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REVIEW
A review of the USEPA's single breath canister (SBC) method for exhaled volatile organic biomarkers

ANDREW B. LINDSTROM* and JOACHIM D. PLEIL

National Exposure Research Laboratory, US Environmental Protection Agency, MD-44, Research Triangle Park, NC 27711, USA

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Exhaled alveolar breath can provide a great deal of information about an individual's health and previous exposure to potentially harmful xenobiotic materials. Because breath can be obtained non-invasively and its constituents directly reflect concentrations in the blood, its use has many potential applications in the field of biomarker research. This paper reviews the utility and application of the single breath canister (SBC) method of alveolar breath collection and analysis first developed by the US Environmental Protection Agency (USEPA) in the 1990s. This review covers the development of the SBC technique in the laboratory and its application in a range of field studies. Together these studies specifically show how the SBC method (and exhaled breath analysis in general) can be used to clearly demonstrate recent exposure to volatile organic compounds, to link particular activities to specific exposures, to determine compound-specific uptake and elimination kinetics, and to assess the relative importance of various routes of exposure (i.e. dermal, ingestion, inhalation) in multipathway scenarios. Specific investigations covered in this overview include an assessment of exposures related to the residential use of contaminated groundwater, exposures to gasoline and fuel additives at self-service gas stations, swimmers' exposures to trihalomethanes, and occupational exposures to jet fuel.

Introduction

Breath collection and analysis has been used in a number of studies to help diagnose illness or assess the severity of disease (Kharitonov *et al*. 1994, Byrnes *et al*. 1997, Risby and Sehnert 1999, Graham and Klein 2000, Paredi *et al*. 2000), to assess the degree of ethanol intoxication (Mason and Dubowski 1976), and to help characterize exposures to potentially harmful xenobiotic compounds (Wallace and Pellizzari 1995). However, compared with the large number of studies that employ blood- or urine-based methods, breath has been relatively neglected as a diagnostic matrix for biomarker analyses. This neglect is puzzling given that breath is freely and continuously available from almost all human study subjects (excluding perhaps those with grave respiratory conditions); it can be obtained non-invasively using a variety of fairly simple collection techniques; it is a relatively clean matrix that does not require extensive preparation before analysis; and, finally, literally thousands of volatile biomarker compounds are present in the breath, directly reflecting concentrations of these materials in the bloodstream.

* Corresponding author: Andrew B. Lindstrom, Exposure Methods and Monitoring Branch, National Exposure Research Laboratory, US Environmental Protection Agency, MD-44, Research Triangle Park, NC 27711, USA. Tel: (+1) 919 541 0551; Fax: (+1) 919 541 3527; e-mail: lindstrom.andrew@epa.gov

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The US Environmental Protection Agency (USEPA)'s interest in the use of breath sampling as an exposure assessment technique began with the Total Exposure Assessment Methodology (TEAM) studies of the 1980s (Wallace *et al.* 1987). The TEAM studies were a series of population-based exposure assessment investigations designed to evaluate the most basic aspects of pollutant exposure. Blood and breath samples were collected from the study subjects to evaluate recent exposures and to help determine which sampling methodology would be most useful in future studies. While the breath collection and analysis techniques used in these studies were quite cumbersome by today's standards, it quickly became apparent that breath sampling was a clean, non-invasive method that was readily accepted by the study subjects. Moreover, breath sampling was found to be more sensitive than traditional blood-based analyses for many of the targeted volatile organic compounds (VOCs) under investigation.

Given these obvious advantages, we set about developing a simplified breath collection and analysis technique that would be easy to deploy in the field while providing maximal sensitivity for a wide range of VOCs. This paper summarizes the development and application of the resulting single breath canister (SBC) method of exhaled breath collection and analysis, which was developed in the mid-1990s at the National Exposure Research Laboratory's Exposure Methods and Monitoring Branch and which continues to find widespread application to the present day. We discuss the basic practical considerations involved with breath sampling and the importance of a rigorous analytical methodology, and we highlight the utility of this method in the context of various human exposure assessment studies. While this review focuses on the past use of the SBC method, it also demonstrates the wide-ranging general applicability of breath sampling in human biomarker research.

Methods

In the SBC method (Pleil and Lindstrom 1995a, b), a specially passivated, evacuated 1 litre SUMMA® stainless steel canister (electropolished interior) is fitted with a small (5 cm length) Teflon® tubing stub (see figure 1). While these canisters come in a wide range of shapes and sizes, we quickly found that the 1 litre size was ideal for collection of a single exhaled breath.

At the end of a normal exhalation, with the dead-space (i.e. non-alveolar) portion of a breath eliminated, the subject places the end of the Teflon® collection tube in his or her mouth and opens the canister valve to fill the volume with 1 litre of the lungs' expiratory reserve (see figure 2). Because the canister is initially evacuated ($< 50\,\mu mHg$), the sample is collected until it comes to atmospheric pressure. The subject then closes the sample valve and is free to resume normal activities. Unlike other end-tidal breath collection devices (e.g. glass Haldane–Priestly tubes), these canisters are extremely durable and no special precautions are needed when shipping samples back to the laboratory. Moreover, most VOCs remain stable in these canisters for periods of 30 days or longer without any appreciable degradation.

After collection, the samples are shipped back to the laboratory where they are inventoried and the absolute pressure of each sample is measured and recorded. Samples are then pressurized using laboratory-grade nitrogen gas and a dilution factor is calculated based on initial and final pressures. CO_2 levels are measured to

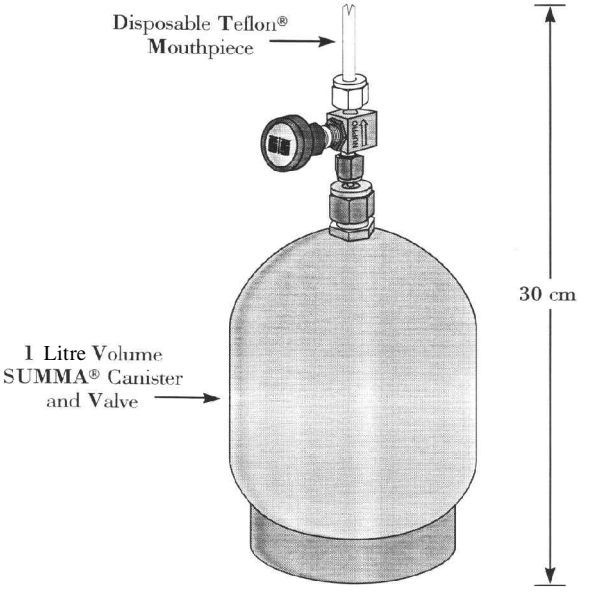


Figure 1. An evacuated 1 litre SBC fitted with a disposable sampling stub.

