An off-line breath sampling and analysis method suitable for large screening studies

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Abstract

We present a new, off-line breath collection and analysis method, suitable for large screening studies. The breath collection system is based on the guidelines of the American Thoracic Society for the sampling of exhaled NO. Breath containing volatile gases is collected in custom-made black-layered Tedlar bags and analyzed by proton-transfer reaction mass spectrometry (PTR-MS). The collection method and data analysis is validated for its accuracy, precision, selectivity, limits of detection, sensitivity and reproducibility. Consecutive fillings of five bags by the same person gave reproducible results to within 12% relative standard deviation (RSD) for methanol, acetaldehyde, acetone and water content from breath, whereas isoprene was constant to within 30% RSD. In an exploratory small-scale case-control study, we monitor the exhaled breath of 11 lung cancer patients on the day before surgery. The control group consisted of 57 age-matched subjects, the so-called ‘healthy smokers’. This study is used as an example of the use of the system presented here.

Keywords: breath sampling, PTR-MS, lung cancer, trace gas analysis, VOCs

1. Introduction

Modern breath analysis started in the 1970s when Pauling isolated over 200 gaseous compounds in human breath (Pauling et al. 1971); currently more than 1200 compounds have been observed (Phillips 1992, 1997). Many case studies were performed to define biomarkers (specific compounds that show the presence or absence of a disease) in breath, varying from lung (Phillips et al. 2003, Poli et al. 2005, Rahman et al. 2002, Alving et al. 1993) to intestinal diseases (Lechner et al. 2005), schizophrenia (Phillips et al. 1995) or ‘general’ UV-induced lipid peroxidation (Harren et al. 1999, Kneepkens et al. 1994). An overview of recent work on breath analysis is given by Amann and Smith (2005).
However, various sampling and/or analysis techniques were used (Kneepkens et al 1994, Risby 2006). Some studies sampled mixed expiratory breath, including dead-space air, whereas others sampled alveolar air only. In the latter, concentrations of endogenous volatile organic compounds (VOCs) can be 2 to 3 times higher (Miekish et al 2004). The air in the conducting airways does not take part in gas exchange with the blood, and therefore mostly resembles the environmental conditions. To avoid another systematic error nasal air contamination has to be prevented. The soft palate must close, which can be achieved via exhalation over a resistance (Poli et al 2005, Kharitonov and Barnes 1997, Silkoff et al 1997, American Thoracic Society Documents 2005). Varying the exhaled flow rates causes differences in contact time between the conducting airway mucosa and the exhaled air, modifying the exhaled concentrations (Jöbsis et al 2001). As a result of these varying collection methods, outcomes are difficult to compare and to extrapolate. Therefore, standardized sampling methods should be implemented (Miekish et al 2004, Risby 2006), such as for the determination of the amount of NO in exhaled air, for which standardized procedures are available (American Thoracic Society Documents 2005).

In screening programs, one tries to identify patients having a certain disease from large amount of people at high risk for developing that disease. A first challenge in such a program would be to find markers for the specified disease. The methods to be used for such research are preferably non-invasive, fast and cheap. Since in a mass screening situation large numbers of patients are tested, the sampling method should be standardized and suited for a high throughput of samples. This is the reason why we choose an off-line breath sampling method for this study.

Based on the ATS guidelines (American Thoracic Society Documents 2005) for off-line measurement of exhaled NO, we present and validate a method for the collection of breath VOCs. Proton-transfer reaction mass spectrometry (PTR-MS) (Boamfa et al 2004, Lindinger et al 1998, Steeghs et al 2006) is used to monitor the breath profile, since it is a versatile, fast and sensitive method, which needs no sample pre-concentration (Steeghs et al 2006, Amann et al 2005, Lirk et al 2004). Here, we present a method that is suitable for such large-scale screening studies and we show a small-scale study on patients with and without lung cancer, indicating how this method works in practice and how a statistical approach can offer insight into differences in the mass spectral fingerprints obtained from human breath.

As an example application, we performed a pilot study on 11 patients with lung cancer, who were about to undergo surgery. The contents of their breath were compared with a control group of 57 ‘healthy smokers’ with a similar smoking behavior, but without cancer, emphysema or an impaired lung function. We applied a statistical analysis on the mass spectra obtained from these 68 subjects to see if we could identify differences between healthy and lung cancer patients in the fingerprints obtained with the method presented here.

