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Application of Novel Method to Measure Endogenous VOCs in Exhaled Breath Condensate Before and After Exposure to Diesel Exhaust

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Abstract

Polar volatile organic compounds (PVOCs) such as aldehydes and alcohols are byproducts of normal human metabolism and thus are found in blood and exhaled breath. Perturbation of the normal patterns of such metabolites may reflect exposures to environmental stressors, disease state, and human activity. Presented herein is a specific methodology for assaying PVOC biomarkers in exhaled breath condensate (EBC) samples with application to a series of samples from a controlled chamber exposure to dilute diesel exhaust (DE) or to purified air. The collection/analysis method is based on condensation of normal (at rest) exhaled breaths for 10 min (resulting in 1 to 2 ml of liquid) with subsequent analyte adsorption onto Tenax® cartridges followed by thermal desorption and analysis by gas chromatography/mass spectrometry (GC/MS). Analytical data have linearity of response ($R^2 > 0.98$) across a range of 0 to 160 ng/mL with a detection limit ranging from 0.2 - 7 ng/ml depending on the compound. Statistical analyses of the results of the controlled exposure study indicate that metabolism, as reflected in simple breath-borne oxygenated species, is not affected by exposure to ambient airborne levels of DE. Linear mixed-effects models showed that PVOC biomarker levels are affected by gender and vary significantly among nominally healthy subjects. Differences among PVOCs analyzed in clinic air, pure chamber air, and chamber air containing dilute DE confirm that most of the compounds are likely of endogenous origin as the exogenous exposure levels did not perturb the EBC measurements.

Keywords: biomarkers, exhaled breath condensate, diesel exhaust

1. Introduction

Exposure assessments provide the independent variables against which environmentally related health outcomes are measured. Traditionally, human exposure is estimated from environmental and personal measurements of various media including air, water, soil, dust, and food; these measurements are then coupled with information regarding human activity patterns and uptake parameters to calculate internal dose. To better understand the sources and routes of human exposure to environmental contaminants, measurements of substances in human biological fluids have been added to the exposure/dose assessment arsenal. Exogenous compounds and their metabolites found in biological tissues or fluids are considered "biomarkers of exposure" and are used to reconstruct and confirm contacts with environmental pollutants. Biological measurements are also used to assess biochemical effects, which include altered or damaged proteins, DNA, RNA, lipids, and carbohydrates, as well as altered biological processes (e.g., protein expression, cell signaling). Because changes in biological components can be interpreted as pre-cursors to cellular and organ damage, these outcomes may be useful surrogate measures of general health status. In this paper we explore the measurement of chemicals that reflect the metabolic perturbations occurring between exposure and effect. These are commonly referred to as "endogenous biomarkers" whose modulation may indicate subtle changes in normal metabolic processes [1]. In this work, we focus on a series of specific low molecular weight (volatile) alcohols and aldehydes.

A wide variety of biomarker molecules can be measured in biological media such as blood, urine, adipose tissue, lung and nasal lavage fluid, and exhaled breath. Although blood is generally considered the gold standard for biomarker assessment, breath analysis provides distinct advantages in that sample collection is non-invasive, trained medical personnel are not required, little potentially biohazardous waste is generated, and the sample supply (breath) is essentially unlimited [2-4]. Breath can be sampled as a gas matrix or in its condensed form as exhaled breath condensate (EBC) [4]. We have explored the use of EBC previously [5]; here we apply an improved methodology for assessing polar volatile organic compounds (PVOCs) in EBC as endogenous biomarkers of diesel exhaust (DE) exposure.

DE is a complex and variable mixture of gases and particles that is classified as a probable human carcinogen [6,7]. Components of DE have been linked with acute respiratory conditions, such as asthma and increased respiratory infections, and decreased lung function [8-14]. In this study, we evaluate changes in metabolite patterns resulting from DE exposures in a controlled environment. Specifically, we present new work in three areas: the improvement of the existing method for EBC collection and analysis; the application of this method to samples collected from a controlled DE exposure study; and the interpretation of levels of PVOCs measured in samples of EBC and inhaled air.

2. Materials and Methods

2.1 Chamber Study Design

The overarching study design was presented in Sobus *et al.* [15] and Sawyer *et al.* [16]. In brief, all chamber exposures were performed at the EPA National Health and Environmental Effects Research Laboratory (NHEERL) located on the campus of the University of North Carolina at Chapel Hill (UNC-CH). Environmental and biological samples were collected with approval from the UNC-CH Biomedical Institutional Review Board (IRB# 99-EPA-283 Physiological, Cellular, and Biochemical Effects of Diesel Exhaust in Healthy Young Adults). Eleven healthy, non-smoking adults (6 females and 5 males, age 18-40) with no history of smoking (<1 pack-year over lifetime), respiratory diseases, active medical conditions, active allergies (within 2 weeks of exposure), respiratory infections or flu-like symptoms (within 6 weeks of exposure) were qualified as subjects. One male subject was lost to followup. Subjects were asked to participate in two 2-hr exposure periods; one in which they were exposed to filtered (high efficiency particulate air [HEPA] filters) and purified (activated charcoal purification) air, and one in which they were exposed to filtered air with the addition of 100 $\mu g/m^3$ of DE (based on tapered element oscillating microbalance [TEOM] measurements). The level of DE was chosen to simulate exposure by a pedestrian at a typical urban intersection [17]. The DE was generated from an idling six-cylinder, 5.9 L-displacement diesel engine (Cummins, Columbus, IN), mounted in a vehicle located outside the human studies facility, which burned a certified diesel fuel (Chevron Phillips Chemical Co., Borger, TX; 0.05 LS Certification Fuel, type II). During DE and purified air exposures, the subjects performed moderate and intermittent bicycle exercise to achieve a desired ventilation rate of approximately 20 $L/min/m^2$ body surface area. Samples of EBC were collected from subjects immediately before, immediately following, and 20 hours post-exposure for a total of 6 samples per subject.

