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Method improvements for real-time mass spectrometric monitoring of exhaled VOCs in the clinical environment

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ABBREVIATIONS

cps	Counts per second
DT	Drift tube
E_{drift}	Drift tube electric field
E/N	Reduced electric field of the drift tube
GC	Gas chromatography
IF	Ion-funnel
ITMS	Ion trap mass spectrometer
LCU	Liquid calibration unit
LOD	Limit of detection
LOQ	Limit of quantification
m/∆m	mass resolution
MS	Mass spectrometry
ms	Millisecond
mbar	Millibar
m/z	Mass-to-charge ratio
N	Buffer gas number density
PA	Proton affinity
P_{Drift}	Drift tube pressure
ppbV	Parts per billion by volume
pptV	Parts per trillion by volume
QMS	Quadrupole mass spectrometer
PEEK	Polyether ether ketone
PTR	Proton transfer reaction
Sccm	Standard cubic centimetre per minute
SIFT-MS	Selected ion flow tube - mass spectrometry
SPME	Solid phase micro extraction

ToF-MS	Time of flight-mass spectrometry
Td	Townsend
T_{Drift}	Drift tube temperature
V	Volt
V_{p-p}	Volt peak-to-peak
U_{Drif}	Drift tube voltage
VOC	Volatile organic compound

1. INTRODUCTION

Analysis of volatile organic compounds (VOCs) from exhaled breath provides an attractive option for disease diagnosis, environmental exposure assessment as well as for metabolic, therapeutic and physiological monitoring because it is non-invasive with the potential to be conducted at the point of care[1–6].

The most widely used technique for determination of VOCs in exhaled breath is gas chromatography (GC) coupled to mass spectrometry (MS) preceded by concentration/extraction steps[7–11]. Although these analytical methods are highly selective and specific, they are labour intensive and time consuming as extraction, pre-concentration, and analysis may require hours. Moreover, each sample preparation step may lead to losses and adulterations of the analytes.

Introduction of direct mass spectrometric techniques such as Proton Transfer Reaction Mass Spectrometry (PTR-MS) and Selected Ion Flow Tube Mass Spectrometry (SIFT-MS) that allow direct analysis and real-time monitoring of exhaled breath, has significantly reduced the aforementioned problems[12–14]. Compared to GC-MS, direct mass spectrometric techniques enable continuous monitoring of VOCs without the need for sample preparation. Thus, even rapid changes in breath concentration profiles occurring during physiological and metabolic processes or inflammation could be traced[15–18]. Recent findings in the field of breath research indicate that changes in concentrations are more important than the yet unproven existence of unique breath biomarkers[4,19,20].

Improved detection and identification of VOCs in breath may provide novel insights and a more in-depth understanding of biochemical processes in the human body. Therefore, instrumental improvements have always been a pursued topic in PTR-MS. High sensitivity and low limits of detection (LODs) are crucial for VOC analysis at ultra-trace levels (ppbV-pptV). Sensitivity of PTR-MS depends on the ability to effectively focus and transmit ions through the small orifice that interfaces the drift tube (DT) and the mass analyser. Ion-funnels represent a type of ion guide that enhances sampling of ions through an orifice[21,22].

In order to accurately monitor changes in VOC concentrations, the time response of the PTR-MS must be faster than the transient event being observed. The high reactivity of some classes of compounds such us nitrogen- and sulphur-containing substances represents a huge obstacle for breath-resolved monitoring by means of

PTR-MS. These compounds may interact with any surface of the analytical system resulting in slow response times and large memory effects. As exhaled highly reactive compounds may be related to clinically relevant factors such as diet, diseases, metabolic processes, and bacterial activity[23–28], their direct breath-resolved real-time monitoring in human breath is of high relevance.

For a reliable and accurate monitoring of breath VOC concentration changes, potential confounders induced by breath matrix and clinical environment have carefully to be taken into account[29]. Recent studies e.g. showed that quantification of breath VOCs by means of PTR-MS is significantly affected by humidity, CO_2 and levels of O_2 content in breath samples[30]. In the clinical environment and under mechanical ventilation or when respiratory devices are used to provide supplementary oxygen to the patient, the amount of oxygen in the breath samples may be substantially higher than in room air and may even reach 100%. Oxygen supply may also vary between subjects or within the course of a measurement or study, e.g. due to therapeutic interventions. This may introduce artificial effects on results of PTR-MS based VOC analysis that may be misinterpreted as physiologically or metabolically relevant if not taken into account.

The main goal of this thesis was to improve the measurement capabilities of PTR-MS for real-time monitoring of breath VOCs in the clinical environment. In the first study, we evaluated the effects of a modular ion-funnel onto PTR-MS performances in terms of sensitivity and LODs. In the second study we optimized instrumental conditions for direct breath-resolved real-time monitoring of highly reactive compounds. In the third study, we evaluated the effects of potential confounders from the clinical environment such as elevated inspired O_2 concentrations onto PTR-MS based VOC analysis.

2. RESEARCH QUESTIONS

To take advantage of the unique features of breath VOC analysis by means of PTR-MS, a sensitive, continuous and breath-resolved monitoring of exhaled VOCs is extremely important. Moreover, breath matrix and clinical environment may have significant effects onto PTR-MS analysis. The following questions were addressed in detail:

1. Can sensitivity of PTR-MS be significantly improved by introducing an IF device?
    a. Does the IF affect the ion chemistry in the DT/IF region of the PTR-MS?
    b. Is the IF technique beneficial for the analysis of complex samples such as human breath?

2. How can PTR-MS performance for monitoring of exhaled highly reactive compounds be improved?
    a. Is breath-resolved monitoring of these compounds possible by means of PTR-MS?
    b. Does protein diet effect the concentration of reactive compounds in exhaled breath?

3. How does oxygen concentration in the sample matrix affect VOC quantification by means of PTR-MS?
    a. Are these effects also occurring in a clinical setting in subjects under varying inspiratory oxygen concentrations?
    b. Could a factor-based correction be applied?

3. METHODS

3.1 PROTON TRANSFER REACTION MASS SPECTROMETRY

PTR-MS is an analytical technique that allows real-time monitoring of VOCs at low concentrations. It is widely used, e.g. in environmental sciences, food chemistry, homeland security, and breath analysis [31,32]. PTR-MS was first introduced in the mid-1990s by the group of Lindinger[33,34] and its working principle is based on proton transfer from hydronium ions (H_3O^+) to the sample molecules. H_3O^+ ions are produced in a hollow cathode glow discharge ion source from electron ionization of water vapour and are drawn by an electric field into the DT. Here the analyte sample is injected and the proton transfer reaction between H_3O^+ and the neutral analyte molecules (M) occurs:

$$M + H_3O^+ \rightarrow MH^+ + H_2O$$

Only molecules with proton affinities (PA) higher than water (PA (H_2O) = 691 kJ mol^{-1}) are ionized. This criterion excludes the major constituents of air such as N_2, O_2, and CO_2 but includes many trace gases such as most VOCs.

The DT is usually ~ 10 cm long and consists of a series of stainless-steel ring electrodes separated by Teflon® spacers. A drift voltage (U_{drift}) can be applied over the entire set of rings to induce an electric field (E_{drift}). The E_{drift} accelerates ions towards a small aperture at the end of the DT, but at the same time collisions with the buffer gas (i.e. the major components of air, N_2 and O_2) tend to slow them down. The energy delivered in these collisions depends both on the E_{drift} strength and the buffer gas number density (N) in the DT. These two operating parameters are extremely important and are commonly combined and expressed in terms of the reduced electric field (E/N) value of the DT. Typical E/N values are in the range 90-150 Td, where 1 Td = 10^{-17} V cm².

The protonated VOCs then enter the mass analyser in which they are measured according to their mass-to-charge ratio (*m/z*). The original PTR-MS configuration used only quadrupole mass spectrometers (QMS) for ion mass analysis and the chemical ionization of VOCs was exclusively based on the proton transfer from hydronium ions (H_3O^+)[34]. However, there have been several important developments in PTR-MS over the years. Modifications of the hollow cathode discharge ion source allowed to successfully use different chemical ionization agents such as NO^+, O_2^+, Kr^+, Xe^+ and

NH_4^+ improving versatility and selectivity of the instrument[35–38]. Ion trap (IT-MS) and Time-of-Flight (ToF-MS) mass analysers have been successfully used in PTR-MS[35,39,40]. Whereas IT-MS never had a breakthrough, PTR-MS instruments developed in the last decade are almost exclusively based on ToF mass analysers. One of the main advantages of using a ToF-MS in comparison to a QMS instrument is its higher mass resolving power (m/Δm), which enables the separation of nominally isobaric compounds and thereby lead to a better identification of specific VOCs[41]. Another important advantage of the ToF-MS is that it allows the acquisition of the whole mass spectrum during each measurement cycle of a theoretically unlimited mass range, which makes it most suitable for real-time monitoring of complex matrices. In contrast, a QMS requires a scan or a pre-selection of the relevant *m/z* values, thus important information could be lost.

In all three studies, we used PTR-ToF-MS (figure 1) instruments provided from Ionicon Analytik GmbH (Innsbruck, Austria). For **Publication 1,** we used a PTR-ToF-MS 1000 (mass resolution ~1500 m/Δm) equipped with a modular IF and commercialized as PTR-ToF 1000 *ultra*. For **publications 2 and 3**, we used a PTR-ToF-MS 8000 (mass resolution ~4000 m/Δm).

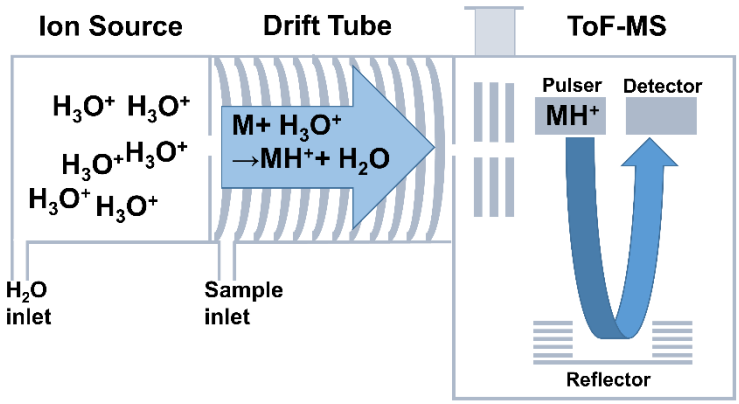

Figure 1. Schematic view of PTR-ToF-MS.

3.2 MODULAR ION-FUNNEL TECHNOLOGY

In **Publication 1** a modular ion-funnel (IF) was placed adjacent to the DT of the PTR-ToF-MS (figure 2). The IF was 2.2 cm long and consisted of 12 electrodes (6 with RF+ and 6 with RF-) with gradually decreasing orifice diameters, from 1 cm to 0.2 cm, placed at 0.1 cm distance of each other. The electrode geometries of the modular IF

used in this work were designed in order to avoid the trapping of ions in axial potential wells, particularly those with low *m/z* [22]. A radio frequency (RF) voltage and a direct current (DC) electric field were then applied to the electrodes. The DC electric field drives the ions axially through the IF toward the exit aperture. An additional alternating current (AC) is superimposed on the electrodes, with the RF on neighboring electrodes being phase-shifted by 180°. In this way, the RF field creates a strongly repulsive potential near the surface of each electrode. In combination with the progressively decreasing aperture size, this serves to focus the ions radially. We investigated the effect of the RF voltage and DC field on the sensitivity of a pattern of VOCs with relevance for breath analysis including alcohols, aldehydes, ketones and aromatics. The signal intensity was recorded for each *m/z* while the settings of the IF region were varied. The DC field was varied in the range of 4.5–27 V/cm while the RF voltage was varied in the range of 40–200 V peak-to-peak (V_{p-p}) at 4.5 MHz. The entire set of experiments was repeated at two different E_{drift} strengths, 66 V/cm and 48 V/cm, and using both dry and humid samples (absolute humidity 47 g m^{-3}).

In a proof of concept study, the instrument operating with IF switched off (DC-mode) and with IF switched on (RF-mode) was applied for breath analysis in 21 healthy human subjects.

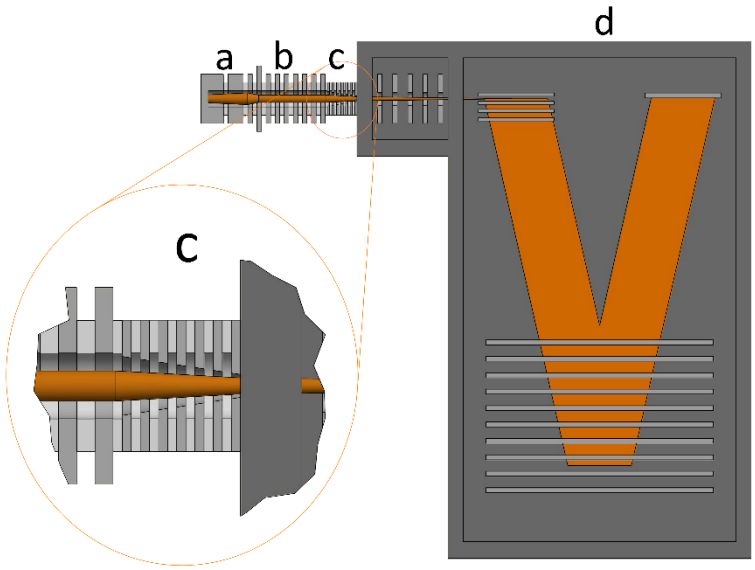

Figure 2. Schematic view of the IF-PTR-ToF-MS setup: a) hollow cathode ion source, b) DT, c) IF, d) ToF mass analyser (from **Publication 1**).

3.3 BREATH-RESOLVED REAL-TIME MONITORING OF HIGHLY REACTIVE COMPOUNDS

In **Publication 2** we optimized PTR-ToF-MS set-up and operating conditions for breath-resolved real-time monitoring of highly reactive compounds. For this purpose, highly reactive aliphatic amines (methyl-, dimethyl- and trimethyl-amine) were used as test substances. Polyether ether ketone (PEEK) tubes of the PTR inlet system (T-pieces and capillary connecting the two T-pieces) were replaced with stainless steel Sulfinert® coated tubes in order to obtain the most inert surface conditions as possible to prevent N- and S-containing compounds from reacting. Moreover, the effects of transfer line and DT temperature (table 1), and E/N of DT onto PTR-ToF-MS response times and sensitivity were systematically evaluated. The instrument operating at the optimal conditions was then applied for breath-resolved measurements of highly reactive compounds in the breath of 17 human healthy subjects following a low and high oral protein challenge.

Table 1. Experimental design conducted for temperature optimization for PTR transfer line and PTR DT (from **Publication 2**).

	T LCU (°C)	T transfer line (°C)	T drift tube (°C)
1	75	75	75
2	75	75	100
3	75	75	120
5	75	100	120
4	75	130	120

3.4 BREATH MATRIX EFFECT: ELEVATED OXYGEN LEVELS

In **Publication 3** we assessed the effect of different O_2 concentrations (up to 90%) in the sample matrix onto PTR-ToF-MS sensitivity for analysis of 23 VOCs including aldehydes, ketones, hydrocarbons and aromatic compounds in dry and humid samples. Finally, we performed real-time breath analysis in two mechanically ventilated animals and in three healthy volunteers under varying oxygen supply to demonstrate effects of varying oxygen content onto VOC profiles under *in vivo* conditions.

3.5 VOC STANDARD GENERATION

For all studies, gaseous VOC standards were generated by means of a liquid calibration unit (LCU; IONICON Analytik GmbH, Innsbruck, Austria). Figure 3 shows a schematic representation of the LCU. The working principle of the LCU involves the introduction of a liquid standard solution into a carrier gas stream, by forcing it through a nebuliser (X175, Burgener Research Inc., United Kingdom) and spraying the solution into an evaporation chamber at a defined temperature. This results in a rapid evaporation. The generated gaseous standard mixture can then be measured or collected directly at the output of the evaporation chamber. Two liquid ports (1–50 µl min^{-1}), one carrier gas port (1–1000 ml min^{-1}) and two additional gas ports (1–100 ml min^{-1}), controlled by mass flow controllers (Bronkhorst High-Tech B.V., Ruurlo, Netherlands), enable the generation of complex standard mixtures at defined concentrations. Trace VOC standards can be prepared from either liquid solutions or gases, or even from both at the same time. In addition, defined amounts of humidity can be added by adding pure water via one of the two liquid ports[30].

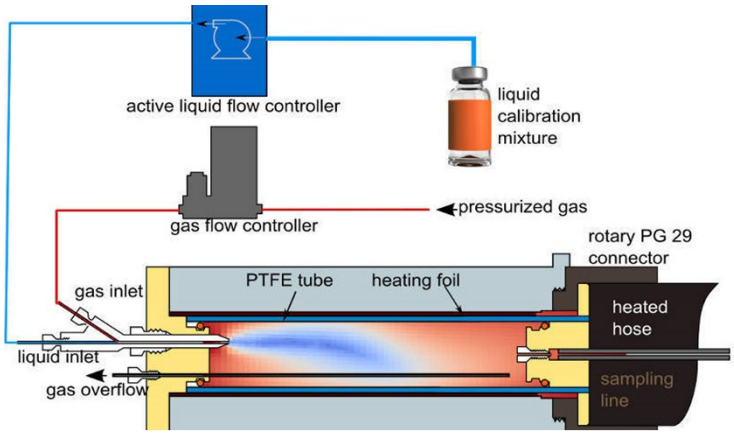

Figure 3. Schematic representation of the LCU (source: https://www.ionicon.com).

In **Publication 1,** the IF characterization was performed using a multicomponent gas VOC mixture (IONICON Analytik GmbH, Innsbruck, Austria) stored in a Silcosteel® canister that was connected to one of the gas ports of the LCU. The gaseous VOC mixture included methanol, acetonitrile, acetaldehyde, ethanol, 2-propenal, acetone, isoprene, 2-butenal, 2-butanone, benzene, toluene, *o*-xylene, chlorobenzene, *α*-pinene and 1,2-dichlorobenzene at concentrations of ~ 1ppmV. The VOC mix was subjected to a 100-fold dynamic dilution with pure nitrogen (purity 5.0, Linde, Pullach,

Germany) to generate a standard mixture with approximately 10 ppbv of each component.

In **Publication 2,** methyl- dimethyl- and trimethyl-amine gaseous standards were generated from liquid stock solutions prepared in ultrapure water (HPLC grade, Carl Roth GmbH, Karlsruhe, Germany). The pH of the solutions was adjusted at physiological pH 7.4 by adding 5 mL of phosphate buffer solution at pH 7.2 (Honeywell Fluka™, Morris Plains, New Jersey, USA). As for the PTR inlet tubes, stainless steel Sulfinert® coated tubes were used for evaporation chamber and capillaries of the LCU in order to have the most inert surface conditions as possible to prevent reaction or decomposition of highly reactive aliphatic amines during gas standard generation.

In **Publication 3,** we used two standard multicomponent gas VOC mixtures. The first standard mixture included n–C_1 to C_{10} aldehydes, 2-propenal, and 2-butenal; and the second contained formaldehyde, acetaldehyde, methanol, ethanol, isoprene, acetone, 2-propenal, acetonitrile, 2-butanone, benzene, 2-butenal, toluene, chlorobenzene, o-xylene, and 1,2-dichlorobenzene. Both mixtures were stored in Silcosteel® canisters and purchased from IONICON Analytik, Austria. The two standard mixtures were analysed separately. Calibrations with different amounts of O_2 in the sample were analysed for all substances over a concentration range from approximately 4 ppbV to 100 ppbV. The amount of O_2 in the sample matrix was varied in five steps between 0 and 90%. An oxygen canister (Air Liquide Deutschland GmbH, Düsseldorf, Germany) connected to the second gas port of the LCU was used for this purpose.

For all studies, the standard mixture was introduced into the PTR DT in continuous mode *via* a 1.5 m long transfer line (ID: 0.75 mm, Restek, Bellafonte, PA, USA) that was directly connected to the outlet of the LCU. A PEEK transfer line was used for **Publication 1**. In contrast, a Silcosteel® transfer line was used for **Publication 2 and 3.**

3.6 CONTINUOUS BREATH SAMPLING

In **Publication 1** and **Publication 2**, 21 and 17 healthy human volunteers were recruited, respectively. Volunteers were asked to sit down on a chair and to breathe evenly through a sterile mouth piece directly connected to the PTR transfer line by means of a T-piece in side stream mode (figure 4).

In **Publication 3** the three healthy human volunteers were breathing through a tightly fitting face mask that was connected to the ventilation system (Breas LTV 1000,

Pulmonetic Systems, USA) and a sterile T-piece that enabled the connection of the PTR transfer line. For the two mechanically ventilated pigs, a sterile T-piece was introduced into the ventilation system as close to the endotracheal tube as possible. PTR transfer line was connected via luer lock adapter and breath sampling was done continuously in side-stream mode.

All studies were performed in accordance with the guidelines laid down in the Declaration of Helsinki and approved by the ethics committee at University Medical Center Rostock.

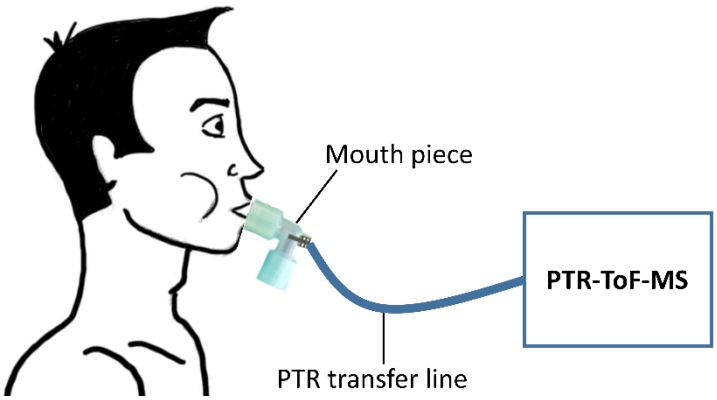

Figure 4. Schematic representation of continuous side-stream breath sampling.

