Real-time versus thermal desorption selected ion flow tube mass spectrometry for quantification of breath volatiles

Slingers Gitte (Orcid ID: 0000-0002-7021-5125)

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Slingers G[1,2,3,*], Vanden Eede M[2,4], Lindekens J[1], Spruyt M[2], Goelen E[2], 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, VITO Health, Boeretang 2400 Mol, Belgium

(3) Jessa Hospital, Paediatrics, Stadsomvaart, 3500 Hasselt, Belgium

(4) University of Antwerp, Laboratory of Experimental Medicine and Paediatrics, Universiteitsplein, 2650 Edegem, Belgium

*** Corresponding author:**
Gitte Slingers
Hasselt University
Faculty of Medicine and Life Sciences
Martelarenlaan 41, 3500 Hasselt, Belgium
Tel: +32 14 33 51 54
e-mail: gitte.slingers@uhasselt.be

Short title
Quantification of breath volatiles: real-time vs off-line SIFT-MS

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Abstract

Rationale: Selected ion flow tube mass spectrometry (SIFT-MS) is versatile, rapidly provides result output, and determines a wide range of volatiles, making it suitable for biomedical applications. When direct sampling onto the SIFT-MS instrument is impractical, combining thermal desorption (TD) and SIFT-MS might offer a solution as it allows sample storage on sorbent tubes for later analysis. This work compares off-line TD SIFT-MS and real-time SIFT-MS for the quantification of selected breath volatiles.

Methods: Ten healthy non-smoking individuals provided 60 breath samples per method. For off-line analysis, breath was collected onto sorbent tubes via a breath sampler provided with filtered inspiratory air. After TD, samples were re-collected in Tedlar bags which were then connected to the SIFT-MS instrument. For real-time analysis breath was sampled directly onto the SIFT-MS instrument. In both cases the analytical method included a total of 155 product ions, and 14 selected volatiles were quantified. The agreement between the methods was assessed using Pearson correlation coefficients and Bland-Altman plots.

Results: Overall, correlations between real-time and off-line analysis were moderate to very strong (r: 0.43 - 0.92) depending on the volatile of interest, except for 2,3-butanedione and styrene. The difference between real-time and off-line measured breath concentrations (average bias) ranged between -14.57 ppbv and 20.48 ppbv. For acetone and isoprene, it was 251.53 ppbv and 31.9 ppbv, respectively.

Conclusions: Real-time SIFT-MS and off-line TD SIFT-MS for quantification of selected breath volatiles did not show optimal agreement. Analyzing a multitude of analytes in breath via direct exhalation onto a SIFT-MS instrument for real-time analysis is challenging. On the other hand, off-line analysis using a breath collection device also has its issues such as possible sample losses due to selective absorption depending on the sorbent used or during desorption and transfer to the SIFT-MS instrument. Despite these drawbacks, both methods were moderately well correlated.

Keywords Thermal desorption, SIFT-MS, real-time vs off-line, breath VOCs

Introduction

Analytical techniques allow the measurement of volatile organic compounds (VOCs) in various media including exhaled breath [1]. Breath analysis is used for the identification, detection and quantification of volatile biomarkers related to disease. Given that the concentrations of most volatile biomarkers in exhaled breath are low, often at parts-per-billion by volume (ppbv) or lower, this is a challenge for the analytical instrumentation which must be sufficiently accurate and precise to be useful [2].

The most widely used technique for analyzing mixtures of volatiles is gas chromatography mass spectrometry (GC/MS). However, non-separative methods based on mass spectrometry have been established as an attractive option for analyzing volatile compounds. These methods are suitable for biomedical applications because of their versatility, rapid output of results, and the wide range of volatiles that can be determined [3]. Selected ion flow tube mass spectrometry (SIFT-MS) is such a non-separative method and offers significant advantages over GC/MS in

a range of scenarios through its simplicity of operation and its capability for real-time analysis of VOCs [4]. Details on the principle of SIFT-MS have been given in several reviews [5, 6]. A reagent ion, selected by a quadrupole mass filter, is injected into fast-flowing helium carrier gas and is used to ionize the volatiles in an air or breath sample that is introduced at a known flow rate into the carrier gas downstream. The chemical ionization of the volatiles by SIFT-MS is based on the reagent ions (H_3O^+, NO^+, O_2^+) and results in characteristic product ions that identify the compounds, while their count rates, determined by mass spectrometry, allow quantification [7].

