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1D one-dimensional

ADME absorption, distribution, metabolism, and excretion

AOP adverse outcome pathway

ARDS acute respiratory distress syndrome

ATD automated thermal desorption

BAL bronchoalveolar lavage

CBA cytometric bead array

CI-MS chemical ionization-mass spectrometry

charge coupled device (CCD)

CO_2 carbon dioxide

COPD chronic obstructive pulmonary disease

EBA exhaled breath aerosol

EBC exhaled breath condensate

EBP exhaled breath particle

EC-QCL external cavity quantum cascade lasers

e-nose electronic nose

GCxGC two-dimensional gas chromatography

HFIP hexafluoroisopropanol

HRMS high resolution mass spectrometry

IL interleukin

IMS ion mobility mass spectrometry

H_2O_2 hydrogen peroxide

LTQ linear trap quadrupole

MCC multicapillary column

MOSFET metal-oxide-semiconductor field-effect transistor

MRSA methicillin=resistant Staphylococcus aureus

MSD mesoscale discovery

MSSA methicillin sensitive S. aureus

MTBE methyl tertiary butyl ether

NH_3 ammonia

NO nitric oxide

OSA obstructive sleep apnea

PCA principal component analysis

pptv part per trillion by volume

PTR proton transfer reaction

SIFDT selected ion flow drift tube

SIFT selected ion flow tube

SIMOA single molecule array

SELDI surface-enhanced laser desorption/ionization

SERS surface enhanced Raman scattering

SESI secondary electrospray ionization

SEV sevoflurane

STRA severe therapy-resistant asthma

TBA tertiary butyl alcohol

TD thermal desorption

Evolution of Clinical and Environmental Health Applications of Exhaled Breath Research: Review of Methods : Instrumentation for Gas-phase, Condensate, and Aerosols

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Abstract

Human breath, along with urine and blood, has long been one of the three major biological media for assessing human health and environmental exposure. In fact, the detection of odor on human breath, as described by Hippocrates in 400 BC, is considered the first analytical health assessment tool. Although less common in comparison to contemporary bio-fluids analyses, breath has become an attractive diagnostic medium as sampling is non-invasive, unlimited in timing and volume, and does not require clinical personnel. Exhaled breath, exhaled breath condensate (EBC), and exhaled breath aerosol (EBA) are different types of breath matrices used to assess human health and disease state. Over the past 20 years, breath research has made many advances in assessing health state, overcoming many of its initial challenges related to sampling and analysis. The wide variety of sampling techniques and collection devices that have been developed for these media are discussed herein. The different types of sensors and mass spectrometry instruments currently available for breath analysis are evaluated as well as emerging breath research topics, such as cytokines, security and airport surveillance, cellular respiration, and canine olfaction.

Graphical abstract

TH T helper

THC tetrahydrocannabinol

TOF time-of-flight

TNF-α tumor necrosis factor α

TNT trinitrotoluene

UFP ultrafine particulate matter

volatile organic compound (VOC)

Conflict of Interest Disclosure

The authors declare no competing financial interest.

Keywords

breath research; exhaled breath condensate (EBC); exhaled breath aerosol (EBA); volatile organic compounds (VOCs); analytical techniques; biomarkers

Abbreviations[1]

1.Introduction

Before the advent of even the simplest laboratory instrumentation, the human nose served as the original chemical detector for disease diagnosis. It all started with Hippocrates (born 460 BC) who taught his students to use breath odor to identify patients with liver disease, uncontrolled diabetes, and failing kidneys.[1]There are a few additional pivotal moments in breath history that bring us to the present. In 1971, Linus Pauling published a seminal article demonstrating analytical methodology used to identify approximately 250 compounds in breath in addition to nitrogen, oxygen, water and carbon dioxide.[2] The final step in ushering in the modern era of breath research came from the work of Lance Wallace of the U.S. Environmental Protection Agency, who recognized that breath analysis could unambiguously document recent exposures to environmental contaminants.[3] Michael Phillips (St. Vincent's Medical Center, New York and Mensanna Research Inc., New Jersey) also contributed by developing case-control tudies starting in 1987 using breath constituents and patterns to discern a variety of adverse health states including lung and breast cancer as well as cardio-pulmonary disease.[4–5]

Over the years, the wide use of different methods for breath media analyses has revealed the need for consistency and standardization in breath research. Researchers have proposed that breath analyses should undergo a series of checks, including standard parameters, specifications, and test methods, as well as risk analysis. These are measurable criteria by which research studies can be characterized and evaluated.[6] A wide variety of compounds, such as volatile organic compounds (VOCs), NO, CO_2, NH_3, cytokines, and hydrogen peroxide (H_2O_2) are commonly analyzed in exhaled breath, exhaled breath condensate (EBC), and exhaled breath aerosol (EBA). These compounds can be analyzed using different techniques, including GC-MS, ion mobility mass spectrometry (IMS), chemical ionization-mass spectrometry (CI-MS), spectroscopy, and sensors.[7] The use of mass spectrometers that measure the integer mass of non- target compounds instead of the exact mass (to the fourth or fifth decimal place) can make it challenging to identify non-target compounds in breath samples since many compounds share the same integer mass despite having different chemical formulas. The use of high-resolution mass spectrometry, including GC × GC-MS and GC-time-of-flight (TOF)-MS, can alleviate this problem for non-targeted analysis.[8] However, the wide range of available analytical techniques and target compounds make it difficult to apply universal standards for sampling and analysis that are suitable across all methods.[7]

[1]Abbreviations:

Despite these challenges, breath research has made many advances in both analytical techniques and health-related applications throughout its history. Traditional gas-phase breath spectrometry, including GC × GC-MS and GC-time-of-flight (TOF)-MS, can alleviate this analysis as well as EBC and EBA have become valuable breath matrices for disease detection and environmental exposure.[9,10,11] The variety of breath sample collection devices and methods that have been developed, ranging from large canisters and polymer bags to easily portable thermal desorption (TD) tubes for VOC analysis, have evolved to suit the needs of the changingfield.[12] The analytical instrumentation for detection of compounds in exhaled breath, EBC, and EBA has also improved to include GC-MS, LC-MS, Direct MS, and a variety of sensors, including electronic noses.[13,14,15,16] These detection methods have been utilized to advance research in human health and disease, including alcohol breath testing,[17,18] lung cancer,[19,20] asthma,[21,22] and chronic obstructive pulmonary disorder (COPD),[22,23,24] as well as to identify biomarkers of health state in breath matrices. These topics are discussed in this review within the context of the history of breath research from 1995–2016. Papers were curated using PubMed searches. The data from these searches were utilized to construct bar graphs for many of the classic breath topics to discern trends in the number of publications over time. All of the topics show an increase in publications, especially in recent years. These data are utilized as a supplement to explain the significance of breath research over time, but investigation of all publications in this time range is outside the scope of this review. This review briefly discusses many of the main topics in breath research, providing an overview of some of the history and interesting applications in recent years. Novel applications of breath research for security, cellular respiration, organ-on-chip, canine olfaction, and crowd breath show the evolution of the field from standard VOC analysis of breath toward cellular and animal models with promising future directions.

2. Publication Curation Methods

Publications reporting breath research data in the various topic areas were gathered using PubMed advanced searches. All keyword searches were limited to the Title/Abstract of the paper to ensure that breath research was the focus of the article. Papers were date restricted from 01/01/1995 to 12/31/2016 for all categories except alcohol, which was searched from 01/01/1960 to 12/31/2016. The year range from 1995–2016 was selected because breath research became more prominent in 1995 in a wider range of applications. For alcohol research, a longer time span was chosen because alcohol research began earlier than many of the other types of breath research. Initial keyword searches were inspected to determine the relevance of the returned articles versus the topic area, and “NOT” statements were added to the search strings to eliminate topics unrelated to the analysis of exhaled breath. These “NOT” statements included “shortness of breath,” “short of breath,” “breath sounds,” and “gastric emptying.” These phrases tended to be present in articles describing medical procedures that did not involve the analysis of exhaled breath. The PubMed database was accessed in July 2017. Aerosol, condensate, and alcohol searches only included the search string for exhaled breath, as these topic areas involved sampling from just humans. Searches for GC-MS, LC-MS, Direct-MS, and sensors also included volatile organic compounds and headspace from biological media, including blood, urine, feces, faeces, milk, cell line, cell culture, bacteria, or saliva. Detailed search strings for all topics that were used to compose

the figures can be found in the accompanying Data in Brief article. The number of manuscripts published per year in each category as well as the appendix of articles curated using each search can also be found elsewhere (Wallace et al. submitted).

3. Types of Breath Matrices

3.1 Gas-Phase Exhaled Breath

The traditional venue of breath testing has always revolved around the gas-phase. After inhalation and exposure, VOCs are retained within different parts of the body, depending on their breath-blood-fat partition coefficients. VOCs located in fatty tissues are released to the blood and are then exchanged into the breath through the alveoli and airways in the lungs. A portion of VOCs are also retained within the respiratory tract after exposure.[25,26] Thus, breath concentrations of VOCs are representative of blood concentrations, but samples can be obtained non-invasively with little discomfort to the individual.[26] VOCs may originate from exogenous environmental exposures through inhalation, ingestion, or skin absorption (e.g., gasoline, food, lotions) or endogenous sources from within the body (e.g., microbe metabolism, byproducts, cancerous tumors).[27] Breath contains both dead space air and alveolar air. Dead space air originates from the mouth, trachea, and bronchi, while alveolar air is from the lungs.[28,26] Alveolar breath only consists of a 350 mL volume at the tail end of breath. Many breath sampling techniques have been designed to capture only the alveolar air, as this contains the VOCs that have been exchanged across the blood-breath barrier.[26] Since VOCs are gases, they are easier to access in the breath compared to a condensed medium.[29]

3.2 Exhaled Breath Condensate (EBC)

The breath, however, is more complex than gas exchange. The lungs humidify exhaled breath, and because the human body is usually warmer than the environment, the exhaled water vapor tends to condense on the way out. Furthermore, the surfaces in all parts of the lung down to the alveoli are coated with an aqueous mucous layer that can be aerosolized and carry along a variety of non-volatile constituents.[9,30] This was originally exploited for assessing lung status via dissolved ions (pH).[31,32] The pH provides an indication of the acidobasic balance of the compounds that compose EBC.[9] Access to this fraction of the breath was developed using a technique wherein multiple breaths were passed through a chilled tube and condensate was recovered for analysis. Termed exhaled breath condensate (EBC) in the literature, it was quickly recognized as a matrix providing a completely new set of analytes that were previously unknown.[11–33] EBC is now used regularly in breath research as a complement to gas-phase analyses.

