Breath Analysis: The Approach Towards Clinical Applications

Anton Amann[1,4,*], Patrik Španěl[2,3] and David Smith[3]

[1]*Department of Anaesthesia and General Intensive Care, Innsbruck Medical University, Anichstr. 35, A-6020 Innsbruck, Austria;* [2]*V. Čermák Laboratory, J. Heyrovský Institute of Physical Chemistry, Academy of Sciences of the Czech Republic, Dolejskova 3, 18223 Prague 8, Czech Republic;* [3]*Institute for Science and Technology in Medicine, Medical School, Keele University, Thornburrow Drive, Hartshill, Stoke-on-Trent, ST4 7QB, UK;* [4]*Department of Environmental Sciences, ETH-Zentrum, P.O. Box CAB 24, CH-8092 Zürich, Switzerland*

Abstract: Exhaled breath analysis for clinical diagnosis and therapeutic monitoring is described with special reference to the techniques used and the underlying chemistry and physics involved. Brief outlines are given of the research carried out to date, and prospects for the future of this potentially valuable non-invasive technique are indicated.

Key Words: Breath analysis, gas chromatography, selected ion flow tube mass spectrometry, proton-transfer reaction mass spectrometry, ion mobility spectrometry, laser spectrometry.

1. INTRODUCTION

The biochemical and molecular biological diagnostic methods used in medicine have developed very rapidly in recent decades, the main focus being on blood and urine analyses. Clearly, these methods require that a sample of the body fluid be taken, which is quite invasive, especially for blood sampling, and uncomfortable for the donor. Nevertheless, these analyses are an essential, irreplaceable tool in clinical diagnosis. Blood analysis is usually concerned with the large molecular weight non-volatile compounds such as proteins and ions that are present and not with the low molecular weight volatile species that are mostly lost when a blood sample is taken. However, in recent years it has emerged that these volatile compounds will be present in exhaled breath, some now known to be present at trace levels in the parts-per-million (ppm) and parts-per-billion (ppb) levels, and that these can be valuable indicators of metabolic status and can distinguish between the healthy and diseased state if their levels can be measured to an acceptable accuracy [1]. So the major challenge is to be able to identify and quantify these volatile compounds to sufficient accuracy to be useful in diagnosis. This is now becoming possible, due to remarkable developments in gas analysis techniques and in sampling methodology that have occurred during the last decade (which are referred to in Section 3). Routine breath analysis would be a valuable addition to the armoury of the clinician, especially since breath sampling is a non-invasive technique, totally painless and agreeable to patients and can be achieved in real time with the results immediately available to the clinician. However, at this time, breath analytical techniques are not so well developed as those for urine and blood analyses and thus are not yet widely utilized in clinical practice, but this new addition to medical diagnostic techniques is gaining momentum as the potential of breath analysis is being realized and more scientists and clinicians are becoming involved in this area of science and medicine. To date, the United States Federal Drug Administration (FDA) has approved only the following compounds for breath testing:

- ethanol (law enforcement)
- hydrogen (carbohydrate metabolism)
- nitric oxide (asthma)
- carbon monoxide (neonate jaundice)
- $^{13}CO_2$ (*H. pylori* infection)
- branched hydrocarbons (heart transplant rejection).

This list is still quite limited even though it has been established in pilot studies that some other breath metabolites, when elevated above normal, are indicative of disease, for example, elevated breath ammonia occurs in kidney and liver dysfunction [2] and breath acetone is elevated in diabetes [3]. Also, there is clear evidence that breath isoprene is related to cholesterol biosynthesis [4] and there are strong indications that the endogenously produced ethanol that is seen in exhaled breath is associated with gut bacterial overgrowth [5]. These pilot studies will be referred to again later. A reason for the few FDA approvals is mostly because of the stringent requirements laid down by the FDA: sampling procedures are critically investigated and measurement reproducibility is considered as paramount to avoid misleading artefacts.

Breath analysis is very attractive, because of its non-invasive nature and because it can easily be realized for sick patients, including children and elderly persons. Multiple breath samples can be collected into bags or onto traps, and now on-line breath sampling can be achieved with the advent of new experimental techniques. Of these new techniques, selected ion flow tube mass spectrometry (SIFT-MS), proton-transfer reaction mass spectrometry (PTR-MS), laser spectrometry and ion mobility spectroscopy (IMS) are particularly promising for real time breath analyses. Also, the well established gas chromatography mass spectrometry (GC-MS) now in conjunction with solid phase micro extraction (SPME), although slower in providing analysis, has a

*Address correspondence to this author at the Department of Anaesthesia and General Intensive Care, Innsbruck Medical University, Anichstr. 35, A-6020 Innsbruck, Austria; E-mail: anton.amann@uibk.ac.at

1389-5575/07 $50.00+.00

valuable role in breath analysis. These techniques are described later to illustrate their potential and the features they must exhibit to become a reliable clinical diagnostic.

2. REQUIREMENTS OF BREATH TEST; ANALYSIS OF TRACE COMPOUNDS

What are the demanding analytical requirements that will allow breath analysis to become a reliable tool for medical diagnostics and therapy? Ideally, a wide range of diverse volatile organic and inorganic compounds need be unambiguously identified and accurately quantified in directly exhaled breath in *real time*, obviating sample collection into bags or onto traps that can compromise the sample by introducing exogenous impurities, selectively favour certain compounds and delay analyses. This is a serious challenge made more demanding by the fact that most of the metabolites will be present at the ppb level or lower, although in the diseased state some will present at the ppm level and greater. One saving grace is that the molecular weights of most volatile compounds do not greatly exceed 200 u (u = atomic mass unit), which eases mass spectrometric complications.

The compounds known to be present in exhaled breath range from the simple diatomic inorganic molecules, nitric oxide, NO, and carbon monoxide, CO, to the small molecules ethane, C_2H_6, carbonyl sulphide, OCS, and ammonia, NH_3, to polyatomic molecules such as dimethylsulphide, $(CH_3)_2S$, alcohols, including methanol, CH_3OH, and ethanol, C_2H_5OH, aldehydes such as acetaldehyde, CH_3CHO, ketones, including acetone, CH_3COCH_3, and higher-order alcohols, aldehydes and ketones, such as 3-heptanone, $CH_3CH_2(CO)(CH_2)_3CH_3$. In this mix there are also high-order hydrocarbons and other types of organic compounds. It has been suggested that there are hundreds of different compounds in breath and - given the complexity of human biology and biochemistry - this may well be true, but relatively few compounds have been positively identified and even fewer quantified to better than an order-of-magnitude in concentration. Given the widely different physical properties of these compounds it is now understood that no single analytical method can be used to detect and quantify all of them. We describe some of the available analytical methods later and their application to exhaled breath analysis.

2.1. A Paradigm: Nitric Oxide (NO) in Breath

The most widely reported exploitation of breath analysis in clinical studies is the detection and quantification of NO. In this sense the NO breath test is a paradigm [6]. It can be measured on-line, even with breath-to-breath resolution. The technique used is fluorescent spectroscopy, which involves the conversion of the NO to excited nitrogen dioxide, NO_2*, in reaction with ozone, and the subsequent fluorescence of the NO_2* is used to establish the NO concentration [7]. NO is produced in almost all organs and is present, in particular, in the lungs and the paranasal sinuses [8]. It shows a dose-dependent increase after a challenge with acetylcholine or bradykinin [9], and allows the therapeutic monitoring of asthma [10-12]. Its concentration can increase within seconds, as, for example, in the paranasal sinuses after humming [13, 14].

However, the measurements of NO in exhaled breath exemplify the potential difficulties in the development of a breath-based *clinical test*. As mentioned above, NO is produced in both the lungs and the paranasal sinuses, and is often seen at much higher concentrations in the latter regions. Therefore, expiratory flow rate and dead space air may influence the NO levels. Consequently, specific guidelines must be followed when measuring NO production in the airways [15]. The difficulties in developing guidelines for NO during the last decade should serve as an example and warning that the development of *clinically applicable tests* can be cumbersome, even when interesting preliminary pilot results are available. The general assumption is that the trace gas compounds present in alveolar breath evolve from the blood and that their concentrations in the exhaled breath stream (gaseous phase) are representative of the respective blood concentrations (liquid phase), the relative gas phase/liquid phase concentrations being determined by the appropriate partition coefficients at the temperature at the alveolar interface. This can be approximated by the (published) Henry's Law coefficients for aqueous solutions [16] but these can be significantly modified due to the complexity of blood [17]. Also, the pH of the blood can have an influence on the partition, this being particularly severe for ammonia [18]. Obviously, if a particular trace gas is also generated within the lungs or the airways, like NO, then the exhaled breath concentrations will not be simply represent the blood levels. However, it should be noted that NO is present at relatively low levels (typically a few ppb) in the exhaled breath of healthy individuals when compared to several other compounds including ammonia, acetone, methanol, ethanol and isoprene (typically > 100 ppb; see the sections on analytical techniques below). Even in diseased states (such as asthma and chronic obstructive pulmonary disease (COPD)) the exhaled NO is still at relatively low concentrations [8, 19]. Although the quantification of the most abundant metabolites is much simpler using the recently developed analytical techniques, a potential complication is the generation of some of them (notably ammonia and ethanol) by mouth flora and their injection into the exhaled breath stream [20]. But these potential complications are becoming well recognised, and as the techniques are developed and more research is carried out into breath analysis and the sampling methodology is refined, breath analysis will become a more reliable and more widely used for clinical diagnosis and therapeutic monitoring.

3. TECHNIQUES FOR BREATH ANALYSIS; RECENT DEVELOPMENTS

Of the commonly used analytical techniques for breath analysis, GC-MS has been most widely used and has the most illustrious history of achievement. Several years ago, this technique had provided most of the data available and had been exploited to identify many of the trace gases (metabolites) present [1] and indicated the presence of many more without identifying them [21]. But GC-MS does have some limitations in that sample collection into bags or onto traps (pre-concentration of trace gases) is necessary, quantification is not straightforward and constant calibration of the GC-MS instruments is required [22-25]. However, recent advances in this technique have extended its value for breath analysis, as described below.

More recently developed techniques for breath analysis include laser optical spectroscopy, which is very molecule specific and can be made accurately quantitative, but it is useful only for low molecular weight compounds, such as NO (also analysed using fluorescence spectroscopy, as described above) and ethane, C_2H_6. Of the newly developed mass spectrometry-based techniques, selected ion flow tube mass spectrometry, SIFT-MS, and the similar proton transfer reaction mass spectrometry, PTR-MS, which use quantitative mass spectrometry coupled with chemical ionisation and flow tube technology, are proving to be very valuable tools for accurate quantitative breath analysis, as will be indicated below. The less precise ion mobility spectrometry (IMS) described below has its place in breath analysis, because IMS instruments can be made very small. So these instruments can be more easily used in the clinical environment, although it would appear that their best role is for monitoring and not analysis. Significantly, SIFT-MS, PTR-MS and IMS can be used on-line to the patient to provide reliable real time breath gas analysis obviating breath sample collection, allowing rapid diagnosis and thus facilitating early treatment. These new developments have gone hand-in-hand with developments in sampling methodology, as will be mentioned throughout the following sections.