3.7 DATA PROCESSING

For all studies, the ion yields of all *m/z* were determined in counts per second (cps) and compounds were identified by means of their protonated monomers and isotopic patterns. Both breath and standard files were processed using the software PTR-MS viewer v. 3.2.8 (IONICON Analytik GmbH., Innsbruck, Austria).

Normalization of the measured ion intensities to the H_3O^+ counts is standard practice in PTR-ToF-MS data treatment[42]. In **Publication 1** the normalization to H_3O^+ was omitted in order to reflect the actual sensitivity of the instrument, which would be masked by normalization. In **Publication 2** all measured intensities were normalised to the H_3O^+ counts. In **Publication 3** we reported both non-normalized data and data normalized onto H_3O^+ counts.

For breath measurements, only compounds for which signals were higher than 3 times the instrumental background signals were considered for further analysis. In addition, compounds with expiratory concentrations lower than the corresponding inspiratory

concentration plus the standard deviation of inspiratory concentration were excluded. Expiratory and inspiratory phases were recognized by means of a custom-made data processing algorithm called ‘breath tracker’ (MATLAB version 7.12.0.635, R2011a). The function of the algorithm has been described previously[29]. Briefly, an endogenous compound that has a sufficiently abundant signal intensity in expiration is used as a tracker to differentiate between alveolar and inspired phases. For all studies, acetone was used for this purpose (figure 5). Expiratory and inspiratory phases determined by means of the algorithm were then applied to all *m/z* of interest.

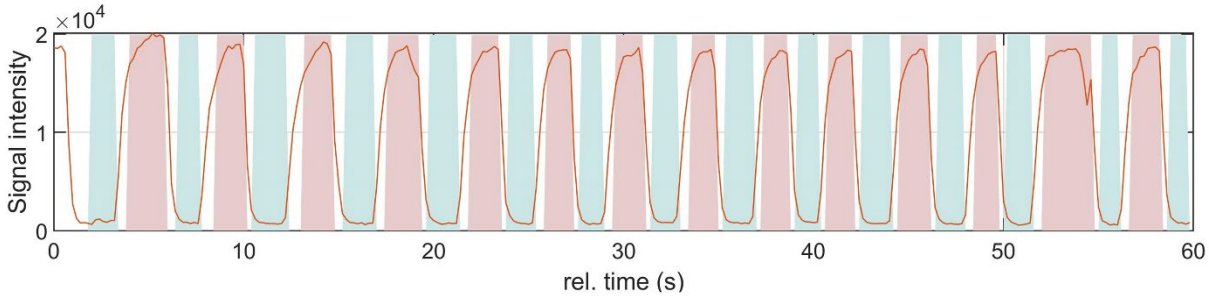

Figure 5. Breath Tracker plot. Acetone was used as a tracker mass. Expiratory and inspiratory phases are marked in pink and green colours, respectively.

4. RESULTS

The results are presented based on three original publications:

Publication 1: ***Effects of modular ion-funnel technology onto analysis of breath VOCs by means of real-time mass spectrometry***
Pugliese G, Piel F, Trefz P, Sulzer P, Schubert JK, Miekisch W.
Anal Bioanal Chem. 2020; 412, 7131–7140.
(Impact-Factor 2020: **3.637**)

Publication 2: ***Extending PTR based breath analysis to real-time monitoring of reactive volatile organic compounds***
Pugliese G, Trefz P, Brock B, Schubert JK, Miekisch W.
Analyst. 2019; 144, 7359–7367.
(Impact-Factor 2019: **4.019**)

Publication 3: ***Effects of elevated oxygen levels on VOC analysis by means of PTR-ToF-MS***
Trefz P, **Pugliese G**, Brock B, Schubert J K, Miekisch W.
J. Breath Res. 2019; 13(4):046004.
(Impact-Factor 2019: **3.000**)

Brief summaries of the published results will be given. The original articles are located in chapter 9: "Original publications of cumulative thesis".

4.1 Publication 1: Effects of modular ion-funnel technology onto analysis of breath VOCs by means of real-time mass spectrometry

Pugliese G, Piel F, Trefz P, Sulzer P, Schubert JK, Miekisch W.
Anal Bioanal Chem. 2020; 412, 7131–7140 **(Impact-Factor: 3.637)**

Most breath VOCs are found at ultra-trace levels, typically ranging from low ppbV to pptV, thus their reliable detection requires highly sensitive analytical methods. The sensitivity of PTR-ToF-MS depends on the ability to effectively focus and transmit ions from the relatively high-pressure DT to the low-pressure mass analyser. As most of the ions crossing the DT do not exit through the small orifice at the MS interface, a large quantity of ion signal is lost. In order to improve focusing and transmission of the ions through this small orifice, a modular IF was installed adjacent to the DT of a PTR-ToF-MS 1000. The IF was 2.2 cm long and consisted of a series of electrodes with gradually decreasing orifice diameters. A radio frequency (RF) voltage and a direct current (DC) electric field are applied to the electrodes to focus the ions.

In the first part of the study, we investigated the effect of the RF voltage and DC field on the sensitivity of a pattern of VOCs including alcohols, aldehydes, ketones and aromatics. Then, in a proof of concept study, the instrument operating as normal DT (DC-mode) and at optimal IF conditions (RF-mode) was applied for breath analysis in 21 healthy human subjects.

Investigated VOCs showed maximum intensities at substance specific DC and RF voltages. In general, an improvement of about one order of magnitude in sensitivity was observed in RF-mode compared to DC-mode. LODs and LOQs could be improved by a factor of 3-4 in RF-mode.
Improved sensitivities and lower LODs in RF-mode considerably enhanced the spectrum of detectable VOCs in real-time breath analysis.

Incorporation of the IF improved the performance of PTR-ToF-MS allowing the real-time monitoring of a broader range of potential breath biomarkers which enhances the potential for VOC profiling in the clinical environment.

4.2 Publication 2: Extending PTR based breath analysis to real-time monitoring of reactive volatile organic compounds

Pugliese G, Trefz P, Brock B, Schubert JK, Miekisch W.
Analyst. 2019; 144, 7359–7367 **(Impact-Factor: 4.019)**

Reliable monitoring of metabolism-induced changes in exhaled VOC concentrations requires short instrumental response times. The high reactivity of N- and S-containing substances represents a huge obstacle for their time-resolved analysis and quantification at trace levels (ppbV- pptV range) by means of PTR-MS. This is because these compounds may interact with any surface of the analytical system resulting in slow response times and large memory effects.

We optimized PTR-ToF-MS set-up and analytical conditions for direct breath-resolved monitoring of reactive compounds. Aliphatic amines (methyl-, dimethyl- and trimethylamine) were used as test substances for this purpose. Gas standards were generated by means of a LCU. Calibration conditions were adapted in terms of materials, temperature and equilibration time. PTR-ToF-MS was optimized in terms of inlet materials, transfer line and DT temperature, and E/N of DT. The method was then applied for breath-resolved monitoring of reactive compounds in 17 healthy subjects after a high and low oral protein challenge.

The interactions of compounds with the surfaces of PTR-ToF-MS were reduced using inert materials and high temperatures for the inlet system and the DT. In this way, good linearity and reproducibility (R^2 > 0.99, RSDs < 5%) with LODs between 0.15 ppbV - 1.23 ppbV and LOQs between 0.24 ppbV - 1.94 ppbV could be achieved.

Exhaled concentrations of trimethylamine, indole, methanethiol, dimethylsulfide, acetone, 2-propanol, 2-butanone and phenol showed significant changes after protein intake. Methanethiol concentrations increased 6-fold within minutes after the protein intake.

Optimization of instrumental set-up and analytical conditions enabled reliable breath-resolved PTR-ToF-MS analysis of reactive VOCs in breath down to the sub-ppbV concentration range. Continuous *in vivo* monitoring of exhaled amines and sulphur containing compounds may provide novel non-invasive insights into protein metabolism.

4.3 Publication 3: Effects of elevated oxygen levels on VOC analysis by means of PTR-ToF-MS

Trefz P, **Pugliese G**, Brock B, Schubert J K, Miekisch W.
J. Breath Res. 2019; 13(4):046004 **(Impact-Factor: 3.000)**

Elevated inspiratory oxygen concentrations are likely to occur in clinical settings, but also when respiratory masks or other devices are used (e.g. high-flow oxygen cannulas). Moreover, inspiratory oxygen concentration may vary between subjects or within the course of a measurement or study and thus bias results of VOC analysis.

In this study, we assessed the effect of high O_2 concentrations (up to 90%) in the sample matrix onto results of PTR-ToF-MS analysis. 23 VOCs including aldehydes, ketones, hydrocarbons, and aromatic compounds were assessed in detail and we investigated whether sensitivity changed with increasing oxygen amount in the sample. Matrix adapted gas standards were generated by means of a LCU and the whole set of experiments was performed with both dry and humid samples. Finally, *in vivo* experiments with three healthy human volunteers and two mechanically ventilated pigs under elevated and varying oxygen levels were done to confirm these effects under real-life conditions.

H_3O^+ and water cluster ($H_2O \cdot H_3O^+$) intensities decreased by more than 40% and 60%, respectively, when the amount of oxygen in the sample matrix was increased. Intensities of most investigated VOCs showed a substantial dependency on oxygen concentrations in the sample. Differences in signal intensities of more than 50% could be observed. The effect was generally more pronounced in dry samples but still significant under humid conditions. A linear dependency of sensitivity on the oxygen concentration in the sample matrix was observed for a number of VOCs (e.g. aldehydes) possibly enabling a factor based-correction.
In vivo experiments involving spontaneously breathing healthy volunteers and mechanically ventilated pigs confirmed the results under real-life conditions.

When breath analysis by means of PTR-ToF-MS is carried out in any situation where supplemental oxygen is supplied, its effect on measured VOC intensities has to be carefully considered for reliable VOC quantification.

5. DISCUSSION

Implementation of the modular IF considerably improved PTR-ToF-MS performances in terms of sensitivity and LODs (**Publication 1**). The use of inert materials and high temperatures for PTR inlets and DT allowed breath-resolved real-time analysis of highly reactive compounds (**Publication 2**). Thus, the range of detectable exhaled VOCs for clinical monitoring was significantly enlarged. Whenever PTR-ToF-MS based breath VOC analysis is carried out in situations where supplemental oxygen is supplied to the subject (e.g. under mechanical ventilation), its effect on VOC intensities has to be carefully considered for reliable quantification (**Publication 3**).

Most of breath VOCs are found at low ppbV-pptV concentrations. Disease states, therapeutic interventions or physiological maneuvers may lead to an under expression of breath VOCs or may generate new breath VOCs at ultra-low concentrations. Highly sensitive analytical methods are therefore crucial for a reliable breath VOC detection and quantification. In all studies, we performed direct online breath sampling in order to be able to monitor fast changes in concentration profiles and to avoid potential biases caused by offline sampling procedures or storage. For direct online analysis, the performances of the analytical method can only be enhanced through instrumental development and optimization. PTR-ToF-MS sensitivity and LODs were improved through the incorporation of the modular IF, which enable the detection of an increased number of VOCs in the breath of healthy human volunteers. These additional breath VOCs could provide further insight into biochemical processes in the human body and may serve as markers for physiological and pathophysiological conditions or even for environmental health risk assessment. VOCs showed maximum intensities at substance specific DC field and RF voltages. Therefore, the IF can be tuned to obtain the best operating conditions for targeted as well as for non-targeted VOC analysis. The sensitivity improvement was uniform, i.e. one order of magnitude for all investigated compounds. This clearly indicates that the IF actually serves only to focus ions as they exit the DT without altering the ion chemistry in the reaction region. Thus, calculated concentrations according to kinetic theory remain accurate. This is a remarkable improvement compared to previously described IF-PTR-MS instruments in which the IF affected the PTR ion chemistry, resulting in vastly different sensitivities for different compounds and unusual fragmentation patterns[43,44]. The IF also induced an increase of background noise. Thus, the gain in sensitivity cannot directly translate

into identical improvements of LODs and LOQs. In contrast to the sensitivity, LODs and LOQs could only be improved by a factor of 2-4 through the IF implementation. For real life applications, e.g. trace gas analysis in breath, LODs and LOQs have to be carefully considered to determine the actual gain in instrumental performances thought the modular IF incorporation.

The interaction of highly reactive VOCs with the surfaces of the analytical system was reduced by using inert materials and high temperatures for PTR inlet and DT. In this way, exhaled reactive N- and S-containing compounds could be monitored in a breath-resolved manner at trace levels (low ppbV-pptV). Considerable fragmentation was observed for dimethylamine and trimethylamine with increasing the E/N of the DT. This is due to the loss of H_2 from the excited protonated parent molecule. Hydrogen elimination is more pronounced for trimethylamine than for dimethylamine[45]. As has been shown for urine and blood[24–26], we expected an increase in the amine breath concentration after protein intake. This was true for trimethylamine and indole in our study. Methylamine and dimethylamine concentrations were always below LODs. Changes in concentration profiles of exhaled trimethylamine, methanethiol, indole, phenol and dimethylsulfide after protein intake may be related to protein degradation by gut and oral bacteria[28,46–50]. As these changes were not statistically significant between the two protein diets, bacterial enzymatic activity may have already been saturated at low protein intake. The huge breath-to-breath variation showed by methanethiol concentrations within the first two minutes after protein intake, can be attributed to its oral origin[51]. Fast changes like this emphasize the great potential of breath-resolved real-time monitoring of exhaled VOCs. In such cases, punctual sampling and offline analysis would lead to misleading results, as compound concentration would largely depend on the time of sampling. Concentration profiles of acetone, 2-propanol and 2-pentanone after protein intake may be attributable to lipolysis and breakdown of glycogenic amino acids[52–56]. Results indicated that lipolysis is apparently more pronounced in the low protein group while, in the high protein group, it is prevented to some extent through breakdown of glycogenic amino acids.

Measured intensities of protonated VOCs decreased with increasing O_2 content in the samples. The formation of additional O_2^+ influences the ionization of analyte molecules within the DT, as reactions of analytes with O_2^+ follow a charge transfer mechanism that could compete with the proton transfer reaction[57]. Furthermore, reactions with O_2^+ lead to increased fragmentation compared to reaction with H_3O^+ [58,59]. Apart from these

effects, changes in the buffer gas composition can influence the mobility of the ions in the DT and hence effect the kinetic energy of ion-molecule collisions[60,61]. This may have altered the sensitivity for certain compounds and thus contributed to the observed effects. For VOCs that showed a linear dependency of sensitivity on sample O_2 content, an O_2-based correction factor could be applied. However, such a factor would have to be experimentally determined and the cross-dependency on sample humidity would have to be considered as well. Results observed from real-time breath analysis in spontaneously breathing healthy volunteers and mechanically ventilated pigs under elevated and varying oxygen supply were in good agreement with results obtained from standard measurements. It was obvious that the observed effects also occur in a clinical setting and not just under laboratory conditions. It is important to notice that expiratory O_2 concentrations are lower compared to inspiratory concentrations due to oxygen uptake in the blood and oxygen consumption in the lungs. Hyperoxic conditions can further complicate this issue as arterial PO_2 will increase over time and as a consequence oxygen uptake will decrease[62]. We cannot fully exclude such effects and the differences in expiratory O_2^+ between the first and second measurement with 21% inspiratory O_2 in healthy volunteers may be explained this way. As the hyperoxic phase was longer in the mechanically ventilated pigs, physiological effects may have been more pronounced. This could explain the opposing behaviour of acetone in the mechanically ventilated animals.

6. SUMMARY

We improved PTR-ToF-MS performances for direct real-time monitoring of VOCs in human breath and we showed that reliable quantification is challenging as artificial VOC concentration changes may easily be introduced by the breath matrix or clinical environment.

The incorporation of the modular IF led to a significant improvement of the PTR-ToF-MS performances without affecting the ion chemistry within the DT itself. Higher sensitivity and lower LODs enlarged the spectrum of detectable compounds from real-time breath analysis, enhancing in this way the potential of breath VOCs screening for biomedical applications.

Optimized PTR conditions in combination with inert materials and high temperatures allowed breath-resolved real-time monitoring of exhaled reactive N- and S-containing compounds at trace concentrations. As shown for methanethiol, breath VOC concentrations may change quickly and distinctly. Hence, only breath-resolved real-time monitoring can provide complete and comprehensive information from breath VOC analysis. Profiles of exhaled reactive VOCs may provide novel non-invasive insights into various aspects of metabolism, energy production and fuel consumption in the human body.

Varying oxygen concentration in the sample matrix significantly influences VOC analysis by means of PTR-ToF-MS. Whenever supplemental oxygen is supplied to a subject, differences in oxygen concentrations will introduce differences in the measured signal intensities. This can affect data from a single subject or introduce a bias when several subjects are analyzed under varying conditions. These artificially induced effects on results of VOC analysis may be misinterpreted as physiologically or metabolically relevant if not carefully taken into account.

The described methods are able to improve the potential of real-time breath VOC analysis for basic research and clinical applications.

7. THESIS

I. Incorporation of the modular IF improved PTR-ToF-MS performances without having major effects onto ion chemistry within the DT itself.

II. Higher sensitivity and lower LODs increased the number of detectable exhaled breath VOCs for biomedical application.

III. The use of inert materials and high temperature allowed breath-resolved real-time monitoring of highly reactive compounds by means of PTR-ToF-MS.

IV. Concentrations of reactive exhaled volatile substances changed when volunteers ingested diets with different protein content.

V. As breath VOC concentrations may change quickly and distinctly, only breath-resolved real-time monitoring can provide complete and comprehensive information from breath VOC analysis.

VI. Oxygen levels in the sample matrix have significant effects on VOC analysis by means of direct PTR-ToF-MS.

VII. The effects of O_2 on PTR VOC analysis observed from standard measurements also occur in a clinical setting in subjects under high and varying oxygen supply.

VIII. For VOCs that showed a linear dependency of sensitivity on sample O_2 content, an O_2 based correction factor could be applied.

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9. ORIGINAL PUBLICATIONS FOR CUMULATIVE THESIS

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RESEARCH PAPER

Effects of modular ion-funnel technology onto analysis of breath VOCs by means of real-time mass spectrometry

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Abstract

Proton transfer reaction time-of-flight mass spectrometry (PTR-ToF-MS) is a powerful tool for real-time monitoring of trace concentrations of volatile organic compounds (VOCs). The sensitivity of PTR-ToF-MS also depends on the ability to effectively focus and transmit ions from the relatively high-pressure drift tube (DT) to the low-pressure mass analyzer. In the present study, a modular ion-funnel (IF) is placed adjacent to the DT of a PTR-ToF-MS instrument to improve the ion-focusing. IF consists of a series of electrodes with gradually decreasing orifice diameters. Radio frequency (RF) voltage and direct current (DC) electric field are then applied to the electrodes to get the ions focused. We investigated the effect of the RF voltage and DC field on the sensitivity of a pattern of VOCs including hydrocarbons, alcohols, aldehydes, ketones, and aromatic compounds. In a proof-of-concept study, the instrument operating both as normal DT (DC-mode) and at optimal IF conditions (RF-mode) was applied for the breath analysis of 21 healthy human subjects. For the range of investigated VOCs, an improvement of one order of magnitude in sensitivity was observed in RF-mode compared with DC-mode. Limits of detection could be improved by a factor of 2–4 in RF-mode compared with DC-mode. Operating the instrument in RF-mode allowed the detection of more compounds in the exhaled air compared with DC-mode. Incorporation of the IF considerably improved the performance of PTR-ToF-MS allowing the real-time monitoring of a larger number of potential breath biomarkers.

Keywords PTR-ToF-MS · Ion-funnel · Real-time mass spectrometry · Breath analysis · VOCs

Introduction

Proton transfer reaction mass spectrometry (PTR-MS) is an analytical technique that allows real-time monitoring of volatile organic compounds (VOCs) at low concentrations. It is widely used, e.g. in environmental sciences, food chemistry, homeland security, and breath analysis [1].

Since its introduction in the 1990s [2], PTR-MS has been improved in many ways. Inclusion of time-of-flight (ToF) mass analyzers has substantially overcome the limitations of the first generation of PTR quadrupole-MS (QMS) such as limited mass range and low mass resolution [3–5]. Modifications of the hollow cathode discharge ion source allowed to successfully use different chemical ionization agents such as H_3O^+, NO^+, O_2^+, Kr^+, Xe^+, and NH_4^+, improving versatility and selectivity of the instrument [6, 7].

