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Dynamic profiles of volatile organic compounds in exhaled breath as determined by a coupled PTR-MS/GC-MS study

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Abstract

In this phenomenological study we focus on dynamic measurements of volatile organic compounds (VOCs) in exhaled breath under exercise conditions. An experimental setup efficiently combining breath-by-breath analyses using proton transfer reaction mass spectrometry (PTR-MS) with data reflecting the behaviour of major hemodynamic and respiratory parameters is presented. Furthermore, a methodology for complementing continuous VOC profiles obtained by PTR-MS with simultaneous SPME/GC-MS measurements is outlined. These investigations aim at evaluating the impact of breathing patterns, cardiac output or blood pressure on the observed breath concentration and allow for the detection and identification of several VOCs revealing characteristic rest-to-work transitions in response to variations in ventilation or perfusion. Examples of such compounds include isoprene, methyl acetate, butane, DMS and 2-pentanone. In particular, both isoprene and methyl acetate exhibit a drastic rise in concentration shortly after the onset of exercise, usually by a factor of about 3–5 within approximately 1 min of pedalling. These specific VOCs might also be interpreted as potentially sensitive indicators for fluctuations of blood or respiratory flow and can therefore be viewed as

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candidate compounds for future assessments of hemodynamics, pulmonary function and gas exchange patterns via observed VOC behaviour.

Keywords: exhaled breath analysis, volatile organic compounds (VOCs), exercise, proton transfer reaction mass spectrometry (PTR-MS), gas chromatography mass spectrometry (GC-MS), solid phase micro-extraction (SPME)

(Some figures in this article are in colour only in the electronic version)

1. Introduction

Exhaled breath contains a number of blood-borne volatile organic compounds (VOCs) with great potential for medical diagnosis and therapeutic monitoring (Miekisch *et al* 2004, Amann *et al* 2004, Amann and Smith 2005, Bajtarevic *et al* 2009). These endogenous VOCs may result from normal metabolic activity as well as from pathological disorders. Exhaled breath analysis is non-invasive, and breath may be sampled as often as it is desirable, even under challenging conditions such as during operations or at an intensive care unit (Pabst *et al* 2007). It may also provide information on infections, e.g., of the lungs or the sinuses, by detecting specific volatiles released by bacteria (Preti *et al* 2009). Therefore, breath analysis could be of great clinical value in the future, introducing valuable diagnostic indices that are complementary to those gained by using more invasive methods (Risby 2002, Schubert *et al* 2005, Amann *et al* 2007).

The concentrations of certain compounds in exhaled breath during exercise may change rapidly (Senthilmohan *et al* 2000, Karl *et al* 2001, King *et al* 2009). Real-time investigations in this context have mainly been based on direct mass spectrometric methods allowing breath-to-breath resolution, such as proton transfer reaction mass spectrometry (PTR-MS) (Lindinger *et al* 1998a, Lindinger *et al* 1998b) or selected ion flow tube mass spectrometry (SIFT-MS) (Smith and Spanel 1996, Spanel and Smith 1996). These analytical techniques permit one to detect and quantify very quick changes in breath composition and hence ensure an efficient tracking of possibly short-lived variations in the acquired concentration profiles. Efforts in the context of real-time breath measurements so far have therefore been limited to breath constituents which can be reliably measured by these devices, such as isoprene, acetone or ammonia.

Isoprene (CAS number 78-79-5) certainly holds a distinguished status, since it can be regarded as the prototype of an exhaled breath VOC exhibiting pronounced rest-to-work transitions (Karl *et al* 2001, Turner *et al* 2006, King *et al* 2009). We recently demonstrated that end-tidal isoprene abruptly increases during moderate workload ergometer challenges at 75 W, reaching a peak value within about 1 min of pedalling. This maximum can differ from the end-tidal steady state concentration at rest by a factor of up to 4 (King *et al* 2009). Since endogenous isoprene synthesis has mainly been attributed to pathways with much larger time constants (Stone *et al* 1993), we do not expect that the aforementioned rise in isoprene concentration is due to an increased production rate in the body, but rather due to changes in pulmonary function or changes in hemodynamics.

With this illustrative example in mind, we may thus expect that the sampling of exhaled breath, even under resting conditions, can strongly be influenced by specific physiological

parameters (Cope *et al* 2004). Hence, the impact of breathing rate, breathing volume, cardiac output or blood pressure on the concentration dynamics of specific compounds in exhaled breath generally merits further investigation. A paradigmatic example within this framework is the small inorganic molecule nitric oxide (NO) (Kharitonov *et al* 1997, Dweik *et al* 1998, Bush 2000, Gustafsson 2005, Horvath *et al* 2003). NO arises in almost every human organ, has a short half-life and may change its concentration quickly. It is also produced in the lungs, the nasal cavity and the sinuses. In the latter it acts by its bactericidal effect (Bush 2000). The concentration in the nasal cavity and the paranasal sinuses is usually much higher than in the lungs, and actually increases in concentration within a few seconds during humming (Lundberg *et al* 2004, Maniscalco *et al* 2004). The use of NO for the therapeutic monitoring of asthma relies on the amount released in the airways. Hence, appropriate measurements of airway released NO are important for its clinical use and therefore careful sampling of breath under controlled conditions is necessary. This led to joint guidelines of the American Thoracic Society (ATS) and the European Respiratory Society (ERS) for the protocol to be used for NO measurements in exhaled breath (ATS 1999, ATS/ERS 2005). We expect that a careful choice of conditions and sampling protocol will be important not only for NO, but also for various other volatile molecular species.

