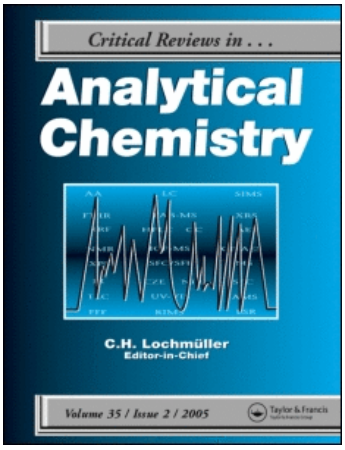

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Current Status of Methods and Techniques for Breath Analysis

Wenqing Cao[a]; Yixiang Duan[a]
[a] Los Alamos National Laboratory, Los Alamos, New Mexico, USA

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Current Status of Methods and Techniques for Breath Analysis

Wenqing Cao and Yixiang Duan
Los Alamos National Laboratory, Los Alamos, New Mexico, USA

Due to the great potentials in clinical diagnosis, disease state monitoring, and environmental exposure assessment, the breath test is becoming one of the most desirable noninvasive procedures for clinical diagnostics. This article reviews the technical aspects of breath analysis including sample collection, analyte preconcentration, vapor desorption, and various measurement techniques, as well as some recent developments in the field. Because the exhaled breath is affected by the composition of ambient air, the elimination of background influence on the analyte detection is also discussed. Both advantages and major obstacles of breath analysis techniques in clinical practice are presented and summarized.

Keywords breath analysis, noninvasive detection, analytical methods, clinical diagnosis

INTRODUCTION

The matrix of breath is a mixture of nitrogen, oxygen, carbon dioxide, water, inert gases, and trace volatile organic compounds (VOCs). The matrix elements in breath vary widely from person to person, both qualitatively and quantitatively, particularly for VOCs (1–4). More than 1,000 trace VOCs have been identified in human breath at concentrations from the ppmv to pptv range. Among these VOCs, only a small number of VOCs are common to everyone. These common VOCs, which include isoprene, acetone, ethane, and methanol, are products of core metabolic processes (4, 5). In addition to these VOCs, exhaled NO, H_2, NH_3, and CO are related to health conditions and can reflect a potential disease of the individual or a recent exposure to a drug or an environmental pollutant (1, 5, 6).

The breath test for the purpose of diagnosis has a long history. The ancient Greek physicians already knew that the aroma of human breath could provide clues to diagnosis. The astute clinician is alert for the sweet, fruity odor or acetone in patients with uncontrolled diabetes, the musty, fishy reek of advanced liver disease, the urine-like smell that accompanies failing kidney, and the putrid stench of a lung abscess (7). Modern breath analysis started in the 1970s when Pauling et al. determined more than 200 components in human breath using gas chromatography (1, 8). For more than one decade, the main problems were the effective separation and identification of exhaled substance. Due to the technical progress of various analytical methods achieved in the 1980s and 1990s, the separation and identification has no longer been a technical obstacle in recent years. In fact, several types of breath tests have been successfully used as diagnostic tools in clinical analysis, such as $^{13/14}$C-urea breath test in the diagnosis of *Helicobacter pylori* infection (9–14) and NO breath test in the diagnosis of airway inflammation (1, 15–18).

Due to the great potential applications in clinical diagnostics and exposure assessment (19), the breath test has become increasingly significant in recent years. It is one of the most desirable noninvasive procedures and is particularly important for patients who have to control daily parameters such as glycemia and urea (1, 20, 21).

This article reviews the technical aspects of the breath test including sample collection, analyte preconcentration, vapor desorption, and various measurement techniques. Because the exhaled breath is affected by the composition of ambient air, the elimination of background influence will be discussed as well. Both advantages and major obstacles of breath analysis in clinical practice are summarized. Some nonvolatile substances such as isoprostanes, peroxynitrite or cytokines, mainly dissolved in water vapor or aerosol particles, can be determined in breath condensate. This part is beyond our discussion and has been reviewed in references (22–30).

COLLECTION, PRECONCENTRATION AND DESORPTION

Because most VOCs and other trace inorganics such as NO, H_2, NH_3, and CO in breath are excreted in ppmv to pptv concentration. Some of these levels are too low to directly detect

Address correspondence to Yixiang Duan, C-CSE, Los Alamos National Laboratory, MS K484, Los Alamos, NM 87545, USA. E-mail: yduan@lanl.gov

for most instruments. Therefore, breath sample collection and preconcentration are required prior to assay in most of the cases.