Figure 2. After a normal exhalation (eliminating the dead-space portion of a breath), the subject places the end of the Teflon® collection tube of the SBC in his mouth and opens the canister valve to fill the volume with 1 litre of the lungs' expiratory reserve. Because the canister is initially evacuated (< 50 μmHg), the sample is collected until it comes to atmospheric pressure.

ensure that the initial sample was indeed alveolar air (at 5.2% CO_2), and an additional normalization factor is determined (if necessary) for samples that deviate significantly from the expected CO_2 level (5.2%/dilution factor). Samples are then analysed using gas chromatography/mass spectrometry (GC/MS) techniques that are based on the EPA Method TO-14 protocol (Winberry *et al.* 1989)

using a Model 3550A cryogenic concentrator (Graseby Nutech, Smyrna, Georgia, USA). This front-end concentrator is also an autosampler for canisters and is used in this mode to perform up to 16 unattended analyses. The analytical system used for most of the work discussed below was an ITS40 (Magnum) GC/MS ion trap instrument (Finnigan MAT, San Jose, California, USA).

Two slightly different analytical schemes have been employed. For analysis of traditional exogenous pollutants such as fuel hydrocarbons or chlorinated 'non-polar' species, a 50 ml aliquot of the sample is cryogenically focused at −165°C in a primary trap, then heated and transferred in a helium stream to a secondary trap and refocused at −190°C on a 0.53 mm precolumn. The precolumn is then rapidly ramped to 150°C, and the analytes are injected in a sharp plug onto a XTI-5 (30 m × 0.25 mm internal diameter [i.d.] × 1 μm phase) analytical column (Restek Corp., Bellefonte, Pennsylvania, USA). When investigating endogenous or 'polar' exogenous compounds containing oxygen or sulphur atoms, up to a 150 ml aliquot of the sample is used with the initial trap temperature at −120°C to pass much of the carbon dioxide, followed by a sequential column arrangement that comprises a SPB-1 precolumn (6 m × 0.53 mm i.d. × 1.0 μm stationary phase) joined with an Rtx-Wax Crossbond-PEG analytical column (60 m × 0.25 mm i.d. × 0.50 μm stationary phase), both from Restek Corp. For either method, the oven temperature profile is −50°C held for 2 min, then ramped at 10°C/min to 220°C and held for 8 min; for some specific separations such as *t*-butyl alcohol, 2-propanol and 3-methyl butanal, the oven temperature profile needs slight adjustment. As only a portion of the total diluted analyte is used per analysis, typical samples may be run as many as four to five times (if necessary) to satisfy quality assurance requirements or to resolve potential chromatographic difficulties. Tentative identification of each analyte is made using conventional mass spectral libraries, but conclusive identification is ensured by injection of authentic standards with verification of both retention times and mass spectra. Quantitative analysis is achieved with multipoint external calibration standards (typically four), which are independently prepared by our onsite contractor (ManTech Environmental Technology, Inc., Research Triangle Park, North Carolina, USA) in a simulated breath matrix (5% CO_2, saturated water vapour) using certified gas standards (e.g. Alphagaz, Morrisville, Pennsylvania, USA, or Scott Specialty Gases, Plumsteadville, Pennsylvania, USA).

Because volatile biomarker concentrations in the breath are directly related to levels in the blood and other tissues, breath analysis has been used in a number of exposure assessment studies to confirm that specific exposures have taken place, to help assess the importance of different routes of exposure (e.g. dermal route versus inhalation route), to establish uptake and elimination kinetics, and to demonstrate the relationships between particular personal activities and their corresponding body burdens (e.g. smoking and increased levels of benzene and other aromatics in the breath and blood). Like many other biomarker methods, breath sampling effectively integrates all routes of exposure into one measure that reflects an individual's total exposure to a given xenobiotic compound.

A model presented in Wallace *et al.* (1993) can be used to help interpret the postexposure breath elimination data. The original model takes the form

$$C_1 = A_1 e^{-k_1 t} + A_2 e^{-k_2 t} + f P_1 C_{\text{air}} \quad (1)$$

where C_1 is the contaminant concentration in the blood at any time t; A_1 and A_2 are complex constants, each a function of the contaminant concentrations and other physiological variables in two hypothetical body compartments; k_i is the exponent associated with the ith term; t is the time in minutes; f is the fraction of the parent compound exhaled at equilibrium; P_1 is the unitless blood/breath partition coefficient; and C_{air} is the ambient air concentration of the contaminant. By dividing this equation through by P_1, we can estimate the alveolar breath concentration at any time t

$$C_{\text{alveolar}} = \beta_1 e^{-k_1 t} + \beta_2 e^{-k_2 t} + f C_{\text{air}} \tag{2}$$

where $C_{alveolar}$ is the alveolar breath concentration at any time during the elimination; the coefficients (β_i) represent the contributions from both unknown body compartments; the inverse of the exponential term ($1/k_i$) represents the residence time (τ_i) of the compound in the ith compartment (i.e. the time it takes the concentration to fall to $1/e$ [≈ 0.37] of its original value); and finally, the half-life ($t_{1/2}$) of the compound in compartment i is equal to (ln 2)/k_i. If the elimination series is collected in an environment where the background level of the contaminant is essentially zero (e.g. outdoors, in many cases), the last term in equations (1) and (2) becomes zero, simplifying elimination calculations.

With the appropriate blood/breath partition coefficient, the alveolar breath concentration can be used to determine the concentration of the contaminant in the blood at any time during the elimination phase

$$C_{\text{blood}} = C_{\text{alveolar}} \times P_1 \times U \tag{3}$$

where C_{blood} is the concentration of the contaminant in the bloodstream ($\mu g\, l^{-1}$); C_{alveolar} is the concentration of the contaminant in the breath ($\mu g\, m^{-3}$); P_1 is the unitless blood/breath partition coefficient; and U is the units conversion factor ($1\, m^3/1000\, l$). If the subject is moved to an area with little or no background contaminant levels immediately after the exposure, the modelled breath values at the y intercept (i.e. $t = 0$) can be used with equation (2) to estimate the maximum blood levels of the contaminant resulting from the exposure. Integrating the area under the elimination curve also provides an alternative measure of total absorbed dose: the resulting quantity in $\mu g\, min^{-1}\, m^{-3}$ can be multiplied by the breathing rate ($m^3\, min^{-1}$) to establish the total mass (μg) of contaminant that leaves the body via exhalation. If it is known what proportion of the compound will be eliminated in this manner (e.g. 10% for trichloroethylene), the unmetabolized mass can be divided by this fraction to provide an indirect estimate of total absorbed dose. A detailed treatment of the analysis of breath elimination data can be found in Pleil and Lindstrom (1998).

