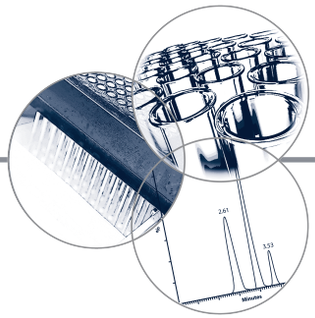

Detection of volatile malodorous compounds in breath: current analytical techniques and implications in human disease

For the last few decades intense scientific research has been placed on the relationship between trace substances found in exhaled breath such as volatile organic compounds (VOC) and a wide range of local or systemic diseases. Although currently there is no general consensus, results imply that VOC have a different profile depending on the organ or disease that generates them. The association between a specific pathology and exhaled breath odor is particularly evident in patients with medical conditions such as liver, renal or oral diseases. In other cases the unpleasant odors can be associated with the whole body and have a genetic underlying cause. The present review describes the current advances in identifying and quantifying VOC used as biomarkers for a number of systemic diseases. A special focus will be placed on volatiles that characterize unpleasant breath 'fingerprints' such as fetor hepaticus; uremic fetor; fetor ex ore or trimethylaminuria.

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Volatile organic compounds: background

Exhaled breath contains mainly water, inert gases, oxygen, nitrogen and carbon dioxide. Aside from these major components, substances such as volatile organic compounds (VOC) are also present in concentrations of parts per million to parts per trillion volume. The first detailed study on VOC in exhaled breath of healthy patients was performed by Pauling *et al.* who reported (but did not identify) 250 different VOC [1]. Since then, hundreds of other VOC have been identified [2,3]. Organic compounds found in exhaled breath are either of exogenous or endogenous origin. Exogenous compounds are inhaled, ingested as food, absorbed through skin, or generated as metabolic products of drugs [4,5]. Endogenous compounds are a result of different biological processes such as oxidative stress [6] or metabolic pathways [7] playing key roles in a wide range of diseases. Being able to analyze VOC in breath can help in assessing a wide range biological events important for human diseases, in a noninvasive manner. In contrast to blood, breath can be sampled as often as it is desirable, even continuously during exertion of an effort at a stationary bicycle [8,9] or during sleep [10]. Analysis can also be done in real-time, even down to breath-to-breath resolution, which represents an advantage in comparison with investigations of blood samples. This approach allows the detection of very fast processes, such as a quick release of isoprene during effort [11].

Several classes of VOC can be identified in exhaled breath [3,12]: saturated hydrocarbons such as ethane and pentane, as well as aldehydes may result from lipid peroxidation of fatty acids, following reactive oxygen species (ROS) accumulation; unsaturated hydrocarbons, such as isoprene – a byproduct of the mevalonate pathway; VOC containing sulfur, such as methyl-mercaptan or dimethyl-sulfide formed along the transamination pathway [13,14]; VOC containing oxygen, such as acetone derived from lipolysis and more specifically produced during acetoacetate decarboxylation [15,16]; and VOC containing nitrogen such as ammonia [17–19] or dimethylamine found in patients with liver or renal dysfunctions [20]. After being produced the volatiles from different organs enter the systemic circulation and are transported by the blood through the lungs where they are exhaled. Some of these **volatile malodorous compounds** are characterized by very distinctive odors that can in some cases be associated with specific pathologies (Table 1). Being able to correctly analyze and use the 'molecular breath print' of each disease has important implications in creating breath tests that can screen, diagnose and follow-up many human pathologies.

Analytical techniques

Analysis of exhaled breath is still a developing field of research. For most volatile compounds

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Table 1. Characteristics of odorous compounds.

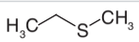
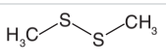
CAS-number	Compound name	Chemical structure formula	Chemical sum formula	Tentative origin	Organoleptic threshold	Oral cavity nmol/l	Normal bad breath (measured in morning)	Odor qualities/ halitosis remarks
7783-06-4	Hydrogen sulfide	H—S—H	H_2S	Produced by bacteria in oral cavity [64]	3.9 nmol/l [70]; 1 µmol/mol [71]; 0.0005 ppm [302]; 0.41 ppb [196,197]; 6.4×10^{-10} mol/ dm^{-3} [198]	1.9 mmol/l [72] as highest concentration in periodontal pockets	11.78 ppb [71]; halitosis vs healthy – 80 ppb vs 2 ppb (median) [199]	Rotten eggs odor. Distinctive role in physiological halitosis [200]
75-18-3	Dimethylsulfide	$H_3C—S—CH_3$	C_2H_6S	Blood-borne [73]	1.0 nmol/l [70]; 0.1 µmol/mol [71]; 2.21 ppb [196]; 0.0030 ppm [197]; 0.001 ppm [303]	ND	20.3 ppb [71]; halitosis vs healthy – 8.49 ppb vs 4.81 ppb (median) [199]	Cabbage-like odor; Increased in extral-oral halitosis [73]
624-89-5	Sulfide, ethyl methyl		C_3H_8S	Blood-borne [201]	ND	ND	ND	ND
10152-76-8	Sulfide, allyl methyl		C_4H_8S	Gut origin [90,202]	0.00014 ppm [197]	ND	Mouth (0 ppb), alveolar (0.10) [71]	Garlic-like odor
624-92-0	Dimethyldisulfide		$C_2H_6S_2$	Blood-borne [70,73,199]	0.007 µmol/mol [71]; 0.0022 ppm [197]; 5.9×10^{-8} mol/dm^{-3} [198]	ND	Mouth (0.061 ppb), alveolar (0 ppb) [71]; halitosis vs healthy – 0.57 ppb vs 0.052 ppb (median) [199]	ND
3658-80-8	Dimethyltrisulfide		$C_2H_6S_3$	Blood-borne [70,73,199]	ND	ND	Mouth and alveolar (0 ppb) [71]; halitosis vs healthy – 0.38 ppb vs 0 ppb (median) [199]	ND
74-93-1	Methanethiol methylsulfide methylmercaptan	$H_3C—SH$	CH_4S	Bacterial interactions with different amino-acids [64]	0.5 nmol/l [70]; 0.035 µmol/mol [71]; 0.001 ppm [302]; 0.07 ppm [196,197] 1.0×10^{-11} mol/dm^{-3} [198]; 0.0021 ppm [303]	0.16 mmol/l [72] as highest concentration in periodontal pockets	9.7 ppb [71]; halitosis vs healthy – 96 ppb vs 0 ppb (median) [199]	Rotten cabbage odor. Involved in oral pathological halitosis [200]
75-15-0	Carbon disulfide	S=C=S	CS_2	Blood-borne [119,203]	0.9 µmol/mol [71]; 0.096 ppm [302]; 210 ppb [196]; 0.21 ppm [197]	ND	Mouth and alveolar (-0.021 ppb) [71]	Sweet ethereal odor. Can be found in breath from patients with schizophrenia [203]

Table 1. Characteristics of odorous compounds (cont.).

