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Pitfalls in the analysis of volatile breath biomarkers; suggested solutions and SIFT-MS quantification of single metabolites

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Abstract

The experimental challenges presented by the analysis of trace volatile organic compounds (VOCs) in exhaled breath with the objective of identifying reliable biomarkers are brought into focus. It is stressed that positive identification and accurate quantification of the VOCs are imperative if they are to be considered as discreet biomarkers. Breath sampling procedures are discussed and it is suggested that for accurate quantification on-line real time sampling and analysis is desirable. Whilst recognizing such real time analysis is not always possible and sample collection is often required, objective recognition of the pitfalls involved in this is essential. It is also emphasized that mouth-exhaled breath is always contaminated to some degree by orally-generated compounds and so, when possible, analysis of nose-exhaled breath should be performed. Some difficulties in breath analysis are mitigated by the choice of analytical instrumentation used, but no single instrument can provide solutions to all the analytical challenges. Analysis and interpretation of breath analysis data, however acquired, needs to be treated circumspectly. In particular, the excessive use of statistics to treat imperfect mass spectrometry/mobility spectra should be avoided, since it can result in unjustifiable conclusions. It is should be understood that recognition of combinations of VOCs in breath that, for example, apparently describe particular cancer states, will not be taken seriously until they are replicated in other laboratories and clinics. Finally, the inhibiting notion that single biomarkers of infection and disease will not be identified and utilized clinically should be dispelled by the exemplary and widely used single biomarkers NO and H_2 and now, as indicated by recent SIFT-MS results, HCN and perhaps pentane and acetic acid. Hopefully, these discoveries will provide encouragement to research workers to be more open-minded on this important and desirable issue.

Introduction

This Perspective is intended to focus thinking on some of the more important issues, the concerns and challenges that face research worker in the essentially virgin field of breath research and, thus, to promote this exciting area of analytical science to true utility as a non-invasive clinical diagnostic and therapeutic monitoring technique. It is based on the talk given at the Breath Analysis 2014 meeting held in Torun, Poland in July 2014. Breath analysis now needs to be approached with the same scientific rigor and care that is practiced in more traditional and well-established sciences on which medicine relies; otherwise there will be reluctance by clinicians and support biomedical scientists to accept breath analysis as a *bone fide* contributor to medical practice. Theory and intuition have their place, but scientific advance is always driven by the acquisition of reliable data and their sound interpretation with a critical eye on previous data and commentaries. Sadly, sometimes it seems that breath analysis research is in danger of losing its way, tending to become a less-than-rigorous academic pursuit that is not sufficiently focused on clinical relevance. Whilst there are laudable and imaginative new analytical techniques being introduced, some are unreliable and involve unnecessarily complex sampling procedures, often requiring over-reliance on statistical analyses of inherently unmanageable experimental data. Surely, there should be greater focus on the search for and the positive identification and quantification of volatile biomarkers present in exhaled breath and biological fluids, which can ultimately be exploited as probes of physiological and clinical status. Relevant published work is often ignored in data assessment, which is partly forgivable in this era of burgeoning published literature, but this can result in misdirected effort and the propagation of misconceptions throughout the literature that can seriously inhibit progress. None of us are immune to these unfortunate tendencies, but they should be recognised and corrected in the interest and progression of breath research. In this short perspective, some of these inadequacies will be identified in the sincere hope that this will help to take breath research towards the next level of rigour and utility.

The experimental challenge of breath analysis; what are the objectives?

The major objectives of breath analysis must be to positively identify, accurately quantify and describe the origins of endogenous volatile compounds appearing in exhaled breath, whilst accounting for any influence of exogenous compounds (see Fig. 1). Endogenous volatile compounds are largely organic, VOCs, and include purely systemic metabolites, i.e. those present in the