Quantification by SIFT-MS is based on reagent ion count rates, product ion count rates and the known reaction kinetics from the SIFT-MS database. Count rates can be corrected for inter-assay variability based on the instrument calibration function (ICF) which is calculated using a gas standard during a validation step, initiated by the startup procedure of the instrument. In cases where the chemical ionization of a compound results in multiple product ions, the product ion branching ratios which indicate the relative proportion of each product ion, are also taken into account to determine absolute concentrations. The appropriate software and kinetics database avoid the need for a daily calibration procedure, as is the case for GC/MS. However, the parameters provided in the kinetics database depend on the instrument's flow tube geometry, temperature and pressure; therefore, a one-time verification of those parameters is needed to increase the accuracy of the instrument [7].

The application of SIFT-MS for direct real-time sampling and analysis is not always feasible, such as in clinical situations. Therefore, the combination of thermal desorption (TD) and SIFT-MS offers a solution as samples can be stored on a sorbent tube for later analysis. Furthermore, parallel analyses of the same samples by GC/MS and SIFT-MS would be possible. It has been previously shown that the TD-SIFT-MS combination provides accurate analysis both for both gas standards in a helium mixture and environmental gaseous samples [9, 10] and for breath concentrations of acetone and isoprene [11]. In this paper we compare real-time SIFT-MS with off-line TD SIFT-MS for the quantification of multiple volatiles in exhaled breath. The work was done in the context of a ring trail in which the ingestion of peppermint oil capsules was used to benchmark different breath sampling and analytical methods [12]. The selected volatiles were acetone, isoprene, acetaldehyde, ammonia, dimethyl sulfide, 2,3-butanedione, pentane, propanal, propanol, benzene, styrene, γ-terpinene, eucalyptol, and menthone.

Methods

Participants

Ten healthy non-smoking adults (1 male and 9 females; aged between 18 and 50 years) without history of chronic or recent acute disease were enrolled in the study after signing an informed consent. According to the peppermint benchmarking protocol [12], the participants provided a baseline breath sample (t=-30 min), ingested a peppermint oil capsule (t=0 min) and provided repeated breath samples (t=60 min, 90 min, 165 min, 285 min, and 360 min). This resulted in 60 real-time breath samples and 60 off-line breath samples. Ethical approval was obtained from the Committee for Medical Ethics University Hospital Antwerp - University of Antwerp, Belgium (EC UZA 18/11/154).

Sampling procedures

First off-line breath samples were collected with the ReCIVA® Breath Sampler (Owlstone Medical, Cambridge, UK) with settings for collection of breath from upper and lower airways. The breath sampler was combined with an active coal scrubber through which inspiratory air was filtered. Using this sampler, a volume of 500 mL of exhaled breath was collected onto each of two sorbent tubes packed with TenaxGR/Carbograph5TD (Markes International, Llantrisant, UK) using a flow rate of 200 mL min^{-1}. On every sampling day filtered air (500 mL) from the scrubber was also sampled using the ReCIVA® in a continuous sampling mode at a flow rate of 200 mL min^{-1}. For this the sampling mask was not worn by a person, but instead placed onto a clean glass curved surface to avoid contamination from ambient air.

Real-time breath analysis was performed immediately after off-line breath collection. For this, the exhaled breath from the participants was sampled directly into the Syft VOICE200 SIFT quadrupole mass spectrometer (Syft Technologies, Christchurch, New Zealand) through a heated (70°C) aluminum three-way mouthpiece with an interchangeable bacterial, viral filter (Medical Device Directive Class IIa). The three-way mouthpiece was mounted on to the heated (110°C) sample inlet of the Syft instrument which used a sampling flow rate of 20 mL min^{-1}. The third opening allowed the breath overflow to escape to the room. Participants were instructed to breathe slowly for 2 minutes at a rhythm of 2 sec inhalation, and 3 sec exhalation. A metronome was used to guide the breathing pattern of the participant. Before each participant provided the breath sample, ambient air was measured through the same sample inlet using the same Syft method.

Analytical method

Volatiles collected onto the sorbent tubes were desorbed using a TD100 thermal desorption unit (Markes International). For this, the tube conditioning mode was used at a temperature of 320°C for 5 min with a split flow of 50 mL min^{-1} and with a pre-purge of 1 minute at 50 mL min^{-1}. After TD, samples were re-collected at the split vent in a cleaned 1-L Tedlar bag, which showed lower background contamination than Nalophan and Teflon bags (data not shown). The Tedlar bag was then manually attached to the Syft sample inlet and its contents were analyzed. Before use, the 1-L Tedlar bag was cleaned by flushing it five times with nitrogen.

Off-line breath samples and real-time breath samples were both measured by the Syft VOICE200 with helium (99.999%, Air Products, Allentown, PA, USA) as carrier gas. The flow tube pressure and temperature were 0.88 Torr and 120°C, respectively. All breath samples were analyzed in multi-ion monitoring (MIM) mode with a scan duration of 2 minutes and a dwell time of 10 msec, meaning that in one scan cycle every ion of a specific *m/z* value was measured for 10 msec by the detector before the next ion was scanned. Twenty-two cycles were run during the 2 minutes scan duration.