EBC consists mostly of condensed water vapor with dispersed nonvolatile molecules from the respiratory tract within it.[11–34] EBC represents a relatively non-invasive sample that provides important information about the constituents in the fluid lining the airway.[35] Hydrogen peroxide, ammonia, adenosine, leukotrienes, isoprostanes, nitrogen oxides, peptides and cytokines are all examples of compounds found in EBC.[10, 34,9] Recently, simple measurements of EBC acidity (pH) have been developed for “at home” collection and monitoring of children’s asthma status.[36,37] Another promising application for EBC

analysis is the collection of condensate from ventilated patients in hospitals. This is of particular interest for preterm infants, as they cannot be regularly sampled for blood or urine analyses in sufficient volumes.[38,39,40,41] Publications of EBC analyzed from patients on respiratory support have continued to grow ever since.[42,20] This is equally important for assessment of EBC from critically ill mechanically ventilated patients with acute respiratory distress syndrome (ARDS).[43,44]

3.3 Exhaled Breath Aerosols (EBA)

EBA represents a fraction of total EBC, and is targeted to larger molecules, such as fatty acids and cytokines, as well as cellular fractions, proteins, viruses, and bacteria instead of the gas-phase.[45,46,47,48,49] This technique has the advantage of simplicity in the field as there are no requirements for freezing out the sample; subjects or patients can simply wear standard hospital/medical masks for a given time (e.g., 10 min) and these can be shipped back to thelaboratory and processed for a variety of targeted compounds analyses.[49,48] EBA has been measured in breath over the past 20 years, with a steady number of articles published per year from 2008–2015 and a significant spike in publications in 2012. Figure 1 compares the publication trend in the number of peer-reviewed articles published featuring EBA versus EBC media. While both EBC and EBA publications have increased over time, EBC has been reported in the literature more frequently than EBA, showing a faster upward trend in the number of publications from 2003–2009 (see Figure 1). The usefulness of EBA for breath research appears to still be evolving, while EBC is a more established technique.

In addition to biological media, particulate matter (PM) and nanoparticles have also been detected using EBA. Exposure to PM from air pollution causes inflammation in the lungs.[50] Exhaled breath particles (EBPs) are formed when closed airways open upon inhalation.[51] EBPs have also been evaluated in individuals with asthma and allergies, children with asthmatic symptoms, patients on mechanical ventilators, smokers, and patients with chronic obstructive pulmonary disease (COPD).[51,50,52,53,54,55] Occupational exposure to titanium dioxide and iron oxide nanoparticles has been investigated using breath analysis. Although particle concentrations in air did not exceed the national airborne exposure limit, employees consistently exposed to nanoparticles showed significantly more titanium and oxidative stress markers in EBC samples compared with controls.[56;57,58]

Breath aerosol sampling has also been applied to develop drug tests. Unlike testing blood or urine, breath sampling represents a noninvasive technique that can be used for routine sampling in work places.[59] For drug testing, bio-aerosols from breath are collected on a filter when the subject breathes through the sampling device. The drug compounds are then released into solution when the filter is immersed in methanol.[60,61] An LC-MS method was developed to detect drugs from breath samples, and the method was employed to monitor amphetamine, methamphetamine, tetrahydrocannabinol (THC), and cocaine.[61] Another study used an ExaBreath sampler to measure methadone in drug addicts to evaluate exposure. The ExaBreath sampler collects aerosols (particles) onto a filter as an individual exhales through the sampling device. However, breath detection was limited to methadone, whereas urine samples collected from the same subjects tested positive for a variety of additional substances, including morphine, neuroleptics, and benzoylecgonine.[60] Breath

samples were found to only reflect the most recent exposure, whereas urine samples were needed to determine drugs that had been in the system for a longer duration.[60,61]

4. Breath Sample Collection

4.1 Polymer Bags

Popular methods for volatile breath sampling include polymer bags, canisters, and thermal desorption tubes. Polymer bags are widely used due to their low cost, easy maneuverability, and potential for reuse. Examples of polymer bags include Tedlar, Teflon, FlexFoil, and Nalophan, although Tedlar is most common.[28] Polymer bags are composed of inert materials to reduce reactions.[28] Some polymer bags are covered with outer layers of black Tedlar to block UV rays that may damage samples.[62] Tedlar bags have been used to analyze acetone as a marker for diabetes,[63] isoprene as a marker of cholesterol synthesis,[64] as well as volatiles from healthy individuals and lung cancer patients to identify biomarkers.[65] While Tedlar bags have been shown to pollute the contents of breath samples, introducing some minor contaminants to the sample that are worse when it is subjected to heat,[28,62] these contaminants have been shown to be less than 10% of the total values from human breath.[62] Common contaminants from Tedlar bags include phenol, N,N-dimethylacetamide, carbon disulfide, and carbonyl sulfide.[28]

4.2 Canisters

Stainless steel inert canisters are used to collect and store breath samples without corrosion, exposure to light, or the potential for interactions with the vessel. Canisters are advantageous for breath sample collection because they are durable and easy to use.[66] However, a disadvantage of canisters is their high cost, the need for prior evacuation before sampling, and requirements for specialized cleaning equipment.[28] Electropolished SUMMA canisters have been used to collect alveolar breath samples in 1 L volumes with parts-per-billion by volume (ppbv) sensitivity.[66] After releasing the dead-space air at the end of a breath, the individual opens the canister valve and expires the reserve air from the lungs into the canister before closing it. Most VOCs are stable in canisters for at least 30 days after sample collection.[67] Canisters have been used to analyze compounds in breath to determine exposure to VOCs in groundwater, gasoline, and swimming pools.[68,66,69,70,71]

4.3 Sorbent Tubes

TD tube sampling is another method commonly used for breath sampling. Individuals exhale end-tidal breath into a device such as a polymer bag or a Markes Bio-VOC concentrator, and the sample is then plunged onto a thermal desorption tube to collect the VOCs.[72,12] Other breath samplers, such as the ReCIVA unit (Owlstone Ltd.) are available, which capture end-tidal breath directly onto sorbent tubes.[73] Modifications of the Haldane-Priestley tube have also been used for such applications, in which insulated aluminum tubes are connected to polymer bags.[28] After sample collection, the tubes are capped and analyzed by automated thermal desorption (ATD)-GC/MS. Many different types of thermal desorption tubes with different sorbents are available, depending upon the volatile compounds of interest. Tenax and carbograph sorbents are commonly utilized in breath research.[74,75,28] TD has been used

to analyze VOCs and other compounds originating from diverse groups such as firefighters, chronic kidney disease patients, and cellular headspace.[68,76,77]

4.4 EBC Collection

Exhaled breath, which consists largely of water vapor and aerosol particles, can be collected through condensation using a breathing tube, and the fractions of EBC and EBA can be used experimentally. EBC is typically collected by 10 min of tidal breathing through a long tube that is cooled with ice (0 °C) or dry ice (−80 °C) in order to condense the aqueous fraction of the exhaled air, depending on the analysis method and the stability of the substances to be measured.[10,9] A volume of 1–2 mL is usually collected at a time.[34,11] Three commercial devices that are often used for collecting EBC are the RTube, Turbodecss, and EcoScreen. The RTube is advantageous because it is a disposable kit with a one-way non-rebreathing valve connected to a collection tube.[37] The Turbodecss is also portable with a disposable collection cell and a non-rebreathing valve, allowing this sampler to also be used for in-home collection.[9] The EcoScreen system has a two-way valve that prevents mixing of inhaled and exhaled air as the exhaled air is cooled in the condenser.[37] However, as opposed to the RTube and Turbodecss, the EcoScreen is not portable, limiting its ability to be used at home.[20] The EcoScreen has therefore been used primarily in research laboratories.[9] A Loccioni instrument can also be used during collection of EBC and EBA samples. This device gives the individual feedback about their breathing pattern during sample collection, reducing variability in sample volumes and biomarker levels that can be caused by hyperventilation.[78,79]

4.5 EBA Collection

EBA can be collected using sampling devices or different forms of filter materials at room temperature. Teflon filters have been used for collection, and EBA particles can be extracted using solvent.[45] Particles can then be analyzed using LC-MS.[46] The Gesundheit II (G- II) sampling device has been utilized to collect breath aerosol while subjects sit in a booth andbreath through the cone of the sampler.[80] Subjects can wear masks or respirators, and breathe, talk, or cough as they normally would. The breath enters a slit impactor, which collects particles larger than 5.0 μm aerodynamic diameter and captures then on a Teflon substrate from which they are extracted and analyzed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). This device is commercially available from SKC BioSampler and has been utilized to assess influenza and levels of other viruses in breath.[80] Other commercially available devices, such as the ExaBreath sampler mentioned above, can also be used for convenient collection of EBA.[60] Silicon wafers with cascade impactors have also been used to collect phospholipids from exhaled breath based on their inertia. This technique was used to compare phospholipid compositions of patients with asthma, cystic fibrosis, and healthy controls.[81] For a more simplistic collection approach, individuals can wear plastic masks/respirators or paper hospital masks for a specified length of time, and the surface of the mask (or the entire paper mask) can be sampled using a moistened piece of filter paper, which can then be extracted with solvent for LC-MS analysis.[82]

5. Detection Methods: Sensors

5.1 Electronic Nose

The electronic nose, or e-nose, detects odors, flavors, and VOCs using non-specific gas sensor arrays and pattern recognition tools.[83] The first e-nose was developed in 1964 by Wilkens and Hatman using redox reactions of odorants on an electrode; in 1965 this design was quickly followed by other designs that utilized conductivity of odorants and variation of odorant contact potential.[84,85 ,86] In 1982, Persaud and Dodd developed an electronic nose using semiconductor transducers that was capable of discriminating between different odorants,[87] and in 1985 Ikegami and Kaneyasu devised an e-nose composed of semiconductor oxide that produced different patterns in response to odors.[88] These publications in the 1980s showcased the e-nose as a sensitive and useful chemical array sensor system, and from this point on the use of e-noses increased.[14]

Sensors in e-noses function by detecting physical or chemical changes within the array in response to the components in exhaled breath. Metal-oxide sensors were first introduced in the 1960s and have low parts-per-million by volume (ppmv) detection levels.[89] Metal oxide sensors have long-term performance and stability, making them advantageous for e-nose designs.[90] Metal oxide sensors are heated with a ceramic support tube coated in metal, typically SnO_2. Gas molecules that interact with the metal cause changes in conductivity due to oxygen exchange.[89] Metal-oxide-semiconductor field-effect transistor (MOSFET) sensor arrays are commonly used to devise e-noses and utilize transistors to amplify and alter electronic signals. Gases, such as hydrogen, enter the sensor array and interact with the metal, changing the electric field inside the device to produce a signal that is electronically recorded.[91] The SpiroNose sensor, which was designed for medical testing, contains five metal-oxide semiconductor sensor arrays, two of which are used as reference arrays to analyze ambient VOCs and three to detect VOCs in breath samples.[92]

Other sensors can be used to develop e-nose devices, such as conducting polymers, polymer composites, acoustic waves, optical fiber arrays, electrochemical gas (EC), and colorimetric sensor arrays.[93] The Cyranose 320 is a widely-used, portable, hand-held e-nose device that utilizes a carbon black conducting polymer sensor array to achieve low ppmv detection limits.[94] Upon exposure to gases or vapors, the polymer undergoes changes in electrical resistance that reduce the number of conducting pathways. Surface acoustic wave sensors are usually developed using piezoelectric substrate materials such as ZnO, lithium niobate or quartz with transmitting and receiving transducers on either side.[91] The surface acoustic wave sensor has been utilized in an e-nose to compare volatile profiles of lung cancer patients with controls.[83] Optical fibers coated in fluorescent dye have been utilized as sensors, in which wavelength changes, intensity alterations, and spectrum changes are tracked as indicators of interaction with a gas or vapor.[91] Metalloporphyrin dye dots have been printed on paper sensor cartridges to mcreate colorimetric sensor arrays, and pattern changes in the dye colors as a result of interactions with the chemicals in the breath samples have been captured by photographs.[93] In EC sensors, volatiles are detected after electrochemical oxidation or reduction at the electrode surface, making these sensors selective for a small number of reactive gases.[89]

