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Original Contribution

A PRACTICAL AND RELIABLE METHOD FOR MEASURING ETHANE AND PENTANE IN EXPIRED AIR FROM HUMANS

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Abstract—We describe a method for the collection of expired air and further document the performance of our analytical technique that is used to measure ethane and pentane simultaneously. Four minutes of breathing hydrocarbon-free air before collection effectively removed high concentrations of residual ambient ethane and pentane from the lungs, with washout times up to 30 min resulting in no further reductions in breath hydrocarbons. Mean (±SE) exhalation rates (pmol/kg b.wt./min) in 11 subjects were 2.4 ± 0.6 for ethane and 1.5 ± 1.3 for pentane. Total intraindividual variability in exhalation rates (as percent coefficient of variation, %CV), measured from 4 subjects on at least 6 different days, was greater for pentane (44% CV) than for ethane (29% CV). Analytical variability contributed 6% to the total %CV. Advantages of the method are described, and reasons for the large variability in values reported in the literature are discussed. © 1999 Elsevier Science Inc.

Keywords—Lipid peroxidation, Oxidative stress, Free radicals, Gas chromatography, Hydrocarbons, Alkanes, Breath tests

INTRODUCTION

The measurement of ethane and/or pentane in expired air, sometimes referred to as the *hydrocarbon breath test* [1], offers a noninvasive means to assess in vivo lipid peroxidation. Elevated levels of ethane and/or pentane have been reported in humans under various conditions of oxidative stress, such as total body irradiation [2], cigarette smoking [3,4], acute aerobic exercise [5], aging [6], and human immunodeficiency virus (HIV) infection [7]. Several studies also show that antioxidant vitamins can mitigate oxidative-stress-related elevations in breath hydrocarbon levels [7–10].

Although these studies demonstrate the potential utility of the hydrocarbon breath test as an index of in vivo lipid peroxidation, there remains much variability in normal adult values reported in the literature [1,11]. For example, ethane values range between 1.7 and 590 pmol/l [3,12] and pentane values range between 11.3 and 880 pmol/l [12,13]. This tremendous variability is likely caused by differences between the various methods that are used to collect and analyze expired air.

A primary difference between methods is whether or not a washout period is used before collecting expired air. A washout period involves having the subject breathe air that has been scrubbed of hydrocarbons (often referred to as hydrocarbon-free air [HCFA]) for a defined length of time in order to flush out residual ambient-air hydrocarbons from the lungs. If no washout period is used, then actual ambient-air hydrocarbon concentrations are subtracted from expired-air hydrocarbons. In a detailed review of the hydrocarbon breath test, Kneepkens et al. [1] noted that hydrocarbon exhalation rates were extremely variable in studies without a washout period, but they correlated reasonably well among studies in which a washout period was used. The removal of ambient air from expired air probably results in greater consistency between studies because ambient-air hydrocarbon concentrations are frequently much greater and more variable than those in expired air [13–15].

Washout periods used by different investigators usually range between 4 and 30 min [1], although periods of up to 2 h have been used [13,16]. The use of different washout periods may contribute to the variability between hydrocarbon values reported in the literature. In the only report on

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the effect of washout on breath hydrocarbon levels, Morita et al. [13] measured breath pentane levels after subjects breathed HCFA for 0, 30, 60, 90, and 120 min. They found that after a 30-min washout, breath pentane values were six times less than those after no washout, and that no significant differences were observed between washout times of 30, 60, 90, and 120 min. Although these results provide evidence that 30 min of breathing HCFA is adequate to flush out relatively high levels of ambient pentane from the lungs, times less than 30 min may be adequate (and more practical).

Another source of variability between studies likely originates from the various analytical procedures that are used to quantitate hydrocarbons in washed-out expired air. For example, most analytical procedures involve a step in which expired-air samples are first concentrated; this is because hydrocarbon levels in washed-out expired air often become lower than the detection limit of most gas chromatographic methods. Hydrocarbons are usually concentrated using "trap-and-purge" techniques in which hydrocarbons are trapped in a cooled adsorbant and purged with heat into a small volume. Although a number of techniques are used for this purpose, we [17] and other investigators [18] have found that certain techniques are ineffective at trapping highly volatile ethane. For measurements of pentane, some investigators [11,19] question many of the current methods, which appear to not discriminate between pentane and isoprene. What is more, few methods provide documentation of analytical performance. Therefore, in response to the need for reliable measurements of ethane and pentane, we developed and validated a method to concentrate and analyze expired-air samples from laboratory rats [17].

Most researchers who perform the hydrocarbon breath test measure only pentane, although few groups measure only ethane or both hydrocarbons [1]. This appears to be due partly to the significantly different physico-chemical properties of ethane and pentane and partly to technical difficulties inherent in concentrating highly volatile ethane. Nevertheless, if only one or both hydrocarbons are measured, then analytical variation, intraindividual variation, and interindividual variation need to be determined in order to assess the reliability of a specific hydrocarbon breath test as a clinical test of in vivo lipid peroxidation.

The purposes of the present study were to (i) set up a practical system for the collection of expired air from resting adults, (ii) evaluate the precision of our analytical technique in measuring ethane and pentane in air samples, (iii) assess the effect of washout times between 4 and 30 min on expired-air ethane and pentane in healthy adult humans, and (iv) assess the day-to-day variability of ethane and pentane in expired air from such subjects and in ambient air.

Table 1. Subject Characteristics

Subject	Sex	Age (y)	Height (cm)	Weight (kg)	BMI[a] (kg/m^2)
1	m	28	183	79.5	23.7
2	f	36	168	63.6	22.5
3	m	66	175	83.2	27.2
4	m	22	170	63.2	21.9
5	f	23	169	58.2	20.4
6	m	23	181	85.0	25.9
7	m	36	188	85.0	24.0
8	m	55	185	73.6	21.5
9	m	57	178	95.5	30.1[b]
10	m	41	170	64.5	22.3
11	f	24	160	50.0	19.5

[a] Body mass index.
[b] BMI in the obese range.

MATERIALS AND METHODS

Subjects

Expired-air samples were collected from 11 adult nonsmokers (Table 1) who reported being healthy. Subjects fasted for at least 8 h before collections. The protocol was approved by the Committee for the Protection of Human Subjects of the University of California at Berkeley.