3.1. Recent Developments in Gas Chromatography Mass Spectrometry, GC-MS

The greatest feature of gas chromatography is that the individual components of a complex gas mixture, like exhaled breath, elute at different times from a column, and thus the many components can be separately analysed using electron impact ionisation (EI) coupled with mass spectrometry if a match can be found with the data contained in the EI cracking pattern library. However, library identification may still be misleading, because different substances may have similar cracking patterns. Therefore, in all critical cases, the elution time of the pure substance itself should be determined to confirm proper identification [26]. Nevertheless, GC-MS is the technique which – as a rule – gives the most detailed information on the composition of breath samples. Even so, *quantification* of a substance in a breath gas sample by GC-MS is not trivial and careful calibration using prepared samples of different concentration of each compound is needed. Additionally, it is difficult to analyse the lowest molecular weight volatile compounds such as formaldehyde and "sticky" compounds like amines. A recently explored extension to GC analysis, which appears to hold great potential, is its combination with SIFT-MS realising the GC-SIFT-MS analytical method [27]. In this combination, SIFT-MS acts as an absolute detector replacing the usual electron impact ionization of the GC-MS instrument. Ionization in SIFT-MS is performed by the reactions of the volatile (trace) substances with the primary ions H_3O^+, NO^+ and O_2^+ and quantification is achieved if the reaction rate coefficients are known. Importantly, regular calibration is unnecessary (see Section 3.3) and quantification by GC-SIFT-MS can be achieved without the need for cumbersome preparation of calibration mixtures.

The GC-MS technique has recently seen enormous developments in sensitivity, software and pre-concentration/ sampling procedures. A ten-year old GC-MS instrument typically acquired data on cracking pattern ions at a rate of about 500 u/sec, whereas now several thousand u/sec (e.g. 13000 u/sec) can be realised. Hence, the individual peaks in a GC-MS spectrum can be much better resolved in time, and the signal-to-noise ratio improved. For sampling and preconcentration of substances the following techniques have been developed:

- solid-phase microextraction (SPME) [28, 29].
- adsorption of volatile substances on adsorbing material like Carbopack X, Carboxen 1018, Carboxen 1019, Carbopack B 60/80, Carbotrap C 20/40 and others, with subsequent thermal desorption and GC-MS measurement [30].
- adsorption of volatile substances onto activated charcoal with subsequent liquid desorption using CS_2 or an appropriate ester like ethyl acetate [31]; this method of "accelerated solvent extraction" (ASE), though not yet widely used in the analysis of volatile compounds, can make use of the high adsorption capacity of activated charcoal.
- sophisticated preconcentration of gaseous samples in a cryogenic trapping system [32].

With all these methods, the handling of the high water content (humidity) of breath samples is a non-trivial matter. With cryogenic trapping, in particular, freezing of the trap may occur (if the breath sample is too voluminous), which in turn restricts the volume of exhaled breath that can be collected. Also, adsorption of volatile substances on the adsorbing material may give rise to the "breakthrough phenomena" due to adsorption of high amounts of water and carbon dioxide present in exhaled breath samples [30]. Perhaps a combination of standard adsorbing materials like Carbopack in sequence with activated charcoal could be used, where the second adsorption minimises "breakthrough" by trapping all the volatiles that eventually pass the first cartridge.

Of these methods, solid phase microextraction (SPME) is very reliable and gives rise to reproducible results [28, 29, 33]. If the pre-concentration of the adsorption volatile components onto the SPME-fibre is high enough, it is highly recommended. In particular, the number of steps in the preparation and analysis (like the passage through different traps) and therefore the possibility of errors and losses of trace compounds are reduced. In addition, costs of solvents and disposal of solvents as required by other techniques can be avoided. Some volatiles (e.g., ethane) cannot be preconcentrated sufficiently with SPME to allow analyses in the ppb concentration range. For such molecular species, preconcentration and the use of adsorbents is still necessary for adequate GC-MS analysis.

3.2. Proton Transfer Reaction Mass Spectrometry, PTR-MS

Proton-transfer reaction mass spectrometry (PTR-MS) is a technique which was first developed during the 1990's [34, 35] and has been used for on-line monitoring of volatile molecular species in environmental research, food control and medical applications [36-42]. The ionisation of a volatile species M, e.g., acetone, is performed by a chemical reaction in which a proton is transferred from a reagent ion, H_3O^+, to M producing the product ion MH^+:

$$H_3O^+ + M \rightarrow MH^+ + H_2O \quad (1)$$

Hence, this ionisation technique is called *proton-transfer-reaction mass spectrometry,* PTR-MS, and it is a special case of the well known *chemical ionisation* technique [43]. This chemical ionisation is performed by injecting a current of H_3O^+ ions into a "drift chamber" (the reaction chamber, some 10 cm long) where these precursor ions react with the trace gas M. Chemical ionisation is a "soft" ionisation technique when compared to EI, since no *pronounced* fragmentation of the MH^+ product ion species occurs. In GC-MS, the components, M, of the gas sample are first separated in the GC column, and then every elution peak (every component) undergoes EI. The fragmentation of the nascent parent molecular ions M^+ is exploited to identify the underlying substance using a library of fragmentation patterns.

In PTR-MS the composite compounds of the sample are not separated in time, but fragmentation of the nascent MH^+ product ion is largely avoided because proton transfer is not greatly exothermic. If a product molecular ion MH^+ does not fragment, it can be observed at a mass-to-charge ratio, m/z, which is determined by the molecular mass of the original molecule M plus 1 atomic mass unit (due to the attached proton). Acetone, for example, with a molecular weight of 58 u, is then observed in the product mass spectrum as an ion with m/z 59. However, even though fragmentation does not occur for many types of MH^+ product ions under PTR-MS conditions, this does occur for some of these MH^+ product ions. For example, if the molecular species M contains a fluorine atom F, then fragmentation often occurs in which an HF molecule is released. An example of this is when M is the volatile anaesthetic sevoflurane with a molecular weight of 200 u, which in PTR-MS mainly gives rise to product ions an m/z value of 181 [40]. (Note that under SIFT-MS conditions, ions at m/z values of 49, 181 and 199 appear as products of this reaction [44]). Total or partial fragmentation also occurs of nascent MH^+ product ions following the protonation of some more common organic molecules by H_3O^+, including some alcohols, aldehydes and esters (see ref [45]).

Volatile compounds can be quantified to a factor of two or so with PTR-MS (see below) when *not* positively identified. If two different compounds give rise to product ions at the same m/z value they cannot readily be distinguished. As an example, consider the substances butanal, 2-methylpropanal, 2-butanone and tetrahydrofuran, which have the same molecular weight of 72 u. Consequently, the respective protonated ions would all have an m/z value of 73 (assuming no fragmentation of the nascent product ions) and so cannot be distinguished by PTR-MS alone. Some of these analysis difficulties are removed using NO^+ and O_2^+ precursor ions in addition to H_3O^+ ions, as used in SIFT-MS, which is described in the next section. Quantification of compounds by PTR-MS is performed by considering the kinetics of the protonation reaction, viz.

$$d/dt\,[MH^+] = k_M * [M] * [H_3O^+] \quad (2)$$

Here, a critical parameter is the compound-specific rate coefficient, k_M, for the proton transfer reaction, which usually takes values between 1.3×10^{-9} cm³/s and 4×10^{-9} cm³/s [46]. Using k_M and the time t_{drift} that the swarm of ions take to traverse the drift chamber, the concentration of species M can be computed (to a first order approximation) as:

$$\text{concentration of M} = \text{counts}(MH^+)/(\text{counts}(H_3O^+) * k_M * t_{drift}) \quad (3)$$

Here the counts(·) are the PTR-MS-measured (and transmission-corrected) counts of ions at a particular m/z value.

Though identification of substances is not always possible with PTR-MS, a few compounds can usually be attributed to certain m/z values of product ions *under prescribed circumstances*, due to assistance from parallel GC-MS measurements:

- When measuring ambient air near a road, the concentrations of benzene (product ion m/z=79), toluene (m/z= 93) or the different isomers of xylene (m/z= 107) can confidently be determined.
- When measuring human breath, methanol (m/z= 33), acetonitrile (m/z= 42, from smoking), acetone (m/z= 59) and isoprene (m/z= 69) can also be attributed with some confidence to the respective m/z values. However, the isoprene protonation reaction leads to some fragmentation producing an ion at m/z= 41 (Alfons Jordan, priv. comm.), although the latter ion may not exclusively originate from protonated isoprene.
- When analysing the breath of patients with propionic academia, an increased level of ions at m/z= 115 can be attributed to an increased level of heptanone in the breath [47].

Such tentative identifications should always be treated with circumspection and checks by GC-MS (or other techniques) are desirable [48]. Also, for proper quantification of volatile substances in gas samples, the respective reaction rate coefficients have to be known (which is only possible after substance identification).

What are the advantages of PTR-MS? The following features are worthy of special mention.

- Breath-gas measurements can be performed on-line [41, 42, 49], and even with breath-to-breath resolution.
- The limit of detection of PTR-MS is very low (down to the ppt-level [50], depending on the measurement time allowed for the particular product ion).
- A medical student can learn to use PTR-MS within a week or so, because the handling of the software is relatively simple and there is no need for method selection (like the choice of column and SPME fibre or temperature ramps etc. for different compounds, as in gas chromatography).

In Fig. (**1**), an on-line measurement of the product ions at m/z= 33 and m/z= 69 obtained as exhaled breath is analysed by PTR-MS together with the breath CO_2 concentration of a human volunteer over a full night. In this experiment, though not shown, all m/z values of ions between 21 and 230 were recorded, with uniform time-slots of 0.5 sec (for each m/z value). CO_2 was measured for control purposes using a standard CO_2 sensor. Measuring 210 different m/z values each

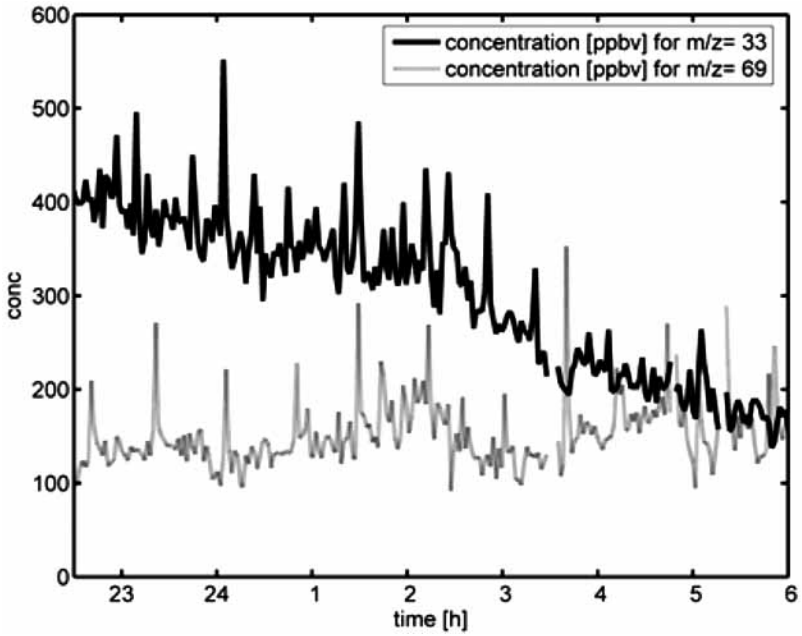

Fig. (1). Time variations of breath concentrations in parts-per-billion, ppb, derived from product ions at m/z= 33 (tentatively identified as methanol) and ions at m/z= 69 (tentatively identified as isoprene) in PTR-MS for a healthy volunteer during a whole night.

for 0.5 sec takes 1.75 min. Hence the concentration of a particular substance is measured about 34 times per hour. For just one particular m/z value the duration of one "cycle" can be reduced to 1 sec (since the count rate of the precursor ions $H_3{}^{18}O^+$ at m/z= 21 have also to be determined).

