A gas-phase standard delivery system for direct breath analysis

Streckenbach, Bettina ; Sakas, Justinas ; Perkins, Nathan ; Kohler, Malcolm ; Moeller, Alexander ; Zenobi, Renato

DOI: https://doi.org/10.1088/1752-7163/acab79

Journal of Breath Research

A gas-phase standard delivery system for direct breath analysis

Journal of Breath Research

PAPER

A gas-phase standard delivery system for direct breath analysis

Bettina Streckenbach[1], Justinas Sakas[1,2], Nathan Perkins[3], Malcolm Kohler[4], Alexander Moeller[5] and Renato Zenobi[1,*]

[1] Department of Chemistry and Applied Biosciences, ETH Zurich, Zurich, Switzerland
[2] EaStCHEM School of Chemistry, University of Edinburgh, Edinburgh, United Kingdom
[3] Division of Clinical Chemistry and Biochemistry, University Children's Hospital Zurich, Zurich, Switzerland
[4] Department of Pulmonology, University Hospital Zurich, Zurich, Switzerland
[5] Division of Respiratory Medicine, University Children's Hospital Zurich, Zurich, Switzerland
[*] Author to whom any correspondence should be addressed.

E-mail: zenobi@org.chem.ethz.ch

Keywords: direct breath analysis, standardization, reference material, multi-component, SESI-HRMS

Supplementary material for this article is available online

OPEN ACCESS

RECEIVED
21 September 2022

REVISED
22 November 2022

ACCEPTED FOR PUBLICATION
14 December 2022

PUBLISHED
29 December 2022

Abstract

Applications for direct breath analysis by mass spectrometry (MS) are rapidly expanding. One of the more recent mass spectrometry-based approaches is secondary electrospray ionization coupled to high-resolution mass spectrometry (SESI-HRMS). Despite increasing usage, the SESI methodology still lacks standardization procedures for quality control and absolute quantification. In this study, we designed and evaluated a custom-built standard delivery system tailored for direct breath analysis. The system enables the simultaneous introduction of multiple gas-phase standard compounds into ambient MS setups in the lower parts-per-million (ppm) to parts-per-billion (ppb) range. To best mimic exhaled breath, the gas flow can be heated (37 °C–40 °C) and humidified (up to 98% relative humidity). Inter-laboratory comparison of the system included various SESI-HRMS setups, i.e. an Orbitrap and a quadrupole time-of-flight mass spectrometer (QTOF), and using both single- as well as multi-component standards. This revealed highly stable and reproducible performances with between-run variation <19% and within-run variation <20%. Independent calibration runs demonstrated high accuracy (96%–111%) and precision (>95%) for the single-compound standard acetone, while compound-specific performances were obtained for the multi-component standard. Similarly, the sensitivity varied for different compounds within the multi-component standard across all SESI-Orbitrap and -QTOF setups, yielding limits of detections from 3.1 ppb (for *p*-xylene) to 0.05 ppb (for 1,8-cineol). Routinely applying the standard system throughout several weeks, allowed us to monitor instrument stability and to identify technical outliers in exhaled breath measurements. Such routine deployment of standards would significantly improve data quality and comparability, which is especially important in longitudinal and multi-center studies. Furthermore, performance validation of the system demonstrated its suitability for reliable absolute quantification while it illustrated compound-dependent behavior for SESI.

1. Introduction

The direct analysis of exhaled breath has met with increasing interest from various research fields, with mass spectrometry (MS) being the primary technology applied today [1]. This is primarily due to the high sensitivity of modern mass spectrometers, which allows to detect volatile organic compounds (VOCs) that are present in breath at low concentration, parts per million (ppm) to parts per trillion (ppt). This enabled breath analysis in real time, bypassing any sample preparation, and together with the wide range of detectable metabolites resulted in expanding applications, including clinical monitoring, phenotyping or diagnostics [2].

Secondary electrospray ionization high-resolution mass spectrometry (SESI-HRMS) is a relatively young technique for direct breath analysis, with applications ranging from basic research to biomarker discovery to clinical trials [3–7]. In contrast to the more established MS-based techniques for direct breath analysis, i.e. selected ion flow tube mass spectrometry (SIFT-MS) [8, 9] and proton transfer reaction mass spectrometry (PTR-MS) [10, 11], the SESI source operates at ambient pressure, which results in a softer analyte ionization. Furthermore, this enables coupling to state-of-the-art mass spectrometers and, as a result, detection with very high mass resolution, as well as compound identification by tandem MS.

One current drawback has been the lack of well-defined standardization procedures for SESI-HRMS. While efforts to standardize exhalation manoeuvres have been made [12], no procedures for identifying technical variations have been agreed upon. Nonetheless, this is fundamental for data comparability from different instruments or sites, and it would strengthen the potential of SESI-HRMS for clinical application. Furthermore, SESI-HRMS-based analysis is currently restricted to relative quantification because its ionization process is not yet fully understood. Particularly in clinical diagnostics, however, absolute quantification is required to define thresholds for breath-derived biomarkers.

MS-based methods that are well established in clinical laboratories all employ routine standardization measures to validate instrument performance, most commonly using internal standards. This is not applicable for direct analysis, as no sample storage is involved to which an internal standard can be added to in a controlled fashion. Thus, external standards are a viable option, particularly when introduced in the gas phase to mimic ionization of breath-derived compounds. This can be realized by commercially available systems (e.g. Owlstone Medical, UK) which are based on permeation tubes [13, 14], but they are limited in the number of standards.

To generate robust and comparable data by SESI-HRMS based breath analysis, methodological standardization procedures are required. Reproducibility and comparability have recently become even more important, since SESI-HRMS is applied in an increasing number of clinical trials and laboratories [15]. This also corresponds to the current need for quantifying potential breath biomarkers, which have been identified in various studies [1, 6, 16], as pointed out recently by Jeerage *et al* [17]. To address this need, we present a standardization approach for routine use in MS-based breath analysis. We developed a custom-designed gas-phase standard delivery system that mimics exhaled breath in the best possible way, including the simultaneous delivery of multiple standard compounds. In this study, the system was thoroughly evaluated on multiple SESI-HRMS setups across different laboratories and using standards that included compound classes commonly detected in breath analysis.

2. Methods

2.1. Technical design of the delivery system

The gas-phase standard was diluted in a stepwise fashion using calibrated mass flow controllers (Bronkhorst High-Tech B.V., Ruurlo, Netherlands). By employing different gas cylinders, various gas-phase standards could be introduced. All stainless-steel tubings and swagelok fittings in contact with the standard were silica coated (SilcoNert®2000, SilcoTek, Bellefonte, PA, USA) to avoid sample adhesion to and reaction with the material (see figure S1 for more details).

