Effects of ventilation on the collection of exhaled breath in humans

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Cope, Keary A., Michael T. Watson, W. Michael Foster, Shelley S. Sehnert, and Terence H. Risby. Effects of ventilation on the collection of exhaled breath in humans. J Appl Physiol 96: 1371–1379, 2004. First published December 12, 2003; 10.1152/japplphysiol.01034.2003.—A computerized system has been developed to monitor tidal volume, respiration rate, mouth pressure, and carbon dioxide during breath collection. This system was used to investigate variability in the production of breath biomarkers over an 8-h period. Hyperventilation occurred when breath was collected from spontaneously breathing study subjects (n = 8). Therefore, breath samples were collected from study subjects whose breathing were paced at a respiration rate of 10 breaths/min and whose tidal volumes were gauged according to body mass. In this “paced breathing” group (n = 16), end-tidal concentrations of isoprene and ethane correlated with end-tidal carbon dioxide levels [Spearman’s rank correlation test (r = 0.64, P = 0.008 and r = 0.50, P = 0.05, respectively)]. Ethane also correlated with heart rate (r = 0.52, P < 0.05). There was an inverse correlation between transcutaneous pulse oximetry and exhaled carbon monoxide (r = −0.64, P = 0.008). Significant differences were identified between men (n = 8) and women (n = 8) in the concentrations of carbon monoxide (4 parts per million in men vs. 3 parts per million in women; P = 0.01) and volatile sulfur-containing compounds (134 parts per billion in men vs. 95 parts per billion in women; P = 0.016). There was a peak in ethanolic concentration directly after food consumption and a significant decrease in exhalation concentration 2 h later (P = 0.01; n = 16). Sulfur-containing molecules increased linearly throughout the study period (β = 7.4, P < 0.003). Ventilation patterns strongly influence quantification of volatile analytes in exhaled breath and thus, accordingly, the breathing pattern should be controlled to ensure representative analyses.

breath analytes; biomarker; ventilation pattern

SAMPLES OF BREATH HAVE BEEN collected noninvasively and analyzed for volatile biomarkers of ambient exposure and disease pathogenesis (for example, Refs 18, 27, 28). Volatile organic compounds can be analyzed after collection of a fraction of breath or detected in real time by using various spectroscopic methods (21, 38). The optimal method for real-time breath analysis requires a controlled expiratory flow rate. For example, reproducible analysis of nitric oxide from the lower respiratory tract depends on monitoring the end-tidal breath at a constant flow rate and mouth pressure (1). Generally, breath molecules that cannot be analyzed in real time are collected in gas sampling bags, in evacuated canisters, or on thermal adsorbents and analyzed by gas chromatography (31). Because the concentrations of breath molecules can exhibit flow rate dependency, it is hypothesized that changes in ventilation can affect the concentration of breath molecules when multiple breaths are collected. Control of tidal breathing is thought to be automatically regulated through respiratory centers localized in the pons and medulla (39). Breathing through a mouthpiece can interrupt regular breathing patterns, presumably by shifting control away from the automatic respiration centers to the cerebral cortex (12). It has been shown that tidal volume and minute ventilation tend to increase during breathing into a mouthpiece (2, 11). Therefore, it may be necessary to regulate voluntary breathing to optimize breath composition for analysis.

The carbon dioxide profile of breath is composed of three phases. Phase I and phase II, characterized by dead space dilution, make up ~20% of the breath volume, and phase III occurs when the end-tidal concentration has been reached (9). Many approaches have been taken to reduce the contribution of dead space to breath samples, such as discarding the first 500 ml of exhaled breath (41). This approach is subject to wide ranges of variability because it assumes that all subjects have the same volume of dead space. Breath holding has been demonstrated to increase exhaled molecule concentrations for the determination of carbon monoxide, carbon dioxide, methane, or hydrogen. However, breath holding can only be used for the analysis of a single breath and may increase nasal contributions for certain molecules (1, 19). Modeling carbon dioxide concentrations in the alveoli and airways suggests that monitoring the concentrations of mixed expired carbon dioxide may provide a close estimation of the anatomic dead space (13, 17, 45). Schubert et al. (35) have reported the use of a carbon dioxide-sensitive switch to allow the capture of the end-tidal fraction when collecting breath from mechanically ventilated patients.

Ambient and/or experimental conditions can interfere with the quantification of endogenously produced molecules that are released into respiratory gas. For example, ethane and ethylene are often more abundant in the room air or in cylinders of breathing air compared with the amounts that are produced endogenously by normal subjects; therefore, it is important to allow the study subject to reach steady state with levels of exogenous molecules present in inspiratory air (26). It has been reported that background levels of ethane can be washed out of the lung in 4 min by breathing hydrocarbon-free air (16). However, others have shown that steady state was not achieved even after 2 h of breathing ethane-free air (48). Recently, von Basum et al. (47) have reported a real-time method for the analysis of breath ethane. They describe a multiexponential

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decay curve over 30 min, after a 5-min period of exposure to 1 parts/million ethane. In our experience, prepurified breathing air can contain as much as 1 parts/million of total hydrocarbons; moreover, most gas cylinders are not easily portable.