Under what circumstances can volatile substance be measured by PTR-MS? The protonation of most compounds, M, by H_3O^+ ions is quite exothermic and so once MH^+ is formed the reverse reaction back to H_3O^+ and M cannot proceed. This is because the proton affinities of most volatile M exceed that of water molecules which is 691.2 kJ/mol and can therefore be detected by PTR-MS. As an example, organic alcohols have proton affinities between about 750 and 880 kJ/mol and can therefore be detected by PTR-MS. The proton affinities of the main components of air (nitrogen, oxygen, carbon dioxide) are smaller than that of water. Hence, significant protonation of these compounds will not occur. Carbon dioxide, for example, has a proton affinity of 540.6 kJ/mol, will therefore show almost no protonation and will not be detected by PTR-MS. However, if the concentration of carbon dioxide (with a molecular weight of 44 u) in a gas sample is very high (as is the case in breath samples), there will be some ion counts at m/z= 45. Hence the concentration of other volatile species in the gas sample with m/z= 45 (like acetaldehyde) cannot be determined in a straightforward way. Similarly, some compounds have proton affinities close to that of water, including formaldehyde, hydrogen sulphide and hydrogen cyanide and then the reverse reaction can result in the loss of the MH^+ product ions in reaction with H_2O molecules in the breath sample giving back the H_3O^+ precursor ions:

$$MH^+ + H_2O \rightarrow M + H_3O^+ \quad (4)$$

The above compounds and some others such as the C_1-C_4 hydrocarbons, methane to pentane, cannot be readily analysed by PTR-MS. A detailed description of this phenomenon has been given following several SIFT-MS studies, as reported in the review paper by Smith and Španěl 1995 [45]. A summary of the characteristics for PTR-MS and other analytical techniques is given in Table **1**.

Recently, there have been some new developments and extensions to the PTR-MS technique, such as the PTR-Time-of-Flight (TOF)-MS technique [51]. These developments could lead to PTR-MS analytical devices which not only allow quantification, but also the positive identification of compounds in gas samples.

3.3. Selected Ion Flow Tube Mass Spectrometry, SIFT-MS

The selected ion flow tube (SIFT) technique was conceived, developed and exploited for many years for the study of the reactions occurring in interstellar clouds [52], and it is only relatively recently that its potential as an analytical tool was realised when it was developed as the novel selected ion flow tube mass spectrometry, SIFT-MS, technique [53, 54]. The broad intention was to develop SIFT-MS as an analytical method for the analysis of trace gases in air with a focus on real time breath analysis for clinical diagnosis and therapeutic monitoring. In this sense, the intention was, and remains, to establish a versatile trace gas analytical method that offered an extra dimension to existing analytical techniques, viz. on-line real time analysis of exhaled breath avoiding sample collection, such as is required for GC-MS analysis.

SIFT-MS has been described in detail previously [53-56] so only a brief summary is provided here. A chosen precursor ion species formed in a microwave discharge (either H_3O^+, NO^+ or O_2^+) is selected from a mixture of ions by a quadrupole mass filter and injected into fast-flowing helium

Table 1. Summary of the Characteristics of Different Analytical Techniques Used for Exhaled Breath Analysis

	GC-MS	PTR-MS	SIFT-MS	IMS	Laser Spectrometry
Ionization method	electron-impact ionisation or chemical ionisation (isobutane, ammonia)	chemical ionisation using H_3O^+	chemical ionisation using H_3O^+, NO^+ and O_2^+	atmospheric pressure chemical ionisation, mostly using H_3O^+ or cluster species	no ionisation necessary
Detection of:	molecular fragments and identification using fragmentation patterns	protonated molecular species	a variety of ionic species characteristic of the precursor ions and reactant molecules	mostly protonated molecular species (but negative ionisation is also possible)	characteristic spectral lines
Fragmentation	unavoidable and desirable (for substance identification by match with library)	is not desirable; fluorinated species or alcohols as well as larger molecules may fragment	does occur but is well characterised for many reactions by SIFT studies	is not desirable; fluorinated species or alcohols as well as larger molecules may fragment	fragmentation is not an issue, however each compound is responsible for a very large number of spectral lines.
Substances which cannot be detected	depends on preconcentration; ethane cannot be detected at ppb-levels using SPME (except with preconcentration and subsequent thermodesorption using appropriate adsorbents)	lower hydrocarbons (methane – butane); formaldehyde and acetaldehyde quantification need further research	lower order hydrocarbons and species of low proton affinity and high ionisation energies	lower hydrocarbons (methane – butane); formaldehyde quantification is difficult	measurement methods have to be developed for every molecular species; substances with higher molecular weight (e.g., pentane) still need method development
Advantages	different substances in gas sample can be identified	on-line measurement is possible; high sensitivity	on-line, real time absolute quantification of several compounds simultaneously to good accuracy. Water vapour as internal calibration	small portable device with high sensitivity	potentially small device; on-line measurement is possible
Potential problems	on-line measurement is only possible in chemical ionisation mode, different protocols for different classes of substances; water in samples may cause problems	formation of water clusters (depending on water content of sample) is not yet fully understood; hence water content of samples may cause problems; identification of compounds from m/z alone sometimes difficult	identification of isomers sometimes difficult	quantification is relatively difficult (ionisation depends on water content of sample)	Specific laser is necessary for each molecular species; wavelength-tunable lasers may be available in the future; water content of samples may cause problems (e.g., Nafion-drying is used)
Limit of detection	depending on preconcentration method and detector (ppb and even ppt)	down to ppt (depending on measurement time slot)	currently at 0.1 ppb/sec	down to ppt (depending on ionisation method; more sensitive using a ^{63}Ni source)	down to ppt using multi pass methods
Calibration	the whole chain of sample preparation and measurement calibrated for each compound separately	Transmission coefficient has still to be determined for a particular instrument setup	once the reaction time and mass discrimination are determined for a given instrument, universal kinetic library can be used for absolute quantification	Calibration for all compounds required.	Absorption spectra must be known for each compound to be detected.

carrier gas. The chosen precursor ion species is used to ionise the trace gases in a breath sample that is introduced into the carrier gas at a known flow rate. The exhaled breath is presented to the entry port of the SIFT-MS instrument *via* a disposable cardboard mouthpiece and a fraction of it (the sample) enters the carrier gas *via* a heated, calibrated capillary. The choice of precursor ion depends on the trace gas compounds in the sample that are to be analysed, characteristic product ions being produced from their reactions with each precursor ion. For example, H_3O^+ is chosen when the trace gas compounds to be analysed are readily protonated by this precursor ion, as described above for the PTR-MS method (see equation (1)). This is the case for many organic compounds, including some that are known to be present in exhaled breath, which include methanol, ethanol, propanol, acetaldehyde, acetone and isoprene (see later) and also ammonia. For example, acetone reacts with H_3O^+ thus:

$$H_3O^+ + CH_3COCH_3 \rightarrow CH_3COCH_3H^+ + H_2O \quad (5)$$

A downstream orifice samples the precursor ions and product ions, which pass into a differentially-pumped quadrupole mass spectrometer and ion counting system for analysis. Hence, a product ion at an m/z value of 59 is observed when acetone is present in the sample of breath. However, in SIFT-MS another ion is always seen at an m/z value of 77, which is the hydrate of the protonated acetone ion formed in the following three-body reaction:

$$CH_3COCH_3H^+ + H_2O + He \rightarrow CH_3COCH_3H^+H_2O + He \quad (6)$$

The production of this adduct ion is facilitated by the presence of the helium carrier gas atoms [57] and is promoted by the relatively high water molecule concentration in the breath sample. The ratio of the total count rates of all the product ions, including the hydrated product ions, to all the count rates of all the precursor ions and the rate coefficients for the reactions of the precursor ions with the particular trace gas compound are the required parameters for the proper quantification of trace gases [45]. Thus, calibration of the instrument for each separate compound, which is difficult at low (ppb) concentrations, is not required. The quantification procedure for particular trace gases is generally along the lines given above for PTR-MS, with the differences that account is taken for the differential diffusive loss to the flow tube walls of the product ion species and the measured rate coefficients for individual reactions are always used. This allows concentrations of trace gas compounds in air and breath to be determined to about 20% or better (see the details and references given in ref [56]).

The ion chemistries that occur when NO^+ and O_2^+ are used as precursor ions are distinctly different than that for the H_3O^+ reactions that essentially involves proton transfer (sometimes with fragmentation), as explained in the PTR-MS section. For NO^+, three different processes dominate the chemistry, these being charge (electron) transfer between NO^+ and the reactant molecule M producing parent radical cations M^+ and neutral NO molecules, hydride ion (H^-) transfer producing $(M\text{-}H)^+$ ions and neutral HNO molecules and adduct formation producing $NO^+.M$ ions. Clearly, charge transfer can only occur when the ionisation potential of M is less than that of NO (which is 9.26 eV). This is the case for most amines and so NO^+ is very suitable for the analysis of these compounds. H^- transfer commonly occurs in the reactions of NO^+ with most alcohols and aldehydes. Adduct formation or association commonly occurs for ketones and carboxylic acids. For acetone the reaction proceeds thus:

$$NO^+ + CH_3COCH_3 + He \rightarrow NO^+.CH_3COCH_3 + He \quad (7)$$

This is analogous to reaction (6) and results in a product ion at an m/z value of 88.

For O_2^+ precursor ions, charge transfer occurs in their reactions with most compound molecules, including acetone, often resulting in partial fragmentation of the nascent parent M^+ radical product ion, e.g.:

$$O_2^+ + CH_3COCH_3 \rightarrow CH_3COCH_3^+ + O_2 \quad (8a)$$

$$\rightarrow CH_3CO^+ + CH_3 + O_2 \quad (8b)$$

In this case and for similar reactions both product ions must be included in the analysis for accurate quantification to be achieved. In general, the ions produced by O_2^+ ionisation are a subset of those observed in electron ionisation (EI) spectra for the reactant molecule [45].