E-noses have been applied to detect pattern differences in a variety of clinical and research samples, including military, food industry, and environmental applications.[83] The e-nose has been utilized to differentiate Alzheimer's disease, Parkinson's disease, and healthy control samples, and organic compounds with significant differences between the disease states were identified by IMS and used to create a predictive model for sample classification.[95] A study of obstructive sleep apnea (OSA) revealed that the e-nose could distinguish OSA breath samples from controls in the morning but not in the evening, indicating that the sampling time may affect the ability of the e-nose to detect the disorder.[96] Breathprints are the chemical signatures of compounds and molecules that are found within an individual's exhaled breath. The SpiroNose was able to discriminate between breathprints of healthy controls and patients with asthma, COPD, and lung cancer with cross-validation values from 78–88%.[92] Recently, *aspergillus fumigatus* colonization was found to produce a distinct breathprint in e-nose samples from cystic fibrosis patients, providing a non-invasive test for infection.[97]

While e-nose publications remain steady in the field, there is still a need for standardization. Some studies have shown that e-noses lack the accuracy and repeatability needed to make them useful for diagnosis. A recent study investigated the effect of different exhalation flow rates and breath holding times on the breathprints of healthy individuals and individuals diagnosed with lung cancer during sample collection using the Cyranose 320 and found that the patterns were significantly altered for healthy adults but not for lung cancer patients.[94] This study shows how inconsistencies in sample collection between individuals and research groups can significantly alter the ability to discriminate healthy versus diseased individuals. In another study, e-noses were found to have greater within-day than between-day repeatability ($P < 0.0001$), but sample transportation and storage on sorbent tubes prior to e-nose analysis has not been found to significantly affect quantitation of target compounds.[98,99]

5.2 Mid-infrared Sensors

Mid-infrared quantum cascade lasers are advantageous for exhaled breath analysis due to their reliability, compactness, tunability, and low power requirements.[100] Mid-infrared lasers and sensors have been utilized over the past fifteen years to detect exhaled compounds in breath. Mid-infrared lasers have a wavelength range from 3–20 μm with a tuning range of greater than 2 μm, giving them high spectral brightness. These properties make them ideal for accurately determining the isotope ratio of $(12)CO_2/(13)CO_2$, which is an important biomarker of glucose metabolism during sepsis.[101,102] $(12)CO_2/(13)CO_2$ isotope ratios have been monitored in mouse breath using hollow waveguide gas cells and external cavity quantum cascade lasers (EC- QCLs).[102,101] A $(13)CO_2$ isotope ratio-meter has also been recently developed to monitor breath levels during moderate exercise for individuals attempting to lose weight, as altered $(13)CO_2$ isotope ratios indicate changes in macronutrient metabolism.[103]

Mid-infrared sensors are often utilized to monitor exhaled levels of volatiles, such as nitric oxide (NO), hydrogen sulfide, isoprene, and acetone. NO is a biomarker for asthma and respiratory disorders and is therefore frequently monitored in clinical settings. Sensitive

sensors capable of real-time measurements of NO in breath have been using the mid-infrared spectral region.[104,105] A miniaturized mid-infrared one-dimensional (1D) photonic crystal cavity sensor with a sensitivity of 10 ppbv with comparable performance to commercial sensors was recently developed.[106] Real time gas sensing of hydroge sulfide was accomplished using substrate- integrated hollow waveguides coupled to a Fourier transform infrared spectrometer with applications for development into a hand-held instrument for industrial monitoring of hydrogen sulfide exposure based on breath levels of this toxic gas.[107] Hollow waveguide mid-infrared sensors have also been utilized to measure isoprene breath levels, and acetone has been detected at sub-ppmv levels using QCL infrared spectrometers.[108,109] Breath profiles of dialysis patients have also been assessed using mid-infrared absorption spectroscopy to quantify levels of volatiles, such as carbon dioxide and ammonia to establish reference values for use in case-control studies.[110]

5.3 Novel Sensors and Sampling Devices

In addition to electronic noses and mid-infrared sensors, a series of novel sensors and sampling devices that use similar technology have also been developed within the last ten years for breath monitoring applications. A gas phase biosensor was developed that can measure breath acetone to determine lipid metabolism after exercise.[111] Another portable breath analyzer capable of measuring acetone levels was developed that utilizes the cavity ringdown spectroscopy technique for online breath analysis.[112] Gold nanoparticle-based flexible sensors that can detect ppbv levels of VOCs have been utilized to distinguish exhaled breath collected from ovarian cancer patients versus healthy controls.[113.]

6. Detection Methods: Mass Spectrometry

Mass spectrometry is a useful analytical tool for breath research that provides essential information about the compounds in different types of breath samples. Breath articles were searched under three main types of mass spectrometry applications: GC-MS, LC-MS, and Direct MS. These breath searches also included applications of gas-phase analysis of biological headspace from blood, urine, milk, saliva, feces, cell lines, and bacteria. A category for sensors, which included electronic noses, laser spectroscopy, and mid-infrared sensors was also included for comparison (discussed in detail in a later section).

As seen in Figure 2, all mass spectrometry and sensor publications show a general upward trend over the past 20 years. Unsurprisingly, the number of publications reporting GC- MS applications has been greater than the number of articles utilizing LC-MS or Direct-MS techniques. This result was expected as breath is a gas-phase medium that easily lends itself to GC-MS analysis. Interestingly, in the past four years, the number of publications utilizing sensors for breath research has caught up to the GC-MS publication output. This indicates that sensors are becoming more popular for breath, which may be due to the wide range and availability of simple and easy to use sensors such as electronic noses and laser spectroscopy.[92,94,101] Direct-MS publications also appear to have steadily increased in the past few years in contrast to LC-MS publications, which remained constant. LC-MS techniques are more commonly utilized for exhaled breath condensate analysis, while Direct MS provides fast, real-time measurements of breath samples in the field, but is not useful

when high resolution data are required.[35,114,13]. Several of the interesting breath research publications curated through these PubMed searches of mass spectrometry applications and their impact on the field are highlighted below.

6.1 GC-MS

6.1.1 GC-TOF-MS—Gas chromatography time-of-flight mass spectrometry (GC-TOF-MS) is faster and provides higher mass resolution than conventional GC-MS instruments and has been used in breath research applications to achieve sub-ppbv limits of detection with high precision.[13,115,116] Ion separation in the TOF analyzer is based on ion mass and the length of the flight tube as well as the electric charge. In addition to the advantage of high mass resolution, the TOF also acquires multiple spectra within a short timeframe.[117] An example of a GC-TOF-MS with an automated thermal desorption (ATD) front end is shown in Figure 3(B), which includes a variety of GC/MS and LC/MS instruments that can be used for breath research analyses of exhaled breath, EBC, and EBA. The GC/MS instruments shown in Figure 3(A) and (B) can be used to analyze sorbent tubes for exhaled breath analysis.

GC-TOF-MS has been utilized in breath research to obtain data with high resolution and accuracy. A method for EBC sample preparation for metabolomic analysis utilizing liquid-liquid extraction and analysis by GC-TOF-MS has been recently developed by Peralbo-Molina et al.,[118] reporting a high percentage of fatty acids, methyl esters, amides, and volatile prenol lipids in analyzed samples. GC-TOF-MS has been applied to detect differences between disease states in patient samples in order to identify biomarkers. Non-targeted metabolomics analysis of EBC samples from lung cancer patients, smokers, and healthy controls was conducted to identify signatures that discirimate the groups.[119] Breath samples from patients with suspected ventilator- associated pneumonia were compared to identify 12 VOCs that differentiated between the diseased and non-diseased groups.[120] In a study of breath biomarkers of Crohn’s disease, 10 VOCs were found to discriminate between active CD and remission using GC-TOF-MS analysis.[121] GC-TOF-MS analysis has also been utilized to analyze the cellular headspace of eosinophils and neutrophils to identify VOCs associated with inflammation and oxidative stress.[122].

6.1.2 GC×GC-TOF-MS—Two-dimensional gas chromatography coupled with time-of-flight mass spectrometry (GC×GC-TOF-MS) is a useful high resolution multidimensional technology that has increased the number of detectable VOCs in breath samples. Multidimensional GC is advantageous compared to one-dimensional GC because it separates co-eluting VOCs using two capillary GC columns and has increased the coverage of VOCs in the metabolome by an order of magnitude.[16] The two columns utilized typically have different polarities and lengths, with the first longer column containing a nonpolar stationary phase and the second shorter column a more polar stationary phase to increase resolution.[123] GC×GC-TOF-MS has been applied to identify and characterize VOCs from healthy volunteers and individuals with medical conditions. In a recent study, 2000 VOCs were identified in alveolar breath samples from healthy volunteers, some of which had not been previously detected in breath.[124] GC×GC-TOF-MS was utilized to analyze the breath of patients undergoing cardiac surgery to identify biomarkers, drugs, and contaminants.[125] VOCs from children with allergic asthma and healthy controls were also characterized using

GC×GC-TOF-MS to identify six alkanes that were unique to the asthmatic group.[126] Gender-specific VOCs have also been identified in breath samples from healthy volunteers using GC×GC-TOF-MS. In this study, eleven VOCs capable of distinguishing between male and female participants were identified.[127] Additionally, biomarkers of radiation exposure have also been identified using GC×GC-TOF-MS technology,[128] demonstrating the potential of this technique for exposure assessment.

6.2 LC-MS

LC-MS has been commonly used to analyze breath and EBC samples in addition to GC-MS, and several LC-MS instruments that can be used for different breath research analyses are shown in Figure 3(C) and (D). These LC-MS instruments, a triple quadrupole-MS (QqQ-MS) and a Q-ToF-MS/MS, can be used to analyze EBC and EBA samples. While gas-phase breath can be analyzed relatively easily using GC-MS instrumentation, EBC and EBA samples often require extensive preparation procedures due to the low concentration of the components within the matrices, making these more difficult samples to manipulate than exhaled breath.[15] As mentioned previously, EBC and EBA samples can both be obtained in liquid form, and therefore are amenable to LC-MS analyses. The samples can be injected directly into the instrument, or extracted for further analyses.[129] More analytes have the potential to be measured using LC/MS than GC/MS; however, LC/MS data can be more variable due to large retention time drifts.[73] High resolution MS instrumentation, such as Q-ToF-MS/MS or orbitrap-MS/MS, provide the opportunity for non-targeted analyses of breath samples, while QqQ-MS can be utilized to quantitate target compounds of interest.[130]

Orbitrap mass analyzers can achieve high resolution and accurate mass, providing excellent platforms for proteomics and metabolomics studies. In 2005, the linear trap quadrupole (LTQ)-Orbitrap mass spectrometer was first introduced, combining linear ion trap with an Orbitrap mass analyzer for sensitive ion detection, stable mass accuracy, and fragmentation capabilities.[130,131] The Exactive mass spectrometer was released in 2008 as a more compact and affordable benchtop instrument.[131] In this Orbitrap instrument, the ion source is directly linked to the C-trap, which is an external ion storage device.[130] A modification of the Exactive instrument, the Q Exactive, is a quadrupole/Orbitrap hybrid with increased accurate mass MS/MS speed compared to the LTQ Orbitrap. The Q Exactive has been utilized for proteomics and high- throughput screening.[130] Introduced in 2011, the Orbitrap Elite instrument has improved MS/MS acquisition rates and resolving power, which was increased almost 4-fold to 240,000 at m/z 400 compared to versions of the LTQ Orbitrap instrument.[130] The Orbitrap Fusion, a more recent combination instrument, contains a quadrupole, orbitrap and linear ion trap mass analyzer, allowing for simultaneous isolation and detection of ions in different mass analyzers.[131] Orbitrap mass analyzers have been successfully utilized for proteomics analysis of breath samples.

