments *FEBS Lett.* **573** 83–92
[37] Hulsen T, de Vlieg J and Alkema W 2008 BioVenn—a web application for the comparison and visualization of biological lists using area-proportional Venn diagrams *BMC Genomics* **9** 488
[38] Hastie T, Tibshirani R and Friedman J 2009 *The Elements of Statistical Learning* (Berlin: Springer)
[39] Wishart D S 2007 HMDB: the human metabolome database *Nucleic Acids Res.* **35** D521–6
[40] Wishart D S 2009 HMDB: a knowledgebase for the human metabolome *Nucleic Acids Res.* **37** D603–10
[41] Wishart D S 2013 HMDB 3.0—the human metabolome database in 2013 *Nucleic Acids Res.* **41** D801–7
[42] Filipiak W, Sponring A, Filipiak A, Ager C, Schubert J, Miekisch W, Amann A and Troppmair J 2010 TD-GC-MS Analysis of volatile metabolites of human lung cancer and normal cells *in vitro Cancer Epidemiol. Biomark. Prev.* **19** 182–95
[43] Chemical D 2012 Dowanol PM vol 110-00617-0812 (http://msdssearch.dow.com/PublishedLiteratureDOWCOM/dh_08ad/0901b803808ad674.pdf?filepath=oxysolvents/pdfs/noreg/110-00617.pdf&fromPage=GetDoc)
[44] Monod A, Sive B C, Avino P, Chen T, Blake D R and Rowland F S 2001 Monoaromatic compounds in ambient air of various cities: a focus on correlations between the xylenes and ethylbenzene *Atmos. Environ.* **35** 135–49
[45] Sepcic K, Josowicz M, Janata J and Selby T 2004 Diagnosis of used engine oil based on gas phase analysis *Analyst* **129** 1070–5
[46] Norback D, Wieslander G, Nordstrom K and Walinder R 2000 Asthma symptoms in relation to measured building dampness in upper concrete floor construction, and 2-ethyl-1-hexanol in indoor air *Int. J. Tuberc. Lung Dis.* **4** 1016–25
[47] de Graaf A A, Maathuis A, de Waard P, Deutz N E P, Dijkema C, de Vos W M and Venema K 2010 Profiling human gut bacterial metabolism and its kinetics using (U–^{13}C) glucose and NMR *NMR Biomed.* **23** 2–12
[48] Kikugawa K and Beppu M 1987 Involvement of lipid oxidation products in the formation of fluorescent and cross-linked proteins *Chem. Phys. Lipids* **44** 277–96
[49] Wardencki W 1998 Problems with the determination of environmental sulphur compounds by gas chromatography *J. Chromatogr.* A **793** 1–19
[50] Tangerman A 1986 Determination of volatile sulphur compounds in air at the parts per trillion level by tenax trapping and gas chromatography *J. Chromatogr.* A **366** 205–16
[51] Harrison R M, Peak J D and Collins G M 1996 Tropospheric cycle of nitrous acid *J. Geophys. Res.* **101** 14429–39
[52] Finlayson-Pitts B J, Wingen L M, Sumner A L, Syomin D and Ramazan K A 2003 The heterogenous hydrolysis of NO_2 in laboratory systems and in outdoor and indoor atmospheres: an integrated mechanism *Phys. Chem. Chem. Phys.* **5** 223–42
[53] Gjolstad M, Bergemalm-Rynell K, Ljungkvist G, Thorud S and Molander P 2004 Comparison of sampling efficiency and storage stability on different sorbents for determination of solvents in occupational air *J. Sep. Sci.* **27** 1531–9
[54] Lee J H, Batterman S A, Jia C and Chernyak S 2006 Ozone artifacts and carbonyl measurements using Tenax GR, Tenax TA, Carbopack B, and Carbopack X adsorbents *J. Air Waste Manag. Assoc.* **56** 1503–17
[55] Kleno J G, Wolkoff P, Clausen P A, Wilkins C K and Pedersen T 2002 Degradation of the adsorbent Tenax TA by nitrogen oxides, ozone, hydrogen peroxide, OH radical, and limonene oxidation products *Environ. Sci. Technol.* **36** 4121–6