2.2. Gas-phase reference material

In this work, a single-component standard containing 1.67 ppm acetone (PanGas AG, Dagmersellen, Switzerland) and a multi-component standard containing different VOCs at 0.1–1 ppm (Apel Riemer Environmental Inc., Miami, FL, USA) served as gas-phase reference standards (table 1). Medical air or VERISEQ® pharmaceutical air (both PanGas AG, Dagmersellen, Switzerland) were applied for stepwise dilution. The standards were humidified by passing a fixed flow of dilution gas (250 ml min^{-1}) through a glass frit placed in optima™ water (Fisher Scientific GmbH, Schwerte, Germany). Humidified standards were subsequently passed through a heated tube to avoid condensation. For performance tests, dilution ratios were fixed: a 3:25 dilution for the multi-component standard and a 1:2 dilution for acetone with dry gas was followed by a 1:6 dilution with humidified gas, resulting in the injection of 15.8 ppb 1,8-cineol (exemplarily for the standard mixture) and 139.2 ppb acetone.

2.3. SESI-HRMS analysis

Exhaled breath, humidified air and humidified gas-phase standards were directly ionized using commercial SESI ion sources (FIT Fossilion Technology, Madrid, Spain) and analyzed in real time on two different high-resolution mass spectrometers coupled to the ion source: an Orbitrap (Q Exactive™ Plus, Thermo Fisher Scientific Inc., Waltham, MA, USA) and a quadrupole time-of-flight (QTOF) MS (TripleTOF 5600^{+}, AB Sciex, Concord, ON, Canada).

To determine the system performance, the standard delivery system was coupled to the respective instrument setups, which were installed at different sites: a SESI-Orbitrap instrument at the University Hospital Zurich (USZ), and two SESI-QTOF instruments, one each at USZ and the University

Table 1. Specifications of the gas-phase standards.

Standard	Compound	Molecular formula	conc.* (ppb)	m/z exp. $[M+H]^+$	Detected
Multi[a]	Dimethyl sulfide	C_2H_6S	938	63.03	Breath [10, 18], SESI [19][c]
	Phenol	C_6H_5OH	763	95.05	Breath [10], SESI[c]
	Styrene	C_8H_8	788	105.07	Breath [20], SESI[c]
	p-Xylene	C_8H_{10}	789	107.09	Breath [21, 22], SESI[d]
	Isobutyl nitrate	$C_4H_9NO_3$	783	120.07	SESI[c]
	α-Pinene	$C_{10}H_{16}$	792	137.13	Breath [23], SESI [12]
	1,8-Cineol	$C_{10}H_{18}O$	789	155.14	Breath [23, 24]
	β-Caryophyllene	$C_{15}H_{24}$	77	205.20	SESI [25]
Single[b]	Acetone	C_3H_6O	1670	59.05	Breath [10, 26–28], SESI [1, 29]

Reference material: *concentration with ±5% uncertainty, supplied by [a]Apel-Riemer and [b]PanGas in nitrogen, m/z: expected (exp.). [c]identity uncertain—assigned based only on exact mass in breath SESI-HRMS spectra (mass error <0.005 Da), [d]detected by SESI-HRMS using a PTR-MS reference gas cylinder [30].

Children's Hospital Zurich. On all sites, the ion source was operated at 130 °C and with a net sample flow of 0.3 l min^{-1}. Further details on operating at the ion source have been described elsewhere [12]. The pure nano electrospray was generated using silica emitters (50 cm length, 20 μm diameter, New Objective Inc., Woburn, MA, USA) and a 0.1% (v/v) aqueous formic acid solution as the primary electrospray solvent. Spectral data were recorded in the mass range from 50 to 500 Da. The spray voltage in positive ionization mode was set to +3.5 kV on the Orbitrap. On the QTOF-MS setups, a spray voltage of +4.5 kV was applied and spectra were recorded with an accumulation time of 0.5 s. MS/MS spectra of the standard mixture components were recorded on the SESI-Orbitrap in positive ion mode with an isolation window of 0.4 Da and compared against library MS/MS spectra available in the human metabolome data base [31].

For exhaled breath analysis, test subjects refrained from eating, drinking, brushing their teeth, or using facial cosmetics for at least one hour prior to the breath measurements. Sitting in an upright position, the subjects exhaled with an overpressure of 10 mbar directly into the SESI-HRMS through a single-use mouthpiece (ACE Handels- und Entwicklungs GmbH, Freilassing, Germany), that was connected to the ion source by a Teflon adapter. Exhaled breath was analyzed in positive ionization mode, recording six consecutive exhalations. The part of this study with human volunteers had been approved by the local ethical committee (KEK-ZH 2019-00030) and was conducted according to the Declaration of Helsinki. Written informed consent was obtained from all subjects.

2.4. Data preprocessing

Raw mass spectral data files were converted into .mzXML files using MSConvert (ProteoWizard v3.0.2) [32] and preprocessed in Matlab® R2020a (MathWorks Inc., Natick, MA, USA). The data preprocessing included interpolation, baseline adjustment, peak signal integration and peak picking. For relative intensities, signal intensities were normalized to the total ion current (TIC). Furthermore, the first 50 scans were excluded to ensure well controlled gas flows. Signal averaging of all scans resulted in the final intensity matrix.

3. Results

3.1. A tailored standard system for breath analysis

The gas standard delivery system developed in this work was primarily used to monitor instrumental fluctuations on different SESI-HRMS breath analysis setups. The system was designed to deliver low mole fractions of humidified gas-phase standards which are introduced from a gas cylinder and diluted in a stepwise fashion to the desired concentration (figure 1). Furthermore, the standards were humidified (98% rel. humidity) and heated (37 °C) before entering the ion source to simulate exhaled breath and the ionization process of breath-borne metabolites in the best possible way. While the first dilution step served to vary gas-phase standard concentrations, the second ensures constant relative humidity.