Several studies have been conducted to analyze the protein profiles of EBC samples using high resolution LC-MS. Muccilli et al.[132] pooled EBC samples from nine healthy subjects and separated the proteins by 1D-SDS-PAGE prior to LC-MS/MS analysis of digested proteins using an Orbitrap-Elite mass spectrometer. A total of 163 gene products were identified, of which cytokeratins were found to be the most abundant.[132] Similarly,

Fumagalli et al.[114] assessed pooled EBC samples from non-smokers, healthy smokers, and individuals with COPD and pulmonary emphysema using a linear trap quadrupole (LTQ)-Orbitrap mass spectrometer. Proteomic analysis was utilized to develop a fingerprint of proteins present in each pooled sample, representing a different disease state.[114] EBC proteins have also been investigated as biomarkers for asthma by comparing protein profiles from healthy and diseased children as well as by monitoring levels of asymmetric dimethylarginine, a contributor to asthma pathogenesis by LC-MS.[133,134]

LC-MS and LC-MS/MS have also been utilized to evaluate drugs of abuse in EBA. A recently developed method was capable of analyzing 28 drugs in EBA with limits of quantification between 1 and 66 pg/filter for most substances of abuse.[135] Cocaine and its metabolites have also been quantified in exhaled breath samples using Q Exactive high resolution LC-MS.[136] SensAbues (Huddinge, Sweden) breath samplers were used to collect EBAm from volunteers and individuals using drugs or participating in elimination studies for sports drug testing. Twelve drugs of interest were characterized by LC-MS/MS with detection limits between 5 and 100 pg/filter.[137] Exhaled drugs including amphetamine, metamphetamine, methadone, and THC have all been detected in exhaled breath samples using LC-MS.[129]

6.3 SESI-MS

Secondary electrospray ionization coupled to high resolution mass spectrometry (SESI-MS) is a fast analysis method whereby proton transfer reactions between the electrospray solution and the volatile components in the sample form ions in the gas phase. This technique is advantageous because it does not require sample pretreatment.[138] SESI-MS was employed to study human exposure to benzothiazoles, a common industrial chemical, in exhaled breath, achieving part-per- trillion by volume (pptv) detection limits for this method.[139] SESI-MS has also been applied to detect volatiles indicative of breast cancer by comparing them to breath samples from healthy 520 controls.[140] Fatty acids have also been identified in human breath samples using SESI-MS.[141] SESI-MS applications have also extended to mouse breath for the determination of bacterial lung infections using volatile fingerprints.[142,143,144]

6.4 Direct MS

6.4.1 PTR-MS—Proton transfer reaction mass spectrometry (PTR-MS) is a fast direct MS method capable of real-time analysis with breath-to-breath resolution.[145] An example of a PTR-MS is shown in Figure 4(A), which includes several other Direct MS instruments and some immunoassay instruments. In PTR-MS, H_3O^+ions undergo non-dissociative proton transfer with compounds with a higher protein affinity than water (VOCs) without reacting with components in air, such as nitrogen, oxygen, and carbon dioxide.[145,146] In PTR-MS, pptv detection limits have been achieved.[13, 146] Some PTR-MS instruments contain quadrupole mass analyzers (PTR-QMS). PTR-QMS has been utilized to assess breath levels of VOCs such as ethanol and acetone,[145] as well as VOCs from carbonic alcohols and short chain fatty acids that are indicative of glucose metabolism.[147] Levels of isoflurane fragment ions have been detected in the breath of anaesthetized patients several days and weeks

following surgery using PTR-QMS, showing that the drug is not eliminated as quickly as expected.[148]

PTR-MS has also been combined with TOF technology to increase mass resolving power.[149] While PTR-QMS has a mass resolution of 1, PTR-TOF-MS has a mass resolution between 4000–5000.[13] PTR-TOF-MS has been applied to assess the effect of body position and breath holding during sampling on the concentrations of VOCs in the samples.[150,151] PTR-TOF- MS is also valuable for biomarker detection. A biomarker of kidney malfunction was identified in kidney transplant patients that showed good correlation with serum creatinine.[152] Exhaled breath from mechanically ventilated patients has also been continuously monitored using PTR- TOF-MS, and over 300 mass traces were evaluated. Ammonia, acetone, isoprene, benzene, and sevoflurane are some of the VOCs that were monitored.[153] Exhaled breath samples from Celiac disease patients on a gluten-free diet were analyzed by PTR-TOF-MS in a discovery mode to look for differences from healthy controls.[154] PTR-MS has been applied to analyze urine headspace to identify differences in metabolism caused by strenuous walking.[155] Exhaled breath samples from mice on different diets and animal cell culture headspace have also been monitored by PTR-MS, demonstrating that PTR-MS applications extend beyond human breath analyses.[156,157]

6.4.2 SIFT-MS—Selected ion flow tube mass spectrometry (SIFT)-MS (Figure 4(C)) provides real-time measurements of volatile compounds in breath using chemical ionization of positive (H_3O^+, NO^+, and O_2^+) or negative reagent ions (O^-, OH^-, O_2^-, NO_2^-, and NO_3^-).[158] These precursor ions react with the molecules present in breath, such as VOCs and inorganic gases, but are unreactive with N_2, O_2, and Ar (common components in air).[159] The product ions are then detected by the mass spectrometer. SIFT-MS can detect pptv levels of volatile compounds in breath, making this a sensitive technique for fast analysis.[159] A variation of SIFT-MS, selection ion flow-drift tube-MS (SIFDT-MS), incorporated an electric field in the axis of the flow tube where the ions interact with one another. A significant advantage of this modified technique is that a lower pump speed is needed for SIFDT-MS compared with SIFT-MS, reducing the analysis cost and the size of the instrument.[160]

SIFT-MS has been used to improve medical diagnostics and provide timely test results. SIFT-MS has been operated in full scan mode to create linear models to estimate chronic kidney disease parameters using principal component analysis with intensity screening.[161] Identification of hydrogen cyanide on the breath of cystic fibrosis patients has been deemed a signature of *Pseudomonas aeruginosa* infection in the respiratory tract.[162] Isoprene has been found to be a specific biomarker of advanced fibrosis in chronic liver disease patient samples analyzed by SIFT-MS.[126] SIFT-MS has also been applied to assess the toxicity of aspartame by monitoring one of its breakdown products, methanol, in the exhaled breath of volunteers and to identify VOCs indicative of obesity in children.[163,164] Exhaled breath VOC profiles have also been found to be capable of distinguishing Crohn's disease, ulcerative colitis, and inflammatory bowel disease.[165] SIFDT-MS has been utilized to measure acetone and isoprene in exhaled breath.[160] In addition to exhaled breath applications, SIFT-MS has also been utilized to monitor bacteria VOCs and feces headspace for colorectal cancer-monitoring applications.[166,167,168].

6.4.3 Ion Mobility Spectrometry—Ion mobility spectrometry (IMS) utilizes a multicapillary column (MCC) to generate two- dimensional peak patterns. IMS instruments are compact, transportable, and advantageous for field research and detection of aldehydes and ketones at sub-ppbv concentrations.[13,169] IMS has also been implemented for use in medical devices. The Exhaled Drug Monitor Edmon®, shown in Figure 4B, can be used to monitor propofol concentrations in the exhaled air of patients under anaesthesia or sedated with propofol during medical procedures.[170,171,172] With quick and reliable detection of compounds in breath, IMS has been utilized to distinguish patients with respiratory disease from healthy controls.[173] An IMS method was recently developed that can detect acetone, acetaldehyde, and acetonitrile in human breath at low ppbv to low ppmv levels.[174] MCC-IMS has been applied to make kinetic measurements, and the results have been shown to be complementary to those obtained by PTR-MS, demonstrating the reliability of the technique, especially considering the small size and portability of the instrument.[175] IMS has also been utilized to identify biomarkers in breath samples by comparing the VOCs of diseased patients versus healthy controls for COPD, lung cancer, Alzheimer's disease and Parkinson's disease by determining differences in breath patterns.[176,177,95] Similarly, IMS has been applied to detect differences in the urinary headspace of samples from patients with Celiac disease and irritable bowel syndrome.[178]

7. Detection Methods: Immunoassay Instrumentation

7.1 Traditional ELISA

While traditionally breath research has focused on characterizing volatile compounds, recent applications have been extended to include the analysis of non-volatile compounds, such as cytokine proteins. Previously only available from blood analyses, this new technology is becoming extremely valuable for assessing inflammatory response directly in the lung.[179,180] EBC and EBA are both utilized as breath media for cytokine analysis. The traditional ELISA, which utilizes a solid-phase immunoassay to detect the presence of an antigen in a liquid sample, was first developed by Eva Engvall and Peter Perlmann in 1971.[181] ELISA has been traditionally applied to cytokine analyses in EBC applications. ELISA has been utilized to measure cytokines in EBC samples of asthma patients to assess correlations between interleukin levels and inflammation.[182,183,184] Cytokine concentrations in EBC have been reported in the ng range, while concentrations in bronchoalveolar lavage (BAL) and sputum have been found in the pg range.[184] ELISA has also been utilized to detect levels of the pro-inflammatory cytokines interleukin (IL)- 6, tumor necrosis factor α (TNF-α), IL-1β, and IL-8 in EBC of children with inflammatory bowel disease.[185] Cytokine levels have also been analyzed in EBC samples of patients with sarcoidosis, allergic rhinitis, hereditary 1,25-dihydroxyvitamin D-resistant rickets, and individuals with tetraplegia to assess inflammatory responses.[186,187,188,182]

7.2 Mesoscale Discovery (MSD)

MSD SECTOR instruments are sensitive electrochemiluminescence plate readers that can be utilized for immunoassay applications (shown in Figure 4(D)). The MSD SECTOR PR 400 and 100 operate using photodiode array technology, while the SECTOR S 600 and QuickPlex SQ 120 utilize charge coupled device (CCD) cameras, enhancing the capacity for

multiplexing. Most MSD instruments can read plates in 1.5–3 min, allowing for high throughput analyses. Additionally, the simplicity of the design lends itself to minimal requirements for routine maintenance, and the instrument does not require calibration. MSD offers a variety of human cytokine and chemokine multiplex assays, including several 4-plex, 7-plex, and 10-plex kits. An advantage of MSD is the small amount of sample volume needed for analysis; the 384-well plates only require 10 μL, while the 96-well plates need 25 μL of sample for analysis (www.mesoscale.com). The sensitivity level for cytokines in EBC samples has been reported in the 0.05–0.10 pg mL^{-1} range, and between-plate variance has been attributed to random error.[179] A comparison of Th1/Th2 cytokine levels in blood plasma, EBC, and urine samples using MSD showed that the cytokine levels among the three different biological media did not share the same pattern, which may be due to biological differences in the location of the three media.[179] Cytokine assays have also been employed in environmental exposure applications to assess ultrafine particulate matter exposure in school children, and cytokine levels in EBC samples in subjects exposed to diesel exhaust, ozone, and a mixture of diesel and ozone in order to elucidate the effects of multi-pollutant exposures.[189,190,180]

