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Maastricht University

Faculteit Geneeskunde en Levenswetenschappen
School voor Levenswetenschappen
master in de biomedische wetenschappen

Masterthesis

Validation of a new method for exhaled breath profiling in noisy breathing infants

Daphne Trippaers

Scriptie ingediend tot het behalen van de graad van master in de biomedische wetenschappen, afstudeerrichting klinische moleculaire wetenschappen

PROMOTOR :
Prof. dr. Tim NAWROT

PROMOTOR :
Prof. Dr. Marc RAES

BEGELEIDER :
Mevrouw Gitte SLINGERS

2017
2018

De transnationale Universiteit Limburg is een uniek samenwerkingsverband van twee universiteiten in twee landen: de Universiteit Hasselt en Maastricht University.

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Preface

Dear,

My thesis with the title "Validation of a new method for exhaled breath profiling in noisy breathing infants" researches the use of breathomics in a peppermint washout experiment. It validates and standardizes a "new method" for breath profiling: selected ion flow tube mass spectrometry. This new method could be used as a non-invasive tool for the diagnosis of multiple diseases, such as noisy breathing diseases in infants. This thesis was the final step in completing my master's degree in Clinical Molecular Sciences at the University of Hasselt.

I want to thank the people who made this internship possible. My daily supervisor, Gitte Slingers, for being my mentor during these few months. Your guidance and support was invaluable for the development of my thesis. My external supervisor and institutional supervisors, doctor Marc Raes and professor Tim Nawrot, for the opportunity of this project and the support of my internship. My second examiner, professor Joy Irobi, for following my progress throughout the year.

Another thank you to the people at VITO (Flemish institute for technological research) for the technical support, especially Rudi Swinnen, Eddy Goelen en Maarten Spruyt. Additionally, I want to thank the Limburg Clinical Research Program (LCRP) and Jessa hospital for their facilities and for making all this possible.

I am grateful for my other colleagues: Joy and Evy. Thank you for being there and cheering me up along the way. My family and friends were also important in keeping me motivated and in helping me see my goal.

Hopefully, you enjoy reading this.

Daphne Trippaers

Hasselt, June 2018

Table of content

Abbreviations

BAL	Bronchoalveolar lavage
BHR	Bronchial hyperreactivity
c/s	Counts per second
CASPER	Clean Air Supply pump
cm^3	Cubic centimeter
COPD	Chronic obstructive pulmonary disease
GC-MS	Gas Chromatography Mass Spectrometry
ICF	Instrument calibration factor
ICS	Inhaled corticosteroids
k	Reaction coefficient
L/min	Liter per minute
m/z	Mass/charge
ms	Millisecond
ppb	Parts per billion
ReCIVA	Respiration Collector for In Vitro Analysis
sd	Standard deviation
SIFT-MS	Selected Ion Flow Tube Mass Spectrometry
SIM	Selected ion method
TD	Thermal desorption
VITO	Flemish Institute for Technological Research
VOC	Volatile organic compound

Abstract

Introduction: Breathomics could be used as a new, objective and reliable tool to differentiate between multiple noisy breathing phenotypes. This could lead to earlier disease control and less mistreatment. Selected ion flow tube-mass spectrometry (SIFT-MS), a relatively new form of breathomics, can identify volatile organic compounds (VOCs) in exhaled breath and has great potential in biomarker research.

Material & methods: The SIFT-MS was validated and compared to the current golden standard of gas analysis and breathomics, gas chromatography-mass spectrometry (GC-MS), using a peppermint washout experiment. After ingestion of a peppermint oil capsule, the washout of three peppermint compounds in exhaled breath was studied over time. Five individuals participated in this study. Validation parameters for the SIFT-MS were calculated prior to the actual benchmark study.

Results: Not all validation parameters were within range. The limit of detection was 9.92, 7.23, and 10.39ppb for eucalyptol, gamma-terpinene, and menthofuran, respectively. The measurement uncertainty for these VOCs was 33.12%, 69.62%, and 6.95%, respectively. These peppermint compounds were calculated during the benchmark study and did not show the typical decline of concentration over time. GC-MS could only detect eucalyptol but showed a better decline.

Discussion & conclusion: The GC-MS showed better results than the SIFT-MS for eucalyptol, questioning the accuracy of this alternative breathomics technique. There is a need for further optimization and testing before the SIFT-MS can be implemented into the medical world.