Expired-air collection apparatus

A representation of the apparatus used for the collection of expired air is shown in Fig. 1. The 100-l and 5-l nondiffusing gas collection bags (Hans Rudolph part no.'s CM1154 and CM1153, respectively) were made of 2-ml thick Tedlar (Hans Rudolph, Kansas City, MO, USA). Each bag has a permanently attached primary bag adaptor (1.375″ bore) to which a three-way valve (2100 series) (Hans Rudolph, Kansas City, MO, USA) can be connected. Tygon tubing (1″ inner diameter × 1 1/4″ outer diameter 1/8″ wall thickness; Fisher Scientific, Santa Clara, CA, USA) connected the three-way valves to the two-way nonrebreathing valve, 2600 series (Hans Rudolph, Kansas City, MO, USA). Copper pipe reducing unions (30 mm OD × 28 mm ID to 24 mm OD × 22 mm ID, 6.35 cm in length) (obtained from local hardware store) were used to facilitate connections between the Tygon tubing and the two-way nonrebreathing valve and the three-way valve. Three-way stopcocks (polycarbonate, large-bore, male lock) (Cat. no. 30600-10; Cole-Parmer, Vernon Hills, IL, USA) were attached to the two sampling ports on each primary bag adaptor and to the nozzle of the 1.5-l acrylic, gas-tight, Hamilton syringe[1] (Supelco Inc., Bellefonte, PA, USA). The three-way

[1] If available, larger gastight syringes may be used instead of a 1.5-l syringe, but they would be more unwieldy and would likely offer considerably more resistance than would a 1.5-l syringe when manipulating large volumes of gases.

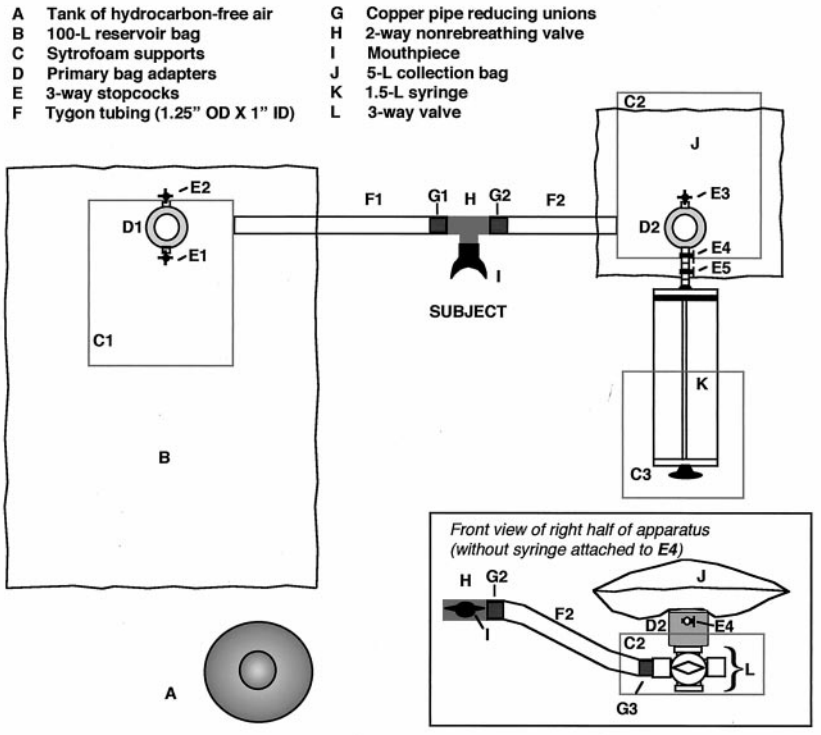

Fig. 1. Representation of the apparatus (viewed from above) used for the collection of expired air. The subject sits in a comfortable chair (not shown, NS) between two tables (NS) that support the 100-l bag (B) and 5-l bag (J). The primary bag adapters (D) of each bag connect to three-way valves (L) that are wedged into styrofoam for support (see insert). The tygon tubes (F1, F2) are supported by string attached to a ceiling hook (NS); this supports and stabilizes the mouthpiece (I). All connections between F–I and F, G, L are secured and made air-tight with four layers of parafilm. Hydrocarbon-free air is added to (B) through the E1 stopcock. Hydrocarbon-free air background samples can be taken from (B) through the E2 stopcock. The subject breathes HCFA for at least 4 min, and a sample is collected by directing the expired air through (L) into (J) (see insert). The 1.5-l syringe (K) is then used to transfer a total of 3.0 l of expired air from (J) to a gas sampling bag (NS), which can be stored for (<12 h) until analysis. For easy transfer, the gas sampling bag is placed between styrofoam supports (C2) and (C3) and connected to the 5″ piece of Tygon tubing (1/4″ OD × 1/8″ ID) (NS) that is attached to the E4 stopcock. The expired air is thus routed through the E4 stopcock (the E5 stopcock is permanently attached to the 1.5-l syringe). Between subjects, the 5-l bag is rinsed 3× with HCFA, which is added through the E4 stopcock, and removed by vacuum through the E3 stopcock. See Materials and Methods for more detail.

stopcocks provide convenient, bidirectional ports through which gases can be transferred effectively without risking the introduction of ambient-air hydrocarbons into the system. In preliminary studies, components of the apparatus were found to neither add nor subtract ethane and pentane. Our breath collection apparatus was always used in the same room, which was separate from the laboratory.

Preparations for expired-air collections

Before collecting from subjects, the 1.5-l syringe was flushed three times with HCFA (USP, zero-grade air, 1.0 ppm total hydrocarbons; Puritan Bennett, Lenexa, KA, USA), and the 5-l collection bag was flushed with HCFA five times. Bags were aspirated using a vacuum pump (Cole Parmer, Niles, IL, USA). The 100-l reservoir bag was filled with HCFA and two 3.0-l HCFA samples were taken with the 1.5-l syringe from the full 100-l bag and transferred into Tedlar gas sampling bags (12″ × 12″ × 2″, with on/off valve; Baxter Diagnostics Inc., McGaw Park, IL, USA) for subsequent ethane and pentane determinations. Hydrocarbon-free air is not truly hydrocarbon "free"; it contains trace amounts of ethane and pentane that must be subtracted from those in expired-air samples. Gas sampling bags were rinsed five times before use with nitrogen gas. Immediately before collecting from the subjects, two 3.0-l ambient-air samples were taken from the room where the expired-air collections took place.