7.3 Luminex

Another alternative to traditional ELISA, Luminex offers bead-based multiplexing xMAP technology with color-coded beads that are read in compact laser or LED-based analyzers to determine individual microsphere interactions. Luminex has a throughput of 1000 samples a day and can multiplex 1 to 500 tests per sample (www.luminexcorp.com). Luminex multiplex assays have been utilized to measure cytokines in EBC of preschool children with asthma and patients with non-small cell lung cancer.[191,192] A Luminex 21-plex assay plate was also used to quantify cytokines in BAL and sputum samples from children with severe therapy-resistant asthma (STRA) to determine if airway inflammation is caused by T helper 2 (TH2) cytokines. However, the cytokine levels in patients with STRA and controls were comparable, indicating that cytokines were not playing a significant role in inflammation.[193]

7.4 Cytometric Bead Array (CBA)

CBA is an emerging immunoassay technique for analyzing cytokines, chemokines, growth factors, and phosphorylated cell signaling proteins. CBA is a flow cytometry technique that utilizes antibody-coated beads to capture analytes and has a reduced analysis time and sample volume requirements compared with traditional ELISA. CBA can be used to analyze up to 30 proteins at the same time using only 25 to 30 μL of sample with detection limits as low as 0.27 pg mL^{-1} in multiplex assays (https://www.bdbiosciences.com/sg/research/cytometricbeadarray/). While CBA can multiplex more proteins than MSD, the detection limit of MSD is lower (0.05–0.10 pg mL^{-1}). CBA has been used for several different applications, including measuring cytokine levels in EBC and BAL fluid samples from mechanically ventilated patients.[194,195]

7.5 Quanterix Single Molecule Array (Simoa) HD-1 Analyzer

The Quanterix Simoa HD-1 Analyzer is an automated robotic immunoassay platform that operates by capturing single protein molecules on magnetic beads and labeling them with standard ELISA reagents (see Figure 4(E)). The beads are combined with the enzyme

substrate, loaded into wells, and sealed with oil. The fluorophores are digitally counted, and the percentage of beads with immunocomplexes is determined using a Poisson distribution (www.quanterix.com). This technique has achieved single-molecule resolution at sub-femtomolar concentrations with as much as 200- to 1000-times as much sensitivity as current multiplex immunoassays.[196,197] While the Simoa HD-1 Analyzer has been previously demonstrated for cytokine analysis in blood plasma and serum samples, the technique was recently utilized to analyze cytokines in EBC samples, which is challenging due to the dilute nature of the medium.[196,197,198,199] An advantage of the Simoa HD-1 Analyzer is that the system is fully automated, eliminating inconsistencies and errors caused by pipetting and sample handling. However, because the instrument requires a minimum sample volume of 100 μL and a sample dead volume of 50 μL, automating the Simoa HD-1 Analyzer requires more sample volume than for manual sample preparation. In a previous study, sample dilution was required prior to analysis, and researchers found that EBC samples should be diluted with buffer, as diluting with water caused signal suppression issues due to clumping of the beads.[199] At the time these experiments were performed, no multiplexed cytokine kits were available from Quanterix, increasing the need for EBC sample dilution in order to analyze more cytokines using the individual assay kits.

8. Applications to Human Health and Disease

8.1 Alcohol Dependence

One traditional application of breath research with which the general public is familiar with is breath testing for blood alcohol levels. Alcohol dependence and abuse remains a major disease that affects 208 million people worldwide.[200] Many individuals who suffer from alcohol dependence struggle to control the amount of alcohol that they consume and often end up driving and performing other tasks while impaired. The use of alcohol breath tests allows law enforcement to identify individuals who have consumed levels of alcohol that are unsafe for driving and operating heavy machinery.

Dr. R. N. Harger developed the first breath alcohol test in 1938, named “The Drunkometer”.[17] The portable breath testing device that most people are more familiar with, the “Breathalyzer”, was developed in 1954 by Robert Borkenstein.[18] While breath applications involving alcohol date back all the way to the 1930s, only a handful of publications were in existence at this time. More breath alcohol applications started appearing in the 1960s-1970s and gained popularity in the 1980s (Figure 5). Publications in the 1960s included basic assessments of breath alcohol levels, with a focus on the development of novel measurement devices for assessing drunk drivers.[201-202] Alcohol breath tests also extended to the clinic in the 1970s to determine the effects of alcoholism on hepatic function and to evaluate the absorption of a tuberculosis drug.[203-204] In 1982, an important publication monitored exhaled breath alcohol concentrations and temperatures to show that ethanol dissolves in mouth membranes and equilibrates with breath.[205] New applications have been steadily developed over the years, with a spike in publications from 2013–2015 (see Figure 5). A significant focus of alcohol-related breath research still revolves around reducing instances of drunk driving, although the focus has shifted away from the development of novel breath

tests toward large-scale surveys of the effects of alcohol consumption using previously established breathalyzers.[206,207]

8.2 Lung Cancer

Another familiar application of breath testing is lung cancer research, which has steadily gained momentum over the past 20 years. Lung cancer research has made headway in both the clinic and the research laboratory, as both patient samples have been investigated and new assays have been developed to detect lung cancer using breath biomarkers. Lung cancer has the highest death rate among cancers,[208] with non-small cell lung cancer (NSCLC) being particularly deadly due to the late stage at which it is typically detected.[209] This makes the search for a simple and accurate diagnostic test for early stage lung cancer a worthy cause.[209] Breath testing for lung cancer is attractive because it is noninvasive, and the breath provides a direct link to the current state of the lungs.

While a majority of the lung cancer research has revolved around exhaled breath analysis, EBC has become a more popular breath medium for early lung cancer testing to measure proteins linked to pathogenesis.[210] In 2002, EBC from NSCLC patients was analyzed to find that interleukin-6 (IL-6) levels were increased compared to healthy controls, indicating that IL-6 could serve as a potential biomarker for early cancer detection.[211] In 2004, increased endothelin- 1 peptide concentrations and mutations in the p53 gene were detected in DNA extracted from the EBC of patients with NSCLC, which provided additional indicators of disease state.[212-213] Canine olfaction has also been utilized to detect biomarkers of lung cancer in EBC and distinguish cancerous and healthy samples.[214,215] These studies have indicated that there are likely only low concentrations of biomarkers in EBC, presenting a challenge for developing a reproducible detection method.[215]

After 2004, the number of lung cancer publications in breath research began to increase rapidly. In addition to EBC analyses, studies were conducted using exhaled breath to compare the VOC profiles of healthy individuals and patients with NSCLC to look for potential biomarkers of disease state.[216,217,218] Wang et al. compared the VOC profiles of breath from non-small cell carcinoma patients before and after tumor resection to healthy controls, finding caprolactam and propanoic acid as potential biomarkers.[216] Poli et al. compared VOCs from NSCLC patients, smokers, non-smokers and patients with COPD. While no individual VOC discriminated the groups, the patterns of the 13 VOCs studies allowed for correct classification of disease state.[217] Fu et al. reported that 2-butanone levels were much higher in the breath of NSCLC patients with stages II-IV compared to stage I.[218] 2-Butanone along with 1-propanol have been identified as a the best discriminators of lung cancer amongst all identified biomarkers.[19] Oral glucose tolerance tests (OGTT) have been performed on LC patients with adenocarcinoma, squamous, small cell carcinoma, and controls, with exhaled breath samples taken before and after testing to obtain the volatile signatures of each disease state. OGTT was found to have little effect on LC VOC signatures, but a significant effect on the VOC patterns of the control group because the cancerous group already exists in a hyperglycolytic state.[219].Extensive lists of VOC breath biomarkers of lung cancer can be found in Saalberg et al.[19] and Dent et al.[214] Additional information about lung cancer biomarkers is available in the Literature [20,19].

Advances in diagnostic technology have also improved lung cancer detection. GC/MS and sensor technologies, including electronic noses and colorimetric sensors, have all been widely used approaches.[208] Gas sensors based on gold nanoparticles have been developed for lung cancer detection,[220,221,214] which are capable of distinguishing the breath of lung cancer patients from negative controls with 100% accuracy.[221] The Cyranose 320, another sensor, has been used to distinguish the VOCs from lung cancer and controls with 90% accuracy.[214,222] More recently, lung cancer research has extended to the evaluation of "cell breath" in addition to human exhaled air, in which volatiles from tumors and cell lines have been studied in an attemptto better understand their contribution to disease state.[223,224,225,226]

8.3 Asthma

Asthma is one of the most prevalent human pulmonary diseases manifested as periodic inflammatory events resulting in swelling of the bronchioles that cause severe restrictions in breathing. This disease has a range of severity and is characterized by wheezing, coughing, and shortness of breath.[9] As a disease of the lung, exhaled breath is considered the most proximate bio-fluid for diagnosing and monitoring asthma. Many biomarkers of asthma have been identified. Exhaled nitric oxide (NO) can be used to identify patients with asthma, as asthmatics end to have elevated NO levels due to reduced airways.[227] Hydrogen peroxide (H_2O_2) is also a classic breath biomarker of asthma that has been discovered in increased levels in the EBC of patients with asthma compared to healthy subjects.[20] More specific biomarkers have also been discovered, including urates as biomarkers of airway inflammation in children.[228] Additionally, 8-iso-prostaglandin E2 has been detected as a biomarker of aspirin hypersensitivity in sensitive asthmatics,[229] and leukotrienes have been used to monitor airway inflammation and determine pharmacological treatment.[230,231] E-noses have also been utilized to detect VOCs from patients with asthma. Classification accuracies of 87–87.5% have been achieved for asthmatics versus healthy controls using this approach.[27,232,92]

Analyses of particulate matter (PM) from EBA and EBC have also been significant for asthma research, as PM often exacerbates asthma symptoms. For example, EBA from individuals with asthma who were exposed to birch pollen showed a reduced number of exhaled particles due to inflammation.[51] EBC analysis in children exposed to ultrafine particulate matter (UFP) showed an increase in particle content with worsening respiratory symptoms and decreased lung function during exercise due to lower nitrate levels in EBC.[50,233] Exposure to diesel exhaust particles has also been shown to increase airway inflammation in individuals with asthma.[234,235] For extensive reviews of breath analysis for asthma research see Zhou et al.[21] and Snell et al.[22]

8.4 COPD

COPD is a progressive respiratory disease that includes both bronchitis and emphysema,[9] and is currently the fifth highest cause of death worldwide.[23] COPD is characterized by coughing, wheezing, excess mucous production, shortness of breath and airway inflammation.[9] The highest contributing factor for developing COPD is smoking, although environmental exposure to chemicals and air pollutants, childhood respiratory infections,

and genetic factors, such as alpha-1 antitrypsin deficiency have also been found to play a role in developing the disease.[236] Case-control studies that look for differences in chemicals, proteins, pH, and other properties in EBC and EBA samples from healthy individuals, patients with COPD, and sometimes smokers without COPD have been performed in order to identify biomarkers of COPD.[237,238,176,239] In one study, breath samples were collected from non-smokers, COPD ex-smokers, smokers without COPD, and smokers with COPD and analyzed by TD-GC-MS to identify 14 COPD-related VOCs, including butanone, toluene, phenol, and *m,p*-cresol. 89.4% of the COPD patients were correctly classified in the non/ex-smoking group, and 82.6% were classified correctly in the actively smoking group.[239] In a GC-atmospheric pressure chemical ionization (APCI)-MS study, 2-pentanone was identified as a VOC biomarker that showed differences between COPD patients and healthy controls [240], although this VOC was not deemed a COPD-related biomarker in the previous study.[239]

