

# Don't waste your breath

RESEARCHERS ARE DEVELOPING  
BREATH TESTS FOR  
DIAGNOSING DISEASES,

**BUT HOW WELL DO THEY WORK?**

## Rajendrani Mukhopadhyay

**B**reath can tell you whether a friend ate garlic for dinner. Police officers use breath to monitor alcohol overindulgence. Are there other molecules in breath that provide information about what is going on in your body?

In 1971, Linus Pauling published gas chromatograms to prove that hundreds of volatile organic compounds (VOCs), from  $C_2$  to greater than  $C_{20}$ , were present in picomolar concentrations in the breath. Inorganic gases, such as NO and CO, are also known to be present.

Breath tests are attractive because they are one of the least invasive ways to monitor a person's physiological state. For this reason, researchers have worked hard to understand how various compounds end up in breath. It is now known, for example, that NO is a marker for chronic airway inflammation and is used in breath tests to monitor asthmatics.

Researchers in favor of breath tests for VOCs, however, have had an uphill struggle. Early attempts were made in the 1980s, but poor experimental design created a lot of confusion. Only recently have researchers begun taking a more systematic and analytical approach to this problem.

Three broad questions challenge researchers studying VOCs in breath. How should breath samples be collected? Given the low concentrations of VOCs in breath, how can they be detected and quantified? And finally, is there a clear link between certain VOCs and particular diseases?

### What's in breath?

Breath consists primarily of  $N_2$ ,  $CO_2$ ,  $O_2$ , water vapor, and inert gases. A tiny fraction of breath is a mix of acetone, isoprene, pentane, and literally hundreds of other compounds. The composition

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of VOCs in breath varies widely from person to person, both qualitatively and quantitatively. And although the final sum of VOCs found in everyone's breath is in the high hundreds, only a small number of VOCs are common to everyone. These common VOCs, which include ethane, acetone, and methanol, are products of core metabolic processes.

A person's physiological state is not the only source of VOCs in breath. Objects like trees, gas stoves, gasoline pumps, and household cleaning products release VOCs into the air, and these exogenous VOCs get inhaled and eliminated from the body by exhalation. Terence Risby, from the Johns Hopkins Bloomberg School of Public Health, explains, "Composition of your breath is related to what you have inhaled, what you have ingested, what is your normal physiology, what is your abnormal physiology."

Exhaled breath consists of two components. The first 150 mL of breath, called "dead-space" air, comes from the trachea and the bronchioles where there isn't any gaseous exchange between the blood and air. The remaining 350 mL is called "alveolar" breath. It comes from deep within the lungs and is the air that has undergone gaseous exchange with the blood. Alveolar air can also be thought of as the headspace of the blood.

### Toolbox for breath analysis

The instruments currently used to collect and measure VOCs in breath are familiar to analytical chemists. But the existing tools have problems, and the search continues for new designs that are more suitable for breath analysis.

Two types of breath tests exist. Real-time monitors, like those for NO, measure levels of the compound as the patient exhales. Aerocrine's NIOX detects NO by chemiluminescence, and Ekips Technologies' instrument detects NO by vibrational IR spectroscopy. Chuji Wang and colleagues at Mississippi State University use cavity ring-down spectroscopy to detect acetone in breath as an indicator of diabetes.

Although most breath tests for VOCs are not performed in real time, researchers aim for portable, real-time instruments. They envision a future where breath tests can be easily administered by nurses or even handled by patients at home. But for now, breath collection and analysis are generally performed in separate steps. A liter of a person's alveolar breath usually is collected and analyzed with a turnaround time of 24–48 h.

Sorbent traps are popular for capturing VOCs from alveolar air. The activated carbon inside the traps adsorbs the VOCs from exhaled breath and concentrates them. Solid-phase microextraction (SPME) is another approach to capturing VOCs. A fused-silica fiber is coated with a polymeric stationary phase that extracts and

concentrates VOCs from breath. In both cases, the VOCs are thermally desorbed from the traps for analysis.

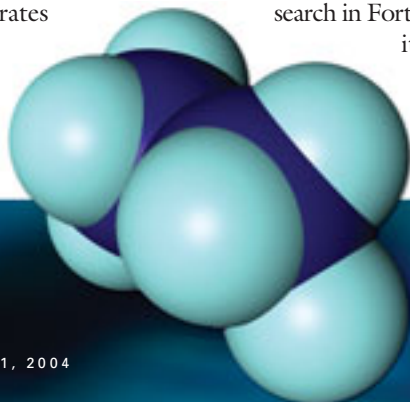
Both sorbent traps and SPME devices initially suffered from water vapor problems. But researchers recognized that moisture in breath had to be removed to prevent condensation inside the collection device, which in turn allowed VOCs to partition from air to the liquid phase. Because VOCs are present in trace concentrations, removal of VOCs from the gas phase was an acute problem.