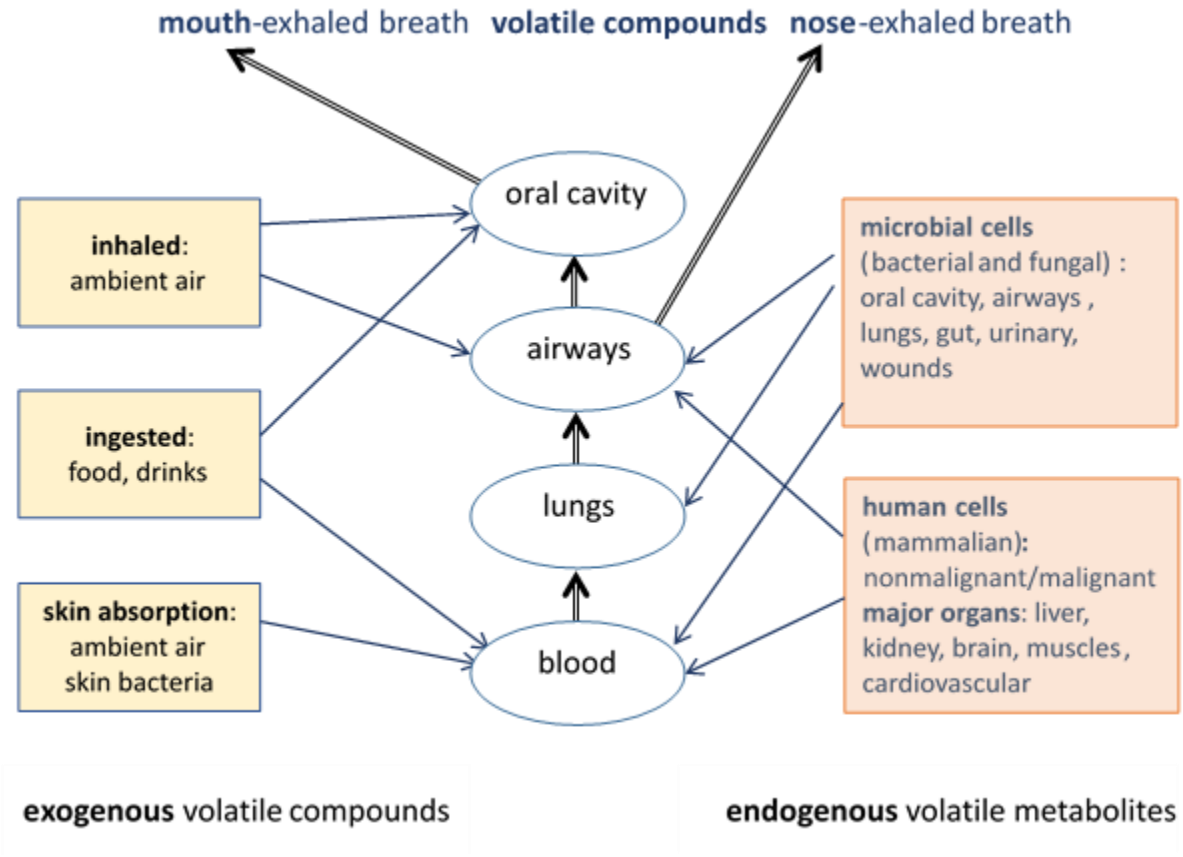

Figure 1. Schematic indicating the origins of exogenous and endogenous volatile compounds and their routes into mouth-exhaled and nose-exhaled breath. Note that the concentrations of trace compounds in both inhaled and exhaled air can be modified as they flow along the airways.

blood stream originating from cellular processes in the major organs, especially the liver and kidneys, those produced in the muscles and fat, those produced in the airways by bacterial and inflammatory processes, and those generated in the oral cavity by bacterial and enzymatic activity. Thus, exhaled breath is a complex mixture of trace compounds, the analysis of which is a real challenge to analytical science, especially because exhaled breath is supersaturated with water vapour. To fulfil the above objectives, the development of reliable analytical methods is a most important and challenging aspect of breath analysis (see later). The hope and expectation is that amongst the many endogenous volatile metabolites there will be **biomarkers** of disease and infection that can be used as non-invasive aids to clinical diagnosis, and it is these biomarkers that are attracting the most interest. In this effort, there is a case for considering those hardly volatile compounds that are present in exhaled aerosols and captured in exhaled breath condensate, EBC, but for the purpose of this commentary they are ignored, since EBC can properly be considered as a sub-branch of breath research.

