

REVIEWS

Volatile Organic Compounds as Diagnostic Biomarkers in Gastrointestinal and Liver Diseases

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Abstract

The assessment of disease activity in various conditions may be performed using a range of different techniques. These include the use of non-invasive tests, such as acute phase inflammatory markers and simple radiological techniques, to more advanced invasive and complex modalities. Over the past two decades the analysis of volatile organic compounds (VOCs) in biological specimens has attracted a considerable amount of clinical interest. The investigation of VOCs, using a variety of analytical techniques, has shown a significant correlation between the pattern and concentration of VOCs and the occurrence of various diseases. This provides a potentially non-invasive means of diagnosis, monitoring of pathological processes and assessment of pharmacological response. It may be rapid, simple and acceptable to patients. In this paper we review the medical literature and research efforts that have been carried out over the past decades, and try to summarize the clinical implications of VOC analysis of various biological emanations including stool, breath and blood samples and their correlation with gastrointestinal and liver diseases.

Key Words

Volatile organic compounds – solid phase microextraction – gas chromatography/mass spectrometer – oxidative stress – inflammation – cirrhosis – encephalopathy – faecal microbiota.

Introduction

Diagnosis and monitoring of disease may be unpleasant, painful and even dangerous. Non-invasive accurate methods

of assessing the disease process are the ‘holy grail’ for research into diagnostics. For patients with gastrointestinal (GI) diseases, assessment may involve blood tests, stool analysis, radiological investigations and invasive endoscopic procedures. Patients with intestinal diseases often report that the odour of their faeces is abnormal and unpleasant during disease relapse, yet little research has been directed towards the composition of faecal gases. Similarly, in patients with liver failure, fetor hepaticus may be observed. Unfortunately not all clinicians can recognise this smell. Early diagnosis of hepatic encephalopathy may be possible if the changes in the odour of patients’ breath could be assessed more reliably. We have investigated the possibility that odours might be used to make the diagnosis of GI and hepatic diseases.

Volatile organic compounds (VOCs) are a diverse group of carbon-based chemicals that are volatile at ambient temperature. They may be odorous and may be emitted from bodily fluids and as a result, VOCs emitted from faeces and breath may include biomarkers of use in the assessment of GI and liver disease [1].

The assessment of VOCs has interested scientists in diverse fields for some decades. VOC analyses are routinely used in the assessment of environmental contamination [2, 3], forensic science [4, 5] and the fragrance and flavour industries [6-8], and developments in such areas may now be applicable to medicine. The analysis of VOCs is complicated by their wide range of abundance, the complexity of the mixtures in which they are present and the presence of water that interferes with analysis. To be useful, analysis must be precise and efficient [9]. The standard approach is a separation step with gas chromatography and analysis with mass spectrometry, electron capture detection or flame ionization detector [10, 11]. The introduction of solid phase microextraction (SPME) by Pawliszyn et al in 1989 [12, 13] improved on the traditional techniques. It is a miniaturized, solvent free, cheap and simple to use sample pre-concentration technique in which all steps of sample preparation are combined in one simple step. The significance of SPME is illustrated by the exponential rise in the articles published since its introduction [Fig. 1] [14].

Normal metabolism generates countless VOCs that

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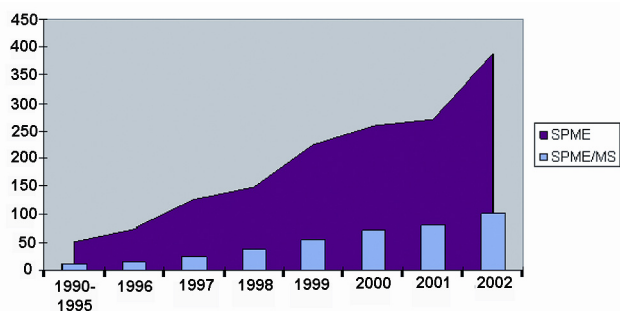


Fig 1. Increase in published articles on solid-phase microextraction (SPME) and SPME / MS (mass spectrometry) since its introduction in 1990.

may be excreted in body fluids to give a specific odour to these excreta. Pathological processes have the potential to influence these VOCs either by producing new VOCs or by the metabolic consumption of VOC substrates that are normally present. It is a change of this kind that underpins the odour on the breath of patients with diabetic ketoacidosis and hepatic encephalopathy [15].