A breath collection system was developed to investigate the variations in the concentrations of selected breath molecules. This protocol allows the subject to visualize each breath in real time and to maintain consistent ventilation patterns. Continuous measurements of mouth pressure, tidal volume, respiration rate, end-tidal carbon dioxide, and mixed expired carbon dioxide measurements are recorded for each breath. To date, this system has been used to adjust, correct, and normalize endogenous concentrations of breath molecules using carbon dioxide measurements. Of particular interest is the effect of different breathing patterns on the carbon dioxide profile and breath molecule concentrations. It was hypothesized that “paced breathing” would decrease variability in breathing parameters compared with “spontaneous breathing” and that breath compounds would correlate with end-tidal carbon dioxide levels. A group of men and women were studied to investigate gender differences and diurnal variation in breath molecules. Pulse rate and transcutaneous oxygen saturation were also monitored during breath collection and correlated to breath compound concentrations. A set of breath molecules, with known endogenous pathways of origin, were selected as biomarkers of metabolism (methanol and acetone), cholesterol biosynthesis (isoprene), intestinal microflora (ethanol), inflammation (nitric oxide), and oxidative stress status (ethane, ethylene, and carbon monoxide). Once volume flow rate is controlled, it is possible to evaluate whether molecule concentrations vary with other physiological processes. It is proposed that ethanol concentration will increase after food consumption as a result of bacterial metabolism. In addition, a new protocol has been developed for the analysis of total volatile sulfur-containing molecules in exhaled breath. Sulfur-containing molecules have been identified as breath markers of liver pathogenesis, lung transplant rejection, colonic fermentation, and nutrient disposition (20, 37, 43, 44).

METHODS

**Human subjects protocol.** The Johns Hopkins University Bloomberg School of Public Health, Committee on Human Research approved these study protocols. Informed verbal consent was obtained from the subjects. The sample group of subjects consisted of 24 healthy, nonsmokers (12 men and 12 women) with an age range of 22–52 yr. Two different studies were conducted to analyze the effects of breathing on the analysis of breathing parameters. In the first study, the subjects with nose clips (n = 8; 4 men, 4 women) breathed spontaneously through a nonrebreathing valve (NRV) for 4 min before the collection of breath. Breath samples were collected three times per day: morning (8:00–9:00 AM), midday (12:00–1:00 PM), and afternoon (4:00–5:00 PM) on 3 days (Monday, Wednesday, and Friday). Tidal volume, carbon dioxide, mouth pressure, and respiration rate were monitored during the collection periods. Five of eight subjects reported taking a daily multivitamin supplement, and one person was receiving cholesterol-reduction therapy.

The second study was a “paced breathing” experiment. During this investigation the subjects (n = 16, 8 male, 8 female) followed a regimented breathing pattern, and respiration was paced at 10 breaths/min. This respiration rate has been found to be most comfortable in both clinical studies of normal and diseased adult subjects (Risby T and Cope K, unpublished data). An audible electronic metronome prompted the study subject to inhale or exhale at a defined interval of 3 s, with equal durations of inspiration and expiration. The depth of breathing (tidal volume) was gauged according to the body mass of the subject and the respiration rate (9). Breath was collected five times per day between the hours of 8:00–9:00 AM, 10:00–11:00 AM, 12:00–1:00 PM, 2:00–3:00 PM, and 4:00–5:00 PM. Carbon monoxide, nitric oxide, and sulfur compounds were also analyzed during the paced breathing study. All subjects reported having eaten before morning and midday breath collections. The breath samples for both protocols were collected in the same research laboratory, and all study subjects were present in the laboratory for at least a 5-min period before breath collection commenced.

**System components and design for the recording of ventilation parameters.** A schematic diagram of the breath collection device is shown in Fig. 1. The breath collection system was designed to process ventilation parameters relevant to quantification and characterization of compounds in exhaled breath. Real-time measurements of carbon dioxide, volume, and inspiratory pressure are displayed on a computer screen during breath collection. This visual display can allow the subject to monitor his or her own tidal volume for each breath and to adjust ventilation to the desired breathing pattern. The system also permits a complete record of respiratory parameters to be saved. Minute ventilation is calculated from the tidal volume and respiration rate. Mouth pressure is monitored to ensure that a subject maintains a tight seal with the mouthpiece for each breath and that complete inhalation and exhalation are conducted through the NRV. This is of particular importance when the inspiratory air is a specialty gas, such as hydrocarbon-free scrubbed air, and it ensures that the breath sample is not contaminated with ambient air. All of the breathing data are stored as ASCII files.

![Fig. 1. A schematic diagram of the breath collection device. The subject breathes tidally through the membrane filter. Exhaled breath flows through pressure and volume transducers, filling the reservoir where end-tidal and steady-state carbon dioxide content are measured. The exhaled breath is pulled through a plastic flow splitter and collected on duplicate thermal desorption tubes. Directional arrows indicate valve orientation during exhalation (dashed arrows) and the digitized signals for volume flow, pressure, and carbon dioxide signals (solid arrows). A/D, analog to digital; PC, personal computer.](http://jap.physiology.org/Downloadedfrom)
The breath collection system contains commercially available components (Kent Scientific, Litchfield, CT). The subject breathes through a disposable low-pressure-drop bacterial filter (Resp-Bac, Princeton, MN) into the mouth port of a three-way NRV (Hans Rudolph, Kansas City, MO). A medium turbine flow transducer with a voltage range of 5–10 V (Interface Associates, Aliso Viejo, CA) was placed between the microbial filter and the NRV. Output from the flow transducer was connected to a personal computer interface (VM4401, Interface Associates, Aliso Viejo, CA). A nondispersive single beam infrared carbon dioxide sensor was placed on the inspiratory port of the NRV directly after the sampling port (Protocol Systems, Beaverton, OR). Capnographic measurements were transmitted to the data-acquisition system via an eight-channel to digital-to-analog converter (D2A-8000, Protocol Systems, Beaverton, OR). A differential pressure transducer (Series 600, Autotran, Eden Prairie, MN), with a pressure range of 0–2.54 cmH₂O, was connected to the inspiratory port of the NRV. The eight-channel digital-to-analog converter, personal computer interface, and pressure transducer were all connected to a terminal panel (Computerboards, Mansfield, MA). A data-acquisition card (PCMDAS16/330, Computerboards) digitized the signal from the terminal panel for analysis in a custom worksheet (Dasylab, Dasytect, NH). Anesthesia tubing was added after the carbon dioxide sensor to reduce infusion of room air. The total dead volume added by the system was 70 ml.