Gas-phase standards were selected for positive ionization mode and to cover compounds of different mass and a range of compound classes (table 1). The compounds are expected to be detectable by SESI-HRMS with some of them having been previously detected in exhaled breath (see table 1 for references) [1, 10, 12, 18–30] . The design of the system allows to deliver standards with concentrations in the parts-per-billion (ppb) range. Taking into account the optimal gas flow rates specified for the SESI source (0.2–0.5 l min^{-1}), this resulted in a working range of about 1.5–300 ppb, well in line with the sensitivity reported for SESI-HRMS

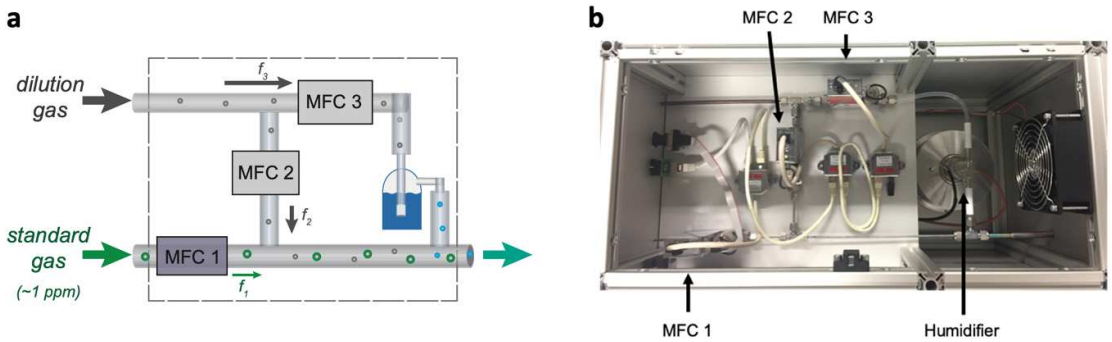

Figure 1. Design of the gas-phase standard delivery system. (a) Schematic illustration, dynamic dilution of the standard controlled by multiple mass flow controllers (MFCs) allows to vary the standard concentration (MFC 1 and 2) but keeping the sample humidity constant (MFC 3). Typical flow rates: $f_1 = 6$ ml min^{-1}, $f_2 = 44$ ml min^{-1} and $f_3 = 250$ ml min^{-1} for a net flow rate of 300 ml min^{-1} introduced to the SESI source. (b) Photograph (top view, opened) of the setup showing the positioning of the MFCs and the humidifier.

[33, 34]. Concentration ranges can be further adjusted by employing more highly concentrated standards or installing an additional dilution step in front of the humidification step.

3.2. System performance evaluation

The selected gas-phase standards were mainly detected as protonated molecular ions ($[M + H]^+$), with two exceptions: 1,8-cineol showed two additional fragments at m/z 81 and m/z 137 with strong signal response (figure S2), as identified by MS/MS analysis (figure S3). This included the base peak at m/z 137, which unfortunately coincides with the protonated molecular ion of α-pinene. Isobutyl nitrate was not detected in the present concentration range (figures S4 and S5).

The performance of the standard system was first assessed by introducing the multi-component standard at fixed concentrations. Over a test period of 1 h, the standard was introduced every 10 min for a minimum of two minutes. The system showed high stability between runs on the SESI-Orbitrap setup with a coefficient of variation (CV) <2% for the prominent 1,8-cineol ions (figure 2) and CV <6% for all mixture compounds (table 2). Repetition on the SESI-QTOF setups for inter-laboratory comparison revealed an increased variation for 1,8-cineol of up to 12% (figure 2(b)). The remaining standard compounds showed comparable variations between the QTOF2 and Orbitrap setup, but increased variability on the QTOF1 setup (table 2). Nevertheless, the obtained between-run variations, with exceptions of that for dimethyl sulfide, phenol and *p*-xylene measured on the QTOF1, were well in line with official guidelines for bioanalytical method validation recommending <15% CV [35, 36]. Within-run variations of <20% CV were obtained for most standard compounds, with the highest stability (<5% CV) for the highest-intensity features m/z 137 (α-pinene/1,8-cineol fragment) and m/z 155 (1,8-cineol) across all laboratories (table S1). Accordingly, the two exceptions showing strong within-run fluctuations (dimethyl sulfide and β-caryophyllene) were also the lowest intensity features.

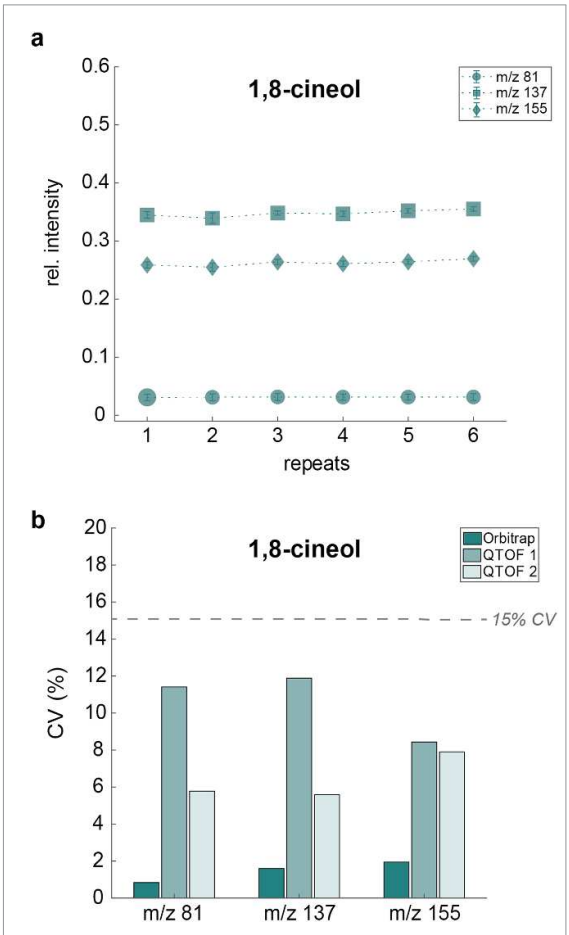

Figure 2. Standard system performance based on between-run variation upon repeated introduction of the standard mixture during 1 h. Performance exemplarily shown for 16 ppb 1,8-cineol. (a) Signal response of 1,8-cineol (m/z 155) and its fragment ions (m/z 81, m/z 137; see figure S3) on a SESI-Orbitrap, intensities normalized to the TIC. (b) Inter-laboratory comparison of the SESI-HRMS setups: Orbitrap, QTOF1 and QTOF2. Dotted line: recommendation of max. 15% CV. Represented data included $n = 6$ timepoints ($\geqslant$180 scans each, every 10 min), averaged CV defined after outlier removal (Tukey's fence).

Table 2. Inter-laboratory comparison of the system performance based on between-run variations of the multi-component standard.

