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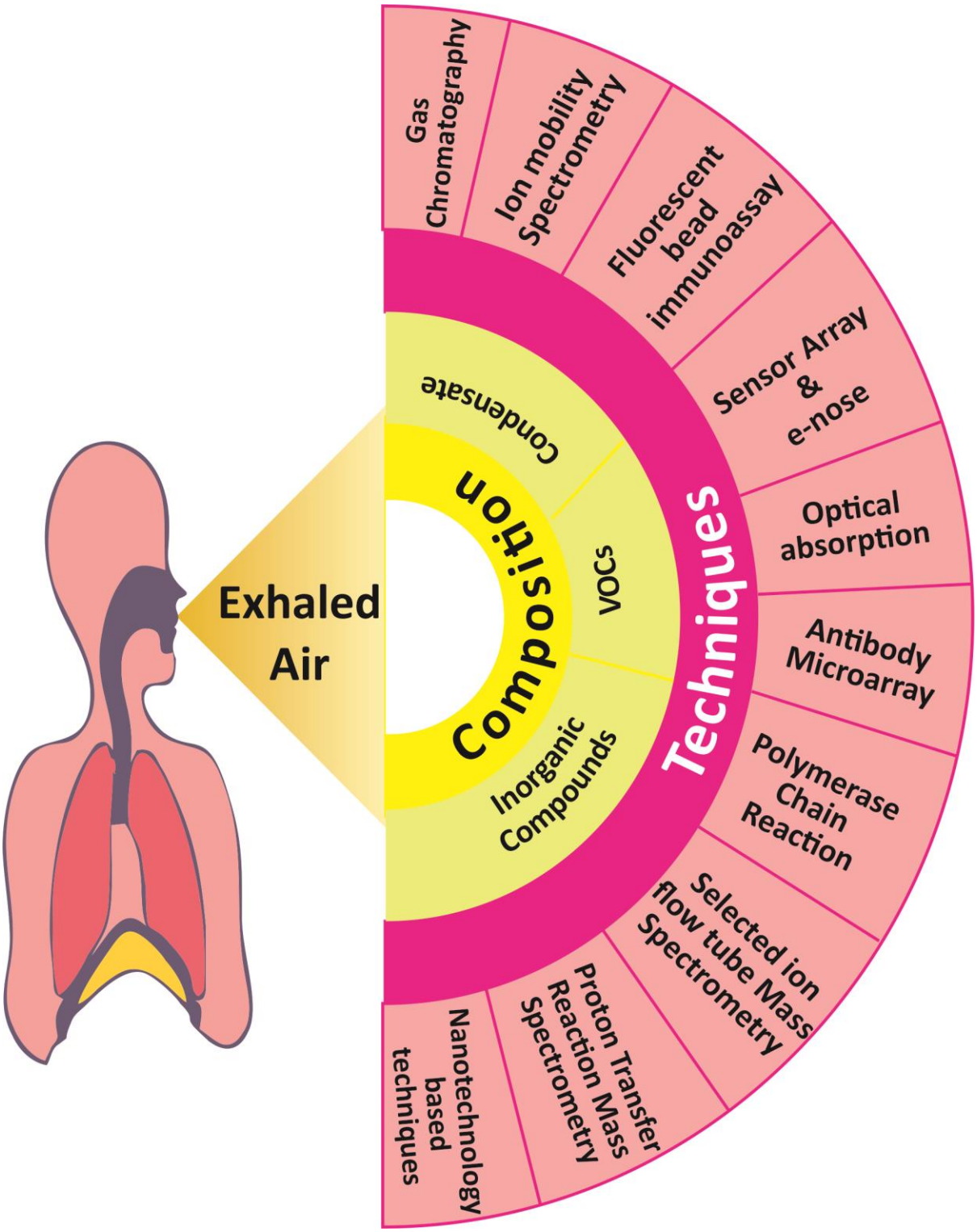
Abstract

The 21st century is witnessing a revolution in understanding the composition of human exhaled breath more deeply, as the "breathe-print" can provide significant information of various diseases and disorders. This review elucidates various breath analytical techniques applied in medical diagnosis. A quick, portable, simple, accurate, non-invasive and relatively inexpensive approach of this technique is the reason of its evolution as a new frontier in disease diagnosis. This review emphasizes on breath biomarkers and traditional approaches in techniques used for breath biosensors like GC-MS, SIFT-MS, PTR-MS, IMS, Optical Absorption, Sensor Arrays and Electronic noses along with other new approaches such as Enzyme Immunoassay, Fluorescent Bead Immunoassays, Polymerase Chain Reaction, Antibody Microarray. It also includes the approaches using nanotechnology to improve the sensitivity and portability of breath biosensors. Still there is a lack of sensitive, standardized and accurate technique for the validation of biosensor. Hence future research is needed to overcome the loopholes in the present techniques.

Keywords: Biosensor; Biomarker; Exhaled Breath; VOCs; Breath Analysis; biodiagnosis

Graphical abstract



Introduction

The study of exhaled breath has captured wide attention from scientists, clinicians and researchers as it can provide clinically significant information for biochemical changes in the human body [1, 2].There are numerous potential bio-analytical techniques available, which are based on blood, serum and plasma for the diagnosis of various diseases such as hepatitis, rheumatoid arthritis, diabetes and cancer. However, these techniques are invasive, time consuming, sometimes costly and some require skilled personnel. Hence among these different techniques, breath analysis becomes an attractive alternative and more acceptable method for the patients, as it is safe, non-invasive as well as quick and easy to carry out [3]. This review article focuses on various techniques used in breath biodiagnostics used for the diagnosis of various diseases.

History

The history of using breath for diagnosis of physical condition is as old as use of medicine. The initiative of breath analysis for diagnosis of disease was first taken by Greek physicians in 460-370 BC where they used the concepts of *fetor oris* and *fetor hepaticus* as described by Hippocrates in his treatise of breath aroma and disease [1, 2, 4-6]. Further, Lavoisier identified carbon dioxide from breath of guinea pigs as the combustion product in the body [1, 6].In 1970, Linus Pauling detected 200 VOCs using gas chromatography [7, 8]identified 1259 VOCs using GC-MS system from 20 healthy subjects. In 1999, Phillips described total 3481 VOCs in the breath of healthy individuals [9]. Also, total 1765 VOCs have been published in healthy humans in recent times [6]. This discovery clarified one thing that the air exhaled by human is a complex mixture of various compounds. In last 30 years, scientists identified thousands of different substances present in breath. Human breath consists of gases such as carbon dioxide, oxygen, nitric oxide and large number of VOCs. Many of the VOCs

are under investigation for characterization. The exhaled breath also contains aerosolized condensates, particles and non-volatile compounds such as proteins.

In today's era, medical practitioners check the smell of the breath of patients in order to determine the disease for example fruity smell of acetone is a characteristic of diabetes and urine like smell was that of renal failure. The detailed composition of the exhaled breath is given in figure 1. [2, 10, 11]

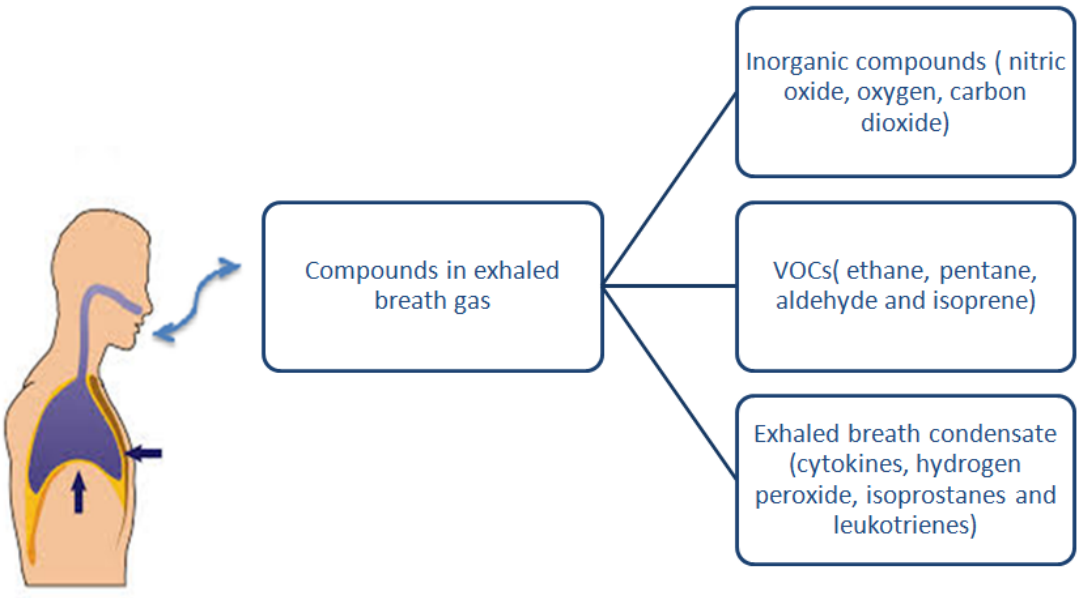


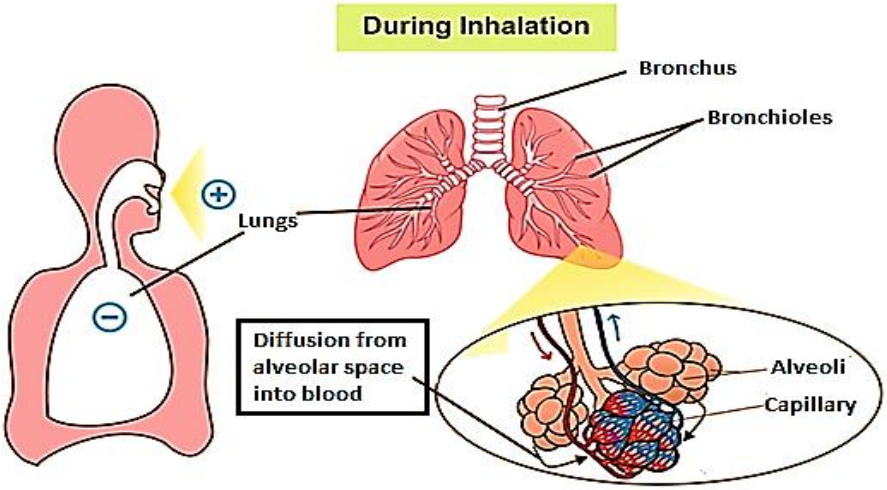
Figure 1. The basic composition of exhaled breath

