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REVIEW

Human exhaled air analytics: biomarkers of diseases

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ABSTRACT: Over the last few years, breath analysis for the routine monitoring of metabolic disorders has attracted a considerable amount of scientific interest, especially since breath sampling is a non-invasive technique, totally painless and agreeable to patients. The investigation of human breath samples with various analytical methods has shown a correlation between the concentration patterns of volatile organic compounds (VOCs) and the occurrence of certain diseases. It has been demonstrated that modern analytical instruments allow the determination of many compounds found in human breath both in normal and anomalous concentrations. The composition of exhaled breath in patients with, for example, lung cancer, inflammatory lung disease, hepatic or renal dysfunction and diabetes contains valuable information. Furthermore, the detection and quantification of oxidative stress, and its monitoring during surgery based on composition of exhaled breath, have made considerable progress. This paper gives an overview of the analytical techniques used for sample collection, preconcentration and analysis of human breath composition. The diagnostic potential of different disease-marking substances in human breath for a selection of diseases and the clinical applications of breath analysis are discussed. Copyright © 2007 John Wiley & Sons, Ltd.

KEYWORDS: volatile organic compounds; exhaled breath; breath analysis; biomarkers of disease; gas chromatography; mass spectrometry

INTRODUCTION

Breath testing dates back to the early history of medicine. Ancient physicians knew that the odour of a patient's breath is associated with some diseases and may give an insight into physiological and pathophysiological processes in the body (Ma *et al.*, 2006). For example, the sweet smell of acetone in breath accompanies uncontrolled diabetes, a fishy smell is a result of liver disease and a urine-like smell is related to kidney failure (Di Francesco *et al.*, 2005; Libardoni *et al.*, 2006). Therefore, they tried to recognize illnesses by the specific smell of human breath. Modern breath testing began in the 1970s when Linus Pauling detected (though without identifying) around 200 different volatile organic compounds (VOCs) in exhaled air by gas chromatography. He proved that normal human breath is a gas of rather complex composition. In the last 30 years, many of these compounds have been identified. It has turned out that exhaled breath may contains traces of many VOCs, like acetone, methanol or isoprene (Miekisch *et al.*, 2004; Zolotov, 2005) and even small inorganic molecules like nitric oxide (Dweik, 2005), carbon monoxide (Kharitonov and Barnes, 2001) or carbonyl sulfide (Studer *et al.*, 2001).

The analysis of exhaled breath has been proposed as convenient and safe complementary method to blood and urine sampling (Abbott *et al.*, 2003; Amann and Smith, 2005). Breath analysis has a number of advantages as compared with traditional diagnostic techniques. It is a non-invasive and painlessness procedure, and its sampling does not require skilled medical staff (Spinhirne *et al.*, 2003). Even though up to 3000 compounds may be detected in different persons' breath (Phillips *et al.*, 1999a,b), the matrix of exhaled air is less complex than that of blood or other body fluids. This makes breath analysis simpler to perform (Prado *et al.*, 2003; Libardoni *et al.*, 2006). Despite the obvious advantages it presents for routine biological monitoring, it has not yet been introduced as a standard tool into clinical diagnosis. Only certain specific breath tests, for example, based on ingestion of ^{13}C-labeled substances and subsequent detection of the $^{13}CO_2$–$^{12}CO_2$ ratio in breath (Modak, 2005) already have a definite place in

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Abbreviations used: DMPP, dimethylallyl pyrophosphate; PUFA, polyunsaturated fatty acid; VOCs, volatile organic compounds.

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clinical applications. Standard capnography, determining the CO_2 concentration pattern with breath-to-breath resolution has also been used for a long time in anesthesia and general intensive care. Many endogenously produced compounds, on the other hand, which would be valuable for medical diagnosis and therapeutic monitoring, do not yet belong to the armoury of the clinician. The reason is the lack of suitable and simple substance separation and identification techniques, and the lack of normalization and standardization methods (Abbott *et al.*, 2003). Moreover, the physiological meaning and biochemical origin of most of volatile compounds are still not known. At present, typical routine applications of breath tests include the evaluation of ethanol and acetaldehyde concentrations (the latter as a metabolic product of ethanol) in expiratory gas after alcohol consumption (Mitsubayashi *et al.*, 2005). The ^{13}C-urea or ammonia breath tests are considered to be the most sensitive and specific techniques for diagnosis of *H. pylori* infection and the nitric oxide test is used to recognize asthma (Ochiai *et al.*, 2001). In addition, there are other volatile compounds that can be utilized to recognize certain diseases (Table 1). Nevertheless, biochemical pathways of their generation, origin and distribution are only partly understood. The biochemical origin of methylated hydrocarbons, for example, is not understood at all.

One of the most interesting clinical applications would be the detection of lung cancer. It is a major cause of death among adult and its incidence is increasing globally. A number of studies have been carried out in order to characterize substances in breath samples from patients with and without lung cancer (O'Neill *et al.*, 1988; Petri *et al.*, 1988; Phillips *et al.*, 1999a,b; Deng *et al.*, 2004a; Poli *et al.*, 2005; Yu *et al.*, 2005; Phillips *et al.*, 2006). In 1988, two groups of researchers separately reported some VOCs as potential lung cancer marking substances (O'Neill *et al.*, 1988; Petri *et al.*, 1988). Later, Phillips *et al.* (1999a,b) suggested a combination of 22 breath VOCs, including methylated alkanes and benzene derivatives, for detection of lung cancer. Considerable evidence supports the hypothesis that oxidative stress may be associated with lung cancer (Phillips *et al.*, 1999a,b). A limitation of this research is that only few clinical samples were studied, and that indoor air-related artefacts may distort the data. Although VOCs as markers of lung cancer differ in different reports, the obtained results have shown that there are significant variations between lung cancer patients' breath and the exhaled breath of healthy volunteers. Therefore, further studies are needed to confirm the usefulness of breath VOCs for detecting lung cancer in general population (Phillips *et al.*, 1999a, b, 2006; Deng *et al.*, 2004a; Yu *et al.*, 2005). The most important biomarkers detected in breath of cancer patients are summarized in Table 2.

There are a few VOCs with relatively high concentrations in exhaled breath (with median concentrations in brackets; Turner *et al.*, 2006a–d): ammonia (833 ppb), acetone (477 ppb), isoprene (106 ppb), methanol (461 ppb), ethanol (112 ppb), propanol (18 ppb) and acetaldehyde (22 ppb). Nevertheless, most of the VOCs in exhaled breath have typical concentration ranges which are lower than 1 ppb. The concentrations of VOCs in breath are *not* generally higher in the diseased state when compared with healthy volunteers, even though this is the case for some prominent molecular species such as for acetone with untreated diabetes or isoprene (in some patients) and ammonia for renal impairment.