The MIM method used H_3O^+, NO^+ and O_2^+ reagent ions, and included some common breath volatiles, environmental compounds benzene and styrene, and typical peppermint-related volatiles. For these volatiles, concentrations in parts per billion by volume (ppbv) were calculated based on the best product ion using the parameters in the Syft kinetics library (Table 1). Criteria used to select the best product ion for quantification were (i) the absence of secondary product ion, and (ii) the preference for product ions without conflicts i.e. more than one of the volatiles measured from the same product ion. If two product ions from one volatile met both criteria, we opted for the product ion resulting in the lowest concentration of the

volatile, which is the same approach as used by the LabSyft software (version 1.4.9; Syft Technologies).

The parameters in the Syft kinetics library were verified against a standard gas only for ɣ-terpinene, eucalyptol and menthone. For other volatiles the parameters were not verified as the goal of the study was to compare the relative volatile concentrations. For the quantification of pentane, *m/z* 42 (O_2^+) was the best quantifying product ion; however this ion was in conflict with propanol. Therefore, the pentane concentrations were calculated by subtracting the propanol concentration calculated from *m/z* 59 (NO^+) from the pentane + propanol concentration calculated from *m/z* 42 (O_2^+). The same approach was used for eucalyptol and menthone (see Table 1).

In addition, the MIM method included unidentified product ions which were strongly present in headspace measurements of the contents of the peppermint oil capsule. As the compounds from which these ions originated were not identified, their reaction kinetics were unknown and their concentrations could not be calculated. These unidentified product ions are beyond the scope of this article. In total 155 product ions were measured (Supplementary Table 1, supporting information). This semi-targeted method was used as a prototype sampling methodology for broad-spectrum screening of volatiles suspected to be present in exhaled breath, allowing multiple product ions to be scanned simultaneously.

Data analysis

Concentrations in ppbv were extracted from the comma separated value files generated by the LabSyft software (version 1.4.9.). The two-minute real-time breath collection included 22 exhalations, approximately. The 10 highest peaks of each product ion were used to calculate the average concentration. The off-line method, which comprised breath collection onto sorbent tubes which were then desorbed into Tedlar bags, resulted in a homogenous mixture providing a more stable signal without pronounced exhalation peaks. Therefore, the average concentration of the entire two-minute measurement was calculated per sorbent tube. The average concentration of both sorbent tubes was used as the final off-line measured breath concentration. The repeatability of the quantification from the two tubes was assessed by calculating the correlations.

Statistical analysis was performed using Prism GraphPad Software (v.5.00, Graphpad Software Inc., San Diego, CA, USA). Pearson correlation coefficients between real-time and off-line measured breath concentrations were calculated based on the entire dataset of all sampling timepoints (N=60 per method). The difference in concentration between the two methods was assessed by the paired T-test. The Bland-Altman method was used to evaluate the agreement between real-time and offline measured breath concentrations: Bland-Altman plots are scatterplots of the difference between two paired measurement of the two methods (real-time – off-line) against the mean of the two measures ((real-time + off-line)/2)[13].

Sample humidity was recorded based on the *m/z* 37 and 55 ions from H_3O^+ reactions. The count rates of these product ions were used to compare the sample humidity between the real-time and off-line method by a paired T-test, which was performed in GraphPad Prism Software.

Results

The breath concentrations of the 14 selected volatiles were measured using real-time and off-line SIFT-MS at different timepoint pre- and post-ingestion of a peppermint oil capsule

(Supplementary Table 2, supporting information). The repeatability of off-line quantification from two sorbent tubes showed significant correlations for all volatiles. Highest correlations between the two sorbent tubes were observed for acetone, isoprene, acetaldehyde, ammonia, pentane, propanal, γ-terpinene, eucalyptol and menthone (r = 0.71 – 0.99). Dimethyl sulfide, 2,3-butanedione, propanol, benzene and styrene showed much lower correlations between the tubes (r = 0.26 – 0.66).

In addition to the breath samples, several background samples for the real-time and off-line method from ambient air and filtered air, respectively, were analyzed. Figure 1 (left) shows the measured breath and background concentrations over time of both methods. All the volatiles showed significant differences in absolute breath concentration between the two methods, except for 2,3-butanedione and styrene. For all three peppermint volatiles – γ-terpinene, eucalyptol, and menthone – the maximum concentrations were reached 60 min after ingestion and then gradually decreased to concentrations similar to the background (although less clear for menthone). The differences in concentration between the real-time and off-line method at 60 minutes after ingestion of the peppermint oil capsule were 9.9 ppbv, 4.6 ppbv and 10.3 ppbv for γ-terpinene, eucalyptol, and menthone, respectively.