A real advantage of SIFT-MS is seen *via* these acetone examples. For the analysis of some compounds in complex samples like exhaled breath, all three precursor ions (H_3O^+, NO^+, O_2^+) can be used on the same sample, thus providing a check on the analyses. This can be especially helpful to distinguish between compounds that have the same molecular weight, like, for example, acetone and propanal (that react with NO^+ producing $NO^+.M$ and $(M\text{-}H)^+$ ions respectively). These aspects of SIFT-MS have been thoroughly discussed in the review paper by Smith and Španěl [45].

There are two distinct analytical modes of operation of SIFT-MS. Firstly, the *full scan mode,* where a conventional ion mass spectrum is obtained over a chosen range of mass-to-charge ratio, m/z, as the breath sample (either from a bag or from directly exhaled breath) flows into the carrier gas, in order to identify all the precursor ions and product ions and to determine their respective count rates. From these and from the well-defined reaction time, the on-line computer immediately calculates the concentrations of those trace gas compounds present in the breath sample exploiting the in-built kinetics database, which comprises the rate coefficients and the product ions of the particular precursor ion/trace gas compound reactions. The SIFT-MS kinetics database has been constructed from numerous detailed selected ion flow tube (SIFT) studies of the reactions of various classes of compounds (alcohols, aldehydes, ketones, hydrocarbons, etc) with the three SIFT-MS precursor ions [45, 58, 59]. Secondly, the *multiple ion monitoring mode* in which the downstream analytical mass spectrometer is rapidly switched between selected m/z values for both the precursor and product ions, in order to quantify both water vapour and the targeted trace compounds. This mode of operation reveals the concentration time profiles of several (targeted) trace gases simultaneously in a single direct breath exhalation and also identifies the alveolar portion of the breath sample, thus providing more accurate quantification of the chosen trace compounds than can be obtained using the broad sweep *full scan mode*. An example of breath analysis data obtained using the

multiple ion monitoring mode is presented in Fig. (**2**). Note, also, the important point that during the breath inhalation cycles the levels of the targeted compounds in the ambient air are determined, which allows an assessment to be made as to whether or not the ambient levels can influence the derived breath levels.

A unique feature of SIFT-MS breath analysis is that the water vapour concentration in the air/breath samples is routinely calculated from the signal levels of the hydrated hydronium ions $H_3O^+(H_2O)_{1,2,3}$, which are formed in three-body reactions involving H_3O^+ and the abundant H_2O molecules, analogous to that shown in equation (6). Such measurements act as an internal calibration of the sampling system since the absolute humidity of exhaled breath is known to be close to 6% (see ref [45]). The accuracy of SIFT-MS analyses has been well established by several investigations (as reviewed in ref [45]) and can be used with confidence for breath analysis.

The great advantage of SIFT-MS breath analysis is that it is a truly non-invasive technique and as such does not hold fears for patients. It is clear that the appearance in breath of volatile metabolites must reflect in some way the normal physiology and pathophysiology of the individual donor. The challenge is to identify the trace gas metabolites, to quantify them to the required precision and accuracy and to understand their biochemical origin and their physiological significance. An initial essential phase of SIFT-MS studies has been to establish the concentration distributions of those breath metabolites that can be unambiguously identified in the healthy population and thus to gain additional insight into normal physiological processes *via* this unique (exhaled breath) window. Critical to this topic is the recent work reported in a series of papers by Turner, Španěl and Smith [60-63] who have carried out longitudinal studies of several common breath metabolites in the exhaled breath of a cohort of 30 healthy volunteers over a six month period, thus establishing their concentration distributions in breath, which were found to be close to log-normal rather than normal and thus they were described by a median and a multiplicative (geometric) standard deviation. The median values (given in the brackets) for these compounds are: ammonia (833 ppb), acetone (477 ppb), isoprene (106 ppb), methanol (461 ppb), ethanol (112 ppb), propanol (18 ppb) and acetaldehyde (22 ppb). The relationships between the levels of these metabolites and factors such as age, gender and body mass index were also investigated. These studies were a follow-up to the more limited pilot study by Diskin *et al.* [64] carried out over a 30 day period. Collectively, these studies have thus established the expected levels on these compounds and their variations in the breath of the healthy population (within the constraints of the limited healthy volunteer cohorts). These studies have also provided some information on the biochemical origins of these common breath metabolites in healthy individuals; for example, it seems most likely that the alcohols originate in the gut *via* bacterial action. It is also clear that exhaled breath ammonia levels increase with the age of the volunteer. But of major value is that with these data it becomes possible *via* breath analysis to recognise departures from the norm and thus to exploit breath analysis for clinical diagnosis.

A number of previously published studies have linked increase levels of specific compounds in exhaled breath and the headspace of body fluids with various diseases as, for

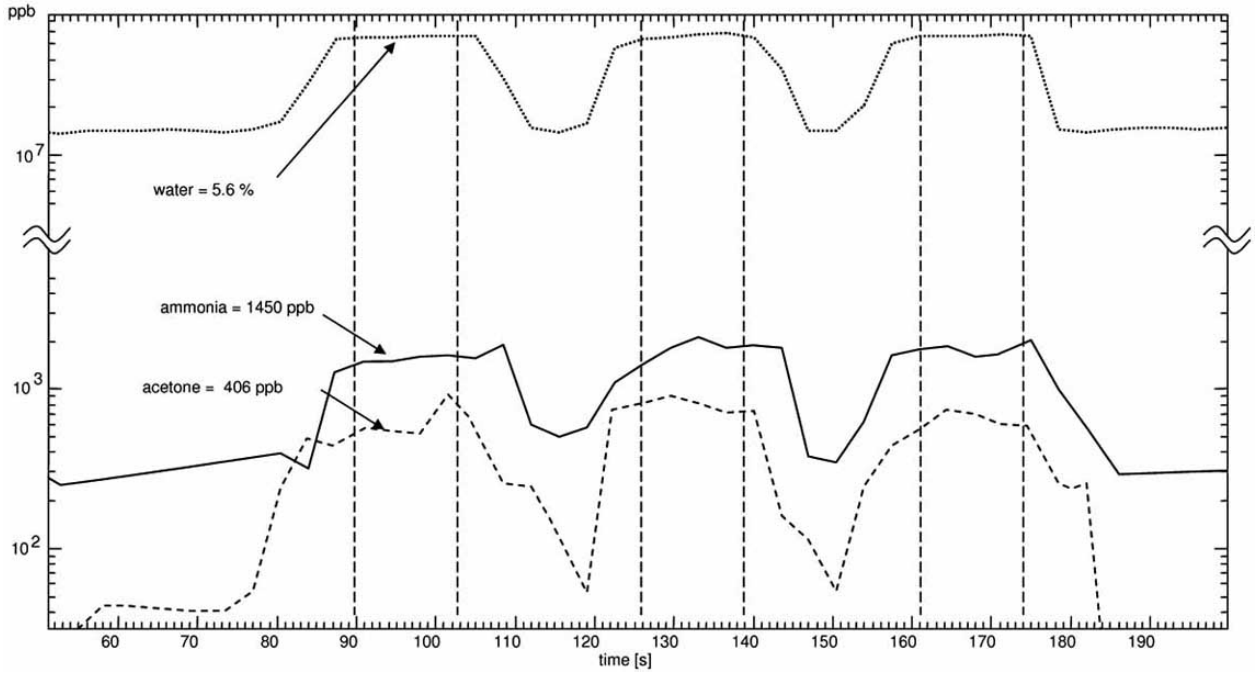

Fig. (2). Time profiles of concentrations of water vapour, ammonia and acetone in three sequential breath exhalations obtained simultaneously using SIFT-MS. The alveolar concentrations are obtained by analysing the indicated portions of the exhalations (vertical dotted lines).

example, elevated breath acetone in diabetics (see the excellent early review by Manolis [1]). As envisaged, SIFT-MS studies have now begun to add significantly to this important topic. A useful approach to breath analysis is that there are two distinct types of breath tests: (i) measuring changes in the breath levels induced by food or a dose of a particular drug and (ii) measuring breath levels of compounds spontaneously generated by the human organism in the healthy and diseased states. Some of the compounds generated naturally by all human being are mentioned above; many other compounds are surely present, but few have been positively identified and certainly not properly quantified. Of the recognised compounds, the levels of some have been shown by SIFT-MS and other studies to be altered in the diseased state. It has been shown that the breath levels of some can be modified by the ingestion of certain foods and drugs. Mouth production has also been investigated using SIFT-MS, as is mentioned below. Much can be written on these SIFT-MS studies, but only headings can be given here; the details can be obtained from the papers cited below.

In the first category (i), modification of the common breath metabolites following the ingestion of protein and carbohydrate meals has been studied, showing that breath ammonia increases following the protein meal but not after the carbohydrate meal, whereas acetone decreased following both of these food types [65]. A study of the loss kinetics of ethanol metabolism in which the formation of acetaldehyde was also tracked, well demonstrates the key feature of on-line breath analysis that samples can be taken every few seconds, allowing accurate definition of the kinetics [66]. This facility was invaluable in the study of breath ammonia increase following a large dose of vitamin B3 (Niacin), a topic of some interest in psychiatry [67]. Increase in breath ammonia was also observed following the ingestion of a small dose of urea by *H. pylori*-positive individuals [53], which offers a simple non-invasive test for the presence of this bacterium in the body. An interesting application of on-line SIFT-MS (and flowing afterglow mass spectrometry (FA-MS) [68]) breath analysis is the determination of the deuterium content of breath water vapour following a measured dose of D_2O, from which total body water can be readily derived [69], a parameter of great importance in nephrology. Finally, it is pertinent to note again that the production of trace gases in the mouth must be guarded against if true blood levels of metabolites are to be derived by breath analysis. However, SIFT-MS also offers the opportunity to study halitosis. In this regard, a SIFT-MS study has shown that mouth washing with sucrose and urea solutions generates copious amounts of ethanol and ammonia respectively [20], and that because of the rapid time resolution of the SIFT-MS analyses, mouth and alveolar production of these trace compounds can be partially distinguished. In this category (i) can be included the appearance in breath and urine of acetonitrile after cigarette smoking; that the measured quantities in breath and urine are seen to be equivalent indicates that the acetonitrile is systemic [16].

In the second category (ii), SIFT-MS studies of note are those involving patients with end-stage renal failure. The first significant observation was the greatly elevated ammonia present in the breath of these patients prior to haemodialysis and the reduction of this metabolite during treatment [2]. Diabetes was also recognised in some of these patients by the elevated levels of acetone in their breath, and in some patients the breath ethanol was also many times greater than normal, which has been attributed to gut bacterial overload. In a subsequent study, it was observed that other breath metabolites were reduced during dialysis, but in a fraction of the patient cohort the breath isoprene actually increased; the speculation is that this is due to bio-incompatibility of blood and the dialysis membranes [70]. These *in vivo* studies are complimented by the investigations of the presence of malignant tumours (cancer) in the body by analysing the volatile compounds emitted into the headspace above urine samples provided by bladder and prostate cancer patients [71]. This *in vitro* study revealed that formaldehyde was present above the urine from the cancer patients but not above urine samples from healthy controls [71]. This observation has spawned studies of the breath of cancer patients [72] and is the focus of an EU project BAMOD involving 13 research groups (cf the homepage of BAMOD at: http://eu-proposal.voc-research.at). Further SIFT-MS studies that are the prelude to breath analysis studies are the study of volatile compounds generated by cancer cell line cultures *in vitro*, which showed the emission of acetaldehyde in close proportion to the number of cancer cells in the culture [73]. Finally, as part of a concerted effort to use SIFT-MS for the study of children with respiratory disease, and especially cystic fibrosis, specimens of Pseudomonas aeruginosa taken from these child patients have been cultured on plates and the volatile emissions studied with the remarkable result that hydrogen cyanide (HCN) gas is emitted from these cultures [74]. This may offer a non-invasive monitor (ideal for children) of the severity of colonisation by this bacterium.

