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FACULTEIT GENEESKUNDE EN LEVENSWETENSCHAPPEN
master in de biomedische wetenschappen

Masterproef

Validation of a new method for molecular phenotyping of noisy breathing in young children

Promotor :
Dr. Marc RAES
dr. Ingrid ARIJS

Copromotor :
dr. GUDRUN KOPPEN

Lotte Broeckx

Scriptie ingediend tot het behalen van de graad van master in de biomedische wetenschappen

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Content

Abbreviation list

ANOVA	Analysis of Variance
COPD	Chronic obstructive pulmonary disease
CASPER	Clean Air Supply pump
ELISA	Enzyme linked immune sorbent assay
FEP	Fluorinated ethylene propylene
GC-MS	Gas-chromatography mass spectrometry
G-CSF	Granulocyte colony stimulating factor
Hz	Hertz
IFN-γ	Interferon-gamma
IL	Interleukin
LTC4	Leukotriene C4
L/min	Litre per minute
m/z	Mass/charge
MSD	Mesoscale discovery
ms	Millisecond
MUC5ac	Mucin 5ac
ppb	Parts per billion
PCR	Polymerase chain reaction
PGD2	Prostaglandin D2
ReCIVA	Respiration collector for *In Vitro* analysis
SIFT-MS	Selected ion flow tube mass spectrometry
SIM	Selected ion method
TNF-α	Tumour necrosis factor alpha
VOC	Volatile organic compounds

Abstract

Introduction: Noisy breathing is a common symptom in young children that can occur as multiple phenotypes. The most common phenotypes are wheezing and rattling. The current method that used to differentiate these phenotypes is subjective. The ongoing project is part of a bigger study which aims to improve and objectify the diagnosis of wheezing and rattling by using nasal mucus protein markers, exhaled breath volatile organic compounds (VOCs) and sound analysis. The current project focusses on VOCs and the Selected Ion Flow Tube mass spectrometry (SIFT-MS) used to analyze them. The aim of this project is to optimize and validate the SIFT-MS for compounds that are linked to lung disorders. We hypothesized that the SIFT-MS technique is a valid method to measure different compounds and that they will be quantified correctly.

Material & methods: Selected compounds, linked to lung disorders, pathogens and oxidative stress, were diluted in methanol (1, 25, 50, 75, 100ppb) and injected in separate Teflon bags. Each concentration level was measured on five different days to determine the validation parameters. The clean air supply pump (CASPER®), used to scrub ambient air, was also tested at different flows of 15 and 10 litres per minute.

Results: The validation for the different compounds gave widespread results. The average trueness was 95.46% (±11.52%). The repeatability and reproducibility gave similar results with averages of 2.74ppb (±1.33 ppb) and 6.55ppb (±3.59ppb), respectively. The residuals of the different compounds at different concentrations varied from 0% to 15%. The average limit of detection and quantification was 3.86ppb (±5.1ppb) and 7.72ppb (±10.21ppb), respectively. The average measurement uncertainty was 31.51% (± 15.82%), which is acceptable. The CASPER® worked best at 15 litres per minute and functioned less at ten litres per minute.

Discussion & conclusions: The technique to produce the gasses in Teflon bags is more variable and depends on various factors. The validation parameters are overall acceptable and the measurement uncertainty of some compounds is comparable with GC-MS parameters. Overall validation results indicated that the SIFT-MS can measure the various selected compounds and quantify them correctly in the expected range. This technique is suited for on-line measurements of different volatile organic compounds.

Samenvatting

Introductie: Luidruchtige ademhaling is een veelvoorkomend symptoom bij jonge kinderen dat zich kan uiten als meerdere fenotypes. De meest voorkomende fenotypes zijn piepen en reutelen. De methode die momenteel gebruikt wordt om deze fenotypes te onderscheiden is subjectief. Het lopende project maakt deel uit van een grotere studie die beoogt om de diagnostisering van piepen en reutelen te verbeteren en deze objectiever te maken. Dit door gebruik te maken van proteïnemerkers in nasaal mucus, volatiele organische stoffen in de uitgeademde lucht en geluidsanalyse. Het huidige project focust op de volatiele organische stoffen en de Selected Ion Flow Tube Mass Spectrometry (SIFT-MS). Het doel van het huidige project was om de SIFT-MS techniek te optimaliseren en te valideren voor volatiele organische stoffen die gelinkt zijn aan verschillende longaandoeningen en ademhalingsproblemen, zoals astma. Onze hypothese stelde dat de SIFT-MS techniek een goede methode is om verschillende volatiele organische stoffen te meten en dat ze correct gekwantificeerd worden.

Materiaal & methoden: De geselecteerde stoffen, gelinkt aan long aandoeningen, pathogenen en oxidatieve stress, werden verdund in methanol (1, 25, 50, 75 en 100 ppb) en geïnjecteerd in aparte Teflon zakken. Elk concentratie niveau werd gemeten op vijf verschillende dagen om de validatie parameters te bepalen. De clean air supply pump (CAPSER®) werd ook getest op verschillende debieten (15 en 10 liter per minuut).

Resultaten: De validatie voor de verschillende stoffen gaf uiteenlopende resultaten. De gemiddelde juistheid was 95,46% (± 11,52%). De herhaalbaarheid en de reproduceerbaarheid gaven gelijkaardige resultaten. De afwijking van de verschillende stoffen op de verschillende concentraties varieerden van 0% tot 15%. De gemiddelde detectielimiet was 3,86 ppb (± 5,1 ppb) en de gemiddelde kwantificatielimiet was 7,72 ppb (± 10,21 ppb). De gemiddelde meetonzekerheid was 31.51% (± 15.82%), wat aanvaardbaar is. De CASPER® werkte beter bij 15 liter per minuut en functioneerde minder goed bij tien liter per minuut.

Discussie & conclusie: De techniek om de gassen te produceren in de Teflon zakken is meer variabel en afhankelijk van verschillende factoren. De validatie parameters zijn over het algemeen aanvaardbaar en de meetonzekerheid van sommige stoffen is vergelijkbaar met die van gas chromatografie (GC) –MS. De validatie parameters tonen aan dat de SIFT-MS de verschillende geselecteerde stoffen kan meten en juist kan kwantificeren in de verwachte range. Deze analytische techniek is geschikt voor online metingen van verschillende volatiele organische stoffen.

1. Introduction

1.1. Noisy breathing

Noisy breathing is a common symptom in young children caused by a turbulent airflow. A turbulent airflow induces the air molecules to collide with each other and with the airway walls which produces a sound (1). This can have different underlying causes such as irregular airway walls or narrowing of the airways (1).

Young children and infants are more susceptible for noisy breathing because their larger airways are relatively soft and more likely to collapse (2). Additionally their airways are smaller and therefore more prone to obstruction caused by bronchoconstriction or excess mucus production (3). A study of Thornton et al. (1990) assessed the symptoms, seen at home, of 298 infants. Thirty percent of the parents reported that their child had experienced mucousy sounds, snuffles, stridors, grunts or wheezes in the previous 24 hours (4).

There are different phenotypes of respiratory noises (table 1) which can have different sites of origin and a different pathophysiology. Two common phenotypes of noisy breathing in infants and toddlers are wheezing and rattling.

Table 1 Common respiratory noises and sites of origin (2).

Noise	Site of Origin
Wheeze	Intrathoracic airways
Rattle	Either or both intra- and extrathoracic airways
Stridor	Extrathoracic airways
Snore	Oro-naso-pharyngeal airway
Snuffle/snort	Nasal passages/naso-pharynx
Grunt	Alveoli/lung parenchyma

1.1.1. Wheezing

Wheezing is common in young children, 28.5% of Dutch children has experienced wheezing in the first year of life (5). It is characterized by a high-pitched continuous sound with a musical quality and a dominant frequency of 400 Hertz (Hz) or more (6, 7). This sound is usually heard over the chest and is caused by bronchial wall vibrations due to an interaction between gas moving through the airway and the airway wall (6, 8).

Wheezing in adults is associated with multiple clinical respiratory conditions such as asthma and chronic obstructive pulmonary disease (COPD) (6). These clinical conditions can induce airway hyperresponsiveness or bronchoconstriction and thereby causing respiratory sounds (9). A study on 826 newborns in Tuscon, Arizona suggests that wheezing is transient in most infants and that they have no increased risk of asthma. But in approximately one third of the infants, wheezing episodes indicate a predisposition for asthma (5, 10, 11).

Wheezing can be treated with corticosteroids and/or bronchodilators. Treatment of this phenotype in pre-school children with corticosteroids will ameliorate wheezing and control the exacerbations of symptoms (12). Bronchodilators will relax the smooth airway muscles and this way dilate the airways (13).

1.1.2. Rattling

Another phenotype of noisy breathing is rattling which is much lower in pitch (200Hz or less) and lacks musical quality (14). This phenotype is also characterized by vibrations over the baby's back which can be felt by the parents and the paediatrician (14). Rattling is caused by a mucus build-up in the airways. The sound is caused by mucus that moves with the normal respiration, but the underlying pathophysiology of rattling is unknown (15, 16).

Rattling can be treated with anticholinergic medication if necessary. This medication will work on the cholinergic nerves which are the main stimulus for mucin secretion (17, 18).

Rattling differs from wheezing in multiple aspects. The respiratory sound of rattling is much lower in pitch than that of wheezing. They have a different underlying pathophysiology and they have to be treated differently (16). Rattling often resolves by the age of five, but approximately half of these children were diagnosed as wheezing or having asthma (2).

1.2. Current diagnostic method

The diagnostic method is the same for the two noisy breathing phenotypes. The paediatrician will examine the child mainly by auscultation of the chest with a stethoscope. The medical history of the child is also an important diagnostic instrument, especially when the child is not experiencing noisy breathing at the time of consultation (7, 19). A few important factors that should be obtained by the paediatrician are: the timing and pattern of noisy breathing, the response to previous treatments, family history and personal history (19).

This method of diagnosing is very subjective and relies on the interpretation of the symptoms by the paediatrician and the parents (19). However, parents often misinterpret the symptoms of their child and will describe any respiratory sound as wheezing (15, 20, 21). Figure 1A describes the use of the term wheezing and ruttling by parents. This figure shows that after receiving additional information about the different respiratory sounds (a detailed questionnaire, a list of example words, an imitation or an instruction video), most of the parents changed the term they used for the respiratory sound of their child (15). Figure 1B shows the proportion of parents agreeing after the question: "Does the word "wheeze" mean the same as any of the following words?" Almost half of the parents that were questioned agreed that rattling is a synonym for wheezing (21). These studies showed the degree of inaccuracy when the terms wheezing and rattling are used in clinical practice.

