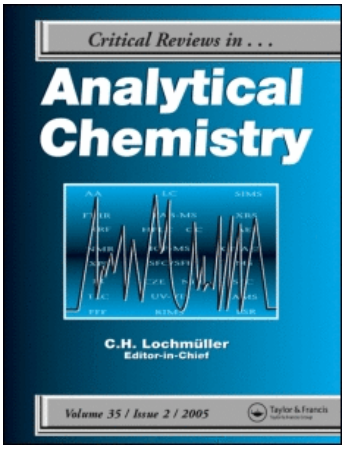

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Analytical Methods for Breath Investigation

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Analytical Methods for Breath Investigation

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Breath analysis is a non-invasive, painless and attractive diagnostic method without risk to the patients. It has been recommended as a convenient and complementary method for blood and urine analysis. Despite the advantages, breath analysis has not been introduced to medical diagnosis. Only few specific breath tests are available: ethanol in breath after alcohol ingestion, detection of $^{13}CO_2$ for diagnosis of H. pylori, NO test for evaluation of asthma and capnography, which is commonly used in intensive care and anesthesia. However, there has been an increase, notice in the interest of exhaled breath analysis, especially for the purpose of early cancer diagnosis. It is mainly the result of chromatographic methods development, which has been achieved in the last twenty years. The up-to-date methods of separation and detection of volatile organic compounds allow for achieving low detection limits and high precision of measurements. Sample pre-concentration methods, such as solid sorbents combined with thermal desorption or solid phase microextraction, also have significant importance. Great expectations are also connected with the development of techniques allowing on-line monitoring of breath, directly at the patient bed. This review depicts the issues connected with breath analysis. The main focus has been placed on technical and apparatus aspects. Analytical procedures concerning sample taking, Pre-concentration and analytical techniques have been also discussed.

Keywords Breath analysis, gas chromatography, mass spectrometry, sample preparation, medical diagnosis

INTRODUCTION

The concept that body fluids such as blood, urine and tissues can be analyzed to yield information for medical diagnosis is fundamental for modern medicine. However, breath analysis is a non-invasive and attractive diagnostic method without risk to the patient. Therefore, breath testing has been proposed as a complementary method to the classical methods, i.e., biopsy, blood, and urine sampling. Smelling the patient to help in the diagnosis of diseases has a long history. The ancient Greeks knew that patients suffering from some diseases emitted specific smells. Consequently, characteristic odors of breath have been used for centuries as indicators of "evil humors" that are now diagnosed as uncontrolled diabetes, liver disease, renal disease, bacterial infection or dental disease (1). The classic example of a symptom of disease is the sweet smell of a person suffering from diabetes resulting from the excessive amount of acetone emitted in the breath. However, the use of smells by doctors for diagnosis is becoming a lost art (2). In 1969, the modern breath analysis was started. Scientist such as Jansson (3) and Pauling (4) applied gas chromatography (GC) to detect more than 200 different volatile organic compounds (VOCs) in breath. They recognize that exhaled breath is a rather complex mixture. Determination of breath compounds attracts an increasing interest in clinical and toxicological analysis. Exhaled breath contains VOCs which can be divided into different chemical classes such as: hydrocarbons (methane, ethane, propane, butane, hexane, isoprene), alcohols (ethanol, propanol, butanol), ketones (acetone, 2-butanone), aldehydes (metanal, etanal, propanal, butanal, pentanal, hexanal), esters (ethyl acetate, butyl acetate), and heterocycles (2-methylfurane, 2-pentylfurane, 2,5-dimethylfurane). Moreover, exhaled straight hydrocarbons, such as ethane, and pentane have been widely investigated as non-invasive biomarkers of oxidative stress status (5–11). These molecules are produced following free radical attack on n-3 or n-6 polyunsaturated fatty acids found in cellular membranes (12).

Exhaled breath contains also inorganic gases such as carbon monoxide, nitric oxide, carbonyl sulfide and ammonia. Nitric oxide is produced in the airways and looked upon as a marker of asthma and other diseases involving airway inflammation. This molecule is a good example of the combination of biochemical

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knowledge, and analytical technique can be seen in the diagnostic application of NO.