Within this context, the primary motivation for the present work was to actively scan exhaled breath for trace gases showing pronounced changes in response to variations in ventilation and perfusion. While on the one hand such compounds would evidently require special attention regarding their sampling procedure, they can also be thought of as sensitive indicators for fluctuations in respiratory/hemodynamic flow. In the same spirit, they might therefore serve as candidate compounds for assessing pulmonary gas exchange patterns via observed VOC profiles. Consequently, a secondary aim was also to supplement and support continuing efforts to base multiple inert gas elimination technique (MIGET) (Wagner 2008, Wagner *et al* 1974)) on endogenous VOCs rather than exogenously administered gases, thereby circumventing invasive infusion and improving patient compliance (Anderson and Hlastala 2010). Furthermore, our measurements are intended to place previous measurements of breath isoprene and acetone in a broader context by comparing their dynamic behaviour during distinct physiological states with synchronized profiles of VOCs expected to show similar exhalation kinetics.

2. Methods

2.1. Sampling procedures

Recently, an experimental setup efficiently combining PTR-MS measurements with continuous data streams reflecting a series of hemodynamic and respiratory factors was developed in our group (King *et al* 2009). This setup serves to assess the behaviour of exhaled breath components in conjunction with decisive physiological driving forces during rest and ergometer-induced workload schemes in *real time*. While its main purpose is to monitor specific, predefined molecular species, the identification problem stated above can only be tackled with alternative analytical techniques, giving detailed information on the composition of the exhaled breath sample. Gas chromatography mass spectrometry (GC-MS) coupled with solid phase micro-extraction (SPME) as a pre-concentration method can be regarded as gold standard within this framework (Amorim and de 2007, Bajtarevic *et al* 2009, Ligor *et al* 2008, 2009, Pawliszyn 1997, Schubert *et al* 2003, 2005, Miekisch *et al* 2008). SPME/GC-MS represents a good trade-off between high resolution of individual breath components with low detection limits and rapid sampling. The main advantages of SPME are its ease of

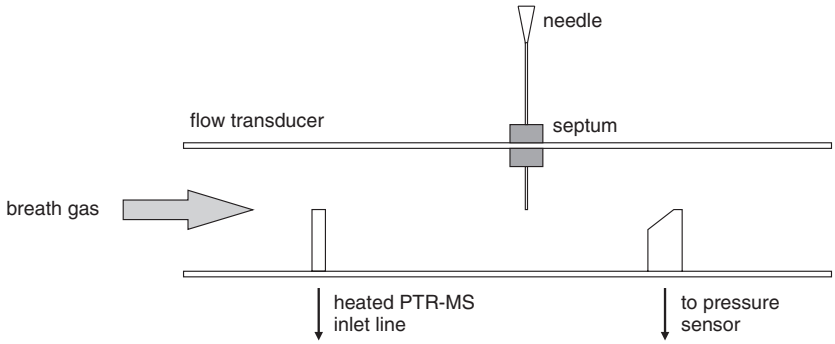

Figure 1. Sketch of the flow transducer mouthpiece from which end-tidal samples are manually extracted for subsequent SPME/GC-MS analysis.

operation and the small amounts of sample gas—usually between 10 and 20 ml—required to perform extraction. An additional benefit from the usage of SPME/GC-MS techniques is the possibility of detecting and quantifying compounds that cannot be measured by the PTR-MS (e.g. alkanes). A suitable choice of specimen storage vessels guarantees high recoveries of the target compounds and ensures reliable GC-MS analyses of breath samples taken within a relatively short period of time. In the following, a methodology for complementing continuous VOC profiles obtained by PTR-MS with simultaneous SPME/GC-MS measurements under workload conditions is developed.

A detailed description of the entire experimental setup used for acquiring hemodynamic and respiratory data in conjunction with PTR-MS variables is given elsewhere (King *et al* 2009). Here we will only discuss the parts that are relevant for the analysis of exhaled breath samples by GC-MS. The test subject freely inhales/exhales through a flow transducer mouthpiece, which is connected to a silicone head mask covering mouth and nose, see figure 1.

From the mouthpiece, gas samples are directed to the PTR-MS via a heated Teflon sampling line. Moreover, respiratory flow is obtained by means of a differential pressure sensor as explained in King *et al* (2009). Breath samples for the GC-MS analyses were taken using a 20 ml gas-tight glass syringe (Roth, Germany) equipped with a replaceable needle. For sampling purposes an additional rubber septum (Supelco, Canada) was installed in the wall of the flow transducer mouthpiece approximately 3 cm from the lips. Sampling was achieved manually by piercing the septum and drawing a volume of 18 ml during one single end-tidal exhalation segment. Care was taken to ensure that the needle tip is located at the centre of the axial mainstream. In order to avoid possible losses of hydrophilic compounds due to condensation, needle and syringe were preheated to about 60 °C shortly before the sampling procedure.