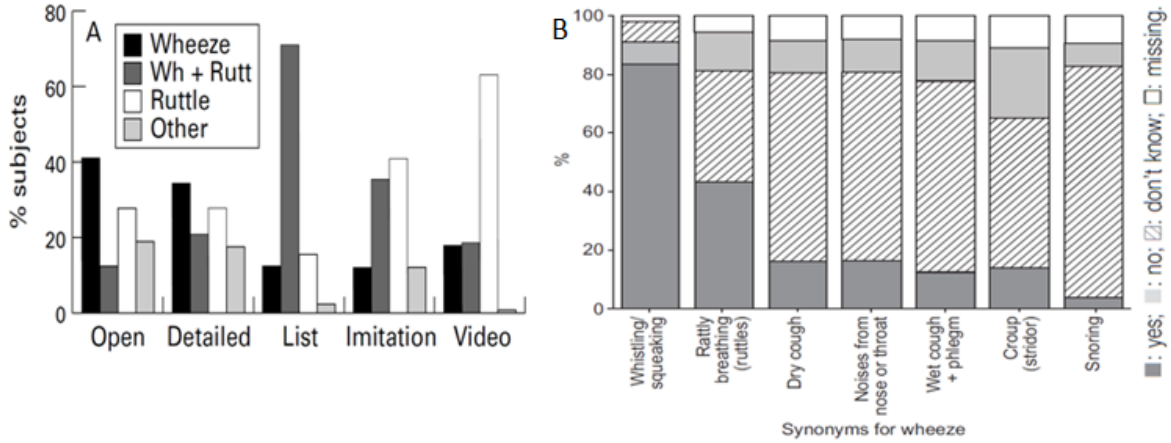

Figure 1 The degree of inaccuracy when using the terms wheezing and rattling in practice. A) Use of the word wheeze and ruttle by parents after different forms of education (a detailed questionnaire, a list of example words, an imitation or an instruction video). B) The proportion of parents agreeing that the following words are synonyms of wheezing (15, 21).

Not only parents can misinterpret the symptoms of their child, also the paediatrician can misdiagnose the noisy breathing child. Different studies suggest that auscultation of the chest has a poor interobserver reliability (22, 23). A study of Spiteri *et al.* (1988) showed that this leads to an incorrect diagnosis in 28% of the cases (24). Elphick *et al.* (2004) even states that the stethoscope is an unreliable method for assessing respiratory sounds in infants (25).

An incorrect interpretation of the symptoms by the parents can lead to an incorrect interpretation by the paediatrician which will have various implications on the diagnosis and treatment process. Wheezing is significantly over-reported by parents and paediatricians when a child has another form of noisy breathing (14). This can lead to overtreatment with corticosteroids which can lead to different side effects in young children such as growth restriction and a decrease in bone density (26, 27).

A more objective test exists for diagnosing wheezing or asthma in older children, from the age of three, or adults. This is the lung function test or spirometry. To perform this test voluntary breathing manoeuvres are needed which is not possible in infants and toddlers (28). There is a need for an objective non-invasive diagnostic method for noisy breathing in infants and toddlers (0-2 years).

1.3. Non-invasive objective methods

To obtain an objective diagnosis, biomarkers can be measured and linked to a certain condition or disease. Invasive techniques such as bronchoalveolar lavages and biopsies are risky in young children and are not well tolerated, so non-invasive techniques are in order (29).

1.3.1. Nasal mucus protein markers

Biomarkers can be measured non-invasively in nasal mucus. These nasal markers are mainly proteins involved in inflammatory processes since mucus production and secretion is controlled by these processes (30).

Nasal mucus is an easy and safe method to obtain information about the situation in the lungs and airways because of a strong functional and immunological relationship between the nose and bronchi (29, 31). Janssens *et al.* (2015) verified that nasal mucus is a good matrix to assess inflammation in the respiratory system in adults and that the sampling technique shows a low variability within multiple samples in the same individual (29). Examples of compounds that can be measured in nasal mucus are interleukin 1 (IL-1), interferon-gamma (IFN-γ) and tumour necrosis factor alpha (TNF-α) (29).

Other interesting compounds to measure, besides inflammatory proteins, are eicosanoids which are produced from arachidonic acid. These eicosanoids can be responsible for bronchoconstriction. Examples of eicosanoids are 8-isoprostane, leukotriene C4 (LTC4) and prostaglandin D2 (PGD2) (32, 33).

1.3.2. Sound analysis

Sound analysis is a non-invasive manner to obtain information about the breathing pattern of the child. Elphick *et al.* (2000) showed that breath sound characteristics during wheezing and rattling can be different (14).

These breath sounds have three characteristics: frequency, intensity and timbre. This way different sounds can be distinguished (1). The frequency is measured in Hz and will determine the pitch of the sound. The intensity is dependent on the energy of the sound and will determine the loudness. The timbre will differentiate sounds made up of the same pitch and loudness (1).

Based on these characteristics an acoustic analysis can be done on the different breath sounds. Previous studies revealed that the waveform signal of a breath sound also can differentiate between the phenotypes. The waveform of a wheezing baby is more sinusoidal. This is in contrast to the breathing pattern of a rattler, which is more variable and irregular (14). Elphick *et al.* (2000) also discovered that the dominant frequency of wheezing (400Hz or more) and rattling (200Hz or less) is different (14).

1.3.3. Volatile organic compounds

Biomarkers can also be measured in a non-invasive manner in exhaled breath samples. These samples can be easily and safely collected in pre-schoolers and the concentration of volatile organic compounds (VOC) can be measured (34). Costello *et al.* (2014) reported 872 different compounds that could be measured in human breath (35). Examples of the most common VOCs that can be measured in breath samples are ammonia, acetone, methanol, ethanol and isoprene (35, 36).

Volatile organic compounds can originate from exogenous sources or from endogenous sources (37). The endogenous VOCs are formed by different inflammatory and metabolic pathways in the body (38). They can diffuse to the alveoli in the lungs and are exhaled afterwards (39). Volatile organic compounds can also originate from local cellular metabolism or can be produced by bacteria (figure 2) (40).

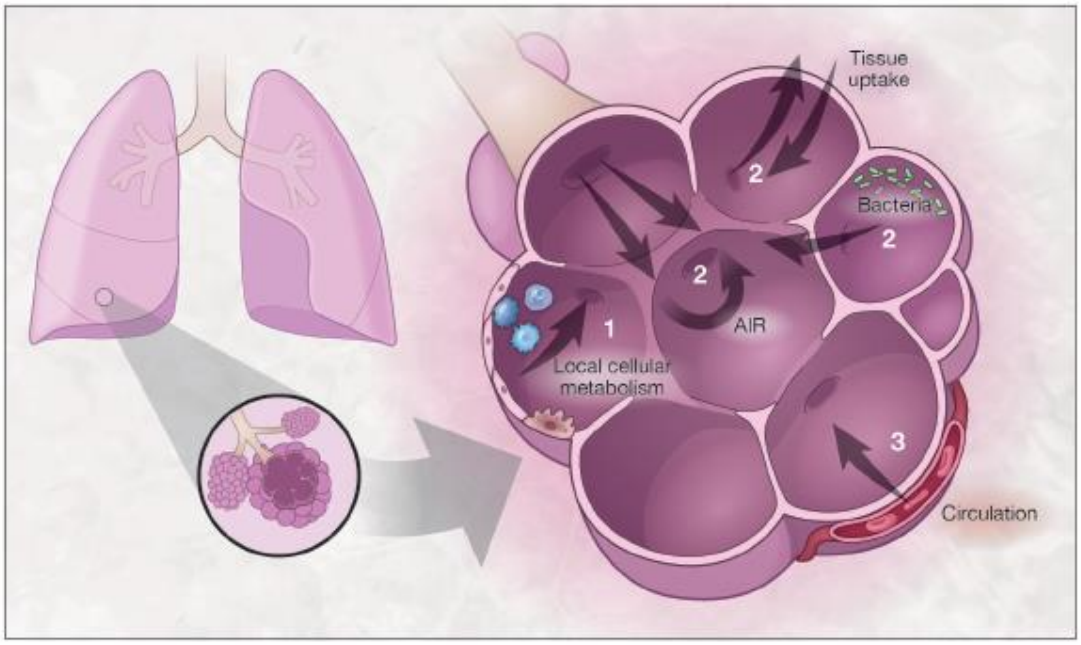

Figure 2 Different sources of exhaled VOCs (40).

Previous studies have reported that VOCs in exhaled breath samples are promising biomarkers for different lung disorders such as asthma, cystic fibrosis and COPD (41, 42). Van de Kant *et al.* (2013) even showed that these compounds can be used to differentiate between wheezers and non-wheezers in 1 to 4 year olds (34). Therefore, exhaled VOCs can be promising biomarkers which can give insights into lower airway inflammation and the phenotype-specific physiological processes present in wheezing and rattling.

Volatile organic compounds can be measured with a selected ion flow tube mass spectrometry (SIFT-MS). This analytical technique is a new method to measure VOCs in exhaled breath. The golden standard for measuring VOCs is the gas-chromatography mass spectrometer (GC-MS), but the SIFT-MS has the advantage that it can measure VOCs in exhaled breath in real time (43). When quantifying compounds in exhaled breath, the SIFT-MS is dependent on different factors. These factors will change under various circumstances, such as temperature, pressure and flow of the carrier gas.

Therefore, the SIFT-MS has to be validated with a standard gas when the aim is to quantify compounds correctly (44).

Before measuring VOCs, the exhaled breath has to be sampled. Different sampling methods exist for obtaining exhaled breath samples. Direct breathing into the analytical instrument is the best method to acquire these samples. However, this is not always possible. When this is the case the exhaled breath samples can be stored into containers. There are different types of containers available for breath sampling such as canisters, adsorbing agents (cartridges) and Teflon or Tedlar bags (45). Two common methods of exhaled breath sampling in research are fluorinated ethylene propylene copolymer (FEP), or Teflon, sampling bags and the Respiration Collector for *In Vitro* Analysis (ReCIVA®) breath sampler combined with cartridges.

Sampling exhaled breath with Teflon sampling bags is relatively easy. These bags are mostly used for research purposes because they do not have the tendency to emit organic compounds and contaminate the samples (46). However, small VOCs can diffuse over the wall of these bags from the outside in causing contamination of the samples or from the inside out causing loss of VOCs. Koziel *et al.* (2005) reported 60.6% loss of VOCs after 24 hours (45).

When exhaled breath samples have to be stored over a longer period or have to be transported, it is best to use cartridges. These are tubes filled with a sorbent which will trap the VOCs on the cartridges. Then, by means of thermal desorption, the VOCs will be released from the sorbent material. Using cartridges is not labour intensive and one cartridge can be used approximately a hundred times which makes this technique very cost-effective. Van der Schee et al. (2012) stated that adsorption, desorption and transportation of VOCs in sorbent tubes does not affect the stability over time for single-molecule compounds over a period of two weeks (47).