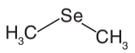
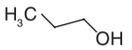
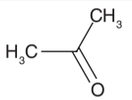
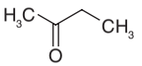
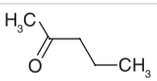
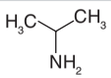
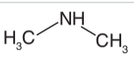
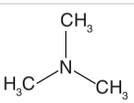
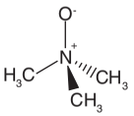
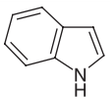
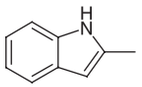
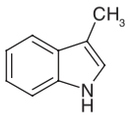
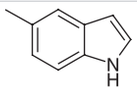
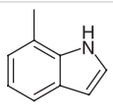
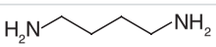
CAS-number	Compound name	Chemical structure formula	Chemical sum formula	Tentative origin	Organoleptic threshold	Oral cavity nmol/l	Normal bad breath (measured in morning)	Odor qualities/ halitosis remarks
593-79-3	Dimethyl selenide		C_2H_6Se	Selenium metabolism; blood-borne [71,122]	ND	ND	Mouth (0.13 ppvb), aveolar (0.56 ppvb) [71]	Garlic odor; Found in alveolar air
71-23-8	1-propanol		C_3H_8O	Blood-borne [204]	45 µmol/mol [71]; 2.6 ppm [302]; 0.094 ppm [197]	ND	Mouth (25.7 ppb), alveolar (7.30 ppb) [71]	Alcoholic fruity odor
67-64-1	Acetone		C_3H_6O	Blood-borne [35,182]	300 µmol/mol [71]; 4.58 ppm [302]; 42 ppm [197]	ND	Mouth (101.67 ppb), alveolar (199.19 ppb) [71]	Fruity or sweet odor associated to diabetic ketoacidosis
78-93-3	2-butanone		C_4H_8O	Blood-borne [122]	30 µmol/mol [71];	ND	Mouth (0.32 ppb), alveolar (0.24) [71]	Can be increased in breath of patients with liver conditions. Odor quality resembles that of acetone
107-87-9	2-pentanone		$C_5H_{10}O$	Blood-borne [122]	8 µmol/mol [71]; 1.55 ppm [302]	ND	Mouth (0.11 ppb), alveolar (0.38 ppb) [71]	Can be increased in breath of patients with liver conditions. Odor quality resembles that of acetone
7664-41-7	Ammonia		NH_3	Blood-borne [128]	5.75 ppm [302]; 1500 ppb [196]; 1.5 ppm [197]; 0.037 ppm [303]	ND	ND	Pungent odor. Specific 'fishy' odor in patients with chronic liver failure
75-31-0	2-propanamine		C_3H_9N	Blood-borne [205]	0.061 ppm [197]	ND	ND	Ammonia-like odor
74-89-5	Methylamine		CH_5N	Blood-borne [20,160,195]	0.019 [302]; 0.035 ppm[197]; 0.021 ppm [302]	ND	ND	Fish, ammonia odor
124-40-3	Dimethylamine		C_2H_7N	Blood-borne [20,160,195]	0.081 208; 0.033 ppm [197]; 0.047 ppm [303]	ND	ND	Fish, ammonia odor
75-50-3	Trimethylamine		C_3H_9N	Blood-borne [141,154]	0.001 [302]; 0.032 ppb [196]; 0.000032 ppm [197]; 1.8×10^{-11} mol/dm^{-3} [198]	ND	ND	Fish-like or ammonia-like odor. Associated with trimethyl-aminuria [154]; may be found in odor hepaticus [115]

Table 1. Characteristics of odorous compounds (cont.).

CAS-number	Compound name	Chemical structure formula	Chemical sum formula	Tentative origin	Organoleptic threshold	Oral cavity nmol/l	Normal bad breath (measured in morning)	Odor qualities/ halitosis remarks
1184-78-7	Trimethylamine-*N*-oxide		C_3H_9NO	Blood-borne [141,154]	ND	ND	ND	Associated with trimethylaminuria
120-72-9	Indole ll 2,3-benzopyrrole		C_8H_7N	Blood-borne [71] or produced by bacteria in oral cavity [88]	0.0003 ppm [197]	Highest concentration detected in oral malodor 34 ppm [88]	Mouth (0.15 ppb), alveolar (0.20 ppb) [71]; halitosis vs healthy – 0.11 ppb vs 0.14 ppb (median) [199]	Fecal odor. May contribute to some cases of halitosis
95-20-5	2-methyl indole		C_9H_9N	Oral cavity [88]	ND	ND	ND	May contribute to some cases of halitosis
83-34-1	3-methyl indole ll skatole		C_9H_9N	Oral cavity [88,206]	0.0000056 ppm [197]; 7.2×10^{-13} mol/dm^{-3} [198]; 0.0012 ppm [302]	ND	Mouth (0.0 ppb), alveolar (0.0 ppb) [71]; halitosis vs healthy – 0 ppb vs 0 ppb (median) [199]	Fecal odor. May contribute to some cases of halitosis
614-96-0	5-methyl indole		C_9H_9N	Oral cavity [88]	ND	ND	ND	May contribute to some cases of halitosis
933-67-5	7-methyl indole		C_9H_9N	Oral cavity [88]	ND	ND	ND	May contribute to some cases of halitosis
462-94-2	Cadaverine		$C_5H_{14}N_2$	Oral cavity [206]	ND	ND	ND	Urine- and semen-like odor. Its association with halitosis is independent of volatile sulfur compounds
110-60-1	Putrescine		$C_4H_{12}N_2$	Oral cavity [206]	9.1×10^{-10} mol/dm^{-3} [198]	ND	ND	Amine-like piperidine odor

the biochemical background is not yet elucidated. The progress in the field is fostered by progress in analytical instrumentation, allowing more sensitive and faster measurements, as well as more reliable compound identification.

The gold standard is GC–MS associated with different preconcentration techniques: solid phase microextraction (SPME) [21,22], SPE, thermodesorption (TD) [2,23–25] and needle-trap devices [26–29]. A critical point in GC–MS analysis is the reliable identification of chromatographic peaks by spectral library identification combined with retention index [2,22–25,27,30]. This reliable identification of peaks is absolutely necessary in elucidating the biochemical background of volatile compounds. Among these different preconcentration techniques, SPME is easiest to handle; on the other hand, SPME is only semi-quantitative and has comparatively low sensitivity (with LOD ~1 ppb). SPE on TD tubes gives much more reliable quantitative results than SPME, and shows higher sensitivity (LOD ~0.01 ppb). Needle trap devices are between SPME and TD with regard to sensitivity; needle trap devices are quite versatile, but care must be taken to calibrate each individual needle trap based on measurement of flow resistance.

GC–TOF-MS allows much faster measurements than conventional GC with quadrupole MS and (depending on the specific TOF mass spectrometer) achieves much better mass resolution. When, for example, 10,000 spectra per second are recorded, deconvolution of peaks can be improved, which leads to better peak identification and allows faster measurements. Better resolution of peaks is provided by GC × GC–TOF-MS (an example is given in [31]), revealing a lot more volatile compounds in exhaled breath (and at lower concentrations) than conventional GC–MS.