Procedure for the collection of expired air

Subjects sat comfortably upright in a chair and breathed through either a secured facemask (Hans Rudolph, Kansas City, MO, USA) or a blue vinyl 109NT mouthpiece (Vacu-Med, Ventura, CA, USA) that connected to the two-way nonrebreathing valve. A facemask was used for studies that required extended

Table 2. Linearity of Calibration Curves

Analyte	Sample processing	Intercept[a] (mm)	Slope[a] (mm/pmol × l^{-1})	Correlation coefficient
Ethane	None[b]	0.40 ± 0.67	8.57 ± 0.09	0.999
Ethane	Trap and purge[c]	−1.65 ± 1.08	8.80 ± 0.14	0.999
Pentane	None	0.05 ± 0.41	2.67 ± 0.05	0.999
Pentane	Trap and purge	0.30 ± 0.52	2.61 ± 0.07	0.998

[a] Values are mean ± SE.
[b] Standards are diluted in 50 ml and then injected directly into gas chromatograph.
[c] Standards were added to 3.0 l of hydrocarbon-free air and then concentrated to a final volume of 100 ml before being injected into gas chromatograph.

periods of breathing (>10 min); in all other studies, the mouthpiece was used along with a disposable noseclip. When expired-air samples were collected from three subjects with either the facemask or the mouthpiece, identical ethane and pentane results were obtained (data not shown). Subjects watched television while they breathed through the system; this helped them to become less aware of their breathing and helped keep them from falling asleep. During collection periods, expired air was directed into the 5-l bag for roughly 30 s. The time required to collect the expired air was recorded with a stopwatch. Using the 1.5-l syringe, 3.0 l of collected expired air was then transferred from the 5-l bag into a gas sampling bag. The 1.5-l syringe was next used to empty (and also measure) the expired air that remained in the 5-l collection bag. The total volume of the collected expired air and the time required for the collection were used for calculations of concentration (pmol alkane/l) and exhalation rate (pmol alkane/kg body wt [b.wt.]/min). Bags were stored at room temperature and analyzed within 12 h. Before collecting from the next subject, the 5-l bag and 1.5-l syringe were again flushed three times with HCFA.

Analysis of expired air for ethane and pentane

The 3.0-l expired-air samples were concentrated to a final volume of 50 ml using a trap-and-purge procedure and then analyzed by gas chromatography (GC) as previously described in detail [17], but with three modifications: (i) For the heating bath, mineral oil was replaced with Heat Transfer Fluid 550 (Fisher Scientific, Santa Clara, CA, USA). (ii) A modified Drierite-soda lime container was constructed to replace the previously used 120 cc in-line gas purifier, because the latter, after extensive use, started to interfere with ethane and pentane recovery. The modified 120-cc Drierite-soda lime container was made out of cylinders from two 60-cc luer-lock plastic syringes, each fitted with a plastic three-way stopcock (Sherwood Medical, St. Louis, MO, USA). Drierite (20 cc) and soda lime (100 cc) were added and secured by glass wool. The full syringes were then placed back-to-back, secured with several layers of parafilm, and purged with a strong stream of nitrogen for 3 min to remove contaminant hydrocarbons. (The soda lime we used in these studies, from Fisher Scientific, Santa Clara, CA, USA, was consistently contaminated with hydrocarbons, especially pentane.) The purged container can be used for the analysis of five expired-air samples before the soda lime becomes saturated and needs to be changed. (iii) Each day before analyzing expired-air samples, the gas chromatograph was run with the column at 200°C for about 20 min. The heating ensured that any isoprene remaining on the column from the previous day's analyses had eluted from the column; it also removed traces of water that may have been introduced into the column.

Calibration curves

Ethane and pentane calibration curves were prepared from a hydrocarbon standard gas mixture (0.493 ppm ethane/0.516 ppm pentane in nitrogen; Praxair, Los Angeles, CA, USA). A concentration of 0.5 ppm is equivalent to approximately 20.5 nmol/l, using the conversion formula: nmol/l = ppm (10^{-6} l/l)/[(24.4 l/mol) × (10^{-9} mol/nmol)]. Various amounts hydrocarbon standard gas mixture (0, 2, 4, and 6 ml—which contained 0, 41, 82, and 123 pmol hydrocarbon, respectively) were measured with a 50-ml gastight, glass syringe (Supelco Inc., Bellefonte, PA, USA) and were then either diluted to a final volume of 50 ml in the syringe or added to 3.0 l of HCFA in a gas sampling bag. The hydrocarbon standards in 50 ml were injected directly into the gas chromatograph (5-ml sample loop), whereas those in 3.0 l were subjected to the trap-and-purge procedure before GC analysis. Calibration curves, representing recorder response (peak height) versus pmol of standard analyzed by GC, were subjected to linear regression analysis using Statview 4.1 (Abacus Concepts Inc., Berkeley, CA, USA).

Analytical precision

Within-day and between-day analytical precisions of ethane and pentane standards were assessed by analyzing five replicates of three different concentrations on four different days. Standard ethane and pentane concentrations were prepared by adding 1, 2, and 4 ml of standard gas mixture to 3.0 l of HCFA. Standard gas mixture was added using a 10-ml gastight, glass syringe (Supelco Inc., Bellefonte, PA, USA). Each of the 20 samples at each concentration was prepared individually. Estimates of within- and between-day variance were obtained by one-way analysis of variance (ANOVA) using SPSS software (SPSS Inc., Chicago, IL, USA). To assess and ensure the reproducibility and stability of the gas chromatography, 8.2 pmol of standard gas mixture was analyzed daily. This amount was prepared by drawing 4 ml of standard gas mixture (82 pmol hydrocarbon) into the 50 ml syringe and then adding nitrogen to a final volume of 50 ml (of which 5 ml was analyzed by GC).

Determination of hydrocarbon elution times

Dilutions of isopentane (2-Methylbutane, >99.7%; Fluka Chemical Corp., Milwaukee, WI, USA), hexane (95+%, high-performance liquid chromatography grade; Sigma-Aldrich, St. Louis, MO, USA), and isoprene (99%, Aldrich Chemical Company, Milwaukee, WI, USA) were prepared by adding 5 μl of each liquid to separate 5-l tedlar bags containing roughly 4 l of nitrogen. Vaporization of liquid hydrocarbons was ensured by placing the mixture at 65°C for 10 min. Dilutions in nitrogen of ethylene (0.1 ppm, balance nitrogen; Matheson Gas Products, Newark, CA, USA) and ethane and pentane standard gas mixture were also prepared. Samples from each hydrocarbon dilution were injected directly into the gas chromatograph and retention times were determined. Next, aliquots of each of these dilutions were mixed in one bag to prepare a mixture of hydrocarbons in concentrations approximating those typically found in 3 l of expired air. A sample from this mixture was then injected directly into the gas chromatograph and retention times were compared (Fig. 2C).