In contrast to GC-MS, e-noses have also been applied to COPD research. For COPD patients, detection of viral versus bacterial infections is critical for proper diagnosis and medical treatment. Van Geffen et al. demonstrated that an Aeonose e-nose could discriminate between COPD patients with viral infections versus controls (sensitivity 83%) and COPD patients with bacterial infections versus controls (sensitivity 73%).[241] A Cyranose 320 e-nose was recently used to distinguish the breathrpints of COPD from those of obstructive sleep apnea (OSA) alone and overlap syndrome, which is a combined syndrome in which OSA is associated with COPD. While the OSA patient breathprints were distinct from those of OVS and COPD, the breathprints from OVS patients were similar to those of COPD patients, indicating that the technique could be utilized to distinguish OSA from COPD.[242] Similarly, another study sought to differentiate the VOC patterns of COPD from those of NSCLC patients and healthy controls using the Cyranose 320. With a cross-validated correct classification rate of 85%, COPD breathprints were differentiated from those of NSCLC.[27,222]

Non-volatile compounds have also been utilized as biomarkers of COPD. C-reactive protein (CRP) levels have been identified as a biomarker capable of predicting risk of death and hospitalization for COPD patients.[22] COPD breath research has included the analysis of cytokines in EBC samples as a measure of airway inflammation, a common symptom of the disease.[243,244,245] Using an EcoScreen device, Ko et al.[244] collected EBC from patients with acute exacerbations of COPD (AECOPD) and monitored levels of TNF-α while patients received steroids and antibiotic treatment, showing that cytokines could be used to non-invasively monitor the severity of the disease. Eosinophils, which are markers of airway inflammation, have been detected in higher levels in COPD patients,[243] as have leukotriene B4 and prostaglandin E2.[245] Airway obstruction in COPD has also been evaluated by analyzing the number and distribution of exhaled particles.[53] Reviews of COPD research can be found in Snell et al.,[22] Cazzola et al.[24] and Christiansen et al.[23]

8.5 Biomarkers of Environmental Exposure

Volatile organic compounds (VOCs) represent a major environmental exposure issue for the human population. VOCs in air have myriad sources including consumer products,

combustion of fossil fuels, water disinfection, and a variety of industrial and manufacturing processes.[246,247,248,249]. Airborne chemicals in the environment such as benzene, toluene, ethylbenzene, xylene,[246] ethyl acetate, propylene glycol monomethyl ether acetate, and inorganic acids [249] impact human health primarily through the inhalation pathway, and as such, are also present during the exhalation cycle. In fact, exhaled breath provides a direct window into blood-borne VOCs through the gas exchange mechanisms of the alveoli.[31–29] Advantages of using breath to assess VOC exposures (in contrast to blood or urine) are that breath can be monitored in any time frame (minutes or hours) or even continuously without reaching a sampling limit. This has provided insight into the classical pharmacokinetics of VOCs exposure with respect to absorption, distribution, metabolism, and excretion (ADME), which are of critical importance in determining acquired dose and ultimate health risk.

8.6 Disease Detection using EBA Patterns

Exhaled aerosol patterns can be utilized to identify fingerprints and signatures of conditions and diseases. Computational fluid dynamics approaches with fractal analyses have been applied to distinguish between aerosol fingerprints of normal, tracheal tumor, bronchial tumor, and different levels of asthma severity in lung models.[250,251] In these physiological-based models, exhaled particles form unique patterns on filters. The fractal, or repetitive, properties of these images have been used to identify disease location and severity.[250] Asthma models created using this technique have been shown to have 100% diagnosis accuracy under perfect conditions, and 99.1% accuracy under more realistic sampling conditions.[251] Fingerprints of bacterial, viral, and healthy individuals can also be distinguished from one another using exhaled breath and volatile profiles. Differences in infected and uninfected fingerprints of bacteria strains have been demonstrated in a mouse model, and methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-sensitive *S. aureus* (MSSA) breathprints were investigated to distinguish features using principal component analysis (PCA).[252] Intensity differences in the shared dominant peaks and the presence of several low abundance peaks were found to be important for distinguishing MRSA and MSSA.[253]

9.0 Emerging Topics in Breath Research

9.1 Security and Airport Surveillance

Breath research has several unique applications toward security and airport surveillance implementations. During disaster scenarios, such as earthquakes, tornadoes, fires, or explosions, individuals may become entrapped inside collapsed buildings. Portable sensors and devices can be utilized to detect VOCs emitted from human breath, sweat, skin, and biological fluids in order to locate lost individuals who may be injured or nonresponsive.[254] Acetone, isoprene, dimethylsulfide, dimethyldisulfide, and trimethylamine have been proposed as target VOCs that could be used to detect individuals within collapsed buildings. These VOCs would be advantageous for detection from exhaled breath, blood, urine, or sweat, all of which would be emitted from entrapped individuals.[255]

Portable sensors also have applications for border security and airport surveillance. Aportable quadrupole mass spectrometer with a heated membrane probe has been utilized to

detect chemical signatures of volunteers in a container simulator. VOCs, ammonia, carbon dioxide, and water were measured from exhaled breath, sweat, and skin excretions. These compounds were all found to be useful probes for discovering humans within confined spaces. Therefore, this portable mass spectrometer could be used to detect humans hiding in boxes or suitcases to increase border control and decrease instances of illegal immigration.[256,257] A method for multiplex surface enhanced Raman scattering (SERS) has recently been developed that has the potential to be utilized in a portable sensor device to detect VOCs, such as acetone and ethanol, emitted from human breath.[258] SERS operates using metallic nanostructures that create an electromagnetic field at surface plasmon hot-spots for single molecule detection.[258] These portable devices could be used in airport security and border control to detect humans hiding in boxes or suitcases to improve safety regulations and decrease instances of illegal immigration.[255,258 ,256,257]

Detection and identification of drugs and other harmful substances could also be accomplished using portable sensors for security applications. The portable membrane inlet mass spectrometer was tested for the ability to detect narcotics, explosives, and chemical warfare agents. Methyl benzoate, a signature of cocaine, and piperidine were successfully detected using the portable spectrometer, indicating that the device can be utilized to determine if individuals have handled or consumed drugs. A breakdown product of trinitrotoluene (TNT), 2-nitrotoluene, and a chemical used in plastic explosives, cyclohexanone were also identified in addition to chemicals used to produce sarin and soman nerve agents and sulfur mustard gas. Low ppbv LODs were determined for each of these compounds.[257] This spectrometer could be utilized as a fast, non-invasive technique to screen for illegal and harmful substances in airports as well as in law enforcement applications. Hand-held screening devices can also be used in law enforcement to monitor substance levels. A new breath alcohol analyzer has been developed that does not require contact with the subject. The device uses carbon dioxide as a biomarker and has been shown to meet industrial standards.[259] This non-invasive breath sampler can be used for law enforcement applications to measure breath alcohol levels and determine levels of consumption to enforce alcohol safety laws.

9.2 Cellular Respiration and Microbiome

Breath VOCs have been frequently used to detect alterations in cellular respiration. While VOC profiles of breath have been extensively investigated, research has recently expanded to include biological and cellular headspace as a useful model of exhaled breath. VOC profiles of breath, saliva, milk, blood, urine, skin secretions, and feces have all been compiled, and clear differences between compound abundance in certain biological media were noted.[260,261] VOCs emitted from breath and biological media can be important indicators of health state, as they may be produced by adverse outcome pathways (AOPs) within the body. An AOP is a model consisting of biological events that result in an adverse effect and can be used to assess exposure risk.[262] Cellular models can be used for *in vitro* systems to identify VOCs that are altered by the presence of external stressors.[263] Headspace samples of four bacteria were analyzed, demonstrating that the microorganisms could be identified based on their unique VOC profiles. Applications such as this show potential for non-invasive breath tests for diagnosis of bacterial infections.[264]

Breath VOCs have also been found to change based on diet and disease onset. Adhering to a gluten-free versus a normal diet for four weeks altered the volatile profiles excreted in breath, suggesting that the gluten-free diet reversibly altered metabolism.[265] The effect of diet on breath VOC profiles was also investigated by feeding mice low and high fat diets, which showed differences in exhaled VOC levels that may be linked to metabolic functions.[266] Exhaled VOCs have also been linked to cancer, and VOCs have been studied for indications of viral infections and pneumonia in mechanically ventilated patients.[267,268,269] Identifying VOCs that are produced by bacteria or fungi, such as hydrogen cyanide, which is produced by *Pseudomonas aeruginosa*, in breath samples can indicate the presence of an infection.[270]

Liver function can also be assessed using breath biomarkers by monitoring data for probe molecules. Probe molecules are exogenous compounds with known kinetic data that can be introduced to an *in vitro* or *in vivo* system to produce an expected metabolite. An example probe molecule is methyl tertiary butyl ether (MTBE), which is metabolized to tertiary butyl alcohol (TBA) by the CYP 2A6 pathway in the liver.[262] Sevoflurane (SEV), an anesthetic used during surgery, has also been demonstrated as a probe molecule for liver function. SEV is metabolized to hexafluoroisopropanol (HFIP), which is eliminated in exhaled breath.[271] Potentially liver toxic compounds of interest can be added to cellular *in vitro* systems in the presence of MTBE or SEV to see if production of TBA or HFIP, respectively, is disrupted by the presence of the test compound.[262,271]

9.3 Canine Olfaction

An interesting application of breath research that has often baffled researchers is the use of canine olfaction for pre-clinical cancer and disease detection. Some dogs can tell the difference between breath samples from healthy and diseased patients, aiding in sample discrimination and diagnosis (Figure 6). The reason for this ability is attributed to a dog's enhanced sense of smell.[272] Dogs have been recently used to differentiate lung cancer patient samples from healthy controls in several different studies.[273,274,275] However, dogs are not perfect diagnosticians, and often generate random results, presumably due to boredom, distraction, hunger, fatigue, or a lack of sufficient training.[274,272] While using dogs as breath samplers for cancer and disease detection is certainly not optimized at this point in time, canine olfaction has promise as a future tool for breath research diagnostics because it is affordable, relatively simple, and does not require complex instrumentation, in contrast to some of the other methods currently available. However, identifying the chemicals that dogs identify as cancerous or attributed to a disease-state would be advantageous for understanding the biological processes undergoing diseases as well as for developing instruments capable of detecting the same compounds. The current state of thought is that dogs must be detecting compounds in EBA, as the VOCs in exhaled air would quickly dissipate within the environment.[276] For an extensive review of canine olfaction for breath research, see Pirrone et al.[277]

9.4 Recent Applications and Future Directions

Several other interesting and unique breath applications have emerged in the last several years. These applications hold promise for future development to extend breath research beyond its current scope. Additional research areas for future development include:

- • Nose breath
- • Organ-on-chip
- • Crowd breath

Pure nose breath has been utilized for breath sampling instead of mouth breath to avoid the contribution of mouth bacteria to the analysis. This is not to be confused with e-nose measurements, which use mouth breath to mimic the sensory functions of the nose.[278] Nasal breath has been typically utilized to measure NO in clinical settings and is a sign of airway inflammation leading to asthma.[279,280] Nose breath can be used in situations where sample purity is critical and contamination is a concern due to illness or other factors.