Principle of exhaled breath analysis

The basic principle of exhaled breath analysis is shown in figure 2[12, 13]. The equation of partition coefficient as shown below is the ratio of concentration of any chemical in blood to that of in gas phase and is unique for each gas [14].

$$(K) = \frac{\text{Concentration of chemicals in the blood}}{\text{Concentration of chemicals in gas phase}}$$

Blood- gas partition coefficient



According to Henry's law, which states that the amount of given gas gets dissolved in a given volume and type of a liquid is directly proportional to the partial pressure of a gas in equilibrium with that liquid at a constant temperature. From this, it can be assured that the quantity of any substance coming out from the lungs changes in proportion to its vapour pressure in lungs.

Figure 2. Diffusion of gases from alveolar space to blood during inhalation following Henry's law

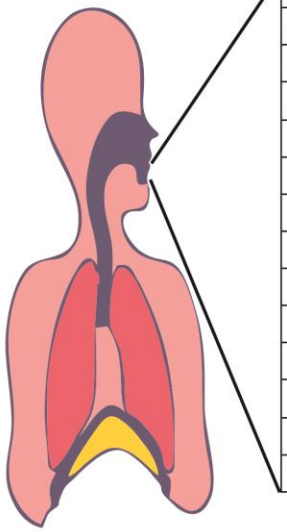
Although the mechanism is simple, there are many factors that need to be considered for bio-monitoring using breath samples. The important advantages and few limitations of breath biodiagnostics are summarized in table 1.

Table1. Advantages and limitations of breath biodiagnostics

Advantages	Limitations
• Non-invasive method • Personalised medicine • Quick and real time analysis is possible • Repeated measurements are allowed • Easy to handle and possibly portable	• Lack of standardisation in sampling of breath • Lack of standardisation in analytical techniques • Storage of samples • Physician's acceptance • Fluctuation in results • Other parameters such as interference of other gases , diet, environment

Biomarkers for diseases

In context of diagnosis of diseases from human breath, there are many biomarkers in exhaled breath. These diseases are categorized into 1) diseases of respiratory system and upper gastrointestinal tract and 2) states in which specific metabolites get accumulated due to progression of disease such as diabetes/hyperglycaemia, kidney failure, extra-oral halitosis, chronic liver disease and hypermethioninemia [15]. The VOCs which are frequently used for diagnosis of various diseases from expired breath comprise compounds containing oxygen like methanol, ethanol and, 2-propanol, acetone and acetaldehyde; nitrogen-containing substances like ammonia, dimethylamine, trimethylamine; hydrocarbons like ethane, isoprene and pentane; sulphur-containing compounds like methyl mercaptanes, ethyl mercaptanes and dimethyl sulfide[2]. The most commonly used biomarkers of diseases are given in figure 3.



Biomarker	Disease
Acetone	Diabetes mellitus
Acetaldehyde	Low activity enzyme polymorphism
Acrylonitrile	Smoke exposure
Benzene	Uraemia, Kidney impairment, *H Pylori* infection
Ethane	Various disease (oxidative stress)
Ethanol	NASH, obesity
Hydrogen sulphide	Periodontal disease
Isoprene	CVD (Cholesterol metabolism)
Methane	Carbohydrate malabsorption
Methylated hydrocarbons	Lung or breast cancer
Nitric oxide	Asthma / allergy
Pentane	Various disease (Oxidative stress)
TMA	Atherosclerosis, CVD

Figure 3. Biomarkers for diagnosis of various diseases

(**Abbreviations:** CVD: cardiovascular disease; TMA: trimethylamine and NASH: Non-alcoholic steatohepatitis)

Metabolic disorder

The major metabolic disorders are diabetes, phenylketonuria, acid–base metabolism, metabolic syndrome or other disorders associated with potassium, magnesium and phosphate [15]. Out of these disorders, diabetes has prominent biomarker for analysis of breath. The metabolic changes that occur in case of diabetes are increased levels of glucose and lipolysis which results in increased acetone levels in exhaled breath [16, 17].The exhaled breath of normal human being contains 0.8 parts per million (ppm) level of acetone which increases in diabetic patient up to 1.7 to 3.7 ppm and hence considered as an important biomarker [18].

Lung Infection

Breath analysis has been widely used for testing of lung disorders like COPD, asthma, interstitial lung disease, bronchiectasis, cystic fibrosis [19-21]. Lung disorders are

characterized by fluctuation in breath biomarkers such as, increase in nitric oxide concentration in case of asthma, cystic fibrosis and COPD [22, 23], elevated levels of hydrocarbons in the cases of COPD [24], asthma, obstructive sleep apnoea [25], pneumonia [26] in exhaled breath. Lung cancer is identified by the changes in levels of different VOCs as compared to normal healthy individual.

The elevated nitric oxide (NO) is observed due to activation of iNOS in case of inflammation or damage to the epithelial cells of airways of lungs [27-30]. These increased NO levels helps in differentiating the asthma from chronic cough which is characterised by common symptoms as that of asthma [31]. So the breath analysis of NO can be used as a diagnostic tool for asthma which is non- invasive. Also NO can be used as a biomarker for pulmonary allograft dysfunction as it is found to be increased in case of lung-transplant recipients with early obliterative as well as lymphocytic bronchiolitis [32]stable COPD patients [33, 34]. Hydrogen peroxide is a biomarker for asthma, COPD and cystic fibrosis whereas isoprostanes are biomarker for COPD and asthma [35]. Several alkanes (C_4-C_{10}) and monomethylated alkanes are used in detection of lung cancer [36].

Oxidative stress

This is caused by chemical reactions taking place due to free radicals which causes the damage to cell walls, genetic material and proteins [37]. Hydrogen peroxide present in exhaled air is a biomarker for oxidative stress [38]. These substances oxidize PUFA to alkanes which are eliminated in exhaled breath [39]. This is a presentation of an idea about severity of disease or risk of complications of disease.

Gastroenteric disorders

The diagnosis of gastroenteric diseases such as *H. Pylori* infection, malabsorption of bile salt, liver dysfunction, pancreatic insufficiency, lactose and fructose intolerance, abnormal small-bowel transit, bacterial overgrowth is possible using UBT. This test depends on its ability of *H. Pylori* to breakdown the urea, which is produced from excess of nitrogen present in the body [40]. Also hydrogen is detected in breath which is produced due to malabsorption of carbohydrates and responsible for disorders such as lactate deficiency, starch malabsorption and bacterial overgrowth [41, 42].

Kidney Disorder

High ammonia content in human exhaled breath is a characteristic of kidney disorders such as End Stage Renal Disease (ESRD). In gastrointestinal tract urease enzyme converts excess of urea into ammonia which is released in exhaled breath [43].

Traditional approaches

The variety of instrumentation platforms have been developed for the detection of volatile as well as non-volatile components in exhaled breath. Depending on the biomarker, different techniques or combinations of techniques are used for diagnosis of disease. Steps in detection of biomarkers are given in figure 4.

