Techniques and issues in breath and clinical sample headspace analysis for disease diagnosis

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Abstract

Analysis of volatile organic compounds (VOCs) from breath or clinical samples for disease diagnosis is an attractive proposition because it is non-invasive and rapid. There are numerous studies showing its potential, yet there are barriers to its development. Sampling and sample handling is difficult, and when coupled with a variety of analytical instrumentation, the same samples can give different results. Background air and the environment a person has been exposed to can greatly affect the VOCs emitted by the body, however this is not an easy problem to solve. This review investigates the use of VOCs in disease diagnosis, the analytical techniques employed and the problems associated with sample handling and standardization. It then suggests the barriers to future development.

Keywords

VOCs, breath analysis, biomarkers, SIFT-MS, GC-MS, headspace analysis, disease diagnosis, PTR-MS, e-nose, spectroscopy,

Executive summary

Introduction

- It has been known for centuries that some diseases have an odour associated with them
- Modern volatile organic compound (VOC) analysis for disease diagnosis has arisen from this

The origin of VOCs

- VOCs arise from normal or abnormal metabolic processes in the body and from the bacteria that live in or on the body
- Some illnesses results in a difference of the profile of VOCs emitted in breath or from other body fluids
- Infectious disease may also produce a change in the profile of VOCs.
- VOCs also arise from the body through exposure to them in the environment

Sampling and handling considerations

- A major difficulty in using VOCs in diagnosing or detecting disease is being able to handle and store them
- Whole breath may be analysed directly if it is possible to get the patient to the instrument
- If it is not possible to do this, samples need to be stored
- Whole breath can be stored in sample bags or evacuated metal canisters
- If whole breath cannot be stored, sorbent methods such as SPME (solid phase micro-extraction) or the use of sorbent tubes can be coupled with analytical techniques such as gas chromatography mass-spectrometry
- These indirect methods are sensitive but not all compounds may be detected and quantified.

Techniques

- Trace gas analysis mass spectrometric techniques offer rapid and direct analysis but are often cumbersome and expensive
- Laser based spectroscopic techniques are rapid and direct and may be used instead of mass spectrometry for some compounds
- Non-specific sensors may be assembled into an array called an electronic nose, which respond to different odours by producing a complex signal. E-noses are rapid, portable and relatively inexpensive but cannot identify individual compounds.
- The most widely used technique is a combination of gas chromatography (GC) and mass spectrometry (MS). Sample components are separated by GC and then identified by MS. This is a powerful technique, but it is slow, cumbersome and expensive.

Backgrounds

- The environment to which a subject has been exposed will contribute its own VOCs, and these will be exhaled or excreted by the body for some time after, depending on their retention co-efficient in the body.
- There needs to be some way of accounting for the variation in background environments to which subjects are exposed.
- There is no perfect way of accounting for background air, but several methods have been tried, for example by analyzing the background and subtracting those VOC concentrations, calculating retention co-efficients for compounds of interest, or selecting matched controls who have lived in a similar environment.

Standardisation

- There are no acknowledged standardized ways of taking, handling, storing and analyzing breath and clinical fluid samples for VOC analysis
- As there are many different methods for taking and analyzing samples, standardization methods need to focus on ensuring that each method should give the same results when analyzing identical samples.
- This can be achieved through using standardised artificial breath test mixtures and validating methods against these.

Future perspective

- VOC analysis for disease diagnosis is promising but progress is slow
- Standardization is necessary
- Profiles from multiple compounds is likely to be more robust at diagnosis than the use of individual marker compounds
- Properly validated statistical methods are needed to ensure findings are robust and repeatable
- This approach has great potential but further work is needed to ensure it is at least as robust and accurate as existing diagnostic techniques

Key references

*References of considerable interest ***

[10], [43]. These articles are of particular interest as they summarise the knowledge available of the range of VOCs that are generated from various body fluids

[128] This article explains the need for standardization in breath sampling.

[112] This shows the huge range of VOCs which may be analysed as instruments have improving sensitivity, however with complex data sets, use of this information is harder.

*References of interest **

These articles are of interest because they give examples of where VOC analysis can be used in diagnosis: [15], [40].

[125] is important because it gives an effective method for dealing with background air, although only for known compounds.