Volatile components in exhaled breath may be produced in the lungs or nasal cavity (such is the case for nitric oxide) or may be of systemic origin and be exhaled after diffusion from blood into the alveoli (Abbott *et al.*, 2003; Di Francesco *et al.*, 2005). Combined analysis of exhaled breath and urine headspace may be useful if a volatile substance is of systemic origin, when it may be expected to be contained in both exhaled breath and urine headspace. Diffusion of VOCs from blood to alveolar air across the alveolar–capillary membrane depends on their physicochemical properties, such as polarity, solubility in fat, Henry partition constant $K_{air/water}$ and volatility. Therefore, analysis of VOCs in breath does not immediately allow determination of the respective concentrations in blood (Abbott *et al.*, 2003). Different classes of substances (e.g. hydrophilic or hydrophobic substances) may show rather different behaviour in this respect (Libardoni *et al.*, 2006).

Table 1. Some examples for disease markers (Amann *et al.*, 2005)

VOCs	Disease
Ethane and pentane	Oxidative stress
Methylated hydrocarbons	Lung or breast cancer
Hydrocarbons (especially ethane and pentane)	Oxidative stress
Isoprene	Cholesterol metabolism
Acetone	Diabetes mellitus, ketonemia
Sulfur-containing compounds (dimethylsulfide, methyl mercaptane, ethyl mercaptane)	Liver impairment
Nitrogen-containing compounds (ammonia, dimethylamine, trimethylamine)	Uremia, kidney impairment

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Table 2. Identified breath biomarkers of cancer

Class	Compounds
Alcohols	2-Propanol (Phillips *et al.*, 2006)
Aldehydes	Formaldehyde (Wehinger *et al.*, 2007), acetaldehyde (Smith *et al.*, 2003), heptanal (Phillips *et al.*, 1999a,b; Deng *et al.*, 2004a,b; Phillips *et al.*, 2006); hexanal (Phillips *et al.*, 1999a,b; Deng *et al.*, 2004a,b)
Ketones	1-Phenylethanone (Phillips *et al.*, 1999a,b)
Hydrocarbons	Benzene (Yu *et al.*, 2005; Phillips *et al.*, 1999a,b; Poli *et al.*, 2005); toluene (Poli *et al.*, 2005); styrene (Yu *et al.*, 2005; Phillips *et al.*, 1999a,b; Poli *et al.*, 2005); xylenes isomers (Phillips *et al.*, 1999a,b; Poli *et al.*, 2005); trimethylbenzenes isomers (Phillips *et al.*, 1999a,b; Poli *et al.*, 2005); propylbenzene (Yu *et al.*, 2005; Phillips *et al.*, 1999a,b); ethylbenzene (Poli *et al.*, 2005) 2-Methylheptane (Phillips *et al.*, 1999a,b); 3-methylnonane (Phillips *et al.*, 1999a,b); 3-methyloctane (Phillips *et al.*, 1999a,b); 2-methylpentane (Poli *et al.*, 2005); 2,2,4,6,6-pentamethylheptane (Phillips *et al.*, 1999a,b; Poli *et al.*, 2005); 2,4-dimethylheptane (Phillips *et al.*, 1999a,b); pentane (Poli *et al.*, 2005); heptane (Poli *et al.*, 2005); octane (Poli *et al.*, 2005); decane (Yu *et al.*, 2005; Phillips *et al.*, 1999a,b; Poli *et al.*, 2005); undecane (Yu *et al.*, 2005; Phillips *et al.*, 1999a,b); methylcyclopentane (Phillips *et al.*, 1999a,b); isoprene (Phillips *et al.*, 1999a,b; Poli *et al.*, 2005); 1-hexene (Phillips *et al.*, 1999a,b) 1-Heptene (Phillips *et al.*, 1999a,b); cyclohexane (Phillips *et al.*, 1999a,b); 1-methyl-2-pentylcyclopropane (Phillips *et al.*, 1999a,b)
Esters	Isopropyl myristate (Phillips *et al.*, 2006)
Heterocycles	2,3-Dihydro-1-phenyl-4(1H)-quinazoline (Phillips *et al.*, 2006)

Table 3. Volatile compounds physiological origin

Compound	Physiological basis
Acetaldehyde	Ethanol metabolism (Norberg *et al.*, 2003; Turner *et al.*, 2006)
Acetone	Decarboxylation of acetoacetate and acetyl-CoA
Ethane	Lipid peroxidation (Schubert *et al.*, 2004)
Ethylene	Lipid peroxidation (Risby, 2005)
Hydrogen	Gut bacteria (Ledochowski *et al.*, 2001)
Isoprene	Cholesterol biosynthesis (Stone *et al.*, 1993)
Methane	Gut bacteria (Ledochowski *et al.*, 2001)
Methylamine	Protein metabolism (Risby, 2005)
Pentane	Lipid peroxidation (Schubert *et al.*, 2004)

Endogenous volatile markers and their clinical applications

There are approximately 3000 VOCs that have been detected at least once in human breath, and most breath samples usually contain more than 200 VOCs (Phillips *et al.*, 1999a,b; Teshima *et al.*, 2005). Exhaled breath includes small inorganic compounds, such as NO, O_2, CO_2, volatile organic compounds (hydrocarbons, alcohols, ketones, aldehydes, esters) and nonvolatile substances such as isoprostanes, cytokines, leukotrienes and hydrogen peroxide, which can be found in breath condensate (Montuschi *et al.*, 2002). These compounds are chemically very diverse. Major VOCs present in human breath gas include isoprene, acetone, ethanol, methanol and other alcohols, as well as alkanes (Libardoni *et al.*, 2006). It is well known that the profiles of breath volatiles in patients suffering from particular diseases are different from the normal volatiles profile.

The source and physiological function of most of VOCs, however, are still not known. In most cases, specific metabolic pathways that give rise to their exhalation are unknown (Zolotov, 2005). Some VOCs in breath gas derive from the environment, because they may be absorbed as contaminants through the skin or taken up via inhalation or ingestion (Ma *et al.*, 2006). These compounds are possibly metabolized in the body, and then excreted by expiration. Other VOCs are generated in the body, as products of metabolic processes or activity of intestinal bacteria. Endogenous biomarkers are not yet commonly used for diagnostic purposes. A summary of physiological origins of selected endogenous breath molecules is presented in Table 3. Factors affecting the great variability in the composition of human breath include physical condition, general health of the subject, food intake, environmental influences and overall lifestyle (Amann *et al.*, 2004; Libardoni *et al.*, 2006).