Pearson correlations between the real-time and off-line measured breath samples are shown in Figure 1 (middle). For all volatiles a significant correlation was observed, with a Pearson correlation coefficient ranging between 0.43 and 0.92, except for 2,3-butanedione and styrene which both did not show a significant correlation. Bland-Altman plots are shown in Figure 1 (right). The average bias ranged between -14.57 ppbv and 20.48 ppbv, except for acetone and isoprene which showed a larger average bias of 251.53 ppbv and 31.9 ppbv, respectively. The sample humidity for both the real-time and the off-line method was estimated. Real-time measured samples showed significantly higher humidity than off-line measured samples, as was seen from the higher count rates of *m/z* 37 and 55 from the H_3O^+ reactions (Figure 2).

Discussion

Real-time and off-line SIFT-MS breath analysis were compared for a selection of volatiles in exhaled breath. Samples were obtained from ten subjects participating in a study in which they provided one baseline sample before ingestion of a peppermint oil capsule and five samples at specific (washout) timepoints after ingestion of the capsule [12]. Real-time SIFT-MS analysis consisted of tidal breathing directly onto the instrument, comparable with previously used protocols [14-19]. For off-line analysis, on the other hand, tidal breath was collected using the ReCIVA® breath sampler and analyzed using an indirect combination of TD and SIFT-MS. In both methods participants were asked to breathe through their mouth, a protocol which has been previously shown to provide similar patterns to nasal breath [20]. The lack of a standardized breath sampling or analysis protocol complicates method comparison. This was the main reason for setting up the peppermint ring trial study to compare different breath sampling and analytical methods [12].

Exhaled breath is diluted with inspiratory gas from anatomic dead spaces and therefore, background correction for the contributions from this inspiratory gas should be performed [21]. However, there is at present no consensus on a standard method and different methods have been proposed to reduce these localized effects [22, 23]. Ensuring that the subject inhales clean air is one possible approach, which was applied in the off-line method of the current study by

using the active coal scrubber to filter the inspiratory air. For the real-time method no filtering of inhaled air was foreseen. However, the participants provided the real-time breath samples immediately after the off-line breath collection, partly still profiting from the cleaned inhaled air during the off-line sampling. It should be noted that, depending on the properties of the compound of interest, longer clean-air inhalation (wash-out periods) could be required [21, 24]. Other approaches to reduce background effects include collecting a background sample at the time of breath collection and calculating the alveolar gradient (concentration exhaled – concentration inhaled) [23] or setting a cutoff (e.g. the concentration in inspiratory air should not be greater than 25% of the breath concentration) [22]. However, caution must be paid to the localized environmental effects because compounds may be inhaled at concentrations greater than those in the general ambient air when they are strongly present at a subject's point of inhalation during sampling [23].

In this study background samples were collected for both the real-time and the off-line method. Using the real-time method all quantified volatiles showed higher breath concentrations than the background concentrations in ambient air, except for 2,3-butanedione, ammonia, benzene and styrene. The Detection of higher concentrations in breath than in background samples suggested these volatiles are produced by endogenous metabolic processes. For benzene and styrene, the breath and background concentrations were similar, indicating they were not formed endogenously, but rather taken up from the environment as expected. The same pattern was observed for the off-line method. However, propanal and propanol concentrations in breath and background were similar when measured offline. This indicated that the off-line method was not optimal for analyzing these volatiles. For 2,3-butanedione a different situation was observed. The real-time measurements were unexpectedly lower than the background concentrations, while the opposite was observed when measured off-line. This could indicate that the background samples from ambient air contained some unidentified volatile(s) which caused a conflict at *m/z* 86 (NO^+), resulting in higher background concentrations. All three peppermint-related volatiles – γ-terpinene, eucalyptol, and menthone – showed higher breath concentrations than in background samples after ingestion of the peppermint oil capsule [12].

Some volatiles showed very high concentrations compared with concentrations previously reported in the literature. For example, we observed higher concentrations in both real-time and off-line measurements for propanal, pentane and benzene than the mean breath concentrations of 18.3 ppbv, 1.8 ppbv and 0.8 ppbv, respectively, reported by Mochalski *et al* [25]. These differences could be caused by conflicting volatiles which arise when the chemical ionization of other volatiles with the same reagent ion result in the same product ions, complicating data interpretation. Adaptation of the parameters provided in the kinetics database which depend on the instrument's flow tube geometry, temperature and pressure, would increase the accuracy of quantification [7]. Since this study aimed to compare the real-time and off-line method, the verification was not carried out for most of the selected breath volatiles. SIFT-MS was used as it offers advantages over GC/MS in speed and simplicity, making it an interesting tool for fast screenings. Langford *et al* showed that GC/MS was better suited than SIFT-MS to monitor samples containing large numbers of volatiles at high concentrations. On the other hand, SIFT-MS proved simpler to use, was linear over a much wider concentration range than GC/MS and provided faster results [4]. In the current study we applied the combination of TD and SIFT-MS offering the possibility to use absorption tubes for both

GC/MS and SIFT-MS analyses in clinical situations for which direct (real-time) sampling and analysis is not feasible [11].