So what is a **biomarker**? A widely accepted definition, used by the National Institute of Health (NIH), [1] is :*"a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to an intervention." Example: cholesterol level. The committee defines "objectively" to mean "reliably and accurately."*. Unfortunately, accurate absolute quantification is too often ignored in breath research, principally because it is challenging and time consuming and also because some of the adopted analytical methods are not suited to accurate quantification of volatile compounds in humid exhaled breath. Vital and valuable information is lost by this deficiency. Even so, compounds are often designated as biomarkers of disease and infection when the acquired data is not adequate to support such designations and when there are previously published data that deny such designations. For example, there is an alarming growth in the number of publication (exemplified by papers published during the last year [2-7] and the previous work reviewed in JBR recently [8]) that persistently suggest that acetone in exhaled breath is a definitive indicator of diabetes in spite of clear warnings of several confounding factors, particularly diet, that effectively disqualify this VOC as a reliable diabetes biomarker [9]. Why should this be happening when it is misleading and distracting, especially to new entrants into breath analysis research? Is it a manifestation of the "publish and (or) be damned" [10] syndrome or attempts to raise the profile of home institutions driven, not by scientists and clinicians, but by managers and institutional leaders? A similar situation surrounds the suggestion that breath ammonia is closely related to the uremic condition, arrived at on the basis of flawed data, notably the failure to recognise that ammonia in the usually sampled mouth-exhaled breath can largely be produced in the oral cavity and, as such, is not strictly systemic. Paradoxically, since the origin of the orally-generated ammonia is most probably salivary urea, then mouth-exhaled ammonia may, indirectly, cautiously be considered as a proxy indicator of uraemia. These aspects of breath ammonia generation and origin are discussed in recent papers [11-13]. So an urgent need is for much more attention to the quantifications of VOCs in exhaled breath and to trace their biochemical origins before they can be truly designated as biomarkers.

Breath sampling and analysis procedures

Three distinct methods for breath sampling are available: (i) direct on-line sampling into the analytical instrument with immediate analysis; (ii) sample collection into flexible (e.g. Tedlar and Nalophan bags)) or inflexible containers (e.g. stainless steel and glass vessels) followed

by off-line analysis; (iii) direct trapping and pre-concentration of trace compounds from exhaled breath samples onto an absorbent for subsequent desorption and off-line analysis. There are advantages and disadvantages in both on-line and off-line methods.

(i) Direct sampling and immediate analysis, as exemplified by selected ion flow tube mass spectrometry, SIFT-MS, and proton transfer reaction mass spectrometry, PTR-MS, is desirable since it avoids the potential losses of trace compounds; also, the exhalation profile of individual compounds can be observed sometimes allowing oral generation of trace compounds can be seen and the end-tidal portion of the exhalation to be identified and selectively analysed [14-16]. However, the time for analysis is limited to the exhalation time, so the precision and sensitivity of quantification of compounds present at low concentrations may be too low to be useful.

(ii) Bag sample collection has the advantage of allowing sample storage, consequently longer analysis times and more certain trace compound identification coupled with increased sensitivity. However, losses of trace compounds from the breath samples can occur by surface adsorption and diffusion through the walls of some collection vessels [17-19].

(iii) Collection and preconcentration onto sorbent traps and subsequent off-line analysis, as exemplified by SPME/GC-MS, involves uncertainty in adsorption and desorption efficiencies, which can lead to inaccurate quantification unless time-consuming calibration procedures are used [20].

The choice of either direct sampling/on-line analysis or sample collection/off-line analysis is influenced, if not determined, by the size/portability and cost of the available analytical instrument and the analytical sensitivity required, as referred to later.

A problem, so often ignored, is the contamination of mouth-exhaled breath due to generation of some compounds in the oral cavity by bacterial and enzymatic activity (see Fig. 1). This is very serious for some compounds such as ammonia and ethanol, as has been demonstrated by real-time SIFT-MS analyses of mouth-exhaled and nose exhaled breath [21]. Deliberate oral generation of copious amounts of these compounds can easily be demonstrated by introducing just traces of urea and sugar into the mouth [22]. It has been revealed by these studies that some of the ubiquitous breath compounds, such as acetone and isoprene, appear to be produced totally systemically [23] and this nose/mouth sampling approach can be extended to distinguish systemic/oral origins of other breath compounds, as required. Such measurements are easily carried out in real time by rapidly switching between mouth-exhaled and nose-exhaled breath sampling. Thus, the largely oral origin of several mouth-exhaled breath trace

compounds has been confirmed [23], including hydrogen sulphide and methanethiol [24, 25]. Yet nose-exhaled breath may also be partially contaminated by bacterial emissions in the sinus cavities, as has been shown by the mouth/nasal studies of exhaled NO and exhalation rate measurements have cast some light on this phenomenon, as mentioned later when further referring to NO measurements.

The mantra is that hundreds, even thousands, of trace VOCs are present in human exhaled breath, and given the growing evidence that numerous VOCs are emitted by bacterial cultures *in vitro* [26-28], it seems very likely that oral cavity bacteria generate many of the VOCs present in mouth-exhaled breath [29, 30]. So when the major objective is to identify and quantify systemic volatile VOCs in relation to metabolic disease and infection, then the likelihood of oral generation must be taken seriously. Unfortunately, this has largely been avoided by most workers in the field, presumably because of the perceived difficulty in collecting samples of nose-exhaled breath for off-line analysis. However, such sampling can be achieved by a pump/bag combination.

