Methodological issues of sample collection and analysis of exhaled breath

Amann A[1,2,*,#], Miekisch W[3,2], Pleil J[4], Risby T[5], Schubert J[3,2]

[1] Univ.-Clinic for Anesthesia, Innsbruck Medical University, Anichstraße 35, A-6020 Innsbruck, Austria

[2] Breath Research Unit of the Austrian Academy of Sciences, Dammstrasse 22, A-6850 Dornbirn

[3] University of Rostock, Department of Anaesthesiology and Intensive Care, Schillingallee 35, 18057 Rostock, Germany

[4] National Exposure Research Laboratory, Office of Research and Development, U.S. Environmental Protection Agency, Research Triangle Park, NC 27711, USA

[5] Department of Environmental Health Sciences, Bloomberg School of Public Health, Johns Hopkins University, 615 N. Wolfe Street, Baltimore, MD 21205, USA

* *Corresponding author:* Anton Amann, Univ.-Clinic for Anesthesia, Anichstr 35, A-6020 Innsbruck, email: anton.amann@i-med.ac.at, anton.amann@oeaw.ac.at.

[#] Anton Amann is representative of Ionimed Analytik GesmbH (Innsbruck)

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Abbreviations used: SPME, solid phase microextraction; GCMS, gas chromatography mass spectrometry; PTR-MS, proton transfer reaction mass spectrometry; SIFT-MS, selected flow tube mass spectrometry; IMS, ion mobility spectrometry; LOD, limit of detection; LOQ, limit of quantification; TOF, time-of-flight; VOCs, volatile organic compounds.

SUMMARY

Recommended standardized procedures have been developed by task forces of the European Respiratory Society and the American Thoracic Society for measurement of exhaled lower respiratory nitric oxide and nasal nitric oxide (NO). These recommendations have paved the way for measurement of nitric oxide to become a diagnostic tool for specific clinical applications. It would be desirable to develop similar guidelines for the sampling of exhaled breath related to other compounds, especially volatile organic compounds (VOCs) reflective of ongoing metabolism. For such systemic VOCs, *CO_2-controlled sampling* is recommended to assure reliable and consistent sample quality for within- and between-subject comparisons. In addition to consistent sampling protocols, the appropriate storage, pre-concentration, and analyses of breath samples require standardized methodology, calibration standards, and laboratory inter-comparisons. There are two basic approaches for analysing VOCs in breath: *real-time analysis* and *off-line* laboratory analysis, each with its particular advantages. Real-time analysis of exhaled breath is most promising for reactive compounds and for compounds that change rapidly as a function of external influence. It is also the best choice when rapid results are of utmost importance in assessing health status for immediate intervention. Real-time direct methods based upon mass-spectrometry, absorption spectrometry using laser sources, or chemical sensors methods generally do not employ pre-concentration of analytes and so may not have sufficient sensitivity for all applications. Furthermore, specificity may suffer as the compounds are not separated prior to entering the detector. Off-line *laboratory analysis* of exhaled breath generally employs some form of pre-concentration of analytes followed by a separation step using high-resolution gas chromatography and mass spectrometer (GC-MS) based detection. GC-MS gives the most detailed and specific results for identifying the VOCs contained in breath, but the processes of sample storage, pre-concentration, injection, and chromatographic separation may limit the detection of reactive or thermally labile metabolites. In either case, identification and quantification of analytes should be performed not only by computer searches of mass spectra, optical spectra, or chemical response, but also by the specific observed behaviour within the instrument in comparison to native calibration standards. This article discusses the state of the art of exhaled breath analyses for clinical/medical applications, presents current concerns about methods implementation for different instruments and techniques,

and provides specific guidance for standardization to introduce non-invasive breath-based technology into clinical practice.

Introduction

The composition of exhaled breath gives valuable information about biochemical processes in the body and offers new possibilities for non-invasive medical diagnostics [1-8]. It is particularly promising because, unlike circulating blood, exhaled breath represents an elimination pathway and can be non-invasively sampled as often as desirable. The time frames available for breath sampling and analysis are essentially limitless. For subsequent off-line laboratory analysis, some techniques rely on the collection of many liters of exhaled whole breath in sampling bags or solid adsorbent cartridges [9-13]; other methods have used single alveolar breaths as collected in small stainless steel canisters [14-17]. Real-time measurements of breath are also possible using direct breathing ports and instrumentation such as proton-transfer-reaction mass spectrometry (PTR-MS), selected ion flow tube mass spectrometry (SIFT-MS), and ion mobility spectrometry (IMS) as well as other analytical techniques including chemical sensors, and various forms of laser spectrometers [18-21]. Such real-time measurements can be performed with high time resolution, e.g., with breath-to-breath resolution [22, 23] or even with within-breath profile resolution [21, 24].

Exhaled breath contains many different molecular species. Among them are small inorganic molecules like nitric oxide (NO) [25-30] or carbon monoxide (CO) [31, 32]. Many organic volatile compounds (VOCs) have been detected in exhaled breath: the highest concentrations of VOCs in breath are observed for acetone [1, 33-36] and isoprene [1, 37, 38]. Many other VOCs are observed at lower concentration levels around a few parts-per-billion or even in the part per trillion range (ppb-ppt) [4-6, 13, 17, 34, 39-43].