Breath collection. After 4 min of relaxed breathing, duplicate breath samples were collected simultaneously via Teflon tubing (3.2 mm OD) from a sampling port on the expiratory port of the NRV. Sampling was performed at constant flow of (80 ml/min per tube) for 1 min by using a pump (Airpro Surveyor 2, BIOS International, Pompton Plains, NJ). Before breath collection, the sampling line was purged of air. Breath samples were collected in Pyrex thermal desorption tubes (Supelco, Bellefonte, PA). These tubes were packed with three beds (2 cm) of carbonaceous adsorbents: a graphitized carbon, Carbopack X, and two carbon molecular sieves, Carboxen 1018, Carboxen 1019. The physical properties of these adsorbent are contained in Table 1. The thermal desorption tubes have unique code numbers stamped onto their surfaces that were used to track the collected gas samples. During breath sampling, heart rate and percent transcutaneous oxygen saturation were monitored by using a transcutaneous pulse oximeter (Nonin Medical, Minneapolis, MN) that was placed on the same finger during breath sampling. Samples of room air (80 ml) were collected at the time of breath collection.

Breath analysis. The collected breath samples were analyzed by using automated two-stage thermal desorption (ATD400, Perkin-Elmer) capillary gas chromatography (Autosystem PEXLM, Perkin-Elmer). The collected breath samples were thermally desorbed at 300°C, in the opposite direction of flow to collection, with ultrarare helium. Separations were performed by using a 60-m fused silica open tubular column (0.32 mm) with a thick-film (5 μm) of cross-linked bonded dimethyl silicone (Rtx-1, Restek, Bellefonte, PA). The temperature protocol was as follows: isothermal at 35°C for 10 min, temperature programmed 35–200°C at 5°C/min, and isothermal at 200°C for 10 min. The column effluent was monitored by using flame ionization for carbon- and hydrogen-containing compounds and flame photometric detectors for sulfur-containing molecules.

Data analysis. A computerized data processing program (Turbochrom, Perkin-Elmer) was used to acquire the chromatographic data. The concentrations of breath molecules were quantified on the basis of response factors generated from calibration curves of known standard gas concentrations. The between-tube standard coefficient of variation for ethane sampled from repeated breath samples was <4%.

Expression of breath data. Mixed expired breath concentrations were adjusted to end-tidal concentrations, and endogenously derived breath molecules were corrected for any background concentrations of molecules present in room air. End-tidal (i.e., alveolar) concentrations of molecules can be represented in generic units of picomoles per liter (pM/liter), expressed as a body mass normalized generation rate in units of picomoles per kilogram per minute or normalized to the amount of carbon dioxide exhale.

Analysis of nitric oxide. Exhaled nitric oxide was measured by using a chemiluminescence analyzer (Sievers NOA 280, Sievers Instruments, Boulder, CO). Subjects exhaled through a disposable low-pressure-drop bacterial filter that was attached to a NRV. The expiratory port of the NRV contains a flow restrictor. Exhaled nitric oxide was measured by using restricted breathing exhalation, and concentrations were recorded from the signal plateau (>4 s) produced at a constant flow of 50 ml/s and at a mouth pressure of 10 cmH₂O (1). This mouth pressure ensured soft palate closure and isolation of nasal passages. The sampling rate of the nitric oxide analyzer was 160 ml/min. During analysis, the reaction cell pressure was ~6.0 Torr, and the temperature of the photomultiplier was maintained at less than −10°C. Repeated measurements of breath nitric oxide had a coefficient of variation <1%.

Analysis of carbon monoxide. Exhaled carbon monoxide was analyzed electrochemically (LR 250, Logan Research, Kent, UK). Subjects exhaled from rest position (i.e., functional vital capacity) into their expiratory reserve volume through a disposable low-pressure-drop biological filter attached to a Teflon chamber with a flow restrictor at the outlet. The carbon monoxide monitor sampled the breath before the restrictor and recorded the maximum concentration generated during exhalation. A visual display on the monitor indicated when the required mouth pressure (5 cmH₂O) was achieved. The sampling rate of the CO analyzer was 200 ml/min. Repeated measurements of breath carbon monoxide had a coefficient of variation <1%.