Experimental work

Exposures to very volatile compounds via contaminated groundwater

In our first example of the utility of the SBC method, we show how it can be used to confirm and quantify exposures to compounds with extremely high volatilities – materials that would otherwise be extraordinarily difficult to measure directly in blood. Unfortunately, the solvent trichloroethylene (TCE) has become a common groundwater contaminant in the US (Wu and Schaum 2000). Once

underground, it is subject to an iron-mediated reductive dehalogenation process (see figure 3) that leads to the formation of vinyl chloride (VC), a potent human carcinogen (Kielhorn *et al.* 2000). If TCE contaminates well water and gives rise to VC, both compounds can often be measured in residences using the contaminated water. Showering and bathing are of particular concern in such circumstances because of the likelihood of simultaneous inhalation and dermal exposures (Weisel and Jo 1996).

In a single-family residence in Rockford, Illinois, USA, that was using TCE- and VC-contaminated well water, we conducted an experiment that specifically examined shower-related exposures (Lindstrom and Pleil 1996a). The volunteer resident of the study home was asked to take his normal 10 min morning shower and then to quickly leave the house to establish a contaminant-free background for subsequent breath sample collection. Samples were then collected 0.5, 1, 2, 4, 8, 16 and 30 min postshower to establish elimination kinetics. Figure 4 represents the VC elimination profile following this 10 min shower using water contaminated with VC at a concentration of 4 $\mu g\,l^{-1}$. Similar profiles were also obtained for TCE and *cis*-1,2-dichloroethylene but are omitted here for clarity. Analysis of the resulting model parameters indicated that VC had a $t_{1/2}$ of 1.83 and 15.0 min for the first and second compartments, respectively. Because VC has such a low

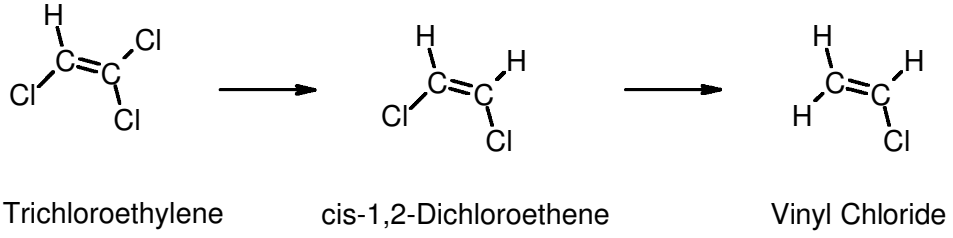
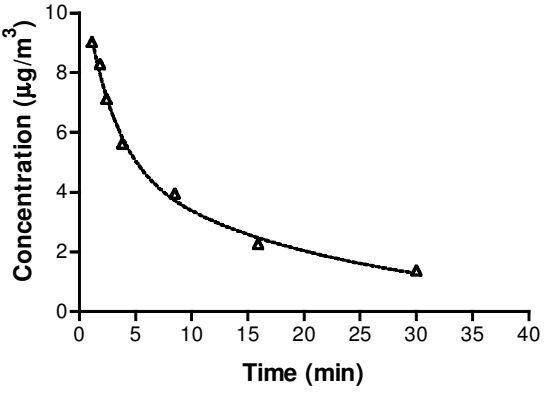


Figure 3. The transformation of trichloroethylene to vinyl chloride in groundwater.



$$C_{alveolar} = 6.54 \times e^{-0.38\,t} + 5.14 \times e^{-0.05\,t}$$

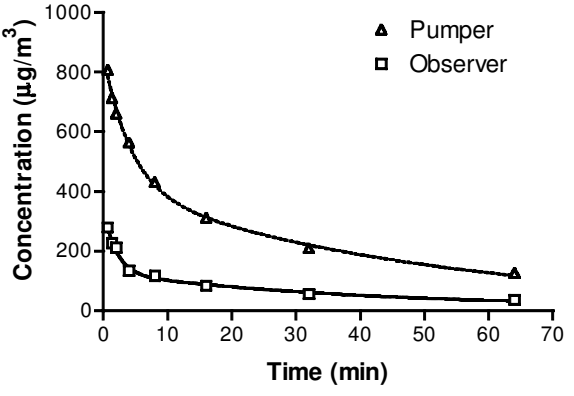
Figure 4. Vinyl chloride elimination in breath after a 10 min shower with water containing vinyl chloride at 4 $\mu g\,l^{-1}$. The symbols correspond to observed data points, while the dotted line represents the resulting model.

boiling point (−14°C) and high vapour pressure (2660 mmHg at 25°C), it is extremely unlikely that conventional blood-based biomonitoring techniques would have been able to confirm exposures to this potent carcinogen. Other biomarker sampling methods, such as *N*-acetyl-S-(2-hydroxyethyl)- L-cysteine and thiodiglycolic acid in urine (Calafat *et al*. 1999), could be used to indicate exposures to VC, but because other low molecular weight xenobiotics (e.g. ethylene oxide, ethylene dibromide) are metabolized via the same pathways, neither method is specific for VC. However, as indicated in figure 4, the SBC method provides definitive proof of recent VC exposure, which can further be linked to a specific activity within the residence.

Exposures to gasoline and fuel additives

In this example, we show how the SBC method can clearly differentiate between two individuals who were exposed to the same atmosphere containing the gasoline additive methyl tertiary butyl ether (MTBE). MTBE is an oxygenate that was first introduced in US gasoline supplies in 1992 to help reduce carbon monoxide emissions. Unfortunately, it is now found as a contaminant in many water supplies across the country due to widespread leakage of underground gasoline storage tanks (Davis and Farland 2001). MTBE has extremely low odour and taste thresholds, making its presence in water supplies obvious to consumers, and recent research has indicated that it is a carcinogen in multiple species of laboratory rodents (Mehlman 2001).

