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Investigation of everyday influencing factors on the variability of exhaled breath profiles in healthy subjects

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Abstract:

Introduction: The human breath is an accurate but complex read-out of many physiological processes in the organism that can be monitored via volatile organic compounds (VOCs) in the exhaled air. However, there are many confounding variables that limit the transfer and application of breath analysis to become a clinical procedure.

Method: This work aims to establish a systematic procedure for sampling and characterization of various everyday influences of healthy subjects using proton transfer reaction-mass spectrometry (PTR-MS). In order to limit the influencing factors on the breath profile, a standard analysis procedure for sampling and evaluation of the exhaled breath samples was developed. The correlations between the selected experimental conditions and the resulting VOC profiles were investigated using a non-parametric Wilcoxon rank sum test.

Results: In addition to the relevant influence of methodological experimental parameters, interesting insights into the effect of everyday factors on the exhalat gas were obtained and discussed. Furthermore, subject and condition-specific differences were found in the exhaled air of male and female subjects.

Conclusion: With a more robust, standardized and reproducible breath sampling protocol, breath analysis is a promising non-invasive tool towards a system-wide understanding and personalized diagnosis and treatment of a wide range of diseases.

Keywords: Breath gas analysis, Breathomics, Proton-transfer-reaction mass spectrometry, Subject variability, Everyday influencing factors

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1 Introduction

In the last decades, the analysis of volatile organic compounds (VOCs) in the exhaled human breath has been studied and developed as a promising technique to identify biomarkers for the diagnosis and monitoring of various diseases. One of the main advantages of this procedure is its non-invasive approach. This is particularly important for populations such as children and elderly people, and for diseases whose current standard diagnoses use invasive techniques such as biopsies and bronchoscopies or imaging methods based on ionizing radiation. Despite of the ongoing advances in this research field, breath gas analysis is not yet a routine clinical tool. Standard clinical procedures have not yet been sufficiently established and validated, especially when it comes to identifying suitable biomarkers and setting comparative values for healthy volunteers [10].

Recent advancements in breath research have led to the identification of biomarkers in a wide range of diseases such as lung and breast cancer, COPD, asthma, diabetes, diseases of the skin barrier and many more [2, 4, 6, 9]. Various methods are available for the measurement of VOCs, which vary in their detection sensitivity and analysis speed e.g. gas chromatography-mass spectrometry (GC-MS), electronic nose, proton transfer reaction-mass spectrometry (PTR-MS), ion mobility spectrometry (IMS), chemiluminescence or optical absorption detection techniques [11].

The work presented in this paper uses PTR-MS, which offers the advantages of a rapid response, a soft chemical ionization principle along with the possibility for an absolute quantification and a high sensitivity down to a parts per trillion (ppt) level [11]. PTR-MS is based on a soft chemical ionization, where a hydronium ion ($H3O^+$) is used to charge VOC molecules R for proton affinities higher than for water molecules [1].

It has been shown that a breath sample contains more than 3.500 different VOCs, mostly in the picomolar range. The gas exchange between the blood system and the external environment can be monitored in the human breath. This process of alveolar gas exchange with the blood facilitates oxygen uptake and releases by-products of metabolic reactions such as

exhaled breath gas volatiles such as methanol, ethanol, acetaldehyde, acetone and isoprene [8]. The specific composition of the individual breath pattern is influenced by the subject's physiological situation, the lifestyle and state of health [8]. However, there is currently a lack of standardization in breath sampling, data acquisition and analysis for the processing of PTR-MS data from clinical study cohorts that are suited for biomarker identification.

The work presented in this paper aims to investigate the influence of everyday lifestyle factors and the volatile biomarker profiles in healthy subjects affecting the reliability, reproducibility and suitability of breath gas analysis for the design of clinical studies. In this setting, several everyday influencing factors such as getting up, brushing teeth and food uptake were investigated for its variability in the VOC abundance along with possible interferences from ambient air samples. In this study, the influence of different everyday factors was determined by measuring the complete range of VOC mass-to-charge ratios between *m/z* 20 to *m/z* 200 [8]. It is important to determine which of the various sampling parameters has a larger influence on the VOC profile and should therefore be avoided in clinical studies to minimize inter- and intra-individual variability of confounding factors.

2 Material and Methods

2.1 Study population

The healthy subject population consisted of 13 voluntary researchers (7 male and 6 female subjects) from the Chair of Medical Systems Technology and the University Clinic for Cardiac and Thoracic Surgery. The informed consent was obtained from the study participants and the study was approved by the institutional ethics committees on human research of the Otto-von-Guericke University Magdeburg (vote 194/20).