GC is a labor-intensive technique with comparatively slow measurements (the time-consuming step is the separation in the GC-capillary). Direct MS methods such as selected ion flow tube (SIFT)-MS [32–36], proton-transfer-reaction (PTR)-MS [4,21,37,38], PTR-TOF-MS [39–41] and secondary ESI–MS (secondary ESI–Q-TOF) [42–44] do not separate the different compounds in the sample prior to measurement and therefore achieve much faster measurements. Measurements can even be done in real-time and down to breath-to-breath resolution [8–11,45–47]. The mere possibility of real-time analysis (e.g., when exerting an effort on a stationary bicycle or during sleep) is an advantage in comparison with investigations of blood samples. It allows the detection of very fast processes, such as a quick release of isoprene during effort. The direct MS techniques are most interesting and versatile, but do not give as much information as GC–MS, since sometimes different compounds are not separated.

The fast development of the analytical possibilities can be illustrated by comparison of a PTR-MS (with quadrupole MS) with PTR-TOF-MS. Both instruments allow for real-time measurements. In PTR-MS the mass resolution is 1, whereas in PTR-TOF-MS the mass resolution is between 4000 and 5000. In PTR-MS the measurement of one ion takes approximately 100 ms. In PTR-TOF-MS, the ionized molecular species 'fly anyway', hence the measurement of one particular molecular species takes the same time as the measurement of the whole spectrum of all ionized species. Typical measurement times are 10–30 s, depending on the sensitivity to be achieved [39,40]. In order to get a sensitivity in the part-per-trillion (ppt) range, longer measurement times of a few minutes are used.

Ion mobility spectrometry (IMS) [48–50] has provided instruments that are smaller than the 'big' research instruments (GC–MS, SIFT-MS, PTR-MS, PTR-TOF-MS). These IMS instruments often couple a multicapillary column (MCC) to ion mobility spectrometric detection [49,50], are easily transportable and can even be used in field experiments [41]. MCC–IMS provides 2D peak-patterns (depending on retention time of the MCC and ion mobility). Careful peak identification (by use of calibration measurements using native standards) is very important. IMS is very sensitive for, for example, aldehydes and ketones (below ppb-level). IMS sensors have been produced mainly for military purposes and can now be used for breath analysis [51]. Modern field asymmetric ion mobility sensors are only a few centimeters wide and use varying compensation voltage.

Laser spectroscopy also has a huge potential for analysis of exhaled breath and allows miniaturization. It is particularly interesting for small molecules such as ethane, carbon monoxide or nitric oxide [52–54]. In the future, tunable lasers will certainly play an important role in analysis of volatile compounds.

Over the last 10 years many different sensors for volatile compounds have been developed for various applications [55,56]. Most of these sensors are nonspecific and are combined in sensor arrays

Key Term

Volatile malodorous compounds: Compounds of endogenous or exogenous origin conferring an unpleasant smell to exhaled breath.

Key Term

Oral malodor: Offensive odors in exhaled breath originating from oral cavity.

with subsequent chemometric analysis of data. Some sensors are specific without much cross-sensitivities, as, for example, the nitric oxide sensor used in the hand-held devices by Aerocrine (Solnaa, Sweden) for asthma monitoring.

A very critical step for all analytical methodologies is the sampling of breath [56,57]. If systemic compounds are of interest, the sampling should be done in a CO_2-controlled manner [58,59]. In this way, preferentially alveolar air is sampled, giving rise to higher concentrations of systemic compounds. In addition, the repeatability of the sampling procedure is improved. An important further issue is the identification of environmental contaminants and food- or beverage-derived artifacts. As an example, 2-ethyl-hexanol may be released by tubing systems. It may appear through metabolism of metabolism of the plasticizer di(2-ethylhexyl) phthalate [60], and further be metabolized to 2-heptanone and 4-heptanone [60,61]. Many cigarette-related compounds are known [2], the most prominent example being acetonitrile. Another example is 2-propanol, which appears in relatively high concentrations in hospital indoor air [21].

Oral halitosis: fetor ex ore

Oral malodor, halitosis, bad breath or fetor ex ore are terms used to describe noticeably offensive odors from exhaled breath. Classification, terminology and treatment options for different aspects of the condition have been reviewed previously [62–64]. The first report on oral compounds causing oral malodor was published more than 30 years ago by Tonzetich [65]. Along with dental caries and periodontal diseases, oral malodor or halitosis ranks as one of the main reasons patients visit dental offices.

Pathological halitosis can have intra- or extra-oral causes. But for the vast majority of people bad breath originates in the oral cavity usually as a result of bacterial metabolism [66]. Microorganisms associated with halitosis mainly include Gram-negative anaerobic flora such as: *Solobacterium moorei, Porphyromonas gingivalis, Prevotella intermedia, Fusobacterium nucleatum and Treponema denticola*. Most of these bacteria are located on the dorsal part of the tongue [67,68]. The volatile compounds responsible for halitosis are metabolic products of anaerobe bacteria interacting with certain substrates such as amino acids cysteine, lysine, arginine or tryptophan (Figure 1A) [69]. From the resulting compounds, oral malodor is caused mainly by volatile sulfur compounds (VSCs). Among them hydrogen sulfide (H_2S) and methyl-mercaptan are the most important components of physiological halitosis with methyl mercaptan concentrations being higher in oral pathologic halitosis. The organoleptic threshold levels for VCS as reported by Tangerman *et al.* [70] are 3.9 nmol/l (93.6 ppb) for H_2S; 0.5 nmol/l (12 ppb) for CH_4S and 1.0 nmol/l (24 ppb) for $(CH_3)_2S$. Another study shows that mean VSCs concentrations in healthy volunteers reporting 'normal' morning bad breath were found to be: 11.78 ppb for H_2S; 9.7 ppb for CH_4S and 20.3 ppb for $(CH_3)_2S$ [71]. Oral malodor is increased in subjects with oral affections such as periodontal complications. In periodontal patients the highest concentrations of H_2S and CH_4S in periodontal pockets were found to be 1.9 mmol/l and 0.16 mmol/l, respectively [72]. Dimethyl sulfide is increased mostly in extra-oral halitosis [73]. In general, the VSC levels in mouth air have been reported to be higher in females than in males, especially in the menstrual or premenstrual phases [74,75]. All three compounds involved in bad breath, H_2S, CH_4S and $(CH_3)_2S$, can be obtained from methionine. Methionine serves as a methyl donor through its derivate *S*-adenosyl methionine. Since sulfur absorption in the organism requires high levels of energy, most organisms adapted specific pathways that can reuse methionine and the sulfur atom, such as methionine salvage pathway, also termed 5′-methylthioadenosine cycle [76]. The pathway is involved in a series of biological processes such as induction of apoptosis or diseases such as: cancer [77], liver diseases, malaria, Trypanosome-related conditions or inflammation [78]. The starting point of the pathway is 5′-methylthioadenosine, which is a product of polyamine synthesis resulting from *S*-adenosyl methionine, that in turn is formed from methionine and ATP [79]. Methionine participation in normal growth is tightly connected with its transulfuration and transamination pathways [80]. Methyl mercaptan is formed either as a product of transamination pathway by the enzyme thiol *S*-methyltransferase out of 3-methyl thiopropionate or directly from methionine by enzyme L-methionine-γ-lyase [81]. It is well-established that methyl mercaptan is a highly toxic VOC playing a key role in oral malodor. Several reports demonstrate the production of methyl mercaptan by different oral bacteria [82]. For example *P. gingivalis* and *T. denticola*, both pathogens involved in initiation and development of periodontal diseases,