Introduction

The ancient Greeks were known to use the odour of volatile organic compounds emitted from breath and body fluids as an aid to diagnosis [1], but it wasn't until Linus Pauling and

co-workers [2] condensed human breath and analysed its constituents using gas chromatography that modern breath analysis began. Linus Pauling was also involved in the early analysis of volatile organic compounds (VOCs) from urine in the 1970s [3,4]. It was still another decade or two before it really took off, but since the mid 1990s, there has been a very rapid development of analytical instrumentation to enable breath analysis to expand [5-8]. In actual fact, VOCs and other trace gases such as ammonia and hydrogen cyanide (which for the purposes of this article are included when VOCs are mentioned) are emitted from all body fluids and tissues, for example breath, urine, faeces, skin, sputum, blood, serum, pus, aspirates, tissue, lavage etc. Selecting the appropriate medium for analysis is important and the choice depends on a number of factors. These include the particular disease or condition, ease of sampling, whether samples can be analysed directly or must be stored, the requirement for measuring individual compounds or a whole range, to name but a few. It is also likely that analysis of more than one sample type yields better results than just looking at breath, for example [9]

The origin of VOCs

A whole range of trace gases and volatile organic compounds are emitted by the body continuously, through exhalation, through skin or from urine or faeces. There are several potential origins of these compounds. Firstly, many VOCs arise from normal metabolism. The body contains thousands of different molecules arising from all the biochemical pathways, and many of these compounds are either gases (for example ammonia) or are volatile enough for form a vapour at body temperature. These compounds travel around the body in blood, and where blood meets the alveoli in the lungs, rapid gas exchange and diffusion means that gases and VOCs are exhaled. Similarly, when capillaries are in contact with skin, gas exchange occurs. VOCs are also excreted as part of the chemical composition of urine or faeces. Thus is can be seen that through normal metabolism, the healthy body produces a whole range of different compounds at different concentrations [10].

When illness occurs, metabolism can alter the profile of trace gases and VOCs [11,12]. For example, untreated diabetes leads to a build-up of blood glucose which cannot enter the cells where it is needed. In response, the body starts to metabolise fat, which then leads to an increase in ketone bodies in blood [13]. Some of these are very volatile, and may be detected on breath, in blood or urine. So it is clear that different profiles of metabolites (including volatile metabolites) occur through illness. Cancer is another condition where the VOC profile may change. This may be because various metabolic pathways are expressed to a greater or lesser extent in a cancer cell compared with a normal cell. In addition, the pH of the cell and its surrounding medium may change, thus rendering the relative acid/base equilibria of various compounds change hence various volatile species may increase or decrease merely as a result of pH. So you would expect to see more organic acids in the volatile form when the pH is lower, for example with acetic acid, CH_3COOH, and its

equilibrium with the acetate ion, CH_3COO^- would be shifted to have more of the CH_3COOH species, which is volatile, while CH_3COO^- is not. Conversely, at a lower pH, the concentration of ammonia as NH_3 would be lower than NH_4^+, for example. There have been numerous studies describing differences in VOC profile in cancer [14] e.g. colo rectal cancer [15-18], lung cancer [19-22], breast cancer [23,24] other cancers [25-29].

VOCs may also be produced as a result of infection. Bacteria, fungi and parasites all have their own metabolism and thus their own profile of VOCs and trace gases. When they infect the body, it is thus reasonable to expect that the VOC profile will change with the degree of infection [30]. In addition, the response of the body (host response) in fighting the infection may also change the volatile metabolites produced [31]. In addition, the host's own metabolism may alter the chemical profile produced by the bacteria. Infection in this case also includes the colonisation of the gastrointestinal tract (and other body cavities and surfaces) by trillions of bacteria which have a major impact on the VOC profile [32]

These bacteria are generally benign, and many are even beneficial, but they produce many of the VOCs and trace gases that may be detected on breath, from skin, or from the headspace of blood, urine and faeces.

Examples of infections causing a change in VOC profile are tuberculosis [33], mycobacteria infection [31], infections causing ventilator associated pneumonia [34], respiratory disease [35]. Gastrointestinal disease may be due to a change in the gut flora, or some pathology of the gut or a combination of both, and these have been shown to give distinct VOC profiles from headspace of urine or faeces as well as breath [36-40] [41].