If the end-tidal portion of exhaled breath can be sampled for analysis, then partial differentiation can be achieved between orally-generated and systemic compounds. In direct real time SIFT-MS analyses this is achieved by analysing the "plateau region" of the water vapour concentration time profile [31, 32]. This has additional value, because the peak water vapour absolute concentration should be that of the saturated vapour pressure of water at body (core) temperature, which is close to 6% by volume of the exhaled gas/vapour, and uniquely and importantly, this provides an internal validation of the quality of direct sampling into the SIFT-MS instrument [32]. Also, when mouth-exhaled water vapour and ammonia are analysed simultaneously, the water vapour profile always shows an increasing water vapour concentration through the exhalation, whereas an abnormally elevated ammonia level is often seen in the early phase of the exhalation that reduces towards the water vapour "plateau portion", and this is indicative of oral generation of ammonia.

An approach to sampling of end-tidal breath adopted by several workers is to include a sensor for exhaled carbon dioxide, CO_2, in the breath sampling line and to use its "peak level" as an indicator of the "plateau region" and thus the end-tidal sampling section [33]. This appears to be a *bone fide* approach, but the use the absolute CO_2 level to normalise concentrations of trace breath compounds is questionable since the breath CO_2 level varies with physical activity and metabolic rate. SIFT-MS measurements have shown that breath acetone, for

example, does not change markedly with exercise whereas breath CO_2 predictably varies significantly [34]. Given that most trace compounds in exhaled breath are present at parts-per-billion by volume, ppbv, concentrations, the losses of trace metabolites and the introduction of contaminants due to sample lines, which include sensors and valves, must be a concern. In any event, the value of this approach to sampling is somewhat overstated given the real challenge of identifying, detecting and quantifying biomarkers at the ppbv level and lower.

A final point worth stressing is that proper account must be made of the influence (inhalation) of exogenous compounds (see Fig. 1 and its caption). Assessments of the fractional influence of exogenous (environmental) compounds have been made [35-38]; in the absence of experimental data this can be the only approach. Clearly, the proper approach is experimental measurement and so a SIFT-MS study was carried out by varying and controlling the inhaled air concentrations (well below toxic levels) of 8 different compounds (each during separate time periods) in the air in a small laboratory in which several healthy volunteers were asked to sit for short periods during which the concentration of the "polluting trace compounds" in several sequential breath exhalation and inhalations (ambient air) were measured [39]. The results obtained are quite remarkable since excellent linear plots of exhaled versus inhaled concentrations for all 8 compounds were realised. Hence, well defined "retention coefficients" have been obtained that express the fraction of the measured inhaled compounds that are retained in the exhaled breath. Interestingly, and fortunately, the inter-individual variations of these coefficients for each compound are insignificant. They are seen to be related to the aqueous solubility of the compound, and hence the solubility in the aqueous mucosa lining the respiratory tract. Thus, the "retention coefficients" are relatively small for water soluble compounds like formaldehyde (0.06) and methanol (0.29) and high for relatively insoluble compounds like pentane (0.76) and isoprene (0.66). These coefficients can be determined experimentally for most compounds and so the guesswork approach to this phenomenon can be avoided. At the least, estimates of these coefficients can also be made from the solubility of the compound as guided by the data reported in the paper [39].

Instrumentation used for breath analysis

Quantitative mass spectrometry offers the best analytical solution to the previously stated major objectives in breath research. Clearly, other techniques have their place, but their strengths should not be overstated and their limitations clearly acknowledged so that clinicians and health workers who, hopefully, will soon be to the fore in the exploitation of

breath analysis, will not be misled and be tempted to acquire inappropriate instrumentation. Ideally, the analytical instrumentation should be capable of accurate VOC identification and quantification and of tracking changes of breath metabolite concentrations over short and long periods in support of pharmacokinetics, longitudinal studies and the efficacy of therapy.

GC-MS instruments, in combination with the sample collection and pre-concentration techniques solid phase micro-extraction, SPME, and automated thermal desorption, ATD [20], are most regularly exploited in breath research. Generally, the instruments are relatively large and expensive, although smaller versions are now appearing that are portable. They cannot yet be considered as on-line, real time analysers, since analysis times remain as seconds to minutes. But SPME(ATD)/GC-MS methods have the precious feature of high sensitivity and (relatively) unambiguous identification of compounds (including the structural isomers of some compounds) present in complex matrices such as exhaled breath. However, accurate quantification of trace compounds is more difficult to achieve without tedious calibration procedures involving standard gas mixtures. Nevertheless, exploitation of these techniques has provided the bulk of data relating to the trace compounds present in exhaled breath.