Sensitivity of PTR-MS is not solely determined by mass analyzers and detectors but it also depends on the ability to effectively focus and transmit ions from the relatively high-pressure drift tube (DT) to the low-pressure mass analyzer. As most of the ions crossing the DT do not exit through the small orifice at the MS interface, a large quantity of ion signal is lost. Ion-funnels (IF) represent a kind of ion guide that enhances sampling of ions through an orifice [8]. In an IF, a radio frequency (RF) voltage and a direct current (DC) electric field are applied to a series of electrodes with decreasing aperture

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sizes. The electrodes provide strong repulsive potentials at the edge of the electrode, radially focusing the ions. The first demonstration of an IF in PTR-MS was shown by Barber et al. [9]. In their instrument, the whole DT was set up as an IF with the first half used as a standard DT reactor running at a lower reduced electric field compared with the traditional DT. The RF electric field was only applied to the second section with decreasing orifice sizes. González-Méndez et al. [10] used the IF to manipulate the ion-molecule reactions and enhance the selectivity of PTR-MS. Brown et al. [11] reported that in this instrument, ion-focusing and proton transfer reaction both occurred in the IF region. This resulted in vastly different sensitivities for different compounds and in unusual fragmentation patterns. Recently, IONICON Analytik implemented a modular IF into proton transfer reaction time-of-flight mass spectrometry (PTR-ToF-MS) instruments. The aim of the present study was to characterize and optimize the IF-PTR-ToF instrument for trace VOC analysis. In a proof-of-concept setup, the instrument was then applied for real-time breath analysis in human subjects. The following questions were addressed in detail:

- How does modification of IF parameters affect primary and VOC product ions?
- Are PTR sensitivity and detection limits in VOC analysis significantly improved by the IF?
- Are benefits of the IF technique suited to support applications such as real-time breath gas analysis in humans?

Methods

Ion-funnel PTR-ToF-MS instrument

All investigations were carried out using an online PTR-ToF-MS instrument equipped with a modular IF (PTR-TOF 1000 ultra, IONICON Analytik GmbH, Innsbruck, Austria; first-generation model (2017)). Figure 1 shows a schematic view of the instrument. The general working principle of PTR-ToF-MS has been described in several studies [4, 5]. Concisely, hydronium ions (H_3O^+) are produced in a hollow cathode glow discharge ion source from electron ionization of water vapour and are drawn by an electric field into the ion-molecule reaction region (DT). Here, the analyte sample is injected and the proton transfer reaction between the formed H_3O^+ and neutral analyte molecules (M) occurs: $M + H_3O^+ \rightarrow MH^+ + H_2O$. Only molecules with proton affinities higher than water (PA (H_2O) = 691 kJ mol^{-1}) are ionized, a criterion that excludes the major constituents of air such as N_2, O_2, and CO_2 but includes many trace gases such as most VOCs. The DT of the PTR-TOF 1000 ultra consists of a 7 cm long tube made of electrically isolated stainless steel rings. The rings are

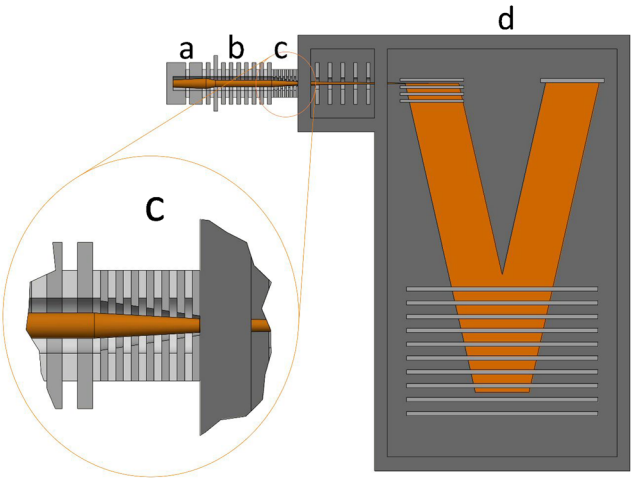

Fig. 1 Schematic view of the PTR-ToF 1000 ultra setup: (**a**) hollow cathode ion source, (**b**) drift tube, (**c**) ion-funnel, (**d**) ToF mass analyzer

connected with resistors, and a drift voltage (U_{drift}) can be applied over the entire set of rings to induce an electric field (E_{drift}) in the DT. The modular IF is 2.2 cm long, it is placed adjacent to the DT and consists of 12 electrodes (6 with RF+ and 6 with RF−) with gradually decreasing orifice diameters, from 1 to 0.2 cm, placed at 0.1 cm distance of each other. In order to avoid the trapping of ions in axial potential wells, particularly those with low m/z, the IF electrode geometries used in this work fulfilled the following conditions:

$$2\pi\frac{\rho}{\delta}\exp\left(\frac{-2\rho}{\delta}\right)\ll 1$$

where ρ is the electrode orifice radius and $\delta = d/\pi$ where d is the electrode spacing [12]. A radio frequency (RF) voltage and a direct current (DC) electric field are then applied to the electrodes. The DC electric field drives the ions axially through the IF toward the exit aperture. An additional alternating current (AC) is superimposed on the electrodes, with the RF on neighbouring electrodes being phase-shifted by 180°. In this way, the RF field creates a strongly repulsive potential near the surface of each electrode. In combination with the progressively decreasing aperture size, this serves to focus the ions radially. Table S1 (see Electronic Supplementary Material, ESM) summarizes details and operating conditions of the modular IF. The protonated VOCs then enter the pulse extraction region of the orthogonal acceleration reflectron ToF analyzer via a transfer lens system. The DT is interfaced to the transfer lens region via a pinhole of ~ 0.1 cm I.D. with the cone toward the transfer lens. The operating pressure in the DT (buffer gas number density, N) and the E_{drift} strength are important parameters, more commonly combined and expressed in terms of the reduced electric field (E/N). E_{drift} accelerates the ions but at the same time collisions with the buffer gas tend to slow them down. The E/N affects the reagent ion distribution. Increasing the E/N ratio results in

more energetic collisions, which reduces the proportion of the water cluster ions ($H_3O^+(H_2O)_n$) in the DT but at the same time can increase the fragmentation of analytes [13]. Typical *E*/*N* values are in the range 90–150 Td, where 1 Td = 10^{-17} V cm^2.

Within the standard PTR DT, E_{drift} variation is about 15% due to the ratio of the inner diameter and axial distance between the drift rings [14]. The additional RF voltage considerably increases this variation and *E*/*N* cannot be properly calculated any more. However, it is possible to define certain voltage settings in RF-mode which enable branching ratios of distinct analytes to be obtained that are comparable with the ones obtained with a classic PTR-ToF-MS instrument operated at a certain actual *E*/*N* ("TRU-*E*/*N*" method). Nevertheless, in the present paper, the *E*/*N* definition is omitted.

Ion-funnel characterization

Gas standard generation

The IF characterization was performed using a multicomponent gas VOC mix (IONICON Analytik GmbH, Innsbruck, Austria) including methanol, acetonitrile, acetaldehyde, ethanol, 2-propenal, acetone, isoprene, 2-butenal, 2-butanone, benzene, toluene, *o*-xylene, chlorobenzene, α-pinene, and 1,2-dichlorobenzene at concentrations of ~ 1ppmV. The VOC mix was subjected to a 100-fold dynamic dilution in pure nitrogen (purity 5.0, Linde, Vienna, Austria) by means of a liquid calibration unit (LCU, IONICON Analytik, Innsbruck, Austria) to generate a standard mixture with approximately 10 ppbV of each component. The working principle of the LCU involves the introduction of a liquid standard solution into a carrier gas stream, by forcing it through a nebuliser (X175, Burgener Research Inc., UK) and spraying the solution into an evaporation chamber at a defined temperature. This results in a rapid evaporation. The generated gaseous standard mixture can then be measured or collected directly at the output of the evaporation chamber. Two liquid ports (1–50 μl min^{-1}), one carrier gas port (1–1000 ml min^{-1}), and two additional gas ports (1–100 ml min^{-1}), controlled by mass flow controllers (Bronkhorst High-Tech B.V., Ruurlo, Netherlands), enable the generation of complex standard mixtures. VOC standards can be prepared from either liquid solutions or gases, or even from both at the same time. In addition, defined amounts of humidity can be added by adding pure water via one of the two liquid ports [15].

In this study, the LCU flow was kept constant at 1000 ml min^{-1} for all experiments, the LCU temperature was 75 °C and the humidity was adjusted by adding pure water (HPLC grade).

Experimental design

The standard mixture was introduced into the DT via a 1.5 m long polyether ether ketone (PEEK) transfer line (ID: 0.75 mm, Restek, Bellafonte, PA) that was directly connected to the outlet of the LCU. The transfer line temperature was 75 °C and the sampling flow was 100 ml min^{-1}. The signal intensity was recorded for each *m*/*z* while the settings of the IF region were varied. Operating the instrument in RF-mode (RF on), the DC electric field applied to the IF was varied in the range of 4.5–27 V/cm while the RF voltage was varied in the range of 40–200 V peak-to-peak (V_{p-p}) at 4.5 MHz. These testing ranges were decided upon after preliminary measurements showed that these settings approximate the best operating conditions. The entire experimental design was repeated at two different E_{drift} strength, 66 V/cm and 48 V/cm, and using both dry and humid samples (absolute humidity 47 g m^{-3}) (ESM Table S2). These two sampling conditions will be referred in the text as "dry" and "humid" conditions, respectively.

When the instrument was operated only with the DC field applied to the IF region (DC-mode), the RF voltage was set to zero and the DC field was set at the same E_{drift} value in order to have a homogeneous electric field along the DT/IF regions.

For the whole experimental design, the DT/IF pressure was 2.3 mbar, the DT temperature was 75 °C, and the integration time was 1 s.

Three replicates were measured for each experimental set-up, then the results were averaged and background signals were subtracted.

Human breath samples

All experiments were performed in accordance with the guidelines laid down in the Declaration of Helsinki and approved by the ethics committee at the University Medical Center Rostock. Informed consent was obtained from 21 healthy human subjects (aged between 20 and 45 years). Demographic parameters such as height, body weight, age, sex, and smoking habits were recorded for each volunteer (ESM Table S3). Volunteers were asked to breathe spontaneously and continuously over 3 min through a sterile mouth piece directly connected to the PTR transfer line in side stream mode by means of a T-piece. During the first minute of measurement, the PTR-ToF-MS instrument was operated in RF mode: E_{drift} was 66 V/cm, RF voltage was 120 V_{p-p}, and DC field was 13.5 V/cm. During the second minute of measurement, the operating mode of the instrument was switched from RF-mode to DC-mode. During the third minute of measurement, the instrument was operated in DC-mode: RF voltage was 0 V_{p-p} and both E_{drift} and DC field were 66 V/cm.

For breath measurements, the PTR transfer line temperature was 75 °C, DT temperature was 75 °C, and DT pressure was 2.3 mbar. For breath measurements, the integration time was 200 ms.

Data processing

The ion yields of all *m/z* were measured in counts per second (cps) and compounds were identified by means of their protonated *m/z* and isotopic patterns. The normalization of the measured ion intensities to the H_3O^+ counts in combination with the water-cluster ion counts is standard practice in PTR-ToF-MS data treatment [16]. However, in the present paper, the normalization to reagent ions was omitted in order to reflect the actual sensitivity of the instrument, which would be masked by normalization.

Both breath and standard files were processed using the software PTR-MS viewer v. 3.2.8 (IONICON Analytik GmbH, Innsbruck, Austria).

For breath measurements, expiratory and inspiratory phases were recognized by means of a custom-made data processing algorithm called "breath tracker" (MATLAB version 7.12.0.635, R2011a). The function of the algorithm has been described previously [17]. Briefly, an endogenous compound that has a sufficiently abundant signal intensity in expiration is used as a tracker to differentiate between alveolar and inspired phases. Acetone, isoprene, or carbon dioxide is usually used for this purpose. Expiratory and inspiratory phases determined by means of the algorithm were then applied to all *m/z* of interest.

Results

Ion-funnel characterization

$H_3O^+·(H_2O)_n$ ($n = 0$, 1, and 2) reagent ions

Figure 2 (a, b) shows the variation of H_3O^+, protonated water clusters, and O_2^+ and NO^+ measured intensities as function of RF voltage. The signal intensities of H_3O^+, $H_2O·H_3O^+$, and O_2^+ are too high to be measured directly because of detector saturation. Therefore, the signal intensities at $m/z = 21$ corresponding to $H_3{}^{18}O^+$, at $m/z = 39$ corresponding to $H_2O·H_3{}^{18}O^+$ and at $m/z = 34$ corresponding to $^{18}O^{16}O^+$ were recorded and corrected by the natural isotope abundances.

H_3O^+ intensity showed its maximum value at RF = 80 V_{p-p} under dry conditions (Fig. 2 (a)) and at RF = 120 V under humid conditions (Fig. 2 (b)). As the RF voltage decreases, the H_3O^+ signal intensity decreases. At the same time, an increase of the water cluster intensities was observed with decreasing RF voltage. $H_3O^+·H_2O$ showed its maximum value at RF = 40 V_{p-p} under dry conditions and at RF = 80 V_{p-p} under humid conditions. Nevertheless, at RF = 40 V_{p-p} under humid conditions, $H_3O^+·H_2O$ showed a higher intensity than H_3O^+.

The protonated water trimer was only observed under humid conditions and at RF = 40 V_{p-p}. Intensities of parasitic ions O_2^+ and NO^+ only increased only at high RF voltages (> 160 V_{p-p}) under both dry and humid conditions. $H_3O^+·(H_2O)_2$ and O_2^+ intensities were up to six orders of magnitude smaller compared with that of the protonated water dimer.

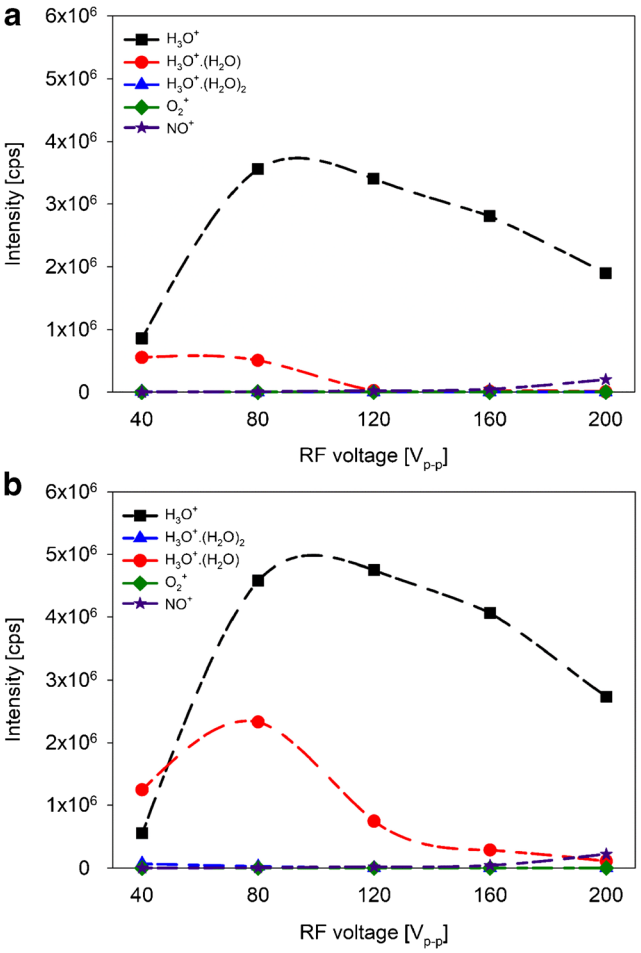

Fig. 2 Ion intensities in counts per second (cps) of the water reagent ions ($H_3O^+·(H_2O)_n$, $n = 0$, 1, and 2) and parasitic ions O_2^+ and NO^+ present in the DT under dry (**a**) and humid (**b**) conditions as a function of RF voltage. DC was 13.5 V/cm and E_{drift} was 66 V/cm

Figure 3 (a, b) shows the variation of the H_3O^+ and $H_3O^+·H_2O$ intensities as function of DC field. Due to the large difference between the intensities of the two reagent ions, $H_3O^+·H_2O$ intensity is displayed on a second *Y*-axis. The H_3O^+ intensity increased with increasing DC field with its maximum value at DC = 27 V/cm under both dry (Fig. 3 (a)) and humid (Fig. 3 (b)) conditions. In contrast, $H_3O^+·H_2O$ showed its maximum value at DC = 22.5 V/cm under both dry and humid conditions.

ESM Fig. S1 (a, b) shows the variation of H_3O^+, protonated water clusters, and O_2^+ and NO^+ measured intensities as function of RF voltage at $E_{drift} = 48$ V/cm. ESM Fig. S2 (a, b) shows the variation of the H_3O^+ and $H_3O^+·H_2O$ intensities as function of DC voltage at $E_{drift} = 48$ V/cm. H_3O^+ intensity showed similar trends of those showed at $E_{drift} = 66$ V/cm. In contrast, substantial differences were found for the protonated

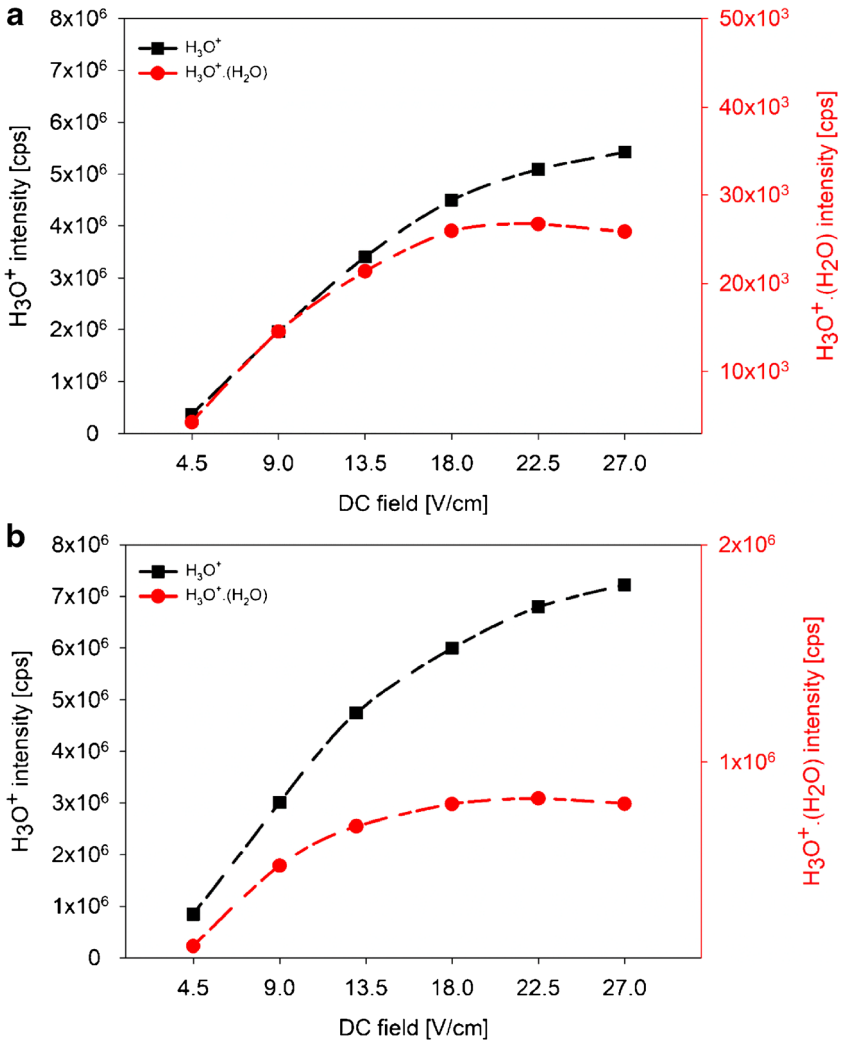

Fig. 3 Ion intensities in counts per second (cps) of H_3O^+ and $H_3O^+{\cdot}(H_2O)$ present in the DT under dry (**a**) and humid (**b**) conditions as a function of the DC field (V/cm). RF voltage was 120 $V_{p\text{-}p}$ and E_{drift} was 66 V/cm

water clusters. Under dry conditions (ESM Fig. S1 (a)) at RF = 40 $V_{p\text{-}p}$ and under humid conditions (ESM Fig. S1 (b)) at RF = 80 $V_{p\text{-}p}$, $H_3O^+{\cdot}H_2O$ became the most abundant reagent ion in the DT. Under humid conditions at RF = 40 $V_{p\text{-}p}$, $H_3O^+{\cdot}(H_2O)_2$ showed a higher intensity than H_3O^+.

In DC-mode at E_{drift} = 48 V/cm under humid conditions, $H_3O^+{\cdot}H_2O$ represent about 65% of the total water reagent ions. In contrast, under dry conditions, they represent about the 15% of the total water reagent ions. In DC-mode at E_{drift} = 66 V/cm, protonated water clusters are present in low concentrations under both dry and humid conditions. Under humid conditions, $H_3O^+{\cdot}H_2O$ represent about 8% of the total water reagent ions; under dry conditions, they represent about 1% of the total water reagent ions.

Effect of RF voltage and DC field on VOC signal intensities

Figure 4 shows effects of RF voltage (40–200 $V_{p\text{-}p}$) and DC field (4.5–27 V/cm) onto intensities of all investigated VOCs.

Figure 5 shows signal intensities of acetaldehyde, acetone, benzene, and dichlorobenzene as a function of RF voltage.

Intensities of acetaldehyde, methanol, ethanol, 2-propenal, and isoprene showed their maximum values at RF = 80 $V_{p\text{-}p}$. In contrast, intensities of acetone, acetonitrile, 2-butenal, and butanone showed their maximum values at RF = 120 $V_{p\text{-}p}$. Aromatic compounds, such as benzene, toluene, *o*-xylene, chlorobenzene, and α-pinene, showed their maximum intensities at RF = 160 $V_{p\text{-}p}$. Intensity of dichlorobenzene showed its maximum at RF = 200 $V_{p\text{-}p}$.

Figure 6 shows the measured intensities of the acetaldehyde, acetone, benzene, and dichlorobenzene as function of DC field. Intensities of most of the investigated compounds showed a substantial increase when the DC field was increased from 4.5 to 13.5 V/cm; when the DC voltage was further increased up to 27 V/cm, they showed variations < 10%. This was with the exception of dichlorobenzene which showed steadily increasing intensity with increasing DC voltage, with its maximum at DC = 27 V/cm.