Some compounds are related to smoking, such as benzene, acetonitrile , 2-methyl furan, 2,5-dimethyl furan, furan, 1,3-cyclohexadiene, 1,3-cyclopentadiene, 2-methyl-1-butene and 1,4-pentadiene [4, 9, 44-50]. Toluene is detected in the breath of many people and shows increased concentrations in smokers [4]. Also exogenous origin of various molecular species is often observed, e.g., for toluene and benzene [51-59] halogenated compounds [12, 39, 60-62], and constituents of aircraft, diesel, and automotive fuels [11, 12, 63-65]. Indoor air in hospitals also contains many different compounds such as isopropanol, ethanol, isoflurane [66], sevoflurane [67] or p-xylene. Compounds contained in cleaning agents, such as limonene, may also be stored in the human body and subsequently released into breath.

The biochemical background of compounds observed in exhaled breath is rarely known [1, 68]: isoprene is produced as a by-product of cholesterol biosynthesis [69-71] and acetone can be formed from acetoacetate (see also refs [72-75]) or from oxidation of isopropanol. Ethane and pentane are produced by lipid peroxidation [13, 76-79]. For other molecules like methylated hydrocarbons [80-82] the origin remains unknown.

A promising method to get information about the biochemical background of compounds relies on headspace investigations of cell cultures [83-86]. Such investigations could be done using ^{13}C-labelled precursors, observing the subsequent release of volatile compounds showing a higher than usual content in ^{13}C.

Collection of breath samples

For those analytical methods, that are not fast enough to realize breath-to-breath sampling and analysis, additional effort is necessary to provide well defined and

reproducible composition of breath samples. Substances in exhaled breath may be blood borne and originate from the alveoli, others, such as NO and CO, are also generated in the airways and still other substances represent contaminants from ambient air. Hence, a thoroughly controlled sampling is a key requirement for reliable analysis of breath biomarkers.

Specific guidance notes have been developed for measurement of exhaled lower respiratory nitric oxide and nasal nitric oxide (NO) [87, 88]. These specific sampling guidelines paved the way for the use of NO concentrations in clinical applications [89-92]. The basic issue for reliable measurement of NO was that its concentrations in the nasal cavity and the paranasal sinuses are much larger than in the lungs.

For compounds other than NO, guidelines for sampling could be equally useful, but have not yet been generally accepted (or even fully developed).

Several confounding factors may impact onto the composition of breath samples and on concentrations of volatile organic biomarkers in exhaled air and, should therefore, be taken into account:

- Dilution and contamination of the sample
- Sampling of single or multiple breaths
- Direct analysis or sampling for storage
- Physiological parameters such as respiratory rate or cardiac output.

As substance concentrations of organic compounds found in exhaled air fall in the range from μmol/l to fmol/l, analytical results are easily affected by impurities from inspired air or by dilution with dead space air. Therefore, sampling procedures may have significant impact onto results of breath analysis [93].

For the ease of its use whole breath samples ("mixed expiratory air) or a part of exhalation sampled after a certain time ("time controlled sampling") are often used in practice. Sampling without controlled identification of the respiratory phases bears the risks of dilution by dead space gas in the case of mixed expiratory sampling or shows large variations in the case of time controlled sampling (due to wide variations of individual dead space volumes and different breathing manoeuvres). Alveolar (end tidal) samples show highest concentrations of systemic and lowest concentration of exogenous substances [94, 95]. Controlled alveolar sampling e.g. by means of expired CO_2 concentrations therefore is the method of choice if blood borne volatile biomarkers are to be assessed.

Exogenous contamination may as well originate from the oral cavity. Examples for compounds released from the oral cavity are ammonia [96] and sulfur-containing compounds like H_2S, methyl sulfide or mercaptans. Often these compounds are produced by bacteria from different niches of the oral cavity, e.g., from an anaerobic layer at the bottom of the tongue [97-101]. Controlled alveolar sampling cannot solve this problem but may help to limit the effects of such contaminations.

Breath sampling can be performed for a single breath or for multiple breath cycles. Sampling of single breath is easier to perform and may be advantageous in rapidly changing situations. On the other hand, the composition of single breaths may considerably vary from each other due to different modes and depth of breathing. For setups such as "screening for biomarker" studies where absolute concentrations and reproducible breath sample composition play an important role sampling of multiple breaths may be preferable.

Direct analysis or sampling for storage

If real-time analysis of volatile compounds in exhaled air is fast enough to achieve breath-to-breath resolution, sampling can be done in the way that whole breath is analyzed and alveolar (or any other) concentrations can be derived from the data measured during expiration. The time resolution typically necessary for that purpose is less than 60ms. Technical details of this kind of sampling strongly depend on the devices used (e.g. Laser spectroscopy, SIFT-MS, PTR-MS).

Direct sampling is preferably used for direct analysis like with laser spectroscopy, ion mobility or direct MS methods. Typical problems like decompostition of the sample or loss of certain compounds (e.g. through decomposition of labile compounds or water loss in Tedlar bags) may be avoided or reduced if direct sampling is applied. Nevertheless, the general recommendations for sampling procedures (see above) have to be respected for direct breath measurements, too. This is especially true, if the time resolution of the direct analytical method is not high enough (< 60 ms) to actually resolve the breath cycle. In this case, additional control of sampling has to be applied e.g. by separating the alveolar phase from the rest of the breath cycle.

A disadvantage of direct sampling is that detection limits cannot be improved by additional pre-concentration of the breath samples.