Standard compound	m/z	CV (Orbitrap)	CV (QTOF1)	CV (QTOF2)
Dimethyl sulfide	63.03	5.2	18.6	2.2
Phenol	95.05	1.7	15.9	3.6
Styrene	105.07	2.2	14.5	1.9
p-Xylene	107.09	3.7	15.9	1.4
α-Pinene/1,8-Cineol fragment	137.13	1.5	10.6	5.1
1,8-Cineol	155.14	1.8	7.5	7.2
β-Caryophyllene	205.20	3.6	8.7	3.4

CV: coefficient of variation in %; different SESI-HRMS setups: Orbitrap = QExactive$^+$ (Thermo Fisher), QTOF = TripleTOF (AB Sciex); $n = 6$ timepoints during 1 h.

3.3. Evaluating multiple delivery units

To achieve comparability between different SESI-HRMS operating sites, standard delivery systems are required in each laboratory. Therefore, three units of the system were manufactured and evaluated on the same SESI-HRMS setup. All three units were tested on a SESI-QTOF setup throughout one day, introducing the single-component standard acetone. Furthermore, the units were applied in randomized order to avoid confounding effects from instrumental drifts, which might occur over time and for which a monitoring system is currently not available.

All three system units performed with similar precisions as previously obtained for the multi-component standard on the QTOF setups. A discrepancy between the units in signal intensity was observed that could not be corrected for by signal normalization to the TIC (figure 3(a)). Nevertheless, the between-run variation for acetone with <10% CV (figure 3(b)) and even lower within-run variabilities (table S2) demonstrated a highly stable and repeatable performance regardless of the units and compounds tested.

3.4. Method validation for quantitative analysis

In addition to the qualitative performance, we evaluated the use case of the standard system for quantification. The concentration-dependent response of the standards was validated by repeated independent runs within one day, between several days and throughout the different laboratories as specified in table 3. The monitored concentration range for each standard was defined by their initial gas-phase concentration.

Isobutyl nitrate was not detected since no change in response was obtained for the expected $[M + H]^+$ ion upon increasing concentrations. This was consistent across all analyzers and laboratories (figures S4 and S5), and could be due to an interference present at the exact same m/z value. Among the detectable standards, different behaviors were identified by least-square regression analysis of the calibration curves (table 3). For example, the acetone response was linear (figures 4(a) and S6(a)), while regression for 1,8-cineol (m/z 155) required a 4th order polynomial fit (figures 4(b) and S6(b)). The linear range for 1,8-cineol was between 13 and 53 ppb. Altogether, lower orders of polynomials were observed for the standards on the SESI-Orbitrap setup, indicating that for some of the standards the present concentration range approached saturation on the SESI-QTOF setup.

Between-run accuracy and precision were assessed based on the independent runs of the calibration curves. Here, three standard samples at low, medium, and high concentration across the concentration range were excluded from curve fitting and used to calculate both accuracy and precision (figures 4(c) and (d)), averaged over the three independent runs and the three selected calibration standards. For most of the tested compounds, we obtained high accuracy and precision (table 3). Specifically, precision values for 1,8-cineol confirmed the between-run variation obtained from the previous stability test results (figure 2). Only dimethyl sulfide showed high variations on both SESI-QTOF and SESI-Orbitrap setups, which have not been observed during the stability test.

For all standard compounds, the limit of detection (LOD) and limit of quantification (LOQ) were determined by three times (LOD, for LOQ: 10 times) the standard deviation of the blank sample divided by the sensitivity, which was derived by dividing absolute signal intensity by the sample's concentration (ppb) and averaging calculated sensitivities across all concentration samples. The blank sample consisted of introducing humidified dilution gas into SESI-HRMS. While the LODs for 1,8-cineol and β-caryophyllene ranged in the lower ppt region (0.05 ppt and 0.06 ppt, respectively) on the SESI-Orbitrap setups, the LODs were significantly higher for the remaining multi-component compounds (2.3–3.1 ppb). The overall LOD range for each standard compound individually was comparable across the SESI-Orbitrap and SESI-QTOF setups.

3.5. Application to monitor technical fluctuations

One of the key motivations for the development of the standard system was to assess technical

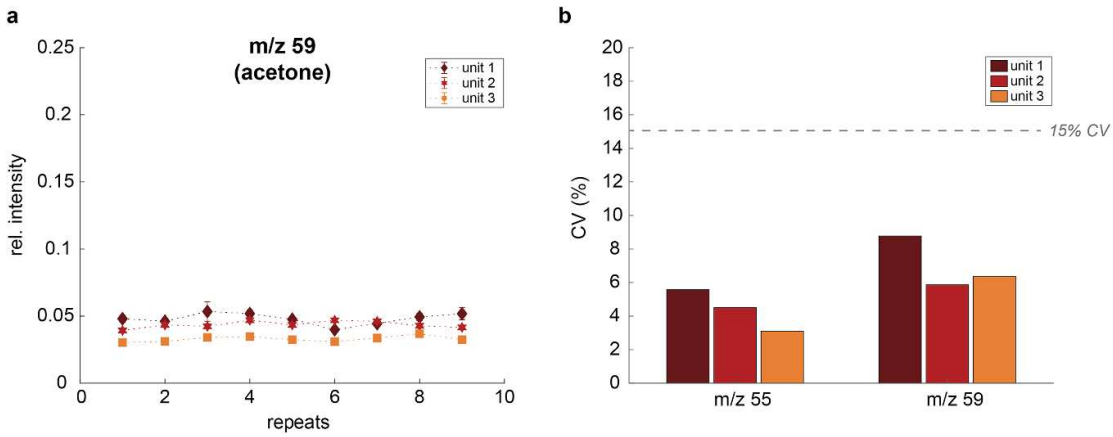

Figure 3. Performance of multiple system units. (a) Signal response of 139 ppb acetone (m/z 59) applying the different system units in randomized order, intensities normalized to the TIC. (b) CV obtained for water cluster (m/z 55) and acetone (m/z 59) on each system unit. Dotted line: recommendation of max. 15% CV, data acquired on a SESI-QTOF with $n = 9$ data points ($\geqslant$180 scans each).

Table 3. Calibration curve characteristics of the standards detected by different SESI-HRMS setups.