Similarly, CO can be produced endogenously and, although it is toxic in high concentrations, it is now known to have various cytoprotective and anti-inflamantory effects in the body (13).

Besides volatile organic and inorganic compounds, breath contains also non, volatile molecules, i.e., leucotrienes, cytokines, prostaglandins, lipoxins, isoprostanes, S-nitrosothiols, and hydrogen peroxide (14). These compounds are supposed to be present as an aerosol. After condensation at sub-ambient temperature, exhaled breath condensate has to be analyzed by means of LC/MS or immuno assays (15).

The analysis of VOC, in the breath is of great importance in clinical chemistry, toxicology, detection of diseases, metabolic processes and for the control of therapy. However, at present, breath analysis encounters many problems such as: exhaled compounds appear in very low concentrations (typically ppb, ppt level) and are found in a matrix where interference with numerous exogenous compounds is expected. Gas chromatography and mass spectrometry (GC/MS) is well-suited technique for investigation of volatiles in exhaled breath. However, more recently introduced techniques such as proton transfer reaction mass spectrometry (PTR-MS) and selected ion flow tube mass spectrometry (SIFT-MS) are very promising for breath research. Both these techniques do not need an additional preconcentration step and separations. Therefore, PTR-MS and SIFT-MS can be very useful for on-line detection of volatiles in breath, also at a patient's bed.

This work will review the current status of clinical breath analysis, suggest reasons for this status and attempt to identify future directions for this field.

Sample Taking

Many volatile substances which exist in blood can be released from blood to exhaled air. However, molecules must exhibit significant vapor pressure. The VOCs detected in breath have both endogenous and exogenous origins. The endogenous compounds derive from normal as well as abnormal metabolism. Hence, the exogenous compounds derive from inspiratory air and also from ingested food and beverages (16). The exhaled air is a mixture of alveolar air and ambient air, which derives from respiratory dead space. It consists of the nose, mouth, pharynx, trachea and bronchi. Alveolar air is a part of air, which has been in contact in blood in alveoli structures. During mixed expiratory breath collection, the exhaled alveolar air containing VOCs is diluted by dead space air. Therefore, variation in analyte concentration and reproducibility of results can occur. This mode of sampling has been often used because it is easy to perform. However, the concentration of endogenous compounds is significantly lower, typically two-to-five fold, than those found in then alveolar breath (17). The concentration of endogenous compounds, which excretion is limited by perfusion increases at the end of expiration, and correlates well with the highest concentration of expired carbon dioxide, when the tidal pressure of carbon dioxide reaches a plateau (6). This approach allows for breath samples with the highest concentration of endogenous molecules. Therefore, during collection of alveolar breath, the real time measurement of the carbon dioxide profile (so called capnography) is necessary (16). The carbon dioxide-controlled sampling was also developed for collection of alveolar air from mechanically ventilated patients (18). Another important problem connected with sample taking involves the failure to distinguish endogenous compounds from inspiratory contaminants. Many compounds, which can be important as biomarkers, are widespread in ambient air, especially in clinic environment. Therefore, ambient air correction to minimize the inspiratory compounds is necessary (6). Typically, two methods of background correction are available. The first requires parallel sampling of breath and ambient air. Afterwards, the difference between concentrations can be calculated by subtracting inspiratory from expiratory level (19). In the second method, lungs wash-out involves breathing pure air for a certain time before sample taking. Although this procedure can be effective, it is also time consuming and uncommon in practice (20).

Containers for Breath Collection

Breath samples can be collected in stainless canisters or bags (21) specially treated to reduce sample losses due to adsorption (e.g., SUMMA canisters or Tedlar bags). An extensive review describing the single breath canisters method of alveolar breath sampling and analysis has been presented in literature (22). The reported application is used to assess exposure related to use of contaminated water, exposure to gasoline, swimmers exposure to trihalomethanes and occupational exposure to jet fuel. Moreover, the fused silica-lined canisters, have been used for the collection of breath prior to the analysis of sulfur compounds (methane thiol, dimethyl sulfide, dimethyl disulfide) (23). Tedlar bags are made of polyvinyl fluoride which is chemically inert and is also relatively resistant to gas permeation. Nevertheless, the carry-over problem should be taken into consideration due to adsorption and permeation of compounds, especially polar (i.e., aldehydes, ketones). Prior to reuse, the bags must be thoroughly cleaned by flushing with pure inert gas. It is recommended to perform analysis of the final flush to ensure that the background present in the bag is acceptable for its intended use. Tedlar bags are usually used for collection of alveolar and mixed exhaled human air, as well as animal breath (24, 25).