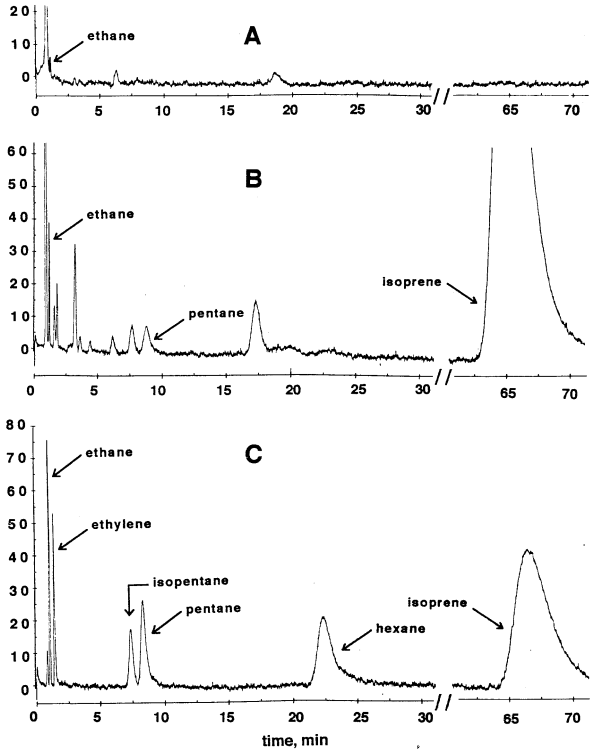

Fig. 2. Chromatograms of: (A) a typical hydrocarbon "free"-air sample; (B) a typical expired-air sample; (C) a mixture of hydrocarbon references gases. Values on the y-axis are peak heights (mm) of the chart-recorder response. Air samples were concentrated (by trap and purge) from 3.0 l to a final volume of 100 ml for the hydrocarbon-free air and 50 ml for the expired air. From these final volumes, 5 ml was analyzed by GC. The reference hydrocarbon mixture was prepared as described in Materials and Methods. No peaks were detected in the omitted interval (30–60 min) on the above three chromatograms.

RESULTS

Recovery, reliability, and stability of measurements

Calibration curves for ethane and pentane were essentially identical if hydrocarbons were injected directly into the gas chromatograph or if they were first trapped and purged from 3.0 l of HCFA (Table 2). The variability of repeated calibration curves is negligible. Indeed within the range of 0–12.3 pmol ethane and pentane, the slopes reported here for the calibration curves differ by <10% from those that we reported previously [17]. We therefore do not prepare a calibration curve each day samples are analyzed. However, in order to ensure reliability of recovery of ethane and pentane on a day-to-day basis, we have made it routine to subject a standard sample (4 ml of calibration gas mixture added to 3.0 l of HCFA) to the trap-and-purge procedure before analyzing expired-air samples. If the results of this sample differ by more than 10% from expected values, either systemic contamination or loss of recovery is suspected. In either event, expired-air samples are not analyzed until acceptable recoveries are obtained. Instrument response was also evaluated on a day-to-day basis by analyzing a standard sample (4 ml of calibration gas mixture in 50 ml of nitrogen). A compilation of instrument-response data for the past 2 years (188 different days; data not shown) yields a coefficient of variation (CV) of 4% for ethane and 7% for pentane. Considering the error in measuring 4 ml with a 50-ml syringe, these low CVs demonstrate long-term stability in instrument response.

Table 3. Precision of Ethane and Pentane Standards in Hydrocarbon-Free Air[a]

Analyte	Mean[b] pmol/l	Within day (SD)	Within day (%CV)	Between day SD	Between day (%CV)
Ethane	8.1	0.66	8.2	0.63	7.8
	13.2	0.68	5.2	0.51	3.9
	26.3	0.90	3.4	1.23	4.7
Pentane	9.2	1.47	15.9	0.58	6.3
	16.0	1.84	11.1	0.00	0.0
	27.7	1.84	6.6	2.58	9.3

[a] Standards were added to 3.0 l of hydrocarbon-free air and concentrated to a final volume of 100 ml as described in Materials and Methods.
[b] $n = 20$ for each concentration.

Analytical precision and limit of detection

Analytical precisions of ethane and pentane standards at several concentrations in HCFA are shown in Table 3. Analytical recoveries of ethane and pentane standard added to expired-air samples from three subjects were: ethane 112% ± 5.5% (mean ± SD) and pentane 97% ± 2.9% (data not shown). The limit of detection for ethane and pentane are 0.22 pmol and 0.77 pmol, respectively. Limits of detection were calculated from the calibration curves using the smallest peak heights for ethane and pentane that could be measured with reasonable certainty (i.e., 2 mm for each peak).

Quantitation of ethane and pentane

A representative chromatogram from the analysis of HCFA is shown in Fig. 2A. In this sample, the peak height of 6 mm is multiplied by a dilution factor of 2 in order to account for a final sample volume of 100 ml obtained after the 3.0 l HCFA was concentrated using the trap-and-purge technique (expired-air samples are concentrated to a final volume of 50 ml). Using the trap-and-purge calibration curve in Table 2, a peak height of 12 corresponds to a total of 15.5 pmol ethane in the 3.0 l HCFA sample (5.2 pmol ethane/l HCFA). No pentane was detectable in this HCFA sample (see Fig. 2A). For HCFA, peak heights >20 mm for ethane and >3 mm for pentane are suggestive of either contamination with ambient air or of insufficient flushing of the Drierite-soda lime. Levels of ethane and pentane measured in 3.0-l samples of nitrogen gas are similar to those obtained from HCFA (data not shown). We have however found several tanks of nitrogen and ultra-purified air to be contaminated with unacceptably high amounts of pentane. We therefore recommend that all tanks be checked before use.