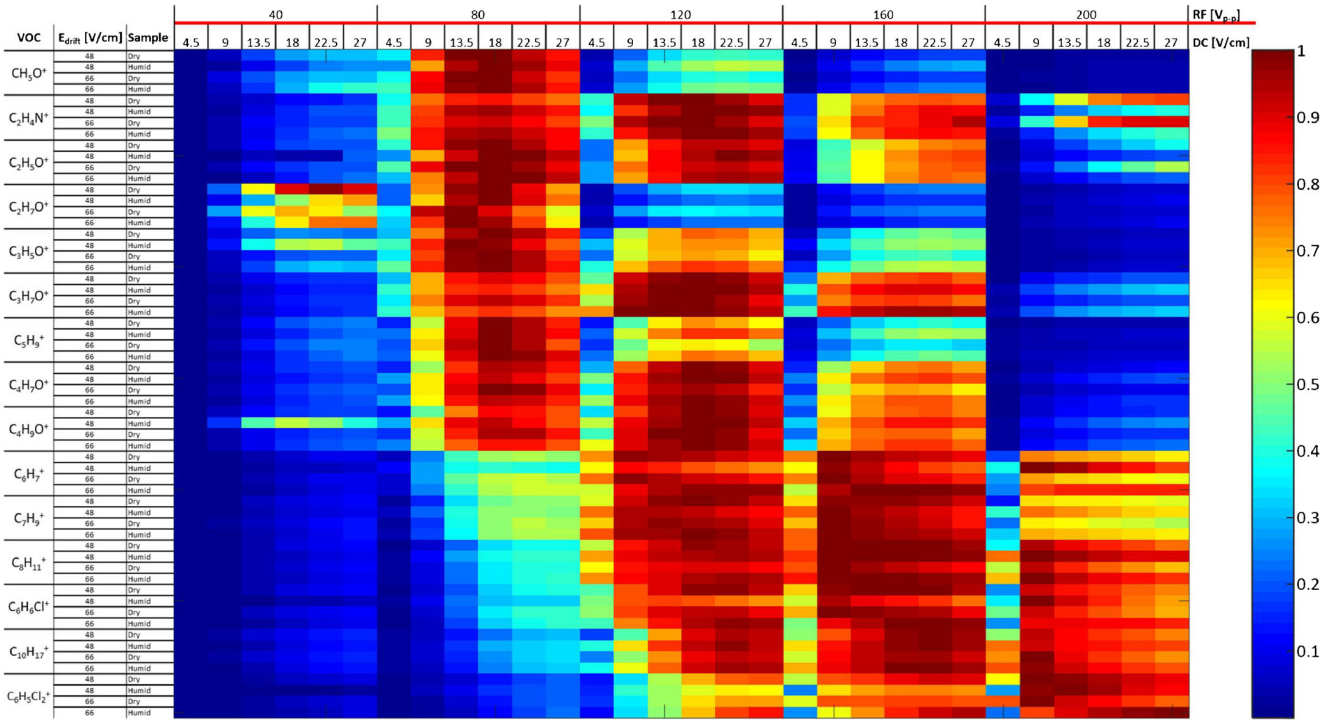

Fig. 4 Effect of RF voltage (40–200 $V_{p\text{-}p}$) and DC field (4.5–27 V/cm) on VOC intensities. The whole experiment was conducted at E_{drift} of 48 V/cm and 66 V/cm, with dry and humid samples (humidity of 47 g m^{-3}). Data were normalized to maximum values to emphasize relative changes

Table 1 compares sensitivities and limits of detection (LODs) calculated for DC-mode and RF-mode for all the investigated VOCs. Sensitivity is expressed as ion count rate per second per part-per-billion volume mixing ratio of supplied analyte (cps/ppbV). LODs were calculated for 1 s of integration time using the "3σ method" with σ being the standard deviation of the background noise level [18]. The DC-mode data were collected at E_{drift} = 66 V/cm, RF = 0 $V_{p\text{-}p}$, and DC = 66 V/cm under humid conditions. The RF-mode data were collected at E_{drift} = 66 V/cm, RF = 120 $V_{p\text{-}p}$, and DC = 13.5 V/cm under humid conditions. At these conditions, switching from DC-mode to RF-mode led to an improvement in sensitivity of about 1 order of magnitude for most of the investigated compounds with the exception of methanol, ethanol, and dichlorobenzene. In contrast, only an improvement by a factor of 2–4 was observed for the LODs in RF-mode compared with DC-mode.

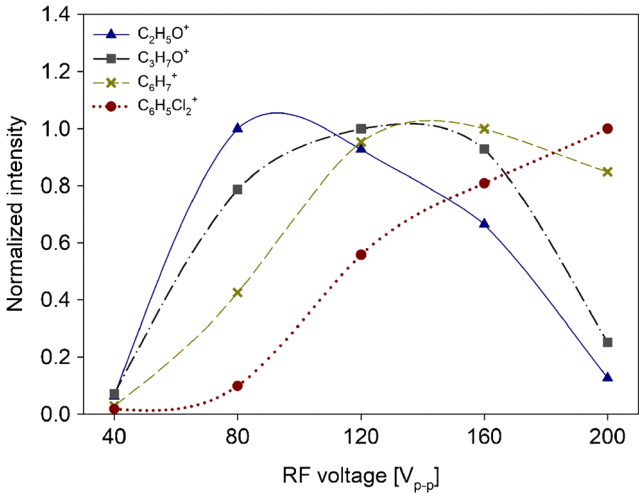

Fig. 5 Intensities of acetaldehyde (blue triangle), acetone (grey square), benzene (yellow cross), and dichlorobenzene (red round) as function of RF voltage. DC field was 13.5 V/cm and E_{drift} was 66 V/cm. Data were normalized onto respective maximum values to emphasize the relative changes

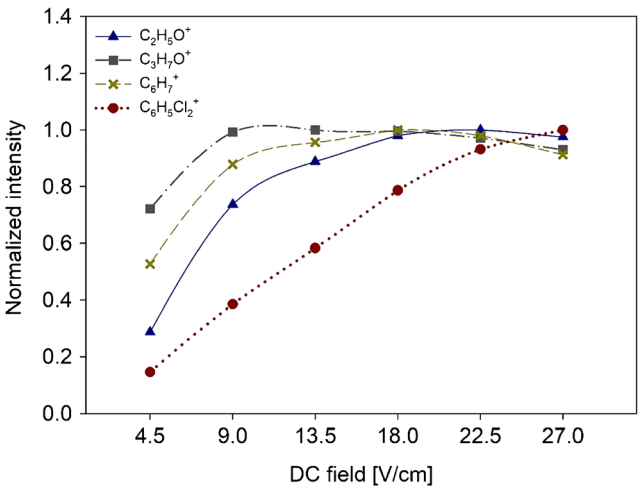

Fig. 6 Intensities of acetaldehyde (blue triangle), acetone (grey square), benzene (yellow cross), and dichlorobenzene (red round) as function of DC voltage. RF voltage was 120 $V_{p\text{-}p}$ and E_{drift} was 66 V/cm. Data were normalized onto respective maximum values to emphasize the relative changes

Table 1 Comparison of sensitivities and LODs for DC-mode and RF-mode of all investigated VOCs. LODs were calculated for 1 s of integration time. The DC-mode data were collected at E_{drift} = 66 V/cm, RF = 0 V_{p-p}, and DC = 66 V/cm under humid conditions. The RF-mode data were collected at E_{drift} = 66 V/cm, RF = 120 V_{p-p}, and DC = 13.5 V/cm under humid conditions

Compound (*m/z*)	Sensitivity (cps $ppbV^{-1}$)			LOD (ppbV)		
	DC-mode	RF-mode	RF-mode/DC-mode	DC-mode	RF-mode	RF-mode/DC-mode
Methanol (33)	27.5	116.5	4.2	0.735	0.432	1.7
Acetonitrile (42)	46.7	418.2	8.6	0.337	0.11	3.1
Acetaldehyde (45)	56.5	458.1	8.1	0.66	0.245	2.7
Ethanol (47)	3.4	18.2	5.3	12.319	8.31	1.5
2-Propenal (57)	43	406.3	9.4	0.301	0.146	2.1
Acetone (59)	103.6	1008.3	9.7	0.26	0.09	2.9
Isoprene (69)	10	120.8	12.1	1.226	0.463	2.6
2-Butenal (71)	65.8	701	10.7	0.163	0.069	2.4
2-Butanone (73)	66.1	750.9	11.4	0.325	0.104	3.1
Benzene (79)	39.2	423.6	10.8	0.22	0.056	3.9
Toluene (93)	50.2	620.3	12.4	0.144	0.06	2.4
o-Xylene (107)	65.4	747.4	11.4	0.115	0.03	3.8
Chlorobenzene (113)	38.2	413.7	10.8	0.155	0.061	2.5
α-Pinene (137)	25.7	252.1	9.8	0.265	0.087	3
1,2-Dichlorobenzene (147)	43.9	264.7	6.0	0.087	0.044	2

Application in human breath samples

In a proof-of-concept study, the instrument operating both in DC-mode and RF-mode was applied for breath analysis of 21 human healthy subjects.

Table 2 contains the list of VOCs that could be detected in exhaled breath. Compounds that could only be detected in RF-mode are labelled using bold italic text. Concentrations of these compounds were below the LODs in DC-mode and above the LODs in RF-mode.

Concentrations and LODs and LOQs were calculated applying the kinetic theory [19, 20].

Discussion

Incorporation of a modular IF adjacent to the DT led to a substantial improvement in sensitivity and LODs of the PTR-ToF-MS instrument. Improved sensitivities allowed the detection of a broader range of VOCs from human breath samples in real-time.

Intensities determined for water reagent ions ($H_3O^+ \cdot (H_2O)_n$, n = 0, 1, and 2) and for protonated VOCs showed a considerable dependence on RF voltage and DC field applied to the IF region. Highest intensities for H_3O^+ were observed in the RF range 80–120 V_{p-p} and at DC = 27 V/cm. At lower and higher RF voltages, the focusing effect of the funnel was lost and ion transfer was less efficient. High RF voltages increase the kinetic energy of molecules. As binding forces in the water clusters are weak when compared with normal chemical bonding, this will lead to collisional decomposition of water clusters long before fragmentation of chemical compounds occurs. Higher H_3O^+ intensities in humid samples were most probably due to back diffusion of sample gas from the DT into the ion source generating additional H_3O^+ [15, 21, 22]. O_2^+ and NO^+ were present in low intensities and were observed in RF-mode only at high RF voltage (> 160 V_{p-p}) as a result of improved ion transmission [13, 23].

VOCs showed maximum intensities at substance-specific DC field and RF voltage. In agreement with IF theory, cut-off values occurred at low (< 50 Vp-p) RF voltages for all VOCs and at high (> 160 Vp-p) RF voltages for low-mass compounds (m/z < 90). At low RF voltages, the focusing effect of IF is lost for low and high masses. In a substance-specific way, higher masses show maximum transmission at high (> 120 Vp-p) RF voltages due to the dependency of effective potential onto m/z. Decreasing transmission of low masses at high RF voltages is attributed to diffusional loss of molecules due to the relatively high kinetic energy of low-mass molecules under these conditions [12, 24]. In addition, fragmentation may contribute to this effect, as we observed a 10% increase in acetaldehyde fragmentation with increasing RF voltage. Up to 50% fragmentation was reported by Barber et al. under similar conditions.

In contrast to oxygen-containing aliphatic substances, aromatic compounds showed increasing ion yields of the protonated monomers even at high RF voltages where non-aromatic substances already exhibited decreasing intensities. Enhanced

Table 2 List of VOCs that could be detected from exhaled breath in real-time. Compounds that could only be detected in RF-mode compared with DC-mode are labelled using bold italic text. The DC-mode data were collected at E_{drift} = 66 V/cm, RF = 0 V_{p-p}, and DC = 66 V/cm. The RF-mode data were collected at E_{drift} = 66 V/cm, RF = 120 V_{p-p}, and DC = 13.5 V/cm

Peak number	Measured mass (*m*/*z*)	Exact mass (*m*/*z*)	Mass accuracy (ppm)	Empirical formula	Concentration range (ppbV)	LOD DC-mode (ppbV)	LOD RF-mode (ppbV)	LOQ DC-mode (ppbV)	LOQ RF-mode (ppbV)	Potential compound
1	33.031	33.033	− 60.55	CH_4O^+	37.401–364.033	0.735	0.432	2.426	1.426	Methanol
2	42.031	42.034	− 71.37	$C_2H_4N^+$	2.443–76.18	0.337	0.11	1.112	0.363	Acetonitrile
3	47.046	47.049	− 63.76	$C_2H_6O^+$	12.332–74.094	12.319	8.31	40.653	27.423	Ethanol
4	59.053	59.049	67.74	$C_3H_7O^+$	133.39–1043.864	0.26	0.09	0.858	0.297	Acetone
5	61.031	61.028	49.16	$C_2H_5O_2^+$	4.328–40.298	0.865	0.763	2.855	2.518	Acetic acid
6	63.028	63.026	31.73	$C_2H_7S^+$	1.435–10.894	0.203	0.12	0.67	0.396	Dimethylsulfide
7	69.072	69.070	28.96	$C_5H_9^+$	31.166–193.766	1.226	0.463	4.046	1.528	Isoprene
8	71.052	71.049	42.22	$C_4H_7O^+$	0.25–6.957	0.163	0.069	0.538	0.228	2-Butenal
9	73.069	73.065	54.75	$C_4H_9O^+$	1.26–3.769	0.325	0.104	1.073	0.343	Butanone
10	79.052	79.054	− 25.30	$C_6H_7^+$	0.309–8.362	0.22	0.056	0.726	0.185	Benzene
11	87.073	87.080	− 80.39	$C_5H_{11}O^+$	0.62–1.643	0.349	0.272	1.152	0.898	Pentanal
12	89.052	89.060	− 89.83	$C_4H_9O_2^+$	0.469–5.146	0.203	0.126	0.67	0.416	Ethylacetate
13	93.073	93.070	32.23	$C_7H_9^+$	0.413–7.61	0.144	0.06	0.475	0.198	Toluene
14	137.129	137.132	− 21.88	$C_{10}H_{17}^+$	0.911–12.413	0.265	0.087	0.875	0.287	Limonene
15	***49.008***	***49.010***	***− 40.81***	***CH_5S^+***	***0.194–2.452***	***0.354***	***0.073***	***1.168***	***0.241***	***Methyl mercaptan***
16	***68.053***	***68.049***	***58.78***	***$C_4H_6N^+$***	***0.067–0.558***	***0.141***	***0.032***	***0.465***	***0.106***	***Pyrrole***
17	***80.047***	***80.049***	***− 24.98***	***$C_5H_6N^+$***	***0.068–0.713***	***0.215***	***0.039***	***0.71***	***0.129***	***Pyridine***
18	***85.063***	***85.065***	***− 23.51***	***$C_5H_9O^+$***	***0.331–1.218***	***0.323***	***0.266***	***1.066***	***0.878***	***3-Penten-2-one***
19	***91.055***	***91.057***	***− 21.96***	***$C_4H_{11}S^+$***	***0.168–1.091***	***0.263***	***0.092***	***0.868***	***0.304***	***Methyl propyl sulfide***
20	***97.061***	***97.065***	***− 41.21***	***$C_6H_9O^+$***	***0.248–3.97***	***0.334***	***0.13***	***1.102***	***0.429***	***2,5-Dimethylfuran***
21	***101.055***	***101.060***	***− 49.48***	***$C_5H_8O_2^+$***	***0.218–0.737***	***0.261***	***0.195***	***0.861***	***0.644***	***Coffee furanone***
22	***105.056***	***105.054***	***19.04***	***$C_4H_9O_3^+$***	***0.142–0.785***	***0.242***	***0.12***	***0.799***	***0.396***	***β-Hydroxybutyric acid***
23	***106.069***	***106.065***	***37.71***	***$C_7H_8N^+$***	***0.017–0.094***	***0.088***	***0.05***	***0.29***	***0.165***	***Vinylpyridine***
24	***108.074***	***108.081***	***− 64.77***	***$C_7H_{10}N^+$***	***0.063–0.242***	***0.202***	***0.077***	***0.667***	***0.254***	***o-Toluidine***
25	***118.065***	***118.058***	***59.29***	***$C_8H_8N^+$***	***0.051–0.569***	***0.084***	***0.014***	***0.277***	***0.046***	***Indole***
26	***125.088***	***125.096***	***− 63.95***	***$C_8H_{13}O^+$***	***0.042–0.343***	***0.168***	***0.089***	***0.554***	***0.294***	***Acetylcyclohexene***
27	***129.077***	***129.070***	***54.23***	***$C_{10}H_9^+$***	***0.07–0.153***	***0.161***	***0.059***	***0.531***	***0.195***	***Naphthalene***
28	***133.058***	***133.065***	***− 52.61***	***$C_9H_9O^+$***	***0.042–0.142***	***0.12***	***0.04***	***0.396***	***0.132***	***Cinnamaldehyde***
29	***135.088***	***135.080***	***59.22***	***$C_9H_{11}O^+$***	***0.074–0.317***	***0.167***	***0.075***	***0.551***	***0.248***	***Cinnamyl alcohol***
30	***149.055***	***149.060***	***− 33.54***	***$C_9H_9O_2^+$***	***0.095–0.561***	***0.223***	***0.126***	***0.736***	***0.416***	***Cinnamic acid***
31	***151.110***	***151.112***	***− 13.24***	***$C_{10}H_{15}O^+$***	***0.054–0.233***	***0.118***	***0.049***	***0.389***	***0.162***	***Carvone***

ability of aromatic systems to stabilize ionic states may explain efficient generation of molecule ions without losses through fragmentation even at high RF voltages. This hypothesis is further confirmed by dichlorobenzene showing an almost linear increase with increasing RF voltages, most probably due to the additional charge-stabilizing effects of the chlorine atoms.

In contrast to previous setups, with the IF used in this study, sensitivity increases were rather uniform, i.e. approximately one order of magnitude for all investigated compounds. This is a strong indicator that the IF does not have major effects onto the ion chemistry within the DT itself. Therefore, the advantages of PTR-MS, e.g. quantification without calibration, are preserved. Although Brown and Barber et al. reported relative increases in sensitivity of 1–2 orders of magnitude for single compounds (acetaldehyde 45×, acetone 200×), absolute sensitivities for a broad range of compounds reported in our study were in general higher, e.g. 10 times higher for methanol and 2 times higher for acetaldehyde. Higher improvements in relative sensitivity as well as higher fragmentation rates

reported by Barber and Brown et al. can thus be attributed to different geometries of IF and DT in their instruments.

Characterization and optimization of DT conditions, RF voltage, and DC field and effects of humidity are of general importance for any IF-PTR-ToF instrument and can therefore be beneficial for the whole community [20]. In addition, the modular IF described in this study can be implemented into several PTR-ToF-MS instruments.

The impact of the IF onto quantification can be seen when LODs and LOQs are looked upon. As the applied IF will improve transmission of the ions, in parallel to the desired effects, increased ion yields will also induce growing background noise. Thus, the "raw" gain in ion counts will not directly translate into identical improvements of LODs and LOQs. LODs and LOQs substantially depend on noise inherent in a PTR-MS signal. This noise can be described by a Poisson distribution: the 1σ error in a measurement that is derived from counting a total of N ions is $\sqrt{N \cdot \tau^{-1}}$, with τ being the integration time [19, 25, 26]. Taking this into account, LODs and LOQs could effectively be improved by a factor of 2–4 when the instrument was switched from DC-mode to RF-mode. Hence, just determining increases in ion yields may lead to overestimation of the instrument performances for quantitative analysis. For real-life applications, e.g. trace gas analysis in breath, LODs and LOQs have to be determined to take into account all effects of DC field and RF voltage applied within the IF.

Especially in diseased states, breath VOC concentrations may change quickly and abruptly. Hence, only real-time monitoring can provide complete and comprehensive information from breath VOC analysis [27–29]. PTR-ToF-MS with integration time of ≥ 200 ms enables breath-resolved continuous monitoring of breath volatiles. In this pilot study, the range of detectable volatile substances was significantly enlarged through application of IF technology.

Conclusion

The spectrum of detectable VOCs in real-time breath analysis was considerably enhanced through the application of IF technology. The IF can be tuned in order either to obtain the best operating conditions for a specific compound of interest or to realize operating conditions which represent the best compromise for the acquisition of a large number of compounds. In contrast to previous setups, the IF used in this study did not have major effects onto ion chemistry within the DT itself and therefore offers optimal conditions for VOC screening in biomedical applications.

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Compliance with ethical standards

Conflict of interest Felix Piel and Philipp Sulzer work for IONICON Analytik GmbH, which provided the instrument used in this study. The other authors declare that they have no conflict of interest.

Research involving human participants All experiments were performed in accordance with the guidelines laid down in the Declaration of Helsinki and approved by the ethics committee at University Medical Center Rostock.

Informed consent Informed consent was obtained from all volunteers.