Filling of the syringe was timed according to the automatic real-time procedure for the selective sampling from specific exhalation phases as described in King *et al* (2009). Adequate algorithmic processing of measured respiratory flow allows for a reliable breath-by-breath detection of each end-tidal segment, with its start and end being marked by an acoustic signal. In order to prevent gas samples from being diluted or contaminated with (fresh) room air at the onset of the next inhalation, the entire sampling process including pressure equilibration within the syringe has to be completed within this time window. Test subjects were therefore asked to slightly prolong their exhalation to about 4 s in the respective breath cycle. Such a

procedure extended the length of the end-tidal phase and turned out to be satisfactory for all test subjects. Some bias might be introduced by this protocol due to the fact that VOC breath concentrations have been demonstrated to increase with the duration of the end-tidal phase (Anderson *et al* 2003, O'Hara *et al* 2008). However, after an examination of the PTR/GC-MS overlays for acetone and isoprene associated with preliminary single expirograms, we decided that the corresponding error will only have a minor impact on quantification. In particular, since the extraction of PTR-MS gas samples is triggered by the same detection mechanism as described above, applying the aforementioned sampling protocol ensures that both probes are drawn from the same portion of exhaled breath.

Immediately after sampling the syringe content was injected into an evacuated SPME vial (20 ml in volume, Gerstel, Germany) sealed with a 1.3 mm butyl/PTFE septum (Macherey-Nagel, Germany). Since the SPME vials also served as storage containers, the type of septa was carefully selected with respect to the background and recovery of the compounds of interest. The applied material guaranteed recoveries better than 90% within the first 12 h of storage. Finally, pressure in the vial was balanced with high-purity nitrogen (of quality 6.0, i.e. with a purity of 99.9999%).

2.2. GC-MS analysis

The gas chromatographic analyses were performed using an Agilent Technologies (USA) type 7890 gas chromatograph equipped with a mass selective detector (MSD) (type 5975C, Agilent, USA). SPME was performed automatically (auto sampler MPS2, Gerstel, Germany) by inserting the SPME fibre coated with 75 μm CAR-PDMS (Supelco, Canada) into the vials and exposing the fibre to the sample for 10 min. The sample temperature during the extraction was kept at 40 °C to avoid the condensation of water vapour. Subsequently, the fibre was immediately introduced into the injector of the gas chromatograph, with thermal desorption at 290 °C in a splitless mode (1 min). The fibre was conditioned at 290 °C for 15 min prior to each analysis.

Analytes under study were separated using a PoraBond Q column (25 m $\times$ 0.32 mm, film thickness 5 μm, Varian USA) working in a constant flow mode (1.7 ml min^{-1}). The column temperature program was chosen as follows: 90 °C for 7 min, increase to 140 °C at a rate of 10 °C min^{-1}, constant temperature of 140 °C for 7 min, increase to 260 °C at a rate of 15 °C min^{-1} and 260 °C for 6 min. The mass spectrometer worked in a combined SCAN/SIM mode. The SCAN, with an associated range set from m/z 35 to m/z 200, was used for the identification of potential target compounds as well as for the quantification of isoprene and acetone. Additionally, major study compounds as discussed below were quantified using SIM (selective ion monitoring mode), with the corresponding m/z ratios and dwell times being presented in table 1.

Calibration graphs and standard retention times were created on the basis of analyses of calibration mixtures prepared from pure compounds. Isoprene (99.5%), methyl acetate (99.5%) and butane (15 ppm C1–C6 hydrocarbon standard) were obtained from Sigma-Aldrich (USA), dimethyl sulfide (99%) from Fluka (USA), 2-pentanone (97%) from Acros Organics (Belgium) and acetone (99.5%) was purchased from Merck (Germany). A primary gas standard was prepared in a 1 litre glass bulb (Supelco, Canada) by injecting 0.5–2 μl (depending on the target concentration) of pure compound into the evacuated bulb. Next, the bulb was heated to 80 °C for 15 min in order to ensure evaporation and subsequently balanced with high-purity nitrogen (6.0–99.9999%). The primary standard was used to prepare six calibration mixtures with concentrations ranging from 20 to 1000 ppb in the case of acetone and isoprene, and 0.1 to 20 ppb for the other species. This was accomplished by transferring

Table 1. Major study compounds together with relevant methodological data. Retention times used for confirming substance identifications based on spectral data are obtained by means of calibration mixtures. Dwell times refer to the SIM mode.

Compound	CAS number	Retention time (min)	m/z (SIM)	Dwell time (ms)	RSD (%)	LOD (ppb)
Isoprene	78-79-5	13.39	–	–	2.1	1.4
Acetone	67-64-1	10.89	–	–	2.6	2.5
DMS	75-18-3	11.54	47, 61, 62	60	5.5	0.05
Methyl acetate	79-20-9	12.28	43, 74	60	4.8	0.04
Butane	106-97-8	8.68	43	180	5.8	0.2
2-pentanone	107-87-9	22.72	86	60	3.8	0.04

0.05–1 ml of primary standard into 3 litre Tedlar bags (SKC Inc., USA) filled in advance with 1500 ml of nitrogen. Final humid calibration mixtures were created in the SPME vials. For this purpose, vials were evacuated with a membrane pump (Vacuubrand, Germany) and heated to 45 °C for 2 min. Next, an amount of 0.8 μl of distilled water—corresponding to the maximal water content in 18 ml of breath (100% relative humidity at 37 °C)—was injected. After 1 min (the time necessary for complete water evaporation), an appropriate volume of dry mixtures was introduced into the vials. During the whole process the vial temperature was maintained at 45 °C to avoid condensation.