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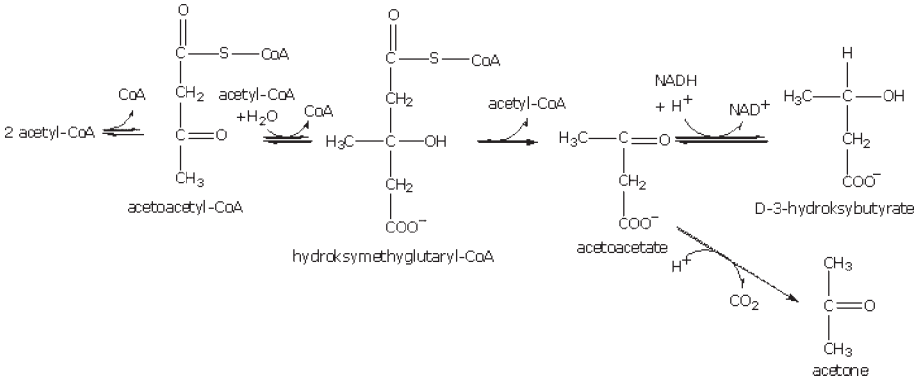

Figure 1. Generation of ketone bodies via decarboxylation of excess acetyl-CoA.

Acetone

Acetone is one of the most abundant VOCs in human breath. Acetone is linked to dextrose metabolism and lipolysis (Miekisch *et al.*, 2004). It is produced by hepatocytes via the decarboxylation of excess acetyl-CoA, which comes from fatty acid β-oxidation (Fig. 1). Acetone is ultimately formed by the decarboxylation of acetoacetate, which is derived from lipolysis or lipid peroxidation (Ma *et al.*, 2006). Ketone bodies in the blood are increased in ketonemic subjects, in patients with uncontrolled diabetes and during starvation (Deng *et al.*, 2004b). Ketone bodies are produced when the body uses fat instead of glucose for energy. For this reason acetyl-CoA cannot enter the Krebs cycle, because of a lack of oxalacetate, which is used to produce glucose in the gluconeogenesis pathway (Libardoni *et al.*, 2006; Ma *et al.*, 2006).

Isoprene

One of the most common VOCs in exhaled air is isoprene, which is present among hydrocarbons in human breath in the highest concentrations. It is synthesized as a precursor of many important compounds and is always present in human breath. Isoprene is formed along the mevalonic pathway of cholesterol synthesis in the cytosolic fraction (Fig. 2; Stone *et al.*, 1993; Hyšpler *et al.*, 2000; Ma *et al.*, 2006).

In rat liver cytosol isoprene is produced by an acid catalysed elimination reaction from dimethylallyl pyrophosphate (DMPP). In certain plants, this reaction is catalysed by an enzyme containing Mg^{2+}. In mammalian tissue isoprene may be produced by a reaction catalysed by the Mg^{2+}-dependent isopentenyl pyrophosphate isomerase. This enzyme catalyses the interconversion of isopentenyl pyrophosphate and dimethylallyl pyrophosphate. Another possible source of isoprene is radically mediated *in vivo* peroxidation of squalene (McGrath *et al.*, 2001). Isoprene has been identified as marker of disorders in cholesterol metabolism such as hypercholesterolemia. Isoprene increases up to an age of about 25 years, its concentrations are significantly lower in children (Miekisch *et al.*, 2004).

It would be interesting to know if the excretion of isoprene by the lungs has some metabolic background (instead of just being an accidental byproduct from biosynthesis of cholesterol). Incidentally, a non-mevalonate pathway for the production of isoprene has been detected in certain plants (Arigoni *et al.*, 1997; Eisenreich *et al.*, 2004).

Hydrocarbons

The presence of the straight-chain hydrocarbons (ethane and pentane) in exhaled air is a result of lipid peroxidation of polyunsaturated fatty acids (PUFAs) found in cellular membranes. Lipid peroxidation is a free radical-mediated process, where PUFAs are prefentially affected, leading to the formation of a wide variety of carbonyl secondary oxidation products, which are successively excreted in the breath (Fig. 3; Handelman *et al.*, 2003; Di Francesco *et al.*, 2005). Oxidative stress refers to a condition where the balance of free radical production and antioxidant systems is disturbed in favour of pro-oxidant free radicals (Spinhirne *et al.*, 2003). Oxidative stress occurs as a pathological mechanism in aging and several diseases (Phillips *et al.*, 2000; Di Francesco *et al.*, 2005). For instance, increased levels of ethane and pentane have been related to oxidative stress in breast cancer, heart transplant rejection, bronchial asthma, rheumatoid arthritis, acute myocardial infarction and schizophrenia (Aghdassi and Allard, 2000; Phillips *et al.*, 2000; Moretti *et al.*, 2004).

Ethane and pentane are the main volatile hydrocarbons formed during the decomposition of ω-3 and ω-6 polyunsaturated fatty acids (linolenic, linoleic and arachidonic acid; Handelman *et al.*, 2003). Pentane can accumulate in human fat depots so is released slowly over a period of several days. Pentane is partially metabolized by the hepatic cytochrome-P450

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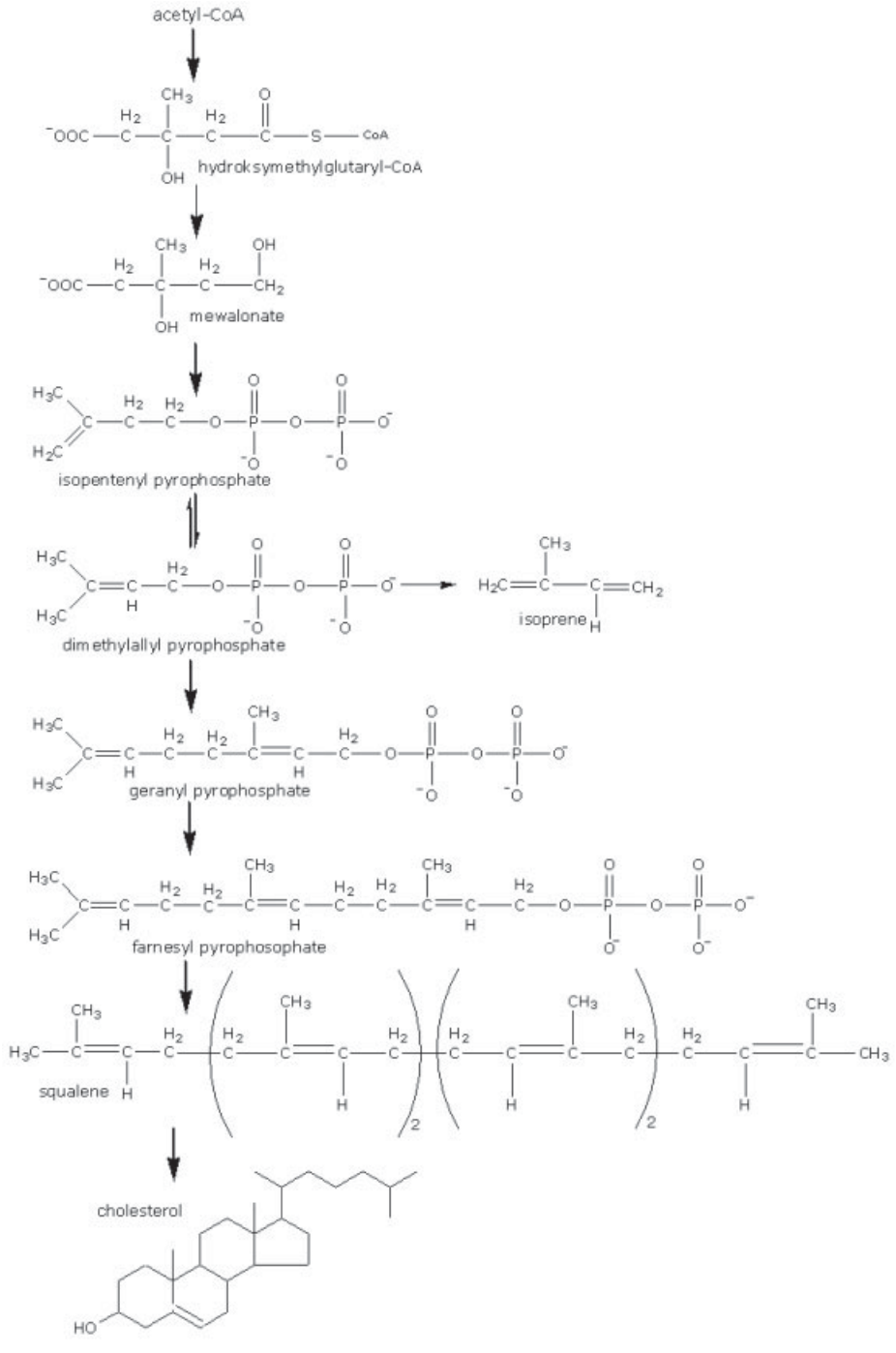