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Extending PTR based breath analysis to real-time monitoring of reactive volatile organic compounds†

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Reactive exhaled volatile organic compounds (VOCs) such as nitrogen- and sulfur-containing substances may be related to diseases, metabolic processes and bacterial activity. As these compounds may interact with any surface of the analytical system, time-resolved monitoring and reliable quantification is difficult. We describe a proton transfer reaction time-of-flight mass spectrometry (PTR-ToF-MS) based analytical method for direct breath-resolved monitoring of reactive compounds. Aliphatic amines were used as test substances. Matrix adapted gas standards were generated by means of a liquid calibration unit. Calibration conditions were adapted in terms of materials, temperature and equilibration time. PTR-ToF-MS conditions were optimized in terms of inlet materials, transfer line and drift tube temperature and drift tube reduced electric field (*E*/*N*). Optimized PTR conditions in combination with inert materials and high temperatures considerably reduced the interactions of compounds with the surfaces of the analytical system. Good linearity (R^2 > 0.99, RSDs < 5%) with LODs between 0.15 ppbV and 1.23 ppbV and LOQs between 0.24 ppbV and 1.94 ppbV could be achieved. The method was then applied to breath-resolved monitoring of reactive compounds in 17 healthy subjects after high and low oral protein challenge. Exhaled concentrations of trimethylamine, indole, methanethiol, dimethylsulfide, acetone, 2-propanol, 2-butanone and phenol showed significant changes after protein intake. Methanethiol concentrations increased 6-fold within minutes after the protein intake. Optimization of methods and instrument design enabled reliable breath-resolved PTR-MS based analysis of exhaled reactive VOCs in the sub-ppbV range. Continuous *in vivo* monitoring of exhaled amines and sulphur containing compounds may provide novel non-invasive insight into endogenous and gut bacteria driven protein metabolism.

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1. Introduction

Analysis of volatile organic compounds (VOCs) in human breath raised high expectations for non-invasive clinical diagnosis as well as for therapeutic, metabolic and pathophysiological monitoring.[1–5] Breath VOCs are exhaled within minutes or even seconds after their generation and may, therefore, mirror the immediate responses of biochemical processes in the whole body. Exhaled N- and S-containing compounds may be related to diet, diseases, metabolic processes and bacterial activity.[6–11] Despite their high relevance for biomedical research and clinical applications, these compounds are rarely described in the literature. This is because their identification and quantitation in breath at trace levels (pptV–low ppbV range) is strongly hampered by their high reactivity.

The most widely used technique for the determination of N- and S-containing compounds in breath is gas chromatography (GC) coupled with mass spectrometry (MS) preceded by a concentration/extraction step. Simenhoff *et al.*[12] used a GC-MS based analytical method for the determination of dimethylamine and trimethylamine in the exhaled breath of patients with end-stage renal disease (ESRD). In this study, aliphatic amines were absorbed in hydrochloric solution. Wzorek *et al.*[13] compared Solid Phase Microextraction (SPME) and Thermal Desorption (TD) techniques for GC-MS determination of dimethylamine and trimethylamine in breath for medical diagnostic purposes. Grabowska-Polanowska *et al.*[14] applied TD-GC-MS for the determination of trimethylamine in the breath of patients suffering from chronic kidney disease. Ochiai *et al.*[15] developed a GC-MS method for the determination of trace volatile sulphur compounds (VSCs) including methanethiol, dimethyl sulfide (DMS) and dimethyl disulfide (DMDS) in which they used a large volume preconcentration

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technique prior to capillary GC–MS analysis. Although they are highly compound specific and allow punctual sampling, these analytical methods are labour intensive and time-consuming as extraction, and pre-concentration, derivatization, and elution steps may require hours. In addition, the high reactivity of N- and S-containing compounds induces problems in sample preparation and chromatographic separation resulting in memory effects and peak tailing.[13,15]

Direct mass spectrometric techniques, such as Proton Transfer Reaction Mass Spectrometry (PTR-ToF-MS) and Selected Ion Flow Tube Mass Spectrometry (SIFT-MS), represent an innovative approach to overcome the above-mentioned problems. As these techniques enable continuous monitoring of VOCs, even rapid changes in breath concentration profiles occurring during physiological and metabolic processes or inflammation could be traced.[16–21]

To take advantage of the unique features of breath biomarkers, a quantitative, continuous and time resolved analysis of these compounds would be highly desirable. As reactive N and S containing VOCs in low concentrations cannot be analyzed by standard techniques reliably,[22,23] the aim of the present study is to establish and optimize a PTR-ToF-MS method for breath-resolved quantitative analysis of these reactive compounds. The optimized method was then applied in a proof of concept study consisting of an oral protein challenge. The following questions were addressed in detail:

• Is breath-resolved monitoring of N- and S-containing compounds possible by means of PTR-ToF-MS?

• How can these compounds be reliably quantified in breath?

• Do concentrations of N- and S-containing compounds change after a protein diet?

2. Experimental

The PTR-ToF analytical conditions for time-resolved analysis of reactive compounds were optimized using highly reactive aliphatic amines as test substances. Methyl-, dimethyl- and trimethyl-amines were analyzed under different PTR transfer line and PTR drift tube temperature conditions and at different reduced electric fields (E/N) of the PTR drift tube. The influence of these factors on the responses and sensitivity of PTR-ToF was systematically evaluated.

2.1. Chemicals and materials

Aqueous solutions of methylamine (40%), dimethylamine (40%) and trimethylamine (25%) were purchased from Sigma-Aldrich (Saint Louis, Missouri, USA). Indole was purchased from TCI Chemicals (Tokyo, Japan). Dimethylsulfide was purchased from Merck Millipore (Burlington, Massachusetts, USA). Acetone was purchased from Ionicon Analytik GmbH (Innsbruck, Austria). 2-Pentanone and phosphate buffer solution, pH 7.2, were purchased from Honeywell Fluka™ (Morris Plains, New Jersey, USA). Nitrogen with a purity of 5.0 (*i.e.* 99.999%) was purchased from Linde (Vienna, Austria).

2.2. Measurements

All investigations on standards and breath were carried out using a PTR-ToF-MS-8000 (Ionicon Analytik GmbH, Innsbruck, Austria). The working principle and operating conditions of the instrument have been described in several studies.[24,25] Concisely, the soft ionisation of VOCs is based on a non-dissociative proton transfer reaction [$VOC + H_3O^+ \rightarrow (VOC)H^+ + H_2O$], which ionises VOCs with a higher proton affinity than water. Protonated VOCs are then detected in a high resolution reflectron time-of-flight mass spectrometer (ToFwerk AG, Thun, Switzerland) according to their mass to charge (m/q) ratio. There is no requirement for pre-concentration and ambient air can be used as buffer gas. After every 60s of measurement, a new file was automatically recorded and the mass scale was calibrated by means of the following masses: H_3O^+-isotope: 21.022 Th; NO^+: 29.998 Th; protonated acetone: 59.049 Th. PTR-ToF time resolution was set to 200 ms for all experiments.

For method optimization, amine gas standards were generated by means of a liquid calibration unit (LCU; Ionicon Analytik, Innsbruck, Austria). The working principle of the LCU involves the introduction of a liquid standard solution into a carrier gas stream, which is forced through a small orifice and sprayed into an evaporation chamber at a defined temperature. This results in immediate evaporation. The generated gaseous standard mixture can then be measured or collected directly at the output of the evaporation chamber. Two liquid ports (1–50 μl min^{-1}), three gas ports (carrier gas (1–1000 ml min^{-1}) and two additional gas ports (1–100 ml min^{-1})), controlled by mass flow controllers, enable the generation of complex standard mixtures. VOC standards can be prepared from either liquid solutions or gases, or even from both at the same time. In addition, defined amounts of humidity can be achieved by adding pure water *via* one of the two liquid ports.[26]

Nitrogen was used as the carrier gas and the LCU flow was kept constant at 1000 ml min^{-1} for all experiments. Aliphatic amine stock solutions were prepared in ultrapure water (HPLC grade) at physiological pH 7.4 by adding 5 mL of phosphate buffer solution at pH 7.2. The absolute humidity in the gas phase was 47 g m^{-3}. Sampling was performed in continuous mode *via* a 1.5 m Silcosteel® transfer line that was directly connected to the outlet of the LCU. The sampling flow rate was 20 ml min^{-1}. Stainless steel Sulfinert® coated tubes were used for the PTR inlet system (T-pieces and capillaries connecting the two T-pieces) and for the LCU (evaporation chamber and capillaries) in order to have the most inert surface conditions as possible to prevent N- and S-containing compounds from reacting. Due to the high voltage applied in the drift tube, it was not possible to use steel materials for the inlet tube directly connected to the drift tube. An electrically isolated polyether ether ketone (PEEK) tube was used for this purpose.

2.3. Data processing

Compounds were identified using their protonated masses. All compounds were determined in counts per seconds (cps) and,

to account for possible variations of the reagent ion signal, their intensities were normalised to the primary ion (H_3O^+) counts.

Both breath and standard files were processed using PTR-MS viewer software (IONICON Analytik GmbH, version 3.2.8). For breath files, the mass scale was recalibrated by using the following masses: H_3O^+-isotope: 21.022 Th; NO^+: 29.998 Th; protonated isoprene: 69.069 Th. Only compounds for which signals were higher than 3 times the blank signal were considered. In addition, compounds with end-tidal concentrations lower than the corresponding room air concentration plus the standard deviation of room air concentration were excluded (ESI Table 1†). Expiratory and inspiratory phases were recognized by means of a custom-made data processing algorithm called 'breath tracker' (MATLAB version 7.12.0.635, R2011a). The function of the algorithm has been described before.[27]

2.4. Optimization of PTR-ToF analytical conditions

Optimization of transfer line and drift tube temperature. The influence of PTR transfer line temperature and PTR drift temperature on PTR-ToF responses was investigated. Table 1 shows the experimental design conducted for temperature optimization. To investigate how the temperature of each region affects PTR-ToF responses, only the temperature of a single region was varied at a time. To avoid condensation effects, the temperatures were increased along the analytical system. The LCU temperature was 75 °C for all experiments.

For the experiments conducted at a drift temperature of 75 °C, the drift voltage was 606 V and the drift tube pressure was 2.3 mbar. For the experiments conducted at a drift temperature of 100 °C, the drift voltage was 560 V and the drift tube pressure was 2.3 mbar. For the experiments conducted at a drift temperature of 120 °C, the drift voltage was 485 V and the drift tube pressure was 2.1 mbar. Under all conditions, the E/N ratio was 138 Td.

For all experiments, amine gas standards with concentrations of 10 ppbV were analyzed for 10 minutes continuously. Three replicates were measured for each experimental set-up.

***E*/*N* optimization.** In this part of the study, we investigated the fragmentation patterns of each compound as a function of reduced electric field (E/N) in the drift tube. We kept N constant by maintaining the pressure and temperature of the drift tube at 2.1 mbar and 120 °C, respectively, whilst varying the voltage across the drift tube over the range of 400–570 V. This leads to values of E/N over the range of approximately 90–160 Td. For each setting, the amine gas standard was measured for one minute. Three replicates were measured for each experimental set-up.

Table 1 Experimental design conducted for temperature optimization for the PTR transfer line and PTR drift tube. The LCU temperature was kept constant at 75 °C for all experiments

	T LCU (°C)	*T* transfer line (°C)	*T* drift tube (°C)
1	75	75	75
2	75	75	100
3	75	75	120
5	75	100	120
4	75	130	120

2.5. Method validation

Calibrations for methylamine, dimethylamine and trimethylamine, indole, dimethylsulfide, phenol, acetone and 2-pentanone were conducted under the optimized conditions over a concentration range of approximately 0.5 ppbV to 100 ppbV. The exact concentrations varied slightly between the different compounds due to their original concentration in the stock solution. Seven concentrations were measured: 0.5, 1, 5, 10, 20, 50 and 100 ppbV. Three replicates were measured for each concentration.

The LCU temperature and PTR transfer line temperature were 130 °C. The drift tube temperature was 120 °C, the drift voltage was 485 V and the drift tube pressure was 2.1 mbar. The E/N ratio was 138 Td.

2.6. Real-time breath analysis after protein intake

All experiments were performed in accordance with the guidelines laid down in the Declaration of Helsinki, and approved by the ethics committee at University Medical Center Rostock. Informed consent was obtained from 17 healthy human subjects (aged between 20 and 47 years) before PTR-ToF-MS measurements. Before measurement, demographic parameters such as height, body weight, age, sex and smoking habits were recorded (ESI Table 2†).

After overnight fasting, subjects were asked to sit down on a chair and breathe evenly through a sterile mouth piece directly connected to the PTR transfer line by means of a t-piece in side stream mode. For each subject, breath was analysed at the baseline and immediately after the ingestion of a low or high oral protein challenge. Breath measurements were performed every 30 minutes for 5 hours. Apart from water, no drinks and food were permitted for the duration of the study. However, in order to avoid washout of VOCs produced in the mouth and in the oral cavity, water ingestion was allowed only until 20 minutes before each breath measurement. Low and high protein oral challenge used 6 g and 60 g of protein, respectively. A commercially available whey protein concentrate powder (Fresubin® Protein POWDER, Fresenius Kabi, Bad Homburg vor der Höhe, Germany) dissolved in 300 mL of water was used. 100 g of protein powder contained 360 kilocalories: 87 g protein, 1 g fat, <1 g carbohydrate and 1.38 g salts. 15 subjects completed the high protein test and 9 of them also completed the low protein test. 2 subjects only completed the low protein test.

The PTR transfer line temperature was 130 °C. The drift tube was operated at 120 °C under 2.1 mbar of pressure and at a drift voltage of 485 V. The resulting E/N ratio was 138 Td.

2.7. Statistical analysis

For each participant, breath VOC concentrations determined at 12 different times were selected to calculate changes from

the baseline. Statistically significant differences from the baseline were determined by means of repeated measures ANOVA on ranks in combination with Dunnett's method for multiple comparisons *versus* the control ($p \leq 0.05$). For each compound, concentrations between the low and high protein groups were compared at the corresponding times applying Mann–Whitney rank sum tests ($p \leq 0.05$ was considered as significant). Statistical analysis was performed using SigmaPlot (version 13) software.

3. Results

3.1. Optimization of PTR-ToF-MS

Optimization of the transfer line and drift tube temperature. Before PTR-ToF optimization, three different temperatures were tested for the LCU: 75 °C, 100 °C and 130 °C. After 7 minutes of measurements, the intensities of all aliphatic amines reached a plateau for all LCU temperature conditions. This is exemplified in Fig. 1 for trimethylamine.

For this reason, only intensities recorded after 7 minutes of measurement were compared for all PTR transfer line and PTR drift tube temperature conditions.

Fig. 2 shows the intensities for the different drift tube (a) and transfer line (b) temperature conditions. Intensities were normalized to the first measurement at 75 °C for both PTR drift tube and PTR transfer line temperature optimization.

All compounds showed an increase in intensities with increasing drift tube temperature. The most pronounced dependence was observed for dimethylamine which increased by 93% and 131% when PTR drift temperatures were increased to 100 °C and 120 °C, respectively. For methylamine and trimethylamine, the highest increase was observed when the PTR drift temperature was increased to 100 °C: by 60% for methylamine and by 51% for trimethylamine. They further increased by another 6% and 5%, respectively, when the PTR drift tube temperature was increased to 120 °C. Compounds did not show any dependence on the PTR transfer line temperature.

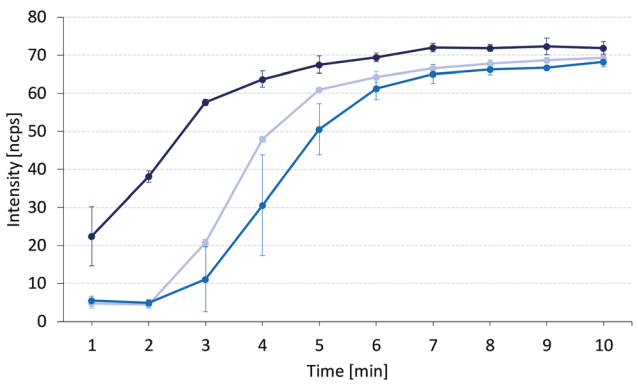

Fig. 1 Time course of PTR-ToF-MS responses for trimethylamine at different LCU temperatures of 75 °C (light blue), 100 °C (medium blue) and 130 °C (dark blue). PTR transfer line and PTR drift tube temperatures were kept constant at 130 °C and 120 °C respectively. The PTR drift tube voltage was 485 V and the drift tube pressure was 2.1 mbar resulting in an *E*/*N* ratio of 138 Td. Aliphatic amine concentrations in the gas standard were approximately 10 ppbV for each amine.

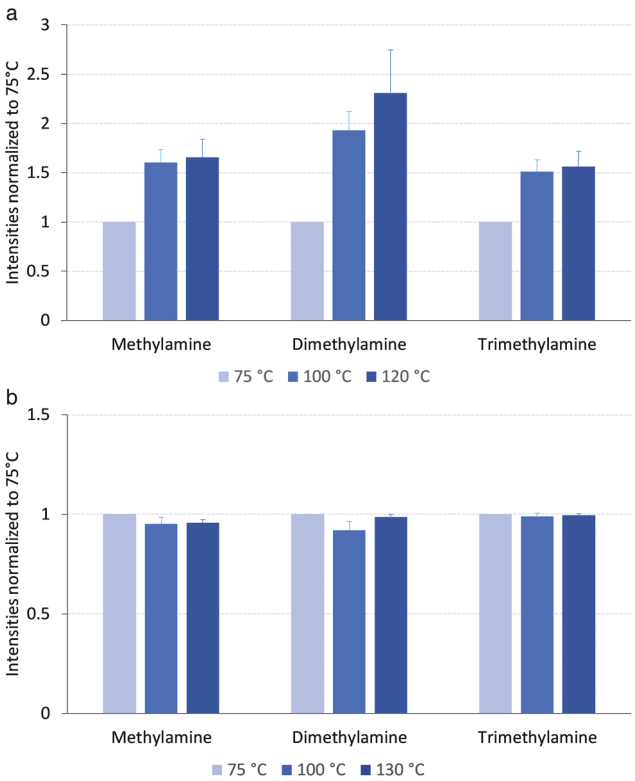

Fig. 2 Changes in PTR-ToF intensities for aliphatic amines after 7 minutes of measurement as a function of: (a) PTR drift tube temperature: 75 °C (light blue bars), 100 °C (medium blue bars), and 120 °C (dark blue bars) and (b) PTR transfer line temperature: 75 °C (light blue bars), 100 °C (medium blue bars), and 130 °C (dark blue bars). Aliphatic amine concentrations in the gas standard were approximately 10 ppbV for each amine. Intensities were normalized to the first measurement at 75 °C. The LCU temperature was kept constant at 75 °C.

***E*/*N* optimization.** Table 2 shows the product ions and ion branching ratios (%) for amines and reagent ions measured at *E*/*N* ratios of 100, 120, 140 and 160 Td. For any *E*/*N* ratio used, the main product ions observed for all amines were the protonated parent ion at *m*/*z* 32 for methylamine, at *m*/*z* 46 for dimethylamine and at *m*/*z* 60 for trimethylamine. No amine water clusters were observed for all three amines.

Increasing the *E*/*N* of the drift tube reduced the proportion of the hydrated hydronium clusters. Up to 120 Td, water clusters are dominant (51%) compared to H_3O^+ ions (49%). At 140 Td, H_3O^+ ions are the most abundant ions in the drift tube and although amine fragmentation occurs, it is not significant. For this reason, compounds were calibrated at the recommended *E*/*N* value of 138 Td.

3.2. Method validation

The method was validated under the optimized conditions: the LCU temperature and PTR transfer line temperature were 130 °C; the drift tube temperature was 120 °C, the drift voltage was 485 V and the drift tube pressure was 2.1 mbar; the *E*/*N* ratio was 138 Td.

Table 2 Product ions and ion branching ratios (%) for amines and reagent ions measured at *E*/*N* values of 100, 120, 140 and 160 Td. Percentage of branching ratios are shown in parentheses; *m*/*z* values are shown in plain text and product ion chemical formulas are given alongside. Note that the signal intensity of H_3O^+ and $H_2O{\cdot}H_3O^+$ are too large to be measured directly using PTR-ToF-MS. Therefore, the signal intensities at *m*/*z* = 21 corresponding to $H_3{}^{18}O^+$ and at *m*/*z* = 39 corresponding to $H_2O{\cdot}H_3{}^{18}O^+$ were recorded for the data presented in the table

	Product ions and branching ratios (%)			
Compound	*E*/*N* = 100	*E*/*N* = 120	*E*/*N* = 140	*E*/*N* = 160
Reagent ion	19 H_3O^+ (40) 37 $H_2O{\cdot}H_3O^+$ (60)	19 H_3O^+ (49) 37 $H_2O{\cdot}H_3O^+$ (51)	19 H_3O^+ (57) 37 $H_2O{\cdot}H_3O^+$ (43)	19 H_3O^+ (67) 37 $H_2O{\cdot}H_3O^+$ (33)
Methylamine	32 CH_5NH^+ (100)	32 CH_5NH^+ (100)	32 CH_5NH^+ (100)	32 CH_5NH^+ (100)
Dimethylamine	46 $C_2H_7NH^+$ (96) 44 $[C_2H_7N{-}H_2]H^+$ (4)	$C_2H_7NH^+$ (96) $[C_2H_7N{-}H_2]H^+$ (4)	$C_2H_7NH^+$ (94) $[C_2H_7N{-}H_2]H^+$ (6)	$C_2H_7NH^+$ (92) $[C_2H_7N{-}H_2]H^+$ (8)
Trimethylamine	60 $C_3H_9NH^+$ (94) 58 $[C_3H_9N{-}H_2]H^+$ (6)	$C_3H_9NH^+$ (90) $[C_3H_9N{-}H_2]H^+$ (10)	$C_3H_9NH^+$ (86) $[C_3H_9N{-}H_2]H^+$ (14)	$C_3H_9NH^+$ (82) $[C_3H_9N{-}H_2]H^+$ (18)

Table 3 Figures of merit for real time analysis of methylamine, dimethylamine, trimethylamine, indole, phenol, acetone and 2-pentanone

Compound	R^2	LOD (ppbV)	LOQ (ppbV)	RSD % (at 10 ppbV)
Methylamine	0.99	0.56	1.94	4.05
Dimethylamine	0.99	0.63	1.40	0.22
Trimethylamine	0.99	0.15	0.24	2.55
Dimethylsulfide	0.99	0.15	0.56	0.86
Indole	0.99	1.23	1.24	4.64
Phenol	1	0.31	0.37	3.66
Acetone	1	0.51	0.99	1.43
2-Pentanone	0.99	0.69	0.71	0.27

Table 3 shows the results for methylamine, dimethylamine and trimethylamine, indole, dimethylsulfide, phenol, acetone and 2-pentanone. Excellent linearities were obtained with correlation coefficients >0.99 for all analytes. Reproducibility was evaluated at the concentration of 10 ppbV by measuring five replicates. Relative standard deviations ranged from 0.22% (dimethylamine) to 4.64% (indole).