The exhaled gas is not homogeneous. For a health individual, the first part of a breath, roughly 150 milliliters, consists of "dead-space" air from the upper airway, where there is no gaseous exchange between the blood and breath air. The following part of a breath, about 350 milliliters, is "alveolar" breath, which comes from lungs where gaseous exchange between the blood and breath air (7). Depending on the type of molecule and the breath test tracks, dead-space air can be a necessity or a contaminant. For NO, the dead-space air is used to quantify the amount of the molecule. If the airways are inflamed, as in an asthmatic patient, high-level NO gets released into the airways and into the dead-space air. But for VOCs exchanged between blood and alveolar air, dead-space air is a contaminant and dilutes the concentrations of VOCs when breath is collected (4). In terms of origin of collected breath gases, there are three basic approaches to breath collection:

1. upper airway collection for NO test means that dead-space gas is collected;
2. alveolar collection means that pure alveolar gas is collected; and
3. mixed expiratory collection means that total breath, including dead-space air and alveolar gas, is collected.

Upper airway collection is only for the NO test. Alveolar collection and mixed expiratory collection are for tests of other inorganic gases and VOCs. Because the mixed expiratory collection method is easy to perform in spontaneously breathing subjects and requires no additional equipment, it has been most frequently used in practical applications. However, concentrations of endogenous substances in alveolar air are two to three times higher than those found in mixed expiratory samples, because there is no dilution by dead-space gas. Collection of breath can be performed for a single breath or for a certain period of time (1). If sample is collected through a single breath, one has to be sure that this single breath is representative for all breaths. Because the breath-to-breath concentrations may vary considerably, averaging of consecutive breaths is required for more accurate measurement.

To get an efficient breath sample, the collection efficiency of alveolar air may be estimated by calculating the ratio of the measured CO_2 concentration in breath samples and the generally accepted CO_2 value (31). The CO_2 concentration in alveolar air is stable in resting healthy subjects (32–37). In Vreman's study (33–35), CO_2 concentration was used to estimate the efficiency of collected samples from adults and neonates. The generally accepted end-tidal CO_2 value for a normal newborn population is $4.2\% \pm 0.5\%$. Franzblau and coworkers (37) analyzed CO_2 in 23 alveolar air samples and found that the concentration of CO_2 is in the range of 6.1–7.6%. Therefore, 6% CO_2 concentration was chosen to justify whether the collection of the breath sample from a normal subject is efficient, because 6% CO_2 represents the plateau level that most normal subjects attain in a true end-tidal air sample by using the respiratory maneuver. Schubert et al. (38) designed a gas-sampling device using end-tidal CO_2 to separate dead space gas from alveolar gas. Alveolar sampling valves were controlled via CO_2 concentration. Compared with mix expiratory sampling, the analytes collected by CO_2-controlled sampling were doubled in concentration. Phillips (39) developed a breath-collecting apparatus (BCA) that was controlled by a portable microprocessor. By settings on the front panel, the duration and flow rate of breath collection were controlled to collect alveolar gas.

In the collection system, the materials of connecting valves, fittings, tubes, and sample containers are another important factor in obtaining reliable breath samples (31, 36). Generally, rubber and some plastics are not suitable for constructing sample collection device, because some VOCs that are released from these materials contaminate breath samples. Stainless steel, brass, glass, or Teflon can reduce this interference. Tedlar bags and Teflon bags can be used for sample storage. Moreover, the collecting system must be airtight and free from chemicals and microorganisms that exist in the ambient air.

There are large amounts of water vapor in human breath. The condensation of water vapor in the collecting apparatus may deplete VOCs that are soluble in water and result in falsely depressed concentrations of some analytes (40). Groves et al. (41) investigated the losses of VOCs to condensed water in Tedlar sampling bag. They found that the loss of VOCs to condensed water is not likely to be significant under typical conditions. However, the presence of water vapor will interfere with the chromatogram and damage the gas chromatograph (GC) column, especially a capillary column (31). Therefore, for collection systems to be used in conjunction with GC, the water vapor has to be removed before the introduction of sample to GC column. The cryogenic method (8, 42, 43) and adsorbent method (44–54) are commonly used for this purpose. Manolis (21) has reviewed these two techniques. Pauling et al. (8) used an isopropyl alcohol/solid CO_2 cooling system to remove water vapor. However, many compounds were lost when water vapor was removed by a water trap. For some soluble VOCs in water, this is a common and tough problem.