Setup	Validation	Standard ($[M+H]^+$)	Polyn. fit order	R^2 run1	R^2 run2	R^2 run3	Acc.* (%)	Prec.* (%)	Sensitivity (a.u./ppb)	LOD (ppb)	LOQ (ppb)
SESI-QTOF	Between-days	Acetone	1	0.9435	0.9932	0.9814	96–111	5	2.75×10^2	1.52	5.08
SESI-QTOF	Within-day	Dimethyl sulfide	4	0.9999	0.9999	0.9998	91–100	25	5.53×10^1	n.a.	n.a.
		Phenol	1	0.9961	0.9937	0.9914	101–109	5	2.53×10^3	1.14	3.80
		Styrene	4	0.9994	0.9991	0.9999	97–107	11	4.19×10^2	2.59	8.64
		p-Xylene	3	0.9833	0.9975	0.9940	93–123	11	5.09×10^2	2.86	9.54
		1,8-Cineol	4	0.9929	0.9991	0.9996	87–101	2	2.22×10^4	0.36	1.19
		β-Caryophyllene	4	0.9976	0.9963	0.9917	91–104	16	2.55×10^3	0.32	1.07
SESI-Orbitrap	Between-days	Dimethyl sulfide	4	0.9876	0.9666	0.9842	88–112	51	4.03×10^2	2.33	7.76
		Phenol	1	0.9954	0.9301	0.9891	97–115	13	3.99×10^4	2.42	8.05
		Styrene	2	0.9961	0.9857	0.9967	90–109	17	1.48×10^4	2.90	9.66
		p-Xylene	1	0.9979	0.9674	0.9963	96–109	16	1.76×10^4	3.07	10.25
		1,8-Cineol	4	0.9987	0.9957	0.9956	87–101	8	1.16×10^7	0.05	0.17
		β-Caryophyllene	4	0.9931	0.9802	0.9963	79–105	13	1.00×10^6	0.06	0.20

Independent runs were performed in triplicates on different SESI-HRMS setups as indicated. *Accuracy (Acc.) and precision (Prec.) were estimated based on the signal response of three calibration standard samples at low, medium, and high concentration, that were excluded from calibration curve fitting, and averaged over three independent runs. LOD: limit of detection, LOQ: limit of quantification. Within-day: runs on the same day, between-days: runs during 3 d (acetone) or 4 d (multi-component standard).

fluctuations on SESI-HRMS setups. Therefore, the multi-component gas-phase standard was introduced into a SESI-QTOF by the standard system over several weeks. In parallel, exhaled breath measurements of one subject were performed shortly before or after the gas-phase standard measurements. This resulted in 40 measurements each for the standard and exhaled breath.

Within-run variability for the most prominent standard 1,8-cineol remained below 10% CV, indicating a stable standard introduction, while more pronounced between-day variations were observed (table S4). To evaluate the standard system's monitoring potential, we focused on the water cluster signal at m/z 55 in exhaled breath, which is detectable on a SESI-QTOF. Because the humidity in breath is expected to be constant, water cluster signal is also assumed to be constant throughout the measurement period and was thus used hereafter to compare breath signal with the standard signal. Outliers in breath samples were defined based on normalization to the most prominent standard signal of the multi-component standard, i.e. 1,8-cineol (figure 5(a)). After outlier removal, Pearson's correlation analysis between the gas-phase standard and exhaled breath (water cluster) of the same subject showed a positive correlation ($R = 0.64$, figure 5(b)), revealing fairly synchronized fluctuations over the test period of several weeks.

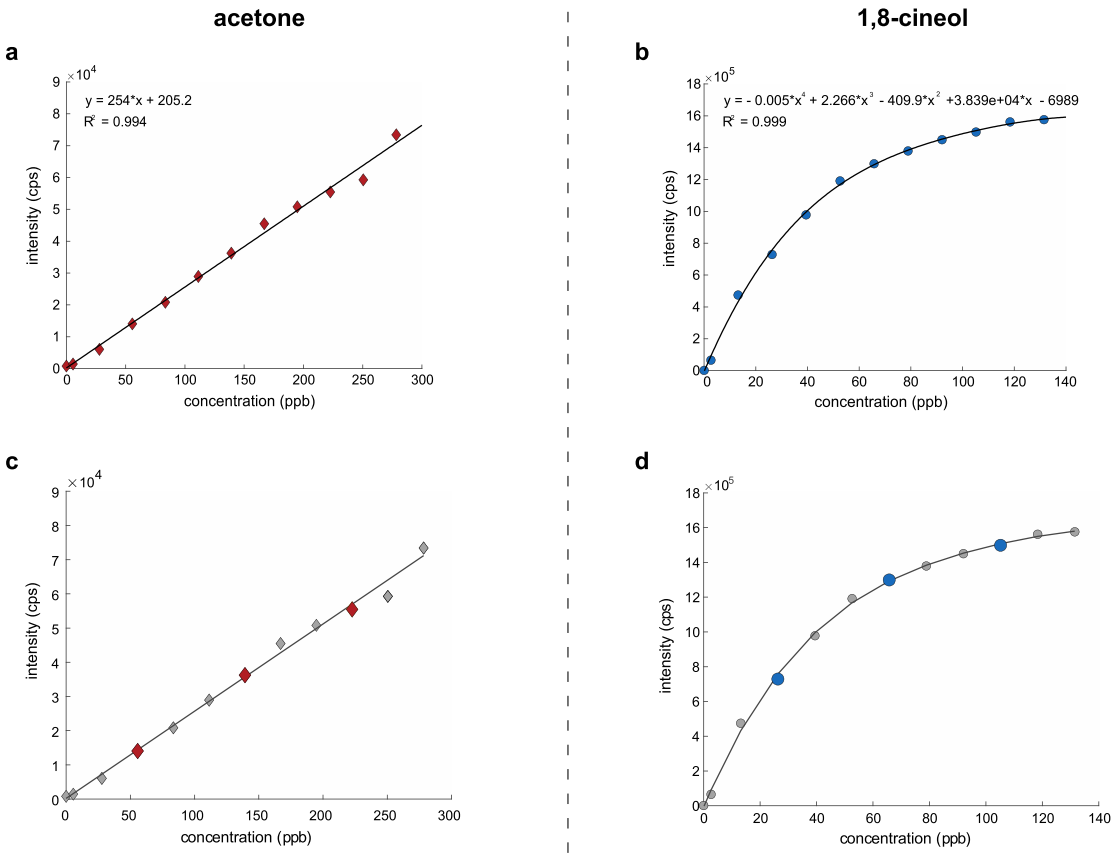

Figure 4. Calibration curves obtained for (a) acetone at m/z 59 (red diamonds) and (b) 1,8-cineol at m/z 155 (blue circles) on the SESI-QTOF setup. Each data point represents the averaged intensity from at least 120 scans per sample. (c) and (d): exemplary standard curves for accuracy and precision calculation. Grey markers: calibration samples used for curve fitting; colored markers: three calibration samples at low, medium, and high concentrations that were excluded from curve fitting and used to estimate accuracy and precision for each standard compound.