The disadvantage of this sampling technique is that it will not trap every VOC in exhaled breath and the type of VOCs that will be trapped is dependent on the type of cartridge. When these tubes are desorbed they will also not release everything. This will cause a gap between the VOCs that can be sampled and the VOCs that can be measured. The composition of the measured samples could be changed due to the selective adsorption onto the sorbent (47). A great advantage of using cartridges is that there can be a preconcentration step. This gives as advantage that low concentrations in exhaled breath can be measured.

1.4. Hypothesis and objective

The current project is part of a bigger study which aims to improve and objectify the current diagnosis of wheezing and rattling. Therefore the hypothesis is that a potentially interesting biomarker profile can be found by analysing nasal mucus and/or exhaled breath samples and/or analysing sound samples and that this biomarker profile can differentiate between wheezing and rattling in young children (0-2 years). To achieve this goal the preparations for a pilot study were started in the current project.

The first step is the optimisation and validation of the SIFT-MS technology for different interesting compound classes. We hypothesize that the SIFT-MS will work correctly and that the different compounds will be quantified correctly. Second, there will be a small setup in which we will determine if the sampling method with the sorbent tubes is the most appropriate for the pilot study. In this setup we will measure the differences between sorbent tubes and Teflon sampling bags. Here, we hypothesize that the VOCs will be measured correctly in the Teflon bags, but with the sorbent tubes and a preconcentration step, the concentrations of VOCs will be higher after thermal desorption. The ReCIVA® breath sampler had to be adjusted for infants and toddlers. Therefore, the flow of the clean air supply pump (CASPER®) had to be changed from 40 Litres per minute (L/min) to ten L/min. Our hypothesis was that the air scrubber of the CASPER® clean air supply still works at ten L/min.

The next step was an extensive literature search to determine which nasal mucus markers could be potential biomarkers to differentiate between wheezing and rattling.

2. Material and methods

2.1. Selected Ion Flow Tube Mass Spectrometry

All the gas measurements were done with the SIFT-MS Voice 200 (Syft technologies, Middleton, New Zealand). More information about the different settings of the SIFT-MS is given in supplement 1.

2.1.1. Clean Air Supply Pump

In our first setup the CASPER® (Owlstone medical, UK) was connected to the SIFT-MS. This CASPER® clean air supply scrubs the ambient air in the room and provides clean, filtered from external VOCs, air to the user. First the ambient air was measured and next the scrubbed clean air to determine if the CASPER clean air supply worked.

These measurements were done in full scan mode for mass/charge (m/z) ratios between 15+ and 200+, with 10 repeats, 100 milliseconds (ms) dwell time, count limit 10,000 and 60 seconds scan time. Each condition was measured 10 times.

Next different flows of the CASPER clean air supply were tested. The standard pump of the CASPER® was replaced with another Bravo H2 pump (Tecora, France) to control the flow that was sent to the CASPER®. The same experiment was repeated for five measurements, but with the flow set at 10 and 15 litres per minute (L/min) instead of the normal 40 L/min.

2.1.2. Dwell time measurements

Dwell time is the time that is set to measure one m/z ratio. This factor will determine the sensitivity of the technique. A higher dwell time will give a higher sensitivity because the SIFT-MS will measure the m/z ratio over a longer period.

The settings of the different dwell times were applied to the measurements of the ambient air. The settings are given in table 2.

Table 2 Settings Selected ion flow tube mass spectrometry.

Dwell time	10 ms	100 ms	400 ms
Type scan	Full scan	Full scan	Full scan
Mass/charge range	15 – 200+	15 – 200+	15 – 200+
Repeats	10	10	10
Inlet	Ambient	Ambient	Ambient
Measurement time	± 2 minutes	± 10 minutes	± 40 minutes

After these measurements the results of the different dwell times were compared for each m/z ratio to determine which dwell time could be used for further experiments.

2.1.3. Selection interesting volatile organic compounds

Different interesting VOCs were selected to determine the different performance characteristics of the SIFT-MS. These VOCs were selected based on literature search and based on the SIFT-MS method. A literature search was done to link different VOCs to asthma, mucus, oxidative stress, inflammation, lung cancer and pathogens. After this the VOCs were selected based on the different chemical classes and on whether these could be measured with the existing SIFT library. The list of selected VOCs is given in table 3. The expected concentrations in breath of these VOCs is presented in table 12 in supplement 2.

Table 3 Selection of interesting VOCs with their chemical class and reference.

Class	Compounds	Reference
Alcohol	1-Hexanol	Qader *et al.* (2015)(48)
Aldehyde	pentanal	Fuch *et al.* (2010)(49)
	Decanal	Rufo *et al.* (2016)(42)
Alkane	Pentane	Rufo *et al.* (2016)(42)
	Dodecane	Rufo *et al.* (2016)(42)
Hydrocarbon	p-xylene	Rufo *et al.* (2016)(42)
Carboxylic acid	Acetic acid	Rufo *et al.* (2016)(42)
	Valeric acid	Rufo *et al.* (2016)(42)
Fatty acid	Hexanoic acid	Syft library
Ketone	Acetophenone	Rufo *et al.* (2016)(42)
Alkene	1-decene	Syft library
	Isoprene	Rufo *et al.* (2016)(42)
Thioether	Dimethyl disulphide	Syft library

The different gasses were collected in Teflon bags using the available Vito facility. For the validation of an instrument, well controlled test gasses are preferred. First stock solutions were made with the selected compounds. These solutions were produces with a balance and with glass Pasteur pipettes. From each stock solution the dilutions were made. The dilutions that were aimed for were 100, 75,

50, 25 and 1 parts per billion (ppb). These dilutions were also made with a balance and with glass Pasteur pipettes.

After these dilutions were made, two µL of the dilutions were injected into separate ten litres Teflon sampling bags. The bags were filled with nitrogen gas until 4.5 litres. Then the two µL of the dilution was injected and then the Teflon bags were filled with nitrogen gas until nine litres. In order to evaporate the dilution with methanol, the bags were placed in an oven at 70° Celsius for approximately five minutes. Then the bags were measured with the SIFT-MS.

2.1.4. Validation parameters

In order to quantify the selected compounds correctly, the branching ratios and k-values were adapted. The branching ratios were adapted based on the measured counts per second and the k-values were determined based on the concentrations measured with adapted branching ratios.

Further calculations were done with these adapted values. Different parameters were determined to validate the SIFT-MS. These include: the trueness, the repeatability, the reproducibility, the limit of detection, the limit of quantification, the linearity and the measurement uncertainty. These parameters were selected from the Flemish compendium for sampling and analysis (CMA/6/A) and is in line with the international standardisation requirements (ISO 17025). The validation parameters were checked for the concentrations expected after a preconcentration step. This means that the expected concentrations in breath (table 12, supplement 2) were multiplied by ten.

To validate the sample production method, these validation parameters were also checked for isoprene with a standard gas and the calibration unit CGM 2000 (Umwelttechnik MCZ GmbH, Bad Nauheim, Germany). Five concentrations (1, 25, 50, 75 and 100 ppb) were measured at five different days.

Next, for each of the selected compounds in the Teflon bags, the five concentrations (1, 25, 50, 75 and 100 ppb) were also measured at five different days. The different measurements for the different parameters are given in table 4.

Each concentration level was measured with a selected ion method (SIM) scan, a dwell time of 100 ms, a settle time of 3 seconds, a count limit of 10,000 counts and a scan time of 60 seconds. An example of the SIM scan is given in figure 6 in supplement 1. Each concentration level was measured seven times. After the measurements the Teflon bags were flushed with nitrogen gas two times.

Table 4 Validation parameters (50).

Parameter	Experiment
Trueness	Based on five analyses under conditions of intra reproducibility at one concentration.
Repeatability	Five analyses on two concentration levels separated at least a factor 10.
Reproducibility	Five analyses of a reference concentration at different times. The instrument is not restarted in this experiment, but revalidated.
Limit of detection and limit of quantification	Multiple analyses at a low concentration level.
Linearity	4 different concentration levels.

2.1.5. Sorbent tubes

When the validation parameters were determined, the sampling method with the cartridges was evaluated.

P-xylene was pumped over the separate Tenax/ carbograph 4 cartridges (Markes international, United Kingdom) with the Gilian LFS-113DC pump (Sensidyne, U.S.A.).

Different options were explored. First the cartridges were spiked with two µL of the different concentrations of p-xylene. Next ten litre Teflon bags of the different concentrations were pumped at a flow of approximately 200ml/min over separate cartridges, the cartridges were then desorbed into one litre Tedlar bags. Next ten litre bags of the different concentrations were pumped over the separate cartridges and desorbed into three litre Teflon bags.

The cartridges were desorbed with the Unity thermal desorber (Markes international, United Kingdom).

2.2. Statistics

The results were analysed with the statistical program R (version 3.3.2). The significance level was set at 5% and the statistics were applied to the mean of different scans. The assumptions of normality and equal variance were tested for all the data. If the assumptions of normality and equal variances were met, a 2-sample t-test statistic was performed to compare the results of the ambient air and the scrubbed air. Otherwise the results were compared with a Wilcoxon rank sum test with continuity correction.

The standard deviations of the dwell times were compared with a Levene's test.

2.3. Literature search mucus markers

Next, a literature search was started to investigate possible interesting nasal mucus markers. Rattling is caused by an overproduction of mucus, so the literature search started with a look into the mucus production and secretion pathways. For each marker in these pathways was checked if they were linked to asthma or other lung diseases, such as cystic fibrosis. If so, it was checked if they were already measured in nasal mucus or nasal lavage. This literature search was conducted at PubMed, Google Scholar and Web of Science.

Next the eicosanoid pathway was checked, because eicosanoids are linked to bronchoconstriction which is a cause of wheezing. For these proteins was also checked if they were already measured in nasal mucus. Different cytokines were also checked, because both wheezing and rattling are linked to inflammation.

After mucus markers were found, they were checked if they could be measured with a multiplex enzyme linked immune sorbent assay (ELISA) (mesoscale discovery, Maryland). This way potential nasal mucus markers were selected.

3. Results

3.1. Selected Ion Flow Tube Mass Spectrometry

3.1.1. Clean Air Supply Pump

To determine if the CASPER® was fully functional, the ambient air from the lab was compared to the scrubbed air from the CASPER®. This comparison was done for each precursor ion. First the number of m/z values that were relevant were counted. This means that the m/z values from the precursor ions were left out and that m/z values with average counts lower than 20 for both clean and ambient air were also left out. The results of this comparison are presented in table 5.

Table 5 Percentages of the number of compounds the CASPER clean air supply was able to scrub from the ambient air. This analysis was done for each precursor ion.