Samenvatting

Introductie: Breathomics kan gebruikt worden als een nieuwe, objectieve en betrouwbare methode om te differentiëren tussen fenotypes van luidruchtig ademhalen. Dit zou leiden tot vroegere controle van de ziekte en minder misbehandeling. Selected ion flow tube-mass spectrometry (SIFT-MS), een relatief nieuwe vorm van breathomics, kan vluchtige organische stoffen (VOCs) in uitgeademde lucht identificeren. Deze technologie heeft veel potentieel in biomarker onderzoek.

Materiaal & methode: De SIFT-MS werd gevalideerd en vergeleken met de huidige gouden standaard voor gasanalyse en breathomics, gas chromatography-mass spectrometry (GC-MS), via een pepermunt afname experiment. Na inname van een pepermuntolie capsule werd de afname van drie pepermunt componenten in de uitgeademde lucht bestudeerd over de tijd. Vijf individuen namen deel in deze studie. Validatie parameters voor de SIFT-MS werden vooraf aan de benchmark studie berekend.

Resultaten: Niet alle validatie parameters waren binnen het bereik. De detectielimiet was 9.92, 7.23, en 10.39ppb voor eucalyptol, gamma-terpinene, en menthofuran, respectievelijk. De meetonzekerheid van deze drie VOCs was 33.12%, 69.62%, en 6.95%, respectievelijk. Deze pepermunt componenten werden gemeten tijdens de benchmark studie en toonden niet de typische daling van concentratie over tijd. De GC-MS kon enkel eucalyptol detecteren, maar deze toonde een betere daling.

Discussie & conclusie: De GC-MS toonde betere resultaten dan de SIFT-MS voor eucalyptol, waardoor de betrouwbaarheid van deze alternatieve techniek in twijfel wordt getrokken. Er is nood aan verdere optimalisatie en testen voor de SIFT-MS gebruikt kan worden in de medische wereld.

1. Introduction

1.1 Noisy breathing

Noisy breathing is a phenomenon that is very common in children less than 2 years old. When there is a narrowing or partial obstruction of the airways, whether it be the nose, mouth, throat, larynx, trachea or the lower airways, it can lead to atypical breathing sounds. Since young children have a developing immune system and small airways, they are very susceptible to noisy breathing. Approximately 45.2% of all infants suffer from at least one episode of wheezing, which is the most common phenotype of noisy breathing (1). However, there is a lot of miscommunication in the medical world about the nomenclature of noisy breathing. Bakker *et al.* stated that when going to two different doctors with the same breathing symptoms, the person would get two different diagnoses (2). This leads to confusion amongst parents, doctors and other medical staff (3, 4).

1.1.1 Wheezing

The most common words used by doctors to describe different types of noisy breathing are: wheezing, rattling, crackling, stridor, and stertor. This study will focus on wheezing and rattling, because these are most often confused with one another. A **wheeze** is a high-pitched peeping sound, mostly heard during expiration, caused by bronchial hyperreactivity (BHR) and could result in difficulty breathing and possibly shortness of breath. Genetic predisposition and environmental factors, such as infections or allergens, influence BHR, which is defined as an excessive bronchial narrowing or bronchoconstriction (5). It can also be caused by airway inflammation (6). Both bronchoconstriction and inflammation are important characteristics of wheezing. A remarkable observation is that 1 in 3 children that start wheezing in their first year of life will later develop asthma (7). Wheezing children are treated with inhaled corticosteroids (ICS), to combat inflammation, and bronchodilators, to relax the airway muscles (8, 9).

Corticosteroids, such as fluticasone, reduce airway hyperresponsiveness by protecting the airways against triggers, such as cold air or allergens, and suppressing airway inflammation. ICS are the first-line therapy for patients with asthma, controlling asthma symptoms and preventing exacerbations (10). Side effects of ICS are impaired growth, reduced bone mass and adrenal function, in addition to elevated intraocular pressure/glaucoma and cataract formation (11, 12).

Salbutamol is a sympathomimetic drug, used as an **airway dilator** in asthma. Salbutamol is a type of rescue medication; it gives quick relief from breathing difficulties and shortness of

breath, and is only used when needed. ICS, however, are maintenance medication, used every day to control the symptoms. Bronchodilators often have trembling and shaking, nervousness, headaches, and tachycardia and other arrhythmia, as side effects (13).

1.1.2 Rattling

Rattling is a different type of noisy breathing. It is a low-pitched coarse sound, caused by excessive mucus in the upper airways. This mucus causes little discomfort for the child and is often induced by a viral airway infection (14). However, some patients are given anticholinergic drugs, depending on the severity of the symptoms. Rattling has two distinct characteristics: firstly, a vibration on the chest and back of the infant can be felt, making palpation useful for diagnosis. Secondly, rattles generally resolve spontaneously after 15-18 months of age (14, 15).