Sample Preparation Methods

The determination of volatile VOCs in exhaled air requires the detection of very low concentrations, hence the analytical methods employed must include a pre-concentration technique. The main methodologies currently utilized for pre-concentration of VOCs are sorption on adsorbent and cold trapping. Both procedures are tedious and require dedicated devices. Additionally, they suffer from particular problems, e.g., excess of water from the breath (26). Water may damage the stationary phase and a

retention time shift may occur. A number of water management procedures have been developed (27). However, the possible loss of polar and more volatile compounds during sampling or drying step needs to be considered.

THERMAL DESORPTION (TD)

TD is an alternative GC inlet system which has been particularly applied to air analysis. However, the analytes subjected to TD must be thermally stable to results in successful analysis. Otherwise, decomposition can occur. Two different modes are available for the concentration of VOCs — direct cry-trapping or trapping on solid sorbent followed by thermal desorption. The direct method involves freezing the volatiles in the cold trap maintained at a low temperature and later thermally released to the GC (28). This procedure consumes cooling medium (liquid nitrogen or carbon dioxide) and water ice can block the cold trap (29). The second method involves sorbent trapping, after which analytes are released from the sorbent by application of high temperature.

This recovery mechanism takes place through the application of heat, however, microwave desorbers are uncommon in practice (30). The desorbed compounds are subsequently refocused on a cold trap from which they are transferred in a narrow band to a chromatographic column for the analysis. The sorbent tubes desorption are made of stainless steel or borosilicate glass with precise dimensions to ensure leak-free connections between tube and desorber. Prior to any sampling, tubes need the conditioning for several hours at high temperature with a flow of ultra pure helium or nitrogen (31). However, reconditioning of tubes immediately before use is often recommended. The proper sorbent selection depends on a few important criteria, such as: adsorbents must first be able to trap target compounds and then release them efficiently during TD, high value of breakthrough volume (BTV), low affinity to water, and high thermal stability.

There are a variety of sorbents which can be used for TD. Table 1 lists some of the common adsorbents and their potential for trapping of analytes (29).

Adsorbent materials can be divided into three groups: organic polymers, inorganic adsorbents and carbon-based adsorbents. Inorganic sorbents (i.e., silica gel, zeolithes and alumina) are not useful in breath analysis due to high affinity to water.

Carbon-based materials such as carbon molecular sieves (CMS) and carbon blacks (CB) are frequently used materials from this family. These materials are commercially available as Carboxen, Carbosphere, Carbosieve and Ambersorb.

CMS have a sharp pore size distribution, high surface area and different groups exist on their surface. The CMS are used for enrichment of low molecular weight compounds (typically C2–C5). Important disadvantage of these adsorbents are absorption of water and strong or irreversible sorption of heavier molecules (> C6) (29).

TABLE 1
Characterization of adsorbent commonly used for enrichment of VOCs [33]

Adsorbent	Sampling range	T_{max} (°C)	Spec. surface area (m^2/g)	V_g H_2O 20°C (mL/g)	Density (g/mL)
Carbon molecuar sieves					
Carboxen 563	C2–C5	>400	510	778	0.53
Carboxen 564	C2–C5	>400	400	276	0.60
Carboxen 569	C2–C5	>400	485	257	0.58
Carboxen 1000	C2–C5	>400	1200	418	0.44
Carboxen 1001	C2–C5	>400	500	234	0.61
Carboxen 1003	C2–C5	>400	1000	79	0.46
Carbosieve SIII	C2–C5	>400	820	387	0.61
Carbospher	C2–C5	400	1000	779	—
Graphitized carbon blacks					
Carbotrap F	>C20	>400	5	—	0.66
Carbotrap C	C12–C20	>400	10	—	0.72
Carbotrap Y	C12–C20	>400	25	—	0.42
Carbotrap X	C3–C5	>400	250	—	0.41
Carbograph 5	C3–C5	>400	560	—	
Porous organic polymers					
Chromosorb 106 (styrene/divinylbenzene)	small molecules	250	750	173	—
Tenax TA poly-(2,6-diphenyl-)-*p*-phenylenoxide	C7–C26	350	35	39	0.25