Analysis of total volatile sulfur-containing compounds. A commercial electrochemical sulfur detector was modified to analyze for total volatile sulfur-containing compounds in breath (RH-17 Halimeter, Interscan, Chatsworth, CA). Subjects exhaled through a disposable low-pressure-drop bacterial filter into a NRV that had a flow restrictor attached to the expiratory port. This monitor sampled the breath before the restrictor and recorded the maximum concentration generated during exhalation. A visual display on the monitor indicated when the required mouth pressure (10 cmH₂O) was achieved. The sampling rate of the sulfur monitor was 400 ml/min. End-tidal concentration of carbon dioxide was measured during sulfur analysis (Novametrix Medical Systems, Wallingford, CT). Repeated measurements of exhaled sulfur-containing molecules had a coefficient of variation <5%.

Statistical analysis. Generalized estimating equation regression models were used to analyze linear time trends for serial measurements. The regression models used an independent correlation matrix with robust standard errors. Variables for which there were no significant changes over time were averaged and treated as a single measurement for each subject. Because of the small number of independent measurements, Mann-Whitney tests were used to compare independent groups. A Wilcoxon matched-pairs signed rank-sum test was used to analyze the difference between paired observations. Correlations between variables were calculated by using Spearman’s rank correlation tests (rₛ). A two-sided P value was considered significant at P < 0.05. All statistical analysis was performed using a computer-based software package (Stata 6.0, Stata, College Station, TX).

Table 1. Physical properties of carbonaceous adsorbents

<table>
<thead>
<tr>
<th>Adsorbent</th>
<th>Surface Area, m²/g</th>
<th>Density, g/cm³</th>
<th>Pore Size, nm</th>
<th>Pore Volume, cm³/g</th>
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<tbody>
<tr>
<td>Carbopack X</td>
<td>240</td>
<td>0.41</td>
<td>Meso, 20</td>
<td>0.62</td>
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<tr>
<td>Carboxen 1018</td>
<td>700</td>
<td>0.6</td>
<td>Micro, 0.6</td>
<td>0.35</td>
</tr>
<tr>
<td>Carboxen 1019</td>
<td>650</td>
<td>0.6</td>
<td>Micro, 0.5</td>
<td>0.30</td>
</tr>
</tbody>
</table>
RESULTS

Effects of ventilation patterns on breath composition. Figure 2 shows a representative profile for data collected from a study subject during a paced breathing (10 breaths/min) experiment. The volume flow (A), the inspiratory pressure trace (B), and the carbon dioxide profile (C) were recorded into an ASCII file (D) over a 1-min interval of breathing. Inspiratory pressure was monitored to ensure that the complete breath was sampled. The carbon dioxide waveform reflects the position of the monitor on the expiratory port of the NRV. Continuous traces for the average end-tidal and steady-state carbon dioxide measurements are also shown. In this position, the steady-state concentration of carbon dioxide reflects the average concentration of carbon dioxide in the collected breath sample. The volume and pressure signals trigger the computer to record measurements for carbon dioxide, respiration rate, tidal volume, and mouth pressure into a spreadsheet (Fig. 2D) after each breath. On inhalation, the pressure signal must drop below the base pressure level setting (Fig. 2B) to record the information for that breath. Similarly, the tidal volume signal (Fig. 2A) must also rise above the breath log setting for the information to be recorded. The breath log trigger line indicates that a breath has been logged. These signals allow a constant ventilatory pattern to be maintained throughout sample collection. Figure 2D shows the data for the calculated respiration rate, the tidal volume, the base mouth pressure reading, the mouth pressure at 100 ms, the change in mouth pressure, and the steady-state and end-tidal carbon dioxide levels stored in a spreadsheet.

During these investigations, it was found that spontaneously breathing individuals hyperventilated. Figure 3 demonstrates online measurement of carbon dioxide content during spontaneous breathing. In this example, a study subject breathed at a respiration rate of 10 breaths/min for 5 min. As the tidal volume increased, the end-tidal carbon dioxide concentration decreased from 4.7 to 3.2%.

Table 2 compares the demographic and ventilation data for subjects evaluated during spontaneous and paced breathing experiments. The study groups for each of these experiments comprised equal numbers of men and women. There were no significant differences between study groups by age, height, weight, body mass index, transcutaneous oxygen saturation, or heart rate. Paced breathing caused mean tidal volume and minute ventilation to be lower than in the spontaneous breathing group. The respiration rate in the spontaneous and paced breathing groups was similar; therefore, the increase in minute ventilation in the spontaneous breathing group is attributed to higher tidal volumes. Shallower tidal breathing in the paced breathing group also resulted in significantly higher end-tidal carbon dioxide levels, but the average steady-state carbon dioxide levels were similar. The end-tidal concentration of carbon dioxide was constant from breath to breath for the paced breathing group.

It was found that the concentrations of breath ethane and isoprene correlated with end-tidal concentration of carbon dioxide levels (carbon dioxide and ethane $r_s = 0.50$, $P = 0.05$, $n = 16$; carbon dioxide and isoprene $r_s = 0.64$, $P = 0.008$, $n = 16$) in the paced breathing group. There was also a correlation between isoprene and end-tidal concentration of carbon dioxide in the spontaneous breathing group ($r_s = 0.71$, $P < 0.05$, $n = 8$). The concentrations of ethylene, methanol, acetone, and ethanol did not correlate with end-tidal concentration of carbon dioxide in either group. Additionally, an increase in the concentration of carbon monoxide was inversely correlated with percent transcutaneous oxygen saturation levels ($r_s = -0.64$, $P = 0.008$, $n = 16$). In the paced breathing group, it was also found that the concentration of ethane correlated with heart rate ($r_s = 0.52$, $P < 0.05$, $n = 16$).