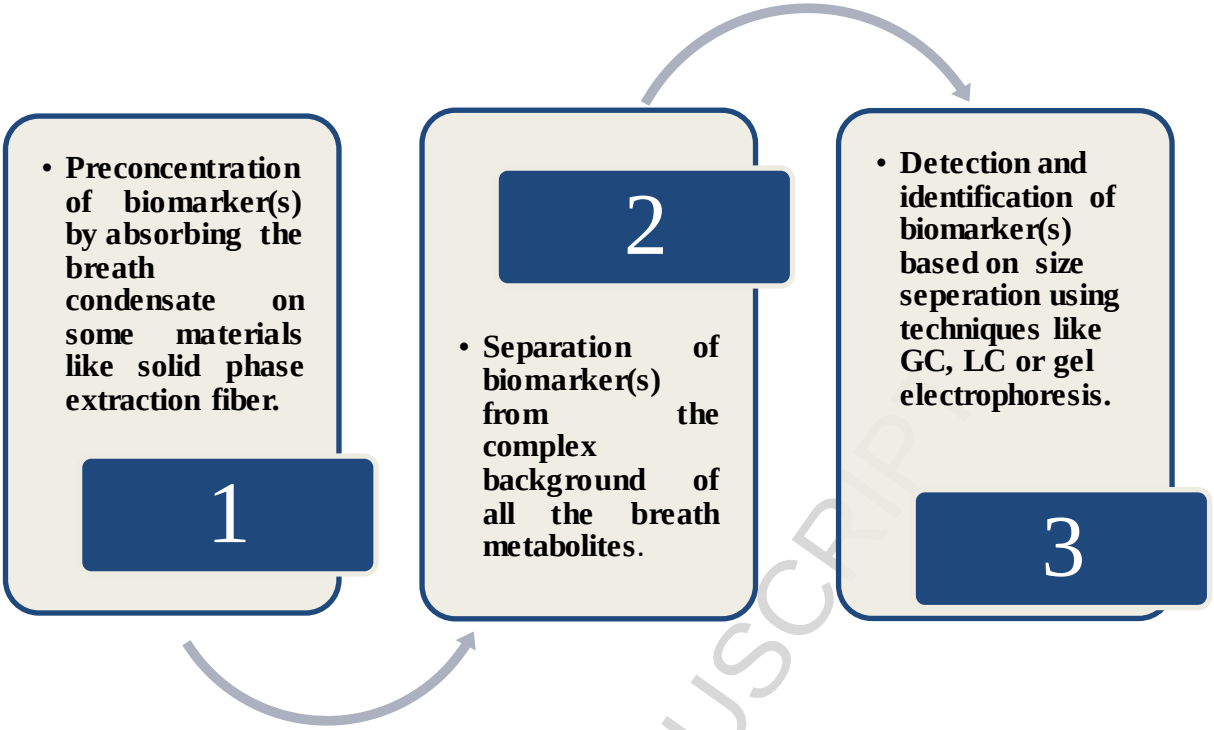


Figure 4. Flow chart for preconcentration of analyte

The techniques for biosensors are under constant development and it's difficult to decide one technique for biosensor to detect even low abundant component of breath. Hence it's important to understand the techniques for biosensor and their advantages and limitations.

Techniques used for bio-sensors for exhaled breath analysis

MS

The MS has been used since 1970's for chemical analysis in different industries such as petrochemical, pharmaceutical, chemical and consumer products. The working principle of MS is that, it ionizes the sample and identifies using suitable detector. Traditional instruments separate ions as per the mass to charge ratio and the ion pattern obtained which is a characteristic of certain chemical is identified by technician or scientist. In modern detecting instruments, other physical parameters such as time of flight etc. are considered. Because of

its high selectivity and sensitivity it is considered as a standard technique for analysis of human breath [44]. These techniques are reviewed in brief below:

GC

The most commonly used method for breath analysis of VOCs is GC or its combination such as GC–MS, FID, IMS and GC with tandem MS (GC– MS^2). [4, 45] The MS systems for analysis of exhaled VOCS are usually associated with other methods like electron impact ionization technique [2], SPE and SPME [1].

For GC analysis, the prepared test sample is injected into the chromatographic column which is transported using gas as a mobile phase. The components are separated using polar or non-polar columns. In case of polar column, the components are separated as per the polarity and in non-polar columns separation takes place according to the boiling point of the compounds.[46] For the entire GC analysis, normal operating voltage is 220 V with the heating rate in between 30 and 60 °C/min in the temperature range of 50-450°C. However such conventional GC-based techniques are unsuitable for real time applications for analysis of breath. [47] GC-MS has been used since 1970s to analyse the composition of breath such as important biomarkers for lung cancer [48], asthma [49], cystic fibrosis [50], interstitial lung diseases [51], type I diabetes mellitus [52], pulmonary tuberculosis [53], organ transplant rejection [53] and various other biomarkers indicating oxidative stress [49].

FID is the one of the most commonly used for detection of organic compounds used in combination with GC. The ions and electrons produced on burning the organic compounds in FID can conduct electric potential which gives qualitative as well as quantitative data of analyte. This method gives a large linear response range and exhibits high sensitivity and low noise. The disadvantage of this system is that it is mass sensitive which do not alter response in response to the change in mobile phase-flow rate. [54, 55]

The basic principle of IMS is that it separates ions through purified gas according to their mobility in an electric field. Various factors affect the velocities and total travel time of ions such as electric field strength; drift length, type of drift gas, atmospheric pressure and temperature [56]. It is a selective detector which provides quantitative data of volatile components in breath. The ethanol and acetone as biomarkers were successfully analysed using GC-IMS technique. [57]

The GC-MS system is generally used for analysis of breath VOCs and based on the principle of difference between mass to charge ratio (m/z) of ionised atoms or molecules [58]. The fragmentation pattern and formation of daughter ions gives quantitative data of target compound [59].The electron impact ionisation technique associated with MS system provides unique fragmentation pattern for each analyte which helps in identification using retention data of chromatography and mass spectral data. [2] This method exhibits high sensitivity, reproducibility and robustness which make it relevant to use as a biosensor [60]. This system is sensitive to quantify breath VOCs of very low concentration such as ppb/ppbv or ppt/pptv [61-63]. One of the established applications of GC-MS system in disease diagnosis using breath is cancer detection as well as rejection of organ transplant in non-invasive way. Philips et al. used human breath samples, to elucidate VOCs pattern which is common in the exhaled air using a GC-MS of patients with lung cancer or experiencing rejection of a transplanted organ or associated, unlike healthy subjects [64, 65].

The GC method is highly sensitive; however it is tedious, costly and unsuitable for real time use and repeated measurements. The poor portability makes it difficult to handle. [3] The GC system is often combined with the pre-concentration system for off-line collection, separation as well as identification of many VOCs in breath and for making instrument portable. [4, 45] Several types of pre-concentration systems are used such as trapping based pre-concentration

[66], adsorption based pre-concentration such as solid phase extraction or micro-extraction [67] and distribution based pre-concentration for the analysis of VOCs in breath [68].

MEMS technology is used instead of above mentioned methods as such preconcentrators achieve level of detection as low as pptv. The low cost and portable size of this technique makes it highly useful as a biosensor [69]. The demand for early disease diagnosis creates a need for development of these GC based analysers. Therefore online and quasi-online biosensors analysing multiple metabolites find many applications in breath [70]. Newly developed GC techniques such as GC-IMS were employed for online analysis of breath for VOCs. GC-IMS with membrane extraction with sorbent interface(MESI) was used to determine the occurrence of ethanol and acetone present in breath as biologically important markers as well as analysing the composition of the 250 mL portion of exhaled air remaining last during the online use [71]. The problems arising due to column bleeding were overcome by using multi-capillary columns (MCC). MCCs separate rapidly while maintaining the desired resolution with LODs in ppbv and pptv range. [72]

Using two dimensional GC helps in identification of unknown compounds as it forms two-dimensional chromatogram of chemically similar compound patterns with LODs in ppt range. [73]. Breath biomarkers such as furan and 2-methylfuran in breath of active smokers were established using such technique. [74]

The first portable GC was developed in 1976 for breath analysis consisting of an apparatus for BHT test which could differentiate lactose absorbers to that from lactose malabsorbers with LOD in ppm range [75].

The newly designed GC works on principle of resistive heating of an electrically conductive solid material with the heating source such as wire. The conduction of heat takes place to the materials and facilitates significantly faster heating up to 800 °C/min. This eliminates the

need for a huge oven thus reducing the size of the column. [76]The smaller surface area reduces heat loss significantly which reduces the power required for heating. This portable instrumentation makes it easy for the real-time analysis of breath [77].

The next generation GC systems used lightweight components and no compressed gas was required for the determination of VOCs in breath [78]. E. T. Zellers and co-workers (2009) designed prototype of portable GC using scrubbed air to determine low levels of VOCs in human breath [79]. A micro GC integrated with PID with a micro GC column and a micro pre-concentrator developed by J. Sun and co-workers in 2014 for non-invasive, fast and cost-effective means for the diagnosis of lung cancer [80].

Hence newly developing GC system is used specially with effective detection techniques to identify VOCs.

PTR-MS

The PTR-MS is quick, accurate and less time consuming technique devoid of pre-concentration step which is associated with GC [81]. Hence it is ideal for complex gas mixtures. It is more sensitive than GC in detection of VOCs from breath with LOD down to ppb [82] to ppt [81] levels. This technique can be easily used to find out the concentrations of 30 VOCs from breath with sensitivity down to ppt level within 2 mins [3]. It is more promising method as it can be used for online as well as multiple measurements. Hence this technique is found to be useful in non-invasive biochemical observations to give insights about metabolic processes especially during sleep.

PTR-MS works on principle of chemical ionisation and analytes are characterized according to mass/charge ratio (m/z). The primary reactant ion is H_3O^+ which is generated in ion source region. [82]

The VOCs have more affinity for proton as compared to water and hence proton transfer takes place during every collision with VOCs. There is no interference of compounds with target compounds which are abundant in nature such as N_2, CO_2 etc. [83]

The studies carried out by Bajtarevic et al. (2009) [84] showed that quantitative data of VOCs of breath of patients with lung cancer using PTR-MS is more accurate than GC-MS. This technique characterises the substance based on only mass/charge ratio (m/z); however identification of compound is not viable. Thus it is often coupled with GC to efficiently separate the complex VOCs [85].