2.2 Sample collection and analysis

2.2.1 EBC sample collection for analytical method optimization

Anonymous biological specimens (n=33, with a minimum of 1.5 mL of EBC per sample) were collected from seven volunteers (with up to nine repeats) to optimize the published methodology of Pleil *et al.* [5]. The seven individuals were nominally healthy, non-smoking adults with unremarkable recent exposure history (no recent bus or air travel, no recent laboratory or hobby exposures to solvents, etc.). A broad group of analytes was initially chosen for method optimization to reflect volatile oxygenated compounds that are potential by-products of human metabolism. Analytes were subsequently restricted to compounds that were repeatedly present at measurable levels in anonymous EBC specimens.

For expediency in analytical methods development, a commercially available sample collection method was used as described previously [5]. Samples of EBC were collected using an RTube™ EBC collector (Respiratory Research, Inc. RTube™ Kit), consisting of a 2.5 cm tube with attached mouthpiece fitted with a non-rebreathing valve. The collector was inserted into an insulated sleeve that was filled with dry ice to surround the RTube™ and rapidly freeze the EBC. After 10 minutes of normal breathing, the apparatus was removed from the insulating sleeve, the mouthpiece was removed, and the end was capped. The collection tube was then set onto a stainless steel plunger

(Respiratory Research, Inc. RTube™ Kit) and the collected contents were allowed to thaw at room temperature. The tube was then pressed down the plunger, which collected the sample in one end of the tube. The sample was removed via syringe, the volume was recorded, and the sample was immediately frozen at -80^{O}C awaiting analysis.

2.2.2 EBC and air sample collection for the chamber exposure study

The chamber study EBC samples had been previously acquired using a different condensation technique (Sawyer *et al.* [16]) designed to collect sufficient sample volumes for multiple analyses. After analysis for large molecules (primarily cytokines and total protein content), unused aliquots became available for analysis of the small PVOC molecules presented here. This method involved the use of 1.5 m of Tygon tubing placed in a cooler filled with a wet ice-salt mixture. The ends of the tubing were allowed to protrude and the subject breathed into the sampling apparatus for 10 minutes. Saliva entrainment was minimized by placing the cooler above the mouth of the seated subject and then cutting off and discarding the first 15 cm of the inlet tubing at the end of the sampling period. The remaining tubing was capped (with a Teflon covered stopper on the mouth end, and a sterile (17 x 120 mm) conical polypropylene tube on the other end) and removed from the dry ice. After thawing, the tubing was tapped to aggregate the fine EBC droplets into larger ones, and the tubing swung overhead, allowing the centrifugal force to collect the EBC into the tube. The sample was immediately removed via syringe, measured, capped, and frozen at -80^{O}C.

To complement the chamber study EBC samples, three groups of indoor air samples were also collected to represent direct PVOC inhalation exposures experienced by the subjects. We sampled clinic air (n = 5) just outside the chamber, purified chamber air (n = 16), and chamber air with diluted diesel exhaust (n = 18). This was accomplished via air sampling directly onto a Tenax® packed sorbent tube (Supelco, Bellefonte, PA) using a portable pump (Dupont Alpha-2, Kennett Square, PA) at 100 mL/min for 120 minutes. Tenax® tubes were subsequently analyzed as described in the Sample Analysis section.

2.3 EBC Sample Preparation

All EBC samples (samples from volunteers and chamber subjects), deionized water blanks, and calibration standards were prepared and analyzed identically. The underlying principle is passive diffusion of PVOCs from the aqueous sample onto a Tenax® sorbent tube within a small sealed volume defined by a glass bulb capped at both ends (Figure 1). The specific procedure is as follows:

- Each 75 mL glass bulb is washed and then heated to 70^{O}C to remove residual solvent.
- Tenax® tubes are cleaned and conditioned with ultra-high purity helium at 290 ^{O}C for 2 hours prior to use.

- Upon cooling bulbs to room temperature, 200 mg of sodium chloride (NaCl, Sigma Aldrich, St. Louis, MO) is added and prepared bulbs are placed on an inclined rack.
- A clean Tenax® sorbent tube is inserted into each bulb.
- EBC samples (or blanks and calibration mixtures) are thawed from -80°C to room temperature and 1 ml aliquots are transferred into each bulb via syringe.
- Five µL of internal standard (0.15 µL/mL of 3-hexanol [Accustandard, New Haven, CT] in water) is added to each bulb via syringe.
- Bulbs are sealed and left at room temperature for 24 hrs. (Figure 1).
- Tenax tube is removed, capped, and stored for subsequent analysis.