Due to the extremely low level of most substances in exhaled breath, preconcentration and desorption are required for most cases to enhance the sensitivity. Generally, there are three approaches to achieve preconcentration: chemical, cryogenic, and adsorptive (7).

Chemical trapping usually uses traditional "wet chemistry": breath is bubbled through a reagent solution that captures a specific compound, such as ethanol or acetone. Henderson et al. (55) reacted acetone with 2,4-dinitrophenylhydrazine to form the corresponding hydrazone for the determination of acetone in human breath. The derivatization mixture was extracted with CCl_4 to separate the hydrazone. However, this reaction takes too much time. Even with a cryotrap cooled with liquid air, the breath sample had to be collected for about 1 hour. Most recently, Teshima

et al. (56) modified the salicylaldehyde chemistry for use in an automated flow system with a reasonable overall measurement cycle of 10 minutes. The method achieved a detection limit as low as 14 ppbv for acetone, which is sufficient for breath acetone analysis. However, memory effects of the reaction system and trace acetone loss are potential problems for the technique in real breath sample analysis (57).

In cryogenic trapping, the volatile compounds are captured by freezing. The breath travels through a tube immersed in such cooling fluids as liquid nitrogen (43). Unfortunately, a cold trap also freezes the water and carbon dioxide in the breath and may rapidly become plugged ice (7).

Comparatively, adsorptive trapping is the most convenient and most widely used method today. It captures volatile compounds by binding them to agents such as sorbent traps, solid phase microextraction (SPME) (58–61). Various adsorptive materials have been used as adsorbent in breath analysis, such as organic polymers (e.g., Tenax TA) (62), activated charcoal (63), graphitized carbon, and carbon molecular sieves (e.g., Carboxen 1021) (64). The physical properties of these adsorbents have been identified and characterized (65). Currently, no adsorptive material can completely capture the VOCs in the breath without loss. The recovery efficiencies of VOCs from preconcentration must be evaluated. For instance, Carbotrap B and Carbotrap C have superior abilities for trapping organic compounds above C5 and C2, respectively.

Carbosieve S-III is a spherical carbon molecular sieve and is useful for trapping small molecules such as vinyl chloride (31). Due to the different boiling points of the VOCs, adsorbents in sorbent, traps have to be selected carefully to avoid breakthrough as well as memory effects. Organic polymers are least affected by high water contents in the samples but have low breakthrough volumes (66), especially for small hydrocarbons. By contrast, carbon molecular sieves and graphitized carbon have high breakthrough volumes for these compounds (e.g., ethane). These advantages have to be paid for by possible memory effects when these compounds are used as the only adsorption material. The problem can be solved by using multibed sorbent traps (5, 64, 67). Memory and breakthrough effects are minimized in the way that the strong sorbents, such as graphitized carbon or molecular sieves, are protected by a layer of a weaker adsorbent consisting, for instance, of an organic polymer such as Tenax (67).

Breath volatiles can also be preconcentrated by means of SPME (58–61). SPME is a technique well suited for breath analysis and has been applied to studying VOCs in the nanomolar concentration range. To extend the concentration range of conventional SPME, Giardina and Olesik (59) developed a novel 4-cm-long, low-temperature glassy carbon (LTGC) macrofiber. The LTGC SPME macrofibers were used to extract five lung cancer-related VOCs (2-methylheptane, styrene, propylbenzene, decane, undecane) in human breath, and they were analyzed via gas chromatography/mass spectrometry. Their detection limits are lower using the SPME macrofibers compared to a conventional SPME fiber, in the low- to sub-picomolar range for the compounds of interest. Also, the LTGC SPME macrofibers demonstrate significantly greater extraction efficiencies, sensitivity, and peak identification accuracy compared to that of commercial PDMS/DVB fibers without excessive chromatographic peak broadening. Due to the physical properties of the available fibers, the number of substances that can be adsorbed is limited (1).

Different trapping methods can also be used in series (40, 68). Phillips et al. (40) captured VOCs in an adsorptive trap containing activated carbon and a molecular sieve. The sample was thermally desorbed from the trap in an automated microprocessor-controlled device, concentrated by two-stage cryofocusing. Such a design permits high concentration and focus that greatly facilitate the assay of trace and complicated breath components by GC (31).

Recently, Lord et al. (69) developed a new technique—membrane extraction with sorbent interface (MESI). This method integrates sampling and preconcentration in one step. It is based on a selective membrane acting as the interface between the respiratory circuit and the analytical system.