Figure 2. Biochemical pathway of isoprene generation.

system (Phillips *et al.*, 1999a,b). By contrast, ethane is highly volatile and relatively insoluble in tissues. Ethane is released quickly after its formation, with minimal metabolism. Thus, ethane reflects the main source of oxidative degradation of PUFAs. Measurements of breath ethane may provide a sensitive marker of *in vivo* lipid peroxidation that directly indicates when physiological events in the subject occur (Shin *et al.*, 1997; Knutson *et al.*, 2000).

Sulfur and nitrogen containing compounds

Volatile sulfur compounds like ethyl mercaptane, dimethylsulfide or dimethyldisulfide cause the characteristic odour of exhaled breath from cirrhotic patients (Di Francesco *et al.*, 2005). Sulfur-containing compounds are generated in humans by incomplete metabolism of methionine in the transamination pathway. The levels of sulfur-containing compounds are elevated in patients

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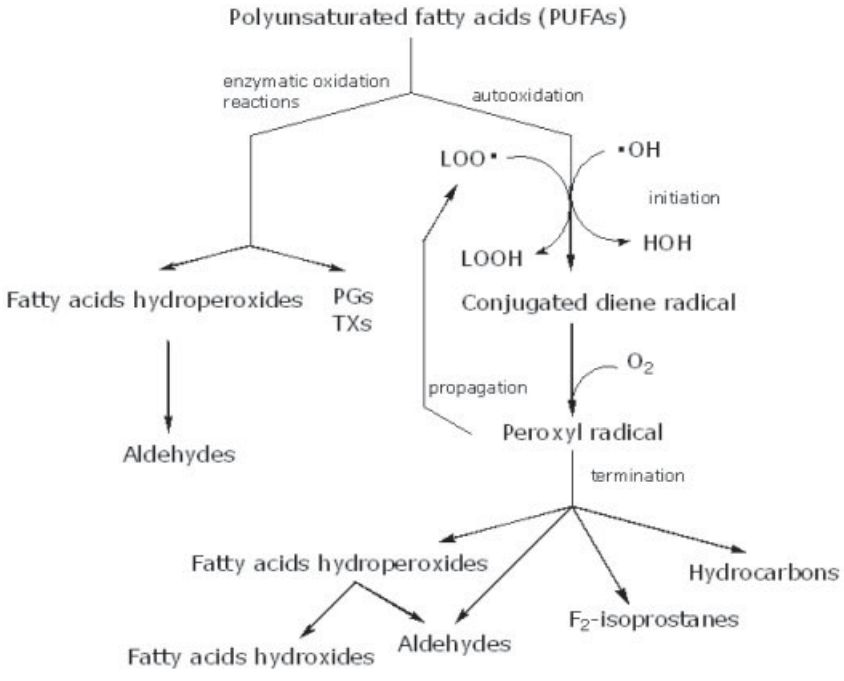

Figure 3. Polyunsaturated fatty acids oxidation products (Kinter, 1995).

who suffer from impairment of liver function (Miekisch *et al.*, 2004). These compounds are associated with hepatic diseases and malodour (Libardoni *et al.*, 2006).

Volatile organic amines, such as dimethylamine and trimethylamine, have been identified as being responsible for the distinctive odour of breath patients with different degrees of renal failure, including uremic ones (Di Francesco *et al.*, 2005). Significant levels of ammonia will appear in the blood if the removal of ammonia through conversion to urea is limited due to an impairment of liver function (Miekisch *et al.*, 2004). Ammonia does not accumulate in the body since it is a natural body product that comes from protein and nucleic acid metabolism. Ammonia is converted to urea and ammonium salts and in these forms is excreted from the body with urine. However, ammonia is also excreted via the lungs (Turner *et al.*, 2006a). Measuring breath ammonia levels can be a fast diagnostic method for patients with disturbed urea balance, e.g. due to kidney disorder or ulcers caused by *H. pylori* bacterial stomach infection (Timmer *et al.*, 2005).

ANALYTICAL TECHNIQUES

Analysis of exhaled air is not currently being used as much as it could be, because of the difficulties in the methodology (sampling and analysis) and lack of basis for data interpretation. There are no normalized systems of sampling, thus making it difficult to interpret the results (Miekisch *et al.*, 2004; Ma *et al.*, 2006).

Sampling

Breath can be collected into various sampling canisters, gas sampling bags, syringes, sorbent tubes containing multiple beds or analysed directly in analytical systems (Abbott *et al.*, 2003). Canisters (1000–3000 dm^3) are made from stainless steel. The inner surface of the canister is electropolished to minimize adsorption and losses of target compounds. Tedlar® sampling bags are made from polyvinyl fluoride (PVF). This material is chemically inert to a wide range of compounds, and resists to some degree gas permeation and adsorption of analyte molecules on its surface. Nevertheless, permeation of certain substances (notably water) should not be underestimated. Tedlar® bags can be reused for most applications. Prior to reuse, the bags must be evacuated and thoroughly cleaned and flushed with purified air or nitrogen (Abbott *et al.*, 2003). It is recommended that an analysis is performed of the final flush to ensure that the background levels present in the bag are acceptable for its intended use. Canisters and bags are used for a single alveolar breath sampling (Deng *et al.*, 2004b). Though useful in many respects, Tedlar® bags release, for example, *N*,*N*-dimethylacetamide and phenol in relatively high concentrations.