Finally, VOCs arise in the environment. They are produced by plants, food, man-made products or processes (diesel exhausts for example) and if inhaled or ingested, they will then circulate in the blood [42]. In the case of environmental origin of VOCs on breath, there is no simple way of dealing with this so that the background air can be excluded in analysis. This is discussed in more detail later.

A major review of all the volatile compounds emanating from the body has recently been produced, and this covers all sources of VOCs described above [43].

Sampling and handling considerations

Capturing, handling and storing VOCs and trace gases is a major challenge [44]. Unless analysed directly, e.g. using an instrument that can analyse breath in real time [45], the VOCs and gases need to be captured, concentrated and then stored. Ideally, storage should be at a very low temperature to reduce the loss of the VOCs, and the samples should be stored as soon after being taken as possible. In the case of liquid or solid samples (e.g. urine, blood, faeces, pus, aspirates etc.), this is fairly straightforward. Samples should

immediately be placed in an appropriate container and frozen, preferably to -80^{o}C or lower. The container should be clean, and should produce no VOCs which could interfere with analysis, and obviously should not change its characteristics with the temperature change and storage. It is known that freezing samples can change their VOC composition [46], but unless every sample can be analysed immediately in the same way, all samples should be frozen immediately.

When breath is to be sampled but cannot be analysed immediately, it is necessary to store it. It can either be stored as whole breath, or if the VOCs are extracted, it can be condensed and stored. If whole breath is stored, there are a number of issues to consider. These include cost, integrity, storage time and simplicity, and also which part of the breath is sampled. Generally, it is desirable to avoid measuring the dead-space of air in the upper respiratory tract and concentrate on end tidal breath. These issues are described in detail in [44]. Probably the simplest and cheapest way of storing whole breath is in breath bags. These can be made of a variety of materials, and range from a few cents/pennies etc. for Nalophan, to the much more expensive Tedlar bags. Other materials such as Kynar and Flexfilm [47], polyvinyl fluoride and polyester aluminium [48] have also been used. Because of the cost, Nalophan is disposable, but as Tedlar is much more expensive, most people try and re-use Tedlar bags, which means a very thorough cleaning regime is required. However, it is difficult to remove all traces of previous samples, even with this. In addition, Tedlar produces a number of VOCs of its own which may contaminate the samples. Despite this, Tedlar is often the sample bag of choice because generally, samples may be stored in Tedlar for longer than in Nalophan or other sample bag materials, as Nalophan tends to be slightly porous so diffusive losses occur. Adsorption onto the walls of the bag also occurs [49]. So if samples cannot be analysed within a few hours, then Tedlar may be better [50]. There have been many studies looking at the relative merits of these sampling bags [47-49,51,52] and the choice of bag will come down to budget, analytes of interest and necessary storage time.

A more expensive option is the use of evacuated metal canisters which have been used in environmental exposure breath analysis [53-55]. Because these are expensive and difficult to clean, they are no longer used much in breath analysis.

If it is not possible or desirable to store whole breath, a sorbent material may be used which extracts the VOCS from the whole breath. There are several sorbent materials that may be used, and this can either be within a thermal desorption (TD) or sorbent tube , or using a technique such as solid phase microextraction (SPME) [56-60] or needle trap device [61] . SPME involves using a very small microfiber and inserting it into the headspace for a fixed amount of time to absorb the VOCs. Although very sensitive, it generally adsorbs some compounds preferentially over others, and as soon as removed from the headspace, may start to desorb the samples. It is also not particularly robust, and great care must be applied in handling the fibre. It is also not quantitative unless very specific steps are taken where

standards are used and the marker compounds are known; the relative concentrations of other compounds present should also be known as they will affect binding. However, SPME may be used to trap very low concentration compounds. Generally the use of TD tubes is more robust, and once samples are collected, the TD tubes may be capped and stored for weeks prior to analysis. TD tubes are also more sensitive [62] and again, accurate quantification is difficult, although slightly easier than with SPME. Great care must be taken in choosing the sorbent, and in many cases, dual or even triple bed sorbents are used in the same tube to capture the range of compounds. Some sorbents are better at lower molecular weight compounds, some higher molecular weight, and others may be better for aromatic or sulphide compounds, for example. From this it follows that to make best use of this technology, some idea of the types of compound expected is needed. Examples of where sorbent tubes techniques have been used to sample breath are in a study of patients with impaired respiratory function [63], or in a study for collecting breath from frail patients [64].

