

## **Detection of Pulmonary Infections in Cystic Fibrosis Patients Using the zNose<sup>®</sup>, an Ultra-fast Gas Chromatograph**

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### **Abstract**

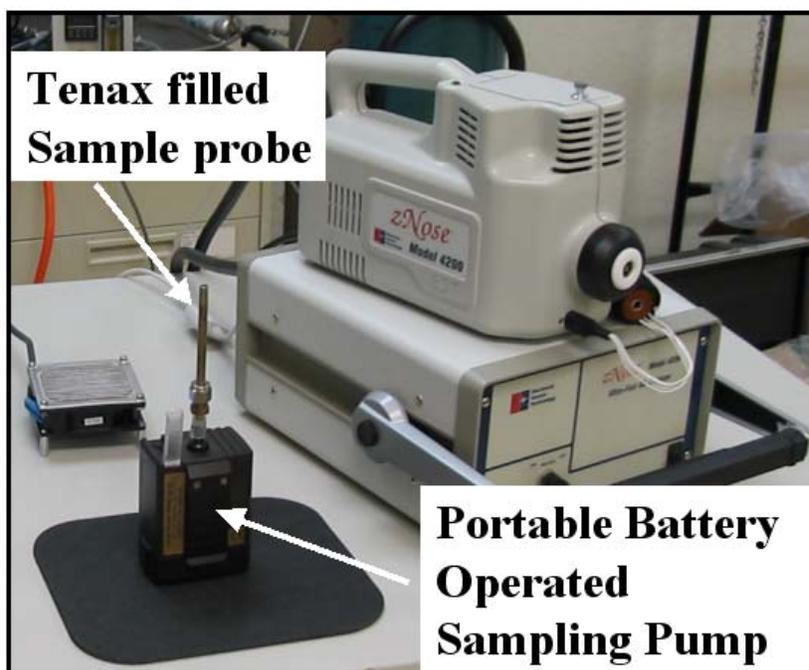
*Pseudomonas aeruginosa* (PA) is associated with a distinctive smell produced by a combination of volatile organic compounds (VOCs). A new electronic nose, which is based upon rapid gas chromatography and called the zNose<sup>®</sup>, may provide a rapid methodology for rapid, accurate detection of trace quantities (parts per billion; ppb) of VOCs associated with infections of PA. In preliminary studies VOCs in breath samples from patients with cystic fibrosis were investigated. Patients with cystic fibrosis also provided sputum and cough swab samples for cultures. These were used to inoculate blood agar (BA) and tryptic soy broth media (TSB) and the headspace vapors from the cultures were analyzed. VOCs may provide specific indicators of PA infection in vivo, and offers promise as a biomarker for noninvasive detection of PA infection by breath analysis. New analytical methods for detection and analysis of infections in cystic fibrosis patients are needed for quick response and treatment.

Electronic noses have interested developers of neural networks and artificial intelligence algorithms for some time, yet physical sensors have limited performance because of overlapping responses and physical instability. A new type of electronic nose, called the zNose<sup>®</sup>, is based upon ultra-fast gas chromatography, simulates an almost unlimited number of specific virtual chemical sensors, and produces olfactory images based upon aroma chemistry. A library of retention time indices for chemicals allows for the creation of hundreds of specific virtual chemical sensors. Virtual chemical sensors combined with odor profiling can be an effective method for recognizing the presence of biomarkers associated with pulmonary infections. The zNose<sup>®</sup> is able to perform analytical measurements of volatile organic vapors and odors in near real time with part-per-trillion sensitivity. Separation and quantification of the individual chemicals within an odor is performed in seconds. Using a unique solid-state mass-sensitive detector, picogram sensitivity, universal non-polar selectivity, and electronically variable sensitivity is achieved. An integrated vapor preconcentrator coupled with the electronically variable detector, allow the instrument to measure vapor concentrations spanning 6+ orders of magnitude. In this paper, preliminary results of quantifying volatile organics in breath samples from patients with cystic fibrosis is presented.

Breath analysis is currently a research procedure, but there is increasing evidence that it may have an important place in the diagnosis and management of lung diseases in the future. This will drive the development of cheaper and more convenient analyzers, which can be used in hospitals, later in family practice settings, and then eventually to the development of personal monitoring devices for use by patients.

## Measuring Chemical Odors with the zNose® *Direct Air Sampling*

An electronic nose called the zNose® shown in Figure 1 is able to directly sample ambient air to perform analytical measurements of volatile organic vapors and odors in near real time. Separation and quantification of the individual chemicals within an odor is performed in seconds. An integrated vapor preconcentrator coupled with the electronically variable sensitivity, allow the instrument to measure vapor concentrations spanning 6+ orders of magnitude. In internal pump samples ambient air at ½ milliliter per second and direct air samples are typically less than 20 milliliters. Using direct air sampling the instrument is able to achieve sensitivity in the ppb and even ppt concentration ranges for hydrocarbons spanning the range C4 to C24.