Precursor ion	Total m/z values	Total significant differences	m/z values scrubbed by CASPER	m/z values not scrubbed by CASPER	Total not significant different
H_3O^+	109	87	81	6	22
	100%	79.82%	74.31%	5.50%	20.18%
NO^+	123	97	96	1	26
	100%	78.86%	78.05%	0.81%	21.14%
O_2^+	125	106	105	1	19
	100%	84.80%	84.00%	0.80%	15.20%

The total of m/z values used in the analysis for H_3O^+, NO^+ and O_2^+ is 109, 123 and 125, respectively. The amount of significant differences are higher than the amount of not significant differences for all three precursor ions. From these significant differences, most m/z values are scrubbed by the CASPER® clean air supply from the ambient lab air.

To test if the CASPER® still scrubs the air efficiently at lower flow volumes a comparison was made between flows of 10 L/min, 15 L/min, the original 40L/min and the ambient air. The results of this comparison are presented in table 6. The least significant differences are between the CASPER® at a flow of ten litres per minute and the ambient air with an average of the three precursor ions of 42.73%. The most significant differences are between the CASPER® at 15 L/min and the ambient air, except for NO^+. The average percentage of m/z scrubbed by the 15 L/min is 55.26%.

Table 6 Comparison of the different flows of the CASPER® clean air supply and the ambient air for the different ions. The different columns present the number of m/z values that were scrubbed by the CASPER® clean air supply at a flow of 10, 15 and 40 litres per minute (L/min).

Ion	Totaal m/z values	Significant differences 10L/min	m/z values scrubbed by CAPSER at 10L/min	Significant differences 15L/min	m/z values scrubbed by CAPSER at 15L/min	Significant differences 40L/min	m/z values scrubbed by CAPSER at 40L/min
H_3O^+	121	52	49	73	71	65	60
	100%	42.98%	40.50%	60.33%	58.68%	53.72%	49.59%
NO^+	139	50	49	69	69	75	75
	100%	35.97%	35.25%	49.64%	49.64%	53.96%	53.96%
O_2^+	146	76	75	93	93	89	89
	100%	53.15%	52.45%	65.03%	65.03%	62.24%	62.24%

3.1.2. Dwell time

In order to determine which settings to use for further experiments, the standard deviations of three different dwell times, 10 ms, 100 ms and 400 ms were compared. The results of this comparison are presented in table 7. The comparison was done for the different precursor ions. There are more significant differences between 10 and 100 ms and 10 and 400 ms than between 100 and 400 ms.

Table 7 Percentages of the number of significant differences between the standard deviation of the different dwell times.

Precursor ion	Total m/z values	Number of 10 vs 100 differences	Number of 10 vs 400 differences	Number of 100 vs 400 differences
H_3O^+	126	74	72	24
	100%	58.73%	57.14%	19.05%
NO^+	140	96	93	27
	100%	68.57%	66.43%	19.29%
O_2^+	153	112	105	17
	100%	73.20%	68.63%	11.11%

3.2. Validation parameters

To determine the quality of the SIFT-MS measurements, different validation parameters were determined. The adapted branching ratios and K-values are presented in table 16 supplement 3.

3.3. Comparing standard gas to Teflon bag measurements

To evaluate the method of gas production, the measurements with the standard gas and the Teflon bags were compared. This comparison of the measurements over the different days is presented in figure 3. The measured concentration in ppb is plotted against the expected concentration in ppb. The different measurement days are plotted in the different colours. The measured concentration decreases stepwise when the expected concentration decreases. There is more variation between

the different days with the measurements done with the Teflon bags, except for the range between 1 and 50 ppb.

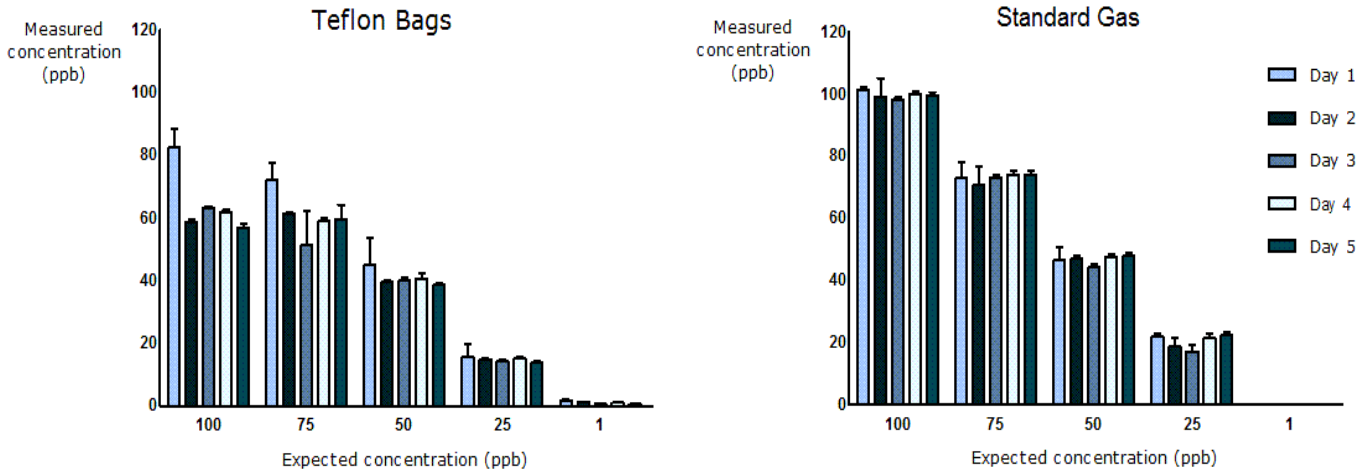

Figure 3 Comparison between the method with Teflon bags and with the standard gas. The measured concentration in ppb is plotted against the expected concentration in ppb for the different days.

The different validation parameters for isoprene in the Teflon bags and in the standard gas are presented in table 8.

Table 8 Validation parameters for isoprene with the Teflon bags and in the standard gas.

Method	relative bias	Trueness	Relative Repeatability	Relative intra-reproducibility	Limit of detection (ppb)	Limit of quantification (ppb)	Measurement uncertainty
Teflon bag	-34.62%	65.38%	4.23%	18.82%	0.16	0.32	72.25%
Standard gas	0.21%	100.21%	0.84%	0.71%	0.31	0.63	1.62%

The relative bias of the standard gas is 0.21% and the trueness is 100.21%. The relative bias of isoprene from the Teflon bags is -34.62% and the trueness is 65.38%. The absolute bias from the Teflon bags is higher than from the standard gas.

The repeatability from the standard gas and from the Teflon bags is 0.84 ppb and 4.23 ppb, respectively. The relative repeatability is 0.84% and 4.23%, respectively. The repeatability of the Teflon bags is higher than with the standard gas.

The reproducibility is 0.70 ppb and 12.36 ppb for the standard gas and the Teflon bags, respectively. The relative reproducibility for the standard gas is 0.71% and for the Teflon bags 18.82%. The reproducibility for the Teflon bags is higher than for the standard gas.

The limit of detection is 0.31 ppb and 0.16 ppb and the limit of quantification is 0.62 ppb and 0.32 ppb for the measurements with the standard gas and with the Teflon bags, respectively.

The measurement uncertainty with the standard gas is 1.62%. The measurement uncertainty with the Teflon bags is higher at 72.25%.

The linearity is presented in table 9. The linearity is presented in the form of relative residuals at a certain concentration level. The absolute residuals are all smaller than 2% for the standard gas. The residuals of the measurements with the Teflon bag are between -6% and 6%. The residuals of the measurements with the Teflon bags are higher than with the standard gas. R square of the standard gas was 0.9992, which is very good. The R square with the Teflon bags was 0.9789, which is still acceptable.

Table 9 Linearity of a standard gas isoprene. Presented as the slope, intercept, R-square and residuals of the linear regression line for the concentrations measured and the concentrations generated.

Compound	Standard gas		Teflon bags	
slope	1.02		0.91	
intercept	-1.82		0.15	
R^2	0.9992		0.9789	
Residual | Concentration level (ppb)	1%	100	-5%	100.47
	0%	75	6%	75.59
	-1%	50	4%	49.57
	-1%	25	-6%	25.92
	1%	1	1%	0.96

3.3.1. Selected volatile organic compounds

A summary of the validation parameters of the selected volatile organic compounds is presented in table 10. More detailed results of the validation parameters is presented in tables 17 to 20 in supplement 4.

Table 10 Summary of the validation parameters of selected volatile organic compounds.

Compound	Bias	Trueness	Relative repeatability	Relative reproducibility	Limit of detection (ppb)	Limit of quantification (ppb)	Measurement uncertainty
Hexanol	1.14%	101.14%	4.13%	10.44%	1.06	2.13	22.02%
Pentanal	2.71%	102.71%	5.67%	16.45%	4.21	8.41	35.61%
Decanal	0.63%	100.63%	17.14%	11.55%	1.44	2.87	23.73%
Pentane	-0.89%	99.11%	3.56%	5.70%	20.49	40.99	12.28%
Dodecane	1.90%	101.90%	7.41%	9.11%	1.37	2.74	20.11%
P-xylene	-5.73%	94.27%	0.89%	5.77%	1.03	2.07	17.28%
Acetic acid	-12.74%	87.26%	1.57%	12.22%	1.98	3.96	37.18%
Valeric acid	7.73%	107.73%	8.95%	21.48%	4.26	8.52	50.70%
Hexanoic acid	-20.22%	79.78%	4.94%	6.47%	5.10	10.21	33.16%
Acetophenone	-5.62%	94.38%	2.03%	12.89%	1.97	3.94	31.39%
1-decene	8.97%	108.97%	10.80%	14.91%	2.71	5.41	38.78%
Isoprene	-34.62%	65.38%	4.23%	18.82%	0.16	0.32	72.25%
Dimethyl disulfide	-2.24%	97.76%	3.10%	6.45%	0.67	1.35	15.14%

To determine the trueness the difference between the measured and the reference concentration was calculated. This is called the bias. The average absolute bias is 8.09% (± 9.37%). The average trueness is 95.46% (± 11.52%) at the expected concentrations in breath after a preconcentration step with the cartridges. The highest absolute bias is from isoprene (-34.62%) and the lowest bias is from decanal (0.63%).

The average repeatability and relative repeatability is 2.74 ppb (± 1.33 ppb) and 5.72% (± 4.30%), respectively. The highest and lowest repeatability is 4.94 ppb for hexanoic acid and 0.89 ppb for p-xylene. The maximum relative repeatability is 17.14% for decanal. The lowest relative repeatability is 0.89% for p-xylene.