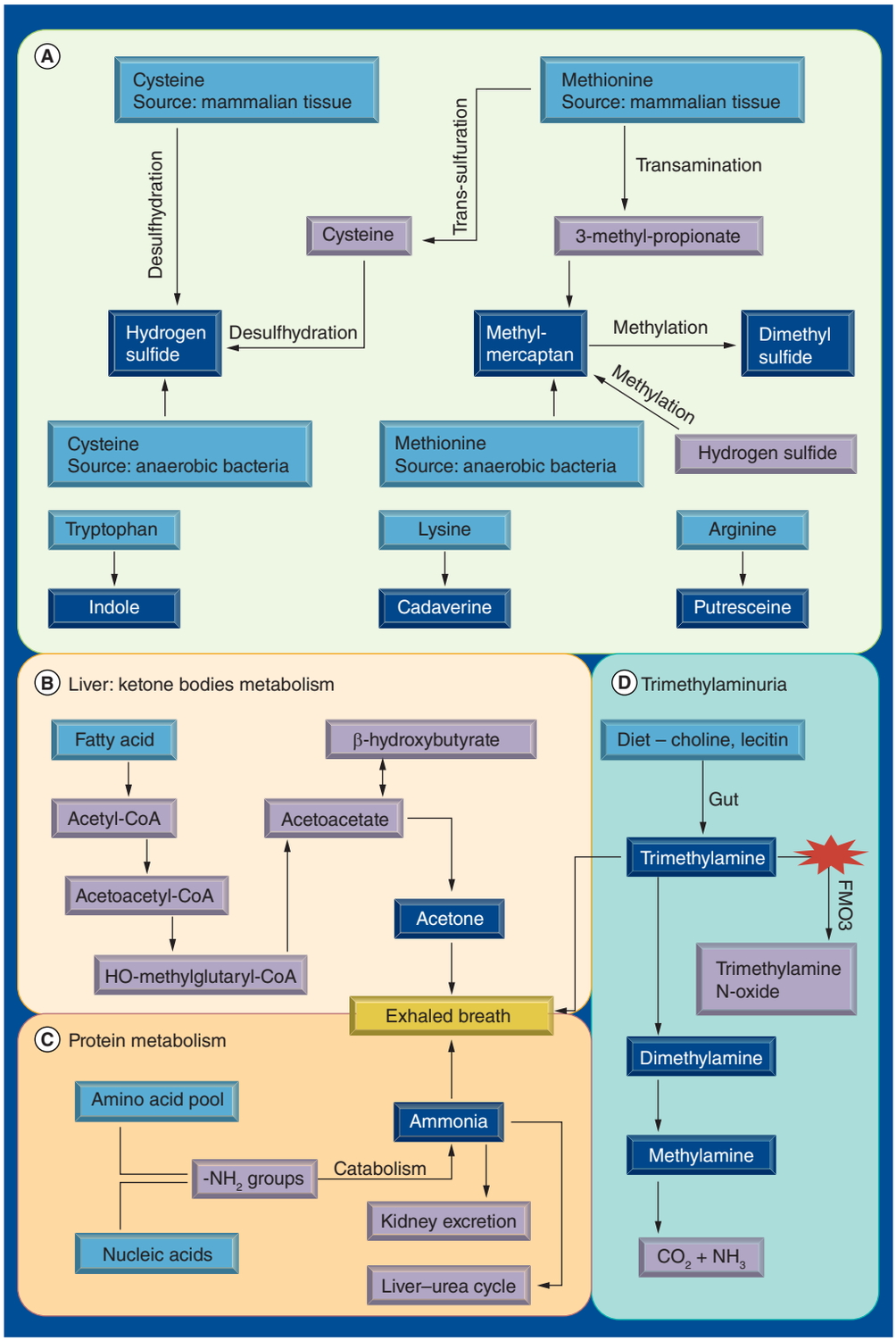


Figure 1. Metabolic pathways of several volatile malodorous compounds. (A) Pathways of volatile sulfur compounds production with roles in oral malodor; **(B)** acetone synthesis as a result of ketone bodies metabolism is increased in chronic liver diseases and diabetes; **(C)** ammonia excretion – ammonia can be detected in exhaled breath of uremic patients with chronic kidney failure; **(D)** trimethylaminuria – a genetic disorder in which trimethylamine (an odorous compound) cannot be transformed in trimethylamine *N*-oxide (an odorless compound) due to FMO3 enzyme deficiency.

are capable of producing methyl mercaptan from L-methionine [83,84]. Dimethyl sulfide is also formed via methionine transamination pathway as a product of methyl mercaptan. From methionine, H_2S is formed through the transulfuration pathway out of cysteine, an intermediate of the reaction. Enzyme thiol *S*-methyltransferase catalyzes the methylation of H_2S to methyl mercaptan and dimethyl sulfide. Since H_2S has a much higher toxicity for humans than the other two compounds, H_2S methylation can be regarded as a possible detoxifying mechanism [81].

Up to now, there is no general consensus regarding the chemical components involved in the chemistry of oral malodor. One reason for the current lack of agreement is the use of different methodologies. As pointed out, GC studies showed that the compounds usually associated with malodor are VSCs, in particular hydrogen sulfide and methylmercaptan. However, some researchers argue that organoleptic scores do not correlate very well with different sulfur compounds concentrations [85]. Other compounds resulted as byproducts of microbial metabolism include [86,87]: amines – putrescine, cadaverine, trimethylamine; short chain fatty acids – propionic, butyric and valeric acids; phenyl compounds – indole, skatole; ketones – acetone; alkanes – 2-methyl-propane; nitrogen compounds – urea and ammonia. The presence of VOC in the mouth may also be a consequence of dietary products such as allyl-methyl-sulfide released from garlic intake. However, VOC presence due to ingestion of certain foods or drinks is usually transitory. Some of these compounds are common for both mouth and lung air [71].

Other research groups have used SIFT-MS in order to analyze all compounds present in oral air. Preliminary studies show that a particular class of compounds that may in some cases be associated with oral malodor are indoles. Indole or 2,3-benzopyrrole is an aromatic heterocycle containing a benzene and a pyrrole ring. Indole is the side-chain of the amino acid tryptophan. In the gastrointestinal tract indole is a direct product of bacterial tryptophan catabolism. It has a particular unpleasant smell associated in high concentrations with fecal matter. Ross and Esarik demonstrated that aside from sulfide compounds, indole and especially various methylindoles may contribute to some cases of halitosis [88]. However, studies on a larger number of patients are needed in order to validate these results.