*Figure 1- Portable zNose® odor analyzing system and battery operated air sampler.*

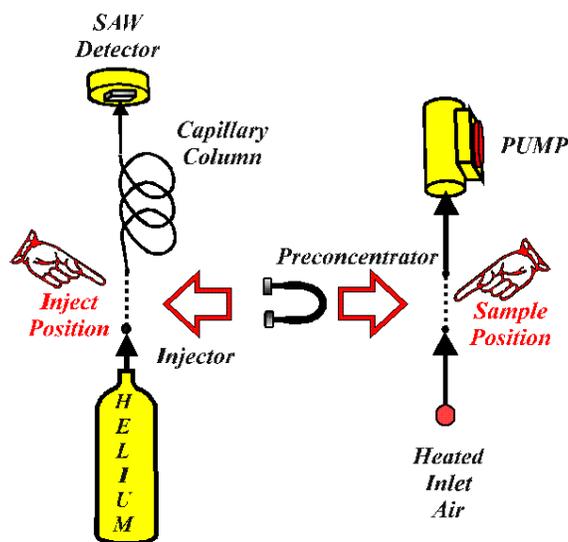
## *Indirect Air Sampling*

Concentration the organics from large air volumes before analyzing is an indirect sampling method, which can increase measurement sensitivity by 100-1000 times that of direct sampling. A handheld air sampler consisting of a battery operated sample pump and a tenax® filled probe enables organic compounds to be collected directly from the air or from tedlar bags containing 1-liter air samples. Using a sample flow of 250-1000 liters/minute allows much larger volumes of air to be tested than with by direct sampling. After sampling the tenax® filled probe is removed from the handheld sampler and inserted into the inlet of the zNose where the collected organic compounds are thermally desorbed and measured.

## How the zNose™ Quantifies the Chemistry of Odors

A simplified diagram of the zNose® system shown in Figure 2 consists of two parts. One section uses helium gas, a capillary tube (GC column) and a solid-state detector. The other section consists of a heated inlet and pump, which samples ambient air. Linking the two sections is a “loop” trap, which acts as a preconcentrator when placed in the air section (sample position) and as an injector when placed in the helium section (inject position). Operation is a two-step process. Ambient air (aroma) is first sampled and organic vapors collected (preconcentrated) on the trap. After sampling the trap is switched into the helium section where the collected organic compounds are injected into the helium gas. The organic compounds pass through a capillary column with different velocities and thus individual chemicals exit the column at characteristic times. As they exit the column they are detected and quantified by a solid-state detector.

An internal high-speed gate array microprocessor controls the taking of sensor data, which is transferred to a user interface or computer using an RS-232 or USB connection. Calibration is accomplished using a single n-alkane vapor standard. A library of retention times of known chemicals indexed to the n-alkane response (Kovats indices) allows for machine independent measurement and compound identification. The time derivative of the sensor spectrum yields the spectrum of column flux, commonly referred to as a chromatogram. The chromatogram responses (Figure 3) of n-alkane vapors (C6 to C14) provides a series of standard retention times, which can be used as an independent reference. .



**Figure 2- Simplified diagram of the zNose® showing an air section on the right and a helium section on the left. A loop trap preconcentrates organics from ambient air in the sample position and injects them into the helium section when in the inject position.**

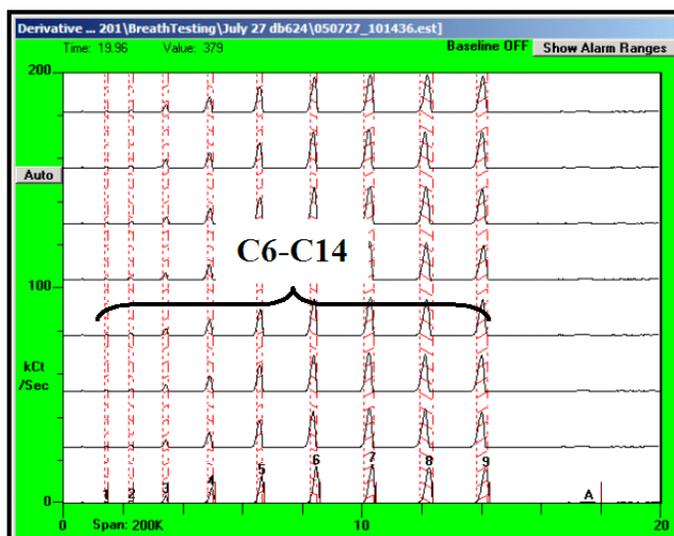


Figure 3- Vertically offset replicate chromatograms of alkane vapors used for referencing chromatogram retention times

