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Real-time selected ion flow tube mass spectrometry to assess short and long term variability in oral and nasal breath

Slingers G (1,2,3), Goossens R (2), Janssens H (2), Spruyt M (2), Goelen E (2), Vanden Eede M (2,4), Raes M (1,3), Koppen G (2)

(1) Hasselt University, Faculty of Medicine and Life Sciences, LCRC, Agoralaan, 3590 Diepenbeek, Belgium
(2) Flemish Institute for Technological Research, Unit Health, Industriezone Vlasmeer, 2400 Mol, Belgium
(3) Jessa Hospital, Paediatrics, Stadsomvaart, 3500 Hasselt
(4) University of Antwerp, Laboratory of Experimental Medicine & Paediatrics, Universiteitsplein, 2650 Edegem

Abstract

Background: Breath based non-invasive diagnostics have the potential to provide valuable information about a person's health status. However, it is not yet widely used in clinical practice due to multiple factors causing variability and the lack of standardized procedures. This study focuses on the comparison of oral and nasal breathing and on variability of volatile metabolites over short and long term. *Methods*: Selected ion flow tube mass spectrometry (SIFT-MS) was used for on-line analysis of selected volatile metabolites in oral and nasal breath of 10 healthy individuals 5 times in one day (short term) and 6 times spread over 3 weeks (long term) resulting in nearly 100 breath samplings. Intra-class correlation coefficients (ICC) were used to assess short and long term biological variability. Additionally, the composition of ambient air was analyzed at different samplings. The selected volatiles common in exhaled breath were propanol, 2,3-butanedione, acetaldehyde, acetone, ammonia, dimethyl sulfide, isoprene, pentane, and propanal. Additionally, environmental compounds benzene and styrene were analyzed as well. *Results:* Volatile metabolite concentrations in ambient air were not correlated with those in exhaled breath and were significantly lower than in breath samples. All volatiles showed significant correlation between oral and nasal breath. Five were significantly higher in oral breath compared to nasal breath, while for acetone, propanal, dimethyl sulfide and ammonia concentrations were similar in both matrices. Variability depended on the volatile metabolite. Most physiologically relevant volatiles (acetone, isoprene, propanol, acetaldehyde) showed good to very good biological reproducibility (ICC > 0.61) mainly in oral breath and over a short term period of one day. *Conclusion*: Both breathing routes showed relatively similar patterns, however bigger differences were expected. Therefore, since sampling from the mouth is practically more easy, the latter might be preferred.

Keywords

Real-time breath analysis, SIFT-MS, oral and nasal breathing, longitudinal follow up, breath volatiles, biological variability

Introduction

The lack of standardization in breath analysis prevents breath based diagnostics in cancer, infectious diseases, and others from being widely used in clinical practice despite its advantages of being non-invasive [1]. Different factors and clinical characteristics of the subject can influence the exhaled breath composition and cause variability. Martinez-Lozano *et al* (2014) reported circadian modulations in human breath metabolome [2]. Day-by-day variation of common breath volatile metabolites was studied by Diskin *et al* (2003) and they concluded that the distribution patterns of these various metabolites for a given individual are like a crude fingerprint [3]. Furthermore, exhaled breath can be influenced by environmental exposures via direct inhalation, dermal exposure or food intake [4]. Additionally, exhaled breath profiles are influenced by the breath sampling protocol itself. Therefore the following parameters should be taken into account: type (nasal/oral), breathing intensity, number of successive breath collections, portion of breath sampled (alveolar, end-tidal, full

breath), sampling duration (single-breath versus fixed-time or fixed-volume breathing), effect of expiratory flow and breath hold, type of collecting materials, sample pretreatment, storage and interference of ambient volatiles from the collection room [5, 6].

The current article focusses on type of breath (nasal/oral) and repeated breath analysis, to discover to which extend there are similarities and differences in the collected breath, using the same breath sampling protocol during the study. To explore this, Selected Ion Flow Tube Mass Spectrometry (SIFT-MS) was used. This technique allows real-time breath analysis, circumventing analyte loss and degradation during collection and storage, selective sorbents adsorbing only part of the volatiles, and possible contamination associated with storage. SIFT-MS (and also a related technique PTR-MS) was already used for quantification of breath volatiles in the late nineties [7]. More recently, Španěl & Smith (2020) reviewed the SIFT-MS measurements of individual breath volatile concentrations and their value as potential biomarkers of normal and adverse clinical status [8]. Soft chemical ionization of the breath sample volatiles is done using the reagent ions H_3O+, NO+ and O_2+, and the product ions formed are counted. This study aims to compare oral and nasal breath concentrations of nine common breath volatiles and two environmental volatiles using real-time SIFT-MS analysis, and to assess biological variability of those volatiles within one day and over a longer period of three weeks.

Methods

Participants

Ethical approval from the Committee for Medical Ethics Committee (CME, University Hospital Antwerp - University of Antwerp, Belgium). All participants (n = 10; 7 male and 3 female; aged between 23 and 56 years) signed an informed consent. Among the participants there was one male smoker and one male type I diabetics patient. Due to practical reasons 2 participants were unable to provide enough breath samples over the long term period of three weeks, therefore they were excluded for data analysis.