<table>
<thead>
<tr>
<th>Resp Rate</th>
<th>TV (L)</th>
<th>Base PR</th>
<th>100me P</th>
<th>Delta $P$</th>
<th>SS CO$_2$</th>
<th>ETCO$_2$</th>
<th>ETCO$_2$</th>
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</thead>
<tbody>
<tr>
<td>17</td>
<td>0.54</td>
<td>0.055</td>
<td>-0.141</td>
<td>0.196</td>
<td>28.706</td>
<td>39.670</td>
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<tr>
<td>10</td>
<td>0.52</td>
<td>0.058</td>
<td>-0.094</td>
<td>0.152</td>
<td>33.531</td>
<td>39.237</td>
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<tr>
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<td>0.55</td>
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<td>-0.069</td>
<td>0.168</td>
<td>30.259</td>
<td>39.548</td>
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<tr>
<td>10</td>
<td>0.50</td>
<td>0.016</td>
<td>-0.041</td>
<td>0.067</td>
<td>32.891</td>
<td>39.752</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.52</td>
<td>0.007</td>
<td>-0.088</td>
<td>0.125</td>
<td>30.950</td>
<td>39.255</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.60</td>
<td>0.062</td>
<td>-0.049</td>
<td>0.111</td>
<td>33.749</td>
<td>39.668</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>0.52</td>
<td>0.042</td>
<td>-0.058</td>
<td>0.100</td>
<td>29.448</td>
<td>40.710</td>
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<tr>
<td>10</td>
<td>0.53</td>
<td>0.058</td>
<td>-0.149</td>
<td>0.208</td>
<td>34.025</td>
<td>39.411</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.54</td>
<td>0.032</td>
<td>-0.102</td>
<td>0.133</td>
<td>29.298</td>
<td>39.127</td>
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</tr>
<tr>
<td>9</td>
<td>0.52</td>
<td>0.048</td>
<td>-0.104</td>
<td>0.152</td>
<td>35.042</td>
<td>39.576</td>
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</tr>
</tbody>
</table>
Correction and normalization of exhaled breath compounds. The concentration gradient in mixed expired breath is the difference between the concentrations of endogenous molecules in the mixed expired breath sample and the concentration in the end-tidal component. Carbon dioxide measurements were used to correct the background concentrations in the dead space and the dilution of the end-tidal breath. A simple way to adjust the mixed expired concentration to the end-tidal concentration is to multiply by the average end-tidal carbon dioxide concentration-to-average mixed expired carbon dioxide concentration ratio. The corrected concentrations ($C_{corr}$) were calculated (in pmol/l) by using the following equation

$$C_{corr} = \frac{ETCO_2 (C_{me} - C_{ra})}{MECO_2}$$

where $ETCO_2$ is the average end-tidal carbon dioxide (in Torr), $MECO_2$ is the average mixed expired carbon dioxide at steady state (in Torr), $C_{me}$ is the concentration of the molecule of interest in mixed expired breath (in pmol/l), and $C_{ra}$ is the room air concentration of the same molecule (in pmol/l). If the concentration in room air is higher than the endogenous production and/or steady state is not reached, this correction will result in a negative value. Negative concentration gradients are not useful in the quantification of the endogenous production of breath biomarkers and were excluded from any data analysis.

To make comparisons between study subjects the concentration of molecules in breath should be normalized. These normalizations minimize contributions due to ventilation and heart rate. Table 3 shows the mean end-tidal, room air-corrected concentrations of the major breath analytes collected from the paced breathing subjects. Data are expressed in units of concentration (in pmol/l), as generation rates normalized to body mass or surface area (in pmol·kg$^{-1}$·min$^{-1}$, pmol·m$^{-2}$·min$^{-1}$), or normalized to carbon dioxide production (in pmol/ml CO$_2$). The end-tidal concentrations of methanol, ethylene, and ethane were all substantially reduced by contributions from room air and show increased variability. Generation rates require measurement of tidal volume, respiration rate, and body weight or body surface area. Normalization for body mass ($C_{norm}$; in pmol·kg$^{-1}$·min$^{-1}$ or pmol·m$^{-2}$·min$^{-1}$) was performed as follows

$$C_{norm} = \frac{C_{corr} \times MV}{b}$$

where $MV$ is the minute ventilation (in l/min) and $b$ is the weight (in kg) or body surface area (in m$^2$). The subject should maintain relaxed steady-state tidal breathing if breath is expressed as a generation rate and the average steady-state carbon dioxide must be determined. However, when there are significant differences in the body masses between subjects carbon dioxide normalization may be more reliable. Carbon dioxide normalization ($C_{norm}$; in pmol/ml CO$_2$) can be obtained as