Glycopyrronium® reduces mucus secretion by inhibiting the action of acetylcholine and working anti-inflammatory. The most common negative effects of **anticholinergics** are coughing, a dry mouth, constipation, and headaches. Sometimes nausea and palpitations can occur (16).

Figure 1 shows the differences between a normal airway and an airway of a wheezer and a rattler. In rattling, the mucus production is strongly increased by the goblet cells and the underlying glands. In wheezing, there is a strong increase of inflammatory cells, such as eosinophils, mast cells and neutrophils, in the lamina propria. The smooth muscle cells have proliferated and show more contraction; thereby narrowing the airways (17).

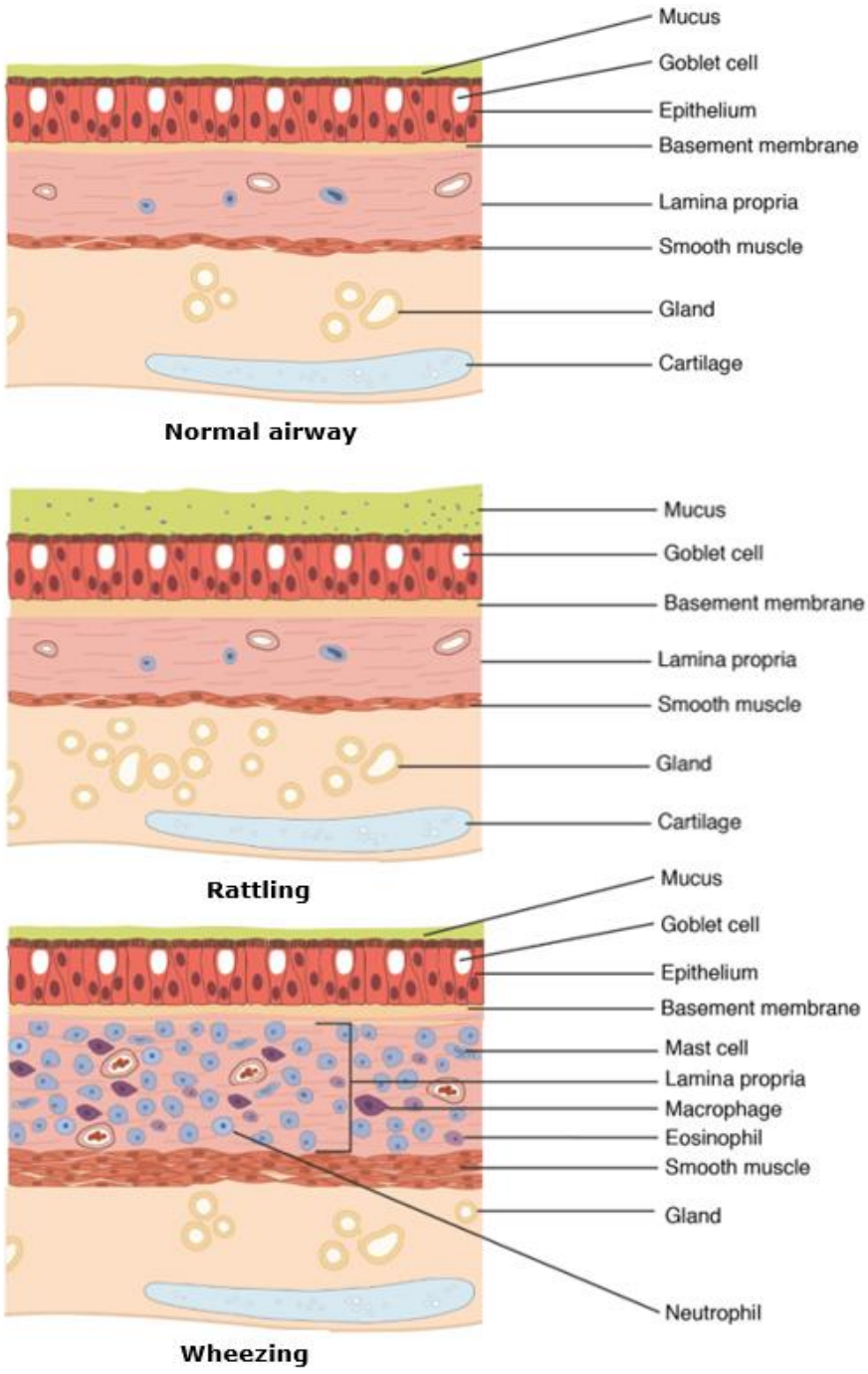

Figure 1: the pathophysiology of wheezing and rattling. In this cross-section of an airway, the difference between wheezing, rattling and the normal state can be seen. Patients with a rattle have more mucus, whereas patients with a wheeze have more inflammation and bronchoconstriction (proliferation of smooth muscle). Figure adapted from OpenStax College (17).