Study protocol

Breath sampling was performed in two different setups: oral breathing and nasal breathing. Both for oral and nasal breathing, participants were sitting upright and were instructed to breath slowly for 2 minutes at a rhythm of 1 sec inhalation, and 3 sec exhalation, resulting in approximately 22 exhalations per minute. A metronome was used to guide the breathing pattern of the participant. Prior to providing breath samples, the participants completed a questionnaire concerning their dietary intake during the previous 2 hours. No dietary constraints were imposed.

First oral breath was sampled via a heated (70°C) aluminum three-way mouthpiece with replaceable bacterial filter through which participants needed to breath. The three-way mouthpiece was mounted on to the heated (110°C) sample inlet of the SIFT-MS instrument which used a sampling flow rate of 20 mL/min. The third opening allowed the breath overflow to escape to the room. No cleaning procedure was adopted before the sampling of oral breath. After a short break, participants were asked to breath slowly via one nostril through a 2 cm Teflon tube with a 5 mm diameter which was connected to the same heated (110°C) sample inlet of the SIFT-MS instrument.

Short term variability was assessed by sampling all participants five times within one working day, starting between 9:15-10:30 (time point 1), and continuing sampling between 11:00-12:00 (time point 2), 13:00-13:45 (time point 3), 14:30-15:30 (time point 4), 16:00-17:45 (time point 5). Per day, one or two participants were sampled. Eight participants were also sampled twice a week for three more weeks in order to assess the long term variability. The latter six samples of each participant were taken at approximately the same time point of the day. Additionally, ambient air was measured at 63 of the

93 breath samplings spread over 17 different days. All measurements were performed in the same ventilated laboratory room.

Analysis with SIFT-MS

Details on the principle of SIFT-MS have been given in several reviews [9, 10]. Briefly, volatiles from the breath sample were ionized in the SIFT-MS flow tube, due to collision with the reagent ions (H3O+, NO+ and O2+), thereby forming product ions, which are quantified by mass spectrometry. Measurements were carried out by a Syft Voice200 instrument (Syft technologies, New Zealand) in multi-ion monitoring (MIM) mode with a scan duration of 2 minutes. Breath samples were analyzed using a dwell time of 10 msec, meaning that in one cycle every ion of a specific m/z was measured for 10 msec by the detector before going to the next. Multiple cycles were run during the 2 minutes scan. For ambient air samples a dwell time of 100 msec was used, and a scan duration of 2 minutes. Every day before the samples were analyzed, a validation step was performed, initiated by the startup procedure of the Syft instrument. This validation run uses a gas standard to calculate the instrument calibration function (ICF), that allows ion counts to be corrected for inter assay variability.

The following common breath volatiles were analyzed: propanol, 2,3-butanedione, acetaldehyde, acetone, ammonia, dimethyl sulfide, isoprene, pentane, and propanal [1, 11]. Additionally, environmental volatiles benzene and styrene were measured as well. The absolute concentrations were semi-quantitatively estimated using the LabSyft software (version 1.4.9.). Different aspects of ion chemistry have been investigated in depth in SIFT-MS and have been accounted for and implemented in the analytical software to obtain accurate quantification without the need for constant calibration [10]. Concentrations of specific volatiles are calculated based on the ICF corrected ion counts, the reaction rate coefficient of the mild ionization reaction (k-value), and in case the compound ionizes in different product ions, also the product ion branching ratios giving the relative proportion of each product ion, that is formed [10]. These parameters are available in the LabSyft software library but depend on the instrument's flow tube geometry, temperature and pressure [12]. An additional calibration (verification) procedure using standard mixtures would allow to adapt the parameters relative to the values included in the software and increase the accuracy of the instruments quantification. Since the volatile concentrations were compared relatively between oral and nasal breath and over the different sample collection time points (short term period of one day and long term period of six days spread over three weeks), this volatile calibration (verification) was not done. Table 1 shows possible endogenous sources and in literature reported concentrations of the common breath volatiles in healthy individuals as well as their measured product ions. Multiple product ions originating from these volatiles, were reported with their respective reagent ion and m/z value. Known conflicting ions, i.e. ions that were formed by more than one of the volatiles measured, were not scanned. Indeed, reagent ion H3O+ was not selected in the measuring method settings, because of that reason. Reducing the amount of scanned m/z, is fortunate in case of online measurements, in which the ion counting needs to be completed in the time frame of an exhalation.

Data analysis

Statistical analysis was performed on the absolute concentrations in parts per billion by volume (ppbv) of compounds calculated from the product ions using STATISTICA 12 (StatSoft, Inc., Tulsa, Oklahoma, USA). Data were extracted from the comma separated value files generated by the LabSyft software. The two minute online breath collection resulted in 22 exhalations, approximately. The 10 highest peaks were used to calculate the median. Spearman rank correlation coefficients were calculated to assess the correlation between ambient air concentrations and breath concentration.