Indirect sampling can be applied at the bedside for a single breath or a certain volume/ time without use of hyphenated (analytical) techniques. Storage and transport of samples bear the risk of decomposition/ loss of compounds (see below) if recipients or pre-concentration methods applied are not suited for the selected analytes. On the other hand transport or storage may be inevitable e.g. if measurements are done in the laboratory or if different clinical centers have to use the same instrumentation. Indirect sampling has the advantage that the collected breath samples can be pre-concentrated, e.g. by means of sorbent traps or solid

phase microextraction (SPME). Sensitivity of the analytical assay applied may be improved, if sample volumes are increased. In addition, instruments applied with indirect sampling need not be approved for use at the bedside.

An estimate of sampling quality may be obtained by comparing end tidal PCO_2 during sampling and PCO_2 in the samples. Whenever samples have to be stored prior to analysis one has to be aware of the following

- Breath samples are saturated with water vapor and condensation effects may affect concentrations of polar substances (alcohols, aldehydes). Condensation effects depending on vessel materials (glass, Tedlar, nalophan) should be taken into account.
- Substances having different physicochemical properties (e.g. boiling points, polarity) will be affected by condensation on vessel walls, losses due to septum materials, quenching reactions in completely different ways.
- Standardization and normalization (of sampling procedures) cannot be achieved using one single parameter or internal standard.

If blood borne endogenous compounds are to be assessed, alveolar sampling has to be applied in any case and documentation of all relevant physiological parameters during sampling is mandatory.

Physiological parameters like cardiac output and ventilation flow may have a considerable influence on the concentrations of volatile compounds in exhaled breath. Fig 1 shows a schematic presentation of an experimental setup that is aimed at investigating the concentration patterns of volatile compounds in exhaled breath under different ergometer challenges. A typical challenge would be 75 W. Fig 2 shows the amounts of isoprene, acetone and carbon dioxide (CO_2) excreted per

minute during three periods of challenge, interrupted by rest phases of 12 min and 3 min, respectively [18, 102]. Acetone and CO_2 show similar behavior, whereas isoprene shows a huge peak at the start of the first ergometer challenge, a moderate peak at the start of the second challenge and no peak at all at the start of the third challenge. The influence of cardiac output and ventilation flow in such experiments can be modelled [18]: the higher the cardiac output, the higher the supply of systemic volatile compounds; the higher the ventilation flow the lower the actual concentration of systemic compounds in exhaled breath (by dilution).

Experiments such as shown in Figs 1 and 2 demonstrate clearly the need of a standardized sampling procedure for exhaled breath. Naturally, different standardized sampling procedures could be developed depending on the origin of the compounds of interest (systemic compounds supplied through blood flow or compounds released in the upper airways or released from the oral cavity). Here we focus on the situation where systemic compounds are supplied through blood flow, diffusing into the alveoli and then being excreted through exhaled breath. To sample such systemic compounds, a CO_2-controlled sampling method is most appropriate [93-95].

Different approaches have been chosen to apply CO_2 controlled methods for breath sampling. Manual sampling (of small volumes) can be performed by use of fast CO_2 mainstream monitors and (silanized) glas syringes [Birken 2006]. CO_2 controlled sampling of larger volumes can be performed by CO_2 triggered automated sampling devices.

Such a device may be used for direct adsorption onto sorbent traps [94] or as an active interface for different methods such as direct measurement by sensors or direct MS techniques or as collection device (e.g. in a conditioned sampling loop with variable volume), see Fig 3 [103]. Given its versatile setup the device works for

spontaneous breathing individuals as well as mechanically ventilated patients. Due to software control of the trigger values different portions of breath (e.g. end tidal phase, dead space, whole breath, inspiration), a single breath or a larger number of exhalations or a fixed volume may be sampled. As a further advantage all sampling parameters such as CO_2 content during sampling, sampling flow and sampling time are continuously recorded during sampling. Fig 3 shows a schematic drawing of such an automatic breath gas sampling device consisting of two CO_2 mainstream monitors, a mainstream T-piece, a mass flow controller and a CO_2 triggered rotary vane pump drawing breath into a (heated) sampling loop or a sorbent trap or filling a bag.

In general all sampling procedures applied in a clinical setting should be approved for patient use and all parts of the sampling device that come into direct contact with the patient must be sterilized and conditioned before use. Direct sampling - to certain analytical devices (e.g. sensors, lasers, direct MS) or to sorbent traps - is preferable since storage of breath samples (in different recipients) may cause further problems (see below).

Fig 4 shows a breath sampling device developed at Innsbruck Medical University and at the Breath Research Institute of the Austrian Academy of Sciences, that samples breath into a Tedlar bag in a CO_2-controlled manner. Only during the end-tidal phase of an exhalation (corresponding to high CO_2-concentration) breath is sampled. This is achieved by an electronically opened and closed Teflon valve, based on information from an IRMA CO_2-sensor (Phasein, Sweden). The threshold of CO_2, above which sampling of exhaled breath occurs, can be freely chosen in the menu of the device. This breath sampler is still a prototype and could be refined and improved in various ways. Incidentally, it could also be used to sample dead-space air (i.e., at low CO_2-concentrations before the end-tidal exhalation phase). The device actually also

indicates the exhalation flow (in arbitrary units), and the patient or volunteer is asked to exhale at a constant flow without hyperventilating.