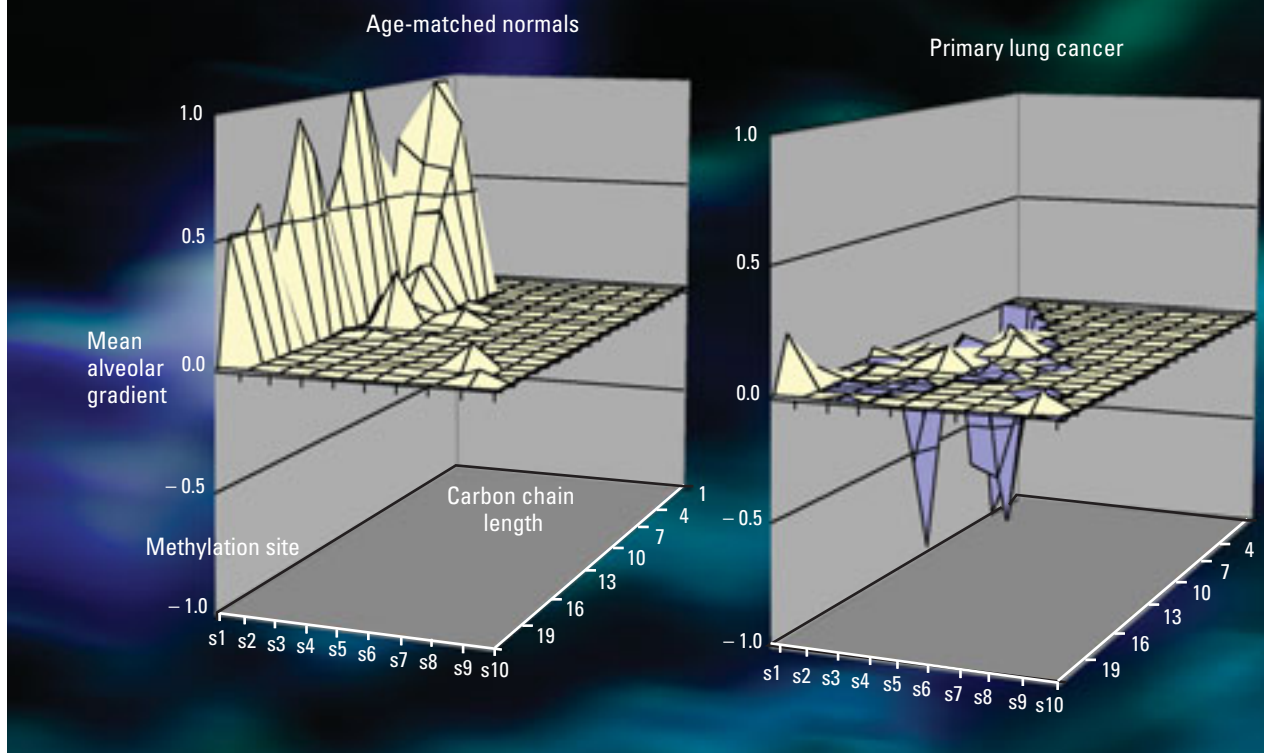
Janusz Pawliszyn and colleagues at the University of Waterloo (Canada) have come up with a solution for some of the SPME coatings that are sensitive to moisture. "The moisture is repelled by [a] hydrophobic membrane so it does not permeate the fiber," explains Pawliszyn. On the other hand, Richard Sacks of the University of Michigan and his group use a series of sorbent traps on their device. He says, "We have a system that is fairly immune to water vapor because of the adsorbents we've chosen."

Sample loss does not occur only through condensation. Some VOCs have short life spans and degrade rapidly. Others may have varying affinities for different adsorbents, which makes it hard to either capture or release them. In addition, researchers have to worry about samples leaking from the collection device and the possibility of the surrounding air contaminating the sample.