In this experiment, two individuals were exposed to fugitive gasoline fumes while they stood side by side refuelling a car (the pumper and an observer) at a conventional service station (Lindstrom and Pleil 1996b). The gasoline used in this process contained 15% MTBE by volume. After refuelling, the study subjects walked from the service station to a secluded sampling location (~100 m) where they gave periodic breath samples for the next hour. In the resulting elimination



$$C_{pumper} = 443\ e^{-0.240\ t} + 410\ e^{-0.020\ t} + (0.7 \times 12.3)$$

$$C_{observer} = 221\ e^{-0.517\ t} + 119\ e^{-0.025\ t} + (0.7 \times 12.3)$$

Figure 5. Elimination of MTBE in the breath of a fuel pumper and a nearby observer following a routine fill-up at a typical commercial service station. The dotted and solid lines represent the models generated for the pumper and observer, respectively.

profiles (see figure 5), it is apparent that the pumper's exhaled MTBE was more than two times higher than the observer's, despite the fact that both individuals stood side by side during the entire refuelling process. If one extrapolates these modelled elimination curves back to the y axis to estimate the exhaled breath concentrations at the end of the exposure period ($t = 0$), we find exhaled breath levels of 862 and 349 $\mu g\,m^{-3}$ for the pumper and observer, respectively. Using these data in equation (3) and a blood/breath partition coefficient of 23.5 (Lee *et al.* 2001), we can calculate maximum bloodborne MTBE levels of 20.3 and 8.20 $\mu g\,l^{-1}$ for the pumper and observer, respectively, immediately following the exposure period.

These data indicate how microenvironmental (or area-based) sampling strategies might lead to inaccuracies in personal exposure assessment. With the SBC method, slight differences in location (relative to sources), breathing rate and individual metabolism are all integrated into one measure that clearly reflects the individual's actual internalized dose. This increase in specificity reduces the potential for exposure misclassification bias, thereby providing the best data possible for epidemiological investigations.

Breath analysis to determine the route of exposure

If an individual is exposed to a constant level of a VOC in the air he or she breathes, the concentration of the VOC in the exhaled breath will rise and, if the exposure continues long enough, will eventually reach an equilibrium with the surrounding atmosphere (see figure 6). This equilibrium concentration of exhaled breath is always a fraction (represented as f) of the surrounding atmosphere because the body maintains some ability to metabolize or sequester the external dose (Wallace *et al.* 1993). Each compound will have its own characteristic f value, which generally ranges between 0.1 and 0.9 depending on the physical properties of the compound and the human body's ability to retain it. The f value can be important in the analysis of exhaled breath data because, among other things, it

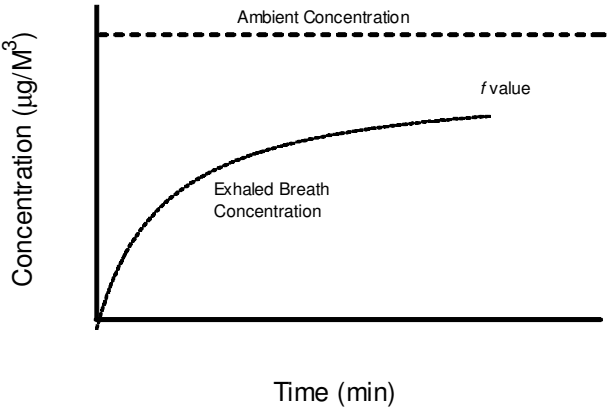


Figure 6. If an individual is exposed to a constant level of airborne VOC, the concentration of that VOC in his exhaled breath will rise and eventually reach an equilibrium with the surrounding atmosphere. This equilibrium concentration of exhaled breath is always a fraction (abbreviated as f) of that in the surrounding atmosphere.

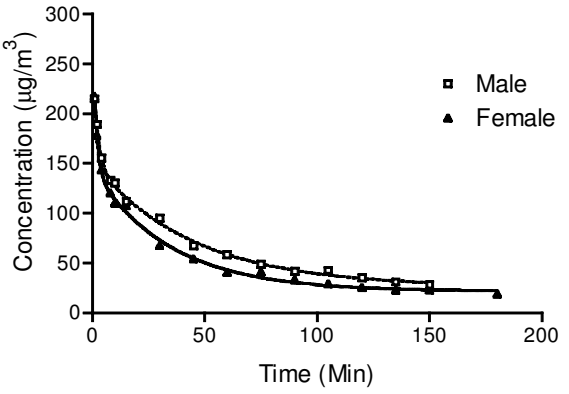
can be used to apportion exposures among various routes (e.g. dermal, inhalation, ingestion), as illustrated in the example below.

When drinking water supplies are treated with chlorine to reduce or eliminate bacterial contamination, a number of potentially hazardous disinfection byproducts are formed, including chloroform, bromodichloromethane and chlorodibromomethane (collectively known as trihalomethanes or THMs), as well as a wide range of other compounds that are present at much lower levels. Bromodichloromethane has been shown to be carcinogenic in a number of rodent species (IARC 1999a), and recent epidemiological studies have linked chloroform and THMs to various cancers and birth defects in human populations (IARC 1999b, Dodds and King 2001). Chlorine-based disinfection compounds are also extensively used in recreational swimming pools to control potential bacterial contamination, and some early studies suggested that THM exposures in pools (particularly indoor swimming pools) might be of some concern (Aiking *et al*. 1994, Aggazzotti *et al*. 1995).

To assess potential THM exposure related to indoor pool use, we designed an experiment to measure exhaled chloroform and bromodichloromethane in two individuals (one male and one female) engaged in vigorous swim training (Lindstrom *et al*. 1997). Both study subjects provided exhaled breath samples before, during and after their regular 2 h swim training workout. Indoor air and pool water samples were also collected to help assess the relative importance of the inhalation and dermal routes of exposure.

Exhaled breath chloroform levels rose rapidly and continuously during the first 90 min of the exposure, with the male subject's breath (160 $\mu g\,m^{-3}$) exceeding the long-term indoor air level (147 $\mu g\,m^{-3}$) after only 8 min of swimming. Peak exhaled chloroform levels of 371 $\mu g\,m^{-3}$ were reached for the male at 90 min, while the female reached a high of 339 $\mu g\,m^{-3}$ at the conclusion of the 2 h workout. With long-term indoor air chloroform levels of 147 $\mu g\,m^{-3}$ and an f value for chloroform estimated at 0.14 (Benoit *et al*. 1998), the highest exhaled breath concentration that we would expect from an inhalation-only exposure would be approximately 21 $\mu g\,m^{-3}$ ($0.14 \times 147\,\mu g\,m^{-3}$). However, the postexposure breath elimination profiles (see figure 7) indicate concentrations were much higher, providing evidence that inhalation alone could not account for the exposures monitored. In fact, with a three-compartment model fitted to these data, breath levels at the very beginning of the elimination phase (the y axis) were found to be over 260 $\mu g\,m^{-3}$ for both individuals, suggesting that greater than 90% of the exposure occurred via the dermal pathway. While this finding was inconsistent with contemporary studies of pool-related chloroform exposure, we hypothesized that a combination of the warm pool water temperatures ($\sim 29^{\circ}C$), vasodilation of dermal capillaries, maximal heart output associated with vigorous exercise, and the complete hydration of the skin all contributed to enhanced dermal input. More recent studies of this phenomenon have supported our initial finding and have clearly demonstrated that dermal transfer of chloroform is greatly enhanced at elevated temperatures, while it is substantially impaired as temperature decreases (Gordon *et al*. 1998).