2.2 Breath gas sampling

Breath gas samples were collected in 3 l Tedlar bags. In order to ensure a minimum level of contaminations in the reused bags, the bags were purged with nitrogen (99.5% purity) twice and a low background VOC level was verified by additional measurements. The exhalation volume of 2-2.5 l was typically reached after 20–30 s containing a mixed fraction of the subject's exhaled breath and the samples were subsequently measured within 2 h after breath collection. Ambient air samples were taken from laboratories and office rooms where the study subjects were located in.

2.3 Measurement of samples with PTR-MS

The breath gas analysis was conducted using a commercial standard PTR-MS with Time-of-Flight (TOF) mass detector (PTR-TOF 2000, Ionicon Analytik, Innsbruck, Austria), which allows very sensitive offline and online measurements in the low ppb to ppt range. The breath sampling was performed as previously described by [1] and [8]. Briefly, the PTR-MS measurements were performed with a drift tube pressure of approximately 2.3 mbar. The VOC masses were analyzed in consecutive scans from a mass-to-charge ratio ranging from *m/z* 20 to 200.

2.4 Statistical analysis

The measured VOC profiles of the healthy volunteers were evaluated using the statistical toolbox in MATLAB (MathWorks, Version R2021a). Descriptive measures included the median and interquartile range (IQR) for the selected breath volatiles. Furthermore, a non-parametric test Wilcoxon rank sum test was performed for comparison of the two independent sampling conditions. This statistical test is well suited for the small evaluated sample size and because the VOC intensities are not normally distributed.

3 Results and discussion

3.1 Influence of everyday factors on the breath profile

The exhaled ethanol concentration (*m/z* 47) increased after the condition "eating cake" as shown in Fig. 3. This is attributed to the function of endogenous ethanol in the carbohydrate metabolism in the small intestine [7]. The breakdown of complex carbohydrates to glucose in the lower gastrointestinal tract is mediated by endogenous ethanol which is known to increase the permability of epithelial and colon cells making it available for glycolysis [7]. In contrast, the condition "eating lunch" had no significant influence on the average ethanol abundance (Fig. 2).

Related to elevated endogenous ethanol abundance that is involved in the intestinal glucose transport, we also found an increased level of breath acetone (*m/z* 59) as a metabolic byproduct (Fig. 3). We observed a slight decrease in acetone abundances with high physiological variation in both male and female subjects.

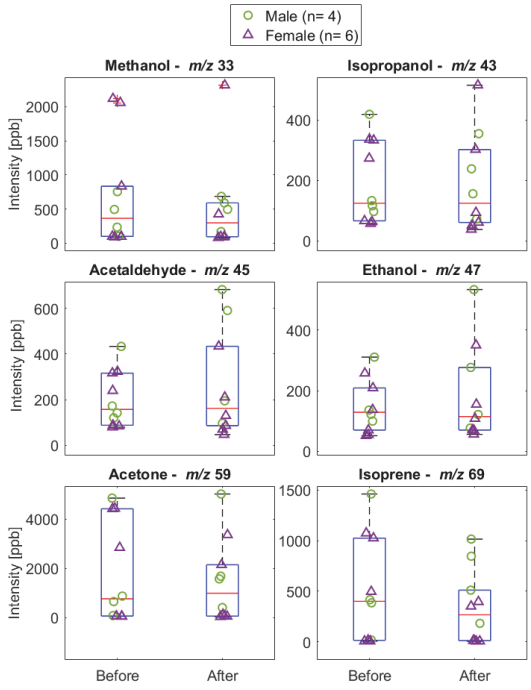

Fig. 1: Comparison of differences in inter-individual variations of selected VOCs after getting up and before brushing teeth related to subject gender.

Previous studies have shown that endogenous breath isoprene is a product of the mevalonate pathway related to the biosynthesis of cholesterol [7]. Under all three investigated conditions, a decrease in endogenous isoprene (*m/z* 69) level compared to the control sample was observed (Fig. 1 - 3). Methanol (*m/z* 33) showed the lowest variabilities in its median and is least sensitive to the influence of the investigated everyday life influencing factors. In addition, we observed higher average concentrations of VOCs for male subjects compared to female subjects. The non-parametric Wilcoxon rank sum test shown in Table 1 illustrates that, apart from *m/z* 33 (Methanol), all other investigated breath components are significantly different between the male and the female volunteers. In contrast, the Wilcoxon rank sum test showed no significant differences of the investigated volatiles before and after eating lunch.
The inter-subject variability of all measurements was compared by calculating the relative standard deviation for all measurements. The lowest relative standard deviation of 68 % was observed for acetaldehyde (*m/z* 45). The endogenous acetone abundance (*m/z* 59) had the highest variability with 115 % relative standard deviation from the mean value.