Whatever adsorption approach is employed, the trapped compounds have to be desorbed by heating the trap (thermal desorption) or by means of microwave energy and introduced into an analytical instrument, such as a GC. Thermodesorption can be done automatically by commercially available devices. An ideal adsorbent should release all adsorbed substances under proper desorption conditions without any reaction and decomposition. Residues in adsorbent will greatly affect the test accuracy.

TECHNIQUES FOR BREATH TEST

Gas Chromatography

The most common method used to analyze trace compounds in human breath is gas chromatography (GC). GC involves a sample being injected onto the head of the chromatographic column. The sample is transported and separated through the column by the flow of inert, gaseous mobile phase. The GC column is the major factor that affects GC separation efficiency. Nonpolar substrates in the GC column, such as the silicones, tend to separate components according to the boiling points of the compounds, whereas polar-column separations are influenced largely by the polarity of the compounds (70). Various detection methods can be employed in GC to identify compounds in human breath, such as flame ionization detection (FID), mass spectrometry (MS), and ion mobility spectrometry (IMS).

GC-FID. The effluent from the column is mixed with hydrogen and air, and ignited. Organic compounds burning in the flame produce ions and electrons that can conduct electricity through the flame. A large electrical potential is applied at the burner tip, and a collector electrode is located above the flame. The current resulting from the pyrolysis of any organic compounds is measured. FIDs are mass sensitive rather than concentration sensitive; this gives the advantage that changes in mobile phase flow rate do not affect the detector's response. The FID is

a useful general detector for the analysis of organic compounds; it has high sensitivity, a large linear response range, and low noise. Therefore, FID is the widely used in GC (71, 72) for the breath test. However, the FID destroys samples through the detection process. Sanchez et al. (5) employed GC-FID for human breath analysis. They combined a nonpolar dimethyl polysiloxane column and a trifluoropropylmethyl polysiloxane column to achieve adequate selectivity for the VOCs in breath. The detection limits of their system are in the low-ppb range. Phillips and Greenberg (73) assayed VOCs in breath by GC-FID. As a quantitative assay for endogenous isoprene in the breath, the method was sensitive, linear, accurate and reproducible.

Gas Chromatograph Mass Spectrometry. Mass spectrometers use the difference in mass-to-charge ratio (m/e) of ionized atoms or molecules to quantitatively analyze compounds. Therefore compounds in the samples can be identified by their fragmentation patterns and quantified by measuring selected daughter ions (31). Currently, GC-MS is the standard technique for determining the composition of VOCs in breath (4). Giardina and Olesik (59) used low-temperature glassy carbon (LTGC) macrofiber to adsorb five lung cancer-related VOCs (2-methylheptane, styrene, propylbenzene, decane, undecane). After desorption, the VOCs were analyzed via GC-MS. Their results show that detection limits are in the low- to sub-picomolar range for the compounds of interest, which should be adequate for the analysis of these compounds in human breath. Pleil and Lindstrom (6) adapted GC-MS for the breath test to assess exposure to halogenated VOCs. Daughtrey et al. (74) compared GC-MS with GC-FID for analysis of low-ppbv level VOCs. The difference between two methods was generally small and relative accuracy between two analysis methods was excellent.

Gas Chromatograph Ion Mobility Spectrometry. Ion mobility spectrometry (IMS) was invented in the late 1960s. The fundamental principle of operation is to separate ions according to mobility as they travel through a purified gas in an electric field at atmospheric pressure. These ions will move at varying velocities as they travel down through the purified gas. The total travel time is a function of the drift length, electric field strength, drift gas (i.e., air or pure nitrogen), temperature, and atmospheric pressure (75). IMS is a highly selective detector capable of quantifying target compounds from a complicated mixture and is relatively portable and inexpensive. By using ionization agents other than water, the sensitivity can be further improved (69). GC-IMS merges two separate technologies to produce a new configuration that incorporates the advantages of the individual technologies. Lord and coworkers (69) used GC-IMS to investigate presence of acetone and ethanol as biologically important markers of human health as well as exposure to volatile compounds. The estimated limits of detection for acetone and ethanol were 0.4 and 0.5 μg/L, respectively.