Validation parameters were estimated using the calibration graphs. Limits of detection, defined as a signal-to-noise ratio of 3:1, are presented in table 1. The system response was found to be linear with correlation coefficients ranging from 0.996 to 0.999. The relative standard deviations (RSDs) were calculated based on consecutive analyses of five separate breath samples taken within 1 min from a single volunteer who had been resting for 15 min. Such a procedure was necessary to include the influence of manual sampling on the RSD values. The estimated RSDs are summarized in table 1.

2.3. Test subjects and protocols

A cohort of seven healthy normal volunteers (four males, 26–28 years; three females 21–28 years; two smokers) were recruited to participate in a single moderate exercise ergometer challenge consisting of an initial resting phase of 5–10 min, followed by a constant workload segment of 75 W for 15 min. The regime ends with a further resting phase of 5 min. No test subject reported any prescribed medication or drug intake. The study was approved by the Ethics Commission of Innsbruck Medical University.

The test subjects were all measured in the morning with an empty stomach. The only exception was drinking of water. Smokers were asked to refrain from smoking on the day of measurement. Volunteers were required to rest at least 15 min prior to analysis. Within this time, they were given general information regarding the experimental protocol and received some training in order to reliably provide a triggered exhalation as discussed in the previous sections. Additional instrumentation and protocols closely followed the general procedure reported in King *et al* (2009).

Regarding real-time VOC analysis, we focused on two major exhaled breath constituents: acetone and isoprene, which can be measured by PTR-MS in their protonated forms at mass-to-charge ratios 59 and 69, respectively (Arendacká *et al* 2008, Schwarz *et al* 2009b). For a series of single experiments we also included m/z 63 (tentatively dimethyl sulfide (DMS))

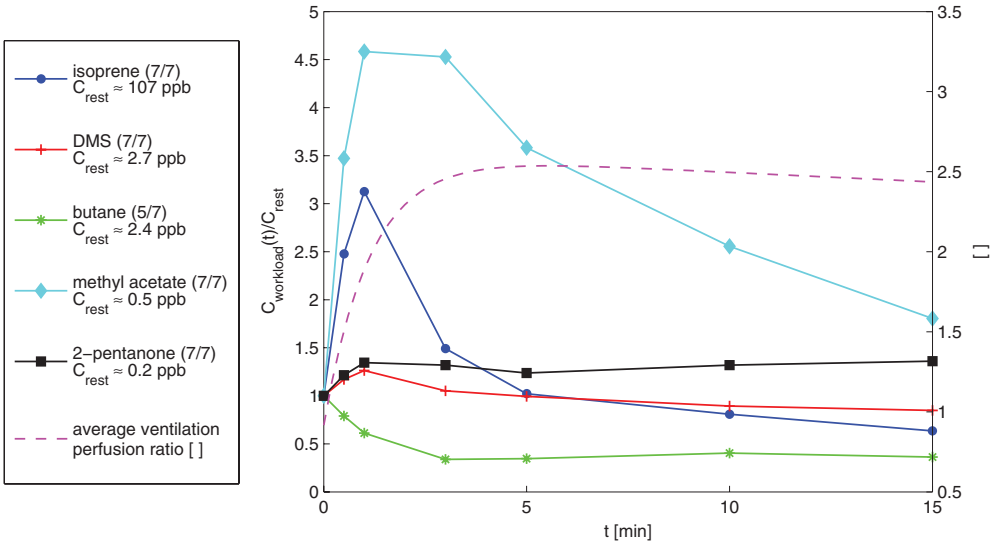

Figure 2. Average relative changes of several VOC concentrations (determined by SPME/GC-MS) during constant load exercise of 75 W as compared to their resting levels ($t = 0$). The graphs presented here correspond to the median values among all seven volunteers investigated. In particular, the concentration profiles were all normalized to the initial resting values C_{rest} prior to averaging.

as well as m/z 75 (tentatively methyl acetate). For purposes of normalization and quality control we additionally monitor m/z 21 (isotopologue of the primary hydronium ions) and m/z 37 (first monohydrate cluster). Further details on subsequent quantification are given in Schwarz *et al* (2009a). Based on our knowledge of isoprene and acetone behaviour, representative time instants for drawing the GC-MS samples were defined as follows: one sample was extracted under resting conditions as soon as the test subject had accustomed to the experimental situation and cardiac output as well as alveolar ventilation had stabilized sufficiently, i.e. about 2 min after the start of our protocol. Subsequent samples were drawn after 0.5, 1, 3, 5, 10 and 15 min had passed since the onset of exercise. Additionally, for the purpose of background correction, a room air sample was taken just before the experiment.