### CalibrationStandards

Injecting known concentrations of chemical standards into a tedlar bag filled with a known volume of air (1liter) yields a useful calibration vapor. Director or indirect sampling of the standard vapor is used to calibrate the method response for each compound. Shown in Figure 4 are three replicate chromatograms used to calibrate the response for benzene, trichloroethylene, toluene, tetrachloroethene, ethylbenzene, o-xylene, and phenol. In this method a direct 5-second sample and a 20°C detector was used. Instrument software automatically displays Kovats retention time indices for all compound peaks.

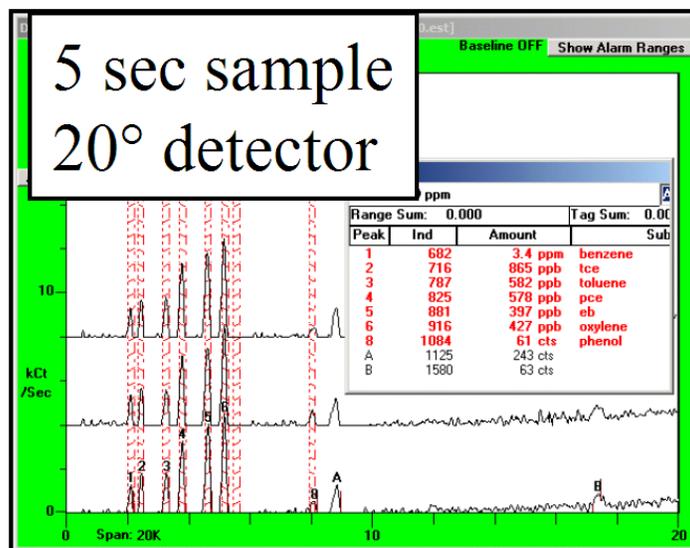


Figure 4- Response to calibration vapors.

## Indirect Odor Measurements

Calibrating indirect sampling methods is shown in Figure 5-A. After a known amount of vapor has been preconcentrated the collection probe is transferred to the inlet of the instrument (5-B) and a temperature controlled heater (5-C) used to desorb the trapped compounds so then can be transferred into the zNose<sup>®</sup> for analysis..

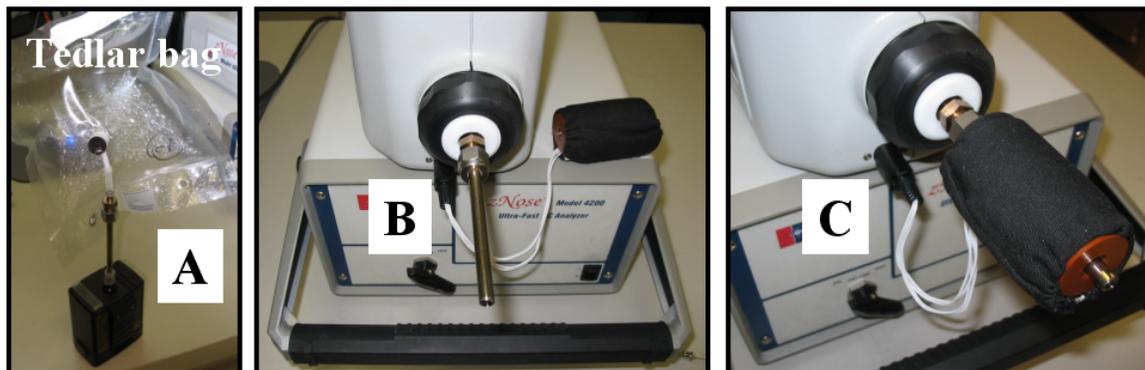


Figure 5- Standard vapors from a tedlar bag are collected by sample probe (A) which is then inserted into the inlet of the instrument (B) and heated to release the absorbed compounds (C) for analysis.

Adding a high flow preconcentrator greatly increases the sensitivity of odor measurements compared to direct sampling. This is demonstrated in Figure 6 where the response in counts from direct and indirect sampling using nearly equal sample times is compared. Using the high flow sampler a sensitivity gain of 30X is achieved.