SIFT-MS

SIFT-MS is a recently developed online quantification tool for VOCs present in breath [4, 45] and it is based on the principle of chemical ionisation. In this technique the breath sample is introduced along with the inert gases such as helium. In this case, generally H_3O^+, $O2^+$, NO^+ is used as positive precursor ions. On reaction with these ions, molecules in this technique generate product ions which are corresponding to each gas that is present in sample [86]. These ions are characterized as per the mass and counted by the detector present downstream. Large amount of molecules are detected and identified at the same time [2].

The direct and quick analysis can be carried out of VOCs in breath samples. This technique has found wide application in analysis of ammonia from breath. Ammonia is a biomarker used for screening the colonization of organism *H. pylori* which splits the urea in gastrointestinal tract [87]. The studies confirmed the increase in ammonia levels in breath samples on oral consumption of 2g of urea by the person infected with *H. Pylori* as compared to uninfected person. SIFT-MS technique can detect the VOCs in ppt/pptv to ppb/ppbv range and analyse small molecules. Hence this technique is preferred over GC-MS [88].

The techniques used for detection of some selected VOCs are given in table 2.

Table 2. The application of GC, GC-MS and SIFT-MS in detection of some selected VOCs

Technique	**Compounds**	**Disease / condition**
GC	TMA	CVD, Atherosclerosis
GC-MS	Pentane	Various states involving lipid peroxidation or oxidative stress
	Ethane	Various states involving lipid peroxidation or oxidative stress
	Methane	Carbohydrate malabsorption
	Hydrogen sulfide	Periodontal disease
	Isoprene	CVD
	Ethanol	NASH, obesity
	2-Propanol	Various states involving 2-Propanol
	Benzene	Lung and breast cancer/smoke exposure
	Acrylonitrile	Smoke exposure
	Acetone	Diabetes mellitus
	Acetaldehyde	Low activity enzyme polymorphism
SIFT-MS	Benzene	Lung and breast cancer/smoke exposure
	Acrylonitrile	Smoke exposure

	Acetone	Diabetes mellitus
	Acetaldehyde	Low activity enzyme polymorphism
	Hydrogen sulfide	Periodontal disease
	Isoprene	CVD
	Ethanol	NASH, obesity
	TMA	CVD, Atherosclerosis
	Pentane	Various states involving lipid peroxidation or oxidative stress

Sensor Arrays and Electronic Noses

The technology of chemical sensors has found many applications in clinical diagnostic tests. One of the techniques based on sensor arrays, one which is widely used is electronic nose (e-nose). It consists of non-selective gas sensors in series along with pattern recognition [89].

The nanosensors present in e-nose undergo reversible reaction when come in contact with specific compounds in exhaled breath [90].The limitation of this technique is its inability to identify the compound to which it reacts. Hence changes in breath pattern are identified however the underlying pathological condition remains undiagnosed. Sensor arrays are affordable, easy to use, easy to fabricate due to modern techniques and exhibit high throughput [89]. The e-nose can be used for diagnosis of disease or infection as well as determination of metabolic pattern or response of the host [91]. It can detect the common pattern in VOCs present in breath samples which can be a biomarker for certain disease. Also it can detect the "smell print" to which it is previously trained, resembling human or animal nose [92].An e-nose, made up of 8QCM sensors, which are coated with several

metalloporphyrins was used to analyse breath of lung cancer patients. This array of non-selective gas sensor could identify diseased individuals [93]. The studies carried out by Machado and co-workers (2005) [94] showed that exhaled air of lung cancer patients exhibit different characteristic pattern of VOCs which can be identified using e-nose [94].Total 11 validated biomarkers were detected both qualitatively and quantitatively using e-nose based on virtual array of SAW gas sensors [95].

This technique can differentiate the lung cancer patients from COPD patients depending on the VOCs pattern [96]. Also the validation of this technique showed high accuracy in distinguishing the asthma and COPD patients [90]. An e-nose is also potential diagnostic adjunct for diagnosis of pneumonia [97]. The validation studies carried out by Bruins and co-workers proved that electronic nose (DiagNose) exhibits high sensitivity and specificity in diagnosis of tuberculosis from exhaled air [98]. An e-nose provides exhaled molecular profiles for diagnosis of pulmonary sarcoidosis [99] and detection of pathogen, uraemia, renal disease and airway inflammation [100]. Among the chemical sensors, use of modified QCM with Ag^{+}-ZSM-5 has been applied in diabetes diagnosis. It showed high sensitivity and specificity against acetone which is an important biomarker of diabetes [101].

IMS

IMS technique estimates the time required for an ion to pass though drift tube [45]. This technique can separate as well as identify different substances from complex mixture under ambient conditions. It is a quick, accurate technique with LOD down to ppm to ppb levels without the need of pre-concentration step for VOCs in breath samples [102].However the limitation of IMS technique is that humidity in breath samples may affect the ion-drift time. Moreover it cannot detect unknown compounds in breath sample [103].

IMS is used mainly for measuring disease specific composition and characterizing the mixture of VOCs and bacteria present in breath for diagnostic purposes. The studies carried out by Ruzsanyi et al. (2005) [104] showed that IMS coupled with multi-capillary columns can be used for detection of different VOCs in breath samples. The handheld miniature devices of IMS are widely used for military purposes for monitoring warfare agents, drugs, explosives and environmental hazardous compounds [105].

Optical Absorption

This technique is used when the particular biomarker of disease or a specific molecule is known which is associated with the disease. It is easy, quick and exhibits high selectivity. It can detect the molecule down to ppb level. Hence it is used for real time diagnosis of disease[45]Analysers based on chemiluminescence, which are used for detection of nitric oxide in breath sample, a biomarker of asthma works on principle of optical absorption. However such instruments are of high cost and exhibit poor portability due to their large size. Hence electrochemical cell technique has been recently developed to improve the performance and for its real time application [106].

Other techniques used in biosensors of breath analysis

Along with the smaller molecules like volatile and fatty acid compounds, larger molecules such as fatty acids and proteins are also present in exhaled breath in aerosol form. Although several hyphenated techniques are available to analyse such big molecules, they are expensive, time consuming and instruments used are not suitable for real time use. Hence apart from these techniques, researchers work with following techniques for the detection of such heavier components in exhaled air.

Fluorescent Bead Immunoassays

It works on the principle of antigen-antibody reaction. The marketed kits available contain different sets of beads smaller in size. They are covered with an antibody. Several such different beads are mixed. If the antigen corresponding to the specific antibody is present, it will bind that particular antigen. The fluorescent intensity of fluorescent reporter antibodies is then analysed using a flow-cytometer. This technique has been used by many researchers for asthma, systemic sclerosis, COPD, and ARDS to discover biomarkers of diseases. [107]

Enzyme Immunoassay

The difference between this technique and Fluorescent Bead Immunoassays is that, in this case beads are replaced by plate and antibodies are conjugated to enzymes which on reaction with reagent gives coloured product. The colour intensity is quantified using spectrophotometer and is directly proportional to antigen present in the sample.

Carpagnano et al. (2003) investigated the levels of leukotriene B4 and IL-6 in smokers and non-smokers. It was found that levels of IL-6 as well as leukotriene B4 increase with increase in number of cigarettes per day in smokers [108]. Also it can be considered as a biomarker for COPD. The studies carried out by Montuschi et al. (2003) also proved that levels of prostaglandin increase in COPD patients than healthy individuals [109].

PCR

This technology analyses the DNA present in exhaled breath condensate. Hence using this technique it is possible to amplify the segments of DNA in exhaled breath. One of the peculiar characteristic of NSCLC is the mutations in p53 gene. The studies carried out by Gessner et al. (2004) found that the PCR technique could identify 4 out of 11 samples from cancer patients with no false positive result in healthy individuals [110]. Also many biomarkers have been observed for COPD, systemic sclerosis, ALI, ARDS, GERD, and

asthma such as leukotrienes and cytokines; however, the difference between the concentrations of biomarkers is very low in disease and healthy subjects. Intra-subject variability is also high for the concentration of biomarkers. Hence to overcome this barrier and for clinical approval for diagnosis of disease using exhaled breath sample, highly specific and sensitive biosensor is required.

Antibody Microarray

This technique consists of printed antibody array and facilitates flexibility in number and type of antigens to be detected. Unlike bead assays, in this technique there is no limit to assay size. The breath samples or exhaled breath condensate are collected and added to this system. The intensity of this reaction is measured using suitable technique such as chemiluminescence. Researchers could find 9 different types of cytokines using this technique in asthma patients as compared to healthy subjects [111]. Also detection and analysis of proteins in exhaled breath also provides significant information regarding gene regulated disease state.