A more recent innovation is ion mobility spectrometry, IMS, in which the composite trace compounds in a breath sample, either input directly into the instrument or released from a SPME collector, are ultimately ionised by precursor (reagent ions) (formed in an electrical discharge or radioactive emanation) thus forming swarms (groups) of characteristic (analyte) ions that are separated in a drift tube according to their differing drift time/mobility [40]. Thus, a drift time spectrum is produced from which compound identification is realised by calibration with known compounds [40]. In principle, IMS analyses can be achieved in real time, but data interpretation is complex, reliance on statistical analyses is usually required and quantification is challenging. Recently, GC separation has been combined with IMS [41], which assists analysis of multi-component mixtures like exhaled breath and the headspace of biological samples such as bacterial cultures. These instruments are relatively small and can easily be located in the clinic, but their value in trace gas analysis must not be overstated.

The available techniques that currently can be exploited for real time analysis and accurate quantification are SIFT-MS and PTR-MS [14-16]. The strengths and weaknesses of these techniques have been discussed in a recent issue of the *Journal of Breath Research* [14] and need not be presented in detail here. It is sufficient to say that they both involve the chemical

ionisation by chosen reagent ions (H_3O^+, NO^+, O_2^+) of the trace compounds in a breath sample that has been introduced directly into a reactor flow tube (SIFT-MS) or a reactor flow-drift tube (PTR-MS). Characteristic analyte ions are produced in the reactions of the reagent ions and the trace analyte molecules in the breath sample and from an in-depth knowledge of this ion chemistry, both the identity of the analyte molecules and, most importantly, their accurate quantification can be achieved even in single breath exhalations. These are versatile and productive analytical instruments, but they are not a panacea, since the complication of overlapping analyte ions (having the same nominal mass-to-charge ratio, *m/z)* can prevent positive identification of some compounds, especially structural isomers. These deficiencies have been partially overcome by the use in PTR-MS of time-of-flight, TOF, mass spectrometers that have greater *m/z* resolution [16]. Notwithstanding, on-line real time simultaneous analyses of several compounds in single breath exhalations can be accomplished, avoiding the complications associated with sample collection. Of course, off-line analyses of breath collected into bags and those compounds collected and released from sorbent tubes can also be carried out [42, 43]. Currently, major deterrents to the exploitation of both SIFT-MS and PTR-MS instrumentation are their size and cost, but developments are in train to reduce both of these restraining factors. Whilst our perspectives on the value of SIFT-MS in breath research are largely borne of experiences on our own instruments, as described in a recent review (Profile 3 [15] [MSR 2011], it should be noted that the SIFT-MS instruments produced in New Zealand (Voice 200, Syft Technologies) have also been used for breath analysis (as reviewed in [44], but are largely used in other areas such as VOC emissions from food [45] and container sampling in ports of entry [46].

A real demand is for small, preferably hand-held instruments for breath analysis that can be operated by health professional in the clinic/general practitioner's surgeries and in the home. Many research groups and commercial companies are attempting to develop such devices, and the accent currently is on the development of surface sensors, especially "gold nanoparticles" sensors [47]. This is a challenging engineering and analytical problem largely because of the complexity of exhaled breath, its high humidity and the low concentrations (ppbv) of potential volatile biomarkers. Some progress has been made towards the development of such sensors, but because of the excessive reliance on statistical analyses their value in breath analysis is yet to be independently verified. Generally, the approach taken is to detect combinations of VOCs (rather than single compounds) to identify differences between breath samples donated by healthy individuals and those inflicted with particular diseases or infections. However, it must

be acknowledged that reliable instruments for single compound detection and quantification in exhaled breath are commercially available, specifically for breath hydrogen [48] and nitric oxide [49]. Perhaps the best hope for hand-held instruments for quantitative breath analysis of single specified polyatomic VOCs are those based on spectroscopy in its various forms, especially laser absorption spectroscopy in the mid-infrared region [50], photoacoustics [51] and cavity enhanced spectroscopy [52].