The average reproducibility and relative reproducibility is 6.55 ppb (± 3.59 ppb) and 11.71% (± 4.92%), respectively. For every compound the reproducibility is slightly higher than the repeatability, except for decanal. The highest and lowest reproducibility is 12.74 ppb for acetophenone and 2.99 ppb for decanal, respectively. The maximum relative reproducibility is 21.48% for valeric acid. The lowest relative reproducibility is 5.70% for pentane.

The limit of detection and limit of quantification is based on the standard deviation of 30 measurements of zero gas. The average limit of detection and limit of quantification is 3.56 ppb (± 5.10 ppb) and 7.12 ppb (10.21 ppb), respectively. The lowest limit of detection and quantification is 0.16 and 0.32 ppb for isoprene. The highest limit of detection and quantification is 20.49 ppb and 40.99 ppb for pentane.

From the bias and relative reproducibility, the measurement uncertainty was calculated. The average measurement uncertainty is 31.51% (± 15.82%). The lowest absolute measurement uncertainty is 12.28% of pentane and the highest is 72.25% of isoprene.

The linearity of the selected volatile organic compounds is presented in table 11. The linearity is presented in the form of relative residuals at a certain concentration level. The lowest absolute residuals are from dodecane, dimethyl disulphide and acetic acid. The highest absolute residuals are form Pentane. R square of the equations varies from 0.7817 of pentane to 0.9962 of 1-decene.

Table 11 Linearity of the selected volatiles. Presented as the slope, intercept, R-square and residuals of the linear regression line for the concentrations measured and the concentrations generated. This is based on the different concentration levels.

Compound	Hexanol		Pentanal		Decanal		Pentane		Dodecane		P-xylene	
Slope	0.86		0.85		0.92		0.57		0.89		0.90	
Intercept	6.93		3.91		3.08		44.26		3.48		5.26	
R²	0.9923		0.9912		0.9767		0.7817		0.9872		0.9953	
Residual | Concentration level (ppb)	-3%	98.67	1%	98.67	4%	101.33	1%	102.82	0%	100.39	-3%	100.85
	4%	74.73	-4%	74.73	-8%	79.33	-14%	78.59	1%	75.91	2%	75.88
	-1%	49.22	3%	49.22	4%	48.73	15%	51.23	1%	50.55	2%	49.50
	2%	24.91	2%	24.91	-2%	25.79	6%	24.56	-2%	25.41	-1%	26.38
	-2%	0.96	-2%	0.96	1%	1.02	-9%	0.97	1%	1.08	-1%	1.20

Table 11 (continued) Linearity of selected volatiles.

Compound	Dimethyl disulfide		Acetic acid		Valeric acid		Hexanoic acid		Acetophenone		1-decene		Isoprene	
Slope	0.99		0.74		0.819		0.62		0.96		0.88		0.91	
Intercept	3.40		18.58		10.710		24.05		3.18		5.29		0.15	
R²	0.9913		0.9934		0.9907		0.9542		0.9938		0.9962		0.9789	
Residual | Concentration level (ppb)	1%	101.79	-1%	101.38	-1%	99.00	-3%	103.11	1%	104.77	-1%	98.09	-5%	100.47
	-2%	74.57	1%	74.56	2%	74.34	6%	75.48	-2%	73.85	-1%	76.01	6%	75.59
	2%	51.36	1%	50.84	0%	48.66	-2%	48.75	-1%	50.22	3%	52.03	4%	49.57
	0%	27.45	1%	24.12	-4%	23.14	-2%	24.65	3%	25.97	-1%	25.45	6%	25.92
	0%	1.19	-2%	1	2%	0.88	1%	1.05	-1%	1.00	-1%	1.07	1%	0.96

3.4. Sorbent tubes

3.4.1. Spiked cartridges

The measured concentration after thermal desorption with the cartridges is presented in table 12. First the expected concentration levels based on the dilutions are given. Then the measured concentrations in the Teflon bags without preconcentration are presented. These are within the expected range. Next the concentrations that were measured with the spiked cartridges after desorption into a one litre Tedlar bags are presented. These concentration are higher than the ones measured directly in the Teflon bags. The concentration factor of the spiked cartridges is given next and ranges from 2.29 to 5.28.

Table 12 Concentrations of the different measurements with the spiked sorbent tubes. Cexpected: the expected concentration based on the dilutions, Cteflon: the concentrations measured in the Teflon bags, Cspiked: the concentrations measured after the spiked cartridges were desorbed into one litre Tedlar bags.

$C_{Expected}$	C_{Teflon}	C_{Spiked}	Concentration factor
100.85	91.64	209.61	2.29
75.88	74.87	245.86	3.28
49.50	51.64	254.29	4.92
26.38	30.18	159.25	5.28
1.20	6.91	27.60	3.99

3.4.2. Nine litre Teflon bags over cartridges

Table 13 presents the concentrations measured after the cartridges were desorbed into one and three litre bags. First the expected concentration and the measured concentration into Teflon bags is given. Then the measured concentration is presented after thermal desorption into one litre Tedlar bags. These concentrations are higher than the concentrations measured directly with the Teflon bags. The concentration factor is stable and the average is 2.6. Next the measured concentrations after thermal desorption into three litre Teflon bags are presented. These are lower than the measured concentrations in the Teflon bags, the concentration factor is lower than one.

Table 13 Concentrations of the different measurements with the sorbent tubes. Cexpected: the expected concentrations based on the dilutions, Cteflon: the concentrations measured in the Teflon bags, C1l: The concentrations measured in one litre Tedlar bags, C3l: concentrations measured in 3L Teflon bags.

$C_{Expected}$	C_{Teflon}	C_{1L}	Concentration factor	C_{3L}	Concentration factor
100.85	91.64	246.03	2.68	74.65	0.81
75.88	74.87	181.29	2.42	63.79	0.85
49.50	51.64	113.92	2.21	48.73	0.94
26.38	30.18	70.34	2.33	26.60	0.88
1.20	6.91	24.41	3.53	8.77	1.27

3.5. Nasal mucus markers

The list of selected nasal mucus markers, together with their pathways and functions, is presented in table 14. Proteins were selected from three pathways: mediators from the mucus production pathway, eicosanoids from the bronchoconstriction pathway and cytokines from the inflammation pathway. In total 18 proteins were selected, six from the mucus production pathway, six from the eicosanoid pathway and six cytokines.

An example of a marker that was selected from the mucus pathway is mucin 5ac (Muc5ac), which could be linked to rattling because it is the main product of the mucus production pathway in airways. Muc5ac is also more produced in case of respiratory disorders such as cystic fibrosis (30). Eicosanoids are selected because leukotrienes and prostaglandins can cause bronchoconstriction, which is linked to wheezing (51). The inflammatory process is linked to both rattling and wheezing, especially the T-helper two pathway. Interleukin 33 is an example of a cytokine that activates this pathway (52).

Table 14 List of prospective nasal mucus markers with the linked pathways and function. M = mucus pathway, C = cytokines, E = eicosanoids pathway.

Protein	Pathway	Function	Reference
Mucin 5ac	M	Product of the mucus production pathway in the airways.	Williams *et al.* (2006) (30)
Interleukin 13	M/C	Pro- and anti-inflammatory cytokine and dominant activator of mucus production.	Williams *et al.* (2006) (30)
Interleukin 17a	M/C	Proinflammatory cytokine and activator of mucus production. Linked with asthma and COPD.	Williams *et al.* (2006) (30)
Interleukin 1b	M/C	Potent proinflammatory cytokine and activator of mucus production and prostaglandin synthesis.	Williams *et al.* (2006) (30)
Forkhead box protein A2	M	Dominant Inhibitor of the mucus production pathway.	Williams *et al.* (2006) (30)
Myristoylated alanine-rich C-kinase substrate	M	Key signalling protein for mucus secretion. Has a high expression in nasal respiratory epithelium.	Williams *et al.* (2006) (30)
8-isoprostane	E	Marker of oxidative stress resulting from airway inflammation.	Funk *et al.* (2001) (33)
Leukotriene C4	E	Linked to increased mucus secretion, severe bronchospasm and increased eosinophil recruitment.	Sanak *et al.* (2016) (51)

Table 14 (continued)

Leukotriene D4	E	Linked to increased mucus secretion, severe bronchospasm and increased eosinophil recruitment.	Sanak *et al.* (2016) (51)
Leukotriene E4	E	Linked to increased mucus secretion, severe bronchospasm and increased eosinophil recruitment.	Sanak *et al.* (2016) (51)
Prostaglandine D2	E	Pro- and anti-inflammatory and linked to bronchoconstriction and eosinophil infiltration.	Funk *et al.* (2001) (33)
Lipoxine A4	E	Pro-resolving eicosanoid, terminates inflammatory reactions.	Sanak *et al.* (2016) (51)
Interleukin 18	C	Proinflammatory cytokine and linked with wheezing.	Malmström *et al.* (2014) (52)
Interleukin 23	C	Stimulate production of IFN-gamma (via STAT4) and linked with wheezing.	Malmström *et al.* (2014) (52)
Interleukin 33	C	Involved in maturation of Th2 cells and activation of mast cells, basophils, eosinophils and natural killer cells.	Malmström *et al.* (2014) (52)
Interleukin 8	C	Major mediator of inflammatory response.	Dougall *et al.* (2015) (53)
Interleukin 6	C	Maturation of B cells.	Dougall *et al.* (2015) (53)
Granulocyte-colony stimulating factor	C	Controls the production, differentiation and function of granulocytes.	Dougall *et al.* (2015) (53)

After this selection was made, there was checked if the selected markers could be measured with the multiplex ELISA from Mesoscale Discovery (MSD). The markers that could be measured in one assay are: IL-13, IL-17a, IL-1b, IL-8, IL-6 and granulocyte-colony stimulating factor (G-CSF). For the other markers, a new assay has to be developed.

4. Discussion

4.1. Clean Air Supply Pump

The function of the CASPER® clean air supply is to scrub the volatiles from the ambient air and deliver clean air without exogenous VOCs to the user. In order for this scrubber to work efficiently the concentrations and amount of VOCs after being scrubbed have to be lower than in the ambient air. For most of the m/z values there was a significant difference between the CASPER® data and ambient air data. From the significant differences, approximately 78% of the m/z values were scrubbed by the CASPER® clean air supply. From these data we can conclude that the CASPER® clean air supply works efficiently. The differences of the m/z values that were not significant or were higher in the CASPER® data than in the ambient air could be explained by the contamination that can happen when long tubing is used. This is the case with the CASPER® clean air supply. Volatile organic compounds can come off the tubing or VOCs from the outside can diffuse into the tubing. Another source of contamination could be the material of the scrubber. The scrubber of the CASPER® is made of airpel 10® (Desotec, Belgium), which is made of active carbon. This material is often used in clean air experiments. In the case of our experiments the number of m/z that were not significant or that were lower in the ambient air are very low and negligible. The CASPER® clean air supply can be used 900 times or one year. This means that it would be appropriate to repeat this test on different time points to determine if the scrubber still works.