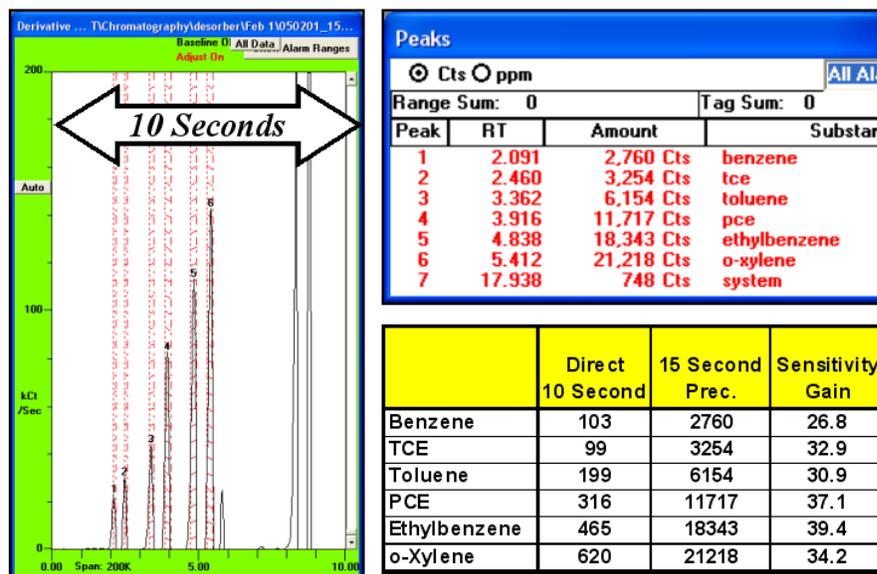


Figure 6- Sensitivity comparison of a 10 second direct sampling measurement with a 15 second high flow sampling measurement using the remote vapor collector.

Even higher sensitivity is possible using increased sample times. Sensitivity and response factors using a 1-minute sample of a low concentration vapor standard with the same six analytes is shown in Figure 7. The minimum detection level of benzene based upon signal to noise is near 1 ppb while that of o-xylene is even lower at 50 ppt. Lower detection levels can be achieved by increasing sample time.

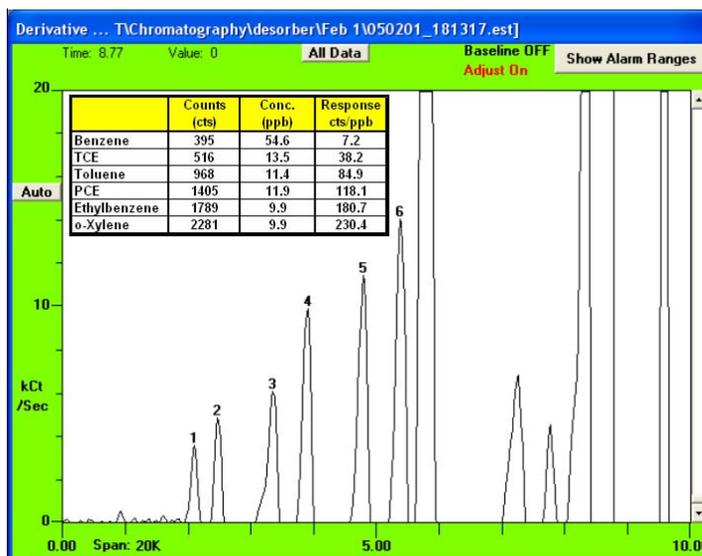


Figure 7-1 min sample of a low concentration 6-compound standard odor mixture from tedlar bag.

## Odors from *Pseudomonas aeruginosa* (PA)

PA is associated with pulmonary infections in cystic fibrosis patients and has a distinctive smell produced by a combination of volatile organic compounds (VOCs). Gas chromatographic profiles of the volatile metabolites of different strains have been shown to be distinctive and sufficient for identification<sup>1,2</sup>. The production of headspace metabolites identified after incubation were as follows: 1-butanol, isopentanol, toluene, 1-undecene, 2-butanone, 2-heptanone, 2-nonanone, 2-undecanone, and 2-aminoacetophenone. The Kovats retention indices<sup>3</sup> for each compound are shown in Table I and enable tentative identification using only n-alkane standards.

<sup>1</sup> Zechman JM, Labows JN Jr. Volatiles of *Pseudomonas aeruginosa* and related species by automated headspace concentration--gas chromatography. *Can J Microbiol.* 1985 Mar;31(3):232-7.

<sup>2</sup> Cox C.D., Parker J. Use of 2-aminoacetophenone production in identification of *Pseudomonas aeruginosa*, *J. Clin. Microbiol* 1979;9:479-84.