The limit of detection (LOD) and the limit of quantitation (LOQ) were calculated following the directives of IUPAC and the American Chemical Society's Committee on Environmental Analytical Chemistry *i.e.* the signal at the LOD and the signal at the LOQ are equal to the signal of the reagent blank plus three and ten times the standard deviation for the reagent blank,

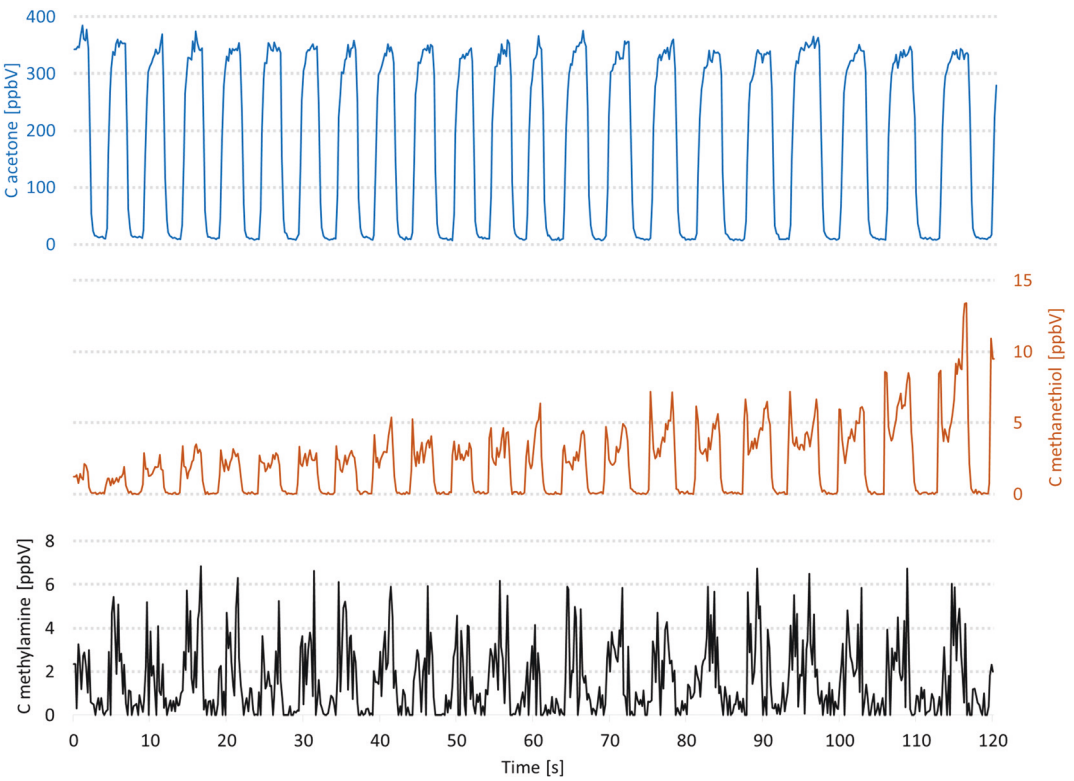

Fig. 3 Two minutes of continuous breath-resolved measurements of trimethylamine (black), methanethiol (orange) and acetone (blue) from a volunteer immediately after ingesting the protein meal.

respectively. LODs ranged from 0.15 ppbV (trimethylamine and dimethylsulfide) to 1.23 ppbV (indole) and LOQs ranged from 0.24 ppbV (trimethylamine) to 1.94 ppbV (methylamine).

3.3. Real-time breath analysis after protein intake

Fig. 3 shows two minutes of continuous breath-resolved measurement of trimethylamine, methanethiol and acetone from a volunteer immediately after ingesting the protein meal. Exhaled concentrations of endogenous substances such as acetone have the highest intensities in expiratory phases and lowest responses in inspiratory phases.

Fig. 4 shows the relative changes in breath VOC concentrations from all volunteers after the oral protein challenge. Methylamine and dimethylamine breath concentrations were

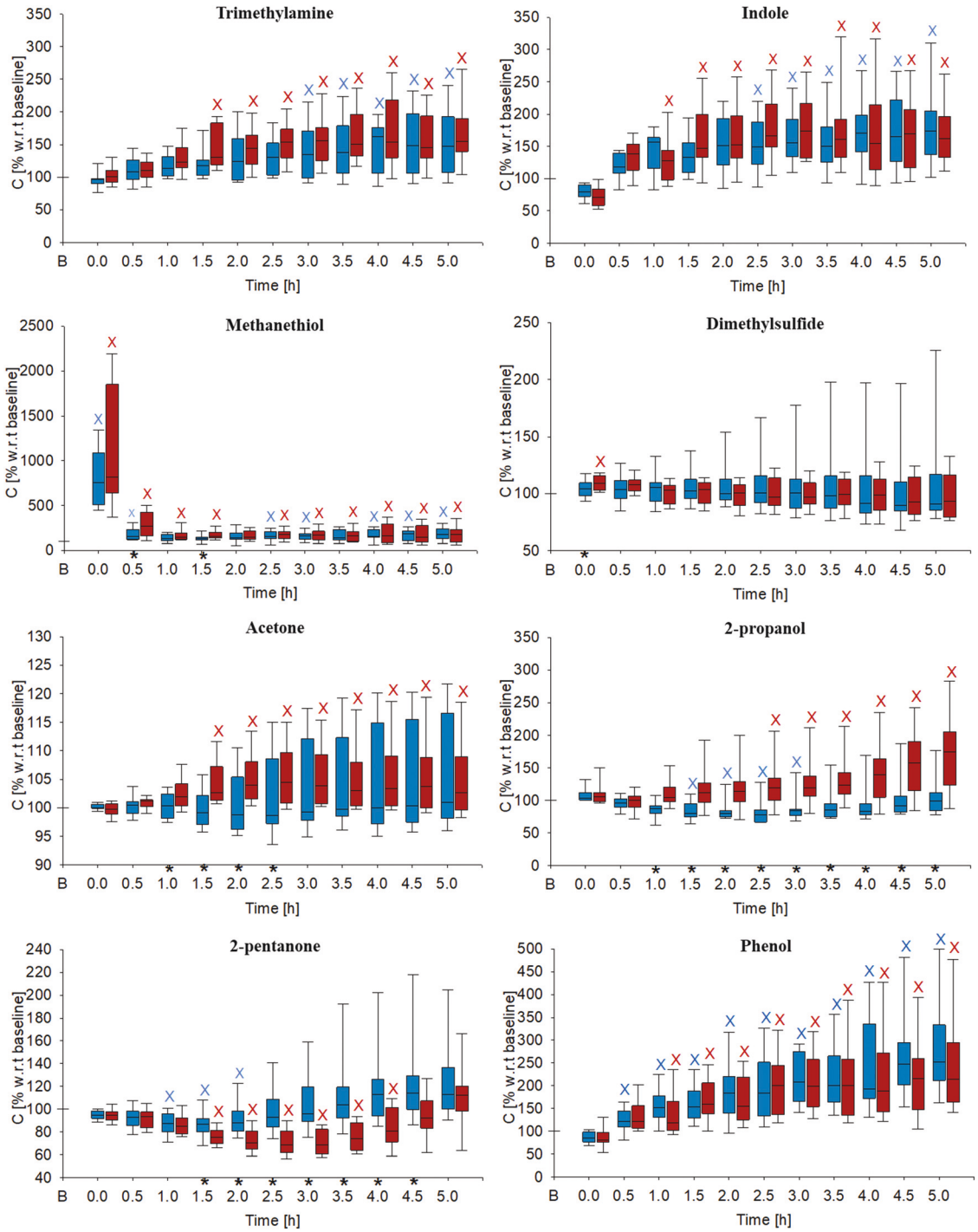

Fig. 4 Breath VOC concentration changes (in %) with respect to the baseline concentrations for the low protein group (blue boxes) and for the high protein group (red boxes). Statistically significant changes from the baseline are marked with a cross. Statistically significant differences between the groups are marked with an asterisk.

below the LOD in both treatment groups. In addition, detection of methylamine was hampered by the saturated O_2^+ peak at m/z 31.989. Trimethylamine concentrations ranged from 0.26 ppbV to 7.17 ppbV and steadily increased over time by 54% ($p \leq 0.001$) in the low protein group and by 67% ($p \leq 0.001$) in the high protein group. No statistically significant differences were found between the high and low protein groups.

Indole concentrations ranged from 1.33 ppbV to 6.06 ppbV and steadily increased over time by 81% ($p \leq 0.001$) after 5 hours in the low protein group; in the high protein group, there was a maximum increase of 83% ($p \leq 0.001$) after 3 hours. No statistically significant differences were found between the high and low protein groups.

Methanethiol concentrations ranged from 0.29 ppbV to 15.57 ppbV and showed a maximum increase immediately after protein intake in both testing groups: by 721% ($p \leq 0.001$) in the low protein group and by 1020% in the high protein group. Significant differences between the two groups were found at 30 min ($p = 0.035$) and at 1.5 hours ($p = 0.021$).

Dimethylsulfide concentrations ranged from 0.19 ppbV to 31.51 ppbV. In the low protein group, its concentrations did not show any statistically significant changes compared to the baseline concentrations. In contrast, in the high protein group, its concentrations significantly increased by 10% compared to the baseline concentrations immediately after protein intake ($p = 0.006$) and then returned to the baseline levels. DMS concentrations in the two groups showed a statistically significant difference immediately after protein intake ($p = 0.045$).

Acetone concentrations ranged from 276.74 ppbV to 418.62 ppbV. In the low protein group, its concentrations decreased by 2% after 2 hours and then steadily increased and reached 106% of the baseline concentration after 5 hours. In the high protein group, acetone concentrations significantly increased by 6% ($p < 0.001$) compared to the baseline concentration after 2 hours and then remained constant. Significant differences between the two groups were found at 1 hour ($p = 0.05$), 1.5 hours ($p = 0.002$), 2 hours ($p = 0.005$) and 2.5 hours ($p = 0.035$).

2-Propanol concentrations were in the range of 84.45–635.95 ppbV. In the low protein group, its concentrations decreased by 19% ($p < 0.001$) after 2.5 hours and then steadily increased up to 105% of the baseline concentration after 5 hours. In contrast, in the high protein group, its concentrations increased steadily over time, with a maximum increase of 72% ($p = 0.003$) after 5 hours. Significant differences between the two groups were found at 1 hour ($p = 0.002$), 1.5 hours ($p = 0.003$), 2 hours ($p = 0.006$), 2.5 hours ($p = 0.004$), 3 hours ($p = 0.004$), 3.5 hours ($p = 0.003$), 4 hours ($p = 0.005$), 4.5 hours ($p = 0.007$) and 5 hours ($p = 0.002$).

2-Pentanone concentrations ranged from 13.96 ppbV to 34.72 ppbV. In the low protein group, its concentrations decreased by 14% ($p = 0.003$) after 1.5 hours and then increased up to 125% of the baseline concentration after 5 hours. In the high protein group, its concentrations steadily decreased to 30% ($p < 0.001$) after 3 hours and then steadily increased over time up to the baseline level after 5 hours. Significant differences between the two groups were found at 1.5 hours ($p = 0.009$), 2 hours ($p < 0.001$), 2.5 hours ($p < 0.001$), 3 hours ($p < 0.001$), 3.5 hours ($p < 0.001$), 4 hours ($p < 0.001$) and 4.5 hours ($p = 0.026$).

Phenol concentrations ranged from 0.36 ppbV to 11.33 ppbV and steadily increased over time, up to 172% ($p < 0.001$) in the low protein group and up to 141% ($p < 0.001$) in the high protein group. No statistically significant differences were found between the high and low protein groups.

4. Discussion

Using inert materials and high temperatures for the inlet system and the drift tube, exhaled reactive N- and S-containing compounds could be monitored in a breath resolved manner at trace levels by means of PTR-ToF-MS. As breath VOC concentrations may change quickly and abruptly, only breath-resolved real-time monitoring provides comprehensive information.

The operating pressure in the drift tube (buffer gas number density, N) and the electric field strength (E) are extremely important parameters, more commonly combined and expressed in terms of the E/N value of the drift tube. The electric field serves the purpose of accelerating the ions, but collisions with the gas tend to slow them down. The net effect is that ions quickly adopt a steady-state velocity as they progress down the drift tube that is determined by the value of E/N. Increasing the E/N ratio results in more energetic collisions, which reduces the proportion of cluster ions such as $H_3O^+(H_2O)_n$ in the drift tube but at the same time increases the fragmentation of the analytes.[28] Depending on the E/N ratio, considerable fragmentation was observed for dimethylamine and trimethylamine. This is due to the loss of H_2 from the excited protonated parent molecule. Hydrogen elimination is more pronounced for trimethylamine than for dimethylamine. This is in agreement with the results shown by Španěl and Smith using selected ion flow tube mass spectrometry (SIFT-MS) and H_3O^+ as the reagent ion.[29]

The developed PTR-ToF method was highly sensitive, with LODs and LOQs considerably lower than those previously reported for a GC-MS based method by Wzorek *et al.*[13] PTR-ToF-MS does not require any sample preparation and immediate monitoring of reactive compounds is possible.

After the protein challenge, up to 20 different compounds could be detected in exhaled air. Some substances showed higher concentrations in room air (ESI Table 1†). As this study is focused on endogenous reactive compounds, we performed detailed investigation only on N, O and S containing substances having high alveolar concentrations. As one could expect for endogenous compounds, room air concentrations of these compounds were always lower than those of the expired ones. Profiles of selected exhaled volatile substances changed when volunteers ingested diets with different protein contents. Significant increases in exhaled trimethylamine, indole and phenol concentrations after the oral protein challenge can be observed, which is attributed to gut bacterial degradation of

choline and of the aromatic amino acids phenylalanine, tyrosine and tryptophan, respectively.[30–34] As these changes were not statistically significant between the two protein diets, one might suppose that bacterial enzymatic activity was saturated already at a low protein intake.

As has been shown for urine and blood,[7–9] we expected an increase in the amine breath concentration after protein intake. This was true for trimethylamine and indole in our study. Methylamine and dimethylamine concentrations were always below the limit of detection.

Within 20 breaths (two minutes), there was a six-fold increase in methanethiol concentrations. Methanethiol is produced from L-methionine by L-methionine-α-deamino-γ-methanethiol-lyase (METase). The oral origin of methanethiol may explain its large increase and its large breath-to-breath variation immediately after protein intake.[35]

Although dimethylsulfide can be formed *via* enzymatic methylation of methanethiol, dimethylsulfide did not show pronounced concentration changes in both groups. This is most probably due to the fact that dimethylsulfide is produced in the gut rather than in the gingiva.[11]

The major source of exhaled acetone is decarboxylation of acetoacetate which in turn may arise from lipolysis, glycolysis and breakdown of ketogenic amino acids.[36–38] The slight decrease in acetone concentrations after two hours in the low protein group may be attributable to a reduced lipolysis due to the protein intake and the following steady increase until the end of the test may be attributable to an increased lipolysis due to the fasting effect. In contrast, the initial increase and later steady state of acetone concentrations in the high protein group may be attributable to acetone production from the breakdown of glycogenic amino acids which increased blood glucose independently from lipolysis. These results are in agreement with those reported in previous studies.[39,40]

2-Propanol is postulated to be a product of an enzyme-mediated reduction of acetone.[41] This may explain why it showed a concentration trend similar to that of acetone.

The major source of 2-pentanone in humans is lipolysis.[42] Then, similarly to acetone, the initial decrease in 2-pentanone concentrations may be attributable to a reduced lipolysis after protein intake and the subsequent increase may be attributable to an increased lipolysis due to the fasting effect. As described for acetone, lipolysis is more pronounced in the low protein group. Apparently, in the high protein group, lipolysis is prevented to some extent through the breakdown of glycogenic amino acids.

Profiles of exhaled reactive VOCs mirrored amino acid degradation by gut bacteria and aspects of energy production in terms of glycolysis and lipolysis.

5. Conclusion

Optimized PTR-ToF-MS enables sensitive time-resolved monitoring of reactive VOCs such as N- and S-containing compounds in human breath. Real-time mass spectrometry based breath analysis may thus provide non-invasive insight into various aspects of metabolism, energy production and fuel consumption in the human body.

Conflicts of interest

There are no conflicts to declare.

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PAPER

Effects of elevated oxygen levels on VOC analysis by means of PTR-ToF-MS

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Supplementary material for this article is available online

Abstract

Proton-transfer-reaction-time-of-flight-mass-spectrometry (PTR-ToF-MS) is a powerful tool for real-time monitoring of volatile organic compound (VOC) profiles in human breath. However, varying oxygen concentrations in the sample matrix may influence results from VOC analysis by PTR-ToF-MS. Elevated oxygen concentrations are likely to occur in clinical settings, but also when respiratory masks or breathing apparatus are used (e.g. in scuba diving, aviation, firefighting). Oxygen concentration may vary between subjects or within the course of a measurement or study and thus bias results. We systematically assessed the effect of high O_2 concentrations (up to 90%) in the sample matrix on the results of PTR-MS analysis for a pattern of VOCs including aromatics, aldehydes and ketones in dry and humid samples. *In vivo* experiments in healthy volunteers and mechanically ventilated animals were done to test the effect under real-life conditions. H_3O^+ count significantly decreased by more than 40% when the amount of oxygen in the sample matrix was increased. Almost all investigated VOCs were significantly effected by varying oxygen concentrations and differences in signal intensities of more than 50% could be observed. The effect was generally more pronounced in dry samples but still significant under humid conditions. A linear dependency of sensitivity on the oxygen concentration in the sample matrix was observed for a number of VOCs (e.g. aldehydes) possibly enabling a factor based correction. VOC intensities were also influenced under *in vivo* conditions, e.g. ethanol decreased up to 71%. When PTR-MS analysis is carried out under oxygen supply, these issues need to be carefully considered.

1. Introduction

The analysis of volatile organic compounds (VOCs) in human breath raised high expectations for the development of non-invasive diagnostic methods. Despite its great potential no unique biomarker for diagnostic purposes could be successfully established yet.

Today, an increasing number of researchers is investigating concentration changes of potential marker substances rather than focusing on the detection of a unique biomarker. Real-time breath analysis, e.g. by means of online mass-spectrometric techniques such as proton-transfer-reaction-mass-spectrometry (PTR-MS) [1–3] or selected-ion-flow-tube mass-spectrometry (SIFT-MS) [4], that allow direct and continuous measurements without the need for sample preparation significantly fostered this development and spawned a number of studies investigating patients with different diseases, therapeutic effects, physiological aspects or even metabolic rates [5–14]. However, the investigation of concentration changes for the purpose of clinical diagnostics requires accurate quantification [15] that is not hampered by artificial effects induced by the analytical technique or the clinical environment.

In a recent publication [16] we have discussed how the quantification of breath VOCs by means of PTR-ToF-MS may be affected by the sample matrix with respect to humidity, CO_2 content and low levels of oxygen (0%–20%). We concluded that the effect induced by the small difference of inspiratory and expiratory oxygen can be neglected when spontaneously breathing volunteers are analyzed. While these settings reflected the breath matrix of a spontaneously breathing volunteer or patient, the amount of

oxygen in the sample matrix may be substantially higher and may even reach 100% in situation where oxygen is supplied to a subject. This can be the case in the clinical environment under mechanical ventilation (during surgery or intensive care) or when respiratory masks are used, but also outside of the clinical environment when breathing apparatus are used for supplementary oxygen supply (e.g. scuba diving, fire-fighting, aviation, mountaineering, military applications). Oxygen supply may also vary between subjects or within the course of a measurement or study, e.g. due to therapeutic interventions. This may introduce artificial effects on results of VOC analysis that may be misinterpreted as physiologically or metabolically relevant if not taken into account.

The objective of this study was thus to systematically investigate the effects of high levels of O_2 on the results of breath analysis under conditions comparable to direct real time monitoring of breath samples. 23 VOCs (including aldehydes, ketones, hydrocarbons and aromatic compounds) in trace concentrations were assessed in detail and it was investigated if the sensitivity changed with increasing oxygen amount in the sample and if a factor based correction is possible. Finally, breath analysis in mechanically ventilated animals and in healthy volunteers under high and varying oxygen supply was done to demonstrate the effect under *in vivo* conditions.

2. Experimental

More than 20 VOCs including aldehydes, ketones, aromatics and hydrocarbons were analyzed at different concentrations. The influence of oxygen on measured VOC intensities, calibrations and sensitivity was systematically evaluated for dry and water saturated samples. Experiments in a large animal model and in healthy volunteers were carried out to investigate the influence of oxygen in the sample within an *in vivo* setting.