The humidity of the samples could affect the measured concentrations. Especially during the analysis of very humid samples such as exhaled breath, the formation of hydrated ions of both the reagent ions and the analyte ions is inevitable, particularly when exploiting H_3O^+ and NO^+ reagent ions. The hydrated reagent ions must be considered as additional reagent ions in the analysis and this is not a trivial task, because these cluster ions are continuously formed along the length of the flow tube and so the reaction times of these additional reagent ions have to be estimated. Smith and Španěl have addressed these complicating aspects of SIFT-MS analysis in detail, and constructed appropriate analytical calculations that involve the rates of these hydration processes [2]. Humidity dependence could be overcome by calibrating for humidity. The validation step in the daily startup procedure of the instrument is based on 2 ppmv of the standard substances in dry air. In comparison with Proton Transfer Reaction (PTR)-MS, SIFT-MS is less humidity dependent and its daily validation step was reported to be more robust against humidity [26]. The influence of humidity on the off-line method, combining TD and SIFT-MS, depends on the sorbents used. Competition between water and VOCs for the active sites of the sorbent makes trapping of VOCs less efficient. This reduced efficiency causes loss of molecules of interest, i.e. breakthrough and poor reproducibility [27]. In this study multi-bed sorbent tubes were used. Tenax GR has a low affinity for water and Carbograph 5TD is hydrophobic, indicating that the influence of humidity was minimal. Sample humidity was recorded based on *m/z* 37 and 55 from H_3O^+ reactions. These data showed significantly higher humidity in real-time measured samples than in off-line measured samples.

All the quantified volatiles showed significantly higher breath concentrations for the real-time method than for the off-line method, except for 2,3-butanedione and styrene. However, the opposite was expected. For the off-line method 500 mL breath was collected and desorbed in 300 mL helium (in the Tedlar bag), which should result in a 1.7x pre-concentration. The lack of such pre-concentration could be caused by the breath sampler if the actually sampled volume through each tube was lower than 500 mL. However, the sampling flow rate of the breath sampler was checked and it did not fall below 200 mL min^{-1}. The breath sampling software indicated when sampling pumps were activated and confirmed activation during exhalation. The lack of pre-concentration observed could also be due to uncertainties in both the real-time and the off-line methods caused by sampling and/or measurement. Using the off-line method, there could have been losses during trapping and desorption stages, in the equipment tubing, or in the Tedlar bag used to re-collect the volatiles after desorption, whereas the real-time methodology requires multiple product ions to be detected by the quadrupole mass spectrometer in every real-time exhalation. In our approach, 155 product ions and 7 reagent ions were selected for scanning in MIM method using a dwell time of 10 msec, resulting in a theoretical scan cycle of 1.62 sec (162 x 10 msec). In practice, however, the time needed for one scan cycle was 5.4 seconds. The breathing cycles must be in line with the scan cycle, otherwise not all exhaled volatiles can be scanned by the quadrupole mass spectrometer. In addition, the real-time methodology causes a higher risk of background contamination as ambient air was inhaled instead of cleaned air.

Despite the differences in absolute concentration between the off-line and the real-time method, the correlation between them was moderate to very strong for most volatiles, except for 2,3-

butanedione and styrene. These moderate to very strong correlations indicated that the two methods were linearly related; however, it did not mean that the two methods agree [28]. The agreement between two quantitative measurements is best described using Bland-Altman plots, which simply represent every difference between two paired methods against the average of the measurement [13]. Acetone showed a remarkably high average bias of 251.5 ppbv, indicating the off-line method resulted in much lower concentration than the real-time method. However, for most other volatiles the average bias was 10 ppbv or less in absolute value. The high average bias could be caused by the suboptimal data processing of the real-time method. Selecting only the 10 highest peaks during the two-minute analyses might cause an overestimation of the breath concentration. A more accurate approach would be to define a time interval corresponding to the end-tidal portion of breath, and to average the concentrations consistently within this interval. For this approach there should be several cycles recorded for each exhalation, which was not the case for the MIM method used due to the larger number of product ions being measured. Using the average concentration of the 10 highest peaks during the two-minute analysis as an estimate for the breath concentration was in our opinion the best approximation, since the highest amounts of breath volatiles are expected during exhalation. Interestingly, the Bland-Altman plots of most volatiles showed a specific trend in the cluster of datapoints, which go from below the average bias at lower concentrations to above the average bias at higher concentrations. This indicates an error proportional to the concentration range, which might be caused by averaging the 10 highest peaks for the real-time measurements as mentioned above.