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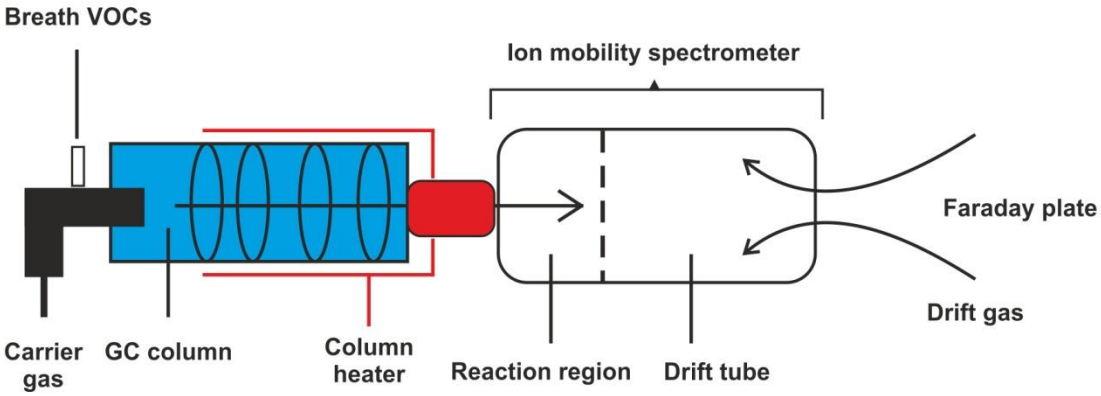

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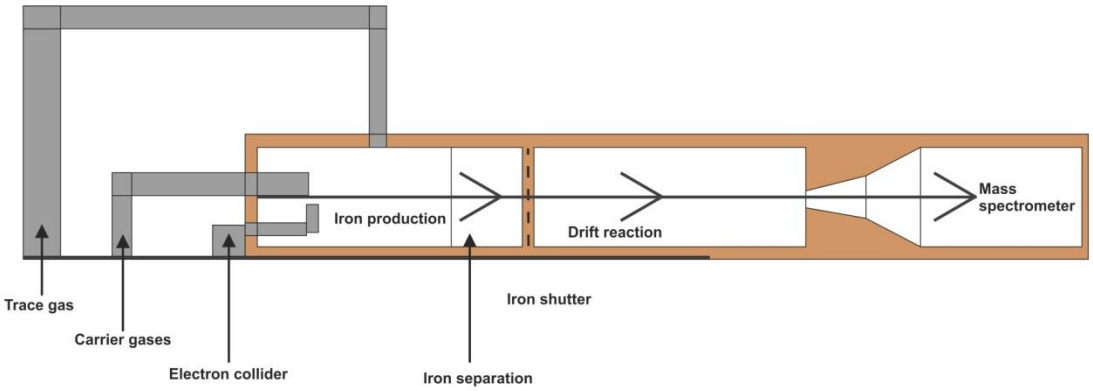

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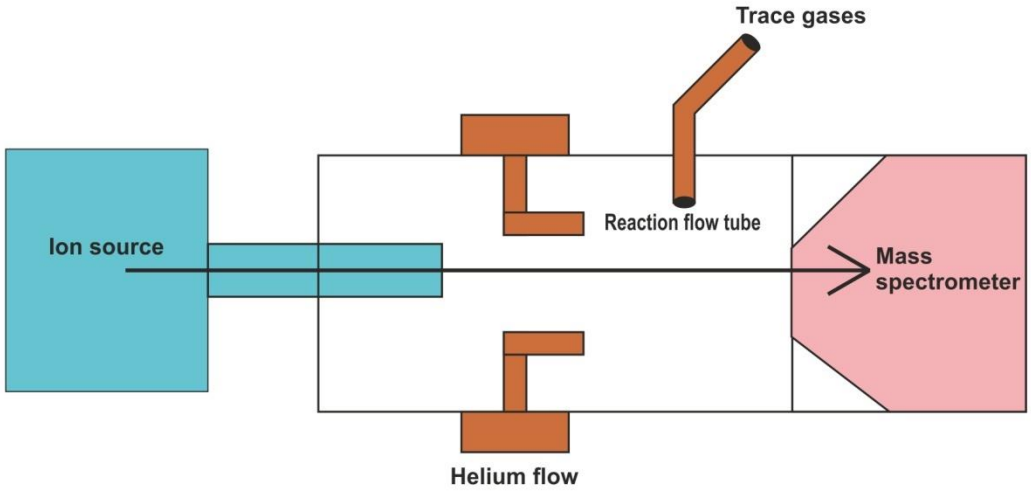

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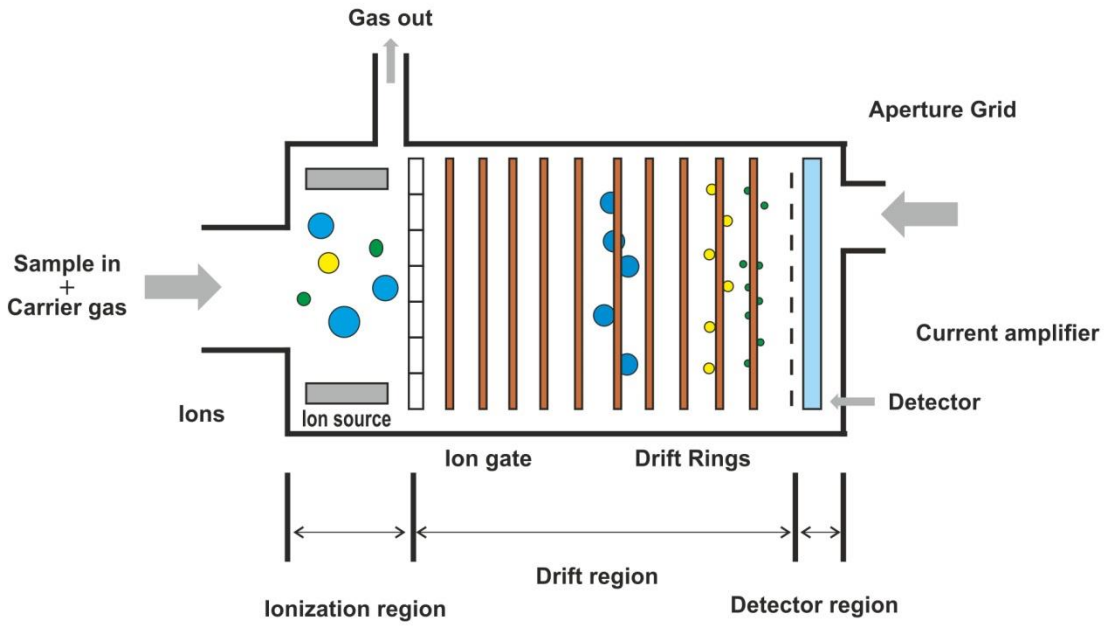

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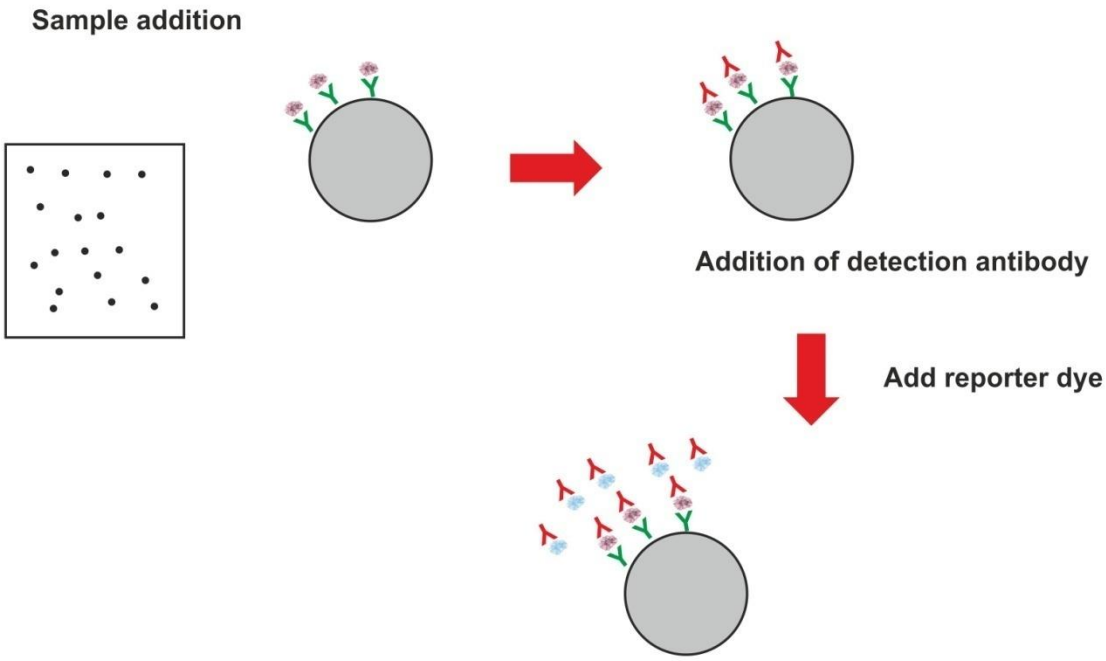