Calibration mixtures are prepared fresh in deinonized water (at 160 ng/ml for each analyte) for each sample set from premixed certified standards (Accustandard, New Haven, CT); blanks consist of deionized water.

2.4 Sample analysis

The Tenax® tubes were placed into an Ultra TD autosampler coupled with a Unity thermal desorber (Markes International, Ltd., Llantrisant, UK) equipped with a water management secondary trap. After desorption, samples were automatically injected into a GC/MS system (6890N GC, 5973N MS, Agilent Technologies, Palo Alto, CA) with an Rtx-Wax (60 m x 0.25 mm i.d. x 0.25 µm) stationary phase column (Restek Corp. Bellefonte, PA). The GC oven was programmed with an initial temperature of 40°C for 2 minutes, followed by a 10°C/min ramp to 250°C, where it was held for 8 minutes. The samples were analyzed in selected ion monitoring (SIM) mode with optimized ion groups and dwell times to achieve a sampling rate of ~2 Hz and a minimum of 10 points per GC peak. To insure that the peaks of interest were correctly identified, peak area for each analyte was calculated for both primary and secondary ions. The ratio of the primary to secondary ion was then compared to the analogous ion ratio in the certified standard and was rejected if the ion ratio in the sample was not within 25% agreement of the standard.

Sample concentrations were calculated through the use of a 6 point standard curve ranging from 0 ng/mL to 160 ng/mL and the use of 3-hexanol as an internal standard. The standard curve (linear regression using Microsoft Excel) was used to assure linearity of response over the concentration range of interest. Sensitivity was determined by the repeat analysis of blank samples, and the analytical limit if detection (LOD) was calculated (for each analyte) as 3 × standard deviation of the blank values. The determination of assay precision through the re-analysis of actual EBC samples was not possible due to insufficient sample volume.

2.5. Statistical Analysis

Descriptive statistics for PVOCs in air and EBC were generated using the Proc UNIVARIATE procedure available from SAS statistical software version 9.1 (SAS Institute, Cary, NC.). The effect of exposure (DE vs. purified air) on the levels of

PVOCs in EBC was evaluated by adjusting post-expoure and 20hr post-exposure values by their respective pre-exposure values. The subject-specific adjusted values, which are the ratios of the post-exposure and 20hr post-exposure values to pre-exposure values, were then evaluated in a pairwise fashion to determine the effect of exposure. The pairwise comparisons of adjusted post-exposure and adjusted 20hr post-exposure were limited to observations from 5 out of 10 subjects, since 5 subjects had at least one missing observation (either pre, post, or 20hr post). Considering the small sample size, a nonparametric Wilcoxon paired signed rank test (available through GraphPad prism software, version 4.03) was used for this analysis; significant differences in adjusted values were determined at a significance level of $\alpha = 0.1$. A value of LOD/$\sqrt{2}$ was imputed for all zero observations. Value imputation was not used for non-zero observations that were below the calculated LOD.

Measurements of PVOCs in EBC were log-transformed (natural log) and further evaluated using linear mixed-effects models (SAS, Proc MIXED). Analyses were restricted to individual analytes that were detected in over 70% of the EBC samples and that displayed an approximate log-normal character. The normality of log-transformed values and potential influential observations were evaluated using the Shapiro-Wilk's W test and by visual inspection of normal probability plots (Proc UNIVARIATE).

The linear mixed-effects models were used to evaluate the effects of time of sample collection (pre-exposure vs. post-exposure vs. 20hr post-exposure), type of exposure (i.e., DE exposure period vs. purified air exposure period), and gender on the levels of PVOCs in EBC. The effect of the 100 $\mu g/m^3$ DE exposure was evaluated by including in the models an interaction effect between time of sample collection and type of exposure. Associations between the independent variables (i.e., time, period, gender, and time×period) and PVOCs were determined at a significance level of $\alpha = 0.05$, and final models were selected using manual backwards stepwise elimination. A compound symmetry covariance matrix was used in each mixed model. Restricted maximum likelihood estimates of within- and between-person variance components were determined after adjusting for significant fixed effects. Estimates of the within- and between-person variance components (i.e., $\hat{\sigma}_w^2$ and $\hat{\sigma}_b^2$, respectively) from the final models were used to calculate intraclass correlation coefficients (ICC) for PVOCs, where $ICC = \hat{\sigma}_b^2 / (\hat{\sigma}_w^2 + \hat{\sigma}_b^2)$.

3. Results and Discussion

3.1 Method optimization and development

The SIM method employed ions to monitor analytes in the volatility (based on retention time) range from 2-methylpropanal to 1-heptanol; common, very volatile endogenous compounds such as acetone, isoprene, formaldehyde, and acetaldehyde were not quantified in this study. Specific analytes measured in samples of EBC and air are presented in Table 1 along with their retention times, primary and confirmatory ions, and coefficient of variation values from multipoint calibration curves. Using the method described by Pleil *et al.* [4] as a starting point for this work, we further optimized the

collection and analysis methodology by exploring a series of parameters and modifications. Specific modifications to the published method include:

- Changed from a "general purpose" to a "water management" secondary trap (U-T4WMT, Markes International, Ltd., Llantrisant, UK)
- Changed from sorbent tubes hand assembled in the laboratory with Tenax® to commercially available Tenax® sorbent tubes
- Implemented immediate freezing step (-80^{O}C) after initial thaw of EBC sample in RTube or Tygon tube method.
- Tested different adsorption temperatures (20^{O}C versus 40^{O}C)
- Implemented confirmatory ions per analyte in GC/MS SIM analysis.
- Implemented internal standard (3-hexanol) addition to samples.