2.1. Chemicals and materials

Two standard mixtures (aldehydes: n-C_1 to C_{10} aldehydes, 2-propenal, and 2-butenal; VOC Mix: formaldehyde, acetaldehyde, methanol, ethanol, isoprene, acetone, 2-propenal, acetonitrile, 2-butanone, benzene, 2-butenal, toluene, chlorobenzene, o-xylene, and 1, 2-dichlorobenzene) stored in silcosteel canisters were purchased from Ionicon Analytik (Innsbruck, Austria). Nitrogen of purity 5.0 (i.e. 99.999%) was purchased from Linde (Vienna, Austria). Oxygen (medical grade) was purchased from Air Liquide Deutschland GmbH (Düsseldorf, Germany). HPLC grade water was purchased from Carl Roth GmbH & Co. KG (Karlsruhe, Germany).

2.2. PTR-ToF-MS measurements

Measurements were carried out by means of a PTR-ToF-MS 8000 (Ionicon Analytik GmbH, Innsbruck, Austria) with experimental conditions chosen as described before [2]. Sampling was done via a 1.6 m long heated silco-steel transfer line directly from the outlet of a liquid calibration unit (LCU; Ionicon Analytik, Innsbruck, Austria). Sampling flow was 20 ml min^{-1} and time resolution of the PTR-ToF-MS measurements was 200 ms. Transfer line and drift tube temperature were maintained at 75 °C. The drift voltage was 610 V and the drift tube pressure was 2.3 mbar. The resulting E/N ratio was 138 Td.

A new data file was recorded every minute and mass scale calibration was done after every run (60 s). Mass calibration was carried out by means of the following four masses: 21.023 (H_3O^+-Isotope), 29.998 (NO^+) and 59.049 (acetone/propanal). PTR-ToF-MS data from all measurements were processed by means of the PTR-MS-Viewer 3 software (Ionicon Analytik GmbH, Innsbruck, Austria).

2.3. VOC standard preparation

Gaseous standards were generated by means of an LCU. The working principle of the LCU has been described before [16]. Briefly, two liquid ports and three gas ports enable the generation of complex standard mixtures. Standards are mixed directly within an evaporation chamber made of silcosteel® at a defined temperature and sampling can be done directly at the outlet of the LCU. Humidity was introduced by adding water into a carrier gas stream that is forced through a small orifice and sprayed into the evaporation chamber.

Nitrogen was used as carrier gas in this study. VOCs were added by means of gas standards. Aldehyde standard and VOC-mix standard were analyzed separately. The amount of oxygen and humidity in the standards was adjusted by adding pure water (HPLC grade) and medical grade oxygen. For humid samples a humidity of 47 g m^{-3} water in the gas phase was used. This corresponds to a relative humidity of 107% under breath conditions (37 °C).

2.3.1. VOC calibrations with different O_2 content

Calibrations with different amounts of O_2 in the sample were analyzed for all substances over a concentration range from approximately 4 to 100 ppbV. As the original concentration in the undiluted gas standard slightly varies between VOCs, the resulting concentrations in the gas phase also slightly vary. Five concentrations were measured (4, 10, 25, 50 and 100 ppbV) in triplicates. The amount of O_2 in the sample matrix was varied in five steps between 0 and 90%. Total gas flow within the LCU (nitrogen, oxygen and VOC standard) was kept constant at 500 sccm and LCU temperature was 75 °C. Calibrations were done for dry samples and for samples with a humidity content of 47 g m^{-3}. Five

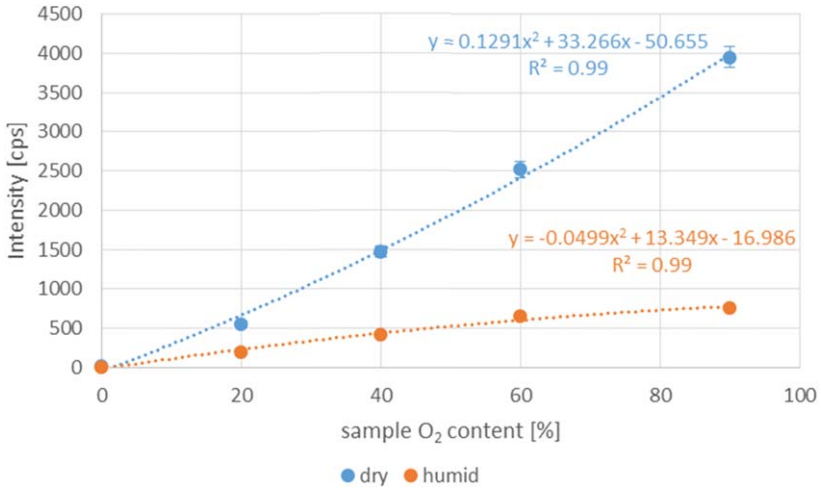


Figure 1. Influence of O_2 in the samples on O_2^+ count during PTR-ToF-MS measurements (via m/z 33.994). Blue dots: dry samples; orange dots: samples with humidity of 47 g m^{-3}.

blanks were measured additionally for each setting in order to investigate the effect of O_2 on H_3O^+ and $(H_2O)H_3O^+$.

2.3.2. In vivo determination of VOCs with varying O_2 amount in inspiratory air

2.3.2.1. In vivo measurements in healthy volunteers

Breath of three healthy adult volunteers was analyzed by means of a PTR-ToF-MS 8000. PTR-ToF-MS sampling, measurement and data analysis was carried out as described before (5, 12). Briefly, volunteers were breathing through a tightly fitting face mask that was connected to the Breas system and a sterile t-piece that enabled the connection of the PTR transfer line. Breath sampling was done continuously in side stream mode. No breathing resistance was introduced through mask and t-piece. PTR settings were as described above. Mass scale was recalibrated after every run of 60 s. Masses used for that purpose were 21.023 (H_3O^+-Isotope), 29.998 (NO^+), 69.069 ($(C_5H_8)H^+$). Expiratory and inspiratory phases were recognized by means of the matlab based 'breath tracker' algorithm [2].

Inspiratory oxygen concentration was adapted by means of a Breas LTV 1000 (Pulmonetic Systems, USA) system. Inspiratory oxygen was increased from 21% to 50% to 90% and then decreased to 21% again. Each concentration was applied for two minutes, resulting in a total analysis time of eight minutes. Only the second minute of each phase was used for data analysis.

2.3.2.2. In vivo measurements in mechanically ventilated animals

Breath from two mechanically ventilated pigs was analyzed by means of a PTR-ToF-MS 8000 (Ionicon Analytik GmbH, Innsbruck, Austria). PTR-ToF-MS sampling, measurement and data analysis was carried out as described before (5, 12). Briefly, a sterile t-piece was introduced into the ventilation system as close to the mouth as possible. PTR transfer line was connected via luer lock adapter and breath sampling was done continuously in side-stream mode. PTR settings were as described above. Mass scale was recalibrated after every run of 60 s. Masses used for that purpose were 21.023 (H_3O^+-Isotope), 29.998 (NO^+), 59.049 ($(C_3H_6O)H^+$). Expiratory and inspiratory phases were recognized by means of the matlab based 'breath tracker' algorithm [2].

Continuous breath analysis was performed over a time course of 20 min. Within the experiment, the inspiratory oxygen concentration of 40% was kept constant for ten minutes and then increased to 100% and kept constant for another ten minutes. A mean over each ten minute phase was used for data analysis. The study was approved by the local University Medical Centre Ethics Committee.

3. Results

3.1. Effect of O_2 on H_3O^+, $(H_2O)H_3O^+$ and O_2^+

Figure 1 shows how the measured intensity of O_2^+ increases with an increasing O_2 content in the sample matrix in both dry and humid samples. The isotope at mass 33.994 was used to determine O_2^+ due to detector saturation at mass 31.989. The increase followed a second order polynomial regression and was much more pronounced in dry samples compared to humid samples.

Figure 2 shows the effect of the O_2 content in the sample matrix on H_3O^+ and $(H_2O)H_3O^+$ ion count. The effect is displayed for both dry (figure 2(a)) and humid samples (figure 2(b)). The H_3O^+ and $(H_2O)H_3O^+$ counts were normalized to the highest value in order to emphasize the differences induced by changing sample O_2 content. Five replicates were analyzed and relative standard deviations were below 2% for H_3O^+ and 5.5% for $(H_2O)H_3O^+$. H_3O^+ decreased

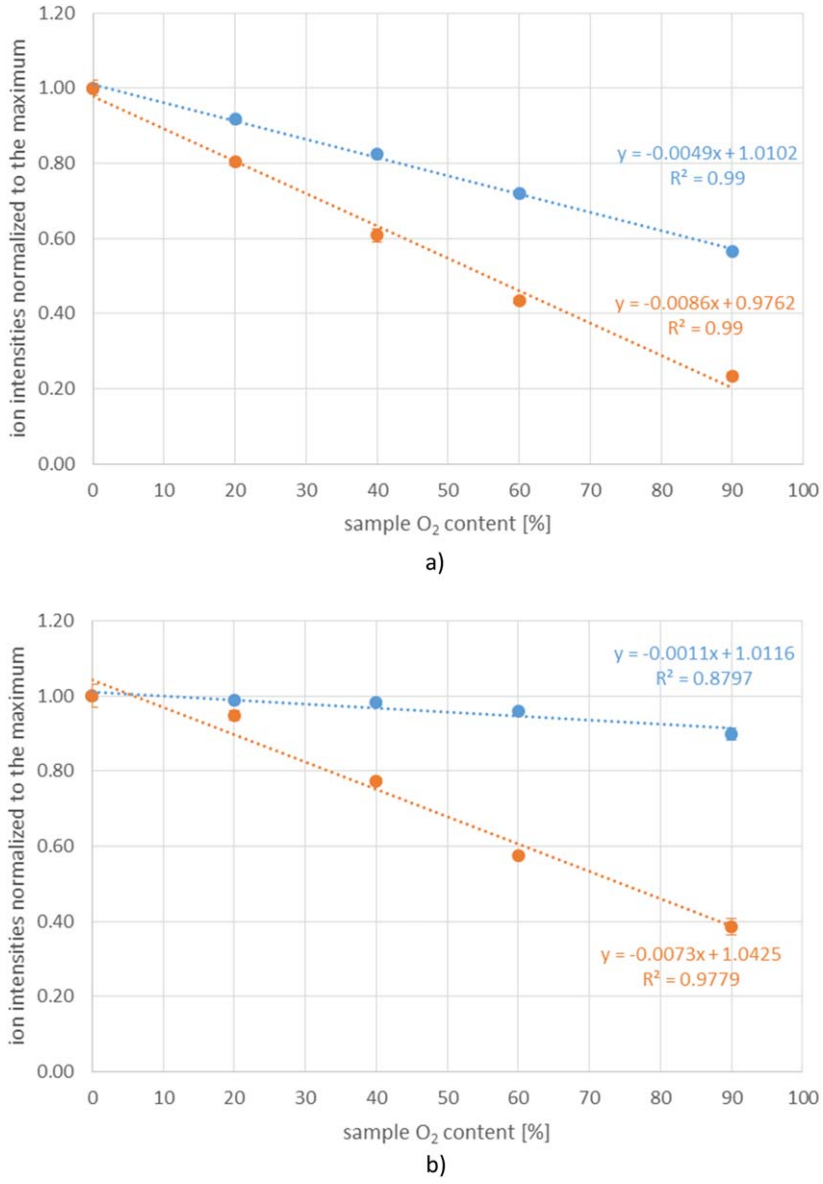


Figure 2. Influence of O_2 in the samples on H_3O^+ (blue dots) and $(H_2O)H_3O^+$ (orange dots) count during PTR-ToF-MS measurements. H_3O^+ and $(H_2O)H_3O^+$ counts (cps) were normalized to their maximum. (a) dry samples (b) samples with humidity of 47 g m^{-3}.

in a linear fashion with increasing sample O_2 in both dry and humid samples. The decrease was more pronounced in dry samples compared to humid samples. $(H_2O)H_3O^+$ also showed a linear decrease that was more pronounced in dry samples compared to humid samples.

The differences were statistically significant for both H_3O^+ and $(H_2O)H_3O^+$ (Kruskal–Wallis one way ANOVA on ranks with Student Newman Keuls post hoc test, $\alpha = 0.05$, $p < 0.05$ was considered as significant), between all groups for dry and humid samples.

3.2. Influence of O_2 on sensitivity

The O_2 content within the sample matrix had a distinct effect on the measured intensities of a variety of VOCs. Table 1 shows the data of 23 VOCs that were analyzed with five different amounts of O_2 in the sample matrix, ranging from 0 to 90%. Concentrations were normalized to maximum values and color coded for emphasis of relative changes. VOC concentrations were approximately 100 ppbV for each compound. The table includes data that was normalized to the H_3O^+ ion count as it is standard practice in PTR data processing. A similar table for the non-normalized data can be found in the supplemental material in table S.1. Standard deviations were generally low (below 4% in most cases) and can be found in table S.2. Results from statistical analysis (ANOVA on ranks in combination with Student Neumann Keuls post-hoc test; $\alpha = 0.05$) are shown in table S.3.

Almost all investigated analytes showed a dependency on O_2, in both dry and humid samples. The effect was generally more pronounced in dry samples. When data was not normalized to H_3O^+ (table S.1), all

Table 1. Effect of different amounts of O_2 in the sample matrix (0%, 20%, 40%, 60% and 90%) on measured VOC intensities (VOC concentration: ~100 ppbV; data were normalized to maximum values to emphasize relative differences; red color represent relatively high values, green color represents relatively low values; as a consequence the presence of green values within a row indicate a large O_2 influence, while the influence is low when only red values are present). Three replicates were analyzed at each O_2 level. Additionally, the whole experiment was done with dry and humid samples (humidity of 47 g m^{-3}) respectively. (a) Dry samples, data normalized to H_3O^+ count; (b) humid samples, data normalized to H_3O^+ count.

a) Dry samples, normalization to H_3O^+

Humidity [g/m³]	0	0	0	0	0
Oxygen content	0%	20%	40%	60%	90%
Formaldehyde	0.88	0.92	0.93	0.95	1.00
Acetaldehyde	0.94	0.99	0.98	0.99	1.00
Acrolein	0.99	1.00	0.96	0.93	0.89
Propanal	1.00	0.95	0.82	0.72	0.58
E-2-Butenal	0.98	1.00	0.95	0.90	0.80
Butanal	1.00	0.97	0.90	0.83	0.75
Pentanal	1.00	0.93	0.82	0.69	0.54
Hexanal	1.00	0.93	0.79	0.67	0.51
Heptanal	1.00	0.93	0.79	0.67	0.53
Octanal	1.00	0.93	0.79	0.68	0.51
Nonanal	1.00	0.93	0.78	0.67	0.50
Decanal	1.00	0.91	0.72	0.62	0.43
Acetonitrile	0.93	0.97	0.98	0.96	1.00
Ethanol	1.00	0.92	0.79	0.64	0.49
Acetone	0.86	0.91	0.93	0.92	1.00
Isoprene	1.00	0.99	0.91	0.81	0.77
2-Butanone	0.96	1.00	0.99	0.96	0.96
Benzene	0.82	0.87	0.90	0.90	1.00
Toluene	0.70	0.76	0.81	0.85	1.00
o-Xylene	0.83	0.88	0.91	0.92	1.00
alpha-Pinene	0.96	1.00	1.00	0.97	0.98
Chlorobenzene	0.96	1.00	1.00	0.96	0.97
1,2-Dichlorobenzene	0.95	0.99	1.00	0.97	0.98

aldehydes, except for formaldehyde showed a decrease in measured intensity with increasing O_2 content in dry and humid samples. Only formaldehyde showed a decreasing trend in dry samples and an opposing trend in humid samples. Ethanol, acetone, 2-butanone, isoprene and acetonitrile showed the same behavior as aldehydes. Aromatic compounds also displayed a decrease with increasing oxygen level in dry samples. In humid samples however, most aromatic compounds showed a slight increase from 0% to 20% or 40% oxygen in the sample, followed by a decreasing tendency when the oxygen level was further increased to 60% or 90%.

Normalization to H_3O^+ led to a decrease of the oxygen induced variation (table 1). This was true for both, dry and humid samples. However, in dry samples, the oxygen induced effect was inverted for certain VOCs (e.g. benzene, toluene, o-xylene, acetone, acetonitrile). I.e. these VOCs showed increasing intensities with increasing sample oxygen content when no normalization was applied but a decreasing trend was observed, when data was normalized to H_3O^+ count.

An increase of the oxygen concentration in the sample matrix led to decreasing intensities of most protonated VOC signals. However, at the same time, increasing intensities of the signals for charged

Table 1. (Continued.)

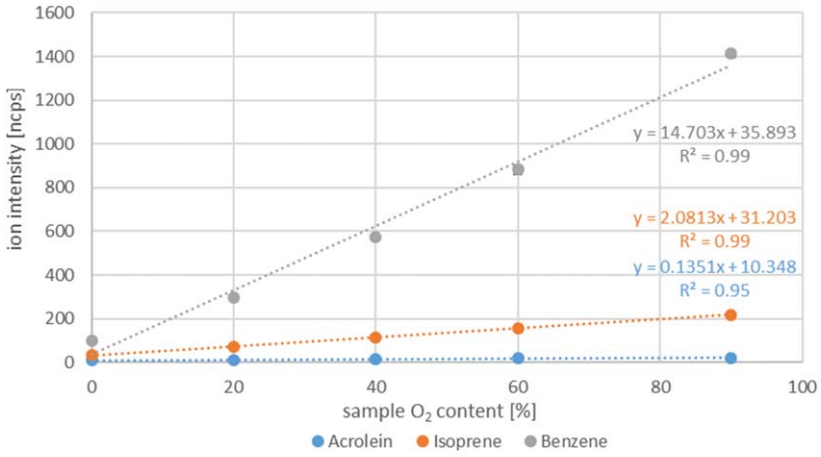
b) Humid samples, normalization to H_3O^+

Humidity [g/m³]	47	47	47	47	47
Oxygen content	0%	20%	40%	60%	90%
Formaldehyde	0.49	0.63	0.76	0.87	1.00
Acetaldehyde	0.99	1.00	0.97	0.93	0.87
Acrolein	1.00	0.99	0.95	0.89	0.81
Propanal	1.00	0.96	0.87	0.78	0.63
E-2-Butenal	0.99	1.00	0.95	0.91	0.82
Butanal	1.00	0.86	0.73	0.59	0.44
Pentanal	1.00	0.87	0.73	0.59	0.42
Hexanal	1.00	0.86	0.72	0.58	0.43
Heptanal	1.00	0.88	0.74	0.60	0.45
Octanal	1.00	0.87	0.73	0.59	0.44
Nonanal	1.00	0.88	0.75	0.60	0.44
Decanal	1.00	0.90	0.77	0.61	0.45
Acetonitrile	0.98	1.00	0.96	0.87	0.78
Ethanol	1.00	0.96	0.82	0.61	0.43
Acetone	0.97	1.00	0.97	0.90	0.83
Isoprene	0.98	1.00	0.95	0.84	0.73
2-Butanone	0.97	1.00	0.97	0.89	0.81
Benzene	0.90	0.97	1.00	0.98	0.96
Toluene	0.91	0.98	1.00	0.98	0.96
o-Xylene	0.95	1.00	0.99	0.94	0.89
alpha-Pinene	0.96	1.00	0.97	0.90	0.83
Chlorobenzene	0.92	0.98	1.00	0.97	0.92
1,2-Dichlorobenzene	0.90	0.97	1.00	0.97	0.94

molecular ions could be observed with increasing of the oxygen concentration in the sample matrix. This is exemplified in figure 3 for isoprene, acrolein and benzene.

Table S.4 shows the effect of sample O_2 on the sensitivity for the investigated VOCs. For dry samples, all aldehydes except for acetaldehyde showed a linearly decreasing sensitivity with increasing oxygen concentration (R^2 ranged from 0.88 to 0.99). Acetaldehyde did not show a linear dependency. Ethanol and isoprene also showed a negative dependency (R^2 of 0.99 and 0.96 respectively), while sensitivities of acetone ($R^2 = 0.89$), benzene ($R^2 = 0.95$), toluene ($R^2 = 0.97$) and o-xylene ($R^2 = 0.97$) showed a linear increase with increasing oxygen concentration. Sensitivities of acetonitrile, 2-butanone, cholorobenzene, 1,2-dichlorobenzene and alpha-pinene did not follow a linear trend.

In humid samples sensitivity of all aldehydes included in the study showed a linear dependency on sample oxygen concentration (R^2 ranged from 0.92 to 0.99). The trend was negative for all aldehydes except for formaldehyde which showed a positive tendency in contrast to the negative trend that was observed in dry samples. Ethanol and isoprene showed the same behavior as for dry samples (R^2 of 0.99 and 0.89 respectively). Acetonitrile also followed a negative trend ($R^2 = 0.87$). In contrast to dry samples, acetone showed a negative tendency with increasing oxygen concentration in humid samples, however the linearity was not as good as for dry samples ($R^2 = 0.80$). Aromatic compounds did not follow a linear trend, a



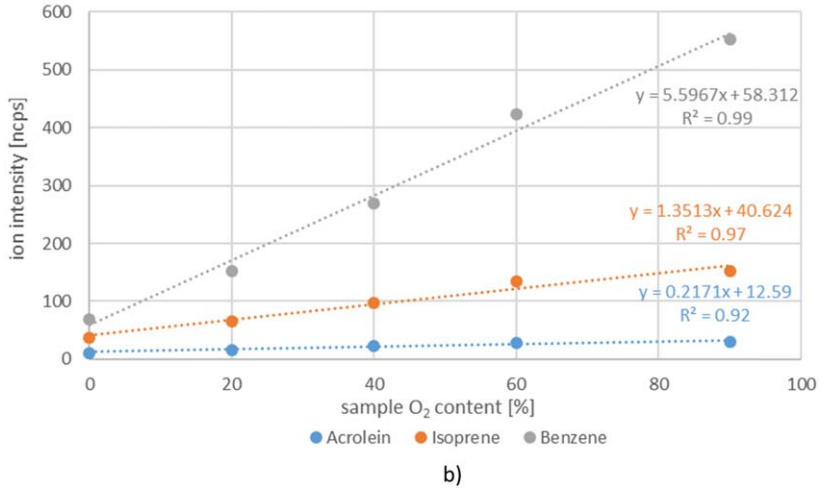


Figure 3. Measured intensities of the charged molecular ions of isoprene (orange dots), acrolein (blue dots) and benzene (grey dots) at five different amounts of O_2 in the sample matrix (0, 20, 40, 60 and 90). Data were not normalized to H_3O^+ count. (a) dry samples (b) humid samples.

negative tendency could only be observed for alpha-pinene ($R^2 = 0.73$).