As shown above, in most cases bad breath is a direct result of metabolic products of oral bacteria and enzymatic activity on salivary components. However, it is important to note that halitosis has systemic causes in around 10% of patients [64]. Therefore, an increasing body of research is focusing on the relationship between volatile compounds in mouth air and systemic diseases [89,90]. Extra-oral conditions that can generate oral malodor include: metabolic disorders such as deficiency in flavin-containing monooxygenase 3 (FMO3) [91,92], gastrointestinal tract diseases, hepatic affections, renal diseases, psychogenic causes and respiratory conditions [86]. Some studies reported different alkanes and alkane derivates connected to oxidative stress increments in oral cavity [93]. Increased levels of H_2S in mouth air have been linked to a history of hypertension or respiratory diseases such as pneumonia, pulmonary emphysema and bronchitis [94]. Methyl-mercaptan (methanethiol) concentrations were also found to be elevated in patients with hypertension, while dimethyl sulfide mouth air concentrations were significantly increased in patients with cerebrovascular affections such as cerebral infarction, intracerebral haemorrhage or subarachnoid hemorrhage [94]. In a further study, Awano *et al.* demonstrated that increased levels of dimethyl sulfide are significantly correlated with high-density lipoprotein levels, cholesterol levels, asthma and medical history of colon polyps [89]. In the same study, increased levels of dimethyl sulfide were found to be associated not only with systemic factors, but also with high oral levels of methyl-mercaptan. These results are consistent with another study [95] showing that dimethyl sulfide in breath can be increased in mouth air of asthma patients due to specific medication. Increased dimethyl sulfide concentrations in mouth air have also been linked to hepatic affections such as hepatic cirrhosis [96].

When sampling for breath tests a careful distinction has to be made between alveolar air and mouth air. This is particularly important as there are differences between composition of mouth air and alveolar air. In order to distinguish between compounds from the oral cavity alone and elements generated from different body systems Wang *et al.* analyzed breath exhaled via mouth, nose and air in the oral cavity separately [35]. Their results, confirmed by Ross *et al.*, showed that certain compounds, such as ammonia or acetone, besides being

found in alveolar air can also originate as end products of microorganisms residing in the oral cavity [97].

Thus, it is important when performing general breath studies to acknowledge the oral cavity contribution to the markers present in total exhaled air. One recommendation is to use nose-exhaled breath for systemic breath analysis in order to bypass mouth air [32].

It is important to note that although there are a wide number of volatile compounds being proposed as possible biomarkers of various diseases, only few of these compounds are currently used on a daily basis in clinical settings. Two of these examples are H_2S, described here in its relation to oral halitosis, and nitric oxide used for monitoring asthma patients and corticosteroids titration [57,58].

The field of bad breath research expanded to focus not only on the esthetic problems generated by VSCs, but also on their toxicity in the oral cavity (Table 2). A number of studies showed that increased levels of VSCs have pathogenic potential and may play an important role in the etiology of periodontitis. H_2S has been shown to inhibit collagen synthesis [98], inhibit proliferation of epithelial cells [99] and the synthesis of basal membranes [100], and decrease total protein production in oral fibroblasts [101]. Osteoclast activation following increased levels of VSCs both *in vitro* and in rat alveolar bone has also been reported [102,103]. Yaegaki *et al.* [104] reported that H_2S inhibited the activity of superoxide dismutase in human gingival fibroblasts. Superoxide dismutase is a critical enzyme responsible for the elimination of the ROS. Elevated intracellular levels of ROS can ultimately lead to DNA damage. Increased levels of ROS associated with DNA damage were also detected in normal keratinocytes [105] and keratinocyte stem cells [106]. In addition, 24 or 48 h of incubation with physiological concentrations of VSCs (specifically H_2S) can induce apoptosis in different oral cellular types. Apoptotic process initiated through the intrinsic mitochondrial pathway is characterized by: mitochondrial membrane depolarization; release of cytochrome C into cytosol; activation of initiator caspase 9 followed by executioner caspase 3 activation [107]. VSCs activate the intrinsic apoptotic pathway in most studied cellular types with the extrinsic ligand-activated pathway being activated only in osteoblasts cells [108]. Furthermore, following H_2S incubation, human oral keratinocyte stem cells express multiple p53-associated genes including programmed cell death, cell cycle control and DNA repair genes [109].

Fetor hepaticus

Worldwide liver diseases are a major health problem with significant mortality and morbidity [110,111]. Biopsy followed by histological assessment of liver tissue plays a key role in managing patients with liver diseases. However, this approach has major disadvantages related to invasive sampling, costs or morbidity [112]. Simple, noninvasive tools would be very helpful for monitoring the outcomes of therapy or for following up the progression of chronic liver disease. Liver is the key organ in detoxification and synthesis and has a major influence on metabolism [113]. As the metabolic functions of the liver are altered, some metabolites will be released in the systemic circulation, reach the lungs and be exhaled through breath (Figure 1B) [114]. Exhalation of these volatile compounds confers a sweet, musty slightly fecal aroma to the breath termed **fetor hepaticus** [96,115]. Therefore, breath tests may be useful in conjunction with other

Key Term

Fetor hepaticus: Exhalation through breath of volatile compounds released as a result of altered metabolic functions of the liver.

Table 2. Volatile malodorous compounds biological effects on different cell types from the oral cavity – *in vitro*.

Tissue	Cells	Origin	Biological event	Ref.
Oral epithelia	Normal keratinocytes	Ca9-22 cell line	Apoptosis – mitochondrial pathway activated; DNA damage	[105]
Oral epithelia	Keratinocyte stem cells	Human skin cell line	Apoptosis – mitochondrial pathway activated; DNA damage; p53 and Bax activity increased	[106]
Oral epithelia	Keratinocyte stem cells	Human oral mucosa	Apoptosis – mitochondrial pathway activated; DNA damage; activation of genes from p53 pathway connected with DNA repair, cell cycle arrest	[109]
Oral dermis	Fibroblasts	Human oral mucosa	Apoptosis – mitochondrial pathway activated; DNA damage	[104]
Dental pulp	Dental pulp stem cells	Human dental pulp	Apoptosis – mitochondrial pathway activated; DNA damage	[207]
Bone	Osteoblasts	Mouse calvaria	Apoptosis – mitochondrial and death ligand pathway activated; DNA damage	[108]

Cells were exposed to H_2S for 24 and/or 48 h at a concentration similar to that found in periodontal pockets of patients with periodontal disease.

Key Terms

Uremic fetor: Modified breath odor characteristic in patients with end-stage renal disease.