A representative chromatogram obtained from the analysis of expired air is shown in Fig. 2B. Peak identification is performed by comparison of retention times to those obtained from the chromatographic analysis of a mixture of reference gases (Fig. 2C). The ethane peak in Fig. 2B has a peak height of 39 mm. Using the trap-and-purge calibration curve in Table 2, this peak height corresponds to a total of 46.2 pmol ethane in the 3.0 l sample (15.4 pmol ethane/l HCFA); and when the background ethane in HCFA (5.2 pmol ethane/l) is subtracted out, the concentration of ethane in this expired-air sample is 10.2 pmol/l. When similar calculations are performed for the pentane peak (height of 7 mm in Fig. 2B), the concentration of pentane in this expired-air sample is 8.6 pmol/l.

In Fig. 2B, the largest peak in this expired air sample (as well as in all other expired air samples we have analyzed) elutes from our column after 60 min. The elution time of this large peak corresponds to the elution time of authentic isoprene (Fig. 2C). The height of this large peak obtained from expired air can be selectively increased by the addition of authentic isoprene (data not shown). The peak that starts to elute at 63 min. in Fig. 2B had a height of 132 mm, a height about 19 times greater than that of the pentane peak.

For the GC analysis of multiple expired-air samples, consecutive samples can be injected into the gas chromatograph 12 min after the preceding injection; this results in no interference of ethane and pentane from the peak that elutes at about 17 min (Fig. 2B), and permits the analysis of up to five samples before the elution of isoprene. Alternatively, possible interference from peaks from preceding injections can be eliminated by ramping the column to 200°C for about 5 min between injections.

Effect of temperature on elution times of pentane and isoprene

The retention time of isoprene on our porasil C column is influenced greatly by column temperature (Fig. 3). When the column temperature was increased progressively from 40–150°C, the retention time of isoprene decreased 34-fold (from 73.3–2.2 min). Between the two extreme temperatures, the retention time of pentane decreased 6-fold (8.2–1.5 min). At 150°C, the retention times of isoprene and pentane were separated by less than 1 min.

Effect of washout time on ethane and pentane values

Expired air was collected from nine subjects at various times after breathing HCFA. Ethane and pentane were determined simultaneously in each collection (Tables 4 and 5, respectively). Using repeated-measures analysis of variance (ANOVA), no significant differ-

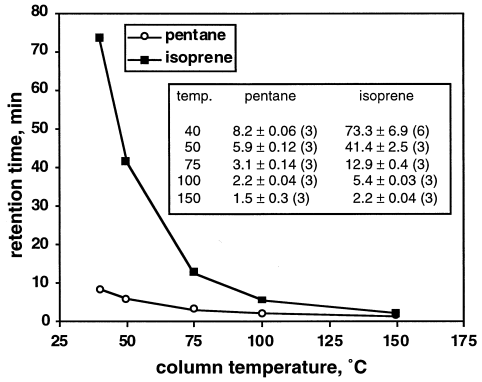

Fig. 3. Effect of column temperature on retention times of pentane and isoprene. Values in the inserted table are mean ± SD with number of determination in parentheses. The column (5 m × 3.18 mm OD stainless steel) was packed with porasil C (80–100 mesh). Nitrogen carrier gas was set at a flow rate of 60 ml/min. Oxygen and hydrogen flow rates to the detector were 50 ml/min and 40 ml/min, respectively. Analysis was performed using a Varian 3700 gas chromatograph fitted with a flame ionization detector.

ences were observed in mean ethane or pentane concentrations or exhalation rates after subjects breathed HCFA for 4, 10, 20, and 30 min. The mean CV was less for ethane than for pentane. In preliminary studies, we found that when subjects breathed ambient air (instead of HCFA) through the collection apparatus, concentrations of ethane and pentane in expired air were very similar to their concentrations in ambient air, because of the high levels of these hydrocarbons in ambient air (data not shown).

Day-to-day variability in ethane and pentane

Intraindividual variability in ethane and pentane exhalation rates were assessed in samples collected from four subjects on at least 6 different days. Ethane and pentane were determined simultaneously in each collection (Tables 6 and 7, respectively). The mean %CV was again less for ethane than for pentane. Ambient-air ethane concentrations were always greater than expired-air ethane concentrations. In contrast, ambient-air pentane concentrations were sometimes lower than expired-air pentane concentrations. When a washout period of at least 4 min was used, no significant correlations were found between ambient air ethane and pentane or between ambient-air and expired-air hydrocarbons. Incidentally, it is worth mentioning that in the course of our studies, we have noted invariably that the consumption of breakfast prior to the collection of expired air did not seem to affect exhalation rates (data not included or shown).

DISCUSSION

Researchers who employ the hydrocarbon breath test generally do not provide data on recovery and precision (see Table 3 in ref. [19]). With our trap-and-purge technique, we recover essentially 100% of ethane and pentane that is added to HCFA (Table 2) or to expired air, although the results were more variable in expired air.

The within- and between-day precisions (Table 3) are most likely overestimates of the variance of our analyt-

Table 4. Effect of Washout Time[a] on Ethane Concentrations in Expired Air and on Ethane Exhalation Rates

Subject	Ethane	Washout time (min) 4	10	20	30	Mean ± SD	%CV	Ambient air ethane (pmol/l)
3	pmol/l	20.3	19.0	24.2	20.3	20.9 ± 2.3	10.9	123.0
	pmol/kg/min	3.0	3.3	2.9	2.2	2.9 ± 0.4	15.6	
4	pmol/l	2.5	5.6	7.4	5.2	5.2 ± 2.0	38.5	68.2
	pmol/kg/min	0.3	0.6	0.9	0.6	0.6 ± 0.2	36.2	
5	pmol/l	13.3	10.0	16.6	13.9	13.4 ± 2.7	20.1	117.5
	pmol/kg/min	1.1	0.8	1.2	1.2	1.1 ± 0.2	16.7	
6	pmol/l	61.7	52.9	32.8	24.0	42.8 ± 17.4	40.7	140.5
	pmol/kg/min	3.7	2.9	3.7	2.2	3.1 ± 0.7	22.9	
7	pmol/l	25.8	24.9	24.0	25.3	25.0 ± 0.8	3.0	199.7
	pmol/kg/min	1.1	1.7	1.4	1.2	1.4 ± 0.3	18.8	
8	pmol/l	28.8	45.0	44.6	42.2	40.2 ± 7.7	19.1	95.6
	pmol/kg/min	4.6	5.9	6.0	4.8	5.3 ± 0.7	13.2	
9	pmol/l	63.9	51.6	70.9	58.6	61.2 ± 8.2	13.3	89.3
	pmol/kg/min	1.2	2.6	1.3	2.7	2.0 ± 0.8	41.4	
10	pmol/l	82.7	54.0	112.9	89.7	84.8 ± 24.3	28.6	157.8
	pmol/kg/min	6.4	8.1	5.9	7.4	7.0 ± 1.0	14.2	
11	pmol/l	23.1	24.9	31.9	26.6	26.6 ± 3.8	14.3	79.4
	pmol/kg/min	1.5	1.6	1.2	1.3	1.4 ± 0.2	12.3	
Mean	pmol/l	35.8	32.0	40.6	34.0		20.9	119.0
Mean	pmol/kg/min	2.5	3.0	2.7	2.6		21.3	

[a] Time the subjects breathed hydrocarbon-free air before sample collection.