### Table 2. Anthropometric and physiological parameters

<table>
<thead>
<tr>
<th></th>
<th>Spontaneous Breathing (n = 8)</th>
<th>Paced Breathing (n = 16)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>30.6 ± 10.8</td>
<td>29.4 ± 5.7</td>
</tr>
<tr>
<td>Body mass, kg</td>
<td>74.0 ± 25</td>
<td>71.0 ± 15</td>
</tr>
<tr>
<td>Height, cm</td>
<td>170.8 ± 10.7</td>
<td>172.7 ± 7.3</td>
</tr>
<tr>
<td>Body mass index, kg/m$^2$</td>
<td>25.2 ± 7.6</td>
<td>23.7 ± 3.9</td>
</tr>
<tr>
<td>Oxygen saturation, %</td>
<td>97.5 ± 0.8</td>
<td>97.1 ± 0.9</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>81.1 ± 11</td>
<td>74 ± 10</td>
</tr>
<tr>
<td>Respiration rate, breaths/min</td>
<td>11 ± 1</td>
<td>10</td>
</tr>
<tr>
<td>Tidal volume, ml</td>
<td>1,080 ± 270</td>
<td>558 ± 86</td>
</tr>
<tr>
<td>Minute ventilation, l/min</td>
<td>11.9 ± 3.0</td>
<td>5.6 ± 0.8</td>
</tr>
<tr>
<td>End-tidal CO$_2$, Torr</td>
<td>32 ± 4.2</td>
<td>36.4 ± 2.0*</td>
</tr>
<tr>
<td>Instantaneous CO$_2$, Torr</td>
<td>28.9 ± 3.3</td>
<td>29.6 ± 2.3</td>
</tr>
</tbody>
</table>

Values are means ± SD; n, no. of subjects. *Significant difference from spontaneous breathing measurements, $P < 0.02$.

### Table 3. Normalized concentrations of ambient air-corrected molecules in breath

<table>
<thead>
<tr>
<th></th>
<th>nmol/l</th>
<th>pmol·kg$^{-1}$·min$^{-1}$</th>
<th>pmol·m$^{-2}$·min$^{-1}$</th>
<th>pmol/ml CO$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethylene</td>
<td>0.157 ± 0.193</td>
<td>7.9 ± 4.8</td>
<td>0.299 ± 0.180</td>
<td>2.5 ± 1.5</td>
</tr>
<tr>
<td>Ethane</td>
<td>0.103 ± 0.095</td>
<td>6.5 ± 4.9</td>
<td>0.230 ± 0.178</td>
<td>1.99 ± 1.45</td>
</tr>
<tr>
<td>Hydrogen sulfide</td>
<td>0.245 ± 0.068</td>
<td>19.1 ± 6.3</td>
<td>0.718 ± 0.201</td>
<td>6.37 ± 1.99</td>
</tr>
<tr>
<td>Carbonyl sulfide</td>
<td>2.03 ± 2.16</td>
<td>121 ± 128</td>
<td>5.48 ± 5.40</td>
<td>50.5 ± 51.4</td>
</tr>
<tr>
<td>Methanol</td>
<td>8.14 ± 3.01</td>
<td>647 ± 258</td>
<td>24.5 ± 8.9</td>
<td>214 ± 87</td>
</tr>
<tr>
<td>Ethanol</td>
<td>6.04 ± 4.97</td>
<td>494 ± 436</td>
<td>18.3 ± 15.2</td>
<td>157 ± 135</td>
</tr>
<tr>
<td>Acetone</td>
<td>19.7 ± 8.2</td>
<td>1,530 ± 640</td>
<td>58.0 ± 22.0</td>
<td>513 ± 225</td>
</tr>
<tr>
<td>Isoprene</td>
<td>6.07 ± 1.75</td>
<td>488 ± 170</td>
<td>18.5 ± 5.9</td>
<td>156 ± 44</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = 16 subjects.
Normalization of breath compounds as a generation rate or to carbon dioxide decreases variability. The interindividual coefficient of variation for the corrected ethane concentrations is improved when the data are normalized to carbon dioxide generation rates: nmol/l (0.92) > nmol·m⁻²·min⁻¹ (0.77) > pmol·kg⁻¹·min⁻¹ (0.75) > pmol/ml CO₂ (0.73).

**Variations of biomarkers as a function of time of day or gender.** The variations of real-time biomarkers, as function of gender, are shown in Table 4. Nitric oxide did not show a variation with gender. However, it should be noted that subjects in our study group showed a wide variation in concentrations of breath nitric oxide. Other biomarkers were found to be gender dependent. Men exhaled significantly higher levels of carbon monoxide and sulfur-containing molecules. Moreover, women had significantly higher heart rates and transcutaneous pulse oxygenation compared with men.

As is shown in Fig. 4, the sulfur signal from a single exhaled breath increased throughout the end-tidal phase, which suggests that these compounds may have endogenous origins. The concentrations of sulfur-containing compounds increased 27% over the 8-h period. This trend, which was investigated by using a multiple linear regression model for repeated measurements, suggests that time is a significant predictor of sulfur concentration (β = 7.4, 95% confidence interval = 2.6–12.1, z = 3.0, P < 0.003). Additionally, time was the only significant predictor of sulfur concentration (β = 5.5, 95% confidence interval = 0.58–10.5, z = 2.2, P < 0.03) in a model that included time, gender, end-tidal carbon dioxide, and heart rate as covariates. Individually, heart rate and gender, which are collinear, were significant predictors of sulfur concentration. Figure 5 shows the variation in the concentration of breath ethanol for each study subject at 12:00–1:00 PM and 2:00–3:00 PM. For 13 of the 16 study subjects, ethanol production was restricted exhaled breath sampling is the gold standard technique for the analysis of breath nitric oxide and carbon monoxide. Controlling expiratory flow reduces the variability of the analytic signal. Moreover, restricted breathing with controlled pressure permits velum closure, preventing nasal contamination of exhaled NO. However, this technique can be used only when molecules are being analyzed in real time. For tidal breath collection, a paced breathing profile, which standardizes the tidal volume according to body mass and respiration rate, provides reduced between-breath variability, resulting in the