These exploratory tests provided information to establish the final method. We note that the "water management" secondary trap improved the noise level, that the immediate freezing step increased collection efficiency, that adsorption (transfer) temperature only moderately improved alcohols signal, and that the use of internal standard improved method dynamic range and linearity as quantified by the coefficient of variation (R^2) of multipoint calibration curves (see Table 1).

3.2 Measurements PVOCs in room and chamber air

In Table 2 we present the measured concentrations of PVOC analytes in the purified chamber air, the clinic air, and the chamber air during DE exposures. Only median levels of 1-butanol and butanal were above the LOD in all three air matrices. Results for 1-butanol, the most abundant analyte measured in these samples of air, suggest that levels in clinic air were approximately 20 times higher than in chamber DE and purified air. This was likely a result of the use of 1-butanol as a common solvent in consumer products including cosmetics, paints, and cleaners. Although median levels of 1-butanol were similar in chamber DE and purified air, levels in the chamber during DE exposures were extremely variable, and ranged from 0.2 $\mu g/m^3$ to over 2000 $\mu g/m^3$. For comparison, levels of 1-butanol in purified chamber air ranged from 1.3 $\mu g/m^3$ to 8.6 $\mu g/m^3$. These results suggest that DE may have been an intermittent source of 1-butanol exposure. In contrast to 1-butanol, median levels of butanal were very similar in chamber DE and clinic air; these levels were approximately 10 times higher than the median butanal level in chamber purified air. These results indicate the effective removal of butanal from source air using HEPA filters and activated charcoal, and suggest that subjects were exposed to greater levels of butanal during DE exposures than during purified air exposures.

Median levels of octanal, 1-heptanol, 1-propanol, heptanal, 2-methylpropanal, pentanal, and 3-methylbutanal were above the LODs in chamber DE and clinic air, but not in chamber purified air (see Table 2), again demonstrating the effective removal of analytes from source air during purification. With the exception of 1-heptanol, which was approximately 5 times higher in DE compared to clinic air (based on median values), median analyte levels were very similar in samples of clinic air and chamber DE. Overall, these results suggest that subjects were exposed to measurable levels of these

PVOCs during DE exposures (albeit low levels based on our comparison to clinic air levels), and considerably lower (in most cases immeasurable) levels during purified air exposures.

For measurements of 2-methyl-1-propanol, 2-ethyl-1-butanol, and 1-hexanol, median levels were above the analytical LOD in chamber DE only. These results highlight DE as an exogenous source of these analytes, and indicate that subjects were exposed to higher levels during DE exposure than during purified air exposure. For the remaining analytes (i.e., hexanal, 4-methyl-2-pentanol, 1-pentanol, and 3-methyl-3-pentanol), median levels in both chamber purified air and chamber DE were below the LOD, indicating that subjects were exposed to levels of these analytes indistinguishable from zero during both exposure periods making it unlikely that presumed endogenous EBC levels were impacted.

3.3 Measurements of PVOCs in EBC

Descriptive statistics for PVOCs measured in the chamber subjects' EBC are shown in Table 3. We note that out of 60 possible samples (6 samples each for 10 subjects), only 44 samples were successfully analyzed for PVOCs. One male subject provided only one EBC sample and was therefore not considered in this analysis. The other 10 missing observations occurred at random generally due to insufficient sample volume. In Table 3 we show that most analytes were observed in EBC samples in the low ng/ml range. Fourteen of sixteen analytes were detected at the 75^{th} percentile, eleven of sixteen analytes were detected at the 50^{th} percentile, and only six of sixteen analytes were detected at the 25^{th} percentile. Generally, these results indicate low levels of PVOCs in the EBC of nominally healthy adults. Two notable exceptions were 1-propanol and 2-methyl-1-propanol which were measured at median levels of 161 and 82.7 ng/ml, respectively. These measurements were over an order of magnitude greater than the median levels of all other analytes. Moreover, 1-propanol was the only analyte to be detected in 100% of the samples (2-methyl-1-propanol was detected in 89% of the samples). To ensure that the compounds behaved linearly across this range, additional standard curves for 1-propanol and 2-methyl-1-propanol were run, spanning from 0 to 500 ng/ml, resulting in associated R^2 values of 0.960 and 0.966, respectively. Aldehydes and alcohols have been measured in EBC at similar concentrations in previous studies. Andreoli *et al.* measured hexanal and heptanal at a median level of 3 ng/ml and 4 ng/ml, respectively in the EBC of healthy subjects [18]. Using SIFT-MS, Cap *et al.* found propanol in the headspace of EBC at a median level of 361 ppb [19].

Previous studies have shown that different EBC collection devices can yield different results due to both the collection efficiency of the device and contaminants introduced by the collector [20-22]. EBC analyte recovery is generally shown to increase with decreasing collection temperature, although comparisons between a similar Tygon-ice method to the one used here and a commercially available condenser have shown interchangeable results [20,21]. It has also been shown that rubber and some plastics in collection devices can contribute to EBC levels [22]. Although there was a potential for contamination, this study focused on changes in patterns of PVOCs in EBC, so any contamination introduced by the collection device would have been distributed approximately equally among samples.