The development of lung-on-chip models have also revolutionized drug discovery, toxicity testing, and evaluation of disease states. Prototypes of human lungs using the lung's air sac linings and blood vessels on flexible membranes have been designed to mimic breathing at the cellular level.[281,282] These lung-on-chips have been used to investigate pulmonary edema, inflammatory disorders, asthma, and lung cancer drugs, among others.[283,284,285] These devices also have the potential to replace animals as more predictable models of human breath.[281]

The analysis of crowd breath as opposed to individual sampling is a future direction of the field of breath research. In crowd breath sampling, the air surrounding large groups of people is measured using a direct-MS approach (e.g., PTR-MS, IMS, or SIFT-MS) as opposed to taking separate breath samples from each individual within the room. Common breath biomarkers, such as carbon dioxide or acetone can be measured in group settings to evaluate trends over time. Changes in the steady state levels of these volatiles can indicate that a chemical exposure has occurred or that a threat has been introduced. Crowd breath technology would be particularly advantageous in national security settings, airports, or for drug and toxicity screening. While it would be unnecessary and time consuming to test each individual separately in such scenarios, a baseline for the crowd breath room air could be established, and individual sampling could be conducted only if a change in the VOC profile was detected that could be indicative of a threat.[286]

10. Conclusion

In the last 20 years, breath research has seen significant improvements in sampling techniques, available instrumentation for analysis of different sample media, and the wide range of applications of breath applications, including investigations of disease state, exposure assessment, environmental research, impairment, and security. While breath research may have started off with a narrow range of relevant applications, such as alcohol breath testing, lung cancer research, and the detection of basic compounds in breath, the field has grown to include EBC and EBA in addition to exhaled breath, widening the range

of relevant applications. The future for breath research is promising, with an increase in health applications, including organ-on-chip and canine olfaction on the horizon. EBA analysis is also likely to be pursued in the future, with an increase in instruments and techniques capable of analyzing these types of samples currently underway. In the midst of all of these advances, one challenge for breath research going forward is the implementation of consistent protocols and standards across research laboratories.

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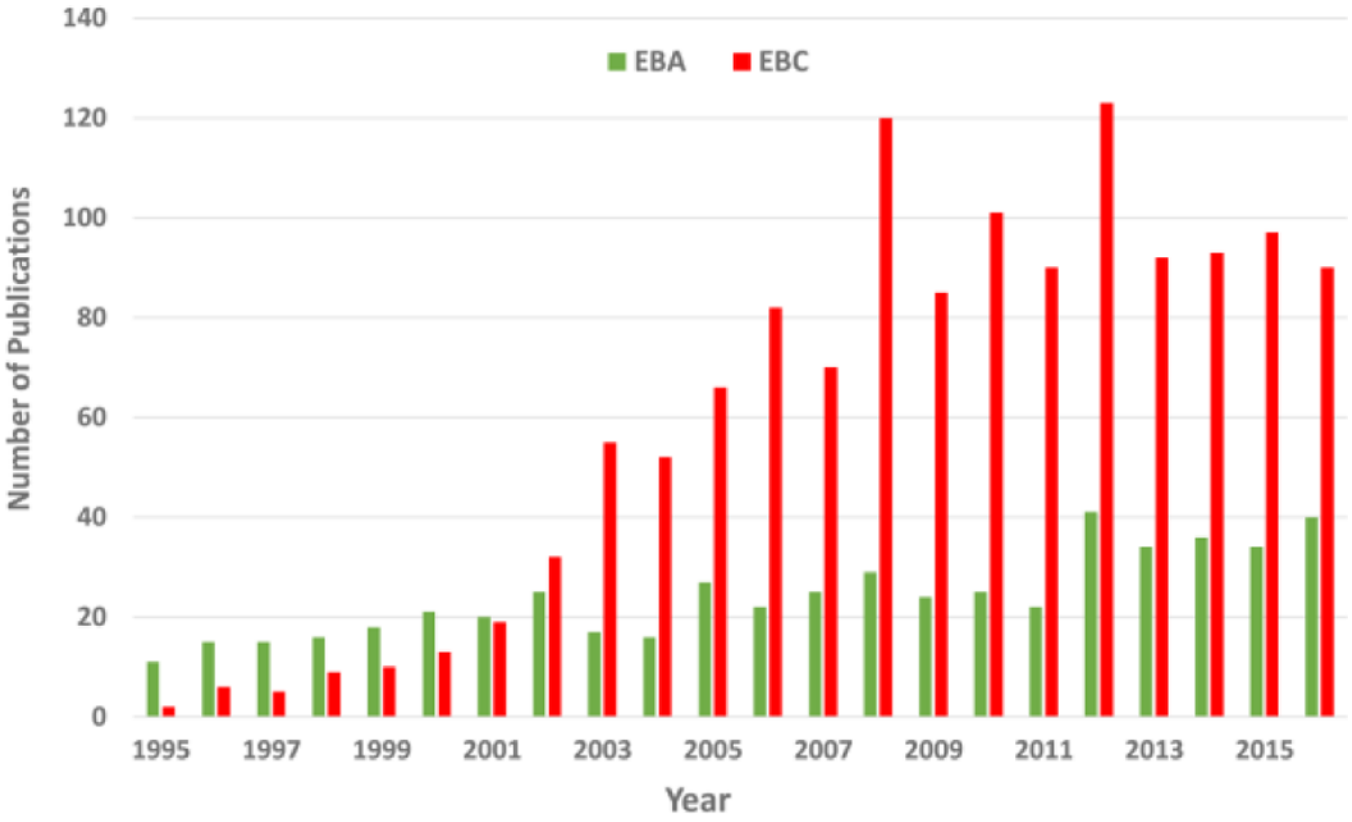

Figure 1:

Comparison of EBC and EBA publication trends

Publications featuring EBA and EBC media have increased over the past 20 years, although EBC publication rates have accelerated past those of EBA, with more than double the number of EBC publications than EBA from 2003–2016.

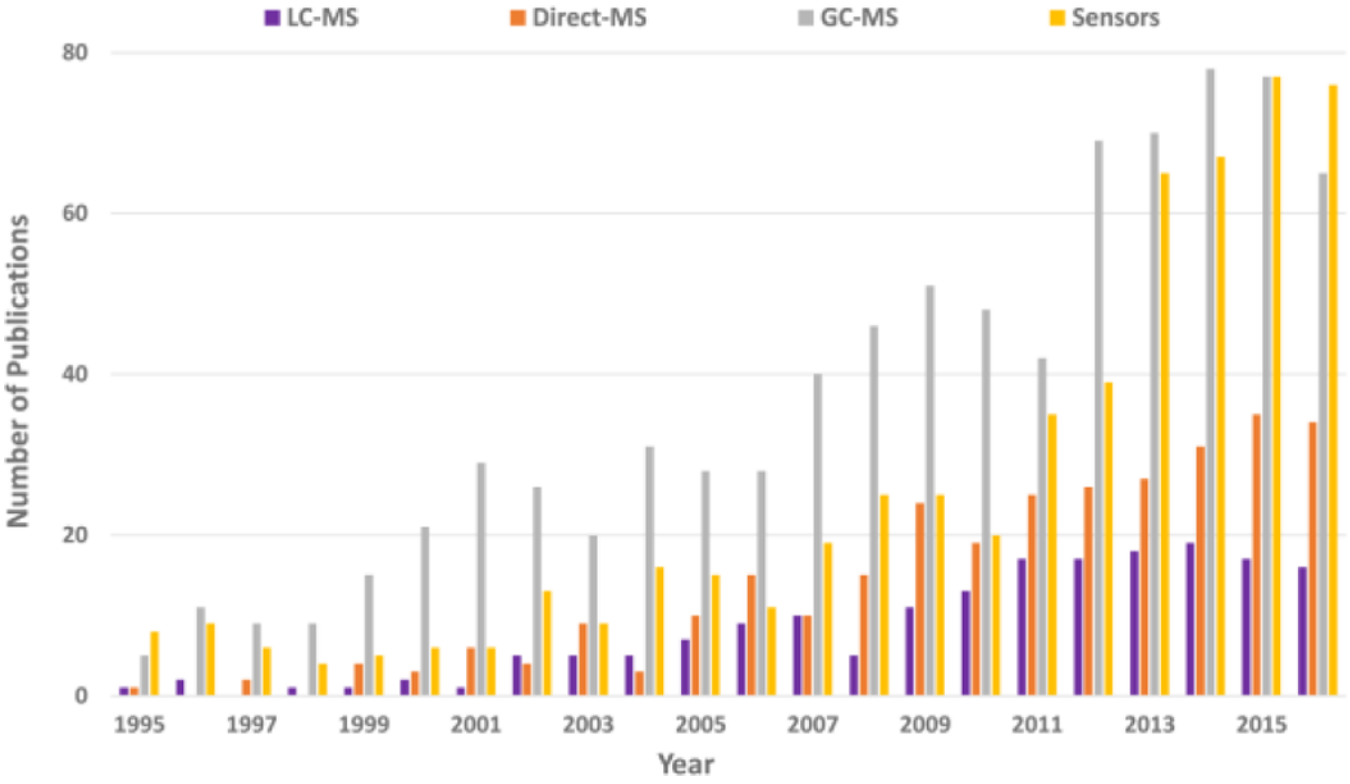

Figure 2:

Mass spectrometry publication comparisons

This chart compares the number of publications for LC-MS, Direct-MS, GC-MS and sensors over the past 20 years. All publications in breath research show an upward trend, with most publications reporting breath research results utilizing GC-MS and sensors in recent years.