3. Results and discussion

On the basis of the underlying SPME-GCMS analysis method discussed before, five compounds were found to substantially increase/decrease in response to the workload sequence: isoprene, butane, methyl acetate, DMS and 2-pentanone. Quantitative effects for these compounds are summarized in figure 2 as well as in table 2.

For comparative reasons, only relative changes are presented in figure 2, i.e. all individual profiles have been normalized to the respective initial steady state value. The measured ventilation–perfusion ratio reflects the applied workload sequence starting at time zero.

Major hemodynamic and respiratory variables generally exhibit a very consistent behaviour among all test subjects (King *et al* 2009). At the onset of exercise, cardiac output rapidly increases from a mean resting value of approximately 5 l min^{-1} at rest to a constant plateau of about 12 l min^{-1} during constant workload of 75 W. Simultaneously, alveolar

Table 2. GC-MS quantification results for the seven volunteers investigated. Time instants correspond to exercise duration in minutes (with time zero referring to resting levels in end-tidal breath).

Volunteer number/time	0	0.5	1	3	5	10	15
				Butane (ppb)			
1	n.d.						
2	5.94	3.93	3.61	3.43	3.45	3.42	3.7
3	n.d.						
4	6.47	8.57	7.83	2.45	1.68	2.02	1.74
5	0.6	0.45	0.4	0.13	0.23	0.29	0.36
6	1.24	0.98	0.76	0.42	0.43	0.5	0.45
7	2.43	2.73	1.43	0.52	0.6	0.79	0.71
				Dimethyl sulfide (ppb)			
1	2.69	3.55	3.48	2.83	2.75	2.37	2.18
2	0.79	0.69	0.82	0.81	0.71	0.76	0.77
3	1.52	1.78	1.92	1.73	1.74	1.54	1.46
4	3.56	5.44	6.05	4.73	3.54	3.18	3.02
5	1.38	1.52	1.5	1.25	1.2	1.18	1.13
6	2.79	4.59	4.52	3.78	3.26	2.77	2.69
7	3.28	3.38	2.93	2.54	2.26	1.94	2.08
				Isoprene (ppb)			
1	163	532	669	324	240	148	124
2	82	122	129	98	79	59	52
3	138	260	342	200	176	130	81
4	107	317	441	159	88	79	67
5	78	192	243	119	79	63	50
6	58	209	212	104	71	47	42
7	154	359	467	229	148	102	79
				Methyl acetate (ppb)			
1	1.66	8.02	9.27	7.7	6.59	4.57	3.31
2	0.53	1.13	1.61	1.31	1.28	1.09	0.9
3	3.23	6.5	8.1	6.99	6.88	5.56	3.86
4	0.22	0.76	1.03	1.02	0.82	0.93	0.96
5	0.04	0.48	0.62	0.59	0.49	0.44	0.37
6	0.81	3.47	3.54	2.86	2.3	1.44	1.05
7	0.36	1.25	1.65	1.63	1.29	0.92	0.65
				2-pentanone (ppb)			
1	0.26	0.33	0.35	0.32	0.31	0.3	0.27
2	0.19	0.18	0.21	0.18	0.19	0.18	0.19
3	0.23	0.2	0.21	0.22	0.23	0.23	0.22
4	0.21	0.25	0.28	0.3	0.26	0.29	0.31
5	0.11	0.15	0.16	0.16	0.15	0.15	0.15
6	0.25	0.34	0.34	0.33	0.31	0.33	0.34
7	0.28	0.34	0.39	0.42	0.41	0.42	0.44

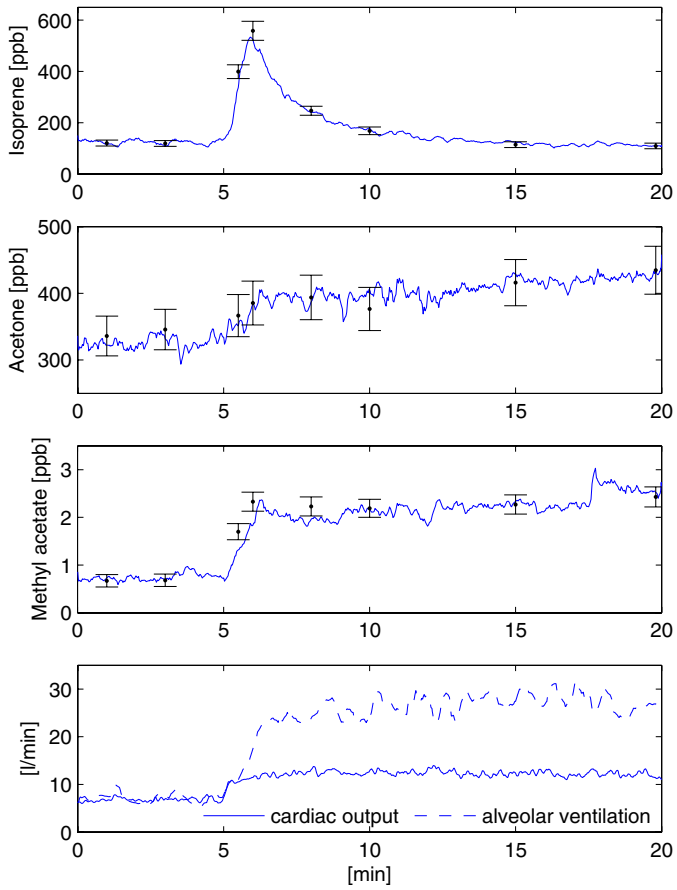

Figure 3. Simultaneous extraction of end-tidal VOC profiles by PTR-MS (continuous signal) and discrete SPME/GC-MS measurements. Error bars for the latter result from taking two times the associated RSD as given in table 1. Data refer to one single volunteer during a constant workload protocol of 75 W after an initial resting phase of 5 min.