3.4 Effects of chamber exposures on analyte levels

Figure 2 shows the adjusted (relative to pre-exposure) post-exposure values of PVOCs in EBC for the purified air and DE exposure periods. In this figure we note considerable variability in adjusted post-exposure levels by subject, such that individual observations ranged from 0.01 (for 2-methyl-1-propanol) to 100 (for heptanal); these observations point to a 100-fold deviation in post-exposure levels from pre-exposure levels after only a 2-hr time period. However, in most instances, observations of individual analytes varied both above and below one, and median values were observed across a much smaller range (0.16 [for butanal] to 2.13 [for 1-propanol]). Median values close to one indicate little difference between pre- and post-exposure analyte levels. Additionally, similar observations of median adjusted values for the purified air and DE exposure periods indicate little effect of DE exposure on analyte levels. This observation is corroborated from the results of the Wilcoxon paired signed rank test which showed no significant difference ($p > 0.1$) in adjusted post-exposure values between the purified air exposure and the DE exposure for all analytes except butanal ($p = 0.1$) and 1-butanol ($p = 0.06$). For these analytes, adjusted post-exposure values were lower on the DE exposure day compared to the purified air exposure day. We note that these results are based on only 5 paired observations, and thus should be interpreted with caution.

Very similar observations to the adjusted post-exposure values were made of the adjusted 20hr post-exposure values. Again, an overall wide range of adjusted values was observed (0.005 – 54), while a considerably smaller range of median values was observed (0.1 – 1.3). Results of the Wilcoxon paired signed rank test showed no significant difference in adjusted 20hr post-exposure values between the purified air exposure and the DE exposure ($0.1 < p \leq 1$). Taken together, these results suggest little effect of the time of collection and exposure conditions on the levels of PVOC in EBC.

3.5 Results from linear mixed-effects models

Mixed model analyses were limited to compounds that were measured in > 70% of all EBC samples and that displayed an approximate lognormal character. These criteria limited the field of 16 potential analytes to 9, namely 2-methylpropanol, butanal, pentanal, 1-propanol, hexanal, 2-methyl-1-propanol, 1-butanol, 1-pentanol, and 1-hexanol. A potential outlier observation of 1-butanol and 1-pentanol was observed in our preliminary analyses; mixed models were therefore evaluated both with and without the outlier. Since only random-effects estimates were affected by the presence or absence of the outlier, the observation was not included in the final model(s).

Results from the final mixed models for these 9 analytes indicate no significant effect of time, type of exposure, or their interaction. These results generally support those from the nonparametric analysis of all of the data (all 16 analytes), and indicate no significant differences between pre-, post-, and 20hr post-exposure measurements, no significant concentration difference in measurements between exposure periods, and no significant effect of the type of exposure (purified air vs. DE) on analyte levels at any

given collection time. The lack of an statistically significant exposure effect (even though airborne concentrations of PVOCs were generally much lower in purified air compared to DE) supports the claim that the PVOCs measured in these EBC samples are primarily endogenous in origin and are not the result of exogenous PVOC exposure.

While no time or exposure effects were observed, a significant gender effect was observed for 1-hexanol ($p = 0.01$), butanal ($p = 0.04$), and pentanal ($p = 0.04$), and a moderate gender effect was observed for 2-methylpropanol ($p = 0.09$); in each case, males produced higher levels then females, as shown by the parameter estimates for fixed effects in Table 4. This result is supported by Ernstgard *et al.* [23], who reported lower levels of 2-propanol in women exposed to identical 2-propanol levels as men, suggesting differences in uptake and metabolism between sexes. Work by Bloemen *at el* [24] also showed significant gender differences in EBC with females having a greater total EBC volume than males, although our study did not show a significant difference between male and female sample volumes. Additionally, measurements in this study were normalized by volume. As such, an unknown mechanism appears to be driving these gender differences.

Table 4 also shows parameter estimates for the random effects in each mixed model along with corresponding ICC values. After adjusting for gender, the majority of the variability in the levels of 1-hexanol was observed between subjects (ICC = 0.76), whereas similar variability in the levels of butanal and pentanal were observed between- and within-subjects (ICC = 0.45 and 0.43, respectively). For each of these analytes, significant random-subject effects were observed indicating subject-specific differences in analyte production. Similar random-subject effects were observed for 1-propanol, 1-butanol, and hexanal, where ICC values ranged from 0.39 to 0.62 (Table 4).

The observed gender effects and random-subject effects are simultaneously displayed in Figure 3, where subject-specific mean analyte levels are arranged by gender (*x-axis*) and in order of overall concentration (*z-axis*). In particular, we observe that 1-hexanol, pentanal, and butanal are lower for female subjects. Together, these gender effects and random-subject effects support the endogenous nature of the measured analytes, and indicate that levels may be differentially expressed based on gender or other unknown factors. Considering the limited number of subjects in this study, future studies should further explore these observed gender and random-subject effects. Further support of these findings may underscore the importance of individual PVOCs as endogenous biomarkers of normal metabolic processes.