Over the past few years the analysis of VOCs in various biological specimens especially in breath, urine and stool to monitor metabolic disorder, has become increasingly popular in clinical settings as numerous studies have evaluated their diagnostic potential in different diseases [16-22].

Faecal VOCs for the diagnosis of gastrointestinal disease

Human faecal flora comprises innumerable species of bacteria which, in normal circumstances, are believed to contribute to the mucosal integrity, protection against invading organisms and maintenance of host health [23]. They are also involved in the colonic fermentation of endogenous amino acids, which produces several putrefactive compounds such as ammonia, aliphatic amines, branched chain fatty acids, indole, phenol and volatile sulphur containing compounds. These compounds are responsible for the specific odour of the faeces. Moore et al [24] and Saurez et al [25, 26] have shown that the sulphur containing compounds namely hydrogen sulphide, dimethyl disulphide, methyl disulphide and dimethyl trisulphide are the main odoriferous compounds of faecal gases. An abnormality in the activity and /or composition of intestinal microbiota may alter the odour of human faeces which has been observed by patients and healthcare staff in various GI disorders. As human stool represents the end-product of diet, digestive and excretory processes, as well as colonic bacterial metabolism, the examination of faeces may be the best non-invasive way of diagnosing GI disease.

Campylobacter jejuni infection from contaminated poultry is the cause of gastroenteritis in 1-2% of Europeans every year. *Campylobacter* is common in poultry faeces. Our group reported the analysis of VOCs from chicken's faeces with and without *Campylobacter jejuni*. This was the first attempt to use VOC techniques to investigate

zoonotic infection. The abundance of six VOCs namely, hexanal, (E)-2-octenal, pyrrole, ethyl ethanoate, methyl alcohol and 2-heptanone was sufficiently different in the two groups of chickens to be considered faecal biomarker for *Campylobacter jejuni* in chicken faeces with a sensitivity of 96% and a specificity of 95% [27].

We have also characterised the human faeces in health and disease [28]. Analysis of faecal samples from 30 asymptomatic individuals identified 297 VOCs, and among these, acids, alcohols and esters were the most commonly occurring. Forty-four compounds were common to 80% of samples; many of these remaining constant in individuals and are shared in health. The distinct pattern of VOCs identified in the stool of patients with *Clostridium difficile*, *Campylobacter jejuni* and ulcerative colitis strongly suggests that specific changes occur in the pattern of VOCs in GI disease and identifying these patterns could be utilised as a diagnostic modality in clinical settings [28]. In addition, an analysis of VOCs from faeces of Bangladeshi patients affected by cholera showed that fewer VOCs were detected in cholera samples in contrast to healthy volunteers [29]. Two compounds namely dimethyl disulphide and p-menth-1-en-8-ol were exclusively detected from cholera samples [29]. Although none of Bangladeshi healthy volunteers had dimethyl disulphide, it was found to be ubiquitous in British control samples [28]. Nevertheless, p-menth-1-en-8-ol remains a promising biomarker.

In an attempt to understand the development and colonisation of infant GI tract, a small study was undertaken by De Lacy Costello et al [30] on the analysis of faecal VOCs from neonates. It showed that fewer VOCs were found from premature neonates (136 compared to 311 from healthy adults). There was a very low frequency of nitrogen compounds, and virtually no sulphides were detected in the neonate's faeces. This reflects the simplicity of neonatal flora compared to the microbiota of adult gut as most of these VOCs are produced by fermentation of dietary substrates by gut microbes. This observation underpinned further work in neonates with necrotising enterocolitis (NEC) in which those infants who developed NEC were shown to have fewer esters among their VOCs than their healthy counterparts; furthermore, these esters were often shown to have 'disappeared' from faeces, having been present a matter of days earlier. The change occurred before NEC was recognised by clinicians, suggesting that VOCs may have a role in the early identification of NEC [31].