Bromodichloromethane exposures were also confirmed during this exposure (to our knowledge for the first time under this scenario) with maximum postexposure breath concentrations reaching approximately 3–4 $\mu g\,m^{-3}$. Postexposure breath



$$C_{male} = 111\ e^{-0.509\ t} + 107\ e^{-0.027\ t} + 45.8\ e^{-0.004\ t}$$

$$C_{female} = 153\ e^{-0.750\ t} + 104\ e^{-0.043\ t} + 49.7\ e^{-0.005\ t}$$

Figure 7. Elimination of chloroform in breath from a male and female study subject following an intensive 2 h swimming workout. The dotted and solid lines represent the models generated for the male and female subject, respectively.

profiles (not shown here) indicated a two-compartment elimination that was measurable for 3 h following the swimming period.

Monitoring exposures to complex mixtures: JP-8 jet fuel

The world's combined civil, military and commercial air fleets are estimated to consume more than 60 billion gallons of jet fuel annually. US- and NATO-led military forces alone use more than 4.5 billion gallons of jet propellant 8 (JP-8) each year. While most of this is used to operate aircraft, JP-8 is also used to fuel heaters, stoves, tanks, generators and many other types of support equipment. This fuel is a complex mixture of aliphatic and aromatic hydrocarbons generally containing a mean of 14.5% aromatic hydrocarbons, with the remainder consisting of most of the possible structural isomers for aliphatic hydrocarbons in the C_6 to C_{18} range (the exact formulation will vary according to the specific refinery operations and its storage and transport conditions). Commercial-grade jet fuel (JA-1) is almost identical in composition, but it lacks a few of the compounds that are blended with JP-8 (at very low levels) to enhance its performance in military operations. While the widespread use of these fuels leads to a large number of potential exposure scenarios in both the military and civilian communities, little is known about the nature of these exposures and even less is understood about their related potential health effects. Studies have, however, shown that major jet fuel constituents are concentrated in adipose and brain tissue of rats exposed to the individual hydrocarbons (Zahlsen *et al.* 1990, 1992), and more recent studies have indicated exposure to the jet fuel itself can adversely affect human neurological (Smith *et al.* 1997) and animal immune (Ullrich and Lyons 2000, Dudley *et al.* 2001) function. These findings are troubling given the widespread potential for exposure within the civilian and military communities and the minimal tolerance for error associated with aircraft operations.

In the first coordinated effort to explore potential JP-8 exposures, the SBC technique was deployed in a series of small studies conducted at 13 US air force bases in 1997–1998 (Pleil *et al*. 2000). As this study was an initial effort to broadly characterize exposures, air and breath samples were collected in a variety of workplace scenarios, including cold-engine start-up, fuel tank entry, fuel system and aircraft maintenance, and other related procedures where some level of fuel exposure could be reasonably anticipated. By the end of the study, 162 breath samples from JP-8-exposed subjects working on air force bases were analysed and contrasted with 19 samples collected from civilians without exposure to aviation fuels.

The total ion chromatogram of JP-8 headspace (see figure 8, top) shows a complex mixture of the thousands of compounds that comprise jet fuel. The four most prominent peaks in this analysis were identified as the C_9–C_{12} *n*-alkanes (nonane, decane, undecane and dodecane), which are the most abundant volatile alkanes amenable to GC/MS analysis. As these four 'fingerprint' compounds comprise about 17% of the total weight of the fuel in the gas phase, their aggregated mass was chosen as a surrogate measure of total fuel. The extracted ion chromatograms of the ions m/z 57 and 84 (see figure 8, bottom), which are common to all four *n*-alkanes, were used to quantify these compounds in all subsequent analyses.

Exposures to jet fuel were unambiguously identified and qualitatively assessed using breath samples collected before and after various work activities. As an example, figure 9A is a total ion chromatogram from a typical breath sample collected before a work shift involving fuel tank maintenance. The sample features isoprene and acetone, the two endogenous compounds that dominate all breath samples, the common industrial solvent methyl ethyl ketone (MEK) (presumably

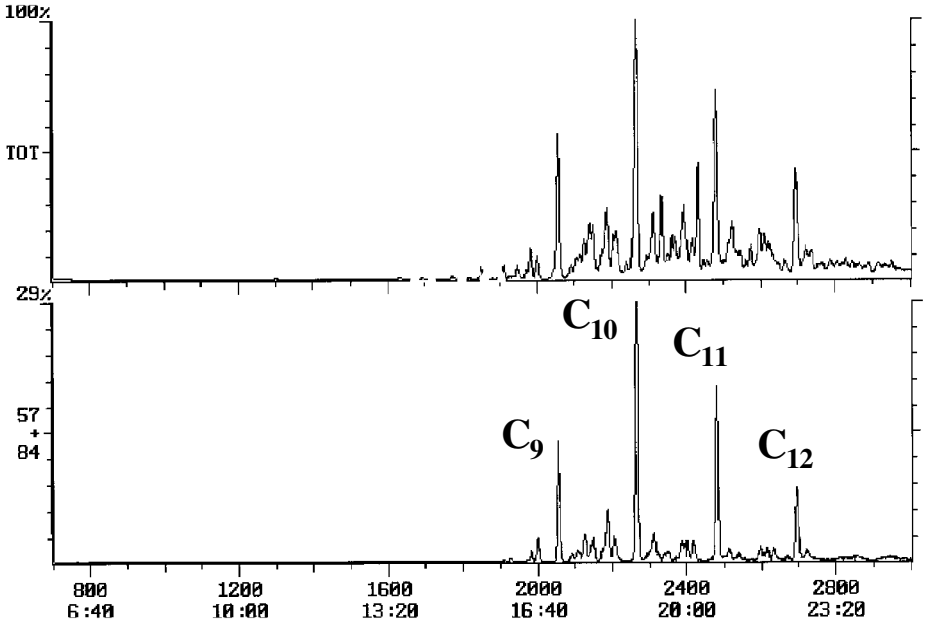


Figure 8. Total (top) and selected (bottom) ion chromatograms of JP-8 jet fuel headspace featuring the prominent C_9–C_{12} *n*-alkanes.