Table 5. Effect of Washout Time[a] on Pentane Concentrations in Expired Air and on Pentane Exhalation Rates

Subject	Pentane	Washout time (min) 4	10	20	30	Mean ± SD	%CV	Ambient air pentane (pmol/l)
3	pmol/l	6.3	7.6	8.2	9.5	7.9 ± 1.3	16.6	14.9
	pmol/kg/min	0.8	0.7	0.7	0.7	0.7 ± 0.05	6.5	
4	pmol/l	3.8	17.7	16.5	12.7	12.7 ± 6.3	49.6	1.9
	pmol/kg/min	0.5	1.9	1.9	1.5	1.4 ± 0.7	46.3	
5	pmol/l	14.6	16.5	17.7	15.2	16.0 ± 1.4	8.8	11.4
	pmol/kg/min	1.2	1.3	1.4	1.3	1.3 ± 0.1	6.3	
6	pmol/l	12.7	27.8	24.0	12.7	19.3 ± 7.8	40.5	31.0
	pmol/kg/min	0.8	1.5	2.7	1.2	1.5 ± 0.9	55.6	
7	pmol/l	8.9	15.2	15.2	11.4	12.7 ± 31	24.5	33.5
	pmol/kg/min	1.1	1.7	1.4	1.2	1.4 ± 0.3	18.8	
8	pmol/l	5.1	7.6	11.4	8.2	8.1 ± 2.6	32.3	41.1
	pmol/kg/min	1.4	1.1	3.0	0.9	1.6 ± 1.0	60.4	
9	pmol/l	na[b]	102.5	320.2	302.5	241.7 ± 120.9	50.0	36.7
	pmol/kg/min	na	5.2	6.0	14.0	8.4 ± 4.9	58.2	
10	pmol/l	10.8	14.6	14.6	27.9	16.9 ± 7.5	44.3	19.3
	pmol/kg/min	0.8	1.4	0.9	2.4	1.4 ± 0.7	51.6	
11	pmol/l	10.1	22.8	15.2	11.4	14.9 ± 5.7	38.4	75.9
	pmol/kg/min	0.7	1.4	0.6	0.6	0.8 ± 0.4	51.9	
Mean[a]	pmol/l	8.0	14.4	13.6	12.1		33.9	29.5
Mean[c]	pmol/kg/min	0.8	1.2	1.4	1.1		39.5	

[a] Time the subjects breathed hydrocarbon-free air before sample collection.
[b] na = not available
[c] Means at all four time points were calculated without subject no. 9's values because of missing 4-min value.

ical technique because each sample was prepared by individual dilutions of small volumes of standards instead of sampling from one sample pool. It would have been ideal to have sampled from one discrete pool, but samples stored for more than 24 h can accumulate ethane and pentane, probably from a slow diffusion of ambient hydrocarbons. In general, measurements of pentane standards showed more variability than ethane standards. This may be partially because we measure peak height instead of peak area, and the ethane peak is narrower than that of pentane (Fig. 2).

It appears that 4 min of breathing HCFA is adequate to flush the lungs of ambient hydrocarbons (Tables 4 and 5). With 4 min of washout, the mean concentration of ethane in expired air was 70% less than the mean ethane concentration in ambient air. Expired-air ethane concentrations varied little when longer washout periods were used, except for subject number six, whose values decreased over time. For pentane washout, expired-air pentane concentrations for all subjects were lowest at the 4-min time point, but after that, the values varied more. The variability may reflect different rates of pentane washout from other body compartments such as fat, muscle, and viscera [1].

Ambient-air ethane and pentane concentrations varied markedly between days. If all the collection days in

Table 6. Day-to-Day Intraindividual Variability in Ethane Concentration in Expired Air and in Ethane Exhalation Rates[a]

Subject	Ethane	Day 1	2	3	4	5	6	7	8	9	10	11	12	13	14	Mean ± SD	%CV
Ambient air	pmol/l	161	197	118	169	193	175	69.2	115	91.1	92.6	224	119	178	71.2	141 ± 50	35.7
1	pmol/l	11.3	13.9	17.0	26.9	8.3	17.9	23.2	15.7	15.3	9.8	19.4	16.4	11.1		15.9 ± 5.3	33.3
	pmol/kg/min	1.0	1.4	1.4	2.6	0.7	1.4	2.1	1.2	1.3	0.8	1.6	1.5	0.9		1.4 ± 0.5	38.0
2	pmol/l									6.3	8.5	10.2	7.8	6.9	3.0	7.1 ± 2.4	34.2
	pmol/kg/min									0.6	0.8	0.9	0.9	0.8	0.3	0.7 ± 0.2	32.3
3	pmol/l	8.3	12.0	13.7	11.7	10.4	8.7	9.8								10.7 ± 1.9	18.1
	pmol/kg/min	1.4	1.9	1.7	1.6	2.1	1.7	2.0								1.8 ± 0.2	13.7
4	pmol/l				9.4	5.9	3.7	7.0	5.6	8.3	7.0	10.4	11.4	11.0		7.9 ± 2.7	34.1
	pmol/kg/min				1.0	0.6	0.4	0.7	0.6	1.0	0.7	1.0	1.2	1.2		0.8 ± 0.3	32.8

[a] Expired-air samples were collected after subjects breathed hydrocarbon-free air for 6 and 10 min. Results represent the mean of these two collections.