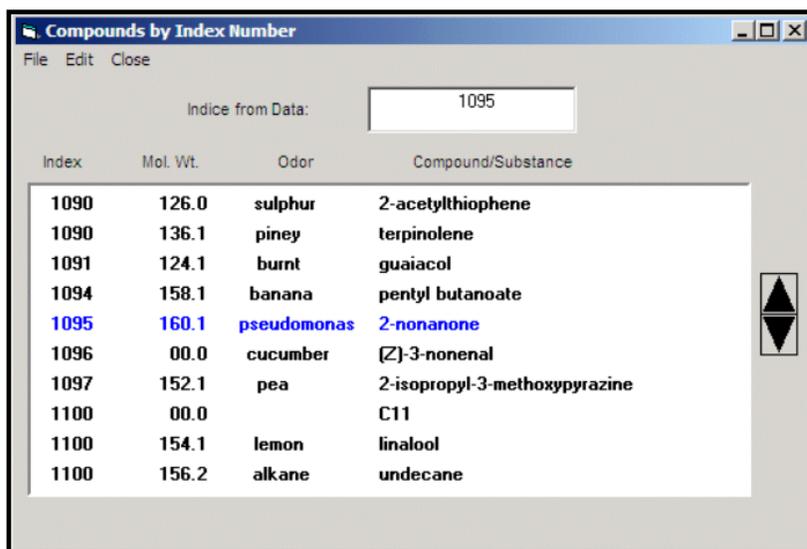
<sup>3</sup> Indices were obtained from National Institute of Standards and Technology (NIST) database see <http://webbook.nist.gov/chemistry/name-ser.html>

*Table I- Pseudomonas biomarkers*

Compounds	Kovats Index
1-butanone	597
1-butanol	675
Isopentanol	736
Toluene	773
2-heptanone	896
1-undecene	1075
2-nonanone	1093
2-undecanone	1296
2-aminoacetophenone	1309

### Compound Identification: Chemical Odor Library

Instrument software contains a library of over 700 chemical compounds and their human odor descriptors based upon Kovat's indices. Clicking on an unknown peak displays the nearest library compound entry. Human odor perceptions for each compound can help in identification of the compound. The library is user-expandable and a typical entry for the pseudomonas biomarker 2-nonanone is shown in Figure 8.



Index	Mol. Wt.	Odor	Compound/Substance
1090	126.0	sulphur	2-acetylthiophene
1090	136.1	piney	terpinolene
1091	124.1	burnt	guaiacol
1094	158.1	banana	pentyl butanoate
1095	160.1	pseudomonas	2-nonanone
1096	00.0	cucumber	(Z)-3-nonenal
1097	152.1	pea	2-isopropyl-3-methoxypprazine
1100	00.0		C11
1100	154.1	lemon	linalool
1100	156.2	alkane	undecane

*Figure 8- A chemical library based upon Kovat's indices and human odor perception is useful for identification of unknown compounds.*

## Analysis of Odors from inoculated Blood-Agar

*Pseudomonas aeruginosa* (PA) was cultured in petri dishes on blood-agar medium. After incubation for 24 hours at 37°C, the chemistry of the headspace vapors was analyzed and compared to blood agar, which had not been inoculated. Offset 10-second chromatograms for each are shown in Figure 9. Four of the nine biomarkers listed in Table I (shown as red bands) were detected at trace levels in both inoculated and un-inoculated agar. Two others, phenol and 2-nonanone, were considered significant markers for the strain *p. aeruginosa* since they only appeared in the inoculated culture.

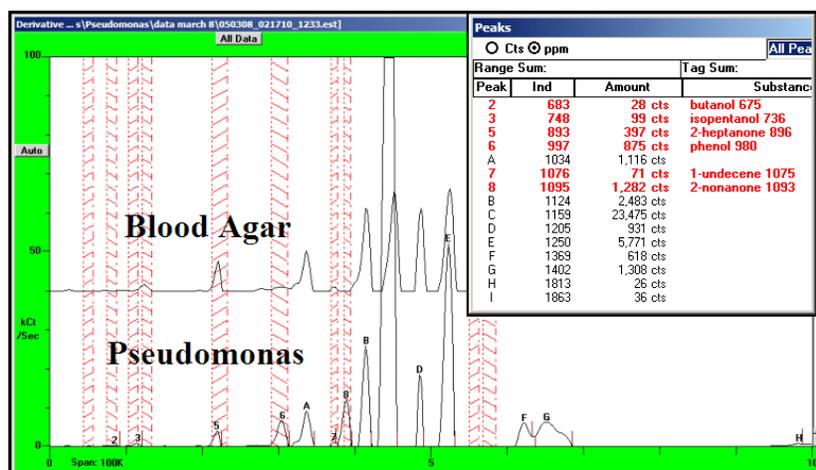


Figure 9- Offset chromatograms for headspace vapors from blood agar inoculated with *p. aeruginosa*

High-speed 10-second chromatograms enable chemical analysis to be performed in near real time. Assigning time slots (red bands) to each biomarker centered at their respective retention time is used to create a virtual sensor array. Display of the biomarker array response shown in Figure 10 is a simpler user interface for identification of bacteria.