Factor analysis with varimax raw rotation was performed to assess the coherence of the different breath compounds in oral and nasal breath. The clustering of product ions was visualized by plotting the factor loadings. Additionally Spearman rank correlation coefficients were calculated to assess the correlation between different volatiles in oral and nasal breath. Single linear regression analysis was performed to assess the correlation between oral and nasal breath and to assess the degree of change based on the b-coefficient. In order to compare oral and nasal breathing analysis of variance (ANOVA) was performed. Normality of the data was checked using the Shapiro-Wilk's W test. The natural logarithm was calculated in case the distribution of a compound product ion did not meet normality. The intra-class correlation coefficient (ICC) was calculated per measured product ion of each volatile for oral and nasal breath over both the short and long term period. The ICC is defined as the ratio of between-individual variance divided by the sum of between-individual and within-individual variance. The underlying concept of the ICC is to compare the variability within an individual's repeated measurements (e.g., how much do exhaled volatiles change in time for one person?) in contrast to the variability between different individuals' measurements (e.g., how do exhaled volatiles differ, on average, among many people?). When ICC is close to 0, repeat measures within an individual vary more compared to the total variation, and when ICC is close to 1, repeat measures are closer to each other [13]. ICC values of 0 to 0.20 are interpreted as a weak reproducibility, 0.21 to 0.40 fair, 0.41 to 0.60 moderate, 0.61 to 0.80 good and 0.81 tot 1 indicate very good reproducibility [14].

Table 1| Common breath volatiles analyzed with real-time SIFT-MS, concentration (C) in ppbv in exhaled breath of healthy individuals reported in literature ([a] mean; [b] mean (range); [c] median; [d] median (range); [e] mean (95%-confidence interval))

Volatile (CAS)	**Possible sources [ref.]**	**C [ref.]**	**Reagent ion, m/z**	**Product ion**
Propanal (123-38-6)	Peroxidation of ω-3 polyunsaturated fatty acid & ethanol metabolism in liver [3]	18.3 (5-66)[b] [15]	NO+, 57	C3H5O+
Acetaldehyde (75-07-0)	Endogenous ethanol metabolism [1]	24 (0-104)[b] [16] 22[c] [17]	O2+, 44	C2H4O+
propanol (71-23-8)	Enzyme-mediated reduction of acetone [18]	22 (0-100)[b] [17] 9.96[a] [19]	NO+, 59	C3H7O+
Acetone (67-64-1)	Lipid & carbohydrate metabolism [3, 20, 21]	477 (148-2744)[b] [17] 950 (281-2525)[b] [15] 469[a] [19]	NO+, 88	NO+.C3H6O
2,3-butanedione (431-03-8)	Fermentation of carbohydrates [4]	29 (1.4-187)[b] [15]	NO+, 86	C4H6O2+
			O2+, 86	C4H6O2+
Dimethyl sulfide (75-18-3)	Incomplete metabolism of sulfur containing amino acids in the transamination pathway [22]	5 (1,4-28)[b] [15] 7.58[a] [19]	O2+, 46	CH2S+
			O2+, 47	CH3S+
			O2+, 62	(CH3)2S+
Isoprene (78-79-5)	Byproduct of mevalonate pathway for synthesis of isoprenoids such as cholesterol [3, 20, 21]	106 (0-474)[d] [17] 131 (31-273)[b] [15] 115[a] [19]	NO+, 68	C5H8+
			O2+, 53	C4H5+
			O2+, 67	C5H7+
			O2+, 68	C5H8+
Pentane (109-66-0)	Lipid peroxidation [20, 21]	1.8 (0.34-22)[b] [15] 0.43[a] [19]	O2+, 72	C5H12+
Ammonia (7664-41-7)	Protein metabolism [3, 21]	833 (376-1846)[e] [17]	O2+, 17	NH3+
Styrene (100-42-5)	Environmental contaminant	0.92 (0.22-4.5)[b] [15]	NO+, 104	C8H8+
			O2+, 104	C8H8+
Benzene (71-42-2)	Environmental contaminant [4]	0.8 (0.16-5.8)[b] [15] 0.13[a] [19]	NO+, 78	C6H6+
			NO+, 108	NO.C6H6+
			O2+, 78	C6H6+

Results

Ambient air influences

Over 17 different days a total of 93 breath samples were collected. Additionally, ambient air was sampled at 63 of the 93 breath samplings. Spearman rank correlation coefficients showed no significant correlation between the VOCs measured in either (oral and nasal) breath and the ambient air, except for oral 2,3-butanedione (O2+, 86) (R=0.39). Additionally, all product ions (from the analyzed volatiles) were significantly higher in breath compared to ambient air.