Table 7. Day-to-Day Intraindividual Variability in Pentane Concentration in Expired Air and in Pentane Exhalation Rates[a]

Subject	Pentane	Day 1	2	3	4	5	6	7	8	9	10	11	12	13	14	Mean ± SD	%CV
Ambient air	pmol/l	23.4	16.5	14	16.5	26.6	22.8	3.4	16.5	13.3	13.3	24.7	22.8	3.8	22.8	17.2 ± 7.3	42.4
1	pmol/l	14.0	9.5	12.1	5.7	7.6	19.6	8.2	10.1	4.5	8.9	12.1	4.5	3.2		9.2 ± 4.5	48.9
	pmol/kg/min	1.2	1.5	1.0	0.6	1.3	1.5	0.7	0.8	0.4	0.7	1.0	0.4	0.3		0.9 ± 0.4	46.8
2	pmol/l									7.0	3.8	13.3	5.1	4.5	1.9	5.9 ± 4.0	67.0
	pmol/kg/min									0.7	0.4	1.2	0.6	0.5	0.2	0.6 ± 0.3	56.8
3	pmol/l	16.5	9.5	7.0	6.4	8.3	8.3	7.0								9.0 ± 3.5	38.6
	pmol/kg/min	2.9	0.9	0.9	0.9	1.7	1.6	1.4								1.5 ± 0.7	48.8
4	pmol/l				8.9	9.5	9.5	6.3	8.9	7.0	5.7	10.8	6.4	10.9		8.4 ± 1.9	22.7
	pmol/kg/min				0.9	1.0	1.0	0.5	0.9	0.8	0.6	1.0	0.7	1.2		0.9 ± 0.2	24.6

[a] Expired-air samples were collected after subjects breathed hydrocarbon-free air for 6 and 10 min. Results represent the mean of these two collections.

Tables 4–7 are combined ($n = 23$), the mean ambient-air ethane concentration was 132 (range, 68–224) pmol/l, and for pentane, 22 (range, 2–76) pmol/l. These ranges are comparable to those obtained by other investigators. Dumelin et al. [14] found the mean ambient-air ethane concentration to be 307 (range, 73–726) pmol/l, and for pentane, 135 (range, 51–209) pmol/l. Zarling and Clapper [15] obtained a mean (± SD, $n = 5$) ambient-air ethane concentration of 800 ± 390 pmol/l, and for pentane, 30 ± 60 pmol/l. Collectively, the reports of ambient-air hydrocarbons reveal that ethane and pentane concentrations are very variable and that ethane concentrations are usually much greater than those in expired air. These facts underscore the need for a washout period in methodologic standardization.

In the literature, hydrocarbon values are often expressed either as a concentration (pmol/l expired air) or as a rate (pmol/kg/min). When expressed as a rate the values take into consideration breathing rate, which may significantly affect hydrocarbon concentrations in expired air [2]. We therefore consider it more accurate to express the values in terms of rate. It is possible that even more accurate rates could be obtained by using an electronic metronome to standardize breathing rates, such as that used by Arterbery et al. [2]. Exhalation rates of ethane in the washout study (Table 4) were relatively stable over time, even in subject number six, whose expired-air ethane concentrations showed a progressive decline. Exhalation rates for pentane in the washout study (Table 5) were lowest at the 4-min time point in six of eight subjects. As a result, in all subsequent studies, we decided to collect two expired-air samples from each subject (one after 6 min and one after 10 min of breathing HCFA) and to express our results in terms of exhalation rates. A 10-min collection period is short enough to be practical for routine investigations and is well tolerated by subjects.

Expired-air ethane exhibited less day-to-day intraindividual variability than did expired-air pentane (Tables 6 and 7). The analytical between-day CV for ethane and pentane (Table 3) is estimated to be 6% at concentrations that correspond to their overall means (10.4 pmol ethane/l; 8.1 pmol pentane/l). If the analytical variation is subtracted out of the total variation, the estimate of interday biological variation in expired-air pentane (38% CV) is greater than that in expired-air ethane (24% CV). Thus it appears that an isolated ethane measurement is more reliable than an isolated pentane measurement. This is the first report of day-to-day intraindividual variation in ethane or pentane in expired air from humans.

It has been reported that [19] a number of commonly used gas chromatographic methods fail to separate pentane and isoprene, the main hydrocarbon in human breath [20]. Among these methods, Kohlmuller and Kochen [19] report that those using analytical columns made of porasil C fail to separate pentane and isoprene (although chromatographic data were not given). This is in contrast to our findings. Our method, which uses a porasil C column at 40°C, is clearly able to separate pentane and isoprene. In the chromatogram of standard hydrocarbons (Fig. 2C), authentic pentane elutes at about 9 min, whereas authentic isoprene elutes at about 64 min—a time that corresponds to the largest peak we obtain from expired air (Fig. 2B). In addition, when expired air was spiked with authentic isoprene, the largest peak increased whereas the pentane peak at 9 min did not (data not shown). Adequate separation of pentane and isoprene on a porasil C column has also been reported by other investigators. Gelmont et al. [20], who used an n-octane porasil C column at 50°C, show a 14.3-min separation between pentane and isoprene. Perhaps the inability of some investigators to adequately separate pentane and isoprene on a porasil C column is due to the use of high column temperatures, for when our column temperatue is raised to 150°C, pentane and isoprene separate by less than one minute (Fig. 3).

Table 8. Ethane and Pentane Exhalation Rates[a] in Healthy Adults as Reported by Various Investigators

Ref.	Subjects (*n*)	Washout[b] (min)	Ethane (pmol/kg/min)	Pentane (pmol/kg/min)	Method of trapping hydrocarbons and recovery
[4]	27	2	1.11 ± 0.26	—	Duplicate cold traps at −70°C; 90% recovery
[8]	3	2	0.36 ± 0.05	—	Same as in ref. [4]
[16]	6	15–120	1.59 ± 0.13	—	Trap at −120°C; 100% recovery
[21]	10	4	—	6.34 ± 0.96	Trap at −85°C for 1 min; no data on recovery
[28]	10	4	—	7.60 ± 0.82	Same as in ref. [21]
[10]	19	4	—	5.8 ± 0.5	Same as in ref. [21]
[29]	5	10–15	—	0.35 ± 0.08[c]	Trap at room temperature; no data on recovery
[13]	15	120	—	1.3 ± 0.3	Trap at −130°C; no data on recovery
[30]	11	90	—	2.02 ± 0.29	Trap at −100°C; 100% recovery
[26]	20	4	10.9 ± 1.4	5.9 ± 0.5	Same as in ref. [21]
[7]	15	4	11.42 ± 0.55	6.06 ± 0.56	Same as in ref. [21]
[22]	3	10	1.4 ± 1.0	1.3 ± 0.5	Trap immersed in liquid oxygen (−183°C)
[23]	5	10	2.95 ± 0.58	1.89 ± 0.21	Trap at 0°C; 100% recovery
This study	11	6–10	2.4 ± 0.6[d]	1.5 ± 1.3[d]	Trap immersed in liquid nitrogen (−196°C); 100% recovery

[a] Values are mean ± SE.
[b] Time the subjects breathed hydrocarbon-free air before sample collection.
[c] Converted from published value (pmol/l) by assuming 70 kg for subject weight and 6 l/min for breathing rate.
[d] For subjects in the washout studies, the 4- and 10-min values were averaged; this is comparable to all other values, which are the average of 6- and 10- min values. For subjects in the day-to-day variability studies, the overall mean value was used.