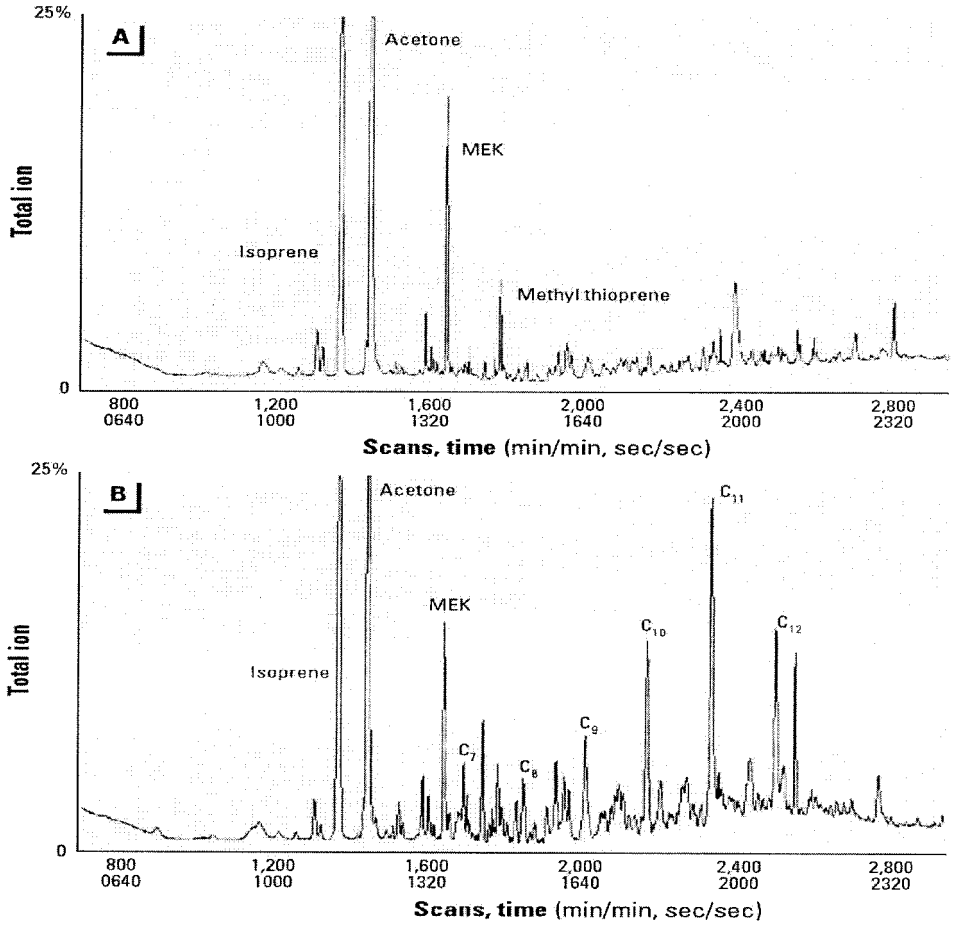


Figure 9. Total ion chromatogram of a breath sample collected before (A) and after (B) a work shift involving exposure to JP-8 jet fuel.

from some prior incidental exposure to this solvent), which is prominent in the middle ranges, and a host of smaller peaks commonly found in the latter portion of the chromatogram. After the work shift, the breath sample reveals a striking increase in the C_9–C_{12} *n*-alkanes (see figure 9B) reminiscent of the fuel headspace analysis and providing clear evidence of exposure to and uptake of jet fuel during the preceding hours.

A summary of the prework and postwork breath data from the fuel maintenance personnel is presented in table 1. Note that the hydrocarbons that are at low concentrations in the fuel (e.g. benzene, hexane, heptane) were at very similar levels in the prework and postwork shift samples, while the fuel-related *n*-alkanes (C_8–C_{12}) were all significantly higher ($p \leq 0.01$) after a work shift with JP-8 exposure.

After analysis of all the resulting breath data by job category, we concluded that the highest overall exposures to JP-8 alkanes were found with fuel systems maintenance workers; these individuals exhibited a chronic elevated level of JP-8 fingerprint compounds in their breath and had the greatest incremental exposure from performing their job functions. Personnel exposed to aircraft exhaust in the

Table 1. Mean ± SEM exhaled breath concentrations of selected VOCs from 40 prework and 45 postwork breath samples collected from fuel maintenance personnel at various US air force bases.

Compound	Exhaled breath concentration ($\mu g\,m^{-3}$)	
	Prework	Postwork
Benzene	11.0 ± 1.69	8.71 ± 1.08
Hexane	2.67 ± 0.27	3.33 ± 0.26
Heptane	3.23 ± 1.72	7.59 ± 1.60
Octane	3.56 ± 0.55	21.5 ± 4.61*
Nonane	22.0 ± 5.55	336 ± 60.5*
Decane	39.9 ± 8.78	412 ± 48.9*
Undecane	28.4 ± 5.56	163 ± 25.9*
Dodecane	20.6 ± 4.43	99.2 ± 25.7*

* Indicates postwork samples were significantly higher than prework samples at $p \leq 0.01$ using a two-tailed *t*-test assuming unequal variances.

typical outdoor scenarios also had measurable exposures, but they were at least 10 times lower than their fuel systems colleagues. When these exhaust workers perform their preflight duties inside a hangar, they exhibit elevated initial exposure levels that then decrease upon opening the doors and starting the aircraft engines. Finally, we also found a slight but measurable elevation in JP-8 fingerprint compounds in subjects at air force bases who had no direct aircraft or jet fuel contact compared with the general population.

These exposure data were used as the basis for an interservice epidemiological study of acute JP-8 exposures among more than 300 fuel systems maintenance personnel at air force bases across the US. During the recently completed field phase of this study, short-term exposure was assessed using a range of JP-8 biomarkers measured in blood, breath and urine samples. Researchers will now attempt to link these exposure assessment data with corresponding measures of immune and neurological function.

Other contemporary breath methods

A variety of other breath collection and analysis methods are also currently in use. They all generally share the same basic steps: samples (preferably the alveolar portion) are collected in some kind of vessel; they are concentrated in some way (sorbent or cryofocus); the constituent compounds are separated (typically via GC); and, finally, the individual components are detected, identified and quantified, most often via flame ionization detection or mass spectrometry. As with all discussions comparing methods, each approach will have its own benefits and limitations that need to be considered before application in a particular study. A short review here of the most prominent methods currently in use further demonstrates the potential utility of exhaled breath as a diagnostic matrix as it provides a comparative context for the SBC studies discussed above.