Proton Transfer Reaction Mass Spectrometry

PTR-MS, the Proton-Transfer-Reaction Mass Spectrometer, was developed by Hansel, Jordan, Lindinger, et al. for online measurements of complex mixtures of trace gas compounds in air with concentrations as low as one part per billion (ppbv) (76–81). The technical details of PTR-MS have been described in references (76) and (81). In brief, chemical ionization is applied based on proton-transfer reactions, with H_3O^+ as the primary reactant ion, which is most suitable when air samples containing a wide variety of trace volatile organic compounds are to be analyzed (76). Almost all VOCs have proton affinities larger than H_2O, and therefore proton transfer occurs on every collision. The ionized VOCs by proton transfer from H_3O^+ are analyzed in mass types rather than concentration.

Analyzing breath gas by PTR-MS has significant advantages: gas mixtures may be readily analyzed without previous concentration and separation procedures; compounds occurring in high concentration like N_2, CO_2, O_2, H_2O do not interfere with measurement; the instrument has very high sensitivity (down to pptv); frequent and rapid measurements are possible. PTR-MS is, therefore, a promising technique for VOC analysis in breath gas particularly suited for online and multiple measurements. However, the PTR-MS characterizes the substances solely according to their mass-to-charge ratio; chemical identification is thus not possible and must be provided by other techniques (82).

Karl et al. (83) measured human breath isoprene by using PTR-MS technique and studied the relation between breath isoprene concentration and blood cholesterol level. In their study, the isoprene concentration in breath varies with the heart rate from a few tens to a few hundreds ppbv. Boschetti et al. (84) used PTR-MS to simultaneously monitor a large number of VOCs in real-time and at high sensitivity. They demonstrated that one could detect the concentration of about 30 VOCs in 2 minutes at a sensitivity of tens of pptv. Reducing the number of compounds could even improve the sensitivity limits up to a few pptv.

Mayr et al. (85) reported on the in vivo breath-by-breath analysis of volatiles released from the mouth during eating of ripe and unripe bananas. The air exhaled through the nose, nosespace (NS), was directly introduced into a proton transfer reaction mass spectrometer and the time-intensity profiles of a series of volatiles are monitored online. Six selected VOCs were measured. Two compounds are characteristic of the aroma of ripe banana, isopentyl acetate at $m/z = 131$ and isobutyl acetate at $m/z = 117$, while two others are characteristic of ripening banana, 2E-hexenal and hexanal. Lirk et al. employed PTR-MS to investigate exhaled concentrations of isoprene, methanol, potential tumor markers, and volatile anesthetics. Their studies demonstrated that PTR-MS offers highly sensitive and rapid determinations of VOC profiles as compared to other methods.

Amann et al. (82) presented results of three studies investigating VOC emission patterns using the PTR-MS technique: (1) long-time, online monitoring of VOC profiles during sleep combined with polysomnography; (2) analysis of VOC patterns in patients suffering from carbohydrate malabsorption; (3) analysis of intra- and inter-subject variability of one particular mass. One of their conclusions is that PTR-MS is a powerful technique for online VOC monitoring. It affords a new opportunity

for noninvasive online observation of biochemical reactions in the body, especially during sleep. This gives an insight into metabolic processes not previously accessible. Moser et al. (86) employed the PTR-MS technique to delineate mass spectrometric characteristics of an average patient sample as possible reference values for the first time.

In PTR-MS, only the masses of the product ions are determined, which is a valuable but certainly not a unique indicator for identifying trace gases. It is clear that different isomers cannot be resolved in this manner. The interpretation of the mass spectra is further complicated by the fragmentation of product ions and the formation of cluster ions, which may lead to additional mass overlap (87). By coupling a GC column to a PTR-MS instrument, GC-PRT-MS can separate the contributions from different VOCs to a single mass channel (88–90).

Selected Ion Flow Tube Mass Spectrometry

SIFT-MS, selected ion flow tube mass spectrometry, is a new analytical technique for real-time quantification of several trace gases simultaneously in air and breath. It involves the chemical ionization of the trace gases in an air/breath sample that is introduced into a flowing inert gas through using selected precursor positive ions. The details of this technique and its application in medicine and other areas are given in several papers (91–94). In short, the analysis occurs through a process of chemical ionization in a reaction tube (or flow tube). To analyze VOCs, a sample is introduced into the flow tube at a precisely controlled rate. Inside the flow tube, precursor ions, usually H_3O^+, NO^+, or $O_2^{+\bullet}$ (95), react with VOCs present in the sample. This reaction results in the formation of product ions, which are analyzed by a quadrupole mass spectrometer to identify and quantify VOCs. Since each organic compound and species of organism produces its own unique profile of VOCs, SIFT-MS makes it possible to identify (fingerprint) which compounds and organisms are present when the sample is collected. Absolute concentrations of trace gases in single breath exhalation can be determined by SIFT-MS down to ppb levels, obviating sample collection and calibration (94). Much of the excitement in the development of SIFT-MS lies in its potential for online and real-time noninvasive breath analysis for clinical diagnosis, therapeutic monitoring, and physiology studies (94).