Trimethylaminuria: Disorder where high levels of trimethylamine accumulate in the body; associated with a specific unpleasant breath odor.

well-accepted serum or imaging techniques for assessing liver diseases. Early studies have highlighted out that in patients with liver disease, breath contains mercaptan, dimethyl-sulfide and hydrogen sulfide as a result of incomplete metabolism of amino acids with sulfur [116]. These results were later confirmed by Hisamura *et al.* [117] and Kaji *et al.* [118]. Using more sophisticated analytical techniques they demonstrated that volatiles containing sulfur such as methylmercaptan or dimethyl sulfide are significantly increased as compared with healthy subjects.

Patients with chronic liver disease could also be differentiated from healthy controls based on carbonyl sulfide, carbon disulfide and isoprene levels in breath. These compounds are also shown to correlate positively with standard clinical blood markers of liver damage [119]. Elevated levels of ethanol have been shown to be associated with hepatic steatosis, while increased breath acetone with nonalcoholic steatohepatitis. At the same time acetone levels were correlated to transaminase values in blood [120]. Other reports combine breath gas and liver enzyme data to propose a model for screening and discrimination of alcoholic fatty liver disease, nonalcoholic fatty liver disease, cirrhosis and cancer based on acetaldehyde and isoprene in combination with three other compounds [121].

More recently, by using GC–MS analysis Van den Velde *et al.* showed that patients with cirrhosis have a distinct pattern of VOC [122]. Their data identifies increased levels of dimethyl-sulfide, acetone, 2-butanone and 2-pentanone, and decreased concentrations of dimethyl selenide and indole. Their proposed model for diagnosing patients with cirrhosis had a sensitivity of 100% and a specificity of 70%. The group expanded the results in a recent study that identified 24 models of eight independent compounds that could discriminate between healthy volunteers and patients with cirrhosis, with sensitivity between 82 and 88%, and specificity between 96 and 100% [123].

Alcohol-induced hepatic toxicity was linked to the presence of volatile aliphatic hydrocarbons released as a byproduct of polyunsaturated fatty acids peroxidation. Therefore, exhaled pentane and ethane appear to be correlated to alcoholic hepatotoxicity [124]. Interestingly in patients with nonalcoholic fatty liver disease, the predominant compound in breath was ethanol, even in the absence of alcohol ingestion [125]. One possible explanation could be the over-production of endogenous ethanol by intestinal microbiota.

Uremic fetor

Chronic kidney disease causes a progressive deterioration of kidney function leading to a terminal phase called end-stage renal disease (ESRD). In the majority of cases, patients with chronic kidney failure require either dialysis or kidney transplantation to sustain life. One characteristic sign of patients with ESRD is the presence of a modified breath odor termed 'uremic breath' or '**uremic fetor**'. Uremia is characterized by retention of various host solutes that would normally be excreted by the kidneys [126]. Some of the molecules retained by uremic patients can also be found in exhaled breath as detectable VOC: methylamine, dimethylamine and trimethylamine.

First studies focusing on the substances characteristic to the 'uremic breath' started more than 40 years ago. In 1977, Simenhoff *et al.* [127] found that exhaled breath from patients with ESRD has increased concentrations of dimethylamine and trimethylamine. Moreover, the levels of these compounds decrease after hemodialysis.

A recent study by Pagonas *et al.* employs IMS coupled to a MCC, to identify specific VOC in patients with ESRD before and after hemodialysis [128]. Several compounds such as ammonia, hydroxyacetone and 3-hydroxybutanone, were significantly increased in ESRD patients as opposed to healthy volunteers. Patients undergoing hemodialysis expressed high levels of 4-heptanal, and 2- and 4-heptanone. In this case, 2-heptanone is a metabolite of 2-ethyl-hexanol and/or 2-ethyl-hexanal, which are contaminants of the dialysis' tubing system. As observed by the authors, the 'uremic fingerprint' that accumulates in renal failure is predominantly lipophilic, with volatile compounds that are able to mirror the state of uremic retention.

Some studies focused on the effect of hemodialysis on the VOC composition in exhaled breath. In one study uremic patients with ESRD undergoing routine hemodialysis were found to show up to eight-times more H_2O_2 in exhaled breath condensate than healthy volunteers [129]. At the same time, H_2O_2 breath levels did not change significantly during or immediately after hemodialysis. However, the endogenous source of the H_2O_2 was not fully investigated. One possible explanation is that H_2O_2 may originate from circulating phagocytes or from

the pulmonary tissue. Another study found that hemodialysis increases isoprene concentration in breath [130]. The study employed 50 patients undergoing dialysis and reported that isoprene levels were significantly higher after the hemodialysis than before the procedure. At the same time, isoprene concentrations did not correlate with other parameters such as blood pressure during hemodialysis, calorie intake or serum lipid levels. Several studies found that hemodialysis influences ammonia levels in breath (FIGURE 1C). Davies *et al.* [131] by SIFT-MS and Narasimhan *et al.* [132] by spectrophotometry, monitored exhaled breath during dialysis and showed that ammonia concentrations in breath are markedly reduced with hemodialysis. A recent study by Endre *et al.* used direct breath SIFT-MS sampling for assessing several volatile biomarkers before, during and after hemodialysis [133]. Their group found that ammonia is also decreased as a result of dialysis, but with a transient increase in some patients during mid-treatment. At the same time another biomarker, trimethylamine, decreased faster than ammonia or acetone, and increased rapidly at the end of hemodialysis. They concluded that ammonia in exhaled breath may be a potentially useful marker of hemodialysis efficacy. Rolla *et al.* showed that following hemodialysis patients with ESRD had a significant decrease of exhaled breath NO [134]. Their results showed that the cause for increased NO metabolites such as NOx, NO_2, and NO_3 in ESRD patients is oxidative stress and not pH. Another volatile compound studied in correlation with hemodialysis is isoprene. Davies *et al.* reported that isoprene concentration is significantly higher for patients on hemodialysis than for normal controls [131]. Also, as measured by SIFT-MS, breath isoprene level was markedly elevated immediately after treatment, as confirmed by Trovarelli *et al.* [135]. Capodicasa *et al.* found that isoprene levels were increased in exhaled air during hemodialysis, although they did not find any change in the exhaled concentrations of alkanes [136]. The same group reconfirmed and broadened the results on isoprene reporting in a subsequent study that in patients on intermittent dialysis isoprene levels in exhaled breath were increased by a factor of 2.7 [137].

Methanol is another compound that is significantly reduced as a result of dialysis in ESRD patients. In a recent study Lee *et al.* [138] point out that dietary restriction of fruits and vegetables can lower methanol production by gut flora making the compound a possible breath marker for monitoring daily diet in ESRD patients. Volatile compounds from human urine were used to detected changes in the head space of urine from patients with prostate or bladder cancer [139]. Studies show that urine from cancer patients contains elevated levels of formaldehyde while patients with urinary infections have higher levels of nitric oxide.