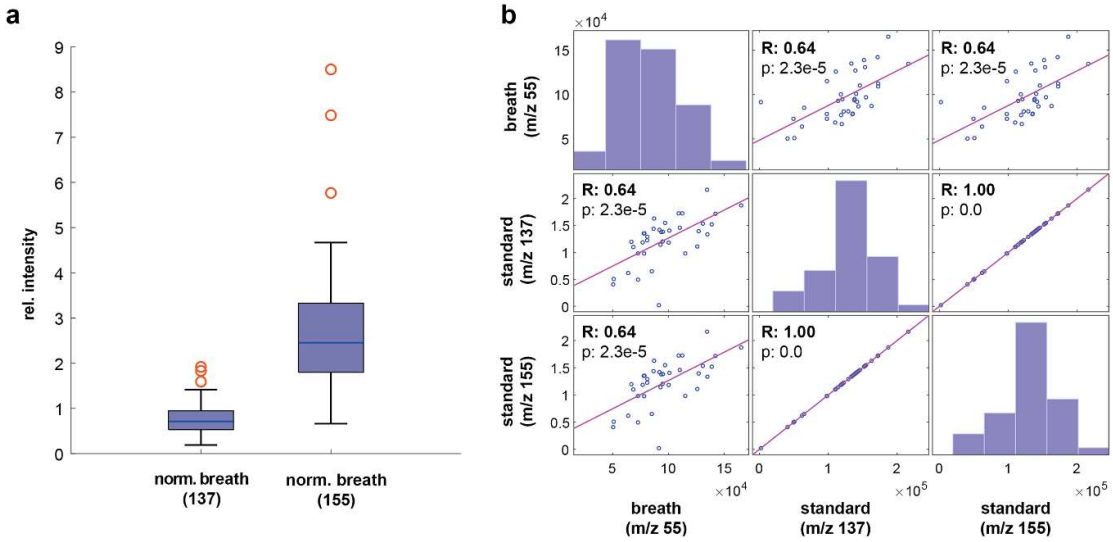

Figure 5. Monitoring of technical fluctuations on the SESI-QTOF setup. (a) Boxplots of normalized water clusters in breath (m/z 55) normalized to the most prominent signals in the multi-component standard (m/z 137 and m/z 155), $n = 40$ independent measurements, outliers marked by orange circles. (b) Correlation matrix of the Pearson´s correlation coefficients for breath samples with parallel measurements of standard compounds ($n = 40$). Along the diagonal: intensity distribution for each m/z feature.

An improved correlation was observed between the water clusters in breath and the water clusters in standard samples ($R = 0.72$, figure S7). Additionally, the correlation analysis revealed that fragmentation of 1,8-cineol (m/z 137) was constant on this instrument.

4. Discussion

The direct analysis of exhaled breath by highly sensitive instruments such as mass spectrometers has gained increasing interest from various research fields [1]. As demonstrated by SESI-HRMS, the scope of ionization technologies is expanding to fully explore and exploit insights provided by the analysis of exhaled breath. However, with new technologies comes the need for standardization procedures to ensure reliable analysis [15]. First suggestions for standardization in SESI-HRMS analysis have already been made [12]. While these focused on standardizing the procedure of breath sampling, quality control approaches for the entire instrumentation have never been tackled. For this reason, we created a standard system that allows for the simultaneous introduction of multiple (more than five) gas-phase standards with the goal of mimicking the complexity and humidity of breath samples [17, 22]. Inter-laboratory evaluation of the standard system revealed highly stable and reproducible delivery of gas-phase standards on various SESI-HRMS setups. Furthermore, estimated accuracy and precision demonstrated reliable performance of the system, as well as its potential for quantitative analysis.

In this work, we were able to thoroughly validate the manufactured gas-phase standard delivery system. Comparing its performance in various laboratories included, for the first time, evaluation on both SESI-Orbitrap and SESI-QTOF setups that are used for breath analysis. We applied a multi-component standard, similar to the one used by Liu *et al* [37], in addition to a single-component standard to extensively evaluate accuracy and precision in multiple independent runs, and were able to demonstrate the system's monitoring suitability over a period of several weeks.

While the standard delivery system was evaluated with an emphasis on exhaled breath measurements, the system is equally applicable for VOC analysis in general, using any direct ambient ionization techniques, particularly SIFT- or PTR-MS. The humidification step in the delivery system is optional and can be replaced by dynamic dilution with dry gas, depending on the humidity-dependence or independence of the respective detection method.

We observed different behaviors for the tested standard compounds based on their calibration curves (table 3). This not only agrees well with previously reported differences in sensitivity for a multi-component standard by SESI-HRMS [37], but also demonstrates the range of possible responses. Interestingly, a discrepancy between the different SESI-setups was obtained: the standard responses on the SESI-QTOF setup required a higher order polynomial fit than on the SESI-Orbitrap, e.g. for *p*-xylene and styrene, which showed a saturation trend. While the absolute signal of both standards is far from the detector saturation limit, this may rather imply a decreasing ionization efficiency for both compounds in the QTOF-connected ion source version as the standard mixture concentration increases. Nonetheless, the different responses observed in this study emphasize the importance of including multiple compound classes in a reference material for breath analysis and, therefore, underlines recently reported recommendations about a multi-component standard for breath analysis [17].

Applying the standard system in parallel to breath samples over several weeks demonstrated its suitability to monitor instrumental fluctuations and define technical outliers in exhaled breath. Therefore, we propose the routine inclusion of such a quality control in standard operating procedures on SESI-HRMS setups. Here, we observed a slightly better correlation for water cluster than for the standard material itself. While water clusters cannot be universally applied to all SESI-setups as they are only detectable on a QTOF with soft ionization settings, this observation highlights the compound-specific response once more. As a result, a different standard compound may perform even better for simple monitoring usage.

The current work was performed in positive ion mode. As a result, a suitable gas-phase standard for negative ion mode remains to be identified in the context of quality control for SESI-HRMS. Ideally, this would lead to a multi-component standard with stable compounds for both positive and negative ion modes as designed for PTR-MS [38]. Its performance for reliable quantification in breath will need to be assessed in the future.