2. Materials and methods

2.1. Breath collection

The breath collection device (figure 1) was designed according to the guidelines of the American Thoracic Society (American Thoracic Society Documents 2005) for the standardized collection of exhaled NO. It consists of a mouthpiece connected to a Teflon tubing ($\frac{1}{4}$" outer diameter) ending in a discard bag and a sample bag. The entrance valve of the sample bag acts as a restrictor that limits the exhaled flows to 50 ml s$^{-1}$. Such a low flow rate lengthens the contact time between exhaled air and the (inflamed) mucosa, increasing the exhaled concentrations. The display of the pressure meter on top of the sampling device helps
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Figure 1. Schematic representation of the breath sampling device. The patient exhales into a mouthpiece (A). The exhaled air goes through a T-piece (B) through a piece of \( \frac{1}{4}'' \) Teflon tubing (C) to a discard bag (D), where the first part of a single breath is discarded. When the discard bag has been filled, pressure builds up and air starts to flow through a resistance, formed by the inlet valve (F) of the bag (G). The pressure in the sampling device is measured by a pressure gauge (E), which continuously feeds back the pressure from an LED display, so the volunteer can keep the flow rate on the desired value.

the subject to maintain a constant exhalation flow. A discard bag is used before the sampling bag to capture the first part (~800 ml) of the breath to prevent interference of air from the conducting airways.

Subjects are asked to take a deep breath and perform one single exhalation into the mouthpiece. No nose-clip is used. First, the low resistance discard bag (Procare, Groningen, the Netherlands) is completely filled, after which the pressure in the device builds up and the collection bag starts to fill. Due to the increased pressure, the soft palate closes (Poli et al 2005, Kharitonov and Barnes 1997, Silkoff et al 1997, American Thoracic Society Documents 2005). Breath samples (~800 ml) were collected in 1 l black-layered custom-made Tedlar bags (SKC Inc., Eighty Four, PA, USA), which are cleaned with a flow of \( \sim 25 \) l h\(^{-1} \) of synthetic air (mixture of purified nitrogen and 20 ± 1% purified oxygen, <3 ppm H\(_2\)O, <0.5 ppm C\(_n\)H\(_m\); Air Liquide BV, Eindhoven, the Netherlands) for more than 2 h before use or re-use.

Before providing the sample for analysis, the subjects are shortly instructed on how the sampling device works and get familiar with it by filling a dummy sample bag. For each subject, a sterilized set of tubing was used. All tubes and connections in the collection device are made out of Teflon (Polyfluor BV, Oosterhout, The Netherlands/Metron Technologies, Wychen, The Netherlands). The samples are transported to Nijmegen, where they are analyzed after a fixed period of time (~54 h).

2.2. Ambient concentrations

Volatile s are present in ambient air and thus influence the composition of the exhaled breath (American Thoracic Society Documents 2005). A washout period, breathing purified air, would therefore be needed (Kneepkens et al 1994, Risby 2006). To minimize the burden for the patients and the contact time per patient, we chose not to do so, but to monitor the composition of the inhaled air (Risby 2006). For each breath sample taken, the inhaled room air is sampled using a Teflonized micro diaphragm gas pump (NMP 830, KNF-Verder B.V.,
Figure 2. Proton-transfer reaction mass spectrometer as used in this study with the automated bag measurement scheme. The PTR-MS consists of an ion source (1), where H$_3$O$^+$ ions are formed in a discharge in helium and water vapor and are extracted into the drift tube (2), where the proton-transfer reaction takes place. After protonation, the ions are led through a buffer chamber (3), which acts as an intermediate pumping stage and is used to refocus and redirect the ion beam into the quadrupole (4) where the ions are mass selected and measured by a secondary electron multiplier (5).

Vleuten, The Netherlands) filling an identical Tedlar bag. These ambient air samples are analyzed and if they are found polluted with high concentrations of one or more VOCs, the specific breath sample is not taken into the analysis.

2.3. VOC analysis


$$H_3O^+ + R \rightarrow RH^+ + H_2O$$  \hspace{1cm} (1)