Exhaled air is a mixture alveolar air and ambient air retained in the respiratory dead space. Alveolar air is a part of exhaled air, which has been in contact with blood inside alveoli. Dead space air does not enter the gas exchange region of the lung, and includes: mouth, nose, pharynx, trachea and bronchi (Teshima *et al.*,

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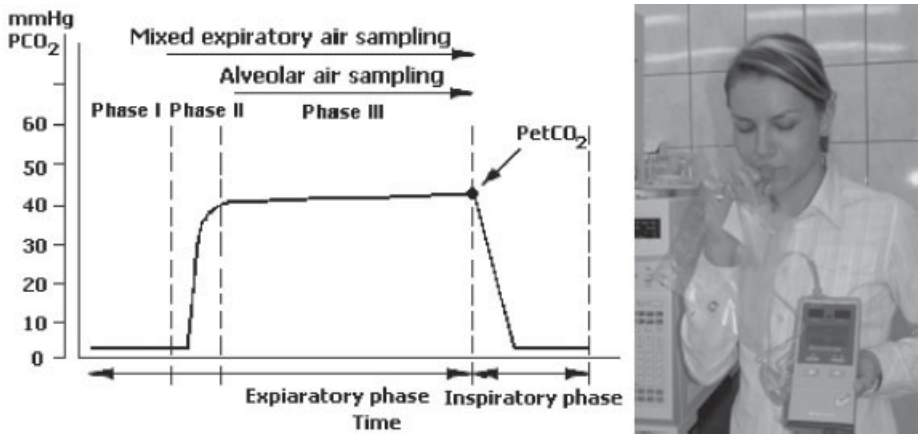

Figure 4. Schematic drawing of a normal capnogram and typical modes of sampling and collection breath samples in association with monitoring CO_2 using of capnograph.

2005; Miekisch and Schubert, 2006). During sample collection dead space air is pulled into the sampling system diluting the alveolar matrix thus affecting quantitation. Collection of breath samples can be done so that end expired air is sampled or mixed expired air is collected (Risby and Sehnert, 1999). The determination of partial pressure of CO_2 in breath is a useful factor to assess the representativeness of the taking samples. It solves the difficulties associated with variations in the analyte concentration in a breath sample that are due to different breathing patterns of different subjects. The concentrations of endogenous molecules will increase at the end of expiration when the end tidal pressure of expired CO_2 reaches a plateau. This increase during expiration can very nicely be observed in on-line measurements using selected ion flow tube mass spectrometry (SIFT-MS). It is therefore recommended that breath samples are collected in association with CO_2 monitoring using a capnograph (Fig. 4; Miekisch and Schubert, 2006), or by direct on-line measurements. The concentration of ammonia in breath is underestimated by a factor of about 4 when collected in Tedlar bags as compared with on-line measurements. Other compounds, like acetone, do not show a large difference when comparing bag-sampled and on-line sampled breath.

A potential error introduced during the breath collection involves the failure to distinguish between endogenous substances and exogenous contaminants. Thus, correction for background concentrations of volatile compounds in inspiratory air is necessary (Risby and Sehnert, 1999). To standardize the sampling procedure and to get rid of many inspiratory contaminants, correction for the actual background concentrations by subtracting them from the exhaled breath concentrations is often employed. This method requires the use of parallel sampling of breath and environmental air so that the concentration differences can be calculated (Libardoni *et al.*, 2006). Whenever a VOC behaves like carbon dioxide, it can be misleading to use the difference of concentration between exhaled and inhaled air: the concentration of CO_2 in exhaled air is about 4%, independent of the inspired concentration of CO_2 (within the normal inspiratory range 0.03–2% in indoor air). Taking the difference of concentrations ($CO_{2,exp} - CO_{2,insp}$) results in entirely different values for the same physiological states.

Another approach, that can be useful to deal with the problem of ambient air is lung washout. It is performed by breathing ultra pure air, which is free of VOCs, for a period of time in order to flush ambient VOCs from the lungs before sampling exhaled air (Handelman *et al.*, 2003). Washout periods used by different investigators usually range from 4 to 30 min (Knutson *et al.*, 2000; Miekisch and Schubert, 2006).

Preconcentration

Because of the very low concentrations of components in breath, preconcentration techniques are sometimes required prior to analysis, e.g. adsorption or cryotrapping, to increase the amount of collected VOCs (Di Francesco *et al.*, 2005; Libardoni *et al.*, 2006). However, high humidity of breath gas and the presence of certain endogenous organic vapours make the collection of breath samples difficult to perform. Improvements in sampling and analytical techniques have partly solved these methodological problems (Ochiai *et al.*, 2001).

There are varieties of enrichment techniques for breath analysis such as adsorption of analytes, followed by their thermodesorption and determination by gas chromatography combined with mass spectrometry (GC/MS). Adsorbents in collection traps have to be selected carefully to avoid both breakthrough and carryover effects. Organic polymers, activated charcoal,

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Table 4. Characterization of adsorbent materials commonly used for enrichment VOCs in human breath (Dettmer and Engewald, 2002)

Adsorbent	Particle size (mesh)	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	20/45	C2–C5	>400	510	778	0.53
Carboxen 564	20/45	C2–C5	>400	400	276	0.60
Carboxen 569	20/45	C2–C5	>400	485	257	0.58
Carboxen 1000	60/80	C2–C5	>400	1200	418	0.44
Carboxen 1001	60/80	C2–C5	>400	500	234	0.61
Carboxen 1003	40/60	C2–C5	>400	1000	79	0.46
Carbosieve SIII	60/80	C2–C5	>400	820	387	0.61
Carbospher	60/80	C2–C5	400	1000	779	—
Graphitized carbon blacks						
Carbotrap F	20/40	>C20	>400	5	—	0.66
Carbotrap C	20/40	C12–C20	>400	10	—	0.72
Carbotrap Y	20/40	C12–C20	>400	25	—	0.42
Carbotrap X	20/40	C3–C5	>400	250	—	0.41
Carbograph 5	20/40	C3–C5	>400	560	—	
Porous organic polymers						
Chromosorb 106 (styrene/divinylbenzene)	60/80	Small molecules	250	750	173	—
Tenax TA poly-(2,6-diphenyl-)-*p*-phenylenoxide	60/80	C7–C26	350	35	39	0.25

different types of graphitized carbon and carbon molecular sieves have been used for enrichment of VOCs in human breath (Table 4).