The standard was introduced externally to the breath sample in this study. While internal standards, often used in routine high-performance liquid chromatography (HPLC)-MS approaches, ensure a more accurate reference value in terms of sample preparation and injection, this is not possible in direct sampling techniques such as breath analysis. Furthermore, since the ionization mechanism for SESI has not yet been fully described, the introduction of a standard into the breath sample may alter the ion efficiency of breath compounds, e.g. due to possible ion suppression.

One of the study's key limitation is the difficulty to manufacture stable and certified VOCs reference materials in the gas phase [17, 39]. While multi-component standards are desired for breath analysis in particular, the availability of low molar fraction gas-phase compounds is limited. A standard mixture designed for SESI-HRMS analysis would benefit both inter-laboratory comparability within the research community and lower manufacturing costs. This is well in line with the 'Peppermint

initiative' that set out for better understanding of differences between the various methods applied in breath analysis [40, 41]. However, as was demonstrated by the PTR-MS initiative for intercomparison using a customized standard for atmospheric science [38], a more complex and diverse reference material tailored for each detection method individually may be more valuable at first to assure comparability within each method.

In conclusion, the stable and reproducible performance of the presented standard system confirmed its suitability to monitor instrument fluctuations. This would allow for on-site quality control and, eventually, comparability between different laboratories. Both are necessary for long-term and multicenter studies. While the system is applicable to any ambient ionization technique, we demonstrated on SESI-HRMS based breath analysis the system's potential for quality monitoring which is critical for untargeted analysis. We propose including standard measurements routinely for SESI-HRMS, as robust and comparable analysis will be invaluable for translating direct breath analysis into clinics, as will absolute quantification of validated breath biomarkers.

Data availability statement

The data that support the findings of this study are openly available at the following URL/DOI: 10.3929/ethz-b-000568385, in a curated data archive at ETH Zurich (www.research-collection.ethz.ch). Data will be available from 09 January 2023.

Acknowledgments

We would like to thank Simona Müller for her fundamental conceptual contribution to the system and its implementation, as well as the gas analysis team at the Federal Institute of Metrology (METAS, Bern-Wabern, Switzerland), Martin Gaugg, Tobias Bruderer, and, especially, Nora Nowak for all scientific discussion. We are grateful for valuable contributions and instrument support of David Bell (PSI, Villigen, Switzerland), Lukas Emmenegger and David Schönenberger (both Empa, Dübendorf, Switzerland) for additional evaluation of the system by PTR-MS and CRDS. This work is part of Zurich Exhalomics, a flagship project of 'Hochschulmedizin Zürich'.

Author contributions

Study design and concept: B S, S M, R Z, M K, A M, S M; Data acquisition: B S, J S; Data processing and analysis: B S, J S; Data evaluation and interpretation: B S, R Z; Drafting of the manuscript: B S, R Z; Resources: A M, R Z, M K, N P; Review and editing of the final manuscript: all authors

Conflict of interest

M K is a founder and board member of and R Z an advisor to Deep Breath Intelligence AG (www.dbi.ch), a company that provides services in the field of breath analysis.

Financial/nonfinancial disclosures

This work was supported by the Heidi Ras foundation, Lotte und Adolf Hotz-Sprenger foundation, Uniscientia foundation and the Evi Diethelm-Winteler foundation.

ORCID iDs

Justinas Sakas https://orcid.org/0000-0002-0914-011X
Alexander Moeller https://orcid.org/0000-0001-7284-4251
Renato Zenobi https://orcid.org/0000-0001-5211-4358

References

[1] Bruderer T, Gaisl T, Gaugg M T, Nowak N, Streckenbach B, Müller S, Moeller A, Kohler M and Zenobi R 2019 On-line analysis of exhaled breath *Chem. Rev.* **119** 10803–28
[2] Beauchamp J, Davis C and Pleil J 2020 *Breathborne Biomarkers and the Human Volatilome* (Amsterdam: Elsevier) 10.1016/C2018-0-04980-4
[3] García-Gómez D, Martínez-Lozano Sinues P, Barrios-Collado C, Vidal-de-Miguel G, Gaugg M and Zenobi R 2015 Identification of 2-Alkenals, 4-Hydroxy-2-alkenals, and 4-Hydroxy-2,6-alkadienals in exhaled breath condensate by UHPLC-HRMS and in breath by real-time HRMS *Anal. Chem.* **87** 3087–93
[4] Gaugg M T, Gomez D G, Barrios-Collado C, Vidal-de-Miguel G, Kohler M, Zenobi R and Martinez-Lozano Sinues P 2016 Expanding metabolite coverage of real-time breath analysis by coupling a universal secondary electrospray ionization source and high resolution mass spectrometry—a pilot study on tobacco smokers *J. Breath Res.* **10** 016010
[5] Nowak N *et al* 2021 Rapid and reversible control of human metabolism by individual sleep states *Cell Rep.* **37** 109903
[6] Schwarz E I *et al* 2016 Effects of CPAP therapy withdrawal on exhaled breath pattern in obstructive sleep apnoea *Thorax* **71** 110–7
[7] Gaugg M T *et al* 2019 Real-time breath analysis reveals specific metabolic signatures of COPD exacerbations *Chest* **156** 269–76
[8] Smith D and Spanel P 2005 Selected ion flow tube mass spectrometry (SIFT-MS) for on-line trace gas analysis *Mass Spectrom. Rev.* **24** 661–700
[9] Smith D and Španěl P 2016 Status of selected ion flow tube MS: accomplishments and challenges in breath analysis and other areas *Bioanalysis* **8** 1183–201
[10] Herbig J, Müller M, Schallhart S, Titzmann T, Graus M and Hansel A 2009 On-line breath analysis with PTR-TOF *J. Breath Res.* **3** 027004
[11] Sulzer P *et al* 2014 a proton transfer reaction-quadrupole interface time-of-flight mass spectrometer (PTR-QiTOF): high speed due to extreme sensitivity *Int. J. Mass Spectrom.* **368** 1–5