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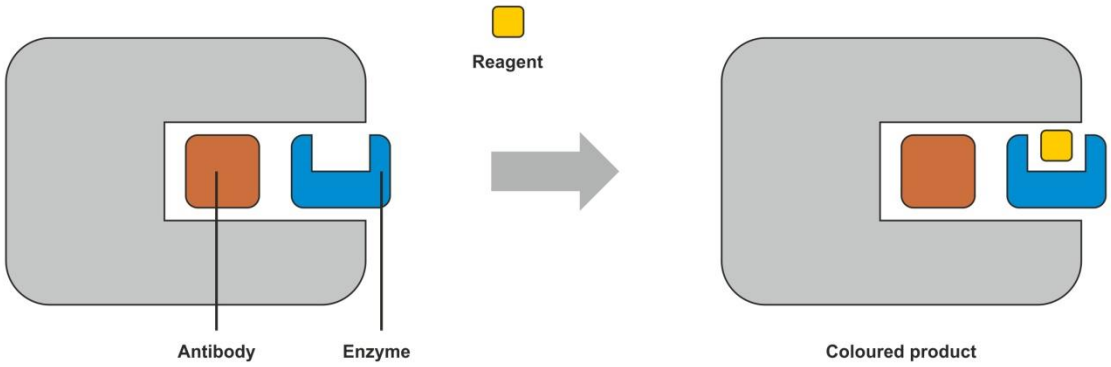

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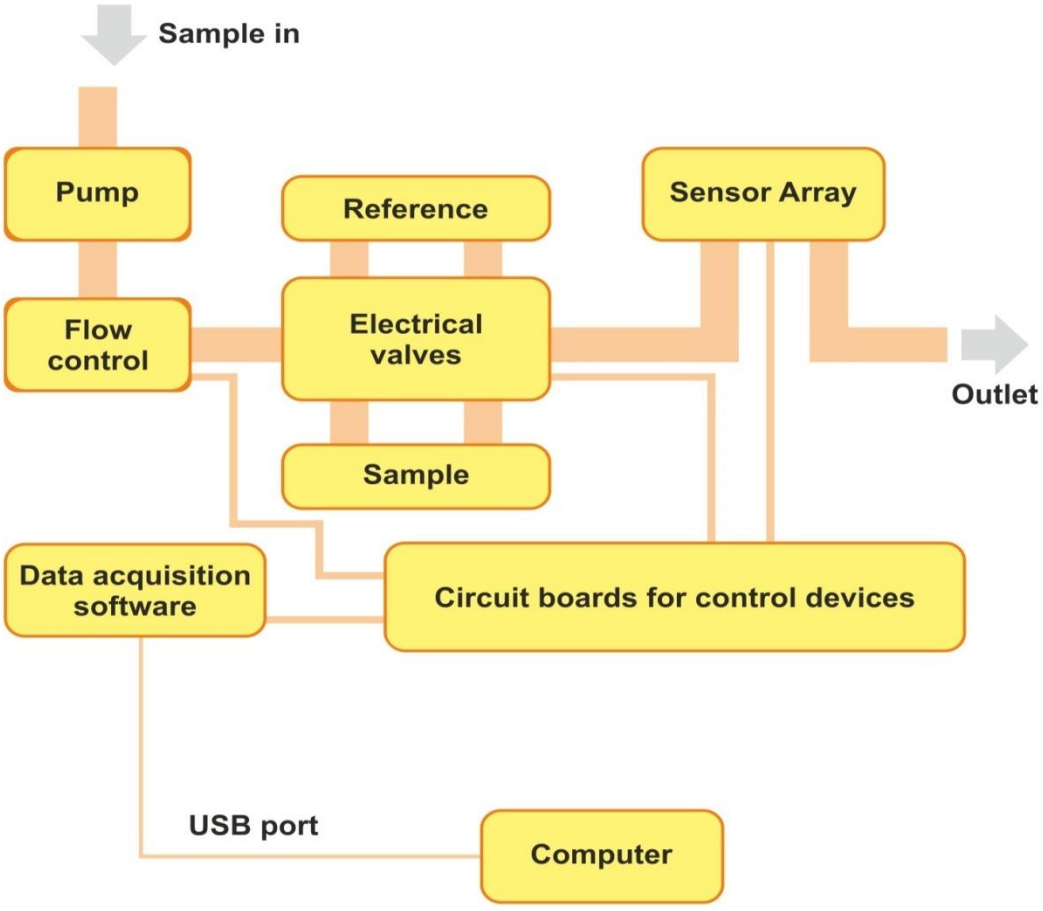

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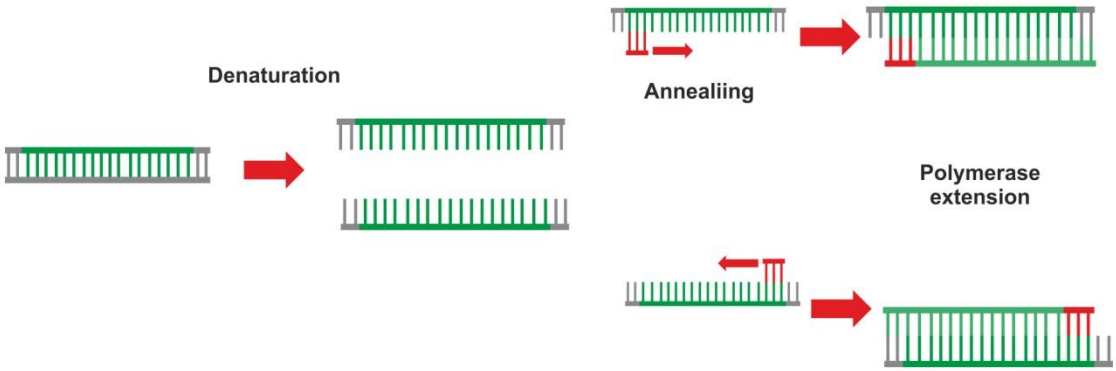

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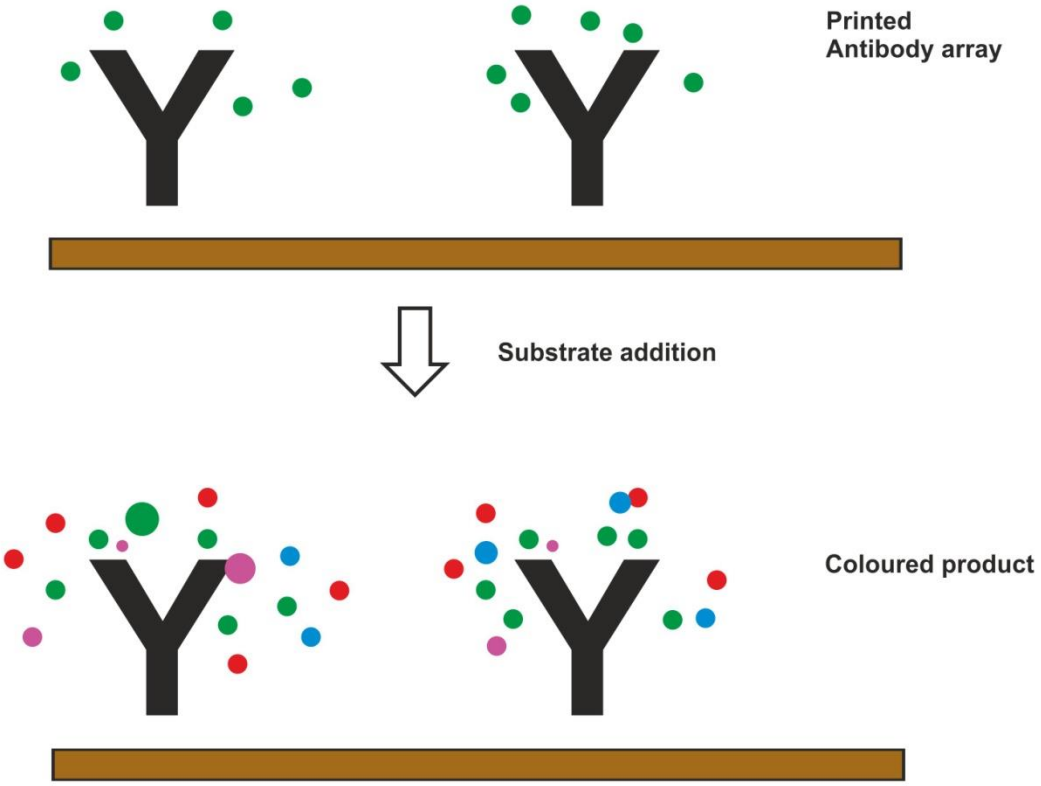