where R is any trace gas component able to react with H$_3$O$^+$. This proton-transfer reaction only takes place if the proton affinity (PA) of the analyte is higher than that of water, meaning that PTR-MS is able to detect most aldehydes (e.g. formaldehyde, acetaldehyde, propanal), ketones, alcohols (e.g. methanol, ethanol, propanol, iso-propanol, n-propanol), acids (e.g. formic acid, acetic acid, propanoic acid) and esters as well as many unsaturated, aromatic and N or S substituted hydrocarbons. The normal constituents of air (NO, CO, CO$_2$, O$_2$ etc) all have a PA lower than that of water, assuring no interference. The reaction product ions are mass analyzed using a quadrupole mass spectrometer and detected by a secondary electron multiplier (SEM). The proton affinity of most organic compounds is in the range of 7–9 eV, whereas the proton affinity of water is 7.16 eV (NIST), which makes the excess energy of the reaction low. This assures that the reaction generally results in only one or two characteristic ions per neutral molecule, of which the ratio can be determined experimentally. The resulting mass spectrum is therefore relatively simple. Besides, the PTR-method is sensitive as compared with electron impact ionization. Unfortunately, alkanes, methylated alkanes and some alkenes cannot be measured with PTR-MS due to their low PA.
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The drift tube of the PTR-MS is operated at 2–2.5 mbar, so no pump is needed to transport the contents of the bags to the measurement device. The bags are simply connected to the gas inlet via a ¼\textsuperscript{′′} Teflon tube. The total flow through the sample inlet in these experiments is about 1.0 l h\textsuperscript{−1} of which 0.5 l h\textsuperscript{−1} enters the drift tube. The drift tube has a total length of 10 cm with an inner diameter of 3.0 cm, resulting in a volume of ∼0.07 l at 2 mbar. With these dimensions and flows, the drift tube is refreshed approximately once every second (see Steeghs et al 2006). All flow-through gas handling parts are made of Teflon to avoid any adsorption of the VOCs to the wall material. From every bag, five mass scans (20–150 amu) are taken and from each mass the area under the peaks (m – 0.5 to m + 0.5) are calculated and averaged over the five scans (the quadrupole mass spectrometer formally only determines the mass-to-charge ratio of ions. Because only singly charged ions play a role in PTR-MS, we simply refer to ions by their mass in this paper). The concentration of compounds measured in this study is always determined with reference to values obtained from a calibration measurement using these same compounds.

2.4. Validation experiments

A screening study’s first goal is the identification of potential marker masses. Therefore, two aspects of the PTR-MS mass scan results need to be validated: the ion intensity and the mass determination.

Accuracy and precision of the mass determination are measured from 35 scans of a preset concentration, where the measured peak position is expressed as a percentage of the expected peak position. Selectivity of the system is determined by measuring the interference of a high ion signal on its neighboring mass signal (m + 1) for the compounds acetaldehyde (interference on mass 46 amu by mass 45 amu), benzene (mass 80/mass 79 amu) and toluene (mass 94/mass 93 amu). Interference is corrected for naturally abundant isotopes.

Accuracy and precision for the ion intensity measurement are determined from mass spectra obtained from a certified gas mixture (Scott Specialty Gases, Breda, the Netherlands) consisting of methanol (600 ppbv), acetaldehyde (800 ppbv), acetone (900 ppbv), isoprene, benzene, toluene and styrene (1000 ppbv). Accuracy and precision are determined from 20 measurements each day of the diluted certified mixture over five different days. The reproducibility of the sampling and analysis ensemble is determined by monitoring the ion intensity at masses 33, 45, 59 and 69 amu, supposed to correspond to methanol, acetaldehyde, acetone and isoprene, respectively. Two series of five bags were filled with breath. One person filled five bags with breath with 5 min intervals between sequential breath samples. Another person filled five bags within 3 min. After 2–3 h, the contents were measured to see how constant the collected breath values were for methanol, acetaldehyde, acetone and isoprene, four of the main VOCs usually present in breath. Additionally, the water content was monitored by the ratio m37/m19 (m19 represents H3O\textsuperscript{+} and m37 represents the cluster ion H3O\textsuperscript{+}·H2O, the ion intensity of which, under constant operating conditions, reflects changes in the relative humidity of the sampled gas). The operating conditions of the drift tube were held constant at 2.3 mbar and 120 Td for all experiments performed for this study.

2.5. Example: case-control study on lung cancer

As an example of the approach adopted here, we studied the breath of subjects with surgically removable lung cancer, who were selected for this case-control study: 11 subjects participated and represent the cases. The 57 controls did not suffer from lung cancer, pulmonary function impairment or emphysema, but had the same smoking burden. These subjects can be
considered as ‘healthy’ smokers. No specific restrictions were applied to the participants regarding food, drinks, diets, exercise, etc.