'The fish malodor syndrome': trimethylaminuria

Trimethylaminuria (TMA) or 'the fish malodor syndrome' is a genetic disorder in which higher than normal trimethylamine levels are accumulated in the body and are excreted in the urine, breath and sweat of the patient. As a result patients have an obvious, unpleasant body odor, associated in many cases with oral malodor. These complications have important implications in the patient social life, professional career, self-esteem, leading to depression disorders or even suicide attempts [140,141]. Interestingly this affection has been described in various cultures and historical ages ranging from ancient Indian epic tales to William Shakespeare [142]. The first clinical report of the syndrome was made by Humbert *et al.* describing a 6-year old girl with multiple systemic affections including a particular 'fish odor' associated to an increased excretion of free amines as a result of defective trimethylamine *N*-oxidizing system [143]. An important step forward was made by Pearson *et al.* who showed that hens producing eggs with a 'fishy' odor had an inherited genetic disorder [144,145]. One autosomal gene was found to be responsible for the inability to *N*-oxidize trimethylamine (FIGURE 1D). Furthermore, genetic studies on human populations showed that dysfunctions in *N*-oxidation of trimethylamine can be fully explained genetically. Studies on large population groups showed that the distribution is around 1% with an over-expression in women [146,147]. The current studies on TMA employ specialized equipment such as MALDI TOF-MS [148]; GC [149]; proton nuclear magnetic resonance spectrometry [150,151] or ESI-MS/MS [152].

The enzyme responsible for oxidizing TMA belongs to the hepatic flavin monooxygenases (FMO) family. The FMO family contains five isozymes (FMO1 to 5) that detoxify a wide range of xenobiotics from the diet. In human liver the isoform that plays a key role in TMA *N*-oxidation is FMO3. The FMO3 gene is located on the long arm of chromosome 1 (1q24.3) encoding a 60 kDa protein [153].

Biochemically, patients with TMA present a difference between the dietary intake of trimethylamine and the liver's ability to process the amines. As a direct result, excess of trimethylamines accumulate in urine, perspiration and can also be detected in the oral cavity. The available scientific literature shows that there are several different types of TMA [142]. The disease can have a primary genetic determinant or can exist in less severe forms at the intersection of environmental, genetic and constitutional parameters. The primary form is the best understood and appears as a result of a genetic dysfunction. Data from different countries is currently available, all pointing to the same underlying problem of inactivating mutations in FMO3 gene [154]. The acquired form is more uncommon and less understood; one possible mechanism may be viral infections that modify normal expression of FMO3 gene [155]. Other types include transient TMA associated with menstruation [156,157] or early childhood [158]; increased amounts of dietary precursors [159] or specific bacterial gut overgrowth [160]. Aside from 'fish malodor syndrome', FMO3 deficiency can also have other metabolic implications. Although there is yet no solid scientific evidence, in theory there might be a connection between FMO3 deficiency and hypertension. One explanation may be due to the fact that the enzyme is responsible for catecholamine inactivation [161]; FMO3 deficiency might lead to higher circulating levels of catecholamines, which can translate in increased blood pressure levels [162]. Another possible systemic effect of FMO3 mutation is possible in patients on medication with several drugs such as: tyramine, morphine, propranolol and chlorpromazine [163]. Scientific literature reports some adverse reactions to tyramine and sulfur-based medication in patients with TMA [164]. Patients suffering from TMA are also known to suffer from affective disorders of different degrees of severity. However, this aspect is probably connected more to the social isolation than to a chemical unbalance.

VOC in other diseases

In vitro

In order to better identify the compounds specific to certain tissues or cell types some studies focused on the VOC profiles released by different cell types cultured *in vitro*. Results of studying different lung cancer cell lines in 2D cultures suggested that altered VOC production can be detected in the early stages of carcinogenesis, and can represent a basis for noninvasive diagnosis of lung cancer.

Therefore, 2D studies on various normal or cancer cell lines showed either an increase or decrease in several volatile compounds classes such as alkanes, aldehydes, aromatic compounds or alcohols [25,165–172]. A recent study uses both cancerous and noncancerous lung cell lines grown in 3D scaffolds of collagen type I hydrogel [167]. Although the study provides limited data by employing only two cell types, the 3D culture method offers clear advantages over classical 2D approaches by better resembling the physiological situation of cells growing *in vivo*. Up to date there is no general consensus regarding the VOC 'fingerprint' of specific cell types. More work is needed to identify specific VOC, not only from cell lines but also from primary cultures isolated from different tissues grown on both 2D and 3D cultures. The release or consumption of VOC in general and VSCs in particular has also been detected in different microbial cultures. Hydrogen sulfide or methyl-mercaptan, malodorous compounds, are released by bacterial metabolism through desulfurization of different amino acids or by removing sulfur from other peptides that contain thiol groups [173]. Oral Gram-negative bacteria are shown to produce more VSCs than oral Gram-positive bacteria [174]. Among other VOC, *Staphylococcus aureus* and *Pseudomonas aeruginosa,* pneumonia-associated bacteria, were found to release VSCs such as dimethyldisulfide and methanethiol but with differences in concentration profiles between the two bacterial species [24]. Other pathogens of the upper airways *Streptococcus pneumoniae* and *Haemophilus influenzae* were also shown to release VSCs such as methanethiol [23]. Reports on *Candida albicans* show that when grown *in vitro*, bacteria releases dimethylsulfide while carbon disulfide and dimethyldisulfide are consumed [30].

Diabetes

Diabetes mellitus is a metabolic disease with prevalence in the general population estimated at 1–6% [175]. The disease is classified in insulin-dependent Type 1 and noninsulin dependent Type 2 diabetes, with the last one representing the majority of cases. A growing body of research shows that the biochemical transformations specific to the onset and development of the diabetic condition may reflect in the pattern of VOC found in the exhaled breath The presence of several specific compounds such as acetone, isoprene, methyl nitrate, ethanol, acetone, xylene or ethyl-benzene have been linked to diabetes and proposed as potential markers of the disease [33].

However, to date the reported data has been inconsistent and suggest that none of the present compounds alone represent a reliable biomarker for the management of diabetes. Acetone in breath is elevated during diabetic ketoacidosis and is an indicator of circulating ketone bodies (FIGURE 1B) [176]. Some studies report that acetone levels in breath are significantly higher in Type 2 diabetes when compared with healthy volunteers [177,178] and may be responsible for the specific 'rotten apple'-like breath print. However, other studies did not confirm these results finding no significant difference between healthy volunteers and Type 2 diabetics [36]. Acetone levels are not affected by gender but differ according to age and fasting state [179] or decrease as a result of glucose intake [180]. No association was observed between acetone in breath and dietary nutrients or capillary blood test [181]. Some reports suggest a positive correlation between acetone in exhaled breath and glucose levels in the blood. Turner *et al.* used a clamp technique to report a positive correlation between acetone in breath and glucose in blood [182]. It was also reported that acetone levels in blood correlate with acetone concentrations in exhaled breath in healthy volunteers [183]. Isoprene (2-methyl-1,3-butadiene) is a byproduct of cholesterol synthesis through the mevalonic acid pathway. Turner *et al.* results show no correlation between isoprene in breath and blood glucose in healthy subjects [184]. Nelson *et al.* also reported no difference in isoprene levels between healthy controls and diabetes patients and no statistical difference between diabetes patients in the fasting and postinsulin state [178]. A few studies showed that methyl nitrate as well as ethanol, acetone and aromatic hydrocarbons have been correlated with blood glucose in patients with Type 1 diabetes [185–187]. Other compounds that can discriminate between healthy controls and patients with Type 2 diabetes include dimethyl sulfide, butanol and pyridine [188]. However, the reasons for these correlations have not yet been explored. Oxidative stress has also been analyzed in relation to diabetes mellitus [189]. Aside from the two main types of diabetes, another study reports a number of volatile biomarkers discovered in the breath of patients with gestational diabetes mellitus [190]. A detailed overview of the selected techniques used for breath analysis in diabetes mellitus was given by Miekisch *et al.* [191].