Graphitized Carbon Black

GCBs are a well-known family of sorbents. These very hydrophobic adsorbents are also known for some positive characteristics, such as excellent thermal stability and low affinity to water. Water condensation problems can be partially prevented by sampling of VOCs, on traps packed with different combinations of hydrophobic materials. GCBs are available in many pore sizes. Adsorbents with low surface area, such as Carbotrap C (10 m^2/g) and Y (25 m^2/g) are more suited for less volatile compounds. Hence, higher specific area gives greater retention capacity due to increased van der Waals interaction forces, i.e., Carbograph X and 5 with surface areas of 250 and 560 m^2/g, respectively. These materials exhibit affinity to very volatile compounds and also enable sampling of low boiling and reactive hydrocarbons (i.e., 1,3-butadiene and isoprene) without decomposition or polymerization (32). Therefore, GCBs could be used for the sampling of VOCs in exhaled breath, which is high in humidity, without additional treatment.

Polymers

Many of the porous organic polymers derive from the stationary phase used in a packed GC column. Tenax is one of the most frequently used trapping materials. This polymer is hydrophobic and does not retain water. Due to its low surface area (30 m^2/g), very volatile compounds are not trapped. Whereby, it is proper material for trapping heavier compounds with carbon numbers higher than four. The co-precipitated GCB with Tenax was introduced on the market as a Tenax GR. This adsorbent should combine advantages of both materials.

Tenax does have drawbacks—upon heating depolymerization occurs and different compounds are emitted, such as: benzene, styrene, benzaldehyde, acetophenone, octanal, nonanal, decanal, 2,6-diphenylquinone and 2,6-diphenylhydroquinone (33). Therefore, emitted products can easily lead to the attribution of false positive data. In contrast, Chromosorb 106 has higher surface area (750 m^2/g) and lower thermal stability. In addition, it also has a higher background level.

Multi-bed Packings

Despite the number of commercially available adsorbents, a universal adsorbent does not exist. The single bed tube contains only one adsorbent, such as Tenax or GCB, generally useful only for a narrow range of organic analyte collection and it works well in limited cases. When analyzing wide molecular weight range samples such as C2-C10 aldehydes, ketones and hydrocarbons in breath, single bed packings fail (34). Weak sorbent (e.g., Tenax) does not trap low molecular weight compounds leading to severe quantitation problems. Carbon-based materials (strong sorbents) collect almost all VOCs but desorption of heavier compounds are very poor or impossible due to irreversible sorption. Since incomplete desorption occurs, the carry-over problem follows. Therefore, the multiple bed packings solve problems connected with low BTV of weak and irreversible sorption of strong adsorbents. These traps are usually packed with two layers—the first at the front of the tube contains weak adsorbents, which should trap heavier compounds. The second layer contains stronger adsorbents to trap more volatile compounds. The strongest adsorbent should be the last and retain the analytes with the highest volatility. Desorption flow is in reverse direction during the releasing process. Typical combinations include Tenax/CMS the or Tenax/medium GCB/CMS or low surface area GCB/medium GCB/CMS (31, 32).

Solid Phase Micro-extraction (SPME)

Among sample preparation methods, the SPME is one of the most frequently used. This technique was used for the determination of VOCs in various matrices. SPME offers a simple (no TD unit and modification of GC are necessary), inexpensive and solvent-free alternative to other pre-concentration techniques. This extraction technique has a number of advantages, such as simplicity, low cost and compatibility with analytical systems.