Clustering of the volatiles' product ions

The coherence between product ions from the exhaled volatiles, including multiple product ions from the same volatiles, was visualized by plotting the factor loadings of all product ions measured in oral and nasal breath (figure 1). Isoprene product ions measured in oral breath cluster together very strongly while the same product ions measured in nasal breath form a separate cluster. Three additional clusters were observed containing the product ions measured in both oral and nasal breath: (1) ammonia; (2) acetone and acetaldehyde; and (3) propanol, 2,3-butanedione (NO+,86), dimethyl sulfide (only 2 product ions), and pentane. Remarkably, oral propanol loaded more strongly into this cluster than nasal propanol did and the same was observed for 2,3-butanedione (NO+, 86). Benzene, styrene and 2,3-butanedione (O2+,86) were only moderately loaded into the last mentioned cluster. Propanal and one dimethyl sulfide ion (O2+, 62) were not strongly loaded onto one of the five factors. Spearman rank correlation coefficient revealed moderate (R = 0.4 – 0.7) to strong (R > 0.7) correlation between acetaldehyde, acetone, propanol, isoprene (all four product ions) and dimethyl sulfide (O2+, 47) in both oral and nasal breath.

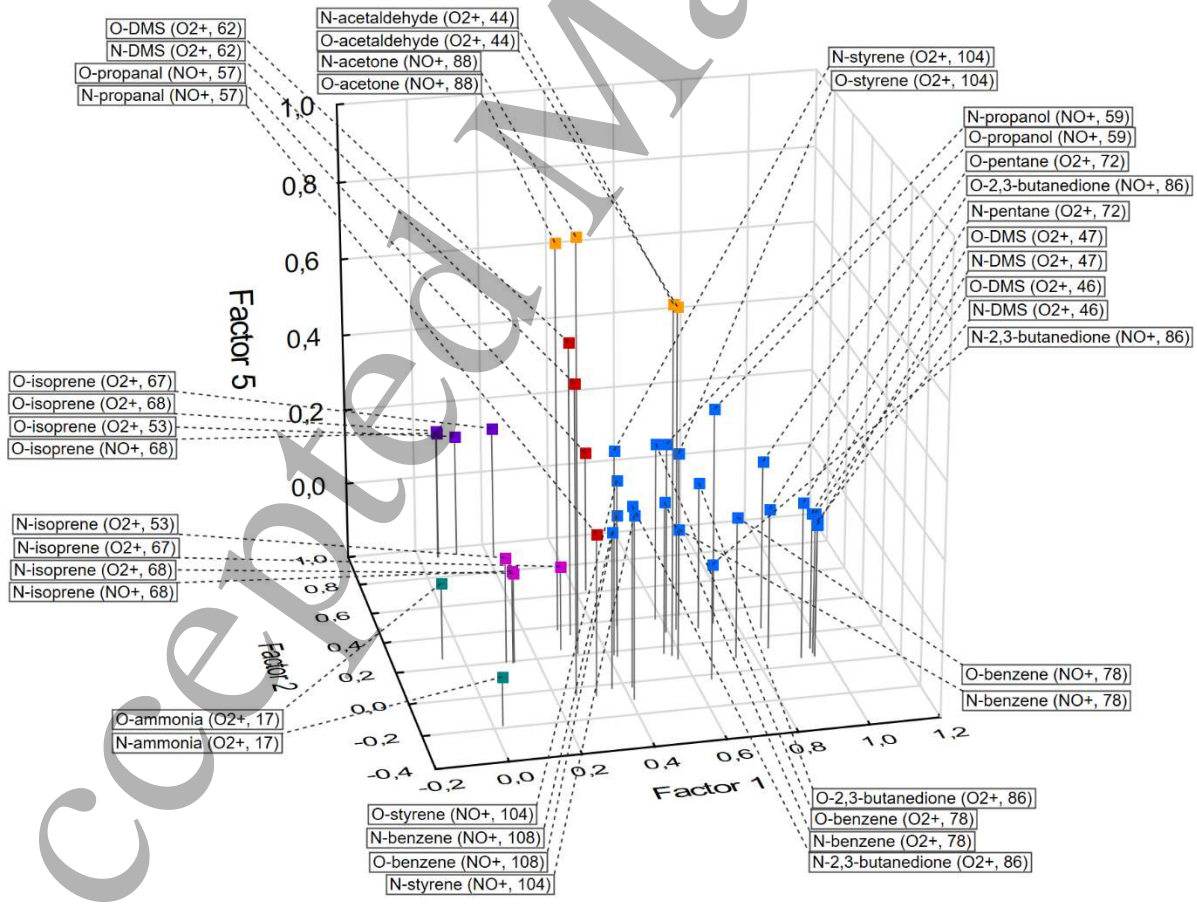

Figure 1| Factor analysis varimax raw rotation of the volatiles' product ions measured in oral (O) and nasal (N) breath; percentage of variance explained by factor 1, 2 and 5 is 30.91%, 13,67% and 10.64%

Oral versus nasal breathing

All product ions of the measured volatiles were either higher or equally present in the oral vs. nasal breath (table 2). Furthermore, linear regression analysis showed significant correlations between oral and nasal breath for all volatiles. For respectively nine, five and six out of twenty measured product ions originating from the measured volatiles this was a strong to very strong (R > 0.6), moderate (R = 0.4 – 0.59) and weak (R < 0.39) correlations. B-coefficients showed that for most volatiles the nasal breath concentration increased by approximately 1 (0.74-1.30) ppbv for every 1 ppbv increase in oral breath. Propanal, 2,3-butanedione, one product ion from isoprene and styrene (both product ions) showed b-coefficients below 0.70 and one product ion from dimethyl sulfide showed a b-coefficient of 1.43. Correlation coefficients and b-coefficients are given in table 2.