The CASPER® is normally coupled to the ReCIVA® breath sampler which was adjusted in our study for infants and toddler (2 – 24 months). Therefore, the normal flow of the scrubber, 40 litres per minute suitable for adults, has to be lowered to ten litres per minute (54). To test if the scrubber still worked efficiently, the experiments were repeated with flow of ten and 15 litres per minute. The amount of m/z values that were scrubbed by the CASPER® were lowest with the flow of ten litres per minute. The highest number of m/z values that were scrubbed was with the flow of fifteen litres per minute. The number of m/z scrubbed were comparable between 15 and 40 L/min. This means that the CASPER® clean air supply works efficiently at a lower flow of fifteen litres per minute, but not at a flow of ten litres per minute.

These conclusions are based on only five measurements which means that more measurements are needed to fully conclude that the CASPER® does not function well at ten litres per minute.

4.2. Dwell time

To determine which settings have to be used during the next experiments, the different dwell times, 10, 100 and 400 ms, were compared. The dwell time determines the sensitivity of the technique. The standard deviations of the different dwell times were compared. The hypothesis was that if the dwell time is higher, the sensitivity and specificity will be higher. This means that the measurements with dwell time 100 and 400 ms will be more correct and thus there would be less significant differences between the standard deviations. This hypothesis was confirmed by the results. The least significant differences were between 100 and 400 ms. The raw data (unpublished) also showed that the standard deviation of 400 ms were lower than those of 100 ms and those of 10 ms. Since there are less significant differences between 100 and 400 ms, the next experiments will be done with 100 ms because this setting is comparable with the 400 ms setting and the measuring time decrease significantly from 40 minutes to ten minutes.

4.3. Validation parameters

In order to validate the SIFT-MS a valid test gas had to be produced. To determine if the variability of the technique was caused by the production method of the gas, a comparison was done between Teflon bags and a standard gas of isoprene. The comparison between the Teflon bags and the standard gas production method showed that the measured concentration decreased when the expected concentration decreased. It also showed that the measurements with the standard gas were more stable over the different days. The variability in the Teflon bags technique was also noticed in the validation parameters that were compared. The measurement uncertainty with the Teflon bag was higher than with the standard gas. The measurement uncertainty of the method with the Teflon was higher than the accepted value of 50%. This parameter is an important value to indicate the quality of the measurements. According to ISO 17025 It is also important to explain the variability in measurements which influences the measurement uncertainty.
The higher measurement uncertainty was caused by the higher reproducibility which was almost 20 times higher with the Teflon bags. A higher reproducibility was expected for the Teflon bags because this technique dependents on different factors, such as pipetting errors when making the dilutions, homogenization in the Teflon bags, diffusion over the walls of the bags and the amount of nitrogen gas in the bags. Koziel *et al.* (2005) also reported the variable volume in the bags as a possible additional source of variability (45). Diffusion of the compounds over the walls of the bags is unlikely because Teflon bags are used because they are inert and have a good durability and reusability (55). Another source of variability could be the syringe that was used to inject the dilutions into the Teflon bags. For this injection two µL was used, which means that the margin for error is not big.
The further measurements and calculations of the validation parameters were done with the Teflon bags. So, this variability has to be taken into account when interpreting the results.

The most important factor in the validation process is the measurement uncertainty and how this measurement uncertainty is explained. The maximum accepted measurement uncertainty was 50%. This guideline was met by all the measured compounds except for Valeric acid and isoprene. For these compounds the trueness and the relative repeatability were within the accepted range of ten percent. The relative reproducibility was slightly higher than the accepted value of 15%. This means that the SIFT-MS measures well under the same circumstances, but that the variability is higher when the factor time is changed. This can be caused by the variability in the production method of the gas. Another cause can be the temperature in the SIFT-MS. One of the five days, during the scan time of Valeric acid, the internal temperature of the device was too high and the SIFT-MS shut down. After cleaning the air filter, the temperature was back to normal. When doing a sensitivity analysis and leaving this day out in the calculation of the reproducibility and measurement uncertainty, the measurement uncertainty decreased to 46%. This is under the accepted limit. Thus, this increased temperature could be the cause of the variability between the measurements of Valeric acid.

Another remarkable compound according to the validation parameters was pentane. This compound had a very high limit of detection of 20.49 ppb and a limit of quantification of 40.99 ppb. The other parameters were within the predetermined accepted range, mentioned above. The measurement uncertainty was also good. The cause of this high limit of quantification could be because the

branching ratios and the K-values were adjusted for the expected values in breath after thermal desorption of the compounds. When adjusting these values for lower concentrations, the detection limit and quantification limit decreased. The residuals of Pentane were also very high for the range between 1 and 75 ppb. For the residuals, the accepted range was between minus five and five percent. The residuals of pentane were higher than six percent. According to CMA6/A this means that the working area of the SIFT-MS for this compound has to be changed or the function between the expected concentration and the response has to be changed (50).

For the other compounds such as Hexanol, Pentanal, P-xylene, etc. the measurement uncertainties were small. This means that the measurements of the SIFT-MS are of good quality and that this technique can quantify the selected compounds correctly. Some of the compounds, such as Pentanal and p-xylene, even had a lower measurement uncertainty than in-house techniques with the gas-chromatography mass spectrometer (GC-MS).

GC-MS is the most common method used to measure VOCs in exhaled breath (56). This technique uses a chromatographic column to separate the different compounds in the sample before identifying and quantifying them with mass spectrometry (56). This technique has a high reproducibility, a high sensitivity and robustness (57). A disadvantage of GC-MS is that the separation of compounds on the column is time consuming and labour intensive. This means that the different compounds in the sample have to be prepared before they can be measured (43). The big advantage of the SIFT-MS is that this technique does not need this pre-separation step and that the samples can be measured in real-time (43). This means that subjects directly can breathe into the analytical instrument and that the concentration of volatiles can be measured immediately. This makes the SIFT-MS the ideal technique in the search for biomarkers in exhaled breath. The only problem with the SIFT-MS technique is that compounds cannot be quantified correctly when they have conflicting m/z values with other compounds. This can be solved by eliminating these conflicting m/z values from the calculations. This is a bigger problem when dealing with unknown compounds in a sample. This can be solved by adding an extra separation step, for example a fast gas chromatography. This means that the advantage of an online measurement disappears when dealing with unknown compounds in a sample (43).

4.4. Sorbent tubes

The breath sampler is used in combination with sorbent tubes. The sorbent tubes in our study were tested in order to determine if the sorbent tubes trapped the VOCs and if a preconcentration step could be done. The results showed that the concentrations were higher after the preconcentration step with the cartridges, except for the preconcentration into the three litre bags. The concentration factors are not as expected. We hypothesized that we could do a preconcentration step with the cartridges but this hypothesis was not entirely confirmed. There was a preconcentration step, but the concentration factors were not as high as expected.

From the validation data we can conclude that the SIFT-MS measures the selected compounds correctly and that this is not the cause of the low concentration factor. Other causes can be a defect in the Unity thermal desorber or that the cartridges don't trap p-xylene entirely. It could be possible that the Unity thermal desorber does not reach the desired temperature or flow causing an incomplete thermal desorption. Sorbents not trapping a compound entirely can be a problem when working with cartridges. The type of sorbent that is used in the cartridges determines the compounds that are trapped. The cartridges used in this study are the Tenax/ Carbograph 4 sorbents. According to ISO 16017 p-xylene will be trapped by Tenax sorbents. The concentration of p-xylene can change due to the sorbent used. This happens when the sorbent does not adsorb everything and does not set everything free after thermal desorption (58). When testing this with a second desorption of the same cartridge of 100 ppb (data not included) the measured concentration was sixteen ppb, which is thirteen times lower than the data from the first desorption. This suggests that only a very small part of the compound has not been released by the cartridge. Walling *et al.* (1986) also concluded that Tenax cartridges are practical to use, but that retention volumes can differ from literature values and that chemical reactions during thermal desorption are possible (59). These chemical reactions were not confirmed by Baroja *et al.* (2007) who also considered a chemical reaction between the VOCs and the Tenax adsorbent (60).

A disadvantage of using cartridges is that the composition of the exhaled breath can change due to selected adsorption by the sorbent material of VOCs. More than one sorbent is needed to trap different compounds in a sample. This is why often multiple sorbent, such as Tenax/Carbograph 4, are used (58). A big advantage of using cartridges is that they can be transported and stored for a long period and that the adsorbed compounds in the cartridge will stay stable. This is an advantage when working with multiple hospitals or research centers or when the samples have to be transported from hospital to laboratory (47).

Future experiments have to point out if this incorrect preconcentration is caused by the Unity thermal desorber or the cartridges and sorbents. These future experiments can include using another thermal desorber, using other cartridges, changing the settings of the thermal desorber or testing other standard gasses. The next step is also to test the ReCIVA breath sampler with a lower flow of the CASPER and test if the flow of fifteen litres per minute is suitable for infants and toddlers.

4.5. Nasal mucus markers

The next step in the set-up of this project was a literature search for biological relevant nasal mucus markers. The selected markers were evenly spread over different pathways which are in some way linked to rattling, wheezing or both. The next step was to determine which markers could be measured with the preselected technique. This technique was chosen because multiple markers can be measured with a small sample volume in a sensitive and precise manner. The advantage of this technique is that the assay can be customized meaning that the markers of interest can be chosen and put together in one assay. For the markers that could not be measured with the multiplex ELISA from Mesoscale Discovery (MSD), a new assay has to be developed. The markers that could be measured with this technique have to be tested in nasal mucus. Nasal mucus is an appropriate matrix to assess inflammation in the respiratory system, but it has not been used very often. Janssens *et al.* (2015) already measured IL-1b, IL-8 and IL-13 in a successful manner, but the other markers in our selection have not been measured yet in nasal mucus (29). For a new assay development, the appropriate antibody pairs have to be selected. So, the next step in this research project is to determine if there are antibody pairs for the selected markers and if these antibodies are suited for this type of ELISA. Then the biotinilation and the SulfoTag of the assay have to be optimized with the selected antibody pairs and it has to be tested if the selected antibody pairs can be combined in one multiplex ELISA assay.

If this multiplex ELISA is not possible for these markers, other options can be considered. Olink proteomics is a proximity extension assay which can detect ideally 92 protein markers in one microliter (µL) of sample. With this technique DNA-tagged antibodies will bind to the same protein which will lead to the amplification of this tag by means of polymerase chain reaction (PCR) (61). The advantage of this technique is that with a minimal amount of sample, a screening can be done for new biomarkers. The disadvantage is that this assay provides a set of markers and they cannot be changed.