GC/MS is currently the standard technique for determining the composition of VOCs in breath. But Sacks says, "Conventional GC with one column doesn't have adequate separating power for the large number of components in a breath sample." His efforts focus on comprehensive 2-D GC (GC × GC) because it has greater separating power and sensitivity. Unfortunately GC × GC in its current state is not portable. GC × GC instrumentation "weighs several hundred pounds, it's wedded to a large dewar of liquid nitrogen, which has to be replaced every couple of weeks, and it needs a big tank of compressed nitrogen once a day," says Sacks. "It's very resource-intensive, but a powerful technique." He adds that his group is working on miniaturization and elimination of resources so that GC × GC can be put into a portable format.

There are other alternatives for VOC detection. The "GasFree" GC with a flame-ionization detector was developed by Aviv Amirav of Tel Aviv University (Israel). The instrument is powered by water electrolysis, has the proportions of a shoebox, and according to Amirav, is "lightweight, sensitive, and analysis takes less than a minute." And Michael Phillips, the founder of Menssana Research in Fort Lee, N.J., suggests that IR spectroscopy may lend itself better to a portable instrument. "We could put [the test] in a box, using IR spectroscopy, and use it at the bedside. It will be cheaper and quicker," he says.





Breath samples from primary lung cancer patients and age-matched healthy individuals show dramatic differences in VOCs.

## Getting a good sample

Breath collection is subject to a number of parameters, including alveolar and dead-space air, method of collection, and exposure to environmental VOCs. Breath researchers agree on the significance of one or two parameters. The rest inspire lively debate.

Depending on the type of molecule 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 levels of NO get 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. Some devices, like those developed by Menssana Research and Pan Diagnostics (Scotland), use one-way valves to discard the dead-space air and capture only the alveolar air.

Background subtraction is a sticky issue in VOC analysis (it's not much of an issue in NO monitoring because NO is very reactive and immediately forms other compounds when inhaled). Some researchers believe that exposure to VOCs in the environment plays a critical role and must be taken into account during the analysis. Amirav says, "A person brings the history of the air [he or she] breathes."

Jochem Schubert of the University of Rostock (Germany) explains that for a breath test to be accurate, the inspired air has to be used as a control to account for any VOCs that may be present in the environment. VOCs in air vary widely and are subject to conditions as whimsical as wind and street traffic. Schubert says, "There are differences between Monday through Friday and Saturday and Sunday because of the [volume] of traffic passing by [a] hospital."

Phillips suggests that the fuss over background subtraction may be unwarranted. He says, "We've done tests all over the USA, several [tests] in Europe, and we've found in rooms where humans congregate, the background [VOCs] are remarkably similar—not identical, but similar."

In his diagnoses, Phillips uses the air from the room where the breath test is performed for the background subtraction. But other experts are uneasy with the idea. Risby says, "It assumes [the patient] immediately reaches equilibrium with the room."

Opinions also differ about the collection method for VOCs. Not everyone thinks that collecting a series of breaths into a device is the best approach because an averaging process takes place. "As you don't know the concentration profile of contaminants in the ambient air, the process of averaging is dangerous because there might be changes in inspired concentrations that might induce changes in the expired concentrations, which you might not see," says Schubert. He advocates performing breath analyses in real time, so that an investigator can have the inspired-air VOC concentrations next to the expired-air concentrations, and see changes immediately. "It's a much more precise way of doing analysis," he says.

Another question arises: Do exhaled VOCs naturally fluctuate, which would then make analysis troublesome? Schubert explains that some VOCs can fluctuate over a wide range, whereas some undergo very little change. Schubert notes that some compounds, such as acetone, fluctuate greatly in breath. Other substances, like ethane, CS<sub>2</sub>, and some aldehydes, are almost undetectable in normal subjects but are increased in certain disease states. For substances with little fluctuation it is sufficient to make one measurement to see whether the substance is present or not. But Schubert does warn, "Problems arise when pa-

tients' groups [have] to be compared with each other. In this case only [VOCs] with low variations can be used."

Phillips says for the types of VOCs Menssana Research analyzes, fluctuation is not an issue. "When we collect two breath samples in a row, the chromatograms are so similar as to be superimposable; [in other words] in a period of a few minutes, there is no fluctuation in breath VOCs."

The rate at which breath is exhaled may also play into the analysis. The rate of exhalation is such a critical factor for monitoring NO in breath that in 1999 the American Thoracic Society established guidelines for sample collection. Although this issue doesn't seem to be as significant for VOCs, standard guidelines for sampling may be beneficial to ensure that tests are consistent.