Another much simpler technique is *buffered end-tidal (BET) sampling* [104]. This is based on work by Haldane and Priestley [105] which demonstrated the geometrical separation of different breath phases via exhalation through a rubber tube about 1 inch in diameter and 4 feet long, with the portion closest to the inlet resembling the end-tidal breath fraction. Incidentally, Haldane and Priestley used this technique to show that the partial pressure of CO_2 in exhaled breath remains almost the same in environments with very different partial pressure of oxygen, such as at the Ben Nevis summit (4406 feet above sea-level) or at the bottom of Dolcoath Mine in Cornwall (2240 feet below sea-level). To use buffered end-tidal sampling for analysis of different volatile compounds, direct mass spectrometric methods (such as proton-transfer-reaction mass spectrometry [104]) are recommended due to the possibility of real-time analysis.

If systemic (blood-borne) volatile biomarkers are to be analyzed, controlled alveolar sampling is mandatory. In addition, important physiological parameters such as respiratory rate, heart rate (a surrogate for cardiac output), end tidal CO_2 and (sampling) flow must be monitored and documented.

Storage and stability of breath samples

Apart from the sampling process, appropriate storage of exhaled breath is also an issue. This is particularly true when direct (bedside) or real-time analysis is not possible or if instruments are not approved for use with patients.

Exhaled breath can be stored in different ways [1], typical examples being:

- transparent or black Tedlar bags (consisting of PTFE – polytetrafluoro ethylene)
- Flex Foil bags (PET/NY/AL/CPE – polyethylene terephthalate / nylon / aluminium foil / chlorinated polyethylene)
- Nalophan bags (PET – polyethylene terephthalate)
- Glass vials (e.g. for analysis with solid phase microextraction, SPME, possibly silanized)
- Thermal desorption tubes (containing different adsorbents such as porous polymer resins based on 2,6-diphenylene oxide, carbon molecular sieves or graphitized carbon blacks)
- Micropacked sorbent traps [106, 107]
- Metal canisters (possibly silanized or electropolished) [14-16, 61, 108].

Depending on the specific molecular species, the loss during storage may be very different. Examples of particularly sensitive substances are biogenic amines like dimethylamine or trimethylamine [109] and sulfur-containing compounds [110]. Very good storage behaviour is achieved using silanized metal canisters or thermal desorption tubes. Many compounds can be stored in them during months or even years. Their disadvantage is the comparatively high price.

Direct adsorption onto sorbent traps or micro-sorbent traps is preferable, especially if stability of certain compounds is an issue. There are, in particular, publications that report the collection of breath directly onto thermal desorption tubes containing three different adsorbent beds [11, 66, 111-115]. Since solid phase extraction (SPE) sorbent traps typically require volumes between 100 and 3000 ml this kind of pre-concentration is time consuming and not appropriate for clinical setting at the bedside. New approaches, therefore, chose micro–adsorption techniques allowing stable storage of labile compounds (such as aldehydes) with much smaller volumes (5-50ml) and improved limits of detection [106].

The stabilities of compounds in polymer bags have been investigated by Beauchamp et al. [116] and Mochalski et al. [117]: Water and some polar compounds diffuse rather quickly through Tedlar bag walls, but other compounds are surprisingly stable. Methanol and benzene, for example, have a recovery rate of 99% after 10 h. Isoprene, an important hydrocarbon in exhaled breath, showed a recovery rate of 81% after 10 h. Acetonitrile and 1-hexanal are at the lower end with recovery rates of 67% and 65% after 10 h. Tedlar bags, on the other hand, are not suitable for storage of biogenic amines like dimethylamine and trimethylamine [109].

Background contaminants and inspired air

An important issue in analysis of exhaled breath are:

- the contaminants in indoor air (e.g. isopropanol, ethanol, isoflurane, sevoflurane and p-xylene in hospital indoor air)
- compounds related to smoking (such as acetonitrile and benzene),
- compounds originating from flavorings, fragrances, cosmetics, cleaning agents and dry cleaning
- BTEX-compounds (benzene, toluene, ethyl-benzene, xylene) appearing in gasoline; BTEX compounds are ubiquitous due to the contamination of soil and groundwater with these compounds.

In addition, the storage or analysis method may have an impact on breath samples:

- Tedlar bags may release N,N-dimethylacetamide and phenol,
- septa often release carbon disulfide (CS_2) and sometimes other compounds like 3-methyl-pentane,
- GCMS column bleed releases various compounds, e.g. silicone-containing molecular species,

- plasticizers from tubings and valves may contaminate exhaled breath samples (e.g., 2,2,4-"trimethyl-1,3-pentanediol diisobutyrate" or "pentanoic acid, 2,2,4-trimethyl-3- carboxyisopropyl, isobutyl ester").

Table 1 presents a list of volatile compounds that might be of exogenous origin (cf. ref [5]) and should therefore be treated with great care.

Particular attention should be paid to very lipophilic compounds, which may be inhaled and stored in the fat compartments of the human body [39, 61, 64, 108, 118-123]. As an example undecane $C_{11}H_{24}$ has a Henry constant ~5.5×10^{-4} M/atm [124], which corresponds to ~0.0135 (mol/Lit)/(mol/Lit). The octanol/water partition coefficient of undecane is ~$10^{-6.54}$= 2.9×10^{-7} [125]. Consequently the air/octanol partition coefficient is ~2.1×10^{-5} (mol/Lit)/(mol/Lit). This implies that a concentration of *1 ppb of undecane in breath* (= 3.9×10^{-11} mol/Lit) in equilibrium state corresponds to *~1.8×10^{-6} mol/Lit of undecane in the fat compartment*. Hence compounds that are very lipophilic should always be treated with great care. The appearance of such compounds in exhaled breath might indicate an earlier exposure of the respective person to this compound (or to other compounds that were subsequently metabolized to the compound observed).