Aggazzotti *et al*. have used a very simple and effective collection and analysis technique that has been applied in a number of their studies over recent years. As an example, in an investigation involving THM exposures in indoor swimming pools, Aggazzotti *et al*. (1998) used a 34 ml glass tube fitted with valves on both ends to collect breath samples: with the valves in the open position, study subjects

are asked to breathe normally into the tube, and at the end of an exhalation both the upper and lower valves are closed, trapping an aliquot of end-expired (alveolar) breath in the tube. Before analysis, the collection tube is heated to 37°C and the sample is transferred to a conventional gas-tight syringe and then injected directly onto a standard GC/MS system. After a 1 h swim period, chloroform, bromodichloromethane and dibromochloromethane were measured in the exhaled breath, with a detection limit of 0.1 $\mu g\,m^{-3}$ for each compound. The rate of uptake was calculated for each compound, and simultaneous breath and blood measurements were compared in the analysis. This group (Fantuzzi *et al.* 2001) has also recently applied these techniques to examine the occupational THM exposures of indoor swimming pool workers (e.g. maintenance staff, lifeguards), and they have been able to document the secondary perchloroethylene exposures of family members of dry-cleaning workers (Aggazzotti *et al.* 1994). The use of this direct, and surprisingly simple, approach demonstrates how easily breath sampling and analysis can be applied in exposure assessment studies.

In a recent study by Egeghy *et al.* (2000), a new self-administered breath sampling method was used to examine benzene exposures at self-service gasoline stations. Subjects ($n = 39$) were asked to provide breath samples before and after refuelling their automobiles, while simultaneous personal exposures were measured using thermally desorbable passive monitors filled with Tenax^{TM} TA sorbent. End-exhaled breath samples were collected in open-ended 75 ml glass bulbs that were sealed with threaded plastic caps lined with inert septa. A total of 130 separate refuelling operations were assessed. After transport to the laboratory, samples were flushed from the glass bulbs with 400 ml of zero-grade air and concentrated in aluminium tubes (90 mm × 6.3 mm outer diameter [o.d.] × 5.0 mm i.d.) containing 100 mg of Tenax^{TM} TA sorbent (the same device used for passive personal monitoring). Samples (breath and personal) were then desorbed using a Perkin-Elmer ATD 400 (Norwalk, Connecticut, USA) automatic desorption system by heating the collection tube to 225°C and transferring the volatile contents to another Tenax-packed cold trap held at −30°C before injection onto a Hewlett-Packard 6890 Series Plus GC/FID (Hewlett-Packard, Palo Alto, California, USA) for analysis. Note that all of the sample was transferred onto the column at this step, thereby providing only one chance for analysis. A CO_2 analysis of a subset of samples indicated a mean CO_2 level of 4.56%, providing evidence that this method primarily captures the alveolar fraction of the breath. Storage stability experiments indicated that benzene remained stable in the sealed glass bulbs for at least 2 weeks, with losses less than 5%.

The resulting benzene exposures (from the personal monitors) were found to vary considerably, with 95% of the measurements covering a 274-fold range. Benzene in exhaled breath concentrations also varied greatly (95% of the measurements within a 41-fold range). Ultimately, benzene exposure, pre-exposure benzene in breath and duration of exposure were all found to be significant predictors of post-exposure benzene in breath. Further analysis indicated that environmental rather than interindividual differences were primarily responsible for benzene exposure.

This was the first general population study to use a self-collected breath sampling technique, and its success suggests that continued application of this technique would be profitable. Compared with the SBC method, the glass bulb collection container is much less expensive than a stainless steel canister (~$12

versus ~$350, respectively). Also, while both sample containers are cleaned and reused in subsequent studies, the lower initial cost of the bulbs may make their use in large-scale studies much more practical. On the other hand, the SBC method offers the advantages of strong transportable sample containers, the potential for multiple analyses of single samples, the collection and analysis of a whole-air matrix that is independent of sorbent-based recovery effects, and unambiguous compound identification via a mass spectroscopy-based analysis.

Given that the constituents of exhaled breath directly reflect the composition of the blood, it is understandable that the relationships between exhaled VOC levels and disease have received increasing attention in recent years. In an effort to explore the potential diagnostic utility of exhaled breath analysis in a hospital setting, Schubert *et al.* (2001) developed a CO_2-dependent sampling method that can be used to collect exhaled alveolar breath from mechanically ventilated patients. Since any potential markers of inflammation, disease or acute medical crisis may only be present in the breath at extremely low levels ($\sim 10^{-12} - 10^{-9}$ mol l^{-1}), Schubert *et al.* (2001) use a CO_2-dependent sampling valve that effectively eliminates contamination or dilution from the dead-space gas typically found in respiratory ventilation circuits. As with the SBC method, they concluded that determination of the sample's CO_2 concentration is vital to ensure optimal sample quality. Their apparatus uses a fast-responding infrared absorption CO_2 analyser that directs a portion of the circuit's expiratory flow into an absorption trap containing 80 mg of activated charcoal after the CO_2 concentration reaches 3.5%. The sample is collected at a rate of 200 ml min^{-1} until the CO_2 level falls to 90% of the preceding maximum concentration, whereupon the sampling valve is closed until the next respiratory cycle. After approximately 1 litre of breath is collected, the charcoal traps are removed, placed in a microwave desorption unit and subsequently analysed via gas chromatography/flame ionization detection (GC/FID).

Schubert *et al.* (2001) used this method to examine the exhaled breath samples collected from 12 patients from the intensive care unit at University Hospital in Freiburg, Germany. They found that when the CO_2-dependent sampling method was used, concentrations of the endogenous compounds isoflurane (present from prior anaesthesia) and isoprene (a byproduct of cholesterol synthesis) increased almost two-fold relative to samples collected using the more typical mixed expiratory method. Use of this sampling technique should make it possible to conduct more rigorous investigations of the potential relationships between exhaled VOCs and specific medical conditions. A similar temperature-dependent sampling inlet is currently being evaluated for use with the SBC method. If successful, we intend to use this sampling system with ventilated hospital patients, normal healthy children, and anyone else who might otherwise be unable to provide samples using the SBC method.