These adsorbents have a relatively hydrophobic surface, which is why they are least affected by high water contents in the samples. Organic polymers have low breakthrough volumes, especially for small and very volatile compounds (in general, those with boiling points <60°C; Ochiai *et al.*, 2001). Tenax is relatively hydrophobic and has a low surface area (S_{BET} = 15 m^2/g). The consequent low adsorption capacity of Tenax limits its application in gas samples of high concentrations. Chromosorb 106 is very hydrophobic and has a greater capacity than Tenax, and can be used at much higher concentrations. Its thermal stability is lower than that of Tenax, and so it is not suitable for semi-volatile compounds. By contrast, carbon molecular sieves and graphitized carbons have high breakthrough volumes for very volatile compounds (Harper, 2000; Miekisch *et el*., 2004). Carboxen (S_{BET} = 1000 m^2/g) is very hydrophobic and is suitable for highly volatile substances (hydrocarbons C2–C8, benzene, styrene). Graphitized activated carbon (S_{BET} = 120 m^2/g) is hydrophobic, adsorbs the smallest amount of water, and is suited for substances with a boiling point above 50°C (Mueller *et al.*, 1998; Risby and Sehnert, 1999). The principal disadvantage of sorbent sampling is that it is not a real-time technique (Harper, 2000).

It is not easy to find a material that allows complete adsorption as well as easy desorption of all volatile components present in exhaled air. Both carryover and breakthrough effects may occur when only a single adsorption material is used. Application sorbent tubes containing multiple beds can solve these problems. Multibed sorbent traps have some advantages over having separate beds, because the arrangement of the sorbents causes that the strong sorbent is protected by a layer of a weaker adsorbent. Therefore, the least volatile compounds are trapped on the weakest sorbent at the front end of the tube, and successively more volatile compounds are trapped by increasingly strong sorbents further down the tube, with the most volatile being trapped at the far end. Typical combinations include Tenax and carbon molecular sieve, and low surface area graphitizied carbon/medium surface area graphitizied carbon/carbon molecular sieve (Harper, 2000). The method for desorption volatile substances from the adsorption materials depends on the interaction strenghtness between analyte and adsorbent molecules. Thermal desorption (TD) technique is commonly used for release VOCs (Miekisch *et al*,. 2004). Thermal desorption is performed in the opposite direction to the sampling flow (Teshima *et al.*, 2005).

Preconcentration of some volatile substances has already been simplified by the introduction of solid-phase microextraction (SPME). SPME was introduced a decade ago by Arthur and Pawliszyn as a rapid extraction technique for the analysis of volatile and semi-volatile compounds from a variety of matrices (Pawliszyn, 1997). Figure 5 shows surface of fused-silica fibre coated with a polymeric phase, mounted in a syringe-like protective holder (Liu *et al.*, 1997). Not all

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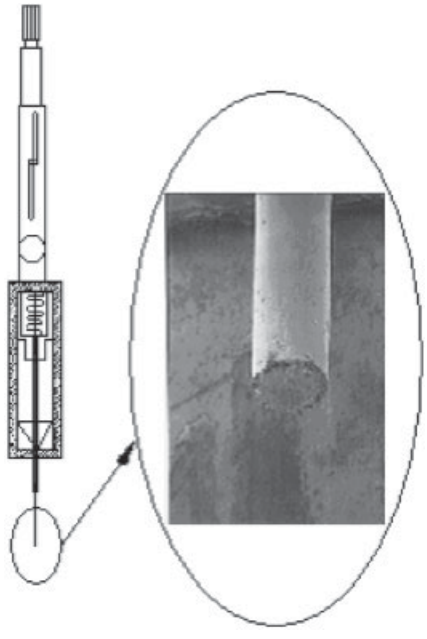

Figure 5. Scheme of a SPME device and scanning electron micrograph polymer film coated fibre (Lin *et al.*, 1997).

breath volatiles can be enriched because of the physical properties of the available fibre coatings. Release of volatile compounds from fibres is done by direct desorption in hot injector of the gas chromatograph. This adsorption technique is not affected by high water contents of the samples. A small volume of the fibre stationary phase constitutes an advantage of this technique in applications where only a limited amount of sample is available (Cha *et al.*, 2006, Miekisch and Schubert, 2006).

Choosing a proper SPME fibre coating is crucial to the effectiveness of the analytical method to a particular analyte types. Before a selection can be made, several determining factors of the analyte should be taken into consideration, i.e. polarity, molecular weight, sample matrix, expected concentration range, sampling temperature and detector type. Non-polar fibre coatings such as PDMS should be used for hydrocarbons with low polarity. Carboxen and divinylbenzene fibres are preferred for polar compounds, such as alcohols, organic acids and other (Spinhirne *et al.*, 2003). Both PDMS and PA phases extract via absorption with analytes dissolving and diffusing into bulk of the coating. The remaining types are mixed coatings and extract via adsorption with analytes staying on the surface of the fibre (Millsa and Walkerb, 2000). Absorptive fibres work well in moderate to high concentrations whereas the adsorptive fibres perform better in low concentration samples (Spinhirne *et al.*, 2003). The type of fibre used affects the selectivity of extraction (in general, polar fibres are used for polar analytes and non-polar types for non-polar analytes). A number of important stationary phases are characterized in Table 5.

Analysis and detection

The actual amounts of the exhaled marker compounds are usually very small and can be detected only using the most sensitive techniques. Separation of volatile compounds is mostly done by gas chromatography. Hydrocarbons in the nanomolar to picomolar per litre (ppbv–pptv) range, such as ethane, pentane or isoprene, are usually determined using gas chromatography coupled to flame ionization (FID) or a mass selective detector. Mass spectrometric detection has to be applied for the identification of unknown substances. GC/MS has been applied to the identification of pathogens from cultures and diagnosing illness from breath samples (Spinhirne *et al.*, 2003). GC/MS has been also used for the identification of metabolic end products pentane, acetone, ethanol, isoprene and other VOCs in normal human breath (Fig. 6).

Table 5. Stationary phases for SPME (Baltussen, 2000)

Stationary phase	Designation	Recommended uses
Polydimethylsiloxane	PDMS	
100 μm		Low molecular nonpolar volatiles
30 μm		Nonpolar semi-volatile compounds
7 μm		Mid- to nonpolar semivolatiles, higher molecular weight
Polydimethylsiloxane/divinylbenzene	PDMS/DVB	
65 μm		Polar volatile compounds (alcohols, amines)
60 μm		general purpose
Carboxen/polydimethylsiloxane	Car/PDMS	
75 μm		Trace volatile organic compounds
Carbowax/Divinylbenzene	CW/DVB	
65 μm		Polar analytes (alcohols)
Polyacrylate	PA	
85 μm		Polar semi-volatiles (phenols)