The results indicated that the proposed indirect combination of TD and SIFT-MS, transferring desorbed compounds via a Tedlar bag, was not optimal. However, Sovova *et al* demonstrated the feasibility of time-integrated TD SIFT-MS for off-line quantitative analysis of three common atmospheric monoterpenes: β-pinene, *R*-limonene and 3-carene [10]. They optimized the type of sorbent, bed length, sampling flow rate, sample volume and the initial desorption temperature for this direct combination of TD and SIFT-MS. Their optimization was based on only three product ions of chemically similar volatiles. Hryniuk *et al* optimized a direct combination of TD and SIFT-MS for targeted analysis of breath acetone and isoprene using *m/z* 88 and *m/z* 68 from the reaction with NO^+, respectively [11].

In our approach, 155 product ions were selected, representing a wider range of volatiles with more diverse chemical properties. This type of broad-spectrum screening requires a longer scan time. Quadrupole mass analyzers are poorly suited for monitoring multiple *m/z* signals over restricted periods of time [29]. The introduction of time-of-flight mass spectrometry, with its high time resolution, represents a considerable step forward in terms of performance with respect to coupling to TD, as shown by Romano *et al*. They developed a direct coupling strategy for TD and PTR-ToF-MS applicable to breath analysis. They tested its performance for the analysis of seven oxygenated VOCs and concluded that this platform displays high throughput and sensitivity [29]. When restricted to a quadrupole mass analyzer, as was the case for the Syft Voice200, transferring the sample from the desorption step directly into the instrument is a point of concern. Using a reservoir for sample transfer, such as the Tedlar bag in our approach, allowed longer scan times. Making sure that all the transfer lines are heated and re-collecting the volatiles after desorption in a heated reservoir to avoid condensation could improve the off-line breath analysis using TD SIFT-MS. That reservoir would need to be inert and it would

need to be ventilated or flushed with nitrogen between two samples to avoid contamination. Further developing SIFT-MS towards the use of SIFT-ToF-MS could also potentially increase the performance and applicability in breath analysis.

Conclusion

In summary, the indirect combination of TD and SIFT-MS for off-line analysis of selected breath volatiles appeared to be challenging. The agreement (on absolute concentrations) between the off-line and real-time method was rather poor. Optimizing experimental parameter for sampling and desorption and other areas of concern such as minimizing sample losses during desorption and transfer to the SIFT-MS instrument, could increase the accuracy of quantification and the agreement between the two methods. As the results from the two methods were overall well correlated, it indicates that optimizing the methodologies is feasible.