4. Conclusions

The DE concentration in this study was set to simulate the exposure by a pedestrian in a typical urban area. Levels of PVOCs measured in chamber DE were greater than those measured in chamber purified air, indicating that subjects were differentially exposed to exogenous PVOCs during the two different exposure periods. However, the short term, low-level DE exposures experienced by ten healthy adults did not result in significant changes in analyte levels in samples of EBC. However, for some compounds, females were observed to have significantly lower levels than males, indicating an effect of gender on the production of these analytes. Significant random-

subject effects were also observed for multiple compounds, suggesting inter-individual differences in the production of these metabolites. Because differences in exogenous DE exposures at ~100 $\mu g/m^3$ did not affect EBC concentrations of PVOCs, and that significant gender and random-subject effects were observed, we conclude that the PVOCs measured in samples of EBC were indeed primarily of endogenous origin. The data collected from this study provides background for future EBC work by building a library of normal EBC patterns. Because this method is performed using thermal-desorption GC/MS, equipment that is relatively readily available, there is capability for this method to be replicated and applied to future studies.

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Table 1. Method Parameters for PVOCs in EBC.

PVOC	Retention Time (min)	Primary Ion	Conf. Ion	R^2
2-methylpropanal	4.83	72	43	0.997
butanal	5.33	44	72	0.992
3-methylbutanal	5.94	44	58	0.997
pentanal	6.60	44	58	0.997
1-propanol	7.46	59	42	0.989
hexanal	8.19	56	72	0.997
2-methyl-1-propanol	8.48	43	74	0.997
3-methyl-3-pentanol	8.95	73	55	0.999
1-butanol	9.30	56	41	0.997
4-methyl-2-pentanol	9.69	45	69	0.999
heptanal	9.96	70	44	0.995
1-pentanol	11.01	42	55	0.998
octanal	11.73	43	57	0.985
2-ethyl-1-butanol	11.96	43	70	0.995
1-hexanol	12.67	56	43	0.998
1-heptanol	14.31	70	56	0.999

Table 2. Median levels of PVOCs ($\mu g/m^3$) in samples of chamber air (purified air and diesel exhaust) and clinic air.

PVOC	LOD[1]	Purified Air	Clinic Air	Diesel Exhaust
1-butanol	0.004	4.33	103	6.23
butanal	0.002	0.061	0.746	0.621
octanal	0.100	< LOD	0.355	0.925
1-heptanol	0.082	< LOD	0.093	0.511
1-propanol	0.252	< LOD	0.449	0.439
heptanal	0.069	< LOD	0.535	0.254
2-methylpropanal	0.007	< LOD	0.083	0.215
pentanal	0.026	< LOD	0.215	0.158
3-methylbutanal	0.009	< LOD	0.042	0.021
2-methyl-1-propanol	0.003	< LOD	< LOD	24.6
2-ethyl-1-butanol	0.056	< LOD	< LOD	0.611
1-hexanol	0.002	< LOD	< LOD	0.214
hexanal	0.012	< LOD	0.422	< LOD
4-methy-2-pentanol	0.078	< LOD	< LOD	< LOD
1-pentanol	0.091	< LOD	< LOD	< LOD
3-methyl-3-pentanol	0.002	< LOD	< LOD	< LOD

[1]Limit of detection ($\mu g/m^3$), defined as the three times the standard deviation of field-blank samples.

Table 3. Descriptive Statistics (min, max and selected percentiles [ng/ml]) for PVOCs in EBC (N = 44).

PVOC	LOD[1]	Percent detected	Minimum	25th	50th	75th	Maximum
1-propanol	1.80	100	8.17	78.0	161	285	634
2-methyl-1-propanol	2.36	88.6	< LOD	14.5	82.7	218	954
2-methylpropanal	1.11	86.4	< LOD	2.00	4.60	13.1	40.7
1-butanol	2.02	84.1	< LOD	3.30	5.42	7.96	19.0
butanal	0.66	81.8	< LOD	0.922	1.89	4.62	10.5
1-pentanol	0.24	79.5	< LOD	0.416	1.04	2.67	7.29
1-hexanol	0.34	75	< LOD	< LOD	0.954	2.42	4.04
pentanal	1.03	72.7	< LOD	< LOD	2.15	4.00	10.5
hexanal	2.69	70.5	< LOD	< LOD	3.80	7.12	19.2
3-methyl-3-pentanol	0.19	63.6	< LOD	< LOD	0.287	1.45	3.47
octanal	3.34	50	< LOD	< LOD	3.71	15.6	41.4
1-heptanol	0.63	45.5	< LOD	< LOD	< LOD	2.61	33.1
4-methyl-2-pentanol	0.53	38.6	< LOD	< LOD	< LOD	0.717	4.98
2-ethyl-1-butanol	1.19	34.1	< LOD	< LOD	< LOD	1.82	4.06
3-methylbutanal	4.27	18.2	< LOD	< LOD	< LOD	< LOD	9.85
heptanal	6.64	18.2	< LOD	< LOD	< LOD	< LOD	13.1

[1]Limit of detection (ng/ml), defined as the three times the standard deviation of 10 blank samples.

Table 4. Results from linear mixed-effects models for PVOCs [ln(ng/ml)] in samples of EBC from chamber subjects.