The collection of the nasal mucus has to be optimized, the right shape of sponges has to be selected and the appropriate sampling protocol has to be determined. Then the assay with the selected markers has to be tested and for the markers that could not be measured with the multiplex ELISA, a new assay has to be developed. When these protocols are optimized and all the assays are ready for usage, the inclusion of infants and toddlers (0-2 years) and the pilot study can be started.

5. Conclusion

We can conclude that the validation parameters for the selected compounds are overall good. This confirms our hypothesis that the SIFT-MS works efficiently and that it can quantify the selected compounds correctly. The measurement uncertainty of the SIFT-MS for some compounds is even comparable with the measurement uncertainty of the GC-MS, which is the standard technique for exhaled breath analysis. The SIFT-MS is an ideal technique for the screening of new biomarkers in exhaled breath.

The CASPER® functions with a flow of 15 L/min, the results were similar to a flow of 40 L/min. More measurements are needed to draw the correct conclusions.

The hypothesis that a preconcentration step is possible with a cartridge sampling method is not yet confirmed. Future experiments will point out what could be changed in the thermal desorption setup in order to optimize the preconcentration step.

The next steps in the start-up of this project can be taken. The analytical technique for breath analysis is optimized and interesting nasal mucus markers are selected. The next steps are optimization of the sampling protocol for the nasal mucus, optimization of the multiplex ELISA in combination with the nasal mucus and the development of a new assay for the remaining nasal mucus markers.

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7. Supplement

7.1. Extra information SIFT-MS

7.1.1. Mechanism SIFT-MS

Selected ion flow tube mass spectrometry is an analytical technique that can be used to quantify volatile organic compounds in the air and in exhaled breath samples. Figure 4 describes the different processes during a SIFT-MS analysis. First the precursor ions are produced in a microwave oven. These precursor ions are H_3O^+, NO^+ and O_2^+. The benefit of using three precursor ions is that the specificity of the technique increases.

In the next step one of the three precursor ions is selected by the quadrupole mass filter. The selected ions flow into the flow tube together with the sample by means of a carrier gas. Here a reaction takes place. The product ions arrive into the second quadrupole mass filter where the products are selected. These selected product ions are detected in the last step by an ion detector mass spectrometer. This ion detector is a particle multiplier where the product ions will collide with a cathode. In this reaction electrons will be produced and these electrons will collide with an electrode. This collision will cause the production of more electrons which will be accelerated to the next electrode. This process is repeated until the signal is strong enough. The amount of electrons is a measure for the amount of product ions at the cathode (62).

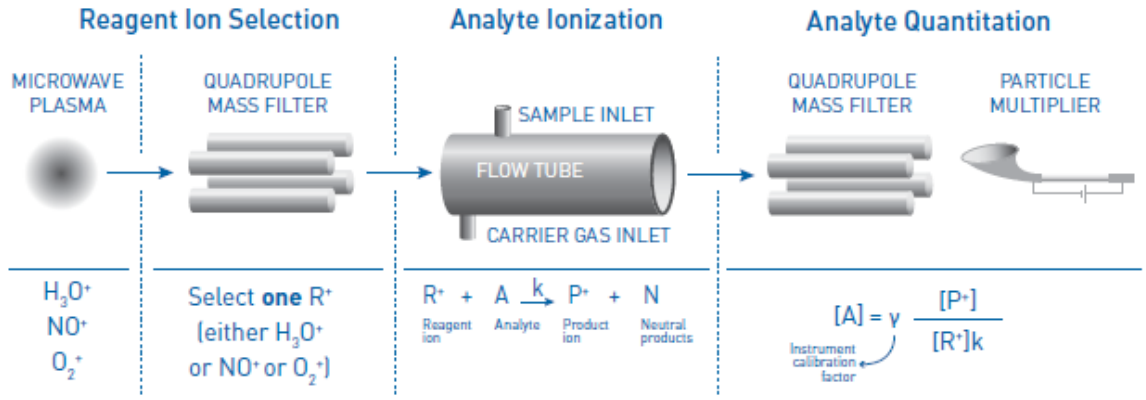

Figure 4 Schematic overview of the SIFT-MS technique (Syft technologies)(62).

In order to quantify the VOCs correctly different factors have to be taken into account. One important factor is the reaction constant or k-value which determines how much product will be formed during a reaction. A higher k-value means a faster reaction which will lead to the production of more product ions. This value is constant when the reaction parameters, such as temperature, pressure and flow of the carrier gas, are the same. The K-value will change when these parameters change. Therefore, the K-values present in the SIFT-MS library have to be checked in a validation process. This validation is done with a standard gas of known concentrations (44).

When a reaction takes place, it is possible that multiple product ions are formed. The distribution of these multiple product ions is indicated by the branching ratio (figure 5). This is another factor that has to be taken into. This will also be checked during the validation process (44).

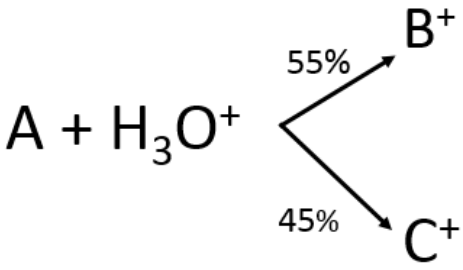

Figure 5 Example of the branching ratio

7.1.2. Scan method

Before analyzing the samples, a correct method has to be selected. There are two types of scans: the SIM scan and the Mass scan. An example of a SIM scan is given in figure 6. During the SIM scan, preselected m/z ratio's will be measured. The SIM scan is an ideal method for the quantification of known compounds in a sample.

During a Mass scan all m/z ratio's between a predefined range will be measured. This type of scan is more ideal for the identification of unknown compounds in a sample.

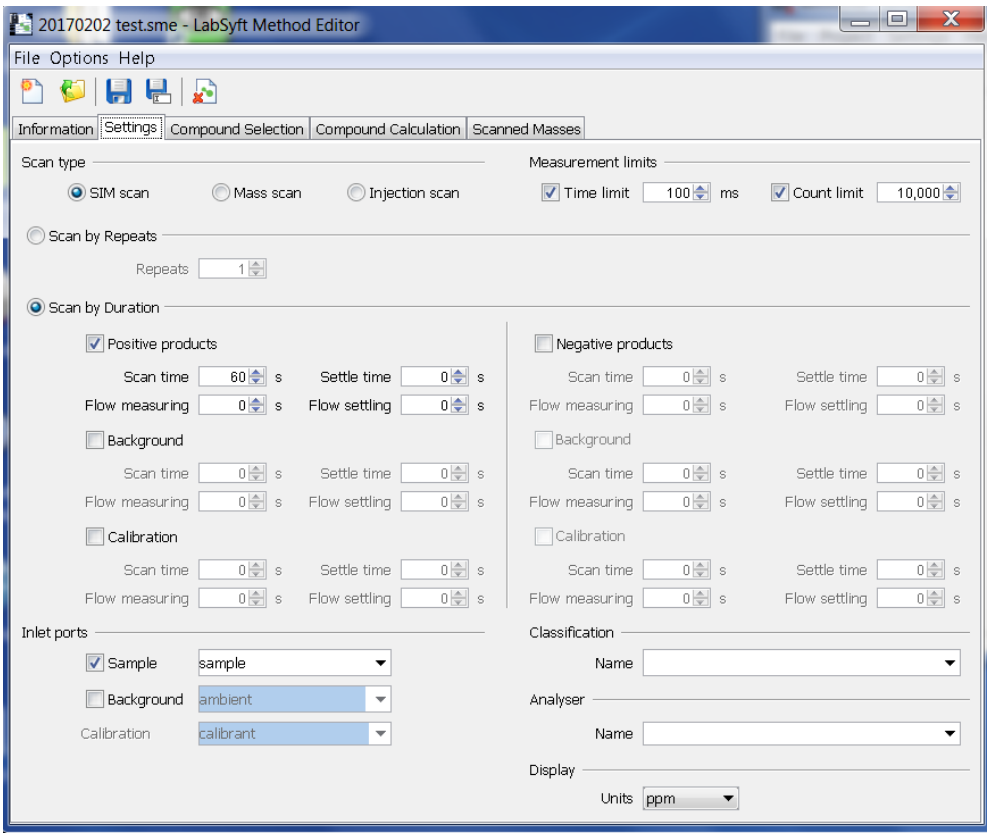

Figure 6 Example of a SIM scan method

7.1.3. Scan settings

The time limit or dwell time is the time that is set to measure one mass/charge ratio (m/z). This factor will determine the sensitivity of the technique. A higher dwell time will give a higher sensitivity because the SIFT-MS will measure the m/z ratio over a longer period. This time limit is generally set at 100 ms. The time limit is set to ensure that masses with a low signal are not measured too long.

The count limit is the maximum number of counts per second the SIFT-MS will measure at one m/z value. This is generally set at 10,000 counts to protect the detector from signals that are too high.

The scan time is the time that is set for one measurement and the settle time is the time at the beginning of the scan that is not taken into account.

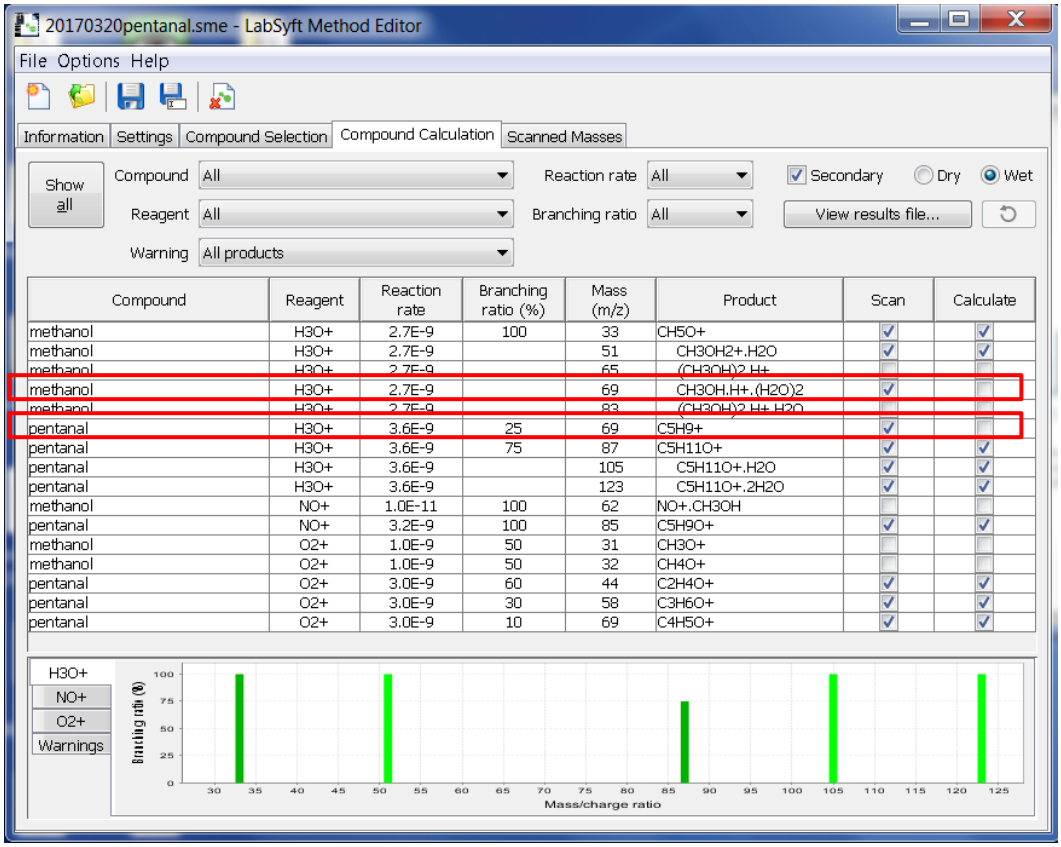

Figure 7 Example compound calculation method. In the red boxes a conflict of m/z 69+ between methanol and Pentanal is given.