3.4. Ion Mobility Spectrometry, IMS

The term ion mobility spectrometry refers to the method of identifying trace compounds in gases by the characteristic mobilities of ions derived from specific compounds. Chemical ionisation of volatile compounds occurs *via* ions like $H^+(H_2O)_n$, $NO^+(H_2O)_x$, $NH_4^+(H_2O)_y$ [75], O_2^- or $O_2^-(H_2O)_n$. Positive ionisation occurs mainly through proton transfer reactions whereas negative ionisation occurs through electron capture or reaction with O_2^-. Halogenated volatiles, MX, for example, can be negatively ionised with subsequent formation of X^- ions. Therefore, ionisation mainly occurs for volatiles having a high enough proton affinity (higher than that for water). Experience demonstrates good ionisation occurs for compounds with proton affinities larger than 750 kJ/mol. Hence, as with PTR-MS and SIFT-MS, low order alkanes (methane – butane) cannot be analysed. Formaldehyde, in particular, has an IMS-detection limit in the ppm range.

The ions in an IMS instrument drift in a buffer gas at ambient pressure under the influence of a weak electric field. The drift times of swarms of ions of specific types formed using suitable ionisation sources (normally ^{63}Ni ß-radiation sources, UV-lamps or discharges) are normally measured exploiting electrical shutters. The drift velocity of the ions, v, is related to the electric field strength, E, by the mobility, μ ($v = \mu \cdot E$). Therefore, μ is inversely proportional to the drift time, which is usually measured at a fixed drift length. The

different ion species that comprises the ion swarm separate in space according to their drift velocities, which are related to their different masses and geometrical structures. Collection of the ions by a Faraday cup results in a time-dependent signal corresponding to the *v* values of the separated ion swarms. Hence, such a *v* spectrum contains information on the nature of the different trace compounds present in the sample gas. Theoretical considerations show that the mobility of the ion is related to their collision rate with the gas molecules in which they are drifting, the gas temperature, the dimensions of the ion (structural dependencies) and the collision integral [76, 77]. Therefore, isomeric forms of ions (and the precursor molecules) should be distinguishable.

IMS was originally developed for the detection of trace compounds, for example gaseous pollutants, in air [77]. It combines both high sensitivity and relatively low technical expenditure with high speed data acquisition. The time to acquire a single drift spectrum is in the range of 10 ms to 50 ms. Thus, an IMS instrument is suitable for process control, but due to the occurrence of ion-molecule reactions in the drift gas and relatively poor mass resolution of the ionic species formed, it is generally not good for identification of unknown compounds. So support is required using other techniques, such as GC-MS and SIFT-MS, to realise positive identification of compounds. Under ideal circumstances, the final IMS spectrum consists of clearly separated peaks, although this is rarely the case unless pre-separation of the separate components of a mixture is carried out. It is necessary to shield the IMS against external electromagnetic disturbances and the gas flow rates (normally in the range of some mL/min) as well as the temperature in the ionisation and drift regions must be carefully controlled.

All parts of the IMS that are in contact with the analytes are constructed from inert materials. Teflon is used for the ionization chamber and the drift tube. Much development work on IMS has been carried out by Baumbach *et al*. [78, 79]. To realize effective pre-separation of a rather complex exhaled breath mixture, long polar multi-capillary chromatographic columns (MCC) are required [80]. The heating of the columns is essential to obtain reproducible results. In the sampling process, the subject blows through a mouthpiece coupled to a brass adapter *via* a Teflon tube, which is connected to a 10 mL stainless steel sample loop of an electric six-port valve. By switching the six-port valve, breath is transported by the carrier gas from the sample loop into the MCC. Then the separated compounds of the breath mixture can be directly analysed by IMS. Therefore, the results can be achieved within 600 s depending on the separation time of the compounds. This construction enables a direct and rapid sampling at a known breath volume. The effective separation of water vapour is one major advantage of using the MCC; using other techniques like humidity sorbents or membrane separation units, some of the breath components may be lost. The potential value of IMS in breath analysis is that the instruments are small and cheap and thus could be readily and widely used as a monitor of known breath compounds in the clinical environment.

Pilot investigations using IMS have been carried out on the breath of 40 subjects, including 22 patients suffering from various pulmonary lung infections [78]. Also, breath analyses for 18 healthy persons were analysed on-line using the MCC-^{63}Ni-IMS combination and the room air was analysed before each breath measurement. To reduce the risk of cross contaminations due to other physiological processes, the subjects had not ingested solids or liquids and not smoked for at least two hours before the breath measurements (for further details see ref [80]). In these studies, acetone was clearly identified in all the breath samples, as is expected even in the healthy state (see section 3.3). Additional peaks in the IMS spectra were also seen when analysing the breath of patients who were taking antibiotics and those suffering from *Pneumonia*. Other peaks were seen when analysing breath of patients suffering from airway inflammation. To identify these characteristic peaks, further measurements will be needed, and larger cohorts of patients and healthy persons will need to be investigated. Data will need to be processed using statistical methods to clearly correlate the characteristic pattern of the IMS-topographic plots with specific diseases. Colon cancer metabolites have also been detected using IMS [81].

In the near future, mass spectrometric methods (such as SIFT-MS) will be used to identify some of the unidentified metabolites detected by MCC-IMS. The opportunity for on-site and short-time analyses using air as the carrier gas at ambient pressure is the most important benefit of this technique. Thus, the use of MCC-IMS directly in the clinical setting will allow results to be obtained within minutes, providing additional information to influence therapeutic strategies and facilitates the building of spectral databases relating to particular illnesses.

3.5. Laser Spectroscopy

This spectroscopic technique is proving to be a promising contributor to breath analysis, especially for compounds that are present in breath at very low levels, such as C_2H_6, NO and CO [82-84] for which PTR-MS, SIFT-MS and ion mobility spectrometry (IMS) cannot be used, and for which GC-MS usually requires pre-concentration from breath samples before analysis meaning that time resolved (breath-to-breath) measurements cannot be made. This detection method is based on absorption spectroscopy and is therefore very molecule specific, providing "fingerprint" spectra in the infrared wavelength region, for example near 3.3 μm for ethane detection and near 4.8 μm for CO detection. The trace gas concentrations are determined from the light absorption observed at the characteristic wavelengths for specific molecules. The analyzer consists of a tunable continuous-wave laser and an absorption cell containing the gas sample of interest. This optical method has great advantages for sensitive and specific trace gas analysis. However, in common with all analytical instruments that essentially have near-static gas samples to analyse, contamination by out-gassing materials can be serious, and so all parts that are in contact with the sample gas must be made of stainless steel, copper or Teflon. Also, the gas sample needs to be dehumidified and (spectroscopically) interfering molecules need to be removed by some form of trap, and this runs the danger of loss of the trace gases that are to be detected and quantified.

Nevertheless, as well as NO, CO, OCS, etc, various organic compounds have been detected in exhaled breath at the

sub ppb level and even at the ppt (parts-per-trillion) level in some cases [83]. In particular, this technique has proved to be very useful for the quantitative on-line detection of C_2H_6 [83, 85], which is considered to be the most important volatile marker of free-radical induced lipid peroxidation and cell damage in the human body and is a promising non-invasive tool for on-line monitoring of oxidative stress status [86]. Ethane has also been detected in the breath of patients suffering from lung cancer [87] and scleroderma [88] using laser spectroscopy. Such optical instruments could be connected to a mechanical ventilator for continuous monitoring. Recently, this technique has been used to investigate carbon monoxide and carbonyl sulphide [89-91]. Exhaled carbon monoxide (eCO) may be a marker for pulmonary diseases [8].

3.6. The Use of ^{13}C-Labelled Substances

^{13}C-labelled compounds have been used as diagnostic probes for some years [92], and there are more than 50 accurate diagnostic ^{13}C breath tests reported. Measurements are carried out, for instance, using isotope selective IR-spectrometers. Both $^{13}CO_2$ and $^{12}CO_2$ have characteristic infrared absorption bands, which allow both absolute and relative quantification of these isotopomers.

Examples of diagnostic tests using ^{13}C-labelled compounds are the following:

- *H. pylori* breath test using ^{13}C-labelled urea [93].
- dihydropyrimidine dehydrogenase deficiency test using uracil-2-^{13}C [94].
- ^{13}C-methacetine-liver function test [95, 96]: cytochromes / fatty liver / cirrhosis rating.
- ^{13}C-n-octanoate liver function test: mitochondrial oxidation [97].
- ^{13}C-aminopyrine liver function test: this is used to assess Non-Alcoholic (NASH) or Alcoholic Steatotic Hepatitis (ASH).
- chronic hepatitis and cirrhosis [98, 99].
- ^{13}C-L-methionine [100, 101] and ^{13}C-isokaproic acid liver function test [102-104]: mitochondrial oxidation.
- ^{13}C-isokaproic acid liver function test [102-104].
- ^{13}C-mixed lipids (cholesteryloctanoate, mixed triglyceride, trioctanoin, triolein, and palmitic acid) for the diagnosis of pancreatic exocrine insufficiency [105-113].
- gastric emptying for liquid food, using ^{13}C-acetic acid sodium salt [114-116].
- gastric emptying for solid food, using ^{13}C-octanoic acid as substrate [117-119].
- lactose ^{13}C-ureide-test to determine oro-coecal transit time [120-124].
- ^{13}C-lactase-deficiency test using naturally ^{13}C-labelled lactose [125].
- ^{13}C-total amylase-test using naturally ^{13}C-labelled cornflakes, to detect chronic pancreatitis [126].
- ^{13}C-phenylalanine and ^{13}C-leucine test for deficiencies in certain enzymes produced in the liver leading to diseases such as PKU (phenylalanine hydroxylase deficiency) or maple syrup disease [127, 128].
- ^{13}C-bicarbonate test for total energy expenditure [129, 130].