Table 2 | Mean concentration in parts per billion by volume (ppbv) and standard deviation of selected volatiles in ambient air, oral and nasal breathing calculated from specific product ions (m/z, indicated in first column together with the reagent ion); and linear regression correlation coefficient (R) and b-coefficient (b) between oral and nasal concentrations

volatile compound (reagent ion, m/z)	**Ambient air**	**Oral breathing**	**Nasal breathing**	**R**	**b**
propanal (NO+, 57) °	3.39 (5.44)	3.51 (0.96)	3.25 (0.77)	0.32	0.38
acetaldehyde (O2+, 44) * °	19.43 (6.2)	37.73 (22.22)	31.75 (13.04)	0.82	1.09
propanol (NO+, 59) **** °	4.52 (11.39)	9.55 (17.21)	5.07 (12.19)	0.74	0.87
acetone (NO+, 88)	253.23 (224.4)	495.31 (416.47)	405.73 (279.42)	0.92	1.18
2,3-butanedione (NO+, 86) ***	1.83 (0.88)	5.58 (2.54)	4.48 (1.88)	0.42	0.38
2,3-butanedione (O2+, 86) ****	23.56 (19.14)	51.09 (17.02)	37.96 (10.26)	0.39	0.50
dimethyl sulfide (O2+, 46) °	38.20 (25.64)	89.45 (214.13)	76.58 (171.21)	0.74	1.30
dimethyl sulfide (O2+, 47) °	28.71 (18.08)	114.55 (506.75)	93.40 (352.66)	0.99	1.43
dimethyl sulfide (O2+, 62)	6.33 (3.48)	13.66 (6.61)	12.22 (4.72)	0.77	1.08
isoprene (NO+, 68) **	27.93 (22.83)	63.62 (35.8)	49.97 (18.1)	0.39	0.77
isoprene (O2+, 53) *	28.78 (20.31)	98.69 (48.72)	84.64 (26.77)	0.32	0.58
isoprene (O2+, 67) ***	41.85 (32.58)	106.79 (58.72)	83.25 (30.88)	0.50	0.95
isoprene (O2+, 68) **	62.65 (44.02)	144.67 (75.97)	115.37 (39.89)	0.39	0.75
pentane (O2+, 72) *** °	64.62 (30.14)	144.01 (103.09)	113.31 (66.71)	0.41	0.94
ammonia (O2+, 17)	25.60 (7.3)	33.63 (6.19)	32.88 (6.17)	0.74	0.74
styrene (NO+, 104)	4.8 (1.8)	8.76 (2.04)	8.29 (2.08)	0.50	0.49
styrene (O2+, 104) **	2.29 (2.65)	6.34 (2.83)	5.33 (1.43)	0.36	0.55
benzene (NO+, 78) *	1.2 (0.67)	3.94 (2.37)	3.27 (1.74)	0.69	0.78
benzene (NO+, 108) ****	9.35 (4.62)	32.16 (13.88)	23.58 (7.78)	0.52	0.88
benzene (O2+, 78)	9.64 (5.32)	17.15 (9.94)	16.79 (8.16)	0.66	1.03
ANOVA: * p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001; ° natural logarithm was taken to obtain normality for ANOVA					

Short and long term variability

Short and long term variability of the selected volatiles was assessed by calculating the ICC for the product ions of those volatiles for oral and nasal breathing separately. Variation in oral and nasal breathing over short and long term was visualized in figure 2. For volatiles which are measured based on multiple product ions, only the product ion with the lowest calculated concentration is shown in figure 2 as the LabSyft software ignores product ions if their concentration exceeds the lowest by more

than 20%. This tolerance ratio can be adjusted if needed, but as the current study evaluates the product ions separately it was not necessary. Supplementary figure 2 shows all product ions of these volatiles. Table 3 shows ICC values for all measured volatiles (per product ion) for oral and nasal breath separately and for both short and long term periods. Over the short term period of one day most volatiles were quite stable in oral breath (ICC > 0.41). Acetone, isoprene (all four product ions), propanol and acetaldehyde showed good to very good biological variability (ICC > 0.61). Additionally, oral breath concentrations were more stable compared to nasal breath (i.e. oral ICC > nasal ICC), with the exception of isoprene (all four ions), propanol, ammonia, dimethyl sulfide (O2+, 47 & 62), and benzene (NO+, 108). Over the long term period of three weeks the opposite is observed. Furthermore, most volatiles showed higher biological variability in oral breath over the long term period compared to the short term period (i.e. short term ICC > long term ICC), with the exception of dimethyl sulfide (O2+, 62), isoprene (all four ions) and styrene (O2+, 104).