SPME was developed in the late 1980s by Arthur and Pawliszyn (35). Moreover, this technique was commercialized in 1993 by Supelco. This method utilizes a fused silica rod coated with a thin layer of stationary phase mounted in a holder. During extraction, the fiber is exposed to the sample, and analytes are adsorbed on the stationary phase and concentrated. Afterwards, the fiber is withdrawn in the holder and then analytes are thermally desorbed in GC. The optimum time of desorption influence recovery has to be found experimentally. The liner also affects SPME analysis. The small diameter liners (0.75 mm ID) for injectors reduce desorption time and improve peak shape (prevent peak broadening) (36). The diameter of liners is less important in the case of mega-bore columns. Moreover, typical septa are easy damaged with SPME needles and chromatographic analysis can be disturbed. Therefore, the septumless injectors are convenient for SPME. Furthermore, compounds which are released from the fiber need refocusing on the column (36). Several types of coatings are commercially available. They consist of polymers such as: PDMS, PA, Carbowax-DVB, Carboxen-PDMS, and PDMS-DVB. The coatings containing DVB polymer consist of porous particles of this material. Fibers prepared with three kinds of materials are also available, e.g., DVB-Carboxen-PDMS (37). Selection of the fiber is mainly based on the principle “like dissolves like”. Non-polar compounds have a high affinity to non-polar PDMS polymer. PA fiber is more polar and therefore it is useful for extraction of more polar species. These two kinds of polymeric materials extract via absorption mechanism. The mixed polymeric coatings such as Carbowax-DVB and PDMS-DVB extract the analytes via adsorption on the surface of the fiber (38) rather than typical absorption. The linear range of these fibers is shorter than that of PDMS. The PDMS-Carboxen coating is another kind of material containing CMS (Carboxen 1006, with surface area approximately 1000 m^2/g) and polymeric PDMS. In this case, two

TABLE 2
Commercial SPME fibers [40]

Coatings	Film thickness [μm]	Recommended application
Polydimethylsiloxane (PDMS)	100	Non-polar, volatile
	30	Non-polar, volatile, semi-volatile
	7	
Polydimethylsiloxane-divinylbenzene	65	Non-polar, semi Volatile
PDMS-DVB	—	Polar, volatile
Polyacrylate (PA)	85	Polar, volatile
Carboxen-polydimethylsiloxane (CAR-PDMS)	75/85	Volatile and very volatile,
Carbowax-divinylbenzene (CW-DVB)	65/75	trace analysis
Carbowax-templated resin (CW-TPR)	50	Volatile, polar
Divinylbenzene-carboxene-polydimethylsiloxane (DVB-CAR-PDMS)	50/30	Polar, HPLC
	—	C3-C20, broad range of polarities

different physicochemical mechanisms operate - adsorption and absorption. The Carboxen-PDMS was designed for determination of trace amounts of VOCs and gases. Comercially available coatings are presented in Table 2 (39).

Initially, the extractions of volatile organic contaminants such as BTEX from water and many other applications were developed during the 1990s. Furthermore, the SPME technique has been applied to environmental analysis (40, 41) and more recently to the analysis of drugs in biological matrices (36, 37, 39), as well as for the analysis of VOCs, in human breath (42). Pawliszyn used a SPME device, which was mounted a short piece of tube and was exposed to the human breath. Different fibers were evaluated for determination of ethanol, isoprene and acetone. The limits of detection (LOD) were of 5.8 nM ethanol, 1.8 nM acetone and 0.3 nM isoprene (42). Spinhirne and co-workwers applied DVB/Carb/ PDMS-coated fibers for determination of acetone, 2-butanone, toluene, tetradecane, pentadecane, nonanal and decanal in breath (43). Isoprene was determined in breath by Hyspler et al. using Carboxen/PDMS (44). The RSD was in the range 4.8–14.6% and the LOD was on the level 0.3 nM. Yu et al. analyzed benzene, styrene, propylobenzene, decane and undecane in breath by means of 100 m PDMS fiber (45). They reported detection limits in 0.012–1.26 ng/mL range, limits of quantitation in the 0.04–4.2 ng/mL range and RSD=3.7–9.8, depending upon the analyte. Recently, Carboxen/SPME was also applied for investigation of emission from stomach cancer and breath of patients suffering from stomach cancer (46).