With all these tests, $^{13}CO_2$ in exhaled breath is measured at several times following intake of the respective ^{13}C-labelled test substance (e.g., 75 mg of ^{13}C-urea). The results are expressed in promille-deviations delta ^{13}C from the Vienna Pee Dee Belemnite standard [131]. The latter was obtained from a Cretaceous marine fossil, *Belemnitella americana*, from the Pee Dee formation in South Carolina. Its ratio, 0.0112372 of $^{13}C/^{12}C$, is higher than nearly all other natural carbon-based substances; for convenience it has been assigned a delta^{13}C value of zero, giving almost all other naturally-occurring samples negative delta values. Exhaled breath gas typically has a delta^{13}C value of about –26, but can increase to –23 for persons consuming maize-derived food. This value of delta^{13}C is measured for each individual patient and taken as the baseline. After ingestion of ^{13}C-labelled substrates, delta^{13}C may increase some 50 times over baseline, and the (delta^{13}C-baseline) time curve can be used for diagnostic purposes, as well as indirect determination of pharmacokinetics and enzyme activity. However, many tests are not routinely used by physicians, mainly due to lack of approval by the FDA. The most used and only ^{13}C-based FDA-approved test assesses *H. Pylori* infection (the stomach bacterium now unambiguously linked with stomach ulcer formation), by the ingestion of ^{13}C urea followed by $^{13}CO_2$ detection.

Breath tests can be used to assess a person's ability to metabolise drugs. In this sense it can be used to adapt or "personalise" drug doses to the individual genetic heritage of each patient. A powerful example of personalized medicine is the breath test to detect dihydropyrimidine dehydrogenase deficiency in patients and adapt 5-fluoruracil (5FU) therapy for cancer therapy [94]. A small proportion of people have severe adverse effects from 5FU and this can be easily avoided by identifying such subjects by a harmless breath test following uracil-2-^{13}C ingestion.

4. ARTEFACTS MAY ARISE: AMBIENT AIR CONTAMINANTS AND ISOMERS

Most exploratory studies of trace gases in exhaled breath have been carried out using mass spectrometric techniques, most commonly GC-MS, and PTR-MS and SIFT-MS, as discussed above. An old observation by Preti [132] of an apparent increase in the concentration of o-toluidine (o-$C_6H_5CH_3NH_2$) in the exhaled breath of lung cancer patients stimulated a follow-up study in Innsbruck using the PTR-MS technique that showed an increase in concentration of a substance, M, in breath that generate product ions at an m/z value of 108 [133]. Thus, it was deduced that a compound of molecular weight of 107 u was indeed present and following the Preti study it was too quickly assumed to be o-toluidine. Clearly, however, ions at m/z= 108 can be generated in PTR-MS from many different substances, such as hydroxybenzyl, N-methyl-aniline, dimethyl-pyridine, ethyl-pyridine, and the other structural isomers of toluidine. Furthermore, a

study of the breath of Innsbruck hospital personnel showed that the concentrations of the substance or substances generating ions at m/z=108 in the PTR-MS instrument could vary widely during the course of a day, with peak values up to 15 ppb as compared to about 2-3 ppb, which was typical for the breath of cancer patients. A subsequent study of the breath of lung cancer patients did not show any increase of the product ion at m/z= 108. This forcibly demonstrates that the ambient air can have a serious influence on breath analysis and so the ambient air in the breath analysis laboratory must be well controlled and carefully monitored at all times, especially when attempts are being made to identify and quantify breath metabolites at the ppb level. Similarly uncertainties have arisen using PTR-MS in that a product ion at m/z= 63 can either be protonated ethanethiol, C_2H_5SH, or dimethylsulfide, $(CH_3)_2S$, both having the same molecular weight of 62 u, considerable concentrations of which can be observed in hospital or laboratory ambient air. Another example is the appearance of an ion at m/z= 107, tentatively due to xylene: in Fig. (**3**) the respective concentrations are shown for a group of lung cancer patients in comparison with healthy volunteers (hospital personnel / group of healthy volunteers). The coincidence in concentrations between the groups of lung cancer patients as compared with hospital personnel is startling and demonstrates that volatiles in ambient air can seriously distort breath analyses. Such disturbing data are by no means restricted to PTR-MS analyses. The earlier inaccurate quantifications of n-pentane and isoprene using GC-MS has earlier flagged up the difficulties of analyzing trace gases in breath samples [134]. However, with the continuous development of the analytical techniques many of these problems are being recognised and resolved.

5. CONCLUDING REMARKS; FURTHER RESEARCH AND CLINICAL HORIZONS

Breath analysis for clinical diagnosis and therapeutic monitoring is in its infancy. In the foregoing, the clinical relevance of this new diagnostic and the experimental techniques that have been developed and exploited to explore its potential as a non-invasive, real time diagnostic have been outlined. These analytical techniques are now sufficiently reliable to make serious clinical research worthwhile, but the development of smaller devices and improvements in sampling methodology will continue. Several companies are attempting to develop hand-held device with the vision of large volume sales sometimes based on naïve hypotheses (e.g. that elevated acetone is a diagnostic for diabetes only). Before these can become a reality, more careful and extensive clinical research must be carried out to establish and strengthen the link between new and/or elevated breath metabolites and particular disease states.

References have also been given in this paper to those pilot investigations carried out to date that begin to demonstrate the value of breath analysis to physiology and medicine. Particularly exciting is that these investigations reveal how valuable real time, rapid analyses can be in providing immediate results to the clinician, which facilitates diagnosis and treatment. Some planned investigations involving breath analysis are:

- Screening of lung and breast cancer - branched-chain hydrocarbons and aldehydes.
- Detection of heart transplant rejection - branched hydrocarbons.

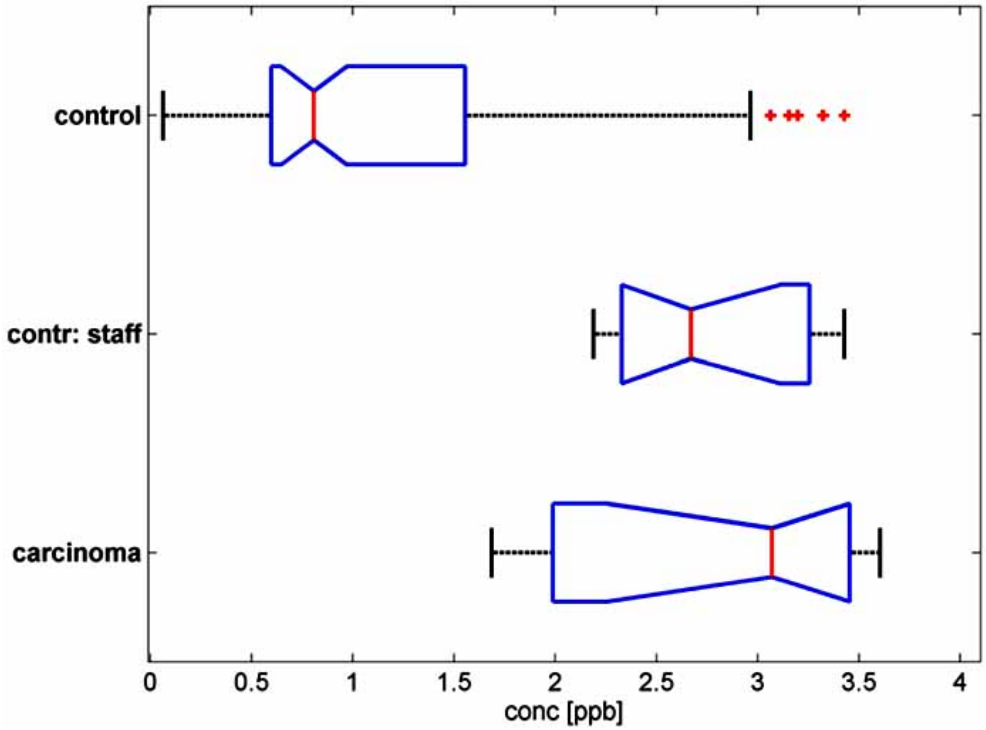

Fig. (3). Comparison of breath gas concentrations derived from product ions at m/z= 107 in PTR-MS for a lung carcinoma group with two control groups (in-hospital staff and healthy volunteers outside hospital). The increased median concentration for the lung carcinoma group seems to be mainly a hospital indoor air effect (unpublished).

- Therapeutic monitoring of asthma - nitric oxide.
- Primary ciliary dyskinesia (PCD, Kartagener's syndrome) - NO in the paranasal sinuses.
- Oro-caecal transit times - ^{13}C-labelled substance ingestion and breath $^{13}CO_2$.
- Carbohydrate malabsorption - breath hydrogen following sugar ingestion.
- Therapeutic monitoring of the critically ill.

These investigations should further indicate the true clinical value of breath analysis and contribute to a clearer understanding of the underlying biochemical processes that produce volatile compounds in breath. Along with these, studies of the trace gases in the breath of healthy individuals will continue, for example following challenges by food and drugs, using the unique opportunity that reliable breath analyses offer to physiology.

ACKNOWLEDGEMENT

A.A. appreciates very much the support by the University Clinics of Innsbruck (TILAK) and its Director Prof. Dr. Herbert Weissenböck. P.S. acknowledges a partial support by GACR project number 202/06/0776. D.S. is pleased to acknowledge the essential collaborations with his clinical colleagues at the University Hospital of North Staffordshire.