	Fixed effects						Random effects						
	Intercept			Gender			$\hat{\sigma}_b^2$			$\hat{\sigma}_w^2$			
PVOC	*Est.*	*(SE)*	*p-value*	*Est.*[1]	*(SE)*	*p-value*	*Est.*	*(SE)*	*p-value*	*Est.*	*(SE)*	*p-value*	ICC
1-hexanol	-0.592	(0.281)	0.07	1.27	(0.483)	0.01	0.438	(0.251)	0.04	0.138	(0.033)	<0.0001	0.76*
butanal	0.135	(0.355)	0.7	1.31	(0.602)	0.04	0.583	(0.406)	0.08	0.710	(0.170)	<0.0001	0.45*
pentanal	0.151	(0.347)	0.7	1.27	(0.588)	0.04	0.545	(0.382)	0.08	0.734	(0.175)	<0.0001	0.43*
2-methylpropanal	1.33	(0.273)	0.002	0.787	(0.453)	0.09	0.206	(0.240)	0.2	1.04	(0.248)	<0.0001	0.17
1-propanol	4.86	(0.304)	<0.0001			NS	0.733	(0.411)	0.04	0.450	(0.107)	<0.0001	0.62*
1-butanol	1.63	(0.191)	<0.0001			NS	0.274	(0.161)	0.05	0.239	(0.058)	<0.0001	0.53*
hexanal	1.32	(0.220)	0.0003			NS	0.325	(0.209)	0.06	0.498	(0.118)	<0.0001	0.39*
1-pentanol	0.079	(0.225)	0.8			NS	0.338	(0.299)	0.1	1.14	(0.275)	<0.0001	0.23
2-methyl 1-propanol	3.95	(0.373)	<0.0001			NS	0.542	(0.640)	0.2	3.34	(0.796)	<0.0001	0.14

[1]Parameter estimates for male subjects. Female subjects were the reference group (i.e., Est. = 0).

*Significant random-subject effects observed ($p \leq 0.05$)

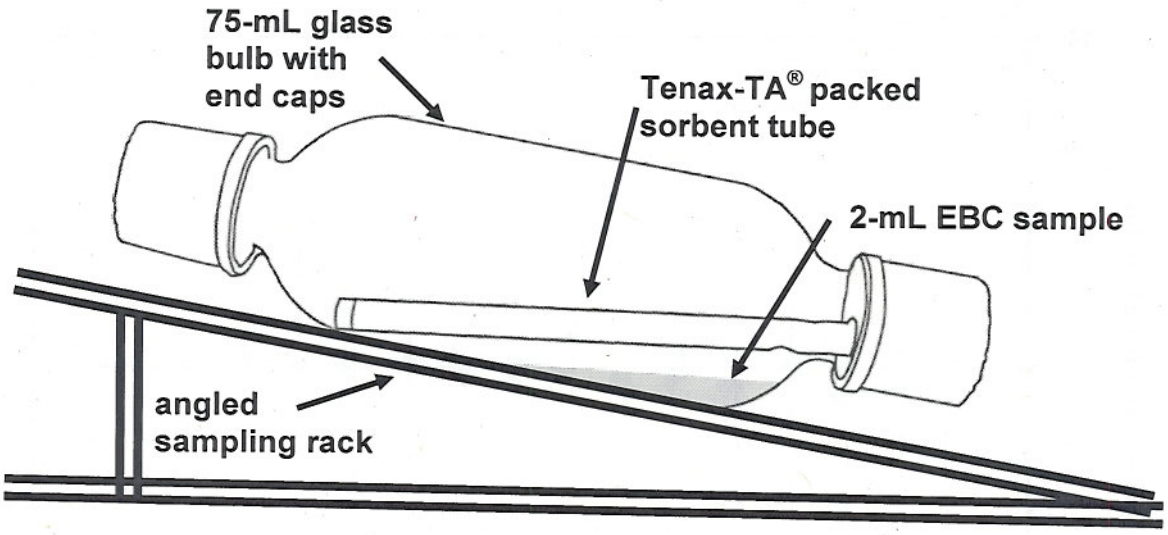

Figure 1. Passive adsorption of sample on to Tenax-TA® packed sorbent tubes [4].