These studies indicate that analysis of faecal VOCs has the potential for the diagnosis of a range of GI disease. With further progress in the analytical techniques and research into this area, the role of faecal VOCs may be defined more specifically in clinical practice. It has the advantage of providing a simple and non-invasive mean of diagnosis and monitoring of disease activity, early detection of complication and tailoring of therapy. Additionally, it is rapid, reproducible and can be performed at the point of patient care.

Breath VOCs

Many VOCs are generated during metabolic and pathological processes which give different odour to the breath. In addition, some of the VOCs may be absorbed from the environment as contaminants. The first modern breath analysis is commonly attributed to Linus Pauling in 1971, who used gas liquid partition chromatography to quantitatively determine about 250 volatile substances in a sample of breath [32].

Although the biochemical origin of these VOCs is mostly unknown, it appears that most of them may not be of endogenous origin. VOCs with positive alveolar gradient (concentration higher in breath than in air) are more likely to have been produced within the body than ingested from external environment through the lung. In an analysis of breath from 50 normal individuals, a total of 340 VOCs were detected and only half of these had a positive alveolar gradient [33]. Further more, only 27 of these VOCs were observed in all 50 individuals, showing a wide inter-individual variation [33, 34]. Analysis of endogenous VOCs in exhaled breath has potential to provide information about underlying pathological process. The recent advances in sample preparation, pre-concentration, storage and analysis have made breath analysis practical and reliable means of collecting clinical information, which is reproducible, acceptable to patient and clinically relevant. A number of breath VOCs have been identified by various studies as markers of different systemic diseases as summarized in Table I.

Liver and gastrointestinal diseases

Liver diseases represent a major health problem with significant morbidity and mortality. Aetiologies are varied and clinical presentation may range from an asymptomatic state to severe liver failure [35]. Since the liver plays a vital and complex role in various metabolic and synthetic functions, damage to its cells results in an increased concentration of toxic metabolites in systemic circulation [36]. Some of these metabolites may be exhaled through the lungs giving rise to malodorous breath. Earlier studies have shown that sulphur containing compounds such as dimethyl sulphide, hydrogen sulphide and mercaptans are increased both in blood as well as in alveolar breath due to incomplete metabolism of sulphur containing amino acids in liver disease [37, 38]. Some of the VOCs may give a characteristic smell to breath, which has a sweet, musty, or even slightly faecal aroma, termed fetor hepaticus [38].

Impaired metabolism of methionine in liver diseases was proposed as a cause of these compounds. Studies by Kinsell et al [39] and later by Chen et al [37] found increased levels of these sulphur containing compounds in breath of liver patients in comparison to healthy individuals when both were fed methionine. In healthy individuals, methionine metabolism produced S-adenosylmethionine (S-AdoMet) which regulates hepatocytes growth, differentiation and death. Low biosynthesis of S-AdoMet, as a result of impaired methionine metabolism, may have a causative role in liver cirrhosis

Table I. VOCs in breath identified by published studies as potential diagnostic markers of oxidative stress in various diseases

Disorders	VOCs detected	Study
Breast cancer	Alkanes, monomethylated alkanes	Phillips M et al 2003 [68]
Asthma	NO, pentane, ethane, 8-isoprostane	Olopade CO 1997 [69], Paredi P 2000 [70], Montuschi P 2000 [71]
Chronic obstructive pulmonary disease (COPD)	NO, CO, H ₂ O ₂ , alkanes, aldehydes, nitro tyrosine	Corradi 1999 [72], 2003, Van Beurden 2002 [73]
Angina, ischemic heart disease	Alkanes, methylated alkanes [22]	Phillips M 2003
Hepatic coma	Methyl-mercaptan, dimethyl sulphide	Hisamura M 1979 [45], Kaji H 1978 [43]
Diabetes mellitus	Acetone, ethanol, methyl nitrate	Novak BJ 2007 [74], Galassetti PR 2005
Schizophrenia	Carbon disulphide, Pentane, ethane	Phillips M 1993 [75]
Cystic fibrosis	Carbonyl sulphide, alkanes	Phillips M 2004 [76]
Allograft rejection	Carbonyl sulphide	Studer SM 2001 [77]
Rheumatoid arthritis	Pentane	Humad S 1988 [78]

[40, 41]. However, a recent systematic review was unable to demonstrate any significant benefit of S-AdoMet replacement in patients with alcoholic liver disease [42].