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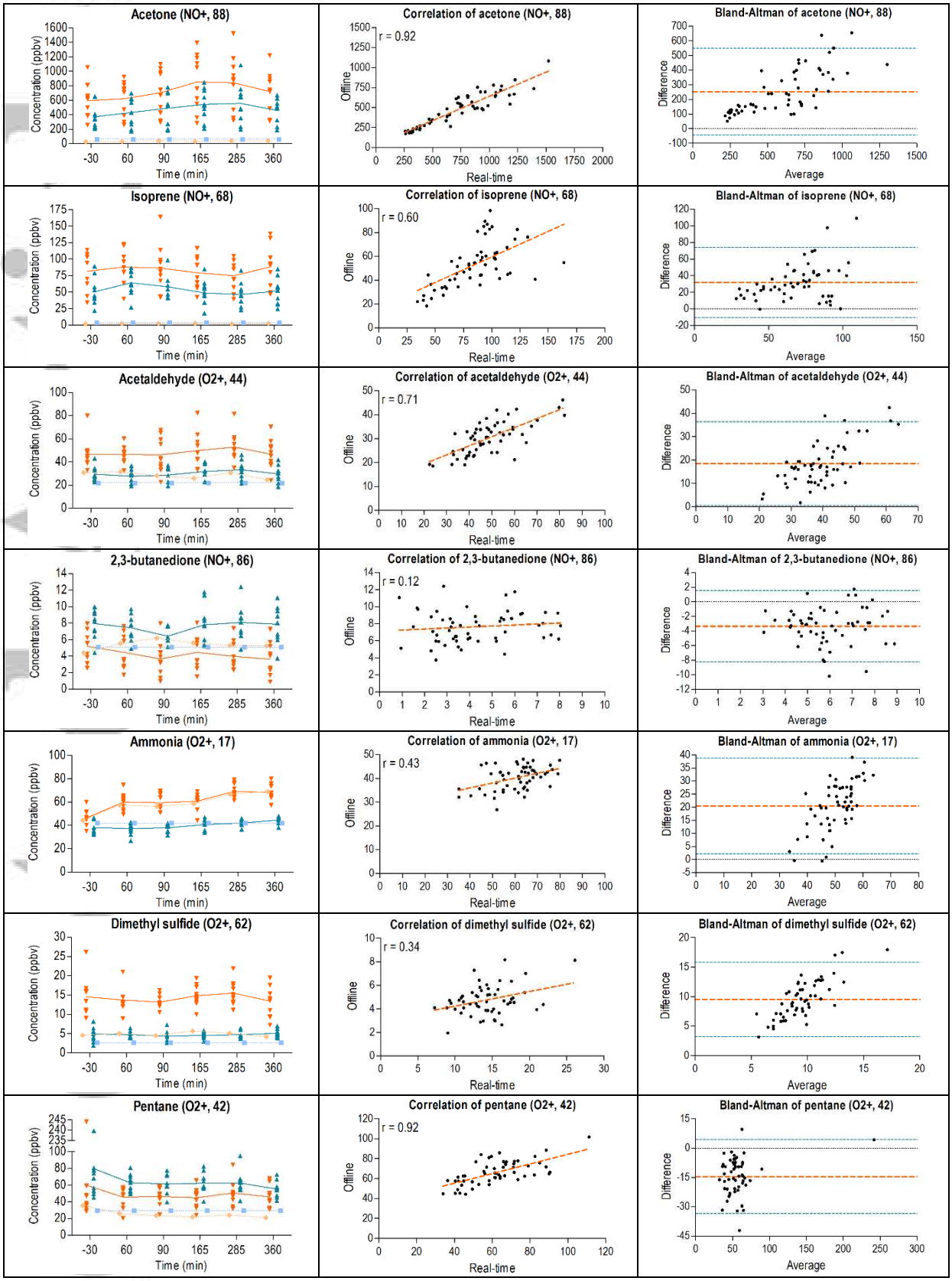
*Table 1: Volatiles quantified in the multi-ion monitoring method, the product ions used to quantify them, and additional remarks about quantification in case of (unavoidable) conflicts; * indicates the m/z overlaps with other known volatiles*

Volatile (CAS)	***m/z* (reagent ion)**	**Additional remarks about quantification**
Acetone (67-64-1)	88 (NO^+)	
Isoprene (78-79-5)	68 (NO^+)	
Acetaldehyde (75-07-0)	44 (O_2^+)	
2,3-butanedione (431-03-8)	86 (NO^+)	
Ammonia (7664-41-7)	17 (O_2^+)	
Dimethyl sulfide (75-18-3)	62 (O_2^+)	
Pentane (109-66-0)	42 (O_2^+)*	[pentane 42 (O_2^+)] = [pentane/propanol 42 (O_2^+)] – [propanol 59 (NO^+)]
Propanal (123-38-6)	57 (NO^+)	
Propanol (71-23-8)	59 (NO^+) 42 (O_2^+)*	Quantified based on 59 (NO^+)
Benzene (71-42-2)	78 (NO^+)	
Styrene (100-42-5)	104 (O_2^+)	
ɣ-terpinene (99-85-4) overlap with monoterpenes	137 (H_3O^+)* 136 (NO^+)	Quantified based on 136 (NO^+)
Eucalyptol (470-82-6)	137 (H_3O^+)* 154 (NO^+)*	[eucalyptol 137 (H_3O^+)] = [eucalyptol/terpenes 137 (H_3O^+)] – [terpenes 136 (NO^+)]
Menthone (14073-97-3)	154 (NO^+)*	[menthone 154 (NO^+)] = [menthone/eucalyptol 154 (NO^+)] – [eucalyptol 137 H_3O^+)]