Table 3 | Intra-class correlation coefficients of all selected volatiles per product ion for oral and nasal breath over both short and long term periods

Volatile (reagent, m/z)	**Short term**		**Long term**	
	Oral	**Nasal**	**Oral**	**Nasal**
Propanal (NO+, 57)	0.59	0.36	0.39	0.52
Acetaldehyde (O2+, 44)	0.75	0.67	0.59	0.79
Propanol (NO+, 59)	0.81	0.87	0.63	0.82
Acetone (NO+, 88)	0.72	0.67	0.47	0.75
2,3-butanedione (NO+, 86)	0.66	0.58	0.50	0.59
2,3-butanedione (O2+, 86)	0.37	0.18	0.54	0.10
Dimethyl sulfide (O2+, 46)	0.41	0.24	0.36	0.14
Dimethyl sulfide (O2+, 47)	0.54	0.77	0.48	0.84
Dimethyl sulfide (O2+, 62)	0.66	0.66	0.71	0.69
Isoprene (NO+, 68)	0.68	0.83	0.80	0.82
Isoprene (O2+, 53)	0.64	0.76	0.73	0.78
Isoprene (O2+, 67)	0.70	0.80	0.76	0.80
Isoprene (O2+, 68)	0.71	0.84	0.76	0.86
Pentane (O2+, 72)	0.63	0.47	0.37	0.58
Ammonia (O2+, 17)	0.24	0.37	0.26	0.37
styrene (NO+, 104)	0.47	0.46	0.16	0.41
styrene (O2+, 104)	0.39	0.36	0.52	0.44
benzene (NO+, 78)	0.49	0.34	0.39	0.50
benzene (NO+, 108)	0.35	0.55	0.30	0.29
benzene (O2+, 78)	0.66	0.49	0.59	0.53