Using SIFT-MS and $O_2^{+\bullet}$ precursor ions, Spanel et al. (96) quantified isoprene in the breath for 29 healthy volunteers over a period of approximately 6 months and at different time periods of the day. Data distribution of breath isoprene indicates that the normal levels of breath isoprene are 83 ppb (SD $\pm$ 45 ppb). A SIFT-MS study was carried out for acetonitrile both in the exhaled breath and the headspace of urine of several cigarette smokers and nonsmokers (97). The results of this study show that acetonitrile is readily detected by SIFT-MS in urinary headspace of smokers at levels dependent on the cigarette consumption, but is practically absent from the breath and urine headspace of nonsmokers. The ability of SIFT-MS to quantify ammonia accurately in exhaled breath has an important application in screening for colonization of the gastrointestinal tract with the urea-splitting organism *Helicobacter pylori* (98). The standard test to detect the presence of this bacterium is to give an oral dose of carbon-13-labeled urea [$^{13}CO(NH_2)_2$] and to observe an increase of the carbon-13 component of the breath carbon dioxide ($^{13}CO_2$) using conventional mass spectrometry (98). The chemistry involved implies that ammonia should also be emitted following the ingestion of urea. Using SIFT-MS, Smith et al. (99) observed a significant increase (4 ppm) of breath level of ammonia 20–40 minutes after an oral dose of 2 g of normal urea [$^{12}CO(NH_2)_2$] in a volunteer known to be infected with *Helicobacter pylori*. Also, when a person who is not infected with *Helicobacter pylori* takes orally the same dose of urea solution, there is no significant rise in breath ammonia observed (99).

Chemical Sensors

In the last two decades, there has been considerable progress in chemical sensor development. To overcome nonselectivity, which is the main drawback of chemical sensors and sensor arrays, now widely known as "electronic noses," the technology has currently been widely studied and developed. This method is based on a series of nonselective gas sensors, coupled with a pattern recognition technique (100–102). Most of the applications are related to the food industry (103). Recently, a few studies have been reported on breath test (104–106) using chemical sensors. Natale et al. (104) investigated the possibility of using an electronic nose to check whether volatile compounds present in expired air may diagnose lung cancer. In their study, an electronic nose, composed by eight quartz microbalance (QMB) gas sensors, coated with different metalloporphyrins, was used. These sensors show a good sensitivity towards alkanes and aromatic compounds that are lung cancer markers in breath.

The application of a "partial least squares-discriminant analysis" (PLS-DA) found that 94% out of 62 subjects were correctly classified. The classes of postsurgery patients were correctly individuated in 44% of the cases, while the other samples were classified as healthy references. The alteration of breath composition induced by the presence of lung cancer was enough to allow a complete identification of the sample of diseased individuals. Based on the similar sensing mechanism, Huang et al. presented an application of quartz crystal microbalance (QCM) modified with Ag^+-ZSM-5 zeolite for diabetes diagnosis (105). Their sensor exhibits high sensitivity and selectivity with good repeatability to acetone in diabetics' breath, which is a marker of diabetes. They claimed that the sensor could markedly distinguish the healthy breath from the diabetic ones. With the same application as above, Fleischer et al. reported an array of QMB-sensors (106). By use of different sensitive layers and pattern recognition algorithms, a discrimination of a broad diversity of compounds can be achieved via QMB, and acetone could be detected in presence of water.

Optical Absorption

Optical absorption spectroscopy systems for gas analysis have created new opportunities in recent years, while the increased sensitivity available with modulation techniques offers particular potential for trace species measurement (107). Although the high-resolution optical techniques are necessary for more specific to targeted species than mass spectrometry and its variants, they offer much faster evaluation of samples with advantage of real-time use.