These sorbent techniques are very sensitive, and when coupled with GC-MS, compounds may be desorbed from the sorbent material (usually by heating), and then separated by gas chromatography (GC), followed by identification and quantification by mass spectrometry (MS). The advantages of doing this are that it is very sensitive, compound identification is possible through separation and mass detection, and samples may readily be collected, concentrated and stored. However, it is slow, indirect requiring several steps, and not always quantitative unless great care has been taken with sorbents and VOC amounts.

A summary of breath sampling techniques may be found in table 1.

Table 1. Summary of exhaled breath sampling techniques giving their main advantages and disadvantages

Technique	Main Advantage(s)	Main Disadvantage(s)	Reference
Direct analysis	Direct so no loss of sample integrity	Need to get equipment to patient	45
Sample bags	Diffusive losses; short storage times	Cheap and simple	47-52
Evacuated metal canisters	Re-useable; longer storage possible	Expensive; difficult to clean	53-55
Thermal desorption	Sensitive; long sample storage times	Choice of sorbent crucial; not all compounds adsorbed; quantification difficult	62-64
SPME	Very sensitive	Fragile; quantification very difficult	56-60

Techniques

There is a very wide range of techniques for the analysis for individual VOCs or VOC profiles from a sample. These range from sophisticated and expensive techniques that can analyse samples of breath or headspace directly in real time, such as selected ion flow tube mass spectrometry (SIFT-MS) [65-80] or proton transfer mass spectrometry (PTR-MS) [67,81-85], to techniques which do not identify or analyse individual components but look at patterns, for example gas sensor arrays (electronic nose) [35,86-93]. If compound identification is required, it is essential to use a mass spectrometric technique, and preferably one that is coupled with a separation technique to avoid complicated spectra, for example gas-chromatography-mass spectrometry.

Direct analysis is difficult but can be done with SIFT-MS [65-68,75,80] and PTR-MS [67,81-84]. Direct analysis using these mass spectrometric methods does not allow absolute identification, because compounds in samples are not separated (unlike in GC-MS, where retention index as well as ions generated aids identification), and the soft chemical ionisation may yield a number of ions which may arise from more than one compound. Despite this, the direct methodology offers the opportunity for quantification where compounds are identified, particularly with SIFT-MS [76,94]. It is a little more complicated with PTR-MS, with the variation in E/N (field strength in the drift tube) but quantification is in some cases possible particularly for low molecular mass compounds, and certainly with the use of calibration gases for specific compounds. In SIFT-MS and PTR-MS, the sample is presented to the instrument, and then reacted with a precursor ion. For SIFT-MS, a choice of H_3O^+, NO^+ or O_2^+ is possible; PTR-MS generally uses hydronium ions, H_3O^+, but newer instruments enable the use of other precursor ions. Ions are generated according to their reactions with the precursors and then these product ions may then be separated by a mass spectrometer, typically a quadrupole for SIFT-MS, and quadrupole or time-of-flight, TOF, for PTR-MS. Whole spectra may be looked at if one is interested in looking for the range of compounds present in either breath or headspace. Alternatively the instrument could be set up to look for one or more specific compounds without scanning the whole spectrum, which would enable more accurate quantification.

Laser based spectroscopic techniques have also been used for real time direct analysis of breath laser based techniques [95-99] and may offer a replacement for mass spectrometric techniques in the future. Similarly, ion mobility spectrometry (IMS) has also been used in real time analysis of breath [20,100,101]. It is relatively low cost, however it cannot identify individual compounds with certainty, although it could indicate the potential identity of species based on how the sample components behave in the electric field. It has also been used by the military in personal equipment for detecting the deployment of chemical weapons [102].

Other gas sensor techniques may also be used in direct analysis of breath or headspace, but these tend to be non-specific. This includes various types of so-called electronic nose, which

use an array of sensors of various types [35,86-93]. Originally, electronic noses contained between 10 and 40 sensors, but newer technology means that a very high number of sensors can be included in a small array. These sensors respond differently to the various components in a sample, and a complex array of signals is generated. By comparing signals from different classes of samples (e.g. breath samples from those with a particular illness and those without), patterns emerge which may enable differences associated with the disease to be identified. There are problems with some sensors in e-nose devices – drift over time, fouling and memory effects [103]. An increasing number of sensor elements or spectral data means increasing complexity in multivariate statistical methods to interrogate and process the data, but such techniques are also developing [15,36,37,104-108].