ventilation shows a monotonic rest-to-work transition from 5–10 l min^{-1} to a steady state level of approximately 25–30 l min^{-1}, thereby increasing the average ventilation–perfusion ratio by a factor of ∼2.5, see figure 2.

Representative PTR-MS/GC-MS results for one single study subject are given in figure 3, displaying continuous end-tidal concentration profiles of isoprene, acetone and methyl acetate as determined by PTR-MS in comparison with discrete measurements obtained from our GC-MS analysis. Error bars result from taking two times the associated RSD value as given in table 1. In particular, the good agreement between both methods for isoprene and acetone can be seen as a cross-validation of phenomenological results related to both compounds which have been published previously (Karl *et al* 2001, King *et al* 2009).

For all test subjects, individual isoprene levels in breath obtained prior to the workload sequence varied within the range of 58–163 ppb, the median concentration being 107 ppb (cf, also Kushch *et al* (2008)). In accordance with earlier findings, end-tidal isoprene concentration abruptly increases by a factor of ∼3–4 within the first minute of the applied workload scenario, followed by a gradual decline back to initial (resting) levels within approximately 15 min of

Table 3. Functional similarities between isoprene and butane with respect to alveolar gas exchange.

	MW	Blood:gas partition coefficient $\lambda_{b:a}$	Octanol:water partition coefficient log (K_{ow})
Isoprene	68	0.75 (Filser *et al* 1996, Karl *et al* 2001)	2.42 (Howard and Meylan 1997)
Butane	58	0.41* (Liu *et al* 1994)	2.89 (Sangster 1997)

*refers to rat blood.

exercise. Excellent agreement between isoprene concentrations acquired by PTR-MS and GC-MS throughout all measurements reconfirms the extraction quality of our manually obtained samples. On the basis of this observation it is deduced that the quantities of additional compounds in these samples are indeed representative for the corresponding end-tidal levels. In this sense, isoprene acts as a practicable control value that can potentially be used for detecting possible error sources and losses in the manual sampling regime.

The marked rise of isoprene at the onset of exercise has mainly been attributed to its low affinity for blood (dimensionless Ostwald blood:gas partition coefficient at body temperature = 0.75 (Filser *et al* 1996)). The classical alveolar gas exchange theory due to Farhi predicts a significant influence of ventilation and perfusion on the observed exhaled breath concentration for compounds with small solubility (Farhi 1967, Farhi and Yokoyama 1967), see also King *et al* (2009) for a derivation. In view of the previously described isoprene exhalation kinetics, natural candidates for preliminary studies of VOC behaviour under workload conditions hence are substances with similar physico-chemical behaviour, e.g. the family of hydrocarbons. Within the ensemble of volunteers investigated, our main focus was on butane, as the respective alveolar gradients (i.e. the difference between end-tidal levels and background values) were generally high enough to allow for a reliable quantification. Butane appeared in the breath of five volunteers with resting levels ranging from 0.6 to 6.5 ppb (median: 2.4 ppb). Blood-borne butane is considered to originate from protein oxidation and/or bacteria production in the colon (Kharitonov and Barnes 2002, Miekisch *et al* 2004) and is particularly interesting due its functional comparability with isoprene. Indeed, the major factors anticipated to affect pulmonary gas exchange show substantial similarity, see table 3 as well as Meulenberg and Vijverberg (2000).

According to these values, by Graham's law, diffusivity (governing the passage through the tissue interfaces separating the respiratory microvasculature from the alveolar space) is expected to be roughly similar for both compounds (West 2005). Moreover, the presented affinities for blood indicate that supply and removal via the pulmonary circulation will be of comparable order.

Despite this agreement, breath concentrations of butane and isoprene exhibit an entirely different qualitative response among the ensemble of volunteers investigated: the behaviour of butane at the onset of exercise resembles the trend as predicted by the classical Farhi equation (i.e. a decrease with higher ventilation–perfusion ratios, see figure 2), whereas the behaviour of isoprene does not. Consequently, from the viewpoint of endogenous MIGET, isoprene (in contrast to butane) appears to be of limited suitability as a potential test gas. The above-mentioned visual discrepancy might be assessed in a more formal manner by employing a Wilcoxon signed rank test (Wilcoxon 1945) at each discrete time instant greater than zero. It tests the null hypothesis that the differences between the respective normalized butane and isoprene levels are drawn from a continuous, symmetric distribution with zero median. Using a 10% confidence level, this null hypothesis might be rejected at all time instants greater than zero with a maximum p-value of 0.0625.