Derivatization

In certain instances, enhancements in the selectivity and sensitivity can be accomplished by derivatization. As in the case of other sample preparation methods the derivatization can be also used in SPME for the chemical transformation of the analyte to a more suitable form for GC. This process reduces the polarity of compounds and improves the extraction efficiency and detection (36). There are several approaches to derivatization in combination with SPME. However, in breath analysis the "on fiber derivatization" is the most suitable. Prior to extraction, the fiber is loaded with derivatization agent (typically vapors or diluted solution). Afterwards, the fiber is exposed to the components of the sample and analytes are simultaneously extracted and converted to derivative. Converted compounds have a high affinity to coating. Martos and Pawliszyn used PDMS/DVB fiber impregnated with O-(2,3,4,5,6-pentafluorobenzyl)-hydroxylamine hydrochloride (PFBHA) for measurement of formaldehyde in the air (47). This involved the formation of oxime derivative of the analyte. The calibration was linear in the range of 15–3200 ppbv ($r^2 = 0.9995$) with LOD of 4.6 ppbv for 300 seconds sampling time. Precision was on the level 2–12%. Recently, derivatization has been applied for investigations of blood of lung cancer patients. Hexanal and heptanal have been analyzed in breath sample, with PDMS/DVB fiber.

The fiber was firstly loaded with PFBHA at 25°C for 10 minutes and afterwards, the fiber was exposed to blood at 60°C, for 8 minutes. The LODs were on the level 0.006 mM for hexanal and 0.005 nM for heptanal, RSD was below 8.5%, and recovery ranged from 89 to 95% (48).

A similar method was also used for determination of carbonyl compounds in human breath. PFBHA has been used for derivatization of acetone by forming oxime. Derivatization agent in solution was firstly adsorbed on PDMS/DVB fiber and then the fiber was exposed to exhaled breath for 4 min. Analysis was performed by means of GC/MS. This method provided low LOD of 0.049 ppbv, RSD of 3.4% and linear range 0.073–6.64 ppmv (49).

At present, SPME is rather limited to the detection of compounds with relatively high concentration in human breath. However, improvement, in sampling devices and introduction of new fibres should help to achieve better sensitivity.

Analytical Techniques

Samples are usually analyzed by means gas chromatography. Different detectors are commonly used, such as: flame ionisation detector (FID) (50), flame photometric (FPD) (51), photoionisation detector (PID) (52, 53) and pulsed discharge helium ionization detector (PDHID) (54). However, these detectors can be used only in limited cases, when identification of compounds has been confirmed. Only GC/MS has the ability to

unambiguously identify many chemicals. Therefore, capillary GC and MS is the method of choice in order to identify exhaled breath constituents. High sensitivity and selectivity are the most prominent advantages of this technique. The GC/MS is often capable of differentiating of co-eluting compounds. However, since breath constituents (especially at ppb level) are identified only by comparing mass spectra to libraries, the GC/MS identification is often tentative or mistaken. In general, two kinds of MS are widely available: quadrupole (Q) and ion trap (IT). In case of IT, the ions are produced in a ion source, stored in the trap and rejected according to their m/z ratio to obtain a spectrum. The IT instrument is more sensitive than Q and useful for MS/MS experiments. However, the main disadvantage is that the spectra are often modified by self-chemical ionization in addition to collision-induced dissociation. Therefore, the interpretation of spectrum can be difficult with regard of these processes, particularly in case of polar compounds (aldehydes, alcohols, ketones, etc.), which are important compounds in exhaled breath analysis (55). During last decade, GC and GC/MS techniques have been widely used for determination of majority of compounds in breath, including hydrocarbons, aldehydes, ketones, alcohols (56–60). GC/MS has been also applied for investigation of oxidative stress, organ rejection, and halithosis (19, 61).

Multi-dimensional Gas Chromatography (GCX GC)