3.3. *In vivo* matrix effect

3.3.1. In vivo matrix effect in healthy volunteers

Breath from three spontaneously breathing healthy human volunteers was analyzed by means of PTR-ToF-MS. Figure 4 shows the effect of the inspiratory O_2 content on normalized mean alveolar intensity of ethanol, acetone, isoprene, pentanal, and α-pinene. Intensities were normalized to the first measurement at 21% of O_2.

Regarding the raw data (figure S.1 is available online at stacks.iop.org/JBR/13/046004/mmedia), H_3O^+ and $(H_2O)H_3O^+$ decreased by 4% and 13% respectively, when the O_2 content was increased to 50%. They further decreased by another 7% and 39%, respectively, when the O_2 content was increased to 90%. Expiratory O_2^+ concentrations also increased with increasing O_2 concentration. All changes were statistically significant (one way repeated measures ANOVA, Student Neuman Keuls post-hoc test, $\alpha = 0.05$, $p < 0.05$). Intensities returned to initial levels when O_2 content was decreased to 21% again. Expiratory O_2^+ remained elevated by 14% when O_2 content was decreased to 21% again compared to the initial levels. However, this difference was not statistically significant. Inspiratory O_2^+ in contrast was identical in both cases.

Normalized mean alveolar intensities of investigated VOCs (figure 4) showed statistical significant changes with increasing O_2 content. Ethanol decreased by 38% and 71%, pentanal decreased by 9% and 24% and α-pinene decreased by 6% and 14% when O_2 content was increased to 50% and to 90%. Changes between 21% and 50% did not reach statistical significance (except for ethanol), changes between 21% and 90% and between 50% and 90% O_2 were statistically significant. Only

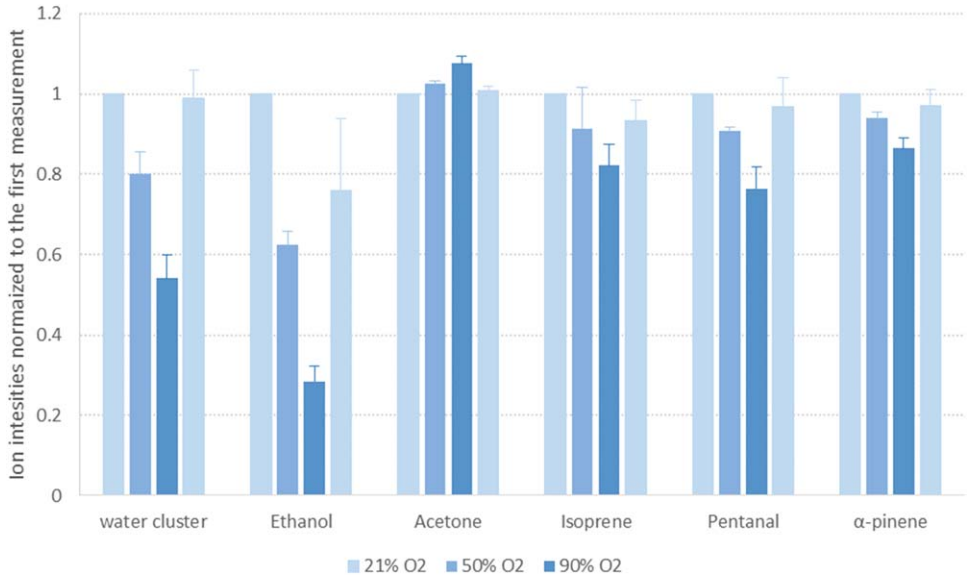


Figure 4. Analysis of breath from three spontaneously breathing healthy volunteers. Normalized intensities of different compounds at three amounts of O_2 in the sample: 21% (light blue), 40% (medium blue), and 90% (dark blue). Inspiratory O_2 was increased from 21% to 50% followed 90% and then decreased to 21% again. VOC Intensities were normalized to the first measurement at 21% of O_2 to emphasize relative differences. Data was normalized to H_3O^+ count.

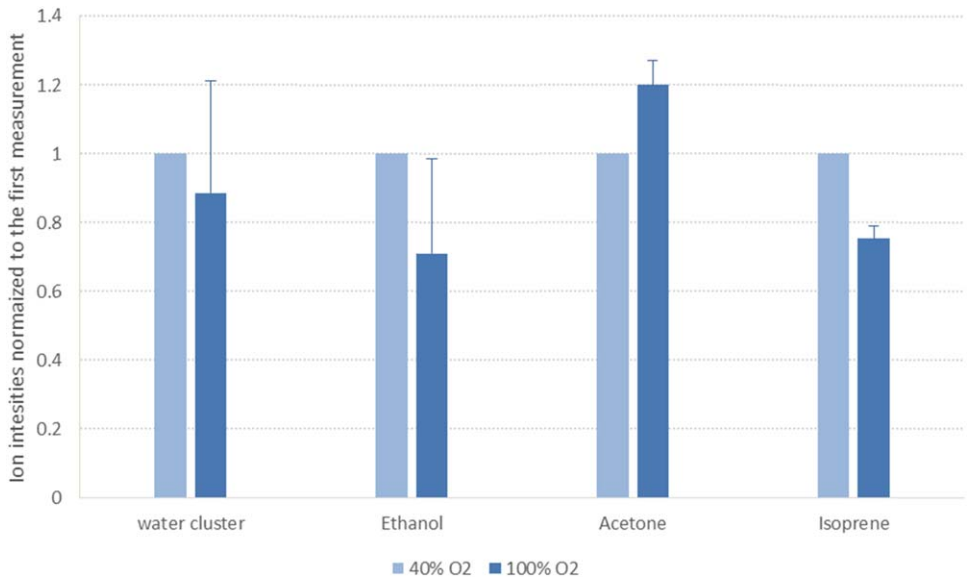


Figure 5. Analysis of breath from three mechanically ventilated pigs. Normalized intensities of different compounds at two amounts of O_2 in inspiratory air (40% and 100%). VOC Intensities were normalized the measurement at 40% of O_2. The data was normalized to H_3O^+ count.

ethanol did not return to baseline levels when O_2 content was decreased again to 21%. However, changes between 21% and 50% O_2 were statistically significant when data was not normalized to H_3O^+.

Acetone showed a significant increase by 8% when O_2 content was increased to 90%, but no statistically significant change when the O_2 content was increased from 21% to 50%. Isoprene also showed a decrease similar to the standard measurements. However, this change was not statistically significant. When data was not normalized to H_3O^+ isoprene showed a significant change between 50% and 90% O_2.

The charged monomers of the five VOCs showed an increasing trend with increasing O_2 content as can be seen in figure S.2.

3.3.2. In vivo matrix effect in mechanically ventilated animals

Breath from three mechanically ventilated pigs was analyzed by means of PTR-ToF-MS at two different inspiratory O_2 concentrations: 40% and 100%. Figure 5 shows the effect of the O_2 content on the normalized mean alveolar intensity of ethanol, acetone

and isoprene. Intensities were normalized to the measurement at 40% of O_2.

A decrease by 11% was observed for H_3O^+ when the O_2 content was increased to 100%. Likewise $(H_2O)H_3O^+$ decreased by 23% when the O_2 content was increased to 100% (figure S.3).

Normalized mean intensities of ethanol and isoprene decreased by 29% and 25%, respectively, when the O_2 content was increased to 100%. Acetone increased by 20% when the O_2 content was increased to 100% (figure 5). However, except for H_3O^+ and isoprene, these changes were not statistically significant.

4. Discussion

Real-time breath analysis in the clinical environment involves considerable challenges with respect to reliable quantification of potential biomarkers. Within this study we showed, that high levels of oxygen that can be present in the sample in a clinical setting have significant effects on VOC analysis by means of direct PTR-TOF-MS.

An increase of the oxygen concentration in the sample matrix led to a significant decrease of H_3O^+ and $(H_2O)H_3O^+$. This effect was more pronounced in dry samples compared to humid samples. At the same time the O_2^+ intensity increased. The increase of O_2^+ was much more pronounced in dry samples compared to humid samples, which is also reflected in the effect on VOCs. The formation of additional O_2^+ can be explained by back diffusion of sample gas into the ion source. Similar effects can be observed for the formation of NO^+ [17] as well as for the formation of additional H_3O^+ and hydrated hydronium ions in humid samples [16]. O_2^+ does not react with H_2O in contrast to N_2^+ (collisions of N_2^+ and H_2O will lead to the formation of H_3O^+). Less N_2^+ will be produced in the ion source when the oxygen concentration in the sample is increased and consequently less H_3O^+ and $(H_2O)H_3O^+$ will be formed [18]. In humid samples, the availability of additional H_2O is counterbalancing this effect partially. We also observed a minor formation of $O_2^+(H_2O)$ cluster with increasing O_2 in the sample matrix. But the maximum intensity was too low (<60 cps) to have a significant influence. The availability of additional O_2^+ and reduced availability of H_3O^+ influences the ionization of analyte molecules within the drift tube as reactions of analytes with O_2^+ follow a charge transfer mechanism that competes with the proton transfer reaction. Such electron transfer reactions are exothermic for analytes with ionization energies below the recombination energy of O_2^+ (12.07 eV) [19]. Ionization energies of the compounds included in this study meet this criteria, except for the ionization energy of acetonitrile which is slightly higher (12.2 eV) [20]. As a consequence, when availability of O_2^+ in the drift tube increases, likelihood of charge transfer reactions increases as well. The result is basically a mixed ionization mode [21]. Further, reactions with O_2^+ lead to increased fragmentation compared to reactions with H_3O^+ [22, 23]. Apart from these effects, changes in the buffer gas composition can influence the mobility of the ions in the drift tube and hence effect the kinetic energy of ion-molecule collisions [24, 25]. This may have altered the sensitivity for certain compounds and thus contributed to the observed effects. However, as the molecular mass and size of O_2 and N_2 are comparable, we do not think that this is the prime reason for the observed effects.

The observed intensities of protonated VOCs in dependency of sample O_2 content showed a decreasing trend for all investigated VOCs in dry samples. At the same time increasing intensities of the charged molecular ions were detected. In humid samples formaldehyde showed an opposing trend to dry samples, with increasing intensities when O_2 was increased, whereas all other aldehydes showed a similar but less pronounced trend compared to dry samples. Aldehydes easily fragment even when soft ionization is applied [26, 27]. From our previous results we know that the protonated signals of aldehydes show a higher abundance in humid samples compared to dry samples [16] as additional proton transfer reactions with hydrated hydronium ions occur which results in less fragmentation [17]. This effect and the less pronounced influence of O_2 on H_3O^+ seems to counterbalance the influence of increasing sample O_2 content in the humid samples to some extent. Consequently, the charged molecule signals showed a less pronounced increase in humid samples.

The proton transfer reaction can be reversible, especially if the proton affinity of the molecule is similar to that of water, as it is the case for formaldehyde. For formaldehyde increasing sample humidity thus increases the rate of the reverse reaction [28]. As a result, intensities of the formaldehyde signal decrease with increasing sample humidity [16]. As for the other aldehydes, the increasing sample O_2 content counterbalances this effect, resulting in increasing formaldehyde signal intensities with increasing O_2.

Aromatic compounds also showed a slightly different behavior in humid samples compared to dry samples. While a constant decrease with increasing O_2 was observed in dry samples, a small increase was observed when O_2 content increased from 0% to 20% or 40%, followed by a decreasing trend when O_2 was further increased. Aromatic compounds are known to show diminished sensitivities in PTR-MS when sample humidity is increased [16, 29, 30]. As increasing O_2 content leads to decreasing $(H_2O)H_3O^+$ concentrations, a minor increase can be observed at first. However, once the charge transfer reaction with O_2^+ dominates over the effect induced by diminishing $(H_2O)H_3O^+$ concentrations, a decrease in VOC intensities can be observed. All other compounds included in the study showed a similar behavior as described for the majority of aldehydes. Overall the observed effects are in good agreement with our previous study were

lower O_2 concentrations ranging from 0% to 20% were investigated [16].

Normalization to H_3O^+ ion count could only partially compensate for the observed effects. While the differences induced by varying O_2 content were smaller for certain VOCs, some compounds such as acetone, acetonitrile and certain aromatic compounds showed opposing trends compared to the raw data in dry samples. This occurred when the effect from O_2 on the VOC was smaller than the effect of O_2 on H_3O^+. As the H_3O^+ ion count is much less effected in humid samples no such inversion was observed in this case.

A number of VOCs showed a linear dependency of sensitivity on sample O_2 content in both, dry and humid samples. For these substances, an O_2 based correction factor could be applied. However, such a factor would have to be experimentally determined and the cross-dependency on sample humidity would have to be considered as well.

Two *in vivo* experiments involving spontaneously breathing healthy volunteers and mechanically ventilated pigs confirmed the results under real-life conditions. A decrease in H_3O^+ and $(H_2O)H_3O^+$ was observed when inspiratory O_2 was increased. VOCs such as ethanol, isoprene, pentanal or α-pinene showed a decrease with increasing inspiratory O_2 as well, while the charged monomers showed an increasing trend. Most changes were statistically significant despite the low number of volunteers and animals and the generally large inter-individual variation. As the results were in good agreement with results obtained from standard measurements, it was obvious that the observed effects also occur in a clinical setting and not just under laboratory conditions. Acetone in contrast showed a less pronounced effect in spontaneously breathing volunteers compared to the effect in the standards and even an opposing trend in the mechanically ventilated animals. It is important to notice that expiratory oxygen concentrations will obviously be slightly lower compared to inspiratory concentrations due to oxygen uptake in the blood and oxygen consumption in the lungs. Hyperoxic conditions can further complicate this issue as arterial PO_2 will increase over time and as a consequence oxygen uptake will decrease [31]. We cannot fully exclude such effects and the differences in expiratory O_2^+ between the first and second measurement with 21% inspiratory O_2 in healthy volunteers (though not statistically significant) may be explained this way. As the hyperoxic phase was longer in the mechanically ventilated pigs, physiological effects may have been more pronounced. This could explain the opposing behavior of acetone.

5. Conclusion

PTR-ToF-MS is a powerful technique to assess breath VOC profiles and monitor physiological changes in real-time, but a reliable quantification is challenging and artificial concentration changes may be easily introduced. Our results show that the varying oxygen concentration in the sample matrix significantly influences VOC analysis by means of PTR-ToF-MS. Whenever oxygen is supplied to a subject, be it under mechanical ventilation or through the use of a respiratory mask, differences in oxygen concentrations will introduce differences in the measured signal intensities. This can effect data from a single subject or introduce a bias when several subjects are analyzed under varying conditions. As a consequence, 'concentration' changes or differences might be observed and assigned to physiologic or metabolic processes, while in fact only the signal intensity is changing due to the analytical conditions and no actual concentration change occurs.

When PTR-MS analysis is carried out in any situation where oxygen is supplied to the subject, its effect on measured VOC intensities has to be carefully considered for reliable quantification.

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Conflict of interest

None

Author contributions

PT designed the study, performed the experiments, analyzed and interpreted the data. GP contributed to the *in vivo* studies and supported data analysis. BB supervised the *in vivo* study under mechanical ventilation. JKS and WM contributed to data analysis and discussion. All authors contributed to the manuscript.

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10. APPENDIX

10.1 Declaration of independence

I, Giovanni Pugliese, born on 25.02.1988 at Cassano Allo Ionio (Italy), hereby declare that the presented thesis entitled

‘‘Method improvements for real-time mass spectrometric monitoring of exhaled VOCs in the clinical environment”

is my own original work and has been composed by me without any assistance.

I further declare that I haven’t used any sources other than those referenced in the manuscript and that I clearly indicated all parts that were taken from these sources be it literally or with respect to the content.

Rostock, 26.10.2020 Giovanni Pugliese

10.2 Acknowledgements

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10.3 Curriculum Vitae

Personal information

Name: Giovanni Pugliese

Birthday: 25.02.1988

Place of birth: Cassano allo Ionio

Nationality: Italian

Professional qualification and research experience

From June 2020	**Research associate** Atmospheric chemistry research group. Max Planck institute for Chemistry in Mainz (Rheinland Pfalz, Germany).
2016 - 2020	**Marie Curie Early Stage Researcher** Horizon 2020 Marie Sklodowska Curie Actions Innovative Training Network (H2020-MSCA-ITN) Ion-Molecule Processes for Analytical Chemistry Technologies (IMPACT).
2016 - 2020	**PhD Student** Rostock Medical Breath Analysis and Technologies research group (RoMBAT), Department of Anaesthesiology and Intensive Care, Rostock University Medical Center (Mecklenburg-Vorpommern, Germany).
2015 - 2016	**Research Associate** Microsystems Nanotechnologies for Chemical Analysis research group (MINOS), Universitat Rovira i Virgili, Tarragona (Catalonia, Spain).
2014 - 2015	**Research Associate** Institute on Membrane Technology - National Research Council (ITM- CNR), Arcavacata di Rende (Calabria, Italy)

Educational qualification

2010 - 2013	**University of Calabria (Calabria, Italy)** Master of Science in Chemistry, GPA: 110/110 *cum laude*
2007 - 2010	**University of Calabria (Calabria, Italy)** Bachelor of Science in Chemistry, GPA: 109/110
2002 - 2007	**Liceo Ginnasio Statale "R. Lombardi Satriani" (Calabria, Italy)** Scientific secondary school diploma, GPA: 82/100

10.4 Complete list of publications

Produced during doctoral research at Rostock University Medical Center:

- Pugliese, G.; Piel, F.; Trefz, P.; Sulzer, P.; Schubert, J.K.; Miekisch, W. Effects of modular ion-funnel technology onto analysis of breath VOCs by means of real-time mass spectrometry. Anal Bioanal Chem. 2020, 412, 7131–7140. https://doi.org/10.1007/s00216-020-02846-8

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Produced during previous research experience:

- Geremariam Welearegay, T.; Durán-Acevedo, C. M.; Lisset Jaimes-Mogollón, A.; Pugliese, G.; Ionescu, F.; Geovanny Perez-Ortiz, O.; Gualdrón-Guerrero, O. E.; Cindemir, U.; Österlund, L.; Ionescu, R. Exhaled Air Analysis as a Potential Fast Method for Early Diagnosis of Dengue Disease. Sensors and Actuators B: Chemical. 2020, 127859. https://doi.org/10.1016/j.snb.2020.127859.

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10.5 Conference Contributions

Oral Talks

- G. Pugliese, F. Piel, P. Trefz, P. Sulzer, J.K. Schubert, W. Miekisch.
  "Characterization and application of an Ion-Funnel-Proton-Transfer-Reaction-Time-of-Flight-Mass-Spectrometry (IF-PTR-ToF-MS) for improved sensitivity in breath analysis".
  PITTCON Conference and Expo 2020, 1st – 5th March 2020, Chicago, USA.

- G. Pugliese, P. Trefz, P. Sukul, M. Weippert, S. Bruhn, J.K. Schubert, W. Miekisch.
  "Real-time breath analysis during exhaustive exercise".
  2nd International SCIMS conference, 10th – 13th June 2019, Prague, Czech.

- G. Pugliese, P. Trefz, B. Brock, J.K. Schubert, W. Miekisch.
  "Real-time analysis of nitrogen containing compounds under varying nutrition"
  PITTCON Conference and Expo 2019, 17th - 21st March 2019, Philadelphia, USA.

- G. Pugliese, P. Trefz, B. Brock, J.K. Schubert, W. Miekisch.
  "Breath-resolved analysis of exhaled amines by PTR-TOF-MS: effects of protein intake"
  8th International PTR-MS Conference 2019, 4th - 8th February 2019, Innsbruck, Austria.

- G. Pugliese, P. Trefz, B. Brock, J.K. Schubert, W. Miekisch.
  "Real-time calibration of aliphatic amines"
  1st International SCIMS conference, 18th – 20th September 2017, Dornbirn, Austria.

Poster Presentations

- G. Pugliese, P. Trefz, B. Brock, J.K. Schubert, W. Miekisch.
  "Real-time analysis of nitrogen containing compounds under varying nutrition"
  PITTCON Conference and Expo 2019, 17th - 21st March 2019, Philadelphia, USA.

- G. Pugliese, P. Trefz, B. Brock, J.K. Schubert, W. Miekisch.
  "Development and optimization of a PTR-ToF-MS analytical method for breath-resolved determination of aliphatic amines"
  PITTCON Conference and Expo 2018, 26th February - 1st March 2018, Orlando, USA.

- G. Pugliese, P. Trefz, B. Brock, J.K. Schubert, W. Miekisch.
  "Real-time calibration of aliphatic amines"
  1st International SCIMS conference, 18th – 20th September 2017, Dornbirn, Austria