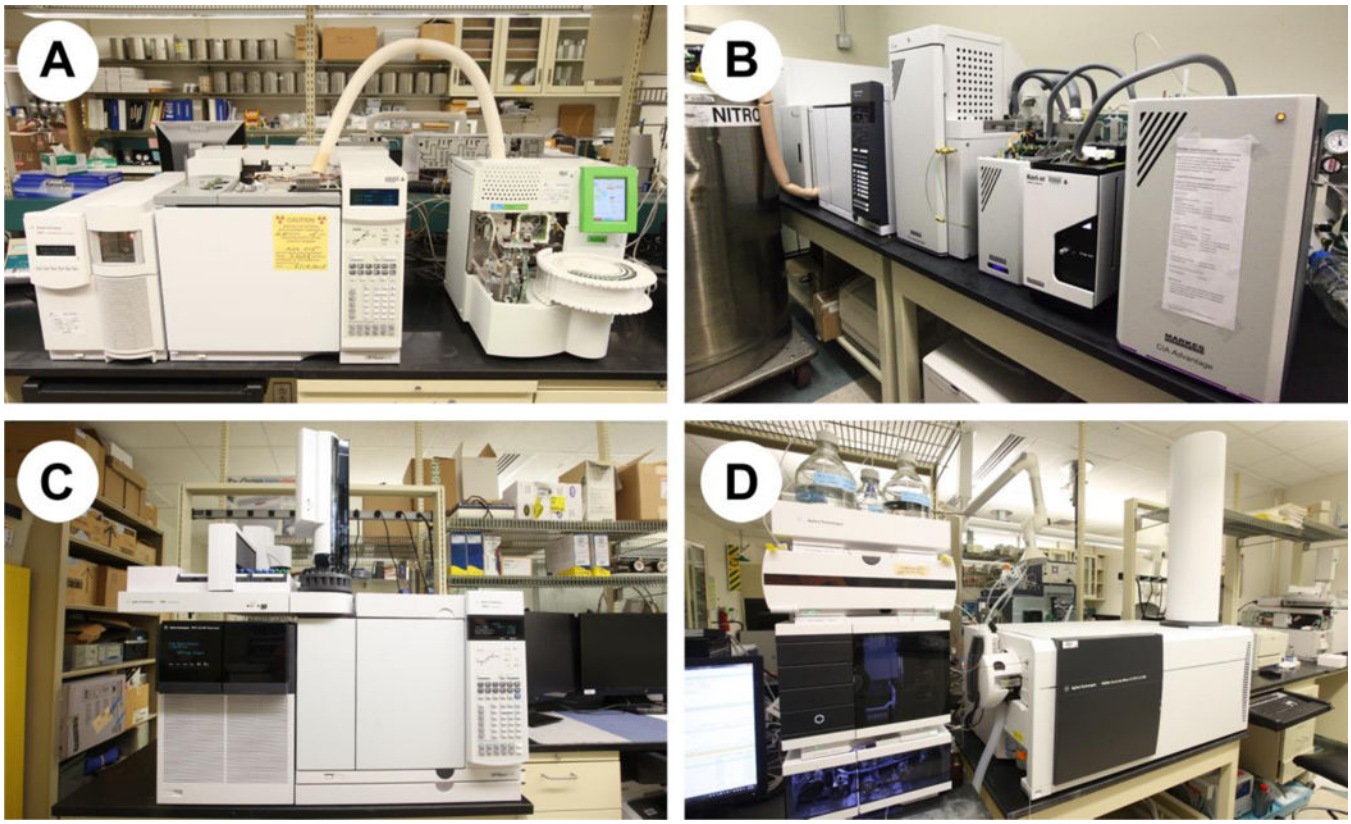

Figure 3:

High-throughput GC-MS and LC-MS instrumentation for analysis of breath matrices A) ATD-GC/MS; B) ATD-GC-TOF/MS; C) QqQ/MS; D) Q-TOF/MS. Instruments A and B have been utilized to analyze sorbent tubes to assess VOC levels in exhaled breath samples, while C and D have been used to analyze liquid filter extractions to detect EBA collected on different types of breathing masks. Photo credits: Chuck Gaul (U.S. EPA).

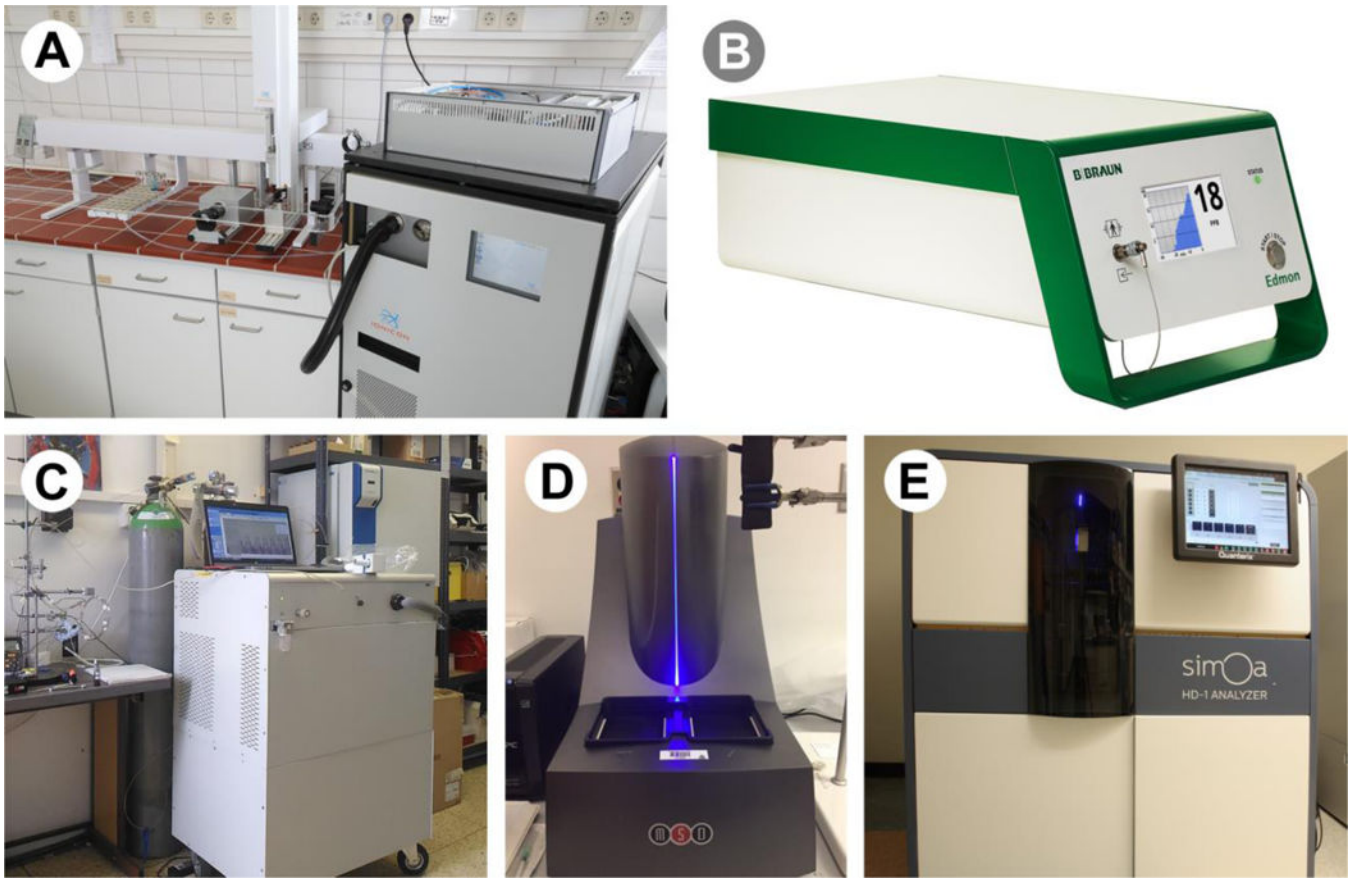

Figure 4:

Direct-MS and immunoassay instrumentation utilized in breath research

A) PTR-MS; B) IMS; C) SIFT-MS; D) MSD; E) SIMOA HD-1 Analyzer. These instruments can be used for fast analysis of breath samples to analyze VOCs (A, B, and C) and cytokines (D and E). Photo credits: A) Dr. Jonathan Beauchamp; B) Dr. Jorg Ingo Baumbach; C) Dr. Patrik Spanel; D) Brett Winters; E) Chuck Gaul (U.S. EPA).

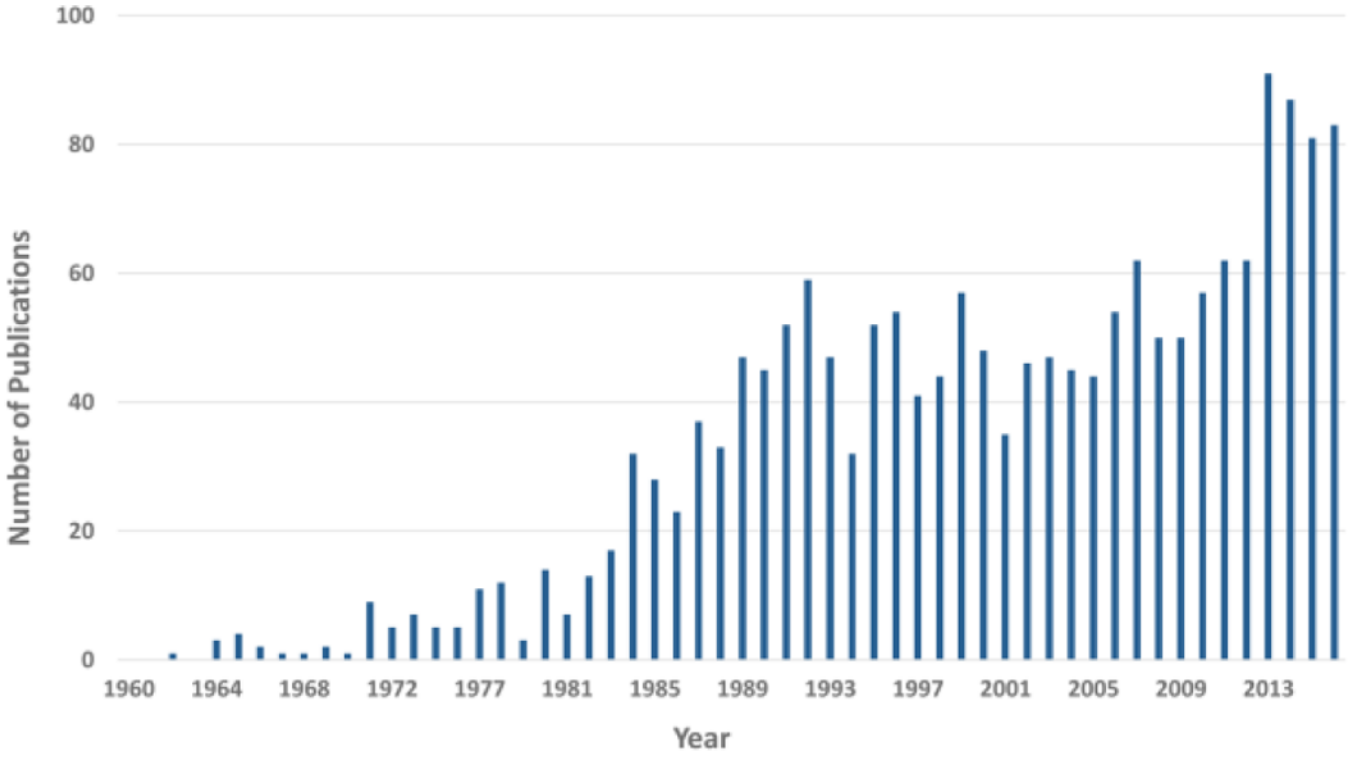

Figure 5:

Trends in breath alcohol research publications

This figure shows the number of papers published from 1960–2016 that discuss breath analysis of alcohol or ethanol. In general, publications have increased over time, with a significant spike in alcohol-related breath publications from 2012–2016.

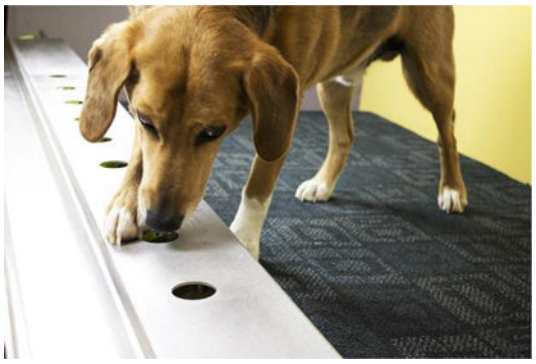

Figure 6:

Canine olfaction in breath sample classification

Canine sniffing of biological samples, including breath, has become a viable method for cancer and disease detection. Dogs are trained to differentiate samples from diseased versus healthy individuals based on scent, providing a potential method for early stage disease diagnosis. Photo credit: Glenn Ferguson from Cancerdogs.