Further developments in sensors means that some relatively low cost sensors are becoming increasingly sophisticated, and they can be made more sensitive and also selective. Long period grating optical fibre sensors may be produced now, which are specific for individual components [109-112]. These can be assembled into an array to produce a low cost alternative to mass spectrometry, although in using a limited number of specific sensor elements means the need to know exactly which compounds should be measured and cannot be used for volatile biomarker discovery. These sensors also enable on-line analysis and could conceivably be used in a point of care device, or even personal breath analysis tool, for instance like one that can monitor asthma and nitric oxide [113].

The most widely used technique for off-line or indirect analysis of samples is probably gas chromatography-mass spectrometry (GC-MS) [11,34,36,59,114-122]. Although this technique is relatively slow and indirect, it is also very powerful. If samples are concentrated, for example by using a sorbent such as a thermal desorption tube or a SPME fibre, desorption of this can then deposit the concentrated sample onto a chromatographic capillary column. This can then separate sample components, which may then be detected sequentially according to chemical and physical properties (e.g. size, volatility and hydrophobicity). A further development in this area is the use of GCxGC MS, which deals with the problem of co-elution of compounds, where it is difficult to identify species. This is a very sensitive technique that can detect many more compounds in any sample [114,123], but it is expensive and generates much more complex spectra

Apart from being able to detect components present in the parts-per-trillion-by volume range (pptv), GC-MS is the best technique for identifying the individual components of a sample. It is quantitative if standards are run for individual compounds, but it is difficult to make it quantitative for compounds during biomarker discovery due to the complexity of the sample and the absorption/desorption differences on the sorbent between individual components.

Choosing the most suitable technique

So which technique should be used? This obviously comes down to a question of availability/budget, but generally if biomarker discovery is desired, then a mass spectrometric technique with a compound separation method, such as GC-MS, is best. However if the aim is to be able to distinguish between volatile profiles from a sample, a technique which can use multiple variables, for instance m/z or sensor array responses to produce a profile of the sample, composed of multiple compounds, then any technique may be used, coupled with suitable multivariate statistical approach. However, even if a diagnostic profile is found and is robust enough for clinical use, knowledge of the major compounds contributing to the differences in profile is highly desirable. This means that use of mass spectrometric methods in the discovery stage is ideal, and then when the compounds responsible for the change in disease state are known, then point of care devices which are less expensive and more portable are better. In the discovery stage, the use of multiple techniques which exploits the advantages of each will give the best results. For instance, the ability to directly analyse a sample and obtain quantitative data (e.g. with SIFT-MS or PTR-MS) can be used in conjunction with GC-MS which is more sensitive and is better at compound identification but is slower and not so directly quantitative.

The choice will also depend on whether the sample must be analysed directly, or whether samples may be taken and stored for subsequent analysis.

For the analysis of a small number of individual compounds, then any technique capable of being sufficiently selective is acceptable. This includes a variety of gas sensors, optic fibre sensors, IMS, mass spectrometry etc. A summary of the main techniques is given in table 2.

Table 2. Summary of analysis techniques giving their main advantages and disadvantages

Technique	Main Advantage(s)	Main Disadvantage(s)	Reference
Direct trace gas mass spectrometry	Direct, rapid	Expensive, not always easy to take to the patient	65-84, 94
Gas sensors (e-nose)	Direct, rapid, inexpensive	Non-specific; cannot identify compounds	35, 86-93, 103
Laser based spectroscopic techniques	Direct, rapid	Relatively expensive	95-99
Ion mobility spectrometry	Rapid; relatively inexpensive	Cannot identify unknown compounds	20, 100-101
Long period grating optic fibre sensors	Can be made specific & low cost; rapid	Not for biomarker discovery	109-112
GC-MS (with TD or SPME)	Sensitive; good for compound identification	Expensive, slow	11, 34, 36, 59, 114-123