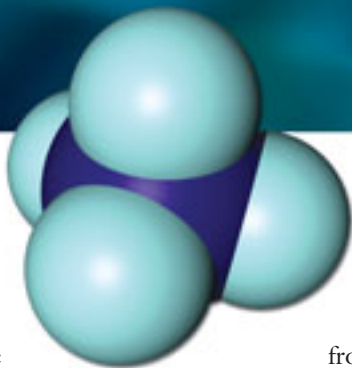
### A link to diseases?

Researchers are focusing on VOCs produced by a phenomenon called oxidative stress. Oxidative stress is a natural pathway that occurs in the body during growth, development, and aging. During oxidative stress, reactive oxidative species attack DNA, proteins, and most importantly for breath analysis, lipids. Products from lipid peroxidation, such as ethane, pentane, and isoprene, can be detected in breath. Oxidative stress has been implicated in a variety of diseases, including diabetes mellitus, rheumatoid arthritis, and chronic obstructive pulmonary disease.

The origin of VOCs in disease is a sensitive topic. Not everyone feels comfortable with solely attributing the presence of certain VOCs in breath to a disease. As Risby explains, "Take ethane for example. Ethane is produced as a result of lipid peroxidation, but ethane in breath could [also] be derived from environmental sources; another source of ethane in breath could be bacteria in the [gastrointestinal] tract. So I cannot, in all conscience, say that all the ethane in your breath is produced by lipid peroxidation. You don't know the complete origin of it."

But others argue that the complete origin doesn't need to be known for a VOC in breath to be an indicator of disease. "You can make a disease marker if it works," says Brian Ross of Pan Diagnostics. "You don't have to know where it's coming from, although it's not very satisfying. [For example] there are many drugs we don't know how they work, but they work."

Phillips says that ample evidence in the literature shows that the subset of VOCs in which he is interested, ranging from  $C_4$  to  $C_{20}$ , consists of direct products of oxidative stress. He explains, "Because we're looking at more than 100 compounds [in the subset], we see very distinctive fingerprints in different diseases. The whole pattern of markers of oxidative stress varies very dramatically from disease to disease. This makes it possible to use breath tests as a diagnostic marker because we see a very distinctive pattern." For example, Phillips adds that in patients with heart-transplant rejection, the pattern is completely different from that in people who don't experience rejection. In fact, in February 2004, the U.S. Food and Drug Administration



approved Menssana Research's Hearts-breath, a breath test for methylated alkanes, which appear when a patient suffers a heart-transplant rejection.

The different VOC patterns Phillips obtains from the various disease states are presented as 3-D contour plots, and he and his colleagues have published VOC plots for breast and lung cancers. They have also recently developed a diabetes breath test. "It's by using this spectrum of compounds in breath that we can look closely and say what the fingerprint is in this particular disease and how it differs from the people of the same age who don't have the disease," Phillips says.

Some experts agree with Phillips. "I think what [Phillips] says is absolutely right," says Peter Barnes of Imperial College, London. "You're looking at a pattern of [various] hydrocarbons, and you can expect to get different patterns from different diseases."

But other experts believe that all the issues of breath collection need to be resolved and that the necessary analytical instruments should be mature before claims can be made for breath tests for diagnosing diseases. Amirav says, "[Phillips] does what he does very carefully and in great detail and length, but it's too complicated. One cannot bring GC/MS to the frontline of medical diagnostics. What we [need to do] is attack the problem from the other side. First, let's do whatever we can to get point-of-care [tools], then we can see how we can [use them] in point-of-care diagnostics."

### To the future of breath tests

The *raison d'être* for breath tests is that they are non-invasive, easily repeated, and don't have the discomfort or embarrassment associated with blood and urine tests. More importantly, researchers believe that VOCs can be very early markers of disease. "It seems when there is oxidative stress occurring in [areas such as] the liver or kidney, the generation of ethane takes place within minutes and [is] seen on exhalation. When the oxidative stress is over, you will see a very rapid decline in the elevated ethane concentrations," says Schubert. "We believe we might find [breath] markers that are quicker than what we have now for components in blood and urine tests."

The true value of breath tests is that they provide an inexpensive way to rapidly screen for certain diseases. A negative result quickly eliminates those who are healthy, but patients who receive a positive result from a breath test can move on to other tests for a more decisive diagnosis. Hazel Ferguson of Pan Diagnostics says, "Breath analysis is not a conclusive diagnostic tool but [to be used] as a part of a range of diagnostics."

Overall, experts believe that breath tests hold promise for the future. Schubert says, "We have problems of standardization, defining markers, and sampling methods. But the prospects are so exciting, I think it's worthwhile to continue and do the basic research."

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