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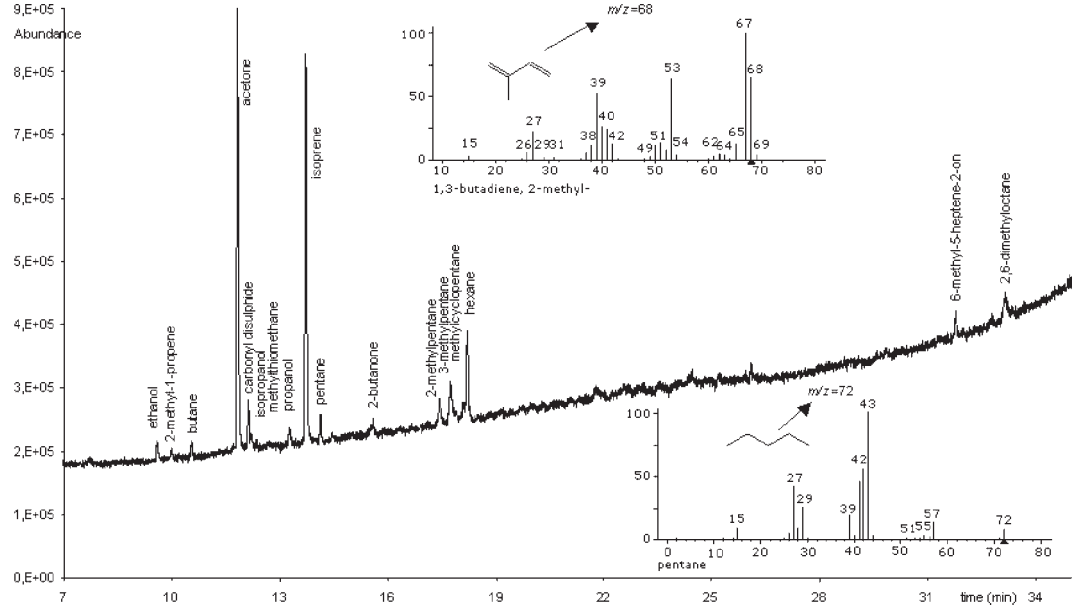

Figure 6. Normal human breath chromatogram.

Preconcentrating breath gas by various means and subsequent analysis by means of gas chromatography combined with mass spectrometry (GC/MS) constitute a reliable and sensitive set of methods for VOCs analysis. Initial results suggest that the GC/MS technique will be able to assist in the diagnosis of diseases in the near future. Despite its high sensitivity, there are a number of limitations associated with its use. This method generally involves both sampling and preconcentration steps prior to the introduction of the collected sample into a gas chromatographic column, which can lead to contamination problems and losses of analytes. The measurements are not taken *in situ*, are not in real time and are time-consuming (Amann *et al.*, 2004).

Paul and Steinwedel described an ion trap in 1960 (Paul and Steinwedel, US Patent 2 939 952, 1960; De Hoffmann *et al.*, 1996). The ions are produced by an electron beam in an ion source, stored in the trap and rejected according to their *m/z* ratio to obtain a spectrum. Electron impact and chemical ionization are available. Ion trap instrument is rather more sensitive than quadrupole. 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 is difficult, particularly in the case of polar compounds (aldehydes, alcohols, ketones, etc.), which are important compounds in exhaled breath analysis.

A relatively new kind of mass spectrometer is a time-of-flight mass spectrometer (TOF) MS, which was described, by Wiley and McLaren in 1955 (De Hoffmann *et al.*, 1996). The TOF instrument provides fast data acquisition rate or high mass resolution. In addition, the ion ratios for a spectrum do not change across the chromatographic peak. Therefore, deconvolution software can be used to deconvolute complex chromatographic coelutions and extract the spectrum of each compound. The deconvoluted spectra can then be used for library identification of unknown analyte (Fig. 7).

Most recently, selected ion flow tube mass spectrometry (SIFT-MS) emerged as a new technology for detection of breath gases in humans. SIFT-MS performs measurements of complex mixtures regardless of the water vapour content in real time without sample preparation. Acetone, acetaldehyde, ammonia, ethanol and water vapour were measured by SIFT-MS at the ppb level in a single-breath exhalation in real time (Spinhirne *et al.*, 2003). In the SIFT instrument reactant ions (precursors) 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 quadrupole mass-selected H_3O^+, NO^+ or O_2^+ primary positive ions in the sample injection region. The resulting product ions are mass selected by a second downstream quadrupole and detected using a particle multiplier producing the resulting mass spectrum. Thus, quantification of particular trace gases in the air sample is achieved. The response time is about 20 ms and hence it is possible to observe real-time fluctuations in trace gas concentrations (Wilson and Monster, 1999). The commonly used reactant ions are H_3O^+, NO^+ and O_2^+ because these do not react rapidly with the major components of air (Abott *et al.*, 2003). Nevertheless, the most commonly used precursor ion is protoned water because this reacts with a wide range of organic species, by non-dissociative

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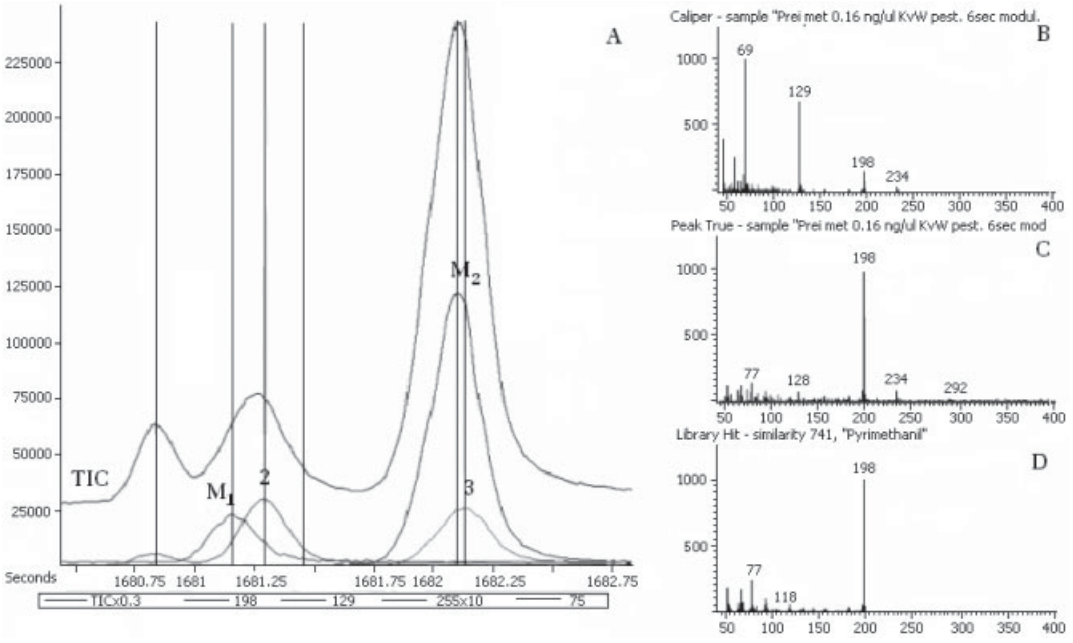

Figure 7. (A) Part of GC × GC-TOF MS chromatogram taken at first-dimension time of 1678 s, used to illustrate peak deconvolution. Peaks: **2**, propyzamide; **3**, pyrimethanil; M_1 and M_2, matrix compounds. (B) Mass spectrum at retention time of peak **3**. (C) The deconvoluted mass spectrum. (D) The library mass spectrum of polymethanil (Dollüge *et al.*, 2003).

proton transfer (Mueller *et al.*, 1998; Risby and Sehnert, 1999; Španěl and Smith, 2001).