Backgrounds

Something that breath analysis researchers in particular have been concerned about for some time is the background air and its effect on the components of breath. It is well known that inhaling compounds from the environment means that these compounds are exhaled for some time after. How long the compounds will be exhaled for depends on factors such as the concentration of the compound inhaled and the duration of exposure, the chemical and physical nature of the compound – its molecular mass, volatility, how fat soluble it is etc.; the body mass index of the individual. Because there are so many variables, it is very difficult to adequately deal with this problem. Different groups have various ways of dealing with this. For instance, Michael Phillips [124-126] uses the concept of alveolar gradient which involves measuring the background air and looking at the difference between the concentrations of various species in the air and in the breath. Although this helps in some way, it is not accurate for all VOCs [127]. Other researchers insist that subjects giving breath samples inhale clean air for a minimum period prior to providing a breath sample, but this cannot reduce the levels of all exogenous compounds in breath. This is much less effective for hydrophobic compounds and where patients have a high BMI, or where the concentration of the compound is high. Schubert et al [128] has shown that the approach of applying a simple background subtraction, where the concentration of the species in background is subtracted from that in breath, is not effective, and substances where concentrations in inspired breath is higher than 5% of expired concentrations should not be used as breath markers. The best, but complicated option, is to apply retention coefficients for individual compounds in the background air [127]. This requires knowledge of the presence of such biomarkers and their retention coefficients. None of these are entirely satisfactory because of the complexity of the problem, and people may have been exposed to a number of different backgrounds with different concentrations of compound in inspired air that may affect the breath prior to a breath sample being given.

Rather than finding a way of dealing with the background directly, an alternative may be the use of appropriate controls. At the same time that a sample is taken from a patient or subject, a sample should be taken from a control person who has been subjected to similar backgrounds, and is as closely as possible be matched to the subject. This could be the partner of the individual, for example. Other studies have used medical personnel for this purpose, but that is less satisfactory as medical facilities typically have high background levels of VOCs, and thus medical personnel may have been subjected to these backgrounds to a greater extent than subjects. It is clear that this is a difficult problem, and backgrounds should always be carefully taken into account when a breath analysis study is conducted. This is also likely to have an impact on blood and urine VOCs as the origin of exogenous VOCs from the headspace of these fluids may also be inhaled air. Table 3 summarises the techniques for dealing with background air.

Table 3. Summary of techniques for dealing with background air, giving their main advantages and disadvantages

Technique	Main Advantage(s)	Main Disadvantage(s)	Reference
Alveolar gradient	Requires simple measurement of background air	Not accurate for many compounds	124-127
Inhaling clean air	Easy to do; requires no further measurements	Ineffective for many compounds	128
Retention coefficients	Effective	Complicated and only useful for known compounds	127
Use of appropriate controls	Will cope with problem of variable retention coefficients	Not easy to recruit appropriate controls; doubles analysis required	

Standardisation

Analysis of breath and the headspace of body fluids has been a growing field of endeavour since the 1970s, and as previously discussed, there are many techniques used. However, results from these investigations often do not correlate with each other, and one reason for this is that there is no accepted standard for sampling and analysis. To make progress in the area of VOC analysis for disease diagnosis, the importance of standardising methods for sampling and analysis of breath is being recognised [129-132]. What has not been noted is the requirement for standardisation of all samples for VOC analysis, but this is equally important.

Breath analysis

There are several aspects to this. The first is where is the sample taken from? Should it be the mouth, or nose or a combination of both? The mouth contains its own flora which produce VOCs, so measuring from the mouth alone will mean that these will change the sample [133-136]. In some cases, mouth VOCs are important, but if systemic VOCs are important, e.g. where a condition at a distant site is to be monitored, then avoiding the contamination from mouth flora is important. This is the case with monitoring HCN in the lungs from *Pseudomonas aeruginosa* in cystic fibrosis patients [137]. Sometimes, the origin of specific VOCs is sought, in which case, both should be analysed in turn [138].

Secondly, which part of the breath should be taken? Should it be whole breath, end-tidal breath? The answer to this depends on the degree of accuracy and precision required. Most methods for analysing VOCs in breath cannot do the analysis with any great accuracy and precision. Repeat samples, even of direct breath, often differ, depending on the compound being analysed and the background [139,140]; factors such as rate and volume of exhalation may also have an effect [141]. The variation between the concentration of VOCs

in whole breath and end tidal may not be close to this, so how important is it that methods require the complexity of a mechanism for excluding dead-space in the respiratory system and consider only end tidal breath? This would depend on the necessary precision for the analysis of a compound. If it is a compound where the presence or absence is important, this matters less, however if small variations in concentration show clinically relevant information, then the additional precision may be important.