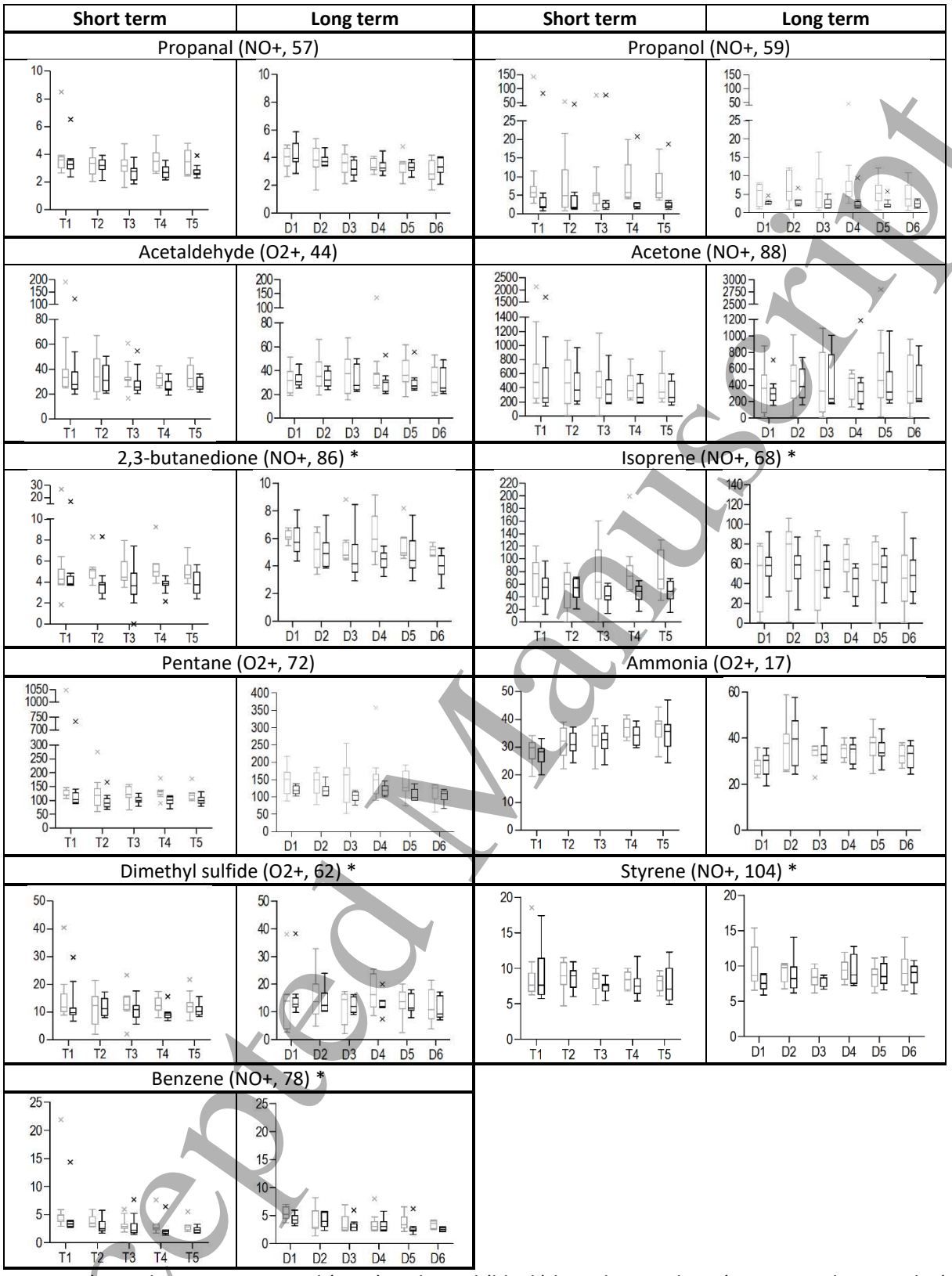

*Figure 2 | Boxplot comparing oral (grey) and nasal (black) breath over short (5 times within one day) and long term (6 times spread over 3 weeks); timepoints (short term) or days (long term) are indicated in x-axis; y-axis indicates concentration (ppbv); * multiple product ions were measured but only the product ion with the lowest calculated concentration is shown*

Discussion

Using real-time SIFT-MS, nine common breath volatile metabolites were analyzed in oral and nasal breath of ten individuals. Short term variability was examined over five different time points within one day. For the long term comparison, six breath samples were taken on the same time point of the day for each individual within a three week period. Additionally, ambient air was analyzed at different time points.

Ambient air influences

Contamination from ambient air is a potential confounder in breath analysis which needs attention [23]. Different methods to reduce localized background effects are proposed: clean air supply for inhalation, equilibrating with ambient air, collect background sample and calculate alveolar gradient or set cutoff (e.g. inspiratory air should not be greater than 25% of the breath concentrations) [23, 24]. However, there is no consensus on a standard method yet. In this study all samples were collected in the same room to avoid background bias. Additionally, measured ambient air concentrations of most volatiles were significantly lower compared to breath and no significant correlations were observed between either (oral and nasal) breath and the ambient air. These results suggest ambient air contamination during sampling was minimal and measured volatiles were predominantly endogenously produced or excreted via the lungs after previous uptake from the environment, e.g. via inhalation or ingestion, and distribution within the body [25].

Clustering of the volatiles' product ions

The breath volatiles were quantified based on product ions (at specific m/z) which did not overlap with other measured volatiles. Such conflicts can also occur with other volatiles in the sample which are not measured. For example, the reaction of both benzene and acetic acid with reagent ion NO+ result in a product ion of m/z 108, therefore this product ion is not best suited to assess either benzene or acetic acid. In the factor analysis product ions originating from the same volatile metabolite (isoprene, 2,3-butanedione, dimethyl sulfide) clustered together, indicating a conflict on their product ions is less likely. Nevertheless, one product ion from dimethyl sulfide loaded into a different cluster than the other two, possibly due to a conflict with other breath volatiles. When quantifying volatiles using SIFT-MS one should be aware of the potential overlapping product ions.

Comparison of oral and nasal breath

Factor analysis revealed strong correlations between oral and nasal breath concentrations for most volatiles – except for isoprene - which was also observed in the linear regression analysis. Oral and nasal breath were quite well correlated. Nasal breath concentrations of most volatiles increased by approximately 1 ppbv for every 1 ppbv increase in oral breath. All measured volatile concentrations calculated based on the product ions were either similar in both matrices or higher in oral breath compared to nasal breath. The latter raises the following question. Can these compounds be

interpreted as being of systemic origin, and having an additional oral origin? Wang *et al* (2008) previously showed that acetone and isoprene concentrations, measured with SIFT-MS, were the same in oral and nasal breath, indicating a systemic origin. They also observed that acetaldehyde and propanol appeared to have both oral and systemic origins which could explain the strong correlation between both breathing routes despite the significant difference. Furthermore, much lower ammonia concentrations were reported in nasal breath than in oral breath and even highest in the oral cavity, indicating ammonia is largely generated in the mouth [26]. Unexpectedly, the presented results for isoprene and ammonia were not in line with the reported results of Wang *et al* (2008). Ammonia showed no significant difference between oral and nasal breath but was quantified based on only one product ion. Therefore, it is not possible to exclude unknown conflicts that might conceal the difference between oral and nasal ammonia concentrations. Isoprene was significantly higher in oral breath, while it is mainly of systemic origin. Perhaps, switching between oral and nasal breath sampling, influenced the concentration of isoprene. Sukul *et al* (2017) studied the effect of switching breathing routes and showed that it induced significant changes in respiratory parameters, which subsequently changed specific volatile patterns [5]. Breath isoprene concentration has been shown to increase within a few seconds after exercise is started as a result of rapid increase in heart rate and then reaches a lower steady state when breath rate stabilizes [27]. Participants had to move to the sampling room which might have increased their heart rate just before providing the oral breath sample. As the nasal breath sample was taken after a short break their heart rate and breath rate would stabilize leading to lower breath isoprene concentrations.