SIFT-MS technology presents an exceptional set of advantages, which include high sensitivity, with detection to lower than ppb in less than 250 ms. Thus, the results are processed and obtained essentially in real time. A particular advantage of SIFT-MS is the quantification of water vapour in the breath samples, which allows us to determine the end-tidal phase during breathing. In contrast to GC-MS, the soft ionization technique requires no additional separation steps so analyses of high moisture samples are easy to perform. This feature is very important when analysing physiological samples. The identification of molecules in breath based only upon the mass-to-charge ratios of chemically ionized molecular ions is difficult (Mueller *et al.*, 1998; Risby and Sehnert, 1999; Wilson and Monster, 1999; Španěl and Smith, 2001; Španěl *et al.*, 2006).

Proton transfer reaction mass spectrometry (PTR-MS) has also been used for rapid and online measurements of breath VOCs measurement (Amann *et al.*, 2004, 2007; Janovsky *et al.*, 2005). PTR-MS instruments consist of four parts: an ion source where H_3O^+ ions are produced, a drift tube section, a transition chamber and an ion detection section containing a quadrupole mass spectrometer or a TOF mass spectrometer (Blake *et al.*, 2004) and a secondary electron multiplier. In the drift tube, the trace gases from the sample gas 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 occurring in high concentration, like NO, O_2, CO, CO_2, N_2, is lower that of H_2O molecules. These compounds do not interfere with the measurement because they do not react with H_3O^+ ions (Španěl and Smith, 2001; Steeghs *et al.*, 2006). Figure 8 shows the concentration–times series

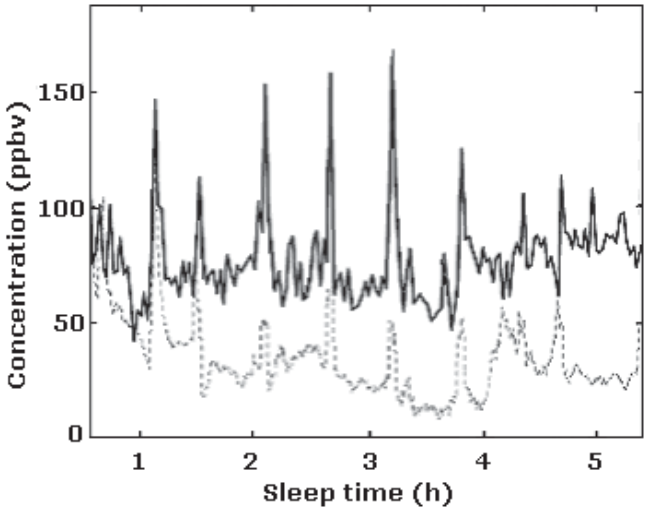

Figure 8. Concentration of mass 69 u (tentatively identified as isoprene) as function of time for one night of a healthy volunteer. The dashed line is the (averaged) heart rate (Amann *et al.*, 2004).

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for ionic mass $m/z = 69$ ($= m_1 + m_H$, tentatively identified as isoprene with molecular mass $m_1 = 68$ u) during one night (Amann *et al.*, 2004, 2007), in comparison with the heart rate. Increasing heart rate leads to an increased blood flow and consequently to an increased concentration of exhaled isoprene (Karl *et al.*, 2001) as long as no wash-out phenomenon occurs.

PTR-MS characterizes the substances only according to their mass-to-charge ratio so the chemical identification is not possible and must be provided by other techniques, i.e. gas chromatogrphy with mass spectrometric detection. VOCs with identical mass cannot be measured separately by using PTR-MS, but PTR-MS or SIFT-MS enable monitoring of metabolic processes over several hours without preconcentration of samples, and therefore may give an insight into physiological and pathophysiological processes. Sleep provides ideal conditions for such long-duration online monitoring (typically up to 8 h), which is difficult to perform on awake individuals due to interference with food intake and other daily activities (Amann *et al.*, 2004). The main advantage of proton transfer chemical ionization is that in most cases it does not induce any significant fragmentation, and when it does only few fragments are produced. Soft-ionization results in only one or two significant characteristic ions, the matrix of signals is much less complicated than with other mass spectrometry techniques (Španěl and Smith, 2001; Steeghs *et al.*, 2006; Amann *et al.*, 2007).

CONCLUSIONS

Chemical analysis of breath gases provides valuable information related to health and well-being. The noninvasive sampling process makes breath collection safe and easy even for nonclinical personnel. Modern analytical instruments enable a good platform to obtain a fingerprint characteristic of human breath. Single substances or sets of exhaled markers were analysed in order to establish correlations between the chemical composition of breath and patients' clinical conditions. Exhaled ethane and pentane concentrations were found to be elevated in inflammatory diseases. Acetone was linked to dextrose metabolism and lipolysis. Exhaled isoprene concentrations showed correlations with cholesterol biosynthesis. Exhaled levels of sulfur-containing compounds were elevated in liver failure and allograft rejection. Looking at a set of volatile markers may enable recognition and diagnosis of complex diseases such as lung or breast cancer.

Breath analysis may be readily accepted for routine screening because it is reliable and robust. The costs for consumables are low or even zero as, for example, in PTR-MS. Hence even though the initial investment in research analytical devices (GC/MS, PTR-MS, SIFT-MS) is high, the actual costs for analysis of samples are low, since no expensive kits like in molecular biological tests are necessary. In addition, matrix effects are minimal and sample preparation prior to analysis is often simpler when compared with the preparation and analysis of biological specimens, such as blood or urine. It requires a professional analytical system in order to establish correlation between the chemical composition of healthy and diseased persons' breath. Though at present available laboratory instruments are comparatively large and expensive, one may expect the development of small, portable and cheap analytical sensors for the detection or particular compounds in the future. Already, instruments for direct breath-to-breath analysis based on mass spectrometry are very useful. Laser spectrometry-based devices for the detection of particular compounds (e.g. ethane carbon monoxide, carbonyl sulfide or nitric oxide) can also be used to investigate exhaled breath on a breath-to-breath basis (von Basum *et al.*, 2003, 2005; Wysocki *et al.*, 2004) and have the potential to become small in the future (e.g. down to the size of a cigarette box). In addition, GC/MS system still can be used for the regular analysis of breath samples, because it is the most economical method for obtaining extensive information about the molecular composition characteristic of humans. MS will remain indispensable in increasing our basic knowledge of volatile markers and to define new markers. Although limited use of breath analysis is attributed to a lack of both bases for data interpretation and methodology, there are indications that in recent years some technical and scientifical progress have been made. Miniaturization of conventional instruments has been the factor responsible for these developments. The development of chemometric methods has made it possible to handle large amounts of quantitative information in a fast and economical way. Well-validated data sets which assist with the interpretation of results are still needed.

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