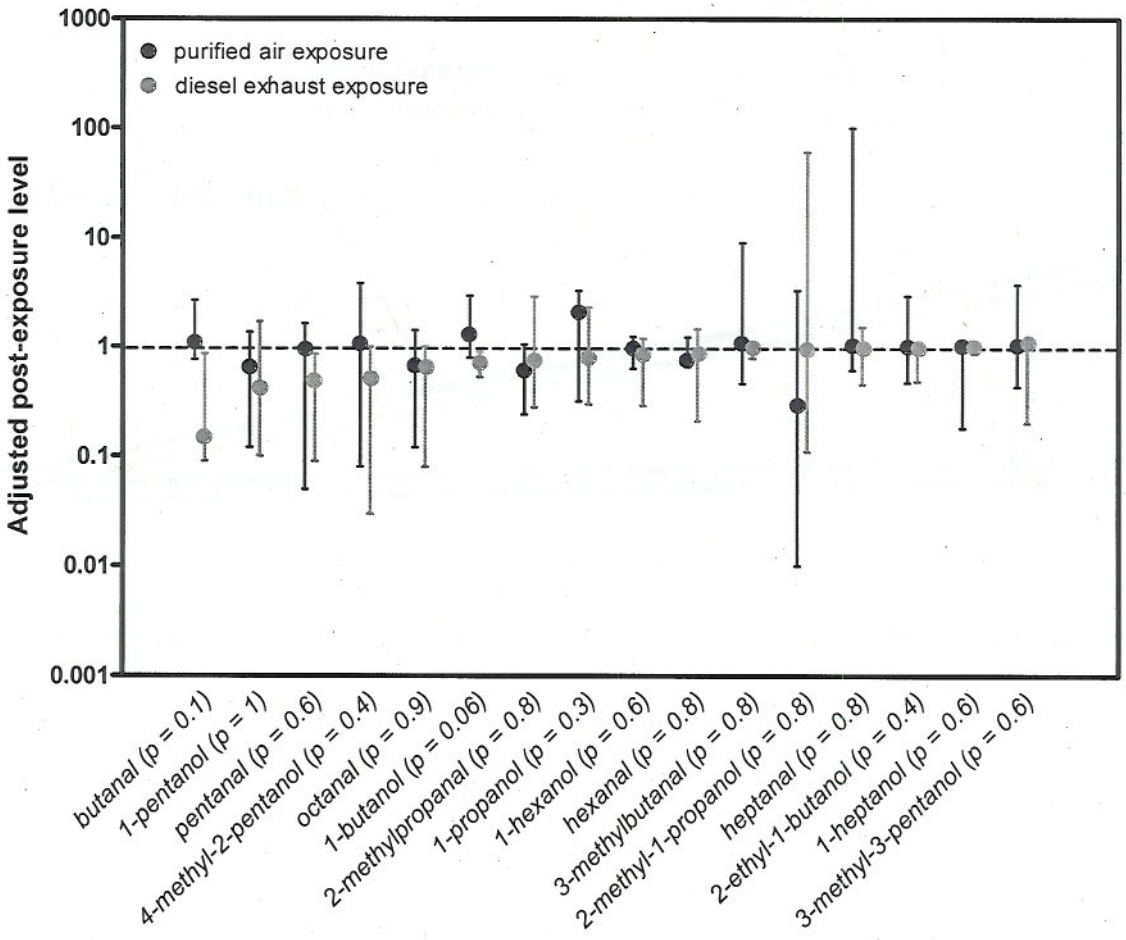

Figure 2. Adjusted post-exposure values of PVOCs in EBC. Blue circles represent the median adjusted values from purified air exposures; red circles represent the median adjusted values from diesel exhaust exposures; error bars represent the range of adjusted values; p-values are from the Wilcoxon paired signed rank test for adjusted purified air *vs.* adjusted DE.

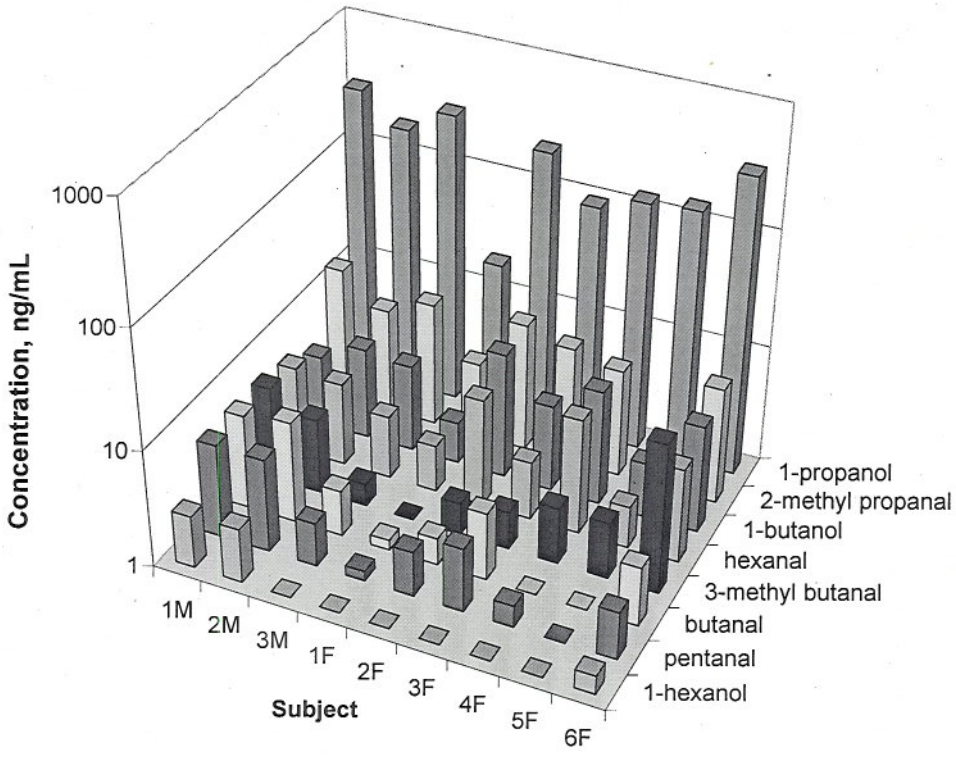

Figure 3. Mean PVOC concentrations by subject for compounds with significant fixed and/or significant random-subject effects (M = male subject; F = female subject).