REFERENCES

[1] Manolis, A. *Clin. Chem.,* **1983**, *29*, 5.
[2] Davies, S.; Španěl, P.; Smith, D. *Kidney Int.,* **1997**, *52*, 223.
[3] Tassopoulos, C.N.; Barnett, D.; Fraser, T.R. *Lancet,* **1969**, *1*, 1282.
[4] Stone, B.G.; Besse, T.J.; Duane, W.C.; Evans, C.D.; DeMaster, E.G. *Lipids,* **1993**, *28*, 705.
[5] Sajjad, A.; Mottershead, M.; Syn, W.K.; Jones, R.; Smith, S.; Nwokolo, C.U. *Aliment. Pharmacol. Ther.,* **2005**, *22*, 291.
[6] Gustafsson, L. In *Breath Analysis for Clinical Diagnosis and Therapeutic Monitoring*, Amann, A.; Smith, D., Eds.; World Scientific: Singapore, **2005**; pp. 103-119.
[7] Hemmingsson, T.; Linnarsson, D.; Gambert, R. *J. Clin. Monit. Comput.,* **2004**, *18*, 379.
[8] Kharitonov, S.A.; Barnes, P.J. *Am. J. Respir. Crit. Care Med.,* **2001**, *163*, 1693.
[9] Malmstrom, R.E.; Tornberg, D.C.; Settergren, G.; Liska, J.; Angdin, M.; Lundberg, J.O.; Weitzberg, E. *Am. J. Respir. Crit. Care Med.,* **2003**, *168*, 114.
[10] Alving, K.; Weitzberg, E.; Lundberg, J.M. *Eur. Respir. J.,* **1993**, *6*, 1368.
[11] Kharitonov, S.A.; Yates, D.; Robbins, R.A.; Logan-Sinclair, R.; Shinebourne, E.A.; Barnes, P.J. *Lancet,* **1994**, *343*, 133.
[12] Persson, M.G.; Zetterstrom, O.; Agrenius, V.; Ihre, E.; Gustafsson, L.E. *Lancet,* **1994**, *343*, 146.
[13] Maniscalco, M.; Sofia, M.; Weitzberg, E.; De Laurentiis, G.; Stanziola, A.; Rossillo, V.; Lundberg, J.O. *Eur. J. Clin. Invest.,* **2004**, *34*, 555.
[14] Weitzberg, E.; Lundberg, J.O. *Am. J. Respir. Crit. Care Med.,* **2002**, *166*, 144.
[15] Kharitonov, S.; Alving, K.; Barnes, P.J. *Eur. Respir. J.,* **1997**, *10*, 1683.
[16] Abbott, S.; Elder, J.; Španěl, P.; Smith, D. *Int. J. Mass Spectrom.,* **2003**, *228*, 655.
[17] Meulenberg, C.J.; Vijverberg, H.P. *Toxicol. Appl. Pharmacol.,* **2000**, *165*, 206.
[18] Wright, P.A. *J. Exp. Biol.,* **1995**, *198*, 273.
[19] Kharitonov, S.A.; Barnes, P.J. *Biomarkers,* **2002**, *7*, 1.
[20] Španěl, P.; Turner, C.; Wang, T.; Bloor, R.; Smith, D. *Physiol. Meas.,* **2006**, *27*, N7.
[21] Phillips, M.; Herrera, J.; Krishnan, S.; Zain, M.; Greenberg, J.; Cataneo, R.N. *J. Chromatogr. B Biomed. Sci. Appl.,* **1999**, *729*, 75.
[22] Hübschmann, H. *Handbook of GC/MS*. Wiley-VCH: Weinheim, **2001**.
[23] Grob, R.; Barry, E. *Modern Practice of Gas Chromatography*. John Wiley & Sons: Hoboken (NJ), **2004**.
[24] Ouyang, G.; Chen, Y.; Setkova, L.; Pawliszyn, J. *J. Chromatogr. A,* **2005**, *1097*, 9.
[25] Poole, C. *The Essence of Chromatography*. Elsevier: Amsterdam, **2003**.
[26] Sojak, L.; Addova, G.; Kubinec, R.; Kraus, A.; Bohac, A. *J. Chromatogr. A,* **2004**, *1025*, 237.
[27] Kubista, J.; Španěl, P.; Dryahina, K.; Workman, C.; Smith, D. *Rapid Commun. Mass Spectrom.,* **2006**, *20*, 563.
[28] Pawliszyn, J. *Solid Phase Microextraction : Theory and Practice*. Wiley-VCH: New York, **1997**.
[29] Vas, G.; Vekey, K. *J. Mass Spectrom.,* **2004**, *39*, 233.
[30] Brown, J.; Shirey, B. *A Tool for Selecting Adsorbent for Thermal Desorption Applications. T402025*. Supelco: Bellefonte (USA).
[31] Weichbrodt, M.; Vetter, W.; Luckas, B. *J. AOAC Int.,* **2000**, *83*, 1334.
[32] Ochiai, N.; Takino, M.; Daishima, S.; Cardin, D.B. *J. Chromatogr. B Biomed. Sci. Appl.,* **2001**, *762*, 67.
[33] Grote, C.; Pawliszyn, J. *Anal. Chem.,* **1997**, *69*, 587.
[34] Lindinger, W.; Hansel, A.; Jordan, A. *Int. J. Mass Spectrom. Ion Processes,* **1998**, *173*, 191.
[35] Lindinger, W.; Hansel, A.; Jordan, A. *Chem. Soc. Rev.,* **1998**, *27*, 347.
[36] Lindinger, W.; Taucher, J.; Jordan, A.; Hansel, A.; Vogel, W. *Alcohol. Clin. Exp. Res.,* **1997**, *21*, 939.
[37] Mayr, D.; Margesin, R.; Klingsbichel, E.; Hartungen, E.; Jenewein, D.; Schinner, F.; Märk, T. *Appl. Environ. Microbiol.,* **2003**, *69*, 4697.
[38] Kreuzwieser, J.; Graus, M.; Wisthaler, A.; Hansel, A.; Rennenberg, H. *New Phytol.,* **2002**, *156*, 171.
[39] Karl, T.; Hansel, A.; Märk, T.; Lindinger, W.; Hoffmann, D. *Int. J. Mass Spec.,* **2003**, *223-224*, 527.
[40] Rieder, J.; Prazeller, P.; Boehler, M.; Lirk, P.; Lindinger, W.; Amann, A. *Anesth. Analg.,* **2001**, *92*, 389.
[41] Amann, A.; Poupart, G.; Telser, S.; Ledochowski, M.; Schmid, A.; Mechtcheriakov, S. *Int. J. Mass Spectrometry,* **2004**, *239*, 227.
[42] Amann, A.; Telser, S.; Hofer, L.; Schmid, A.; Hinterhuber, H. In *Breath Analysis for Clinical Diagnosis and Therapeutic Monitoring*, Amann, A.; Smith, D., Eds.; World Scientific: Singapore, **2005**; pp. 305-316.
[43] Harrison, A. *Chemical Ionization Mass Spectrometry*. CRC Press: Boca Raton, **1992**.
[44] Wang, T.; Smith, D.; Španěl, P. *Rapid Commun. Mass Spectrom.,* **2002**, *16*, 1860.
[45] Smith, D.; Španěl, P. *Mass Spectrom. Rev.,* **2005**, *24*, 661.
[46] Ikezoe, A.; Matsuoka, S.; Takebe, M.; Viggiano, A. *Gas Phase Ion-Molecule Reaction Rate Constants Through 1986*. Maruzen Company Ltd: Tokyo, **1987**.
[47] Janovsky, U.; Scholl-Bürgi, S.; Karall, D.; Beauchamp, J.; Hansel, A.; Poupart, G.; Schmid, A.; Amann, A. In *Breath Analysis for Clinical Diagnosis and Therapeutic Monitoring*, Amann, A.; Smith, D., Eds.; World Scientific: Singapore, **2005**; pp. 401-407.
[48] Lindinger, C.; Pollien, P.; Ali, S.; Yeretzian, C.; Blank, I.; Mark, T. *Anal. Chem.,* **2005**, *77*, 4117.
[49] Karl, T.; Prazeller, P.; Mayr, D.; Jordan, A.; Rieder, J.; Fall, R.; Lindinger, W. *J. Appl. Physiol.,* **2001**, *91*, 762.
[50] Hansel, A.; Jordan, A.; Warneke, C.; Holzinger, R.; Lindinger, W. *Rapid Commun. Mass Spectrom.,* **1998**, *12*, 871.
[51] Ennis, C.; Reynolds, J.; Keely, B.; Carpenter, L. *Int. J. Mass Spectrom.,* **2005**, *247*, 72.
[52] Smith, D.; Grief, D.; Adams, N. *Int. J. Mass Spectrom. Ion Processes,* **1979**, *30*, 271.
[53] Smith, D.; Španěl, P. *Rapid Commun. Mass Spectrom.,* **1996**, *10*, 1183.
[54] Španěl, P.; Smith, D. *Med. Biol. Eng. Comput.,* **1996**, *34*, 409.
[55] Španěl, P.; Rolfe, P.; Rajan, B.; Smith, D. *Ann. Occup. Hyg.,* **1996**, *40*, 615.
[56] Smith, D.; Španěl, P. In *Breath Analysis for Clinical Diagnosis and Therapeutic Monitoring*, Amann, A.; Smith, D., Eds.; World Scientific: Singapore, **2005**.
[57] Španěl, P.; Smith, D. *Rapid Commun. Mass Spectrom.,* **2000**, *14*, 1898.