Acquisition of data and its interpretation

The purest and currently the most meaningful breath analysis diagnostic compounds are the diatomic nitric oxide (inflammation in asthma) [53-56], hydrogen (gastrointestinal disorders such as carbohydrate intolerance and malabsorption and intestinal bacteria overgrowth) [57] and carbon monoxide (smoking) [58]. There is a powerful case to add to this short list the triatomic hydrogen cyanide, HCN, as a discreet biomarker of lung infection by the bacterium *Pseudomonas aeruginosa* [59] and possibly acetonitrile (CH_3CN) as a further marker of smoking [60, 61]. We briefly discuss NO and HCN in a later section that expounds the desirability of identifying discrete biomarkers. Most commonly in breath research, data mining by GC-MS has been used for the parallel detection and identification of collections of polyatomic VOCs present in breath and the headspace of biological samples. The usual objective is the differentiation between samples obtained from healthy and diseased persons as indicated by the appearance of additional VOCs and/or differences in the concentration distributions of the composite VOCs. Some such work has been carried out using the *Voice 200* SIFT-MS instrumentation, including a recent study of the differences in concentration of some breath VOCs in acute decompensated heart failure compared to healthy controls [62]. Such differences are generally revealed by statistical analyses based on multivariate methods (e.g. principal component analysis, PCA), but excessive reliance is often placed on such analyses resulting in unjustifiable conclusions. Quantification of individual VOCs present in the breath matrix is rarely attempted, which is unfortunate because some of the compounds might have been designated as genuine biomarkers if accurately quantified. Mass spectral differentiation by PCA has also been used to reveal differences in the VOCs emitted by bacteria cultured *in vitro* [26-28]. A similar approach has been taken to the analysis of IMS data [40].

It is probably fair to say that data which do not provide quantification of breath VOCs will be of minimal value to clinicians. However, such data mining can be useful in recognising

different trace compounds in samples (e.g., healthy versus diseased) and this can guide the search and identification of true biomarkers, but there is yet little evidence that this is being usefully exploited clinically. It must also be forcibly stated that even when true biomarkers appear to have been identified, the findings must be replicated in different laboratories. Also, their clinical value in diagnosis must be independently and objectively tested and verified, preferably by multicentre studies, as carried out to establish NO as a reliable biomarker of asthma [55, 56, 63], and of exhaled hydrogen cyanide in cystic fibrosis patients, as described later.

The real advantages of PTR-MS and SIFT-MS are in the simplicity and rapidity of breath sampling, the real time on-line immediate quantification of the trace compounds (metabolites) in single breath exhalations and the high data flow rate. Of course, the analysis requires that characteristic analyte ions must be unambiguously identified and related to specific analyte compounds, and that the analyte ion signal levels be high enough to provide sufficiently accurate and precise quantification. These conditions have been satisfied in SIFT-MS for many breath trace VOCs by exploiting an extensive kinetics library that has been built from detailed studies of the reactions of the three available reagent ions with numerous VOCs [64]. Unfortunately, some workers exploiting PTR-MS persist in erroneously designating *m/z* values of product (analyte) ions in the analytical spectra (disease/healthy samples) as "compounds". Obviously, this is unsatisfactory; it is important to properly identify the characteristic product ions and then to assign them to the neutral analyte compounds. This has been discussed in a recent *Comment* in the *Journal of Breath Research* [65] that, mystifyingly, has largely been ignored.

Table 1 Concentrations in parts-per-billion by volume of exhaled breath, ppbv, of volatile metabolites in exhaled breath measured using SIFT-MS.

compound	**Breath concentration (ppbv)**[a]	**Ref.**	**Notes**[b]
acetaldehyde	(2-5) 24±17, (0-104) *23 (7-51) *7(3-51)	[66] [67] [68] [23] [69]	 nose (3-4)
acetic acid	*48 (22-76)	[70]	GERD *85 (32-145)
acetone	293-870 *477 (72-1024) *363 *297	[66] [71] [72] [73] [68]	

	(200- 600) (348-882) *256(172-2598)	[9] [23] [69]	 nose (366-912)
ammonia	(422-2389) *833(248-2935) (223-2091) *317 (885- 1088) *628 *537(137-15450	[66] [71] [72] [73] [74] [68] [69]	 nose (83-110)
carbon disulphide	(0-50)	[24]	
dimethyldisulphide	1.6	[24]	
dimethylsulphide	*2.5	[24]	
ethanol	27-153 196±244 (0-1663) *104 *187 (64-236)	[66] [67] [73] [68] [23]	 nose (26-28)
formaldehyde	(4-7) *1(0-12)	[23] [69]	nose (5-7)
hydrogen cyanide	*10 (6-15) *8 (0-2) *0 (0-3.2) (4-14) *6(1-38)	[72] [73] [68] [75] [23] [69]	 infected *11 (0.8-18) nose (1-2)
hydrogen sulphide	*2.37	[24]	
isoprene	89±36 (55-121) 118±68 (0-474) *37 (114-124) 58(13-126)	[76] [66] [77] [78] [23] [69]	(pre-HD, 138±63, post-HD, 184±95) nose (107-134)
methane	6000 (2000-30000)	[79]	
methanethiol	*2.8	[24]	
methanol	*461(32-1684) *238 *193 (178-399) *189(102-2319)	[80] [73] [68] [23] [69]	 nose (167-396)
pentane	*38 (15-80)	[81]	CD *113(50-220), UC *73(49-135)
propanol	*18 (0-135) *13 *16 (33-64) *40(16-349)	[66] [73] [68] [23] [69]	 nose (6-7)