In general, inspired substance concentrations (e.g. originating from room air or ventilation systems) must be determined and have to be taken into account. High inspired substance concentrations may also impact on concentrations of endogenous compounds. As inspired substance concentrations increase, the correlations between blood and breath levels will be decreased [126].

Analytical methods for analysis of exhaled breath

Apart from collection of breath, its analysis is also an important methodological issue [127]. Typical measurement techniques for exhaled breath are:

- gas chromatography with different preconcentration and detection methods (GC-MS) [4, 5, 7, 8, 11, 16, 42, 62, 66, 80-82, 93, 111-115, 126, 128-136]
- direct mass spectrometry with different ionisation processes (PTR-MS and SIFT-MS) [5, 18-20, 35, 37, 38, 48, 50, 104, 137-146]
- absorption spectrometry using laser sources [21, 23, 68, 147-150]
- ion mobility spectrometry (IMS) [151-154]
- photoacoustic spectroscopy [155, 156] and
- chemical and semiconductor sensors or sensor arrays [92, 157-161].

PTR-MS and SIFT-MS are direct mass spectrometric methods that do not use pre-concentration or chromatographic separation. These analytical techniques allow real-time measurement [18, 20, 35, 37, 104, 139-141, 144, 145] of a number of compounds. Due to the chemical ionisation process, not all compounds are detectable (or detectable in the required concentration range) e.g. small hydrocarbons cannot be detected due to their low proton affinity. In addition the proper identification of compounds is difficult with these techniques, since multiple molecular species as well as fragment ions may appear at a particular mass-to-charge ratio. In SIFT-MS this problem is addressed by the use of different reactant ions (H_3O^+, O_2^+ and NO^+) exhibiting different ion-molecule reactions. Due to the different precursor ion generation, sensitivity of SIFT-MS is lower than PTR-MS. PTR-MS has recently been developed as a time-of-flight (TOF) instrument [139, 141] that has attractive features:

- the mass resolution is improved from 1 amu (in the PTR-quadrupole instrument) to 1/5000 (in the PTR-TOF),
- the time needed for measurement of compounds at "all" mass-to-charge ratios is reduced from about 5 min (in the PTR-quadrupole instrument) to ~10 sec,

- the sensitivity at higher mass-to-charge ratios (>150) is rapidly decreasing in the PTR-quadrupol instrument whereas it remains good in the PTR-TOF instrument.

In addition, PTR-instruments are available with "switchable reagent ions" (SRI), such as O_2^+ and NO^+ in addition to the typical primary reactant ion H_3O^+. The greatly improved mass resolution of the PTR-TOF now allows separation of isobaric compounds such as methyl-vinyl-ketone and 2-methyl-1-butene on m/z 71.

Gas chromatography with mass spectrometric detection (GCMS) provides inherently much more information than the direct mass spectrometric methods since the data have a temporal resolution due to chromatographic separation. Due to the high capacity of commercially available capillary columns up to some 1000 compounds may be separated prior to detection. In addition, fragmentation of the compounds generates a mass spectrometric fingerprint and therefore – in combination with large spectral libraries like NIST and consideration of retention time – allows unequivocal identification of the detected compounds.

Recent developments such as GCMS-TOF (time-of-flight mass spectrometer), or GC×GC-techniques coupled to fast MS have greatly improved the analytical possibilities of GCMS technology. Instead of a few spectra per second (with a quadrupole MS) now around 500 spectra/sec are possible, and offer the possibility of a reduction in analysis time. A GCMS investigation may give information on hundreds or even thousands of different compounds.

A very important issue in GCMS-measurements is the appropriate *substance identification*. In GCMS spectral library identification of a chromatographic peak (using the NIST spectral library, for example) a *list of different compounds* is suggested to be the (one) compound represented by the peak. The best validation

consists in the preparation of calibration samples for the expected compounds with subsequent comparison of the retention times of the peaks in the breath sample and the calibration sample. If the spectrum *and* the retention time coincide (comparing the unknown sample with the calibration sample), then the identification is sound. This sort of identification check using calibration samples has been, for example, performed in refs. [4, 5] using about 250 pure compounds which were commercially available.

Besides GCMS, PTR-MS and SIFT-MS, there are other very promising techniques available for analysis of exhaled breath samples. Ion mobility spectrometry [151-154], absorption spectroscopy using laser sources [21, 23, 68, 147-150, 162] and photoacoustic spectroscopy [155, 156] are most promising, not only because of their sensitivity for certain compounds but also their great potential for miniaturization.

Since all analytical techniques may be affected by confounding variables, calibration and validation with respect to measurement conditions and general GLP requirements is mandatory. It is important to use calibration samples with varying humidity and CO_2-content, testing

- the linear range of the method,
- limit of detection (LOD) and limit of quantification (LOQ),
- confounding effects through high humidity and CO_2-content (as is the case in breath samples)
- potentially confounding constituents of the breath sample such as high concentrations of drugs (e.g. anaesthetic drugs) disinfectants or environmental contaminations
- reliability and reproducibility of analytical methods applied
- cross-sensitivities (e.g., for sensors or sensor arrays) between different compounds.

Guidelines should include a definition for the way the results of breath analysis are expressed [163]. These guidelines will allow intra-subject and inter-subject breath analyses to be compared and contrasted. Breath analysis could be expressed in terms of concentration units that are dimensionless (i.e., parts-per-million, etc) or in terms of moles per unit volume (pmol/l). Alternately, breath analyses could be normalized to a physiological based parameter such as carbon dioxide production (i.e., pmol/ml of CO_2) or oxygen consumption (i.e., pmol/ml of O_2). Normalization to carbon dioxide or oxygen allows breath analysis data for subjects with widely different body masses to be compared. This latter method of data expression should definitely be used for reporting analysis of breath collected after breath holding [164].