[58] Španěl, P.; Smith, D. *Int. J. Mass Spectrom. Ion Processes,* **1997**, *167/168*, 375.
[59] Španěl, P.; Ji, Y.; Smith, D. *Int. J. Mass Spectrom. Ion Processes,* **1997**, *165/166*, 25.
[60] Turner, C.; Španěl, P.; Smith, D. *Physiol. Meas.,* **2006**, *27*, 321.
[61] Turner, C.; Španěl, P.; Smith, D. *Physiol. Meas.,* **2006**, *27*, 13.
[62] Turner, C.; Španěl, P.; Smith, D. *Rapid Commun. Mass Spectrom.,* **2006**, *20*, 61.
[63] Turner, C.; Španěl, P.; Smith, D. *Physiol. Meas.,* **2006**, *27*, 637.
[64] Diskin, A.M.; Španěl, P.; Smith, D. *Physiol. Meas.,* **2003**, *24*, 107.
[65] Smith, D.; Španěl, P.; Davies, S. *J. Appl. Physiol.,* **1999**, *87*, 1584.
[66] Španěl, P.; Wang, T.; Smith, D. *Physiol. Meas.,* **2005**, *26*, 447.
[67] Smith, D.; Wang, T.; Španěl, P.; Bloor, R. *Physiol. Meas.,* **2006**, *27*, 437.
[68] Španěl, P.; Smith, D. *Rapid Commun. Mass Spectrom.,* **2001**, *15*, 867.
[69] Španěl, P.; Smith, D. In *Handbook of Stable Isotope Analytical Techniques (Volume I)*. de Groot, P., Ed.; Elsevier: Amsterdam, **2004**.
[70] Davies, S.; Španěl, P.; Smith, D. *Nephrol. Dial. Transplant.,* **2001**, *16*, 836.
[71] Španěl, P.; Smith, D.; Holland, T.A.; Al Singary, W.; Elder, J.B. *Rapid Commun. Mass Spectrom.,* **1999**, *13*, 1354.
[72] Wehinger, A.; Schmid, A.; Mechtcheriakov, S.; Ledochowski, M.; Gastl, G.; Amann, A. *Lung Cancer Detection by Proton Transfer Reaction Mass-Spectrometric Analysis of Human Breath Gas*, preprint **2006**.
[73] Smith, D.; Wang, T.; Sule-Suso, J.; Španěl, P.; Haj, A.E. *Rapid Commun. Mass Spectrom.,* **2003**, *17*, 845.
[74] Carroll, W.; Lenney, W.; Wang, T.; Španěl, P.; Alcock, A.; Smith, D. *Pediatr. Pulmonol.,* **2005**, *39*, 452.
[75] Good, A.; Durden, D.; Kebarle, P. *J. Chem. Phys.,* **1970**, *52*, 212.
[76] Baumbach, J.; Eiceman, G. *Appl. Spectrosc.,* **1999**, *53*, 338.
[77] Eiceman, G.; Karpas, Z. *Ion Mobility Spectrometry*, 2nd ed. CRC Press: Boca Raton, **2005**.
[78] Baumbach, J.; Vautz, W.; Ruzsanyi, V.; Freitag, L. In *Breath Analysis for Clinical Diagnosis and Therapeutic Monitoring*, Amann, A.; Smith, D., Eds.; World Scientific: Singapore, **2005**; pp. 53-66.
[79] Ruzsanyi, V. *Analyse flüchtiger Metaboliten von der Ausatemluft mittels Ionenmobilitätsspektrometer*. Universität Dortmund, Dortmund, **2005**.
[80] Ruzsanyi, V.; Baumbach, J.I.; Sielemann, S.; Litterst, P.; Westhoff, M.; Freitag, L. *J. Chromatogr. A,* **2005**, *1084*, 145.
[81] Zimmermann, D.; Hartmann, M.; Nolte, J.; Baumbach, J. *Intern. J. for Ion Mobility Spectrometry,* **2005**, *8*, 1.
[82] Dahnke, H.; Kleine, D.; Hering, P.; Mürtz, M. *Appl. Phys. B,* **2001**, *72*, 971.
[83] von Basum, G.; Dahnke, H.; Halmer, D.; Hering, P.; Mürtz, M. *J. Appl. Physiol.,* **2003**, *95*, 2583.
[84] Mürtz, P.; Menzel, L.; Bloch, W.; Hess, A.; Michel, O.; Urban, W. *J. Appl. Physiol.,* **1999**, *86*, 1075.
[85] Wyse, C.; Cathcart, A.; Sutherland, R.; Ward, S.; McMillan, L.; Gibson, G.; Padgett, M.; Skeldon, K. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.,* **2005**, *141*, 239.
[86] Andreoni, K.A.; Kazui, M.; Cameron, D.E.; Nyhan, D.; Sehnert, S.S.; Rohde, C.A.; Bulkley, G.B.; Risby, T.H. *Free Radic. Biol. Med.,* **1999**, *26*, 439.
[87] Skeldon, K.D.; McMillan, L.C.; Wyse, C.A.; Monk, S.D.; Gibson, G.; Patterson, C.; France, T.; Longbottom, C.; Padgett, M.J. *Respir. Med.,* **2006**, *100*, 300.
[88] Cope, K.A.; Solga, S.F.; Hummers, L.K.; Wigley, F.M.; Diehl, A.M.; Risby, T.H. *Biomarkers,* **2006**, *11*, 70.
[89] Halmer, D.; von Basum, G.; Hering, P.; Mürtz, M. *Opt. Lett.,* **2005**, *30*, 2314.
[90] Frech, B.; Mürtz, M.; Palm, P.; Lotze, R.; Urban, W.; Maki, A.G. *J. Mol. Spectrosc.,* **1998**, *190*, 91.
[91] Mürtz, M.; Frech, B.; Palm, P.; Lotze, R.; Urban, W. *Opt. Lett.,* **1998**, *23*, 58.
[92] Modak, A. In *Breath Analysis for Clinical Diagnosis and Therapeutic Monitoring*, Amann, A.; Smith, D., Eds.; World Scientific: Singapore, **2005**; pp. 457-478.
[93] Koletzko, S.; Haisch, M.; Seeboth, I.; Braden, B.; Hengels, K.; Koletzko, B.; Hering, P. *Lancet,* **1995**, *345*, 961.
[94] Mattison, L.; Johnson, M.; Saif, W.; Modak, A.; Hirao, Y.; Koga, T.; Shimizu, T.; Diasio, R. *J. Clin. Oncol.,* **2004**, *22*(14S), 2130.
[95] Pfaffenbach, B.; Gotze, O.; Szymanski, C.; Hagemann, D.; Adamek, R.J. *Dtsch. Med. Wochenschr.,* **1998**, *123*, 1467.
[96] Ciccocioppo, R.; Candelli, M.; Di Francesco, D.; Ciocca, F.; Taglieri, G.; Armuzzi, A.; Gasbarrini, G.; Gasbarrini, A. *Aliment. Pharmacol. Ther.,* **2003**, *17*, 271.
[97] Miele, L.; Grieco, A.; Armuzzi, A.; Candelli, M.; Forgione, A.; Gasbarrini, A.; Gasbarrini, G. *Am. J. Gastroenterol.,* **2003**, *98*, 2335.
[98] Bastien, M.C.; Leblond, F.; Pichette, V.; Villeneuve, J.P. *Can. J. Physiol. Pharmacol.,* **2000**, *78*, 912.
[99] Fasoli, A.; Giannini, E.; Botta, F.; Romagnoli, P.; Risso, D.; Celle, G.; Testa, R. *Hepatogastroenterology,* **2000**, *47*, 234.
[100] Armuzzi, A.; Marcoccia, S.; Zocco, M.A.; De Lorenzo, A.; Grieco, A.; Tondi, P.; Pola, P.; Gasbarrini, G.; Gasbarrini, A. *Scand. J. Gastroenterol.,* **2000**, *35*, 650.
[101] Spahr, L.; Negro, F.; Leandro, G.; Marinescu, O.; Goodman, K.J.; Rubbia-Brandt, L.; Jordan, M.; Hadengue, A. *Med. Sci. Monit.,* **2003**, *9*, CR6.
[102] Mion, F.; Rousseau, M.; Brazier, J.; Minaire, Y. *Metabolism* **1995**, *44*, 699.
[103] Gabe, S.; Williams, R.; Silk, D. *Gastroenterology,* **1997**, *112*, A1268.
[104] Candelli, M.; Armuzzi, A.; Nista, E.; Zocco, M.; Grieco, A.; Miele, L.; Pola, P.; Gasbarrini, G.; Gasbarrini, A. *J. Hepatol.,* **2001**, *34*, 198.
[105] Kato, H.; Nakao, A.; Kishimoto, W.; Nonami, T.; Harada, A.; Hayakawa, T.; Takagi, H. *Am. J. Gastroenterol.,* **1993**, *88*, 64.
[106] Lembcke, B.; Braden, B.; Caspary, W.F. *Gut.,* **1996**, *39*, 668.
[107] Braden, B.; Picard, H.; Caspary, W.F.; Posselt, H.G.; Lembcke, B. *Z. Gastroenterol.,* **1997**, *35*, 123.
[108] Amarri, S.; Harding, M.; Coward, W.A.; Evans, T.J.; Weaver, L.T. *Arch. Dis. Child.,* **1997**, *76*, 349.
[109] Thomas, A.; Super, M.; Sood, M.; Hambleton, G.; Akbar, A.; Weller, P. *J. Pediatr. Gastroenterol. Nutr.,* **1999**, *28*, 594.
[110] Ventrucci, M.; Cipolla, A.; Ubalducci, G.M.; Roda, A.; Roda, E. *Gut,* **1998**, *42*, 81.
[111] Veereman-Wauters, G.; van der Schoor, S.; van Dijk-van Aalst, K.; van't Westeinde, T.; Y, G.; Schiffelers, S.; Van Den Driessche, M. *J. Pediatr. Gastroenterol. Nutr.,* **2001**, *32*, 579.
[112] Loser, C.; Brauer, C.; Aygen, S.; Hennemann, O.; Folsch, U.R. *Scand. J. Gastroenterol.,* **1998**, *33*, 327.
[113] Sun, D.Y.; Jiang, Y.B.; Rong, L.; Jin, S.J.; Xie, W.Z. *Hepatobiliary Pancreat. Dis. Int.,* **2003**, *2*, 449.
[114] Braden, B.; Adams, S.; Duan, L.P.; Orth, K.H.; Maul, F.D.; Lembcke, B.; Hor, G.; Caspary, W.F. *Gastroenterology,* **1995**, *108*, 1048.
[115] Shimamoto, C.; Hirata, I.; Hiraike, Y.; Takeuchi, N.; Nomura, T.; Katsu, K. *Gerontology,* **2002**, *48*, 381.
[116] Pfluger, T.; Antos, D.; Demmelmair, J.; Hahn, K.; Koletzko, S. *J. Nuclear Med.,* **2004**, *44*, 348P.
[117] Lee, J.S.; Camilleri, M.; Zinsmeister, A.R.; Burton, D.D.; Choi, M.G.; Nair, K.S.; Verlinden, M. *Am. J. Gastroenterol.,* **2000**, *95*, 2751.
[118] Folwaczny, C.; Wawarta, R.; Otto, B.; Friedrich, S.; Landgraf, R.; Riepl, R.L. *Exp. Clin. Endocrinol. Diabetes,* **2003**, *111*, 223.
[119] Zahn, A.; Langhans, C.D.; Hoffner, S.; Haberkorn, U.; Rating, D.; Haass, M.; Enck, P.; Stremmel, W.; Ruhl, A. *Z. Gastroenterol.,* **2003**, *41*, 383.
[120] Morrison, D.J.; Zavoshy, R.; Edwards, C.A.; Dodson, B.; Preston, T.; Weaver, L.T. *Biochem. Soc. Trans.,* **1998**, *26*, S184.
[121] Morrison, D.J.; Dodson, B.; Preston, T. *Rapid Commun. Mass Spectrom.,* **1999**, *13*, 1252.
[122] Christian, M.; Morrison, D.; Dodson, B.; Preston, T.; Amarri, S.; Franchini, F.; Edwards, C.; Weaver, L. *J. Pediatr. Gastroenterol. Nutr.,* **2002**, *34*, 570.
[123] Morrison, D.J.; Dodson, B.; Preston, T.; Weaver, L.T. *Eur. J. Clin. Nutr.,* **2003**, *57*, 1017.
[124] Coremans, G.; Geypens, B.; Vos, R.; Tack, J.; Margaritis, V.; Ghoos, Y.; Janssens, J. *Neurogastroenterol. Motil.,* **2004**, *16*, 107.
[125] Zhong, Y.; Huang, C.Y.; Yin, W.Y.; Vonk, R.J. *Wei Sheng Yan Jiu,* **2005**, *34*, 312.
[126] Jonderko, K.; Kasicka-Jonderko, A.; Syrkiewicz-Trepiak, D.; Blonska-Fajfrowska, B. *J. Gastroenterol. Hepatol.,* **2005**, *20*, 1228.

[127] Thompson, G.N.; Bresson, J.L.; Pacy, P.J.; Bonnefont, J.P.; Walter, J.H.; Leonard, J.V.; Saudubray, J.M.; Halliday, D. *Am. J. Physiol.,* **1990**, *258*, E654.

[128] Okano, Y.; Hase, Y.; Kawajiri, M.; Nishi, Y.; Inui, K.; Sakai, N.; Tanaka, Y.; Takatori, K.; Kajiwara, M.; Yamano, T. *Pediatr. Res.,* **2004**, *56*, 714.

[129] Elia, M.; Fuller, N.; Murgatroyd, P. *Proc. Nutr. Soc.,* **1988**, *47*, 247.

[130] Junghans, P.; Derno, M.; Gehre, M.; Hofling; Kowski, P.; Strauch, G.; Jentsch, W.; Voigt, J.; Hennig, U. *Z. Ernahrungswiss.,* **1997**, *36*, 268.

[131] Coplen, T.B.; Brand, W.A.; Gehre, M.; Groning, M.; Meijer, H.A.; Toman, B.; Verkouteren, R.M. *Anal. Chem.,* **2006**, *78*, 2439.

[132] Preti, G.; Labows, J.N.; Kostelc, J.G.; Aldinger, S.; Daniele, R. *J. Chromatogr.,* **1988**, *432*, 1.

[133] Rieder, J.; Lirk, P.; Ebenbichler, C.; Gruber, G.; Prazeller, P.; Lindinger, W.; Amann, A. *Wien. Klin. Wochenschr.,* **2001**, *113*, 181.

[134] Mendis, S.; Sobotka, P.A.; Euler, D.E. *Clin. Chem.,* **1994**, *40*, 1485.

Received: 04 July, 2006 **Revised: 01 August, 2006** **Accepted: 02 August, 2006**