**DISCUSSION**

The breath collection system presented herein allows complete characterization of breathing during sampling. In our experience, spontaneously breathing subjects tend to hyperventilate during breath collection; the use of visual displays and paced breathing results in less variation in individual breaths. It has been found that a respiration rate of 10 breaths/min is well tolerated by subjects breathing through a mouthpiece. Restricted exhaled breath sampling is the "gold standard" technique for the analysis of breath nitric oxide and carbon monoxide. Controlling expiratory flow reduces the variability of the analytic signal. Moreover, restricted breathing with controlled pressure permits velum closure, preventing nasal contamination of exhaled NO. However, this technique can be used only when molecules are being analyzed in real time. For tidal breath collection, a paced breathing profile, which standardizes the tidal volume according to body mass and respiration rate, provides reduced between-breath variability, resulting in the

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**Table 4. Gender differences in biomarkers determined in real time**

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Women (n = 8)</th>
<th>Men (n = 8)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxygen saturation, %</td>
<td>97.7±0.5</td>
<td>96.5±0.8</td>
<td>0.004</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>80.2±10.5</td>
<td>66.9±4.7</td>
<td>0.006</td>
</tr>
<tr>
<td>Sulfur-containing molecules, ppb</td>
<td>94.8±29.2</td>
<td>135±24.6</td>
<td>0.016</td>
</tr>
<tr>
<td>Carbon monoxide, ppm</td>
<td>3.0±0.5</td>
<td>4.0±0.7</td>
<td>0.01</td>
</tr>
<tr>
<td>Nitric oxide, ppb</td>
<td>28.4±29.9</td>
<td>26.8±22.3</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are means ± SD; n, no. of subjects. Significant differences were assessed by Mann-Whitney rank-sum test. ppm, Parts per million; ppb, parts per billion; NS, not significant.
collection of a more reproducible and physiologically relevant breath sample.

There were significant changes in breath molecule concentrations as a result of changes in ventilation parameters. As expected, increased minute ventilation was associated with decreased end-tidal carbon dioxide output. Our data suggest that the concentrations of isoprene and ethane are dependent on the end-tidal concentration of carbon dioxide. In the paced breathing study, isoprene was significantly correlated with end-tidal carbon dioxide levels. One hypothesis investigated in these studies was that controlled breathing would prevent hyperventilation, thereby reducing variability in ventilation that affects the concentrations of breath molecules. Correlations between end-tidal carbon dioxide and isoprene were found in both the paced and spontaneous breathing groups, but the dependence of breath ethane on end-tidal carbon dioxide and heart rate was only found in the paced breathing group. It may be that the paced breathing causes the study subject to achieve a steady-state concentration of ethane from exogenous and endogenous sources more rapidly.

There was a significant inverse correlation between oxygen saturation and carbon monoxide levels in our subjects, who were all nonsmokers. This correlation may reflect increased carboxyhemoglobin levels in the blood, a decreased oxyhemoglobin pool, or an increase in heme turnover (42). It has been stated that control of breath molecules such as garlic and onions may explain the difference in exhaled sulfur-containing molecules; however, the gender-dependent relationship remains unclear. It has been suggested that carboxyhemoglobin cannot be differentiated from oxyhemoglobin by using transthecutaneous pulse oximetry, it is not possible to quantify carboxyhemoglobin levels by this method.

Gender differences were also identified for carbon monoxide, sulfur-containing molecules, heart rate, and percent transcutaneous oxygen saturation. It has been shown that transcutaneous oxygen saturation and heart rate are higher in normal and diabetic women than in men (36). Carbon monoxide and sulfur-containing molecules were higher in men than women: the basis of this difference is currently unknown. Increased consumption of foods with a high content of organic sulfur molecules such as garlic and onions may explain the difference in exhaled sulfur-containing molecules; however, the gender-dependent relationship remains unclear. It has been suggested that exhaled nitric oxide is higher in men than in women (14, 46). However, the present study agrees with a previous report that found no gender difference in exhaled nitric oxide (8).

A major criticism of breath analysis has been the lack of a standard way to express concentrations of breath molecules suitable for interindividual comparisons. Concentrations have been expressed generically as parts per billion (nl/l) or picomoles per liter, but these units are volume dependent and can be influenced by depth of breathing. Therefore, it is necessary to normalize the concentrations of molecules to some other parameter to make interindividual comparisons. Breath molecules can be expressed as body mass- or body surface area-normalized generation rates; however, these parameters will overnormalize the data for obese individuals and are susceptible to changes in tidal volume if depth of breathing is not constant. Therefore, we propose that in healthy individuals breath molecules should be normalized to the production of carbon dioxide. It has been shown that carbon dioxide normalization is a superior method when monitoring breath molecules in mice and rats when there are large differences in body mass (6, 30). Carbon dioxide normalization has also been shown to reduce variability in breath hydrogen, methane, and carbon monoxide measurements (19, 25). Herein it is shown that interindividual variability in exhaled ethane decreased by >20% when normalized to carbon dioxide compared with per liter volume.