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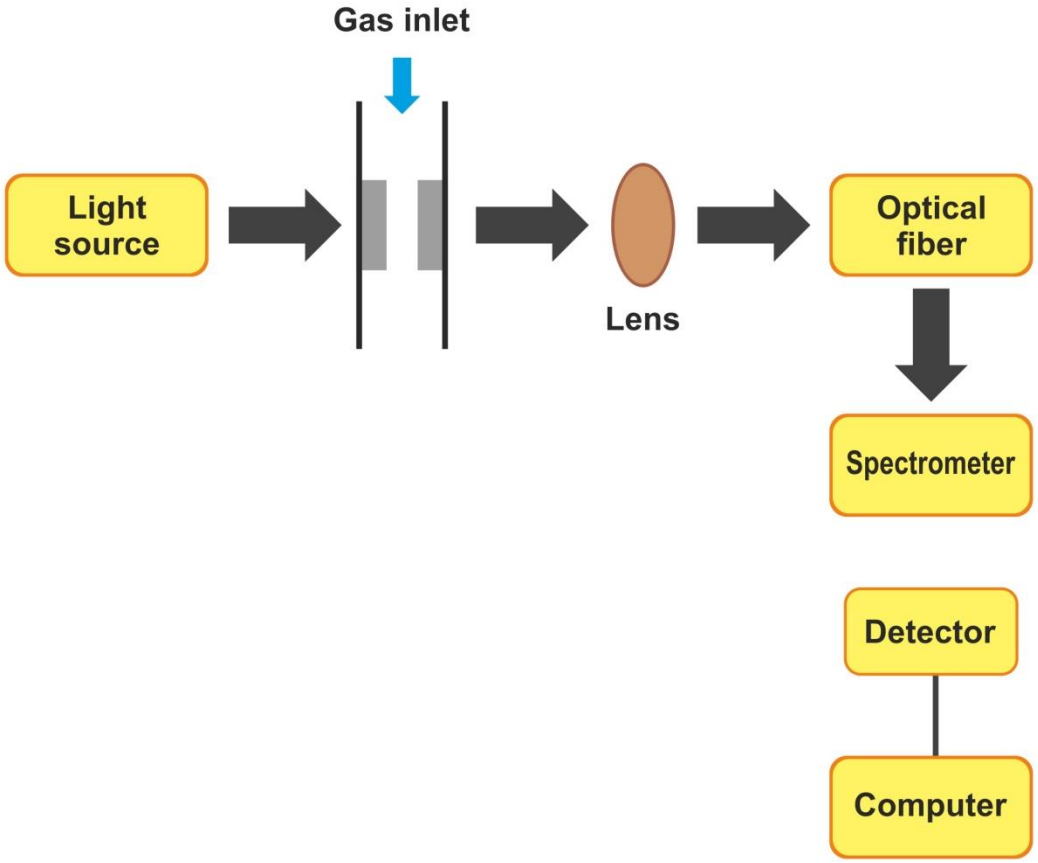

Figure 5. Diagrammatic representation of techniques a. GC-IMS, b. PTR-MS, c. SIFT-MS, d. Ion-Mobility Spectrometry, e. Fluorescent Bead Immunoassays, f. Enzyme Immunoassay, g. Sensor Arrays and Electronic Noses, h. Polymerase Chain Reaction (PCR), i. Antibody Microarray and j. Optical Absorption

Nanotechnology for improving sensitivity

The nanotechnology is the study and optimization of systems with dimensions less than 100 nm and has been commonly used in exhaled air analysis in recent times. The nanoscale (1-100 nm) of the material makes the system sensitive to the molecules of similar size. The large surface-to-volume ratio and other exclusive physical, optical, electrical properties, more

active interfaces are the reasons behind the higher sensitivity of these materials. The properties like surface effect, quantum size effect, mini size effect, macro quantum tunnel effect make the system more sensitive towards biomarkers. The devices which are already developed using this technology are portable, cost-effective and easy to handle and use in diagnosis and screening. In recent times, nanoparticles, nanowires, carbon nanotubes have been used in analysis of exhaled breath mainly due to their higher sensitivity, rapid responsiveness and cost-effectiveness. These nanomaterials can be tailored as per the required specificity to certain biomarkers in accurate diagnosis of disease from exhaled breath.

In case of chemiresistive gas sensors, nanostructures of one dimensional metal oxide are considered as sensitive technique as they possess large surface to volume ratio. Also it is highly permeable to gas as well as exhibits good thermal stability. For the detection of aldehydes in exhaled breath, the bifunctional magnetic nanoparticles have been prepared by solid phase extraction method. They work on the principle of extracting aldehydes in the form of hydrozones by derivatizing with 2, 4-dinitrohydrazne using nanoparticles. The further analysis is carried out using HPLC-photo diode array detector. The limit of detection of this technique is 2.9- 21.5 nmol/L [112].

The use of GNs which are organically capped on the array of sensors in kidney injury helps in detection of small changes in concentration of VOCs from exhaled breath. This makes the system rapid, inexpensive and non-invasive in diagnosis and screening of lung cancer. Zhen et al. (2013) developed single walled carbon nanotubes and organically stabilized spherical gold nanoparticles based sensor array to discern the gastric cancer from benign gastric disorders through VOCs in exhaled breath using GC-MS [113]. Also the use of gold nanoparticles in detecting biochemical pathways or toxins involved in CKD through kidney function loss from exhaled breath proved the potential of the same as a rapid, reliable and

cost-effective test for the detection of CKD and monitoring the progression of disease. The sensors based on thiol derivatization of GN were developed to detect acetone and ethanol from exhaled breath. Total 14 GN based sensor with different organic functional groups were prepared and mounted on PTFE circuit board to provide single array of nanosensors. The Peng et al. (2010) showed the detection of lung, prostate and colorectal cancers from exhaled breath using this system. [114]

Wu et al. (2011) demonstrated the use of ZrO_2 nanoparticles in gaseous sensor of traces of 2 propanal using CTL detector in improving the sensitivity and stability [115]. The acetone sensors based on Si-doped monoclinic tungsten trioxide (WO_3) nanoparticles showed higher sensitivity while monitoring among different test persons. This technique provides portable, cost-effective alternative for diagnosis of diabetes by detecting acetone in exhaled breath. The Cr-WO_3 nanopowder coated platinum-alumina substrate was used in a sensor along with heated pair to analyse gases form exhaled breath such as nitric oxide, acetone ammonia, and some other trace gases among which acetone shows higher sensitivity.

The three nanosensor based microarray system was developed by Goumaet al. (2011) [116] for handheld breath biosensor for ammonia, carbon dioxide and isoprene. Also nanomaterial can be used for short- term follow up in case of lung tumour resection. Ionescuet et al. (2011) showed single-wall carbon nanotubes based array system to discriminate the VOCs in exhaled breath associated with multiple sclerosis through polycyclic aromatic hydrocarbons derivatives. [117]

The biosensors based on MGPNs, Platinum (Pt) nanoparticles and bio-recognition elements have been developed [118]. The performance of biosensors can be improved by modifying the morphology, size and density of Pt nanoparticles which are electrodeposited. The LOD of this biosensor is 0.3 μM, a long stable shelf-life (more than 1 month), 0.01–50 mM linear

sensing range and high selectivity. The stannic oxide (SnO_2) fibers (10 nm to 500 nm in length) were synthesized by Jungwoo Shin et al., (2013) using microphase separation technique between tin precursors and polymers [119]. Coating with Pt nanoparticles improves the acetone response of SnO_2 to acetone. The same biosensor can be used for lung cancer in diagnosis which this biomarker levels are increased.

Organic- layer capped gold nanoparticle (3-4 nm) based biosensors have been developed by Nakhleh et al. (2014) which shows reversible change in the resistance on exposure to ammonia. This technique is more sensitive as compared to GC-MS system and techniques using blood or urine for diagnosis of disease from exhaled breath [120].

The pilot study carried out by Gruber et al. (2014) proved the discriminating power of nanomaterial based array by demonstrating the VOCs pattern of HNSCC such as ethanol, undecane and 2-Propenenitrile [121]. This technique can differentiate HNSCC from benign tumors. The comparative study was performed by Bachar et al. (2013) between chemiresistor and QCM sensors in detecting polycyclic aromatic hydrocarbons [122]. It proved that chemiresistor arrays perform better at controlled humidity levels especially in dry atmosphere whereas latter under extremely variable humidity levels. The WO_3 nanostructured probe can selectively detect low level nitric oxide concentration in the presence of other VOCs.

A sensor for ammonia detection was developed using nanosized Si-doped Molybdenum trioxide particles (Si-doped α-MoO_3) [123]. Different nanomaterials used in biosensors for detecting different gases in exhaled breath are summarized in table 3.

Table 3. Nanomaterials used in different breath sensors

Materials	**Gases**	**Reference**
Gold nanoparticles (GN)	VOCs	[124]
GN and SWCN	VOCs	[125]
Functionalized gold nanoparticles	VOCs	[126]
Gold nanoparticles sensors (Thiol derivatized)	Ethanol, Acetone	[127]
MoO_3Nanosensor	Isoprene, CO_2, NH_3	[128]
Si-doped WO_3 Nanoparticle	Acetone	[129]
GN and Platinum nanoparticles based sensor	VOCs	[130]
Gold nanoparticles decorated polyaniline	VOCs	[131]
Carbon Nanotubes (Chemically functionalized)	Nitric Oxide	[132]
Hemi tubes of Pt-WO_3	Acetone	[133]
Si-doped α-MoO_3	Ammonia	[134]

Parameters used for characterization and validation

Sensitivity

For the diagnosis of disease based on presence of certain biomarkers require assays that can identify the molecules of interest in breath even as low as ppb levels. There are two ways by which the sensitivity of biosensors is increased by researchers: one through target based amplification as increase in target molecule concentration results in more fast and accurate detection. Another means is by signal based amplification where some catalytic entity increases the signal. Both of these techniques help to achieve higher sensitivity.

Selectivity and versatility

In case of biosensors, it is crucial that the target compound or biomarker of particular disease needs to be identified separately in presence of other molecules. A biosensor should be applicable in multiple diseases. It should also allow the personalized diagnosis.

Robustness and portability

The robustness, portability and cost-effectiveness of breath biosensors are very important to use it effectively and reliably by the healthcare professionals as well as patients. It is imperative to develop the biosensors which are handheld, easy-to-use and portable which will facilitate online application rather than large, complex instruments that are mainly used in laboratories and require trained personnel to handle it.