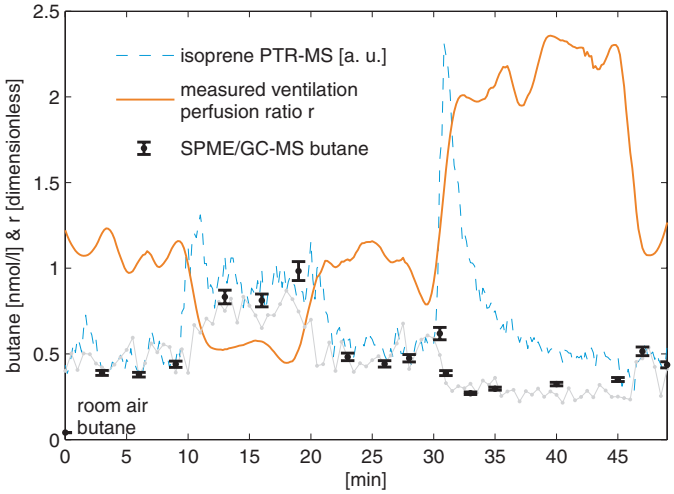

Figure 4. SPME/GC-MS profile of butane ($\lambda_{b:a} = 0.41$; 1 nmol l^{-1} approximately equals 25 ppb under ambient conditions) compared with the synchronized and scaled PTR-MS response for isoprene ($\lambda_{b:a} = 0.75$). The experimental protocol is as follows: 0–10 min rest; 10–20 min supine position; 20–30 min rest; 30–45 min exercise at 75 W; 45–49 min rest. The dotted light grey line represents an eye guide for the expected alveolar concentration of butane as predicted by the Farhi equation (postulating a constant mixed venous blood concentration of 0.7 nmol l^{-1}).

In a series of separate experiments we exclusively focused on the simultaneous dynamics of breath isoprene and butane in response to changes in ventilation and perfusion. Typical results referring to one single test subject are given in figure 4. Here, in addition to the exercise protocol discussed before, the ventilation–perfusion ratio is altered by a sudden change in body posture from semi-supine to supine position (corresponding to the time interval between 10 and 20 min).

As has already been shown in King *et al* (2009), such a manoeuvre will result in only minor changes of alveolar ventilation, while the associated rise in cardiac output (mainly due to an increase in stroke volume) can be utilized for assessing the individual contribution of pulmonary blood flow on the alveolar gas exchange process. Both isoprene and butane concentrations in end-tidal breath approach an apparently stable steady state in supine position that is about a factor of 1.5 higher compared to the resting level. This is the qualitative behaviour expected from the Farhi equation. Particularly, a markedly peak shaped response of isoprene as in the case of ergometer scenarios could not be observed.

Combining the above-mentioned findings it might thus be inferred that some isoprene-specific (release) mechanism has to be taken into account for capturing the exhalation kinetics of this important compound at the onset of physical exercise. The isoprene sources and exact stimuli for such a workload-induced process, however, remain an object of speculation. Potential candidates for instance include the working muscle compartment, which receives disproportionately high fractions of cardiac output during an ergometer challenge and moreover undergoes a rapid change in metabolic activity from which isoprene might be derived. Another alternative, which might be compared to the flow-induced release of NO in the cardiovascular system, is an increased diffusion from potential storage sites of lipophilic compounds such as the endothelial lining or muscle cells (Miekisch *et al* 2001).

A notable rise in concentration was also detected for methyl acetate. The possible endogenous origins of this ester have not yet been explored. However, it has recently been

demonstrated that methyl acetate is released by human bronchial epithelial primary cells *in vitro* (Filipiak *et al* 2010). Exogenous uptake can result from its widespread use as a solvent as well as from different types of food (e.g. coffee (Lindinger *et al* 1998b)). This compound was detected in the breath of all volunteers at concentrations of 0.04–3.2 ppb prior to the workload (median: 0.5 ppb). In the case of all individuals we observed an abrupt increase in concentration, usually by a factor of about 2–5 within approximately 1 min of pedalling (see figure 3). Subsequently, either a new plateau was reached or concentrations slightly decreased with the duration of exercise.

DMS appeared in the breath of all volunteers at concentrations ranging from 0.8 to 3.6 ppb during rest (median: 2.7 ppb) and was found to increase during exercise in four cases. This rise in concentration, however, was not so pronounced like in the case of isoprene or methyl acetate and amounted to 20–60%. Despite the relatively small response to exercise, DMS remains a very interesting compound in our study. DMS is a relatively stable volatile sulphur compound present in human breath. Endogenous production has been ascribed to an incomplete metabolism of sulphur-containing amino acids, methionine and cysteine, in the transamination pathway (Miekisch *et al* 2004). DMS is formed by the enzyme thiol S-methyltransferase via the methylation of H_2S and methyl mercaptane (Tangerman 2009). This process can be considered as a detoxification mechanism, removing toxic sulphur species from the tissues. DMS is the main cause of extra-oral halitosis (Tangerman 2009, Tangerman and Winkel 2007) and elevated breath levels were observed in patients with cirrhosis, hepatitis and hypermethioninemia.