Skeldon et al. (108) have developed a wavelength modulation method-based ethane spectroscopy system for applications in biomedical science. The system performs absorption spectroscopy at $\sim$3.4 μm using a cryogenically cooled, tunable, lead-salt diode laser. The ability to measure ethane at 100 pptv in a second response time and a portable device affords an unprecedented opportunity for assessment of oxidative stress in a range of clinical applications. Roller et al. (109, 110) used a high-resolution mid-IR tunable-laser absorption spectroscopy (TLAS) system with a single IV–VI laser operating near 5.2 μm to measure exhaled nitric oxide (eNO) and carbon dioxide (CO_2) simultaneously in human breath over a single exhalation. The NO absorption feature at 1912.79 cm^{-1} and the CO_2 absorption feature at either 1912.69 cm^{-1} or 1912.97 cm^{-1} were measured simultaneously.

The detection limit for NO was estimated to be 1.5 ppb for a 4-second integration time. Such measurements can help in evaluating airway inflammation and in monitoring the effectiveness of anti-inflammatory therapies. Although variation in exhalation flow is a major factor affecting the determination of accurate eNO concentrations, simultaneous CO_2 measurement provides an internal calibration parameter that accounts for any variation in flow. In addition, IR absorption spectroscopy has been widely used for urea breath tests as well (11, 111–116). The patients swallow a capsule containing urea made from an isotope of carbon, ^{13}C or ^{14}C. If *H. pylori* is present in the stomach, the urea is broken up into nitrogen and carbon (as carbon dioxide). The carbon dioxide is absorbed across the lining of the stomach and into the blood. It is then excreted from the lungs in the breath. If the labeled isotope is detected in the breath, it means that *H. pylori* is present in the stomach. When the *H. pylori* is effectively treated (eradicated) by antibiotics, the test changes from positive (isotope present) to negative (isotope absent) response (117). The urea breath test is also an ideal method for monitoring treatment success following antibiotic therapy, because it needs no endoscopy and can avoid the false negative result from biopsies due to focal colonization of bacteria (118). Even though there are different methods for the test, such as isotope ratio mass spectrometer (IRMS) and laser-assisted ratio analyzer (LARA), measurement based on IR absorption spectroscopy has the great advantage of being much cheaper than others. Several studies have demonstrated excellent results with this technique (11, 111–116). However, an important disadvantage of this testing method is that it can sequentially process only a few breath samples (9).

Besides IR absorption spectroscopy, UV has also been used for breath analysis. Baum et al. (119) reported an instrument based on UV absorption spectroscopy for measuring C_2H_2 in breath for noninvasive cardiac output monitoring. Their analyzer afforded fast (276 $\pm$ 43 ms, 0%–90%, at 2 L min^{-1} flow rates), interference-free detection of C_2H_2, with signal-to-noise ratios in excess of 50. Comparison tests with a MS using calibration gas samples gave an excellent correlation, which validated the linearity and accuracy of the UV system. Recently, Wang et al. (120) applied cavity ringdown spectroscopy for detecting acetone at ultraviolet and near-infrared wavelength ranges, and claimed a detection limit of 1.5 ppmv for acetone gas. Their research demonstrated the potential of developing a portable breath analyzer for medical applications using the cavity ringdown spectroscopy technique. However, some significant challenges have to be minimized before the technology can be used for real breath samples, such as heavy moisture in the breath gas.

Colorimetric Analysis

Some studies used a colorimetric method to measure specific VOCs in human breath such as hydrogen cyanide (121) and acetone (56). Teshima and coworkers (56) presented a method to determine acetone in breath through chemical reactions and preconcentration steps. The detection chemistry is based on the reaction of acetone with alkaline salicylaldehyde to form a colored product, which absorbs in the blue region and can be monitored with GaN-based LEDs with emission centered at 465 nm. The method is sufficiently sensitive for breath acetone analysis. It can also be used to detect a specific compound in human breath, but it cannot be used to detect several compounds simultaneously.

Micro-Plasma

Since the plasma gas has high excitation potentials and generates highly energized metastable species, the plasma source can provide sufficient energy to excite targeted chemical species through Penning ionization and energy transfer. At the same time, plasma sources also emit high-energy photons that provide further excitation of the targeted chemicals. With molecular emission detection, Duan et al. have successfully obtained varied spectra for various chemicals (122, 123). It was revealed from their experiments that different chemicals gave varied spectra in the wide wavelength range from UV to Visible. Careful selection and identification of some particular peaks may have potential for recognizing particular chemical components in an unknown sample without any separation work.