VOC research: limitations

Some authors [192,193] argue that although in the last few years intense efforts have been made to identify potential volatile markers for different diseases, so far only a few compounds are specific and have actually been used in clinical applications. Some drawbacks to breath analysis include:

- Very large variations in the concentrations of the volatiles with each subject (ranging from ppb to ppt per volume). Moreover, preconcentration of samples and also effective standardization of analytical methods is often difficult;
- Cautious interpretation of results from studies that employ small sample populations – the wide inter- and intra-personal variability of VOC concentrations asks that the sampling design is carried out on statistically significant samples from the target population;
- Compounds are also present in the environment and sometimes in higher concentrations than in the body;
- Another concern is the presence of water in exhaled breath, which affects isolation and detection of specific compounds;
- The equipment used for breath analysis are expensive, time-consuming and require specialized operators;
- The metabolic pathways that generate these compounds are also still unclear.

Future perspective

Tests designed to analyze exhaled breath represent an important noninvasive tool for assessing various pathologies. Nevertheless, only very few tests have yet been approved by the FDA and the European Medicines Agency. Among them are the breath carbon dioxide test for capnography, the breath nitric oxide test for monitoring asthma therapy, a breath test for detection of heart transplant rejection (see [194]), the breath ethanol test for blood alcohol (law enforcement) and the ^{13}C-urea breath test for detection of gastric infection by *Helicobacter pylori*. Incidentally, the ^{13}C-urea breath test is the only FDA-approved test using ^{13}C-labeled substrates. The breath tests based on ^{13}C-uracil, ^{13}C-dextromethorphan and ^{13}C-pantoprazol, in particular, are not FDA-approved, mainly due to the high costs for approval.

Breath tests using malodorous substances could rely on different compounds: ammonia (for liver and renal disease), dimethyl-sulfide (cirrhosis, bacterial infections of the airways),

trimethylamine (ESRD and trimethylaminuria), hydrogen sulfide, dimethyl sulfide, methanethiol, cadaverine and putrescine (oral malodor), and 3-(methylthio)propanal (produced by *S. pneumoniae*) [23]. Most of these compounds are not yet easily measurable. For amines, in particular, one has to take into account interaction with the walls of storage containers or tubing [59,195].

Currently, human breath analysis usually requires sophisticated equipment and skilled personnel. It may be expected that hand-held instrumentation for point-of-care settings will be developed in the future. Miniaturization is possible, though not imminent, for detection of the most interesting compounds for medical diagnostics. As an example, a field asymmetric ion mobility sensor has been developed by Owlstone Nanotech (CT, USA) [301], which is a few centimeters wide and allows identification of compounds through a pattern of ion mobilities that are produced by application of different compensation voltages.

In order for breath measurements to be used to their full potential in routine clinical settings a number of requirements have to be met. A future breath analyzer would have to meet several characteristics such as: miniaturization compared with current instruments; reliability; economically viable (low costs for consumables and for actual analyzing procedures); simple sample preparation – comparable with current biochemical and molecular biology tests; solid base for data interpretation and safety. IMS, laser spectrometry and sensors or sensor arrays have considerable potential to result in point-of-care devices in the future.

Current research demonstrates that different pathologies can have their own breath signature given by the presence of specific volatile odorous compounds. It is a long accepted fact that clinical physicians can presume the existence of a disease just by smelling the patient's breath. If the unpleasant odor is coming from the oral cavity the treatment is mostly focused on treating dental problems and improving oral hygiene. However, if malodor is suspected to have an extra-oral cause such as fetor hepaticus, uremic fetor, TMA or diabetes, a precise diagnostic together with treatment of the underlying disease is essential. Although a wide number of volatiles have been proposed as possible biomarkers, no general consensus regarding the odorous compounds that characterize each disease has yet been reached. Further research is needed in order to identify which groups of compounds define the odor signature associated with different pathologies. Another line of research will involve *in vitro* studies in order to clearly elucidate the origin and the biochemical pathways of odorous compounds in particular and volatile compounds in general.

Financial & competing interests disclosure

A Amann appreciates funding from the Austrian Federal Ministry for Transport, Innovation and Technology (BMVIT/BMWA, project 836308, KIRAS) and support from the Oncotyrol-project 2.1.1. The Competence Center Oncotyrol is funded within the scope of the COMET – Competence Centers for Excellent Technologies through BMVIT, BMWFJ, through the province of Salzburg and the Tiroler Zukunftsstiftung/Standortagentur Tirol. The COMET Program is conducted by the Austrian Research Promotion Agency (FFG). A Amann thanks the government of Vorarlberg (Austria) for its generous support.

B Calenic thanks for the funding received from Executive Agency for Higher Education, Research, Development and Innovation Funding, Romania (Postdoctoral grant – PN-II-RU-PD-2011–3–0105). B Calenic also

Executive summary

- Volatile organic compounds are trace elements found in exhaled breath that can be used as biomarkers for various diseases.
- Malodorous compounds with potential for clinical diagnosis and therapeutic monitoring are ammonia, trimethylamine, hydrogen sulfide, dimethylsulfide, methanethiol, cadaverine, putrescine or 3-(methylthio)propanal. Hydrogen sulfide is also an emerging gasotransmiter acting as a physiologic mediator of hypoxia.
- Analytical techniques currently employed in exhaled breath research include: GC–MS, proton-transfer-reaction-MS, selected ion flow tube-MS, ion mobility spectrometry, laser spectroscopy and sensors for volatile compounds.
- Oral halitosis or fetor ex ore is usually associated with volatile sulfur compounds such as hydrogen sulfide or methyl-mercaptan.
- Fetor hepaticus is present in patients with chronic liver disease; exhaled breath contains volatiles most often associated with incomplete metabolism of amino acids with sulfur.
- Uremic fetor is a characteristic sign in patients with chronic kidney disease; several molecules that are normally excreted by kidneys are retained by uremic patients and can be detected in exhaled breath.
- Trimethylaminuria (the fish odor syndrome) is a genetic disorder associated with a defect in the trimethylamine *N*-oxidizing system leading to high levels of free amines accumulating in the body.
- *In vitro* studies are needed for identifying volatile compounds specific to certain cell types and biological processes.

acknowledges the Young Scientist Grant 2011–2013 from University of Medicine and Pharmacy Carol Davila, Bucharest, Romania.

A Amann and B Calenic appreciate travel support within the Erasmus Staff Training program during the period 2012/2013. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

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