With the development of more sophisticated analytical techniques, it has become possible to separate these volatile molecules in breath based on their masses. Consequently, studies from Kaji et al [43], Tangerman et al [44] and Hisamura et al [45] demonstrated the higher levels of these sulphur containing volatiles in the breath of patients with liver disease by using modern analytical methods. More recently, Van den Velde et al [46], in a small study, analyzed the breath from 50 patients with established liver cirrhosis by using GS-MS techniques. They found that dimethyl sulfide, acetone, 2-pentanone and 2-butanone were significantly higher in alveolar air of patients with hepatic cirrhosis and were able to discriminate the cirrhotic group from normal individual with a sensitivity of 100% and specificity of 70% [46].

In a study from Netzer et al [47], a group of four breath markers was identified with the use of ion molecular reaction-mass spectrometry (IMR-MS). Their study group consisted of patients with alcoholic fatty liver disease (AFLD), non-alcoholic fatty liver disease (NAFLD), cirrhosis and healthy controls. Among the detected markers, acetaldehyde, M103, isoprene, M67 and M60 (where Mx indicate unannotated compound mass) were found to discriminate between the diseased group and healthy controls. It is important to note that none of these studies was able to demonstrate whether a particular disease might have a unique breath volatile pattern.

A number of studies support the crucial involvement of oxidative stress in the pathogenesis of liver disease including alcoholic and non-alcoholic hepatotoxicity, infections, iron overload and autoimmune liver damage [48, 49]. The peroxidation of polyunsaturated fatty acids, such as linoleic acid and linolenic acid which are cell membrane components, induces the formation of volatile alkanes that are excreted in the breath. These straight chain aliphatic hydrocarbons have been advocated as noninvasive markers of free-radical induced lipid peroxidation in humans [50, 51]. Exhaled hydrocarbons especially breath ethane and pentane, appear to be better correlated with alcohol induced hepatic injury than to other aetiologies. For example, Letteron et al [52] measured the ethane levels in the breath of patients with alcoholic and non-alcoholic hepatitis and found it significantly higher in alcohol abuser than other groups, although it was weakly correlated with level of alcohol use, histological scoring or other complications. This stronger correlation of breath ethane with alcohol might be due to increased induction of cytochrome P450 by alcohol leading to increased production of oxygen radicals.

In contrast to alcoholic induced liver injury where alkanes were the predominant volatiles in breath, in NAFLD, which is more prevalent in obese patients, ethanol levels were found to be raised in exhaled breath of patients even in the absence of ethanol ingestion [53]. The hypothesis of increased endogenous production of ethanol in obese individuals was supported by preliminary animal models followed by human studies showing the role of intestinal microbiota in the production of ethanol in obese patients [54, 55]. However, it remains unclear whether increased intestinal permeability with secondary endotoxin mediated damage, in addition to increased endogenous ethanol, contribute to the development of steatohepatitis in obese individuals [56, 57].

Studies reporting breath VOCs in inflammatory bowel disease (IBD) are scarce. Lipid peroxidation has been proposed repeatedly in the pathophysiology of IBD, breath alkanes have been studied as a measure of lipid peroxidation and have been correlated with disease activity [58]. In an initial animal study, Ondrula et al [59] showed increased exhalation of pentane by rats after induction of colonic inflammation. Pentane levels in exhaled air rapidly normalized with the resolution of inflammation. This was later supported by a human study undertaken by Kokoszka et al [60] who demonstrated a good correlation of breath alkanes with IBD activity. Further more, Pelli et al [61] also measured breath alkanes in patients with active IBD and found that ethane, propane and pentane were significantly elevated compared to healthy controls. A similar observation was made by Sedghi et al [62] who also demonstrated a positive correlation of breath ethane with endoscopic appearance, symptoms and disease activity score.