Breath samples were obtained as described above and evaluated via stepwise forward logistic regression (Hosmer and Lemeshow 2000). However, the large number of parameters (130 m/z ratios; ‘masses’) delivers an unfavorable case/variable ratio: a rule of thumb requires a ratio of 10:1, a problem shared with micro-array studies. This requirement is not fulfilled, and hence the outcome of the stepwise forward logistic regression can be unreliable. This problem was approached via bootstrapping, a well-known re-sampling technique (Mooney and Duval 1993), which takes a sample with replacement from the database and the stepwise forward logistic regression is run on that sample. The outcome in terms of a set of masses discriminating between ‘cancer’ and ‘no cancer’ is stored. This procedure is repeated 500 times, and a database of 500 sets of parameters is generated.

Suitable markers appear often in the bootstrap samples, but a high prevalence is not an absolute guarantee for a good marker: borderline significant ones can still be present often and these contribute little. We removed these via a confirmatory logistic regression: as the goal of experiments as this is only to define a possible set of markers, we choose $p = 0.10$ as an inclusion level and $p = 0.30$ as an exclusion level.

The probability to make a correct diagnose of the presence or absence of lung cancer is reflected by the area under the ROC (receiver operating characteristic) curve. The ROC curve is a plot of the true positive rate against the false positive rate for different possible cut points (that result above which a person is decided to be called ‘diseased’ or ‘healthy’ according to the test) of a diagnostic test; it shows the trade-off between sensitivity and specificity. The area under the curve is a measure of the quality of the test, where an area of 0.5 is the result of ‘flipping a coin’ and 1.0 is the ideal result.

All statistics were calculated with SPSS statistical software package version 13 (SPSS, Chicago, IL, USA). $P$-values $<0.05$ were considered significant.

3. Results

3.1. Validation of the approach

The gas sample bags have been tested for their storage and collection abilities for various different compounds previously (Steeghs et al 2007). To validate the whole method, which includes breath sampling and analysis, several properties of the system are analyzed.

3.1.1. Linearity. In a mass scan, ion intensities are measured at all m/z ratios from 20 to 150 amu. The ion signal is linear with the concentration of the trace gas component (R) and with the initial number of H$_3$O$^+$ ions (Lindinger et al 1998), as reflected by equation (1). The range of linearity of this system is determined by the total concentration of all detectable compounds and their respective sensitivities. The response of a PTR-MS system is linear up to the point where the primary ion signal has decreased with $\sim$10% of its value without any VOC present. Assuming a primary ion intensity of $1 \times 10^6$ a linear range of five orders of magnitude is achieved (Lindinger et al 1998).

3.1.2. Reproducibility. The methanol, acetaldehyde and acetone signals from the breath of two volunteers, collected in $2 \times 5$ bags are displayed in figure 3. The average values and corresponding relative standard deviation (RSD) of four compounds and the water content are given in table 1. For subject 1 the highest variability is found for isoprene, which was found
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Figure 3. Ion intensities for two series of five bags filled consecutively. Each series has been filled by one volunteer, the first series with time intervals of 5 min between sequential bags, whereas the second series has been filled within 5 min.

Table 1. Results for the repeatability experiments. Concentrations of five compounds in breath are compared over five bags filled consecutively. The water content is monitored by the ratio between mass 37 and mass 21 amu.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mean over five bags, person 1 (ppbv)</th>
<th>RSD person 1 (%)</th>
<th>Mean over five bags, person 2 (ppbv)</th>
<th>RSD person 2 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>32.1</td>
<td>5.6</td>
<td>67</td>
<td>4.9</td>
</tr>
<tr>
<td>Acetaldehyde</td>
<td>25.6</td>
<td>5.3</td>
<td>29</td>
<td>8.1</td>
</tr>
<tr>
<td>Acetone</td>
<td>158</td>
<td>11</td>
<td>80</td>
<td>5.6</td>
</tr>
<tr>
<td>Isoprene</td>
<td>1.8</td>
<td>29</td>
<td>17.9</td>
<td>6.6</td>
</tr>
<tr>
<td>Water content</td>
<td>0.21⁺</td>
<td>8.6</td>
<td>0.189</td>
<td>6.3</td>
</tr>
</tbody>
</table>

⁺ Water content is expressed as the ratio between masses 37 and 19 amu.

at the lowest ion intensities of all compounds. For subject 2 the variability for all compounds is similar and low.

3.1.3. Selectivity. The interference on a certain mass by the neighboring mass is determined for three masses (table 2), but increases with increasing concentration of the interfering compound. The concentrations of the compounds used in this measurement are ~60 ppbv, which is a moderate to high concentration for most VOCs in breath. Interference in all cases was below 2%. In general, the interference will therefore be less than this (except for acetone and isoprene).