Data handling and statistical methods

Data handling and statistical evaluation is a very important topic in analytical sciences. The issues are:

i. choice of group sizes of patients or volunteers, which are sufficiently large to give statistically valid results,
ii. consideration of different control groups (not only healthy volunteers, but also related diseases and also hospital personnel)
iii. appropriate dealing with inspired concentrations (the difference of expired and inspired concentrations does not always make sense),
iv. use of logarithmic scale for concentrations (of some compound) before applying statistical comparisons between different groups of patients and volunteers,
v. careful choice of statistical tests (e.g., tests not requiring normal distribution of concentrations),
vi. consideration of errors not only in the response variable of a regression analysis, but also in the predictor variables.

The use of hospital personnel as an additional control group (ii) can often help to avoid misinterpretations due to inspired indoor air components. Such misinterpretations could, for example, arise for compounds like isopropanol, ethanol, anesthetic gases, or p-xylene which are common in hospital indoor air.

The use of concentrations differences ($concentration_{expiratory} - concentration_{inspiratory}$) between exhaled breath and indoor air can sometimes be misleading (iii). This is particularly an issue for compounds behaving like carbon dioxide: the concentration of CO_2 in exhaled (whole) air is about 4% and in exhaled alveolar air about 5.3%, but is observed to be independent of the CO_2 concentration in inhaled air (0.03% to 2% in indoor air) [105, 165]. Only the exhaled concentration of CO_2 (and not the difference of concentrations in exhaled breath and indoor air) refers to the physiological state of the human body.

The concentrations of any particular compound in a group of healthy volunteers may be influenced *"in a multiplicative way"* by different influential factors. This implies that the distribution of these concentrations is not Gaussian (as would be the case if different *additive* influential factors would be involved). Therefore it is expected and often observed [38, 166], that concentrations of certain compounds are log-normally distributed (i.e., the logarithms of the concentrations are normally distributed) in a group of healthy volunteers. Before applying statistical tests to the concentrations of two different groups (e.g., patients and volunteers), it is therefore advisable to take the logarithm of the observed concentrations. However, log-normal distribution may not always be the correct assumption when the normal distribution fails. For example, important subgroups may appear creating a bimodal distribution, such as with compounds influenced by smoking behaviour. A histogram (distribution graph) of acetonitrile concentration may exhibit two peaks within the group of all healthy

volunteers corresponding to the subgroups of smokers and non-smokers, respectively [5].

(vi): When performing calibration measurements for a particular compound (using different concentrations of the prepared calibration samples), there does not only appear an error in the response variable (= measurement error), but also an error in the predictor variable (= concentrations of the prepared calibration mixtures). Both types of errors have to be taken into account. This is actually clearly stated in the International Norm ISO 6143:2001(E) "Gas analysis – Comparison methods for determining and checking the composition of calibration gas mixtures". Note that the usual formula for computing the slope in a linear regression depends on the assumption that the errors of the response variable have equal variance. If this assumption is not fulfilled, *weighted* linear regression has to be used [167]. This is again part of the ISO 6143:2001(E). A typical situation where this assumption of equal variance is not fulfilled is the situation where the measurements are based on measured counts (which are Poisson distributed, and where the standard deviation is equal to the square root of counts).

Conclusions

Analysis of volatile organic compounds in human breath bears an enormous potential for new diagnostic and environmental tests and enlargement of basic physiological knowledge. The biochemical background of many of the compounds appearing in exhaled breath still remains unknown. Some of the compounds may result from earlier exposure and storage in the human body. This possibility should, in particular, be taken into consideration for strongly lipophilic compounds. In addition, volatile compounds may be produced by bacteria in the gut, transported to and excreted by

the lungs. As a consequence, increased (or decreased) concentrations of certain compounds in a patient group as compared to a group of healthy volunteers should not be over interpreted. Taking into account a group of hospital personnel or a group of patients with some other disease can be helpful in avoiding misinterpretations. In any case inspired substance concentrations have to be determined and taken into account when data are interpreted in terms of biomarker recognition.

Even though breath analysis has been performed for some decades [34, 168], it is still a young field of research. Many of the modern analytical methods have been developed (or considerably improved in terms of sensitivity and reliability) during the last decade. We are still just observing results, and not so much understanding them. Even though these results are interesting and open up fascinating possibilities, many potential applications (e.g. in cancer research) are far from the stage of clinical usefulness.

In vitro investigation of cell cultures [84-86] or bacterial cultures [169] as well as *in vivo* data from well defined settings in animals [129, 170] or controlled settings in humans (e.g. with ergometer tests [18]) may be helpful in the future to get a better insight into the biochemical background of some of the compounds observed in exhaled breath. Also ^{13}C-labelling of precursors for cell cultures or microorganisms and observation of the changes in headspace of the respective cells may give additional information on the specific metabolic pathways involved and into their kinetics.

Breath analysis is a young field of research with great future potential for clinical application and therapeutic monitoring. The analytical methods used in the field have been developed (or greatly improved) in the last decade.