In short, breath testing for volatile compounds in various types and stages of liver disease and GI diseases seem promising and appear to fulfil the demand and desire for a

non invasive investigation due to ease of sample collection, repeatability, reproducibility and acceptance by the patients group especially children and younger adults. Despite their various advantages, few breath tests have been able to gain the confidence of the clinicians to be integrated in everyday clinical practice although they have been used experimentally for many decades. Among the more frequently used breath tests are: glucose-hydrogen breath test for small bowel bacterial overgrowth, hydrogen breath test for lactose intolerance, and the urea breath test to detect *Helicobacter pylori* infection in the stomach [63]. Breath testing still remains an under estimated marker for various GI and liver diseases which deserves further attention.

Blood VOCs

Although breath analysis of VOCs offers a painless, simple and rapid way of assessing underlying pathological conditions, there is a speculation that a significant proportion of these VOCs may be inhaled from the external environment and have the potential to interfere with accurate breath analysis. Therefore, studies have looked into the analysis of VOCs from blood which represent more internal environment of biological activities. Goldberg et al [64] analyzed the serum VOCs of patients with hepatic cirrhosis by using a direct injection capillary column gas chromatography method. They found raised levels of 3-methylbutanal in chronic encephalopathy which correlated well with severity of the disease. In contrast, Marshall et al [65, 66] found no difference in the level of 3-methylbutanal in cirrhotic patients when compared to normal controls. This aldehyde results from breakdown of leucine by bacteria however, when researchers fed leucine to cirrhotic patients, no change in the clinical conditions were observed even when the value of 3-methylbutanal rose to 700% above the base level [66].

More recently, a small study published by Ruyi et al [67] reported the analysis of VOCs in blood of patients with liver cancer. By using the GC-MS technique, they found hexanal, 1-octen-3-ol and octane as possible biomarkers of liver cancer with good sensitivity and specificity. However, further studies are needed to evaluate these markers in more details and their correlation with liver cancer.

Caveats

Dimethyl disulphide is one of two potential markers of cholera in Bangladeshis, however it is commonly found in the faeces of healthy Europeans. This emphasises the need for appropriate controls in every clinical study. For example, VOCs related to oxidative stress were studied in breast cancer and their absence had a higher negative predictive value than mammography. This suggests such VOCs are associated with breast cancer; however the samples group of VOCs are also positively associated with chronic obstructive pulmonary disease (COPD) and pre-eclampsia. In short, disease samples as well as healthy controls must be investigated or else false positive diagnoses will be made.

Conclusion

In summary, as our knowledge about pathophysiological mechanisms of disease processes has increased, the focus started to tilt towards non-invasive, rapid and inexpensive diagnostic modalities which are simple to perform, painless and more agreeable to patients. In the past two decades, the understanding of VOC analysis in biological fluids has grown significantly. The characteristic patterns of VOCs in faeces have been reported for diverse causes of diarrhoea. Since it is potentially fast and convenient, it opens up a new promising area for use as a noninvasive diagnostic tool with various advantages: it can be performed repeatedly, can be applied to children including neonates, to patients with severe disease in whom more invasive procedures are not possible. Despite the progress, VOC analysis has not yet been introduced into diagnostic armamentarium in clinical practice. This is mainly due to the lack of standardization and normalization of sampling procedures and the absence of generally accepted evaluation criteria for data.

In the future, it is likely that the advent of more sensitive analytical techniques will extend the diagnostic value of VOCs analysis and that these may provide a much-needed reliable, real-time and point-of-care diagnosis and monitoring of various GI and liver disorders.

Conflicts of interest

Chris SJ Probert, Iftikhar Ahmed, Tanzeela Khalid, Emmanuel Johnson, Steve Smith and Norman Ratcliffe declare that they do not have anything to disclose regarding funding from industries or conflict of interest with respect to this manuscript.

*Disclaimer

Chris SJ Probert and Iftikhar Ahmed contributed equally in Literature search, writing the main review, revising, editing and finalizing the whole manuscript. Other authors contributed in revising and proof reading.

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