Methods for ensuring only end-tidal breath is taken involve switching mechanisms which may check CO_2 composition of breath and then use a valve system to divert the required part of breath, discarding the dead space [34,142]. These methods are more complicated but can ensure that only a specific part of the breath is taken. However, one study [143] shows comparatively low relative standard deviation between successive bag fills of whole breath, so perhaps accepting whole breath, with apparently better reproducibility but less emphasis on control, is an acceptable option.

Aside from standardising which part of breath is taken, there are other factors that will affect the measurement. This includes the mechanism and material that transports the breath to the analytical instrument. Even if it is direct analysis, breath will start to condense on any surface which is cool enough, so the pipes/tubing/sampling port should be at a standardised (warm) temperature. The material used should also minimise "sticking" of compounds. Some molecules, for example ammonia, are very "sticky" [144] so the longer the tube/pipe etc., the more the compound will stick and thus not be available for analysis. This can also contaminate later samples.

For breath samples that are taken and then stored for subsequent analysis, further standardisation is required. It is not reasonable to expect that every researcher will use exactly the same sampling mask or mouthpiece; instead a way of checking that each method delivers the same results is required. One way of doing this is to use standardised artificial breath. This could involve special calibration vapours which are humid, as is breath, but which deliver known amounts of each analyte at a given temperature; 37^{o}C is best as this is that of breath. Calibration standards can be purchased or standard artificial headspaces or breath can be produced by making aqueous solutions of breath VOCs, putting them in an enclosed sample bag and allowing the aqueous solution and the headspace above it to reach equilibrium. According to Henry's law, a given concentration in the aqueous phase will be in equilibrium with the headspace above it at a given temperature. Knowing the Henry's law coefficient for each compound of interest, these artificial headspaces can easily be generated, which will deliver standard concentrations of compounds in a headspace. These artificial breath samples or headspaces can then be presented to analytical devices and the responses assessed against each other.

Headspace of body fluids

Generating headspace of body fluids can also yield very different results depending upon how they are treated. In some cases, samples can be analysed immediately; they will need be put in a suitable receptacle, clean gas/air added and a headspace equilibrium allowed to develop. However, generally samples of urine, blood, pus, faeces etc. are collected from a hospital or clinic and cannot be processed immediately, but will quickly degrade if not stored appropriately. In this case, standardised samples treatment and storage protocols should be developed and followed. Freezing samples at -80^{o}C as soon as they are taken will reduce loss of VOCs, however samples can degrade under these conditions [46]. Hence standardised protocols should be developed for the sample type, duration of storage, temperature of storage and storage container. In addition, the protocol for defrosting and preparing the sample for subsequent analysis should also be standardised.

Standardisation of sample treatment, storage and use of calibration standards will enable a comparison between studies which should enable this field to be driven further. One of the main issues in the field of VOC analysis for disease diagnosis is that studies do not always give the same results; the lack of standardised protocols means that these different studies are essentially measuring different things.

Future perspective

There is an increasing number of studies on the use of VOCs in diagnosing disease, and there are now very many examples of how VOCs can be used to detect various cancers, infections, metabolic conditions, gastro-intestinal disease etc. Despite this, there are very few of these tests that are used routinely in the clinic. Given the potential advantages of VOC profiling for disease diagnosis i.e. that it is non-invasive or minimally invasive, rapid, potentially cost-effective, etc., why have these apparent diagnostic successes not translated to routine clinical analysis? There are likely to be several reasons for this. One is that mentioned above, i.e. there is no single standardised method for breath or clinical fluid headspace sampling and analysis. Another possible reason is that in some cases, there is no real attempt to get clinical buy-in for the method. Clinicians are responsible for the well-being of their patients so would need to be convinced of the effectiveness of a new test. One reason why they haven't been convinced is because studies often only involve clinicians in sample collection and not in the development the technique itself. Secondly, the output of a breath or headspace analysis may be a complex profile which needs interpreting using multivariate statistics rather than with unique individual breath biomarkers. Although the complex profiles may be fully statistically validated, they are often hard to explain, and thus effort and care needs to be taken in communicating their use. In addition, in order for a method to replace an existing screening or diagnostic method, it needs to be at least as good as the method it is replacing in every respect, and superior in at least one respect. Most studies published do not address this but it is essential for progression of this field.

However, with the vast increase in published studies showing the use of VOC profiling, surely this is only a matter of time.

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