Isoprene is a stable elimination product in the mevalonate pathway of cholesterol biosynthesis (7). Statin therapy and cholesterol consumption have been shown to reduce breath isoprene levels (15, 40). Moreover, Foster et al. (10) demonstrated that isoprene exhalation rates can be modified by controlled exposure to ozone during exercise. A diurnal variation for exhaled isoprene has been reported, and the levels of isoprene were highest in the early morning (2:00–6:00 AM) (4). However, in the present study, there was no apparent change in isoprene concentration over the time period studied when ventilation was paced. Karl et al. (15) propose that a decline in breath and blood isoprene during controlled exercise is due to increasing respiration rate. These investigators modeled blood isoprene during controlled exercise using the breath measurements, heart rate, respiration rate, and the blood-air partition coefficient for isoprene. After a transient increase in isoprene concentration, exhaled isoprene tended to decrease as respiration rate and heart rate increased. These authors attributed the decrease in exhaled isoprene to an increase in respiration rate and decline in steady-state concentration of isoprene in the bloodstream. Similar results have been reported by others (10). However, it has been demonstrated herein that levels of ethane or isoprene correlated with end-tidal carbon dioxide. Steady-state levels of carbon dioxide may drop during exercise owing to hyperventilation, which will also affect the concentration of isoprene. When a subject breathes tidally, the tidal volume, the end-tidal concentration of carbon dioxide level, and the heart rate do not vary significantly. Thus variability in isoprene concentration may also be limited by controlling the tidal volume and respiration rate.

The data presented herein show that background levels can contribute significantly to the exhaled concentration of some endogenously produced molecules, such as ethane and ethylene. In the present study, it was found that some of the corrected breath ethane and ethylene concentrations were negative, which indicated that the background concentration of ethane or ethylene exceeded the endogenously produced concentration. Therefore, measurements of breath ethane or ethylene should only be performed after the study subject has reached steady state with ambient levels of these molecules. The average ethane generation rate was approximately two times greater than that estimated by Knutson et al. (16) when breath was collected from subjects breathing on a hydrocarbon-free breathing air source. Because ethane exhalation correlates with carbon dioxide levels and heart rate, the exhaled quantity of ethane may also be affected by differences in breathing patterns. Therefore, it is suggested that, when using this breath collection system, molecules should be corrected and quantified by estimating the dilution with carbon dioxide measurements and normalizing to carbon dioxide output while monitoring heart rate.
The lunchtime peak in ethanol may be related to the consumption of foods that contained small quantities of ethanol or result from fermentation of carbohydrates by intestinal bacteria. Oxygen tension is low in the colon and anaerobic bacteria can flourish. During alcoholic fermentation, anaerobic bacteria reduce acetaldehyde to ethanol (34). Thus it is conceivable that ethanol levels may peak quickly after the consumption of food. For example, it has been shown that gastric emptying of liquid diet can occur within <30 min in normal adults (22). Moreover, the \( K_{m} \) of bacterial acetaldehyde dehydrogenase enzymes has been found to be approximately seven times lower than the level of endogenous ethanol in the large intestine; therefore, the unabsorbed carbohydrates provide ample substrate for the production of ethanol in breath through the metabolism of gut bacteria (26). There are studies that have reported the endogenous ethanol production from bacterial overgrowth in obese mice (6). Also, it has been reported that gender and body mass index are predictors of high breath ethanol in patients with nonalcoholic steatohepatitis (24). The increase in ethanol after meals suggests that bacterial activity may play a role in the production of ethanol and that endogenous ethanol may be a useful biomarker of anaerobic microflora in the gastrointestinal tract.

Sulfur-containing compounds changed linearly as a function of time. Even when other parameters, such as heart rate, gender, and end-tidal carbon dioxide were controlled statistically, there remained a significant increase in the concentration of sulfur-containing compounds over time. Exhaled volatile sulfur-containing molecules have been suggested to result from sulfate-reducing bacteria in the colon and from the metabolism of allyl sulfur compounds (20, 32). It has previously been shown that both oral and gut bacteria may be significant sources of the volatile sulfur species in breath (44). Moreover, specific sulfur compounds such as carbonyl sulfide and dimethyl sulfide are known markers of liver disease pathogenesis and carbonyl sulfide has been suggested to be an early marker of acute rejection in lung transplant patients (37, 43). Because the profile for the total sulfur-containing compounds tracked end-tidal carbon dioxide output, these molecules are probably generated systemically and not derived from the mouth. Many different sulfur-containing molecules may make up the group of compounds that are detected by using this “breathalyzer.” Several of these compounds may be related to food consumption; however, the reason for the increase over time is not yet known and requires further investigation.

The endogenous source of methanol and ethylene in humans has not yet been fully characterized. It has been suggested that methanol may be generated by hydrolysis of \( S \)-adenosyl methionine forming homocysteine and methanol as products (3). Methanol is commonly found in room air but at levels below those found in breath (28, 38). Ethylene may be a direct or indirect marker of oxidative stress. Ethylene is generated during lipid peroxidation, but it has also been shown to be formed from the reaction of methione with peroxynitrite (33, 29).

In summary, these investigations indicate that if breath is collected during paced breathing and is normalized to carbon dioxide the results can be used for intersubject investigations. Diurnal variation does not appear to affect any of the molecules measured here except for the increased postprandial ethanol production and increased production of sulfur-containing molecules. Therefore, breath samples collected at different times on different days can be compared and contrasted. Finally, the collection of ventilation parameters, including carbon dioxide, is instrumental in documenting breath collections.

GRANTS
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REFERENCES