PTR-MS monitors concentrations of VOCs in airstreams by accurately determining the $m/z$ value of an ion and the ion intensity at that specific $m/z$ value. Since PTR-MS is equipped with a quadrupole mass spectrometer, which cannot distinguish between different isomers, the ion intensity that is monitored is the intensity of the sum of all ions with the same $m/z$ ratio. PTR-MS is therefore not selective in the compounds measured (De Gouw et al 2003, Steeghs et al 2004, Warneke et al 2003).
Table 2. Results of precision and accuracy of the mass determination.

<table>
<thead>
<tr>
<th>Real mass (amu)</th>
<th>Measured mass (amu) ± SD</th>
<th>RSD (%)</th>
<th>Accuracy (%)</th>
<th>Interference on m + 1 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>45.05</td>
<td>44.96 ± 0.028</td>
<td>0.062</td>
<td>99.66</td>
<td></td>
</tr>
<tr>
<td>59.08</td>
<td>58.00 ± 0.01</td>
<td>0.017</td>
<td>99.66 +1.7</td>
<td></td>
</tr>
<tr>
<td>69.12</td>
<td>69.01 ± 0.07</td>
<td>0.1</td>
<td>99.64</td>
<td></td>
</tr>
<tr>
<td>79.11</td>
<td>79.0 ± 0.0</td>
<td>0</td>
<td>100</td>
<td>+1.3</td>
</tr>
<tr>
<td>93.14</td>
<td>93.00 ± 0.01</td>
<td>0.011</td>
<td>99.65 +2.4</td>
<td></td>
</tr>
<tr>
<td>105.15</td>
<td>105.01 ± 0.05</td>
<td>0.047</td>
<td>99.67</td>
<td></td>
</tr>
</tbody>
</table>

Table 3. Results of precision and accuracy of the ion intensity determination of the PTR-MS analysis instrument determined through the measurement of a dilution of the certified gas mixture (N = 100 measurements). Inaccuracy in the concentration in the calibrated mixture is around 10%. Additionally, the limits of detection (LOD) and sensitivity are given.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (±10%)</th>
<th>Accuracy (%)</th>
<th>RSD (%)</th>
<th>LOD (ppbv)</th>
<th>Sensitivity (ncps/ppbv)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanol</td>
<td>300</td>
<td>104</td>
<td>14.7</td>
<td>1</td>
<td>28.6</td>
</tr>
<tr>
<td>Acetaldehyde</td>
<td>400</td>
<td>103</td>
<td>8.7</td>
<td>0.85</td>
<td>42.9</td>
</tr>
<tr>
<td>Acetone</td>
<td>450</td>
<td>102</td>
<td>7.9</td>
<td>0.6</td>
<td>51.7</td>
</tr>
<tr>
<td>Benzene</td>
<td>500</td>
<td>97</td>
<td>8.0</td>
<td>1</td>
<td>7.68</td>
</tr>
<tr>
<td>Toluene</td>
<td>500</td>
<td>96</td>
<td>18.1</td>
<td>0.6</td>
<td>7.75</td>
</tr>
<tr>
<td>Styrene</td>
<td>500</td>
<td>89.2</td>
<td>22.2</td>
<td>1</td>
<td>3.86</td>
</tr>
</tbody>
</table>

3.1.4. Accuracy. For six compounds the precision and accuracy of the mass determination is given in table 2. It should be noted that the mass scale is scanned in steps of 0.06 amu so the peak position is determined on this discontinuous scale, decreasing the accuracy somewhat. The precision and accuracy of the ion intensity is given in table 3. The accuracy is found to be between 89 and 104%.

3.1.5. Limits of detection and sensitivity. The sensitivity of the method for the seven different gases from our certified mixture and the corresponding limits of detection are listed in table 3, based on signal-to-noise = 2 and 1 s measurement time (no averaging). The sensitivity for other compounds will be comparable and can be estimated by taking the differences in collision rate constant and fragmentation ratio into account, since the sensitivity of a PTR-MS is proportional to the reaction rate constant (De Gouw et al 2003).

3.2. Application of the method to a small-scale control-case study to find markers for lung cancer

In this study, 57 male ‘healthy smokers’ were found. Their mean age was 59.8 ± 5.0 years, they smoked 40.8 ± 15.9 pack years. The 11 cancer subjects were 56.4 ± 4.5 years and smoked 42.6 ± 16.2 pack years.