Exhaled breath is sampled with different techniques: as a consequence results from different laboratories (often also achieved using different analytical devices) cannot easily be compared. There is a need and strong desire for standardized sampling and inter-laboratory comparability. *CO_2-controlled sampling* and a thorough documentation of physiological parameters is recommended for systemic compounds, which are transported to and excreted by the lungs. Since both sampling and sample storage may impact onto results, effects of these procedures have to be carefully taken into account and adapted to the problem under investigation. *Real-time analysis* of exhaled breath without preconcentration can be performed by direct mass-spectrometric methods (PTR-MS and SIFT-MS), as well as sensors and absorption spectrometry using laser sources. Gas chromatographic analysis (GCMS) still gives the most detailed information on the different volatile compounds contained in breath. Identification of compounds should be done in GCMS not only by spectral library match, but also by comparison of retention times based on native calibration standards. Time-of-flight (TOF) mass spectrometry has greatly improved the quality of data obtained by PTR-MS and GCMS. Since all analytical techniques may be affected by various confounding variables, e.g. through sampling and storage procedures, meticulous calibration and validation of all analytical methods applied is mandatory.

Further improvement of analytical techniques and a generally accepted standardization of sampling and analytical processes will help to enhance the potential of breath biomarker analysis.

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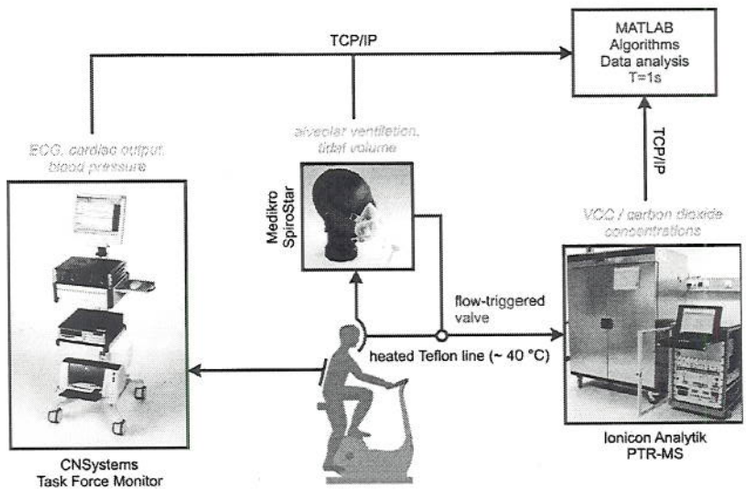

Fig 1: Experimental setting which allows to test the influence of different physiological parameters like cardiac output or lung minute volume. Exhaled breath from a volunteer – sitting on an ergometer – is collected through a mask and analyzed in real-time by proton-transfer-reaction mass spectrometry (PTR-MS). This figure from ref [18] is reproduced with permission of the Institute of Physics (IOP Publishing, Bristol).

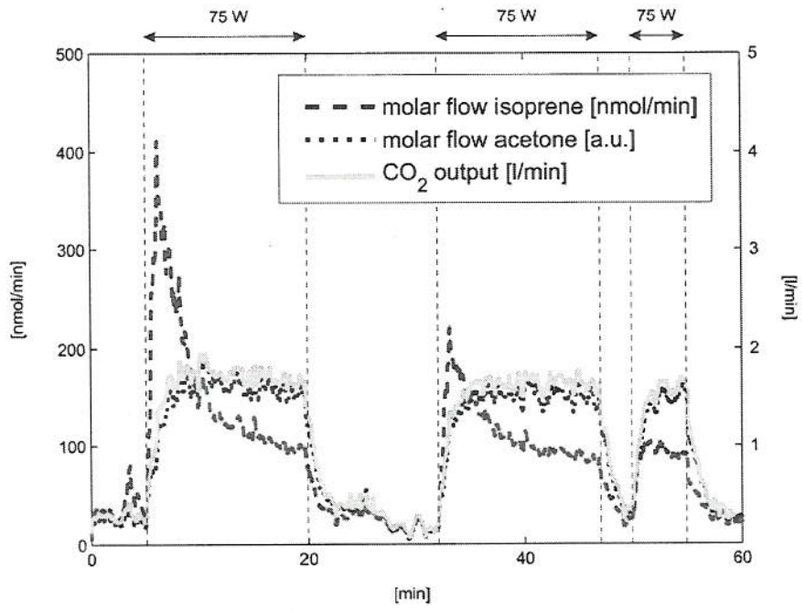

Fig 2: Amounts of isoprene, acetone and carbon dioxide excreted per minute through the lungs for a healthy volunteer pedalling at an ergometer (with a workload of 75 W). Three different phases of pedalling are interrupted by pauses of 12 min and 3 min, respectively. Acetone (shown in arbirary units) parallels the ouput of CO_2, whereas isoprene shows characteristic peaks at the beginning of the first two ergometer challenges. This figure from ref [18] is reproduced with permission of the Institute of Physics (IOP Publishing, Bristol).

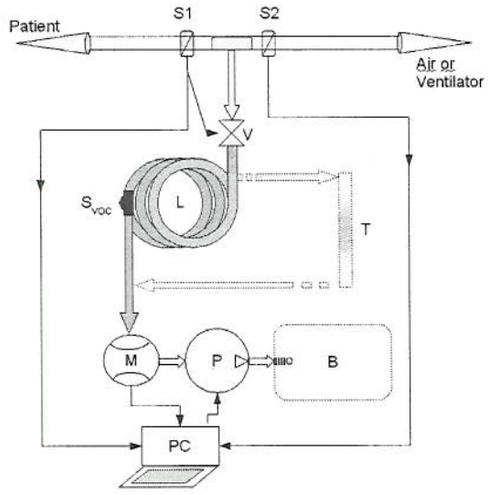

Fig 3: Schematic drawing of the automatic breath gas sampling device [103]. M = mass flow controller; L = sampling loop; PC = computer with A/D-D/A board; T = sorbent trap (optional); P = pump; S1,2= CO_2 sensors; V = Valve; S_{VOC} = Adapter for Syringe or Sensor; B = tedlar bag (optional). Simple arrows describe electrical or electronic pathways. Double contoured arrows described the passage of gas through the device.