[a] End tidal concentrations in mouth exhaled breath are given as ranges in parentheses (min-max); asterisk indicates *median. Where medians are not given, means are quoted before the range.

[b] In the right column labelled **Notes**, are some equivalent nose-exhaled breath concentrations; CD- Crohn's disease; UC – ulcerative colitis; GERD – gastro-esophageal reflux disease. Details of all these studies can be found in the cited references.

Accurate quantifications of many breath trace gases (metabolites) have been made by several SIFT-MS studies, some involving significant sized cohorts of healthy volunteers. A list of these compounds, and references to the published papers that report them, is given in Table 1. This work has allowed reference ranges (intra-individual population distributions) to be constructed for some of these breath compounds [67, 71, 77, 80, 82]. Such reference ranges are important if abnormal concentrations of these metabolites are to be recognised in the breath of individuals that, perhaps, may be indicative of pathophysiology, disease and infection. Too little effort has been given to this important aspect of breath analysis. SIFT-MS studies have also been carried out to define inter-individual breath compound distributions, which, as expected, are seen to be much narrower than the intra-individual distributions [66].

SIFT-MS studies have investigated the volatile compounds emitted by *in vitro* cell cultures of some common respiratory bacteria [27, 83, 84] and fungi [26]. These studies were primarily undertaken as a prelude and a guide to the search for biomarkers of these pathogens in the airways and lungs of patients, especially cystic fibrosis patients. In these studies it was judged important to both recognise and to quantify the VOCs present in the headspace of the cell cultures compared to those present in the headspace of the culture media alone (no cells). The major VOCs released were immediately identified on the SIFT-MS spectra because of the relatively large signal levels of their analyte ions. Necessarily, the numbers of cells in the cultures were roughly estimated and controlled. It was seen that easily measurable amounts of some of the common metabolites present in exhaled breath (including methanol, ethanol, acetaldehyde) were released by cultures of *Staphylococcus aureus* (SA), *Streptococcus pneumoniae* (SP), and *Haemophilus influenzae* (HI) bacteria, which suggests that abnormally high levels of these compounds in exhaled breath might be indicative of infection of the airways and lungs by these pathogen. Cultures of *Aspergillus fumigatus* (AF) fungus emitted some organosulphur compounds; this differentiates this fungus from the SA and SP bacteria. PCA analysis was used to recognise the differences in the VOCs and their concentrations

emitted by media alone and bacterial cultures; the results obtained by this approach are both interesting and revealing [26, 27].

Desirability of single breath biomarkers

As mentioned previously, the most reliable and currently the most utilized in medicine and physiology are the diatomic compounds nitric oxide (NO), hydrogen (H_2) and carbon monoxide (CO). Yet before they became acceptable as biomarkers, it was necessary to validate their relation to disease and inflammation by numerous studies in separate laboratories and by multicenter studies. The most extensive studies have been of breath NO, which has been the subject of review by three different international task forces sponsored by the American Thoracic Society and the European Respiratory Society and no other breath biomarkers have received such reviews [55]. Moreover, the clinical value of breath NO would not have been revealed if researchers had not identified the importance of careful control of breathing and sampling protocols in the analysis of breath NO [56]. Additionally, mouth/nasal collection of breath NO has demonstrated that if the breath donor hums when exhaling via the nose a reproducible analysis of breath nitric oxide can be obtained [63], but this is most probably unique to NO as an indicator of airways inflammation although, ultimately even this must be checked for other supposed volatile biomarkers.