The separation of breath constituents in a conventional one-dimensional gas chromatography can be difficult. Multidimensional GC is used for separation of compounds that coelute, e.g., polar compounds that are not resolved on nonpolar column. The simplest form of multidimensional GC is the heart cutting technique in which a portion of a chromatogram from a first column is introduced to a second column with a different stationary phase. To make the approach more comprehensive, all of the sample should be subjected to two independent separation mechanisms. One such approach is the use of a high speed second column that successively processes all of the samples as it elutes from the first column. The use of on column thermal modulation as means of introducing a sample to a second column has been demonstrated. Such a system has two different columns, which are connected in series. Peaks from the first column are chopped in series of concentration pulses as they are introduced in the second column. The second column is operated under fast GC conditions in which a chromatogram is generated in a few seconds (62). Principles of comprehensive GC have been recently described (63) by Mariott and Shelie. Comprehensive GC was applied for complex hydrocarbon samples (64), environmental (65), and drugs analysis (66). Recently, Libardoni et al. have developed comprehensive GC for the analysis of exhaled breath (67). The FID with 200 Hz electrometer was used as a detection system. Samples (800 mL) were collected in Tedlar bags, and samples were passed through the multi-bed sorption trap (Carbopack Y, B, X and Carboxen 1, 000), afterwards, volatiles were thermally released to GC. Two different columns such as Rtx-1 and Rtx-WAX have been used for separation. The air cooled thermal modulator, instead of a typical liquid nitrogen cooler was applied for modulation. A standard test mixture containing 13 compounds was used for evaluation of the method. A linear dynamic range of three orders of magnitude, LODs in the range 148 – 274 ppt, and RSD below 1% have been demonstrated. Authors used comprehensive GC for the analysis of real breath samples VOCs and identified mainly hydrocarbons.

Time of Flight Mass Spectrometry (TOF-MS)

With conventional MS, the scan time is comparable to the elution bandwidth and the concentration of the compound in the ion source will change during the scan. Thus, the spectrum is skewed for individual compounds. A rapid MS which is based on TOF technology can be an excellent detector for GC. The TOF instrument provides fast data acquisition rate (20 – 500 spectra/second) or high mass resolution (7, 000 fwhm) (68). In addition, the ion ratios for a spectrum do not change across the chromatographic peak. Fast GC can be also applied with TOF. It is possible due to rapid mass spectra acquisition, which provides complete mass spectra in a period shorter than the elution bandwidths of from the GC. Hence, the mass spectrum of each compound has the same ion ratio of the fragments. The software de-convolutes chromatographic co-elutions and extracts the spectrum of each compound. Pure spectra can be used for library identification of individual compounds. Both, fast and high resolution GC-TOF-MS instruments have been applied in various fields such as flavors, drugs, and pesticides analysis (69, 70). Hence, the TOF spectrometers have great potential as a tool for breath investigation.

Proton Transfer Reaction Mass Spectrometry (PTR-MS)

GC cannot be used for continuous measurement of VOCs and also needs the sample preparation methods. PTR-MS is a relatively new technique for rapid and online measurements of VOCs in breath measurement (71, 72). PTR-MS instruments consist of few parts: an ion source where H3O+ ions are produced, a drift tube, a transition chamber, mass analyzer, and detector of ions (73). In the drift tube, compounds from the sample are ionized by proton transfer reactions with H_3O^+ primary ions. This reaction takes place when the proton affinity of the trace compound is higher than that of water (E = 166.5 kcal/mol = 7.16 eV). A major advantage of using H_3O^+ as the primary reactant ion is that the proton affinity of the normal constituents of air (NO, O_2, CO, CO_2, N_2) is lower than that of H_2O molecules. Therefore, these compounds do not interfere with the measurement because they do not react with H_3O+ ions (74).

PTR-MS characterizes the substances only according to their mass-to-charge ratio. Hence, the identification of unknown compounds must be provided by other techniques, e.g. GC-MS. Additionally, compounds with identical molecular weight cannot be measured independently by using PTR-MS.

This technique enables on-line measurement of molecules in breath without pre-concentration or chromatographic

separation, and it can be a excellent tool for investigation of physiological and pathophysiological processes. PTR-MS was applied for profiling of exhaled breath (75), detection of sulphur compounds (diallyl sulphide, disulphide, allyl methyl disulphide) in breath (76), and for monitoring of breath during sleep (71).