Breath concentrations of 2-pentanone during rest were spread around a median value of 0.23 ppb and tended to approach a new steady state level during constant load exercise that was 10–60% higher than the initial value prior to workload, see figure 2. This behaviour closely resembles the profile of acetone (King *et al* 2009) (see also figure 3), which is not unexpected due to the functional similarities of these two ketones. The endogenous source of 2-pentanone is still disputed. Similarly like methyl acetate, 2-pentanone could be shown to be produced by human bronchial epithelial primary cells *in vitro* (Filipiak *et al* 2010). Elevated breath levels have been associated with fasting (Statheropoulos *et al* 2006) and liver diseases (Van den Velde *et al* 2008).

The concentration profiles of compounds identified by GC-MS analysis might be reconfirmed by monitoring their expected PTR-MS signal (i.e. count rate at a mass-to-charge ratio equal to the respective molecular weight + 1). Such an approach is limited to species that are protonated in PTR-MS, i.e. which have higher proton affinities than water (166.5 kcal mol^{-1}). Representative results for methyl acetate (MW 74) are shown in figure 3. Here, PTR-MS count rates were converted to pseudo concentrations (Schwarz *et al* 2009a) and scaled to match the initial GC-MS results at the start of measurement. For correction purposes, room air levels were subtracted from the corresponding signals before conversion. Apart from methyl acetate, propionic acid and butanol appear at m/z 75 in PTR-MS measurements. The agreement between PTR-MS and GC-MS supports the view that in the framework of breath gas analysis of normal volunteers a major part of PTR-MS *signal variability* at m/z 75 can be attributed to the dynamics of methyl acetate.

4. Conclusion

In general, we believe that several valuable pieces of information can be distilled from the phenomenological study of breath VOC behaviour during distinct physiological states.

As has been indicated in the introduction, rather specific but yet very promising fields of application are pulmonary function tests based on the joint exhalation kinetics of an ensemble

of pre-selected blood-borne inert gases. Within this context, a major focus lies on developing a less invasive extension of standard MIGET methodology, which aims at avoiding the exogenous infusion of test gases and replacing them with endogenous compounds originating from normal metabolic activity. The success of such an approach will primarily depend on the extent to which possible test compounds proposed for this purpose can be considered to follow the underlying Farhi description. For instance, it has recently been pointed out that highly water-soluble VOCs (including the standard MIGET test gas acetone) will not represent an adequate choice within this framework since the associated end-tidal concentrations can be expected to differ drastically from the respective alveolar levels (Anderson and Hlastala 2010). This is due to substantial interactions of such compounds with the water-like mucus layer lining the conducting airways, commonly referred to as the wash-in/wash-out effect (Anderson *et al* 2003, 2006). As a phenomenological consequence of this fact, it has been demonstrated that end-tidal exhalation dynamics of highly water-soluble compounds in response to distinct experimental conditions (e.g. exercise, hyperventilation or isothermal rebreathing) will substantially depart from the trend predicted by the Farhi equation (King *et al* 2009, O'Hara *et al* 2008). For instance, acetone concentrations in end-tidal breath tend to increase in response to increased ventilation (see, e.g., figure 3), rather than showing a roughly stable behaviour as anticipated from the classical theory by Farhi.

Analogous tests for low (blood) soluble compounds are straightforward and can be used for directly revealing deviations from the assumptions underlying MIGET methodology (see also the comparison between isoprene and butane presented above). In this sense, profiles of VOCs acquired in the course of dynamic experiments offer the possibility of actively scanning for breath constituents that qualify as (endogenous) MIGET gases as well as to assess the adequacy of potential test compounds.

In the same spirit, we stress the fact that investigations covering dynamic VOC behaviour are a general and necessary tool for gaining novel quantitative perspectives on the inherent variability of VOC concentrations stemming from (short-term) physiological changes. This knowledge is of utmost importance for everyday measurement practice in exhaled breath analysis, as slightly changing experimental conditions (regarding, e.g., body posture, breathing patterns, etc) or even pre-measurement history (stress, physical exhaustion) can have a substantial impact on the observed breath concentration (Cope *et al* 2004). It moreover will be helpful for devising appropriate sampling regimes as well as for comparing results obtained under different experimental protocols (Cope *et al* 2004, Miekisch *et al* 2008, O'Hara *et al* 2008).

Even though instruments for *real-time* breath analysis can be seen as canonical choice for the parallel assessment of physiological (e.g. hemodynamic and respiratory) factors and VOC time response, explorative measurements within this framework necessarily require a combination with GC-MS, ensuring an unambiguous identification of the detected compounds. Here, we presented a methodology for direct manual breath sampling and subsequent SPME/GC-MS analysis during free tidal breathing under dynamic conditions. Moreover, we investigated a limited list of compounds revealing interesting rest-to-work transitions in response to moderate exercise. Continuous PTR-MS isoprene profiles were used as reliable control for confirming the quality of the manually extracted samples. Further efforts will need to take into account a larger variety of trace gases as well as experimental conditions (e.g. isothermal rebreathing (Ohlsson *et al* 1990)). In this sense, we recognize that our data only reflect very preliminary results that hopefully will guide future investigations in this framework.

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