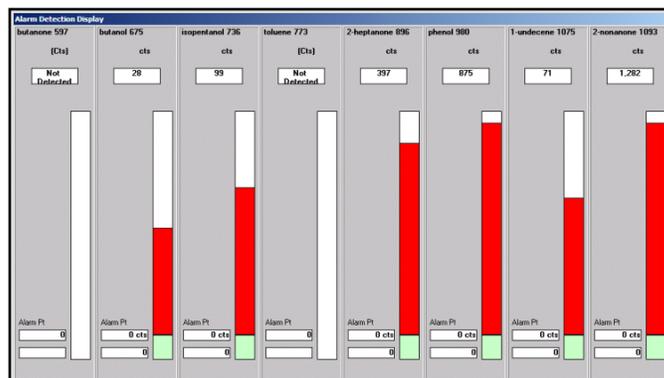


Figure 10- Biomarker virtual sensor array.

## Analysis of Odors from inoculated Tryptic Soy Broth

*Pseudomonas aeruginosa* (PA) was cultured in a tryptic Soy broth containing (g/liter) Peptone from casein 17.0, peptone from soy meal 3.0, D(+)glucose 2.5, sodium chloride 5.0, and dipotassium hydrogen phosphate 2.5. After incubation for 24 hours at 37°C, the chemistry of broth headspace vapors was analyzed and compared to broth without inoculation. Offset 10-second chromatograms for each are shown in Figure 11. Four of the nine biomarkers listed in Table I (shown as red bands) were detected at trace levels in both inoculated and un-inoculated soy broth. As in the case of blood agar cultures, two others, phenol and 2-nonanone were significant biomarkers because they only appeared in significant amounts in the inoculated broth.

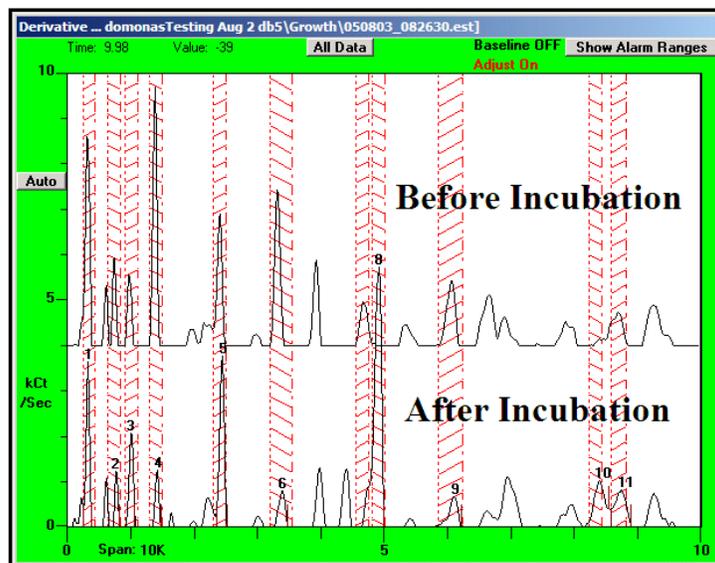


Figure 11- Offset chromatograms for headspace vapors from soy broth inoculated with *p. aeruginosa*

High-speed chromatography was used to measure the concentration of one biomarker, 2-nonanone, at 10-minute intervals throughout the incubation process. Plotting the concentration vs. incubation time, Figure 12, shows logarithmic growth commenced after a short lag and cell counts reached a peak after 8 hours of incubation.