Selected Ion Flow Tube Mass Spectrometry (SIFT-MS)

Disken et al. described the use of the SIFT-MS for real time measurement of VOCs in breath (77). In the SIFT instrument, reactant ions (pre-cursors) are formed by electron impact (EI) or microwave discharge in a carrier gas in a separate ionization region. The trace gases in the sample react with selected precursors (H_3O^+, NO^+ or O_2^+) in the sample injection region leading to characteristic product ions. The most commonly used precursor ion is protoned water because this reacts with a wide range of organic species, by non-dissociative proton transfer. The product ions are sampled to Q=MS. This mass filter can work in two modes: full scan (FS), which provides mass spectra of compounds, or multiple ion monitoring (MIM), which detects only selected ions with higher sensitivity (78). The FS mode is principally used to identify the compounds present in the sample, whereby MIM can be applied for the target studies because it is more sensitive and accurate for quantification. The response time is close to 20 milli seconds, and hence, it is possible to observe real time fluctuations in trace gas concentrations (79). The PTR-MS technique was applied for the monitoring of ammonia, acetone, isoprene and ethanol in breath of volunteers (80, 81). This technique is used for investigation of ethanol metabolism (82), control of ovulatory cycle (83) and for smokers' and passive smokers breath, (84) exposure to volatile solvents (85).

Diode Lasers

Laser spectroscopy based on tunable diode laser adsorption spectroscopy (TDLAS) and leak cavity-out spectroscopy has been used in the real time measurement of gases in breath at ppb level. The operating principle of TDLAS is based on the ability of certain semi-conductors to act as IR laser sources when a p-n junction is formed in the crystal and electrical current is applied to the diode. By changing the temperature of semiconductor, the laser can be tuned over several hundred wave numbers.

As the line widths of tunable diode lasers are rather narrow, absorbencies due to a single rotation line can be measured. Especially in combination with quantum cascade lasers, this technique can be applied in biomedical areas' for simultaneous detection of CO, CO_2, NH_3, NO, ethane, and COS (86–93).

Micro-sensors

Sensors are well known in clinical practice (e.g., CO_2/O_2 blood and breath gases analysis). Sensor arrays are used to distinguish between different gases by the pattern of response of different individual sensors. Several micro-sensors are currently being used for the detection of VOCs, including the surface acoustic wave arrays, and electrochemical arrays, ionic conductors.

This technology has been used in the breath analysis area for monitoring of hydrogen peroxide in exhaled breath (94), analysis of diabetic patient's breath (95), malodour detection (96), NO and acetone detection (97). Zhang et al. investigated light addressable potentiometric sensors (LAPS) as a tool for the diagnosis of diabetes (98). The measured acetone concentrations in breath were also compared with the GC method. However, surface acoustic wave sensor seems to one of the most promising solution in this field (99). Electronic noses (e-noses) have been extensively used to analyze complex odorous foodstuffs, drinks, as well as for identification of pathogens infections (100). E-noses mainly employ metal oxide, conducting polymer resistive gas sensors and quartz microbalance. Sensors promise a number of advantages as compared to chromatographic and spectroscopic techniques which are currently used in breath analysis: they are easy to use and specific for certain applications.

CONCLUSIONS

Analysis of VOCs in breath can provides valuable information related to health and diseases and unlike other existing methods, it is non-invasive. Exhaled breath is a rather simpler matrix when compared to the preparation and analysis of other biological specimens, such as blood and urine. In addition, sampling procedures make breath collection safe and easy even for non-clinical personnel. Modern equipment (GC, GC/MS, SIFT-MS, PTR MS, laser spectroscopy) allows the fingerprint of human breath to be obtaines. However, sample preparation methods, analitycal instruments and skilled laboratory staff are necessary to obtain reliable date. At the present time the GC/MS technique is still " the gold standard" for breath analysis due to its flexibility and powerful identification potential.

The current pre-concentration and separation methods suffer from particular problems (i.e., poor sensitivity, coelution, carry-over problem, etc). Therefore, new kinds of adsorbents, fast and comprehensive GC, and automatization can help to achieve progress. The important technique is the membrane extraction with sorbent interface (MESI) introduced to breath analysis by Pawliszyn (101). This method combines sampling and pre-concentration in one step.

However, development of real time measurement methods such as laser spectroscopy or sensors for direct detection of disease markers should be valuable for medical diagnostics. Nevertheless, breath analysis is not used in common medical practice, due to lack of standarized sampling methodology and evaluation of data. In addition, the biochemical pathway of exhaled VOCs is usually unknown, with the exception of few examples.

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