Phillips *et al.* (1999a) have made an ambitious attempt to catalogue and prioritize the thousands of different compounds that can be observed in exhaled breath. Samples for this effort were collected from 50 normal individuals using breath collection apparatus consisting of a disposable mouthpiece, a heated (40°C, to prevent condensation) wide-bore tubular reservoir, and a pumping system that directs an aliquot of the sample (~1 litre) onto a sorbent-filled (Carbotrap^{TM} C and Carbopack^{TM} B, Supelco, Bellefonte, Pennsylvania, USA) tube. Samples were desorbed and concentrated using an ATD 400 automated thermal desorption unit

(Perkin Elmer) followed by separation and analysis using standard GC/MS techniques (Hewlett-Packard HP6890 and 5973 MSD). A total of 3481 different compounds were detected and identified using an automated mass spectral library search routine. After subtracting the ambient background levels from the exhaled measurements, it was determined that approximately half of the compounds were primarily endogenous in origin, while the remainder were apparently attributable to materials found in the ambient air. A typical breath sample contained a mean of 204 different compounds, but only 27 VOCs (e.g. isoprene, naphthalene, benzene, decane and hexane) were observed in all the subjects. While this effort to begin characterizing the essential nature of exhaled breath is laudable, a compound identification procedure that is based solely on matching mass spectral fragmentation patterns from a commercial library cannot possibly be used to identify large numbers of unknown compounds. Because mass spectra can vary considerably depending on instrument-specific ionization conditions and various matrix effects, identification must be based on the use of authentic external standards that demonstrate a combination of identical chromatographic retention times and mass spectra (as discussed with the SBC method above). Despite the use of this ambiguous compound identification method, this group has completed a number of compelling studies, chief among which are those indicating that various patterns of exhaled VOCs are related to lung cancer (Phillips *et al*. 1999b) and age-related increases in oxidative stress (Phillips *et al*. 2000).

Gordon *et al*. (1998) used one of the most interesting new breath analysis methods available in an investigation of chloroform exposure during bathing. In this study, 10 individuals bathed in chlorinated water held at various temperatures between 30 and 40°C. The subjects wore face masks fitted with a two-way non-rebreathing valve set. Subjects inhaled purified breathing air through one of the one-way valves, while exhalations were directed through the second valve into a mixing chamber and inlet system connected to a glow discharge ionization source interfaced with an ion trap mass spectrometer (Teledyne Electronic Technologies 3DQ Discovery, Mountain View, California, USA). Samples were drawn into the ionizer at 190 ml min^{-1}, allowing measurements of chloroform in breath to be made in the MS/MS mode every 12 s, which essentially provides single-breath resolution. As discussed above (see SBC section on chloroform exposure during swimming), Gordon *et al*. (1998) found a significant relationship between exhaled chloroform and the temperature of the bath, with a 30-fold increase in exhaled chloroform noted as the temperature increased from 30 to 40°C. These researchers postulated that at lower temperatures blood flow to the skin is restricted to conserve body heat, thereby increasing the diffusion distance chloroform must travel from the surrounding water into the blood. Conversely, at higher temperatures the skin is more highly perfused with blood as the body attempts to dump heat into the surrounding environment, effectively reducing the diffusion distance chloroform must travel to be absorbed. This sensitive and immediate method of collection and analysis should have many uses in laboratory-based investigations of uptake and elimination kinetics.

A recent paper by Risby and Sehnert (1999) recounts a decade-long series of investigations exploring the utility of exhaled breath analysis in various clinical applications. This group from Johns Hopkins University has used an evolving breath collection and analysis approach to explore various aspects of oxidant stress status in normal and diseased humans. Their current method involves the

collection of total exhaled breath with one of the most sophisticated collection devices now in use. Flow rate, CO_2 concentration and pressure are all recorded to allow calculation of tidal volume, breath frequency, end-tidal and average CO_2 concentration, and minute ventilation rate for each sample collected. These data are used to calculate a dead-space correction factor that is eventually applied to all the compound concentrations determined. Breath is initially collected in a holding volume (22 litre inert gas sampling bag), and a 60 ml (minimum) aliquot is removed and concentrated on a glass thermal desorption tube filled with equal amounts of graphitized carbon (Carbopack^{TM} X) and carbon molecular sieve (Carboxen^{TM}-1018). Samples are then desorbed using an automated two-stage thermal desorption system (Perkin Elmer) at 300°C and analysed using GC/FID.

As this method represents the culmination of many years of work and consistent refinement, its various precursors have been applied in a wide range of studies:

(i) to relate vitamin E deficiency to increased breath ethane in paediatric patients with severe chronic liver disease (Refat *et al.* 1991);
(ii) to demonstrate that radiotherapy (total body irradiation prior to bone marrow transplant) may induce oxidant stresses that lead to measurable increases in exhaled breath ethane (Arterbery *et al.* 1994);
(iii) to demonstrate that hepatic ischaemia (in a porcine liver transplant model) leads to increases in exhaled ethane and traditional markers of hepatic tissue injury (serum aspartate aminotransferase and serum alanine aminotransferase), which can be subsequently reduced with superoxide dismutase treatment (Kazui *et al.* 1992);
(iv) in numerous other studies relating various oxidant stressors to changes in exhaled VOC levels (Miller *et al.* 1998, Andreoni *et al.* 1999, Risby *et al.* 1999).

Each of the methods discussed above clearly has its own advantages and limitations. Selection of the most appropriate method will of course be dictated by the type of study, the budget and the analytical support available. The SBC method compares favourably with the other methods discussed above and we expect that it will continue to find widespread applications in the future.

Conclusions

The SBC method of exhaled breath collection and analysis is particularly well suited for biomarker studies involving xenobiotic VOCs. The sample collection method is simple and adaptable to a wide range of potential applications. Once collected, samples are stable and easily transported to the laboratory for analysis. The two-stage cryogenic preconcentration removes the bulk of the matrix (i.e. nitrogen, oxygen and other fixed gases), while ensuring that even the most volatile VOCs are collected efficiently. GC/MS analysis provides excellent sensitivity, generally in the parts per trillion to billion range (0.1–3.0 $\mu g\,m^{-3}$), with complete assurance of analyte identification if authentic standards are run in parallel to confirm retention times and mass spectra. When properly applied, the SBC method can be used to clearly demonstrate recent exposures to VOCs, to link specific activities to a recently incurred body burden of xenobiotic materials, to determine compound-specific uptake and elimination kinetics, and to assess the

relative importance of various routes (i.e. dermal, ingestion, inhalation) in multi-pathway exposure scenarios. Like other biomarkers, breath sampling techniques effectively integrate all routes of exposure and provide a single measure that reflects all previous exposures.

Because the SBC method effectively collects all VOCs, it is ideal for assessing exposures to complex mixtures.

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