Most recently, Duan et al. (57) thoroughly investigated acetone emission spectrum generated within a tiny microplasma, and a particular emission peak/band was identified for the breath acetone detection. System testing and calibration were performed with an industrial standard acetone gas and the method/device was validated through real human breath acetone

detection. A detection limit for breath acetone at low ppbv level was obtained without any preseparation and concentration. The advantages of the new method/device include less matrix effects, no preconcentration necessary, and no influence of the large amount of water vapor in breath gas. In addition, the system could be built and integrated into a handheld device with low construction and maintenance cost, and has a potential for routine and daily diabetes monitoring.

CORRECTION OF BACKGROUND

To distinguish between endogenous substances and exogenous contaminants, the background concentrations of the tested gases have to be considered. As mentioned in previous section, most VOCs and other trace inorganics such as NO, H_2, NH_3, and CO in breath are excreted in ppmv to pptv concentrations. Inspired air may critically affect measurement results of exhaled concentration of the testing gases, with the exception of NO because NO is very reactive and immediately forms other compounds when inhaled (15).

Generally, there are two approaches to solve this problem. A straightforward and effective way to eliminate ambient concentrations is to have patients or volunteers breathe pure air for a certain time before measurement (124). But this method is time consuming and will not be applicable for clinical routine purposes. The second approach is to subtract substance concentrations in ambient or inspiratory air from exhaled substance concentrations (39). The difference between the concentration in breath and concentration in air, termed the "alveolar gradient," provides an indication of whether a particular VOC is endogenous or exogenous in origin.

Generally, the alveolar gradient is positive for VOCs manufactured in the body since more is excreted in the breath than is inspired from air. Conversely, the alveolar gradient is negative for air pollutants that are excreted or metabolized by extrapulmonary pathway. There are a number of advantages to this approach: First, it frees investigators from laborious attempts to provide subjects with VOC-free air. Second, it indicates whether a breath VOC was endogenous or exogenous in origin. Third, it provides a new indicator of individual differences in disposing of air pollutants from the body. Fourth, it permits the design and construction of a breath-collecting apparatus (BCA) that is portable and suitable for field use (39). However, this method does not take into account the complexity of pulmonary adsorption and exhalation of volatile substances.

Especially when inspiratory concentrations are of the same order of magnitude as expired concentrations, results will be affected. Expired samples may be diluted or contaminated by inspiratory and/or dead space gas depending on the ratio of alveolar and dead space ventilation, which itself depends on the breathing pattern (125). In addition, excretion and intake of volatile substances depend on the ventilation/perfusion ratio in the lung and on the alveolar concentration gradients of the substances (126). These complex interdependencies cannot be accounted through simply subtracting inspiratory from expired substance concentrations. The more a patient's lung is affected, and the more these problems will be (8).

Because VOCs in air vary widely day-by-day and area-by-area, a subject brings the history of the air breathed. Therefore, as Mukhopadhyay stated, "Background subtraction is a sticky issue in VOC analysis" (4). More research is required to solve this problem.

SUMMARY

Breath analysis has attracted a considerable attention of scientific and clinical studies because breath analysis offers advantages over existing serum or urine analysis (4, 21):

- Breath test is noninvasive, easily repeated, and does not have the discomfort or embarrassment that is associated with blood and urine tests.
- Breath samples closely reflect the arterial concentration of biological substances and may obviate the collection of arterial blood samples, which is much more difficult. Breath analysis would be particularly advantageous where many arterial blood samples are required, e.g., in monitoring a patient.
- Breath is a much less complicated mixture than serum or urine and is amenable to complete analysis of all the compounds present. There is no work-up of a breath sample required, in contrast to many analyses performed on serum or urine samples.
- Breath analysis provides direct information on respiratory function that is not obtainable by other means.
- Breath analysis can dynamically real-time monitor the decay of volatile toxic substances in the body.

Currently, breath tests are not widely applied yet in clinical practice as expectation. The main obstacles include:

- in-depth understanding of the links between the diseases and biomarkers in breath;
- acceptable standardization and normalization of procedures for sampling; preconcentration, analysis, and background correction;
- normalization, expression, and evaluation criteria of data.

Regarding techniques of the breath test, they have progressed considerably in recent years. However, in order to introduce more breath tests into clinical practice, the test techniques/devices are expected to possess following characteristics: (1) high selectivity, high sensitivity, and fast response to test gas; (2) immunity from the interference of water vapor in breath; (3) handheld size and ease of operation; and (4) low construction and maintenance cost. With these features, the breath test will make a very positive contribution to clinical practice.

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