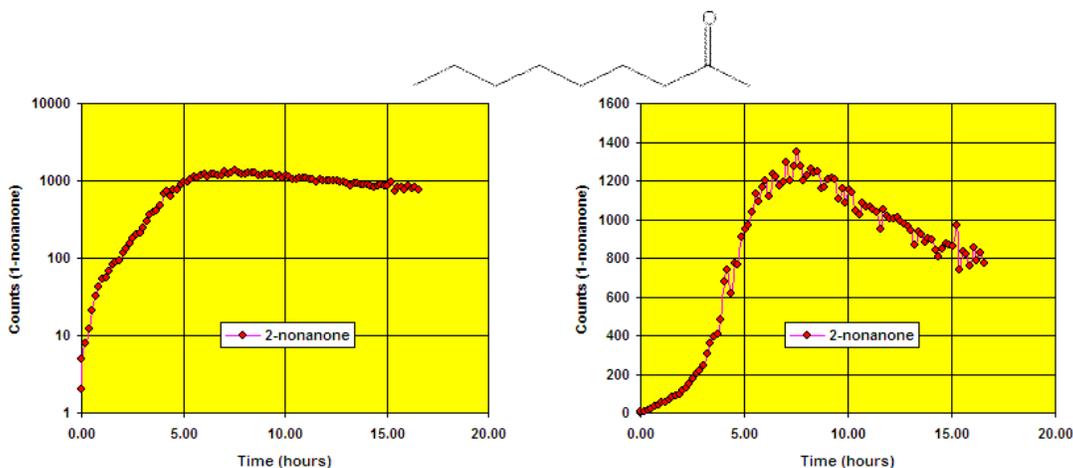


Figure 12- Concentration of 2-nonanone vs. incubation time.

## Testing of Breath from Cystic Fibrosis Patients

Analysis of breath chemistry is difficult because the VOCs are at very low concentrations, typically ppt and lower levels. Contamination of breath samples by VOCs in ambient air and collection vessels requires careful preparation and complicated measurement protocols for accurate measurements. In addition there are a large number of VOCs already present in the breath of normal individuals, which may interfere or mask targeted biomarkers. The number of VOCs detected in breath samples from healthy individuals<sup>4</sup> ranges from 157 to 241. Although the number of different compounds, which may be detected, spans more than 3000, only a few are observed in all subjects. The highest concentration compounds found in normal breath and their Kovats indices are shown in Table II.

Table II

Compound	alveolar gradient	subjects %	Kovats Index
acetone	27.91	92	500
Isoprene	60	100	520
benzene	-0.48	100	655
tetrachloroethene	7.7	76	815
Styrene	1	98	892
benzene, (1-methylethenyl)	4.77	100	988
dl-limonene	1.79	68	1033
acetophenone	1.49	94	1040
naphthalene	4.07	100	1180
benzothiazole	0.93	100	1224

Testing of indoor air revealed high levels of VOCs while testing of outdoor air showed few if any significant VOCs. Based upon these findings breath samples from patients were collected in new 1-liter tedlar bags in an outdoor setting. The breath testing protocol consisted of an initial calibration of both direct and indirect test methods using tedlar bag vapor standards of n-alkanes and the calibration mix described previously (See Figure 4). The test protocol first required purge cleaning and testing of tenax® filled tubes prior to their use. Approximately 500 ccm of the patient's tedlar bag breath sample was then passed through the clean probe, which trapped the VOCs. The probe was then attached to the inlet of the zNose analyzer and heated to 200°C to release the trapped VOCs. The zNose® analysis method settings were selected so optimize collection of volatile compounds. VOCs in the tenax® probe were collected using a 5 second sample time and analyzed on a db624 column temperature programmed 40-120°C at 5°C/second.

A comparison of VOCs collected from a normal individual and a patient with CF are shown in Figure 13. Red bands delineate retention times of pseudomonas biomarkers. An expanded comparison of the more volatile compounds is shown in Figure 14.

<sup>4</sup> M. Phillips, J. Herrera, S. Krishnan, M. Zain, J. Greenberg, and R. Cataneo, "Variation in volatile organic compounds in the breath of normal humans", *Journal of Chromatography B*, 729(1999) 75-88

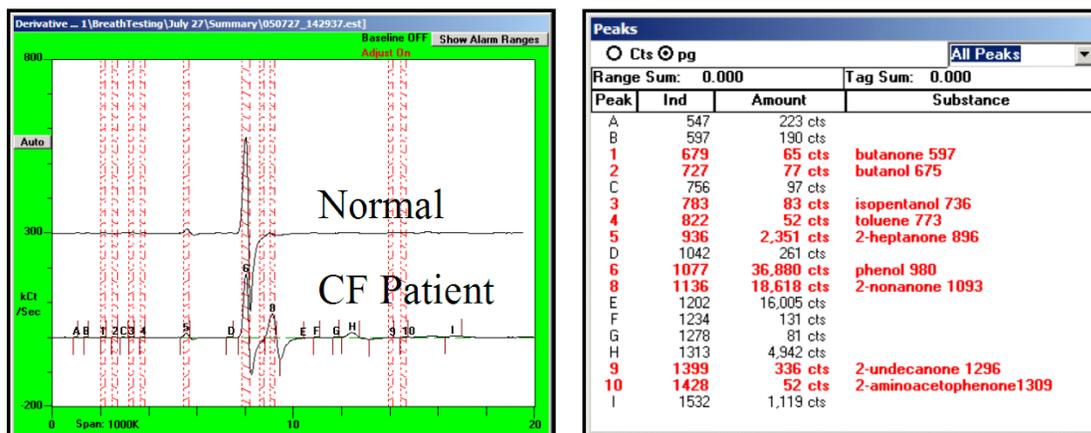


Figure 13- Breath chemistry of normal and CF patient.