Challenges

The fluorescence-based techniques have replaced radioactivity-based assays used in biosensors to tackle the issues of safety, cost and field use. Although fluorescence-based assays allow point-of-care use; photo bleaching and cost-effectiveness are big challenges. Even though quantum dots have shown their applicability in different biosensors, other advantages must be identified. Also techniques used for making these materials in an

environmentally acceptable manner are required to translate it into commercially viable medical diagnostic applications.

Comparison on various parameters of different techniques

The VOCs in exhaled air is in the range of ppb or ppt levels for which highly sensitive and accurate system such as GC-MS is required. However its bulkiness, expenses, difficulty in use due to pre-concentration step and requirement of trained analysts to operate limits the use. Hence this technique is not useful for online detection of VOCs in exhaled air. Hence now techniques like SIFT –MS and PTR-MS are used for real time analysis however these techniques are less sensitive and specific as compared to GC-MS. The IMS requires high vacuum which increases the cost of the technique as well as sensitivity. The use of nanomaterial in breath analysing technique makes the sensor portable, inexpensive and applicable to real time use. Among this, chemiresistive type sensors or semiconductor- based sensor arrays have capacity to detect the VOCs in the range of ppm which is less as compared to other methods. The comparison of these techniques including their advantages, disadvantages along with detection limits are given in table 4.

Table 4. The advantages and disadvantages of different techniques and detection limits

Technique	**Advantages**	**Disadvantages**	**Limit of detection**
GC-MS	Highly selective and sensitive	Pre-concentration step is	ppt

		required. Space-consuming Not suitable for on-site analysis. Expensive	
PTR-MS	Real time analysis is possible	It cannot identify the compounds and range of detection is narrow. Space-consuming. Not suitable for on-site analysis. Expensive	ppb
SIFT-MS	Detection range is broad	Identification of compounds is not possible. Space-consuming Not suitable for on-site analysis Expensive	ppb to ppt
Sensor arrays	Portable and miniature. Real time analysis of compounds is possible.	Identification of compounds is not possible due to recognition of pattern.	-

IMS	Quick, accurate technique. Miniature, portable Low cost of analysis as compared to other techniques.	Humidity in breath samples may affect the ion-drift time. It cannot detect unknown compounds in breath sample	ppm to ppb

Regulatory approval of breath diagnostics

USFDA has the authority of reviewing such breath test products to CDRH. The premarket approval provisions are mentioned in the Act 21 CFR 814 which allows the agency to determine safety and effectiveness of such products. The 510k process is premarket submission which is made to FDA to show the safety and effectiveness of devices that are equivalent to legally marketed devices and do not need pre-market approval. CDRH has now collaborated with CDER. The CFR gives a list of classification of medical devices (Class I, II and III) depending on low, moderate or high risk associated with their use. The regulatory approval process is given below in figure 6.Also the list of FDA approved bio-diagnostics is given table 5.

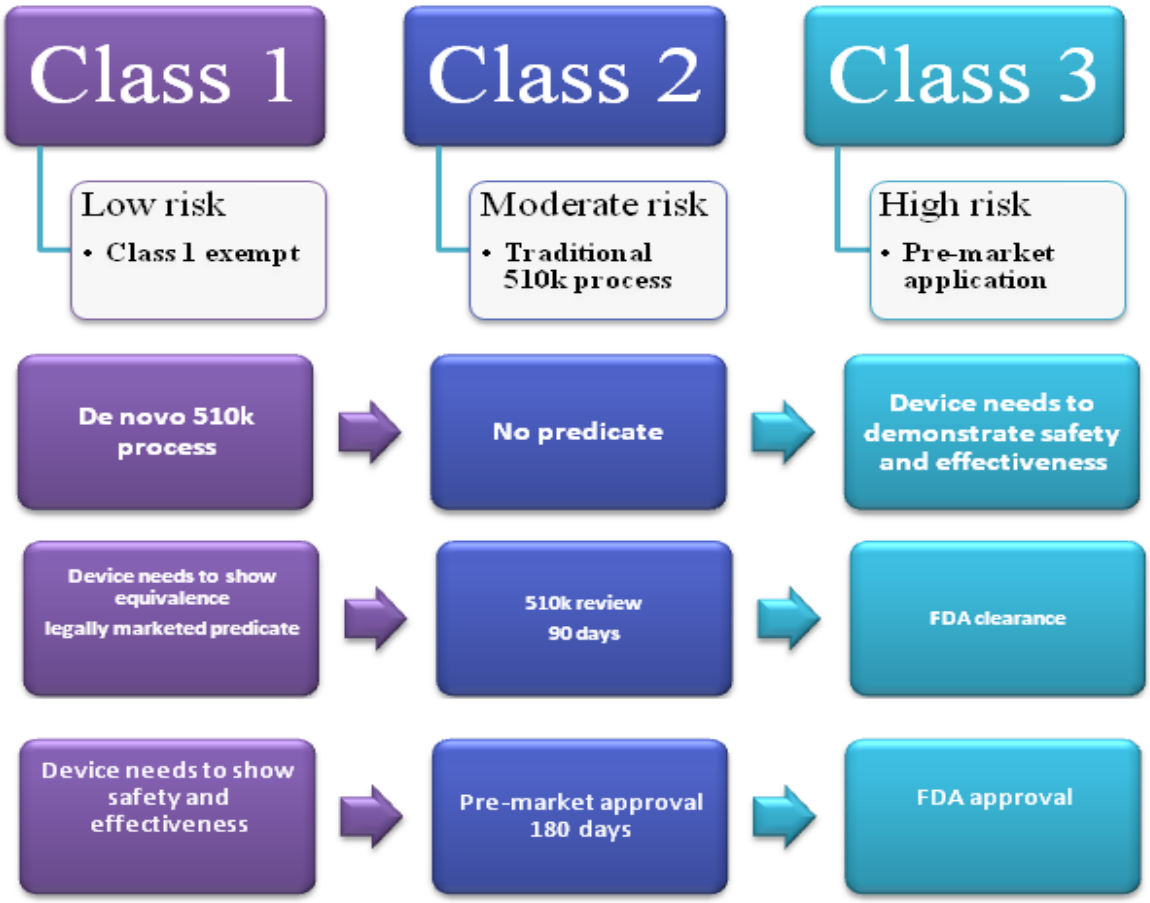

Figure 6. Procedure for Regulatory approval of biosensors

Table 5. List of FDA approved biodiagnostic test

Name of product	**Technique**	**Molecules of Interest**	**Use**	**Manufacturer**	**Year of approval**
Gastric Emptying Breath Test (GEBT)	GC-MS	Non-radioactive material in Spirulina	Gastro paresis	Advanced Breath Diagnostics, LLC	2015

BreathTek Urea BratheTest for *H. pylori* Kit Pediatric Urea Hydrolysis Rate Calculation Application (pUHR-CA), Version 1.0	Infrared spectroscopy	$^{13}CO_2/^{12}CO_2$	*Helicobacter pylori* infection	Otsuka America Pharmaceutical, Inc California, USA	2012
NIOX MINO	Electrochemical sensor	NO	Asthma and airway inflammation	Aerocrine (Solna, Sweden)	2008
ToxCO	Electrochemical sensor	CO	CO poisoning	BedfontScientific Ltd, United Kingdom	2008
Micro H_2 Breath Monitoring Device with	Electrochemical sensor	H_2	Lactose malabsorption	Micro Medical Ltd (Basingstoke, United	2004

Hydra Software Utility				Kingdom)	
Heartsbreath	GC-MS	Alkanes (C_4-C_{20})	Grade 3 heart allograft rejection	Menssana Research, Inc, New Jersey, USA	2004
NIOX^R	Chemiluminescense	NO	Asthma and airway inflammation	Aerocrine, Sweden	2003

Limitations of breath analysis

The analytical sensors that are available can measure low concentrations of the biomarkers for the detecting normal as well as abnormal function in human body, however fabricating a sensor which can detect small amount of VOCs in exhaled air to detect any metabolic disorder is very important. Still there are some technical problems associated with the analysis and sampling and lack of accurate standardization and variations in the results of different pilot studies. Some techniques require pre-concentration or cryophilization for detection down to pmol/L levels. And these steps are considered to be difficult to carry out as

well as standardise as that for blood or serum. Also, the procedures used for collecting samples should be validated against aerosols of known concentration [135]. Not all the upcoming techniques are marketable since the process for taking a breakthrough technique, materializing and developing it, is not straightforward process. Moreover, breath biodiagnosis can be expensive as compared to normal urine or blood analysis. Due to this, new breath biodiagnostics have found limited applications in medical practice.

Future scope

It is distinctly possible that the field of breath biodiagnostics will keep expanding in future. The cost-effectiveness, miniaturization of device and robustness are the key requirements on which studies are being carried out now. Moreover, the standardization of procedures to collect sample, analyze them and data reporting is something to focus on. For more advancement, the issues related to intellectual property and regulatory bodies should be solved and the critical needs in breath biodiagnostics should be addressed. For the successful research on new breath biodiagnostic requires transdisciplinary approach (collaboration of technical experts, health practitioners, regulatory agencies and commercial experts along with the inputs from the end users). These breath biodiagnostics hold great potential in revolutionizing the field of personalized diagnostics in future.

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