At our column-temperature setting of 40°C, the retention time for isoprene was quite variable, with retention times of authentic isoprene ranging from 63–81 min, with a mean of 73.3 min (Fig. 3). Most of this variability is likely due to small temperature differences that are not reflected by the temperature reading, for even a temperature change of 1°C could reduce the retention time of isoprene by 3 min (based on interpolation between 40–50°C). Small day-to-day differences in carrier-gas flow rates may also contribute to the variability. The variability of pentane, however, is minimal at 40°C.

Springfield and Levitt [11] have reported that passing expired-air samples through a precolumn containing anhydrous Drierite (calcium sulfate) removes virtually all the isoprene in the sample. Using our trap-and-purge technique, we have determined that only about 50% of the isoprene is removed when we analyze 3-l samples of nitrogen that contain isoprene at concentrations approaching those typically found in expired air (data not shown). Thus, although some isoprene is removed by the passage of samples through Drierite in our method, the removal is far from complete. Differences between our results and those of Springfield and Levitt are most likely due to marked differences in sample processing. Springfield and Levitt pass 10 ml of expired air through a small Drierite precolumn (60 × 6 mm) "slowly," whereas with our method, 3.0 l of expired air are passed through a 120-cc precolumn (containing 20 cc Drierite and 100 cc soda lime) with an approximate flow rate of 43 ml/s. In any case, the partial removal of isoprene by Drierite has no consequence on the measurement of ethane and pentane by our method.

We identify peaks by comparison of retention times versus those of authentic standards. This method of identification is used by the great majority of investigators in this field [1], but without additional verification (e.g., mass spectrometry), peaks can be misidentified. This being the case, we have taken care to verify adequate chromatographic separation between significant breath constituents that could possibly coelute with ethane and pentane, i.e., ethylene, propane [17], butane [17], isopentane, hexane, and isoprene (Fig. 2). Nevertheless, because our ethane and pentane chromatographic peaks may still represent mixtures of gases with similar retention times, additional verification by mass spectrometry is warranted. Unfortunately, the coupling of our gas chromatographic technique, which uses a packed column with relatively fast flow rates, to a mass spectrometer would be very difficult.

Among studies that use a washout period (Table 8), ethane exhalation rates vary about 30-fold (0.36–11.42 pmol ethane/kg b.wt./min) whereas those for pentane vary about 20-fold (0.35–7.6 pmol pentane/kg b.wt./min). However, if the values obtained using the method of Lemoyne et al. [21] are excluded, there is remarkable consistency in ethane and pentane values reported by various investigators (five different groups). The relatively high ethane values obtained using the Lemoyne method has been recently questioned by Habib et al. [4]. It is difficult to postulate on the reason for the relatively high ethane and pentane values obtained by the use of the Lemoyne method, for essentially no data on method performance (linearity, recovery, precision) are supplied; the noteworthy consistency between studies however suggests a systematic error. When only the methods that provide some documentation of performance are consid-

ered in Table 8, normal hydrocarbon exhalation rates from healthy adults range between 0.3–3.0 pmol/kg/min, with ethane being slightly greater than pentane when both are measured. The mean rates we report here for the 11 subjects were 2.4 pmol ethane/kg/min and 1.5 pmol pentane/kg/min (Table 8). Similarly in a group of 20 healthy women (ages 20–50 years; mean of 26.4 years), we have recently determined mean (± SE) exhalation rates (pmol/kg/min) of ethane and pentane to be 2.5 ± 0.25 and 1.9 ± 0.16, respectively (data not shown).

The method we describe here has the primary advantage of being able to quantitate both ethane and pentane simultaneously in expired air that is "uncontaminated" with ambient hydrocarbons. We are aware of three other published methods that appear to perform similarly. Of these methods, the method by Wispé et al. [22] has the disadvantage of using potentially hazardous liquid oxygen to trap hydrocarbons. The method described by Wade and van Rij [16] seems to be well validated, but the collection process utilizes a rebreathing circuit which requires breathing HCFA for up to 2 h; this precludes the determination of pentane exhalation rates, for pentane in rebreathed air is metabolized extensively. The method described by Seabra et al. [23], which has been optimized and well validated, has the main drawback of being time consuming: it takes 25 min to trap hydrocarbons from 1 l of expired air and 33 min for GC analysis. Their adsorbant trap is also not very robust; it needs to be replaced after 80 samples have been passed through it. Our method has the advantage over these methods by being exhaustively validated and by being relatively rapid: it takes 10 min to collect two 3-l expired-air samples, less than 2 min to trap hydrocarbons from 3 l of expired air, and less than 10 min for GC analysis. Moreover, we have used our hydrocarbon adsorbant trap for the analysis of well over a thousand samples with no detectable changes in quantitation.

Although there is no consensus as to which hydrocarbon, ethane or pentane, is a better indicator of lipid peroxidation, there is general agreement that ethane is a more direct measurement [1]. This is because ethane is poorly soluble in tissues and is metabolized slowly by the liver when compared to pentane [24]. The apparent advantage of measuring pentane is because pentane originates from n-6 fatty acids [25], the predominant lipid class in the body. From a methodologic point of view, our data indicate that the measurement of ethane is more reliable. In any case, the measurement of both ethane and pentane seems preferable, especially in view of several studies that have shown both indicators to behave differently under various conditions of oxidative stress [12,26,27].

In conclusion, the performance of the technique reported here for the collection and analysis of expired-air samples has been amply validated and has several significant advantages over existing techniques. By including a detailed description of the breath collection apparatus, as well as studies of washout times and within- and between-day variation in hydrocarbon exhalation rates in healthy adults, we hope to help advance the hydrocarbon breath test to become a more practical and reliable tool for the noninvasive assessment of lipid peroxidation in humans.

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ABBREVIATION

HCFA—hydrocarbon-free air