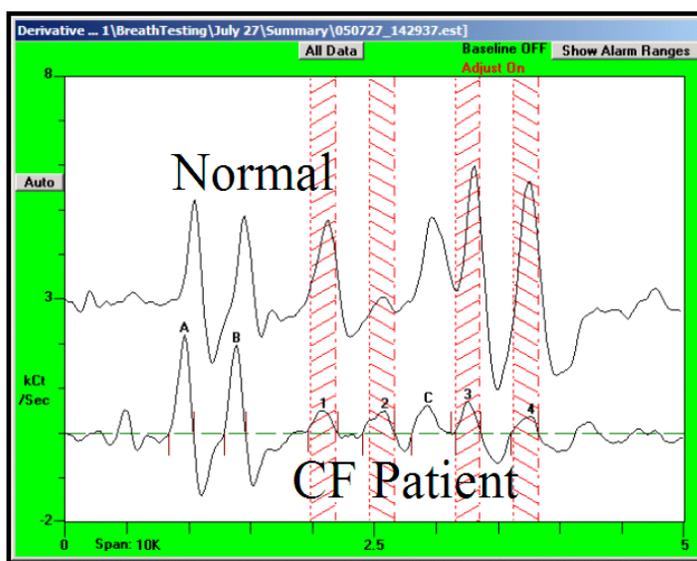


Figure 14- Expanded analysis of normal and CF patient breath.

From these comparisons and other preliminary findings it appears the most notable biomarker for pseudomonas infections in CF patients is the ketone, 2-nonanone, which appears prominently in Figure 13. Evaluations of the test methods and protocol also showed the need for removal of contaminants such as dimethyl acetamide and phenol which are produced by the tedlar bags and which tend to overload the instrument and mask important biomarkers. The need for a clean environment in which to collect breath samples was also found to be an important part of the measurement.

## Summary

There has recently an increased interest in the analysis of breath constituents as a way of monitoring and diagnosing lung diseases<sup>5</sup>. Here we are focused on the use of exhaled breath analysis in the diagnosis and monitoring of lung infections in cystic fibrosis (CF) patients, especially children. Because *Pseudomonas aeruginosa* (PA) colonization is most common in patients with CF, this work focused on detecting the biomarkers of PA in the breath of CF patients.

It is difficult to perform invasive measurements in small children and in patients with severe disease. A conventional approach is to use induced sputum to detect inflammation. This method is relatively reproducible and allows the quantification of inflammatory cells by culturing and bacteria counts. However, this technique is somewhat invasive as it involves inhalation of hypertonic saline, which may induce coughing and bronchoconstriction, and it is difficult to use in small children. Furthermore, the technique is time consuming and may itself induce an inflammatory response making it difficult to repeat measurements in less than 24 h.

Noninvasive real time monitoring of breath chemistry may assist in diagnosis of pulmonary diseases, assessment of disease severity and allow quick response and treatment. Because these techniques are noninvasive, it can be used repeatedly to give information about kinetics, in patients with severe disease, and to monitor lung infections in children, including infants. Breath analysis is currently a research procedure, but there is increasing evidence that it may have an important place in the diagnosis and management of lung diseases in the future. This will drive the development of cheaper and more convenient analyzers, which can be used in a hospital and later in a family practice setting, then eventually to the development of personal monitoring devices for use by patients.

A promising new tool for detection and analysis of breath chemistry based upon ultra-high speed gas chromatography was tested with headspace odors from bacteria cultures and on actual breath samples from CF patients. Using a battery powered remote sampler to collect and concentrate breath samples, ppt and lower sensitivity was achieved in near real time. The zNose® was able to separate and quantify the concentration of biomarker compounds over a wide range of compound concentrations and molecular weights in seconds. Because the measurement is based upon gas chromatography, independent laboratory validation can be used to validate findings. Preliminary results show that biomarkers for pseudomonas can be detected in the breath of CF patients. Additional efforts will focus on removal of contaminants produced by the tedlar bags and the development of a direct collection method. The need for a clean environment in which to collect breath samples was also found to be an important part of the measurement and will be addressed in future efforts.

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<sup>5</sup> A. Sergei., V. Kharitonov, and P. J. Barnes , Exhaled Markers of Pulmonary Disease ,Am. J. Respir. Crit. Care Med., Volume 163, Number 7, June 2001, 1693-1722