Remarkably, absolute concentrations calculated from different product ions originating from the same volatile (isoprene, 2,3-butanedione, dimethyl sulfide, benzene) differed to some extent. This could be due to the fact that the instrument's library parameters (k-value, branching ratios) were not verified with standard mixtures of the measured volatiles. This was not needed as our main aim was to compare oral and nasal breath and to assess the variability in breath samples over a short and long term period.

Short and long term variability

The variability of both breathing routes within one day and between different days spread over three weeks was assessed by calculating the ICC which seemed to vary per volatile compound. Propanol, acetaldehyde, acetone, isoprene (all four product ions) and dimethyl sulfide (only product ion O2+, 62) showed good to very good reproducibility (ICC > 0.61) in both oral and nasal breath over the short term period. Furthermore, Spearman rank correlations between these metabolic volatiles were moderate to strong in both breathing routes. The high ICC values indicated a larger inter-individual difference, compared to a relative little diurnal variation in most of the individuals. Causes for higher between-individual variance could be attributed to differences with respect to age, gender, BMI, diet,

physiology, or illness [28]. Isoprene, however, showed in some individuals quite some within-day variability in the oral breath pattern, but not in the nasal breath. Assuming the oral breath is more representative for the systemic volatiles, which was to be expected. A circadian rhythm in exhaled isoprene concentrations with a maximum in the morning was reported by Wilkinson *et al* (2019) [29], possibly caused by heart rate increases when raising up [27]. As the participants in our study were monitored during a working day, that effect was not observable anymore, but differences in heart rate and exhalation rate could still cause within-day variations.

Over the long term, similar to the short term, the ICC was above 0.61 in both breathing routes for propanol, dimethyl sulfide (O2+, 62) and isoprene (all four product ions). Acetaldehyde and acetone only showed good long term reproducibility (ICC > 0.61) in nasal breath. These results suggest that the above mentioned exhaled volatiles are quite stable both over short and long term periods. This may indicate the stability of the participants' basal metabolic rate. All other product ions were more variable over the long term period of three weeks compared to the short term. This was to be expected as the day-to-day individual physiology and exposure could change and cause more biological variability [28]. Discussing the data on an individual level was beyond the scope of this paper. If subject comparison is desired the normalization of 'patient factors', such as smoking, food intake and diseases, should be considered.

In the current study, participants were not limited in their food and drink consumption, nor were they limited in their working activities but both could have an effect on the breath profile. Acetone was demonstrated to increase during fasting and to rapidly decrease in response to food intake [30, 31]. However, following a ketogenic diet, which is high in fat and low in carbohydrates, appears to elevate breath acetone levels [31, 32]. Isoprene concentrations on the other hand in breath did not change significantly in response to feeding after fasting for 12 hours [30]. Ammonia has been shown to increase after an oral protein challenge both in breath and blood [33]. Additionally, different food diets affect the ammonia concentration in breath differently. Adopting a raw vegan diet for ten days decreased ammonia concentrations in breath which returned approximately to the previous value after three days return to the mixed diet. Persons following the Dukan diet presented high levels of breath ammonia compared to the mixed diet, as did vegetarians [34]. Acetaldehyde concentrations will increase after alcohol consumption because alcohol is largely metabolized to acetaldehyde [35]. All these examples demonstrate how dietary factors may have an important impact on breath compounds and more have been briefly outlined previously by Ajibola *et al* (2013) [36].

Ideally this type of follow-up studies is performed in a large, homogenous study population following some dietary constraints. Asking the participants not to eat food at least one hour before the breath sampling, would reduce the impact of contaminations given by food. This was hardly feasible for the current study. Furthermore, the sampling times and days of the participants were not all similar. For

the short term measurements one or two participants were sampled on the same day, but the time points within the day were specified as time slots of approximately 1 to 1,5 hours. For the long term measurements not all participants were sampled on the same days as well, but to lower variability, all long term measurements of each participant were performed at approximately the same time point of the day. However, this was not the same for all participants. The participants from our research facility adjusted their working schedule to provide the breath samples. The various sampling days and times could introduce variation in the data due to environmental influences or confounders. Therefore, extensive background volatile sampling was done. However, the breath volatile concentrations were not correlated with sampling room ambient air concentrations. Therefore, they were assumed to be of minimal influence.

Conclusion

Short and long term follow up was done in ten healthy individuals for nine common breath volatiles in oral and nasal breath, using real-time SIFT-MS sampling and analytics. The absolute concentrations of the measured volatiles in oral breath were either higher (acetaldehyde, propanol, 2,3-butanedione, isoprene and pentane) or similar (propanal, acetone, dimethyl sulfide and ammonia) to those in nasal breath. Acetaldehyde, propanol, acetone, isoprene – originating from basal metabolic processes – showed little biological variability both over the short term period of one day and over the long term period of three weeks the difference. Larger differences between oral and nasal breath were expected but both breathing routes showed relatively similar patterns. Therefore, since sampling from the mouth is practically more easy, the latter might be preferred.

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