The next factor that has to be taken into account in the method settings are the conflicts between the different m/z. when different masses have the same m/z value, they cannot be quantified because of this conflict. This has to be specified the compound calculation tab. An example of this is given in figure 7. Here we can see that m/z 69+ of pentanal has a conflict with the same mass of methanol. These two masses have to be checked off of calculations.

In the last tab an overview is given of all the scanned masses.

7.2. Expected concentrations in exhaled breath

Table 15 presents the expected concentration in exhaled breath.

Table 15 Expected concentrations in exhaled breath of the selected volatile organic compounds in. ppb: parts per billion.

Compound	Expected in exhaled breath (ppb)
Hexanol	5
Pentanal	2
Decanal	2
Pentane	22
Dodecane	0.13
P-xylene	7.3
Acetic acid	50
Valeric acid	6
Hexanoic acid	10
Acetophenone	10.81
1-Decene	22
Isoprene	31-273
Dimethyl disulfide	4

7.3. Adjusted branching ratios and K-values

Table 16 presents the K-values, old and adjusted, and the branching ratios, old and adjusted, for each m/z ratio of the selected compounds.

Table 16 Adjusted branching ratios and K-values for the selected volatile organic compounds. m/z = mass/ charge ratio, Br_0 = old branching ratio, Br_N = adjusted branching ratio, K_0 = old K-value, K_N = adjusted K-value, Sec produc = secondary product.

compound	ion	m/z	Br_O	Br_N	K_O	K_N
Pentane	O2+	42	0.4	0.15	1.60E-09	1.42E-09
	O2+	43	0.45	0.77		
	O2+	72	0.1	0.08		
Hexanol	H3O+	85	1	1	2.90E-09	5.13E-09
	NO+	101	1	1	2.40E-09	1.01E-09
	O2+	42	0.2	0.07	2.60E-09	3.23E-09
	O2+	43	0.1	0.52		
	O2+	56	0.4	0.23		
	O2+	70	0.1	0.04		
	O2+	84	0.2	0.19		
Pentanal	H3O+	87	0.75	0.75	3.60E-09	8.80E-09
	H3O+	105	Sec prod			
	H3O+	123	Sec prod			
	NO+	85	1	1	3.20E-09	3.55E-09

Table 16 (continued)

	O2+	44	0.6	0.28	3.00E-09	5.98E-09
	O2+	58	0.3	0.28		
	O2+	69	0.1	0.44		
Decanal	H3O+	157	0.97	0.97	3.90E-09	3.05E-09
	H3O+	175	Sec prod			
	H3O+	193	Sec prod			
	NO+	155	1	1	3.30E-09	1.68E-09
	O2+	68	0.1	0.11		
	O2+	70	0.1	0.12		
	O2+	71	0.1	0.11		
	O2+	82	0.1	0.14	3.20E-09	1.96E-09
	O2+	96	0.1	0.1		
	O2+	110	0.1	0.09		
	O2+	112	0.2	0.20		
	O2+	138	0.15	0.13		
Dodecane	H3O+	189	1	1	2.80E-09	4.18E-10
	NO+	169	1	1	1.50E-09	1.63E-09
	O2+	170	1	1	1.50E-09	2.89E-09
p-xylene	H3O+	107	1	1	2.20E-09	4.36E-09
	NO+	106	1	1	1.80E-09	1.78E-09
	O2+	91	0.2	0.17	1.80E-09	2.04E-09
	O2+	106	0.8	0.83		
Acetophenone	H3O+	121	1	1	4.30E-09	3.88E-09
	H3O+	139	Sec prod			
	NO+	150	0.95	\	3.60E-09	1.5E-09
	O2+	105	0.75	0.7	3.40E-09	1.75E-09
	O2+	120	0.25	0.3		
decene	H3O+	57	0.1	0.16	2.60E-09	4.08E-09
	H3O+	71	0.2	0.14		
	H3O+	85	0.15	0.13		
	H3O+	99	0.1	0.12		
	H3O+	141	0.45	0.44		
	NO+	86	0.15	0.16	2.10E-09	1.42E-09
	NO+	100	0.15	0.22		
	NO+	114	0.2	0.23		
	NO+	170	0.45	0.39		
Hexanoic acid	H3O+	99	0.25	0.35	3.00E-09	1.50E-09
	H3O+	117	0.75	0.6550		
	NO+	99	0.1	0.2796	2.50E-09	8.25E-10
	NO+	146	0.9	0.72		
Valeric acid	H3O+	85	0.1	0.1	2.90E-09	2.41E-09
	H3O+	103	0.9	0.9		

Table 16 (continued)

	NO+	85	0.7	0.31	2.40E-09	8.45E-10
	NO+	132	0.3	0.69		
	O2+	60	0.8	0.68	2.40E-09	1.10E-09
	O2+	73	0.2	0.31		
Dimethyl disulfide	H3O+	95	1	1	2.60E-09	3.79E-09
	NO+	94	1	1	2.40E-09	1.73E-09
	O2+	61	0.1	0.1	2.30E-09	2.07E-09
	O2+	94	0.8	0.8		
Acetic acid	H3O+	61	1	1	2.60E-09	3.00E-08
	H3O+	79	Sec prod			
	H3O+	97	Sec prod			
	NO+	90	1	1	9.00E-10	4.70E-10
	NO+	108	Sec prod			
	O2+	43	0.5	0.5	2.30E-09	3.36E-09
	O2+	60	0.5	0.5		
	O2+	61	Sec prod			
	O2+	79	Sec prod			
Isoprene	H3O+	69	1	1	2.00E-09	2.00E+09
	NO+	68	1	1	1.70E-09	1.29E-09
	O2+	53	0.1	0.05	1.70E-09	1.29E-09
	O2+	67	0.45	0.36		
	O2+	68	0.45	0.58		

7.4. Detailed tables validation parameters

Table 17 Trueness of the selected volatile organic compounds. This is based on the bias when comparing the measured concentration in parts per billion (ppb) with the reference concentration in ppb.

Compound	Reference concentration (ppb)	Measured concentration (ppb)	Bias	Trueness
Hexanol	49.22	49.78	1.14%	101.14%
Pentanal	24.91	25.58	2.71%	102.71%
Decanal	25.79	25.96	0.63%	100.63%
Pentane	102.82	101.91	-0.89%	99.11%
Dodecane	25.41	25.89	1.90%	101.90%
P-xylene	100.85	95.07	-5.73%	94.27%
Acetic acid	101.38	88.47	-12.74%	87.26%
Valeric acid	48.66	52.42	7.73%	107.73%
Hexanoic acid	103.11	82.26	-20.22%	79.78%
Acetophenone	104.77	98.88	-5.62%	94.38%
1-decene	25.45	27.74	8.97%	108.97%
Isoprene	100.47	65.68	-34.62%	65.38%
Dimethyl disulfide	51.36	50.21	-2.24%	97.76%

Table 18 The repeatability in parts per billion (ppb) and the relative repeatability. This is based on different measurements at a low and high level.

Compound	Low level (ppb)	High level (ppb)	Repeatability (ppb)	Relative repeatability
Hexanol	30.16	88.13	2.06	4.13%
Pentanal	27.50	87.62	1.42	5.67%
Decanal	24.47	95.84	4.28	17.14%
Pentane	66.33	106.67	3.56	3.56%
Dodecane	27.45	97.73	1.85	7.41%
P-xylene	27.86	99.60	0.89	0.89%
Acetic acid	38.16	89.41	1.57	1.57%
Valeric acid	42.28	87.28	4.47	8.95%
Hexanoic acid	44.15	86.21	4.94	4.94%
Acetophenone	31.85	105.85	2.03	2.03%
1-decene	27.98	79.05	2.70	10.80%
Isoprene	17.98	83.34	4.23	4.23%
Dimethyl disulfide	28.21	92.97	1.55	3.10%

Table 19 The reproducibility in parts per billion (ppb) and the relative repeatability. This is based on the standard deviation of different measurements of a reference concentration at different days.

Compound	Reference concentration (ppb)	Measured concentration (ppb)	Reproducibility (ppb)	Relative reproducibility
Hexanol	49.22	51.10	5.33	10.44%
Pentanal	24.91	25.58	4.21	16.45%
Decanal	25.79	25.87	2.99	11.55%
Pentane	102.82	101.91	5.81	5.70%
Dodecane	25.41	25.89	2.36	9.11%
P-xylene	100.85	75.74	4.37	5.77%
Acetic acid	101.38	85.07	10.40	12.22%
Valeric acid	48.66	52.42	11.26	21.48%
Hexanoic acid	103.11	78.19	5.32	6.47%
Acetophenone	104.77	98.88	12.74	12.89%
1-decene	25.45	31.52	4.70	14.91%
Isoprene	100.47	65.68	12.36	18.82%
Dimethyl disulfide	51.36	50.21	3.24	6.45%

Table 20 The limit of detection in parts per billion (ppb) and the limit of quantification in ppb. This is based on the standard deviation of 30 measurements of a zero gas.

Compound	Standard deviation (ppb)	Limit of detection (ppb)	Limit of quantification (ppb)
Hexanol	0.35	1.06	2.13
Pentanal	1.40	4.21	8.41
Decanal	0.48	1.44	2.87
Pentane	6.83	20.49	40.99
Dodecane	0.46	1.37	2.74
P-xylene	0.34	1.03	2.07
Acetic acid	0.66	1.98	3.96
Valeric acid	1.42	4.26	8.52
Hexanoic acid	1.70	5.10	10.21
Acetophenone	0.66	1.97	3.94
1-decene	0.90	2.71	5.41
Isoprene	0.00	0.00	0.00
Dimethyl disulfide	0.22	0.67	1.35

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Datum: **8/06/2017**