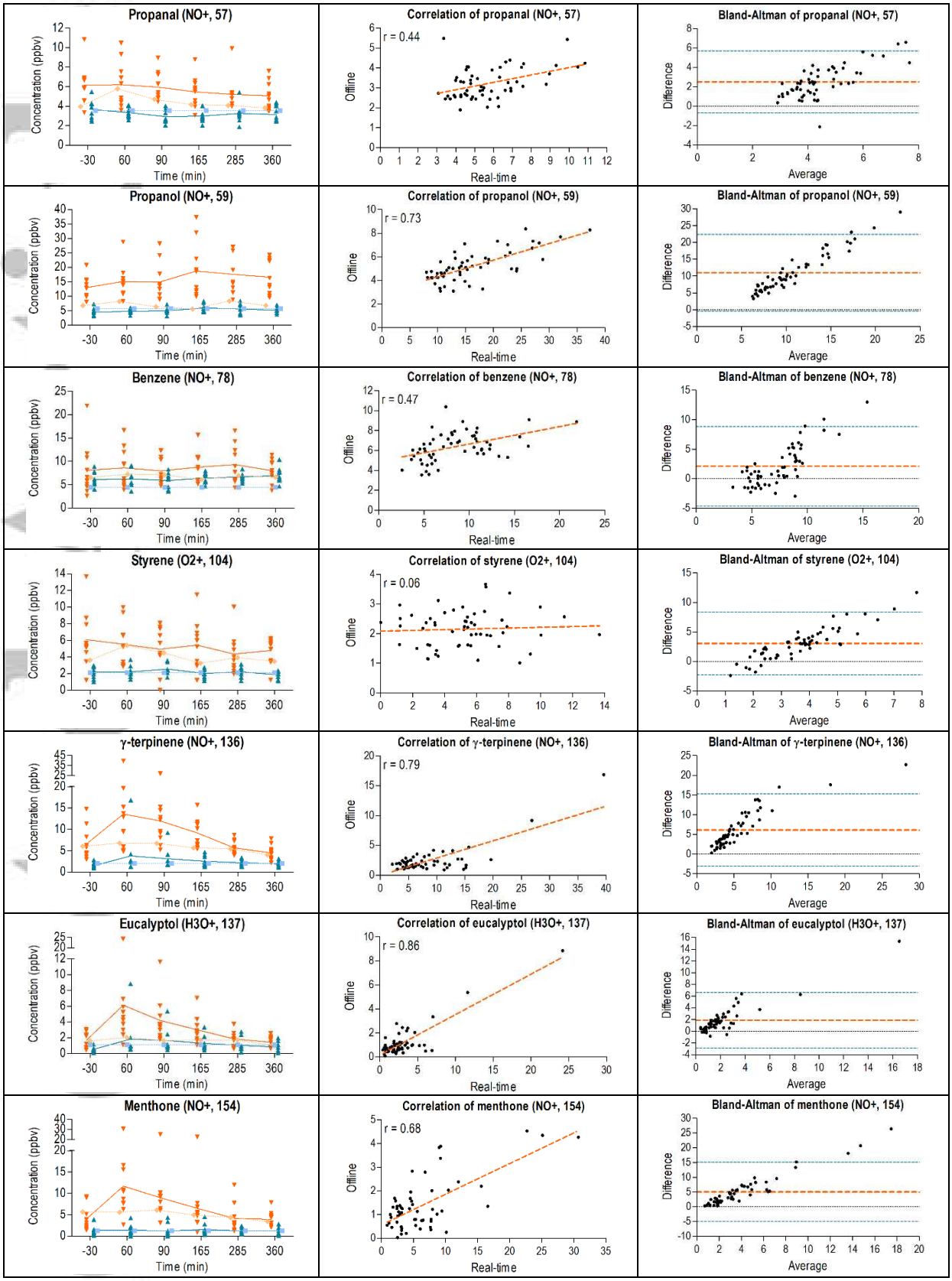


Figure 1: volatile concentrations in breath and background over time measured in real-time and off-line (left) (dark orange: real-time breath; light orange: average real-time background; dark blue: off-line breath; light blue: average off-line background); Pearson correlation between real-time and off-line measured breath concentrations (middle); Bland-Altman plots

for real-time and off-line measured breath concentrations (right) (orange dotted line: average bias; blue dotted lines: 95% CI)

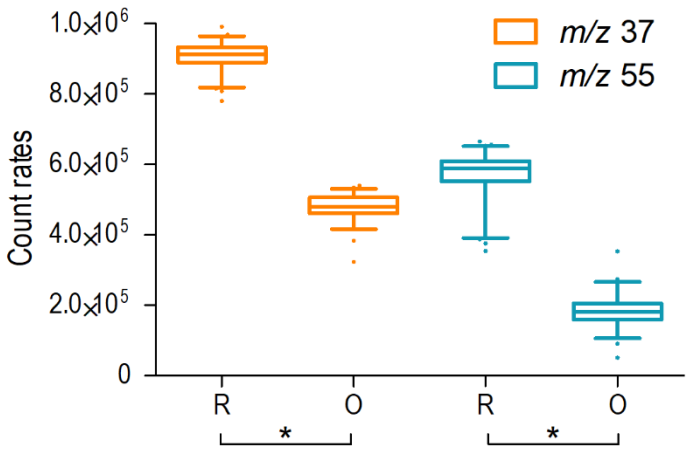


Figure 2: Sample humidity based on count rates of product ions 37 and 55 from H3O+ reactions for both the real-time (R) and off-line method (O); * indicates a significant ($p < 0.05$) difference