The bootstrapped stepwise forward logistic regression defined masses 25 and 69 to discriminate between the mass spectral fingerprints of both groups. The prevalence in 500 bootstrapped samples was 17.6% (p = 0.087) and 24.0% (p = 0.041) respectively. The area under the ROC curve (figure 4) for this test was 0.81. The median m25 value was 18.2 in the controls and 7.40 in the cases, for m69 these values are 15.7 resp. 94.4 normalized counts per second (ncps).
4. Discussion and conclusions

This study presents the design and validation of a single-breath sampling and analysis method suitable for large screening studies.

Usually, gas chromatography mass spectrometry (GC-MS) is used to analyze the composition of breath. However, GC-MS is time consuming, needs breath pre-concentration and cannot provide on-line and multiple breath profiles. Sample pre-concentration can cause errors and requires a prolonged breathing time. In screening studies a large throughput of patients is required, demanding the collection and analysis method to be fast. PTR-MS is sensitive, versatile and needs no sample pre-concentration. Additionally, it has the time resolution needed for online breath profiling and requires only one single breath stroke for repeated measurements of selected volatiles. Therefore, this technique is very suited to be used in clinical situations, where the breath of the patients can be sampled and where an outcome of the diagnostic test can be received within a few minutes in a simple way without any discomfort to the patient.

Validation of the mass determination as well as the ion intensity measurements are performed and accuracy, precision and selectivity are excellent. The breath sample obtained using this single-breath sampling technique is shown to be representative and reproducible. The relative standard deviations for methanol, acetaldehyde, acetone and the relative water content in the breath of two subjects are within 12% in five consecutively sampled bags. The relative standard deviation of isoprene is within 30% in five consecutively sampled bags. Lindinger et al (1998) measured the isoprene content in breath and found variations of up
to 50% in exhaled concentrations. Reproducibility of the values of isoprene is better for the second series than for the first series. In the first series, longer time intervals were used between successive fillings. This shows that the sampling is reproducible and reliable.

As an example, we applied this method on a small-scale control-case study to find differences in mass spectra of healthy volunteers and people suffering from lung cancer. Two parameters were found to be different for ‘healthy smokers’ than for cancer patients. The area under the ROC curve was 0.81.

It should be noted that the number of subjects with cancer \((N = 11)\) in this pilot study is too small for a reliable statistical analysis. Therefore, the outcome of this study should be considered as highly tentative and it can best be regarded as an illustration of the presented method. In a PTR-MS instrument, usually zero concentration is measured on mass 25 amu; otherwise, mass 25 is regarded to reflect instrumental noise. However, at this mass we do find a non-zero contribution and a difference between the values for the two groups (healthy and diseased). Our PTR-MS system uses a different ionization source than the commercial systems. While this source produces high amounts of ions and hardly requires any maintenance, we also see some pollution we currently do not understand. Additionally, the source drift region (which increases \(\text{H}_3\text{O}^+\) purity and circumvents back diffusion of VOCs into the source) was not yet mounted during the measurements performed in this study.

Mass 69 usually represents isoprene, which is a marker of inflammation or lipid peroxidation and is present in breath in relatively high concentrations. Also furan is measured on this mass and is reported in breath as a marker for smoking (Sanchez and Sacks 2006).

An important aspect that needs to be considered is the low prevalence of these two parameters. When are parameters considered to distinguish between two groups? This shows how difficult it is to find a marker for a complex disease such as lung cancer. Additionally, the appearance of an unexpected parameter in general (e.g. mass 25 in our example) represents both a problem and a challenge for the interpretation of the data. With this example, we would like to stress that concluding that a breath VOC is a marker for a disease is far from straightforward. The results of this study will have to be validated. Therefore, we aim for large scale, population-based studies. This is, in our opinion, the only way breath analysis will ever be able to outgrow its infancy status. We currently are performing such a large-scale screening study, hoping more of these will follow in the future.

In the pilot study, we use an approach that is unique in the sense that the control group consisted of ‘healthy smokers’, which represents the group of patients most likely to be encountered in a regular clinical situation. The differences between this control and diseased group are probably much smaller when compared to a control group of ‘healthy volunteers’. Of course, this might further obscure the differences between both groups and complicate the analysis. This experimental setup forms challenging ‘uncontrolled’ (no restrictions on food, diet, exercise, etc) conditions, very much resembling a normal clinical setting.

This method presented here is suitable for collection and analysis of breath via single-breath sampling. Due to the high reproducibility, short operating time, ease of use and versatile detection technique, this method is highly suited for screening studies where a high throughput and therefore a short contact time with the patient are required.

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