[12] Singh K D et al 2019 Standardization procedures for real-time breath analysis by secondary electrospray ionization high-resolution mass spectrometry *Anal. Bioanal. Chem.* **411** 4883–98
[13] Lucero D P 1971 Performance characteristics of permeation tubes *Anal. Chem.* **43** 1744–9
[14] Tumbiolo S, Vincent L, Gal J-F and Maria P-C 2005 Thermogravimetric calibration of permeation tubes used for the preparation of gas standards for air pollution analysis *Analyst* **130** 1369–74
[15] Singh K D, Del Miguel G V, Gaugg M T, Ibañez A J, Zenobi R, Kohler M, Frey U and Sinues P M-L 2018 Translating secondary electrospray ionization–high-resolution mass spectrometry to the clinical environment *J. Breath Res.* **12** 027113
[16] Weber R, Haas N, Baghdasaryan A, Bruderer T, Inci D, Micic S, Perkins N, Spinas R, Zenobi R and Moeller A 2020 Volatile organic compound breath signatures of children with cystic fibrosis by real-time SESI-HRMS *ERJ Open Res.* **6** 171–2019
[17] Jeerage K M, Berry J L, Murray J A, Goodman C A, Piotrowski P K, Jones C M, Cecelski C E, Carney J, Lippa K A and Lovestead T M 2022 The need for multicomponent gas standards for breath biomarker analysis *J. Breath Res.* **16** 044001
[18] Halbritter S, Fedrigo M, Höllriegl V, Szymczak W, Maier J M, Ziegler A-G and Hummel M 2012 Human breath gas analysis in the screening of gestational diabetes mellitus *Diabetes Technol. Ther.* **14** 917–25
[19] Reynolds J C, Jimoh M A, Guallar-Hoyas C, Creaser C S, Siddiqui S and Paul Thomas C L 2014 Analysis of human breath samples using a modified thermal desorption: gas chromatography electrospray ionization interface *J. Breath Res.* **8** 037105
[20] Silva L I B, Freitas A C, Rocha-Santos T A P, Pereira M E and Duarte A C 2011 Breath analysis by optical fiber sensor for the determination of exhaled organic compounds with a view to diagnostics *Talanta* **83** 1586–94
[21] Storer M, Salmond J, Dirks K N, Kingham S and Epton M 2014 Mobile selected ion flow tube mass spectrometry (SIFT-MS) devices and their use for pollution exposure monitoring in breath and ambient air–pilot study *J. Breath Res.* **8** 037106
[22] Trefz P, Schubert J K and Miekisch W 2018 Effects of humidity, CO_2 and O_2 on real-time quantitation of breath biomarkers by means of PTR-ToF-MS *J. Breath Res.* **12** 026016
[23] Malásková M, Henderson B, Chellayah P D, Ruzsanyi V, Mochalski P, Cristescu S M and Mayhew C A 2019 Proton transfer reaction time-of-flight mass spectrometric measurements of volatile compounds contained in peppermint oil capsules of relevance to real-time pharmacokinetic breath studies *J. Breath Res.* **13** 046009
[24] Beauchamp J, Kirsch F and Buettner A 2010 Real-time breath gas analysis for pharmacokinetics: monitoring exhaled breath by on-line proton-transfer-reaction mass spectrometry after ingestion of eucalyptol-containing capsules *J. Breath Res.* **4** 026006
[25] Barrios-Collado C, García-Gómez D, Zenobi R, Vidal-de-Miguel G, Ibáñez A J and Martinez-Lozano Sinues P 2016 Capturing *in vivo* plant metabolism by real-time analysis of low to high molecular weight volatiles *Anal. Chem.* **88** 2406–12
[26] de Lacy Costello B, Amann A, Al-Kateb H, Flynn C, Filipiak W, Khalid T, Osborne D and Ratcliffe N M 2014 A review of the volatiles from the healthy human body *J. Breath Res.* **8** 014001
[27] Mochalski P, King J, Klieber M, Unterkofler K, Hinterhuber H, Baumann M and Amann A 2013 Blood and breath levels of selected volatile organic compounds in healthy volunteers *Analyst* **138** 2134–45
[28] Drabinska N, Flynn C, Ratcliffe N, Belluomo I, Myridakis A, Gould O, Fois M, Smart A, Devine T and Costello B D L 2021 A literature survey of volatiles from the healthy human breath and bodily fluids: the human volatilome *J. Breath Res.* **15** 034001
[29] Martinez-Lozano Sinues P, Tarokh L, Li X, Kohler M, Brown S A, Zenobi R and Dallmann R 2015 Circadian variation of the human metabolome captured by real-time breath analysis *PLoS One* **9** e114422
[30] Bruderer T, Gaugg M T, Cappellin L, Lopez-Hilfiker F, Hutterli M, Perkins N, Zenobi R and Moeller A 2020 Detection of volatile organic compounds with secondary electrospray ionization and proton transfer reaction high-resolution mass spectrometry: a feature comparison *J. Am. Soc. Mass Spectrom.* **31** 1632–40
[31] Wishart D S et al 2007 HMDB: the human metabolome database *Nucleic Acids Res.* **35** D521–6
[32] Holman J D, Tabb D L and Mallick P 2014 Employing proteowizard to convert raw mass spectrometry data *Curr. Protoc. Bioinform.* **46** 13.24.1–9
[33] Chen H, Hu B, Hu Y, Huan Y, Zhou Z and Qiao X 2009 Neutral desorption using a sealed enclosure to sample explosives on human skin for rapid detection by EESI-MS *J. Am. Soc. Mass Spectrom.* **20** 719–22
[34] Martínez-Lozano P, Rus J, Fernández de la Mora G, Hernández M and Fernández de la Mora J 2009 Secondary electrospray ionization (SESI) of ambient vapors for explosive detection at concentrations below parts per trillion *J. Am. Soc. Mass Spectrom.* **20** 287–94
[35] European Medicines Agency 2011 Guideline on bioanalytical method validation (available at: www.ema.europa.eu/en/documents/scientific-guideline/guideline-bioanalytical-method-validation_en.pdf)
[36] U.S. Food and Drug Administration 2018 Bioanalytical method validation guidance for industry (available at: www.fda.gov/files/drugs/published/Bioanalytical-Method-Validation-Guidance-for-Industry.pdf)
[37] Liu C, Zeng J, Sinues P, Fang M, Zhou Z and Li X 2021 Quantification of volatile organic compounds by secondary electrospray ionization-high resolution mass spectrometry *Anal. Chim. Acta* **1180** 338876
[38] Holzinger R et al 2019 Validity and limitations of simple reaction kinetics to calculate concentrations of organic compounds from ion counts in PTR-MS *Atmos. Meas. Tech.* **12** 6193–208
[39] Rhoderick G C 2010 Stability assessment of gas mixtures containing terpenes at nominal 5 nmol/mol contained in treated aluminum gas cylinders *Anal. Bioanal. Chem.* **398** 1417–25
[40] Henderson B et al 2020 A benchmarking protocol for breath analysis: the peppermint experiment *J. Breath Res.* **14** 046008
[41] Henderson B et al 2021 The peppermint breath test benchmark for PTR-MS and SIFT-MS *J. Breath Res.* **15** 046005