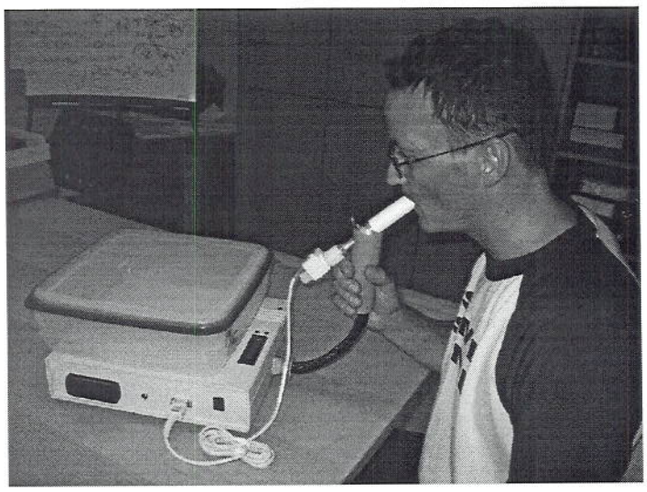

Fig 4: Breath sampling device developed at Innsbruck Medical University and at the Breath Research Institute of the Austrian Academy of Sciences. Exhaled breath is collected in a CO_2-controlled manner into a Tedlar bag. Only during the end-tidal phase of an exhalation (corresponding to high CO_2-concentration) breath is sampled. This is achieved by an electronically opened and closed Teflon valve. The threshold of CO_2, above which sampling of exhaled breath occurs, can be freely chosen in the menu of the device.

Table 1: List of compounds with possible exogenous origin (indoor air, candies, toothpaste, foodstuff and cleaning agents), some being related to smoking behavior. Specific attention is necessary for compounds released by Tedlar bags such as N,N-dimethyl-acetamide and phenol. Carbon disulfide (CS_2) is often released by GCMS-septa. Some compounds need a more detailed investigation, such as the ester methyl acetate. This compound might appear in exhaled breath of healthy volunteers at low concentrations (ca. 1 ppb), and has been demonstrated to increase in concentration with increasing cardiac output (in one volunteer, only, results not shown). Other compounds like -methyl pentane and 3-methyl pentane are potentially interesting for cancer screening, but might be released from certain types of GCMS-septa. The list given here is far from being complete.

compound name	**comment**
1,1-difluoroethane	used as a refrigerant, hence an exogenous origin is possible
1,3-cyclohexadiene	related to smoking behavior
1,3-cyclopentadiene	related to smoking behavior
1,4-pentadiene	related to smoking behavior
1-butene, 2-methyl-	related to smoking behavior
2-propanol	indoor air component in hospital rooms
2-propanol, 1,1,1-trichloro-2-methyl-	exogenous origin ?
acetamide, N,N-dimethyl-	is released by Tedlar bags
acetonitrile	related to smoking behavior
benzene	related to smoking behavior
benzene, ethyl-	potentially interesting compound, but one of the volatile BTEX-compounds (= benzene, toluene, ethyl-benzene, xylene) appearing in gasoline; BTEX compounds are ubiquitous due to the contamination of soil and groundwater with these compounds
carbon disulfide	is released by GCMS septa
cineole	used in flavorings, fragrances, and cosmetics
diethyl ether	suspected to be an indoor air component in hospital rooms

ethanol	could be of exogenous origin
ethylene, tetrachloro-	used in dry cleaning, hence an exogenous origin is possible
formamide, N,N -dimethyl-	suspected to be an indoor air component in hospital rooms
furan	related to smoking behavior
furan, 2,5-dimethyl-	related to smoking behavior
furan, 2-methyl-	related to smoking behavior
isobutane	exogenous origin (propellant)
limonene	exogenous origin (is used in food manufacturing, cosmetics and cleansing agents)
p-cymene	is contained in essential oils (e.g., in cumin and thyme)
m-cymene	misidentification possible (mix-up with natural isomer p-cymene)
menthol mix of isomers	might be contained in candies, toothpaste or foodstuff
methyl acetate	is observed in healthy volunteers in low concentration (ca. 1 ppb), and increases with increased cardiac output
n-hexane	there is an ubiquitous pollution with n-hexane in the environment
p-xylene	indoor air component in hospital rooms
pentane, 2-methyl-	potentially interesting compound, but might be released by GCMS septa
pentane, 3-methyl-	potentially interesting compound, but might be released by GCMS septa
phenol	released by Tedlar bags
silicon-containing compounds (e.g., benzoic acid, 4-methyl-2-trimethylsilyloxy-, trimethylsilyl ester with CAS-number 910614-28-7)	may origin from GCMS column bleeding

styrene	styrene is sometimes added to the BTEX-compounds (see ethyl-benzene above), making it BTEXS
toluene	related to smoking behavior
trichloroethylene	trichloroethylene=TCE, groundwater contamination by TCE is an important environmental concern, hence an exogenous origin is possible

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