In spite of the progress made in establishing these diatomic compounds as reliable biomarkers, it is curious that most workers in breath research are persuaded that other single volatile breath biomarkers of disease or infection will not be discovered. So the almost universal approach is to attempt to recognise differences in combinations of volatile compounds detected in breath samples (e.g. from healthy and diseased people). However, not deterred, we have persisted with the search for single breath biomarkers and following careful experimentation involving both detailed *in vitro* and *in vivo* studies, we have identified hydrogen cyanide, HCN, alone as a valuable biomarker of *Pseudomonas aeruginosa*, PA, bacteria. The *in vitro* studies have involved many genetically identified strains of PA; the *in vivo* studies have included measurements of HCN (see Table 1) in the exhaled breath of cystic fibrosis, CF, patients whose airways are often colonised with PA. The detailed results of these studies, carried out over a decade, are summarised in paper recently published in the *Journal of Breath Research* [85]. This determined enterprise illustrates the rigor and effort required to identify and establish a volatile compound as an exhaled breath biomarker. Nevertheless, this kind of challenge must be accepted if breath research is to progress to the next level. As a

necessary further extension of the HCN/PA/CF work, and as demanded by clinicians, a multicentre study has been carried out in 8 hospitals in the Midlands of England involving 233 children and young adults, which has given support and verification of our contention that HCN is indeed a single biomarker of PA infection in the lungs and airways. The results of this exciting multicentre study will soon be published (Gilchrist et al. 2015).

Further to the above, there is a case for considering n-pentane as a single and valuable biomarker. This and other volatile hydrocarbons (ethylene and ethane), were demonstrated to be markers of lipid peroxidation as long ago as the mid-1960s [86]. However, the pathogenesis of almost every disease, and also ageing, involve the generations of free radicals [87]. So the presence of pentane in exhaled breath cannot be assigned to a single disease, but it could be used to probe the severity of particular diseases and track longitudinal changes resulting from therapy if its concentration in breath can be measured accurately. Very recent ion-chemistry studies have focused on the challenging analysis of pentane in humid exhaled breath that have provided the means of accurately measuring pentane by SIFT-MS on-line and in real time. Thus, a detailed study has been carried out of pentane in the breath of patients suffering from inflammatory bowel disease, IBD [81]. This involved the analysis of mouth-exhaled breath for pentane of a large cohort of healthy individuals and significant cohorts of patients suffering from Crohn's disease (CD) and ulcerative colitis (UC) and a clear and significant elevation was seen between the levels of this hydrocarbon in the breath of both the CD and UC cohorts compared with the healthy controls (see Table 1).

Very recent pilot studies using SIFT-MS have also indicated that acetic acid in mouth-exhaled breath is elevated in gastro-esophageal reflux disease, GERD, [70]. This study involved patients suffering from reflux disease and healthy controls and revealed that the acetic acid concentration was significantly higher in the breath of the patients (Table 1). It is interesting to note that fatty acids were detected in the breath of patients with liver disease decades ago in the very early phase of breath analysis [88]. They are known from much more recent work to be produced by gut bacteria [89], presumably, they will appear in exhaled breath, but none have been accurately quantified as has been achieved in this GERD study. However, to repeat, whilst acetic acid and pentane are unlikely to be discreet single biomarkers of GERD and IBD they could be exploited as non-invasive monitors of the therapy administered to alleviate these conditions.

Concluding remarks.

We have attempted to reveal some of the weaknesses in the current approaches to breath analysis research and to indicate some ways towards improvements in data acquisition and analysis techniques that will both accelerate progress and engender confidence in this new and potentially valuable addition to the armoury of clinicians. We are conscious that some of these criticisms and proposals will be challenged, and some even rejected, but this would be no bad thing, since debate and questioning are vital for the promotion of breath analysis and all areas of science and research. In the present commentary, major focus is placed on breath sampling procedures, reliable identification of trace compounds and especially on their accurate quantification, as is required for the establishment of biomarkers of disease and infection. Key to all of these issues is the instrumentation adopted for breath analysis, astute observation and inference by the scientist/clinician and the adoption of proper data analysis procedures. Whilst multivariant statistical analysis procedures are valuable in revealing differences in VOCs in exhaled breath and other bodily fluids, and can assist in recognising specific potential biomarker VOCs, excessive reliance should not be placed on them for diagnostic purposes. It must be understood that reports of discreet VOCs or combinations of identified VOCs, which apparently discriminate between samples and are claimed to be detectors of various clinical conditions such as the sites of tumours, will not be taken too seriously until they are replicated in other laboratories and verified by multicentre studies. Otherwise, rightly sceptical clinicians will not accept the findings as important contributions to the very serious procedure of disease diagnosis. In such pursuits, the inevitable biological variability must be appreciated; thus, reference ranges of breath compound concentrations are necessary. The search for single breath biomarkers should not be abandoned, since, when recognised, the relative simplicity of their exploitation is clear.

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