3.2 Intra- vs. inter-individual differences of breath biomarkers

Each subject has its own “breath fingerprint”, a characteristic profile of exhaled VOCs, which is influenced by exogenous and endogenous factors. To check whether the inter-individual differences between the subjects are greater than the intra-individual differences of each individual subject, the variability between the measurements was analyzed. The results indicated that the intra-subject differences for isopropanol (*m/z* 43), acetaldehyde (*m/z* 45) and isoprene (*m/z* 69) predominate, while the inter-subject differences for methanol (*m/z* 33), ethanol (*m/z* 47) and acetone (*m/z* 69) were larger. Methanol (*m/z* 33) and acetone (*m/z* 59) were found to show higher inter- than intra-individual differences [8], which can be confirmed by our results.

3.3 Implications for the design of clinical studies using PTR-MS

The experimental condition "eating lunch" led to a higher variability among the study subjects due to the intake of different meals and should therefore be avoided in clinical studies when confounding factors should be limited to a minimum. To further reduce the variability for ethanol (*m/z* 47) and acetaldehyde (*m/z* 45), food intake with a high sugar content should be avoided prior to sampling. The experimental condition "brushing teeth" had only a minor impact on the VOC variability compared to the condition "getting up" and might not have a significant impact on the sampling protocol in future clinical studies (Fig. 1).

4 Summary

In summary, this work provides a tool to systematically evaluate the influence of everyday influencing factors on the breath profile of healthy subjects. This is important for future clinical studies to limit the effect of confounding factors and to identify a robust set of clinically relevant biomarkers for diagnostic and therapeutic monitoring. With the simple, fast and non-invasive technique of breath gas analysis based on PTR-MS, it gives the opportunity to develop a possible application of breathomics as a diagnostic and therapeutic monitoring tool.

Author Statement

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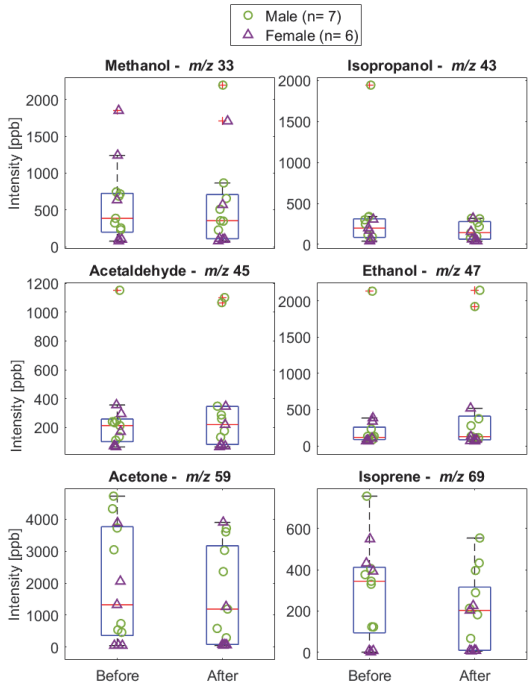

Fig. 2: Comparison of differences in inter-individual variations of selected VOCs before and after eating lunch related to subject gender.

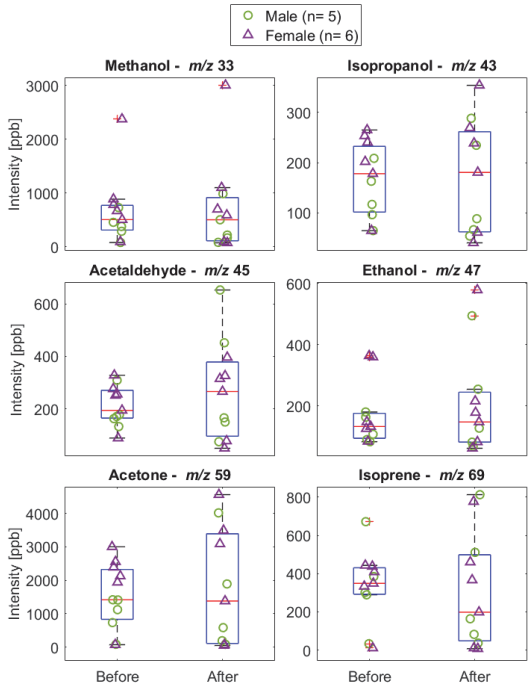

Fig. 3: Comparison of differences in inter-individual variations of selected VOCs before and after eating cake related to subject gender.

Tab. 1: Significance testing of breath metabolites for discrimation between subject gender and influencing factors.

Ion (*m/z*)	Tentative compound	p values for female vs. male volunteers	p values for before and after lunch
33	Methanol	0.14	0.84
43	Isopropanol	0.01	0.38
45	Acetaldehyde	0.02	0.76
45	Ethanol	0.02	0.61
45	Acetone	0.03	0.68
45	Isoprene	0.03	0.44

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