1.2 Misdiagnosis

Currently, noisy breathers are often misdiagnosed due to a lack of objective diagnostic tools resulting in an **overdiagnosis** of wheezers (4). The pediatrician uses the parents' report on the symptoms of their child, palpation of the chest and back, and auscultation to obtain a diagnosis. Sometimes the child doesn't experience any physical symptoms during the doctor's visit and the doctor can only rely on the information provided by the parents. They often don't have a medical background and do not know how to describe the breathing sounds their child made. Figure 2 shows the results of an experiment performed on the link between the use of wheeze and ruttle (rattle), and the increasingly detailed questioning of the parents. When the parents were openly asked to describe the breathing sound of their child, 53% (sum of wheeze and wheeze + ruttle) choose the word wheeze. At the end, the interviewer used a video to show the difference between wheezing and rattling, and the use of rattle increased from 40% to 81%; the use of wheeze declined to 36% (14).

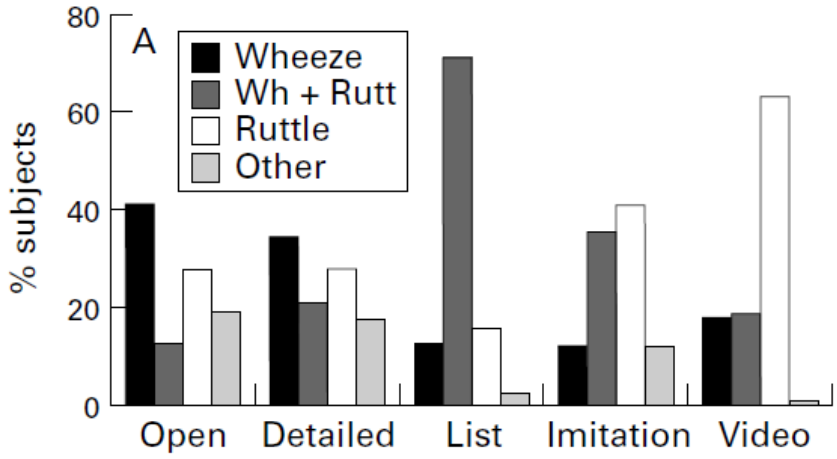

Figure 2: the changing use of the words wheeze and ruttle (rattle) with increasingly detailed interviewing of the parents (14).

This misdiagnosis of rattlers has three negative effects: firstly, the inhaled corticosteroids have some serious **side effects.** Secondly, this leads to a **burden on** the **healthcare** system. And finally, **parents** are often very **concerned** when they don't know what's wrong with their infant (13).

1.3 Methods for objective diagnosis

Since wheezing and rattling have a lot of common ground with asthma, the golden standard for diagnosis of asthma in young children could be used to objectively diagnose noisy breathing as well. This golden standard is a combination of bronchoscopy and bronchoalveolar lavage

(BAL). Bronchoscopy uses endoscopy to visualize differences in the inside of the airways, such as reticular basement membrane thickening and eosinophilic inflammation. During a BAL procedure, a bronchoscope is inserted into the lungs via the mouth or nose. Fluid is squirted into the lung and this fluid is collected and examined. The fluid shows predominantly eosinophilic inflammation in children with asthma. In preschool wheezers however, there is a lack of eosinophilia and it mainly consists of neutrophilic inflammation. These techniques are very invasive on young infants and have some ethical considerations because of the necessary sedation and/or anesthetics (18). Thus, an alternative is necessary. In the search for **non-invasive techniques**, exhaled breath seems very promising.

1.3.1 Exhaled breath

Exhaled breath contains a multitude of **volatile organic compounds** (VOCs) originating from exogenous and endogenous sources. The latter is of interest because they are produced by biological processes in the body itself, both locally in the lungs as elsewhere in the body. Some examples of such processes are inflammation and oxidative stress. After their production, the VOCs are released into the bloodstream, after which they diffuse into the lungs and are exhaled (19, 20). There are some exogenous factors that may influence the exhaled VOC concentrations, such as food, environmental contaminants, pathogens, composition of ambient air, and pharmacological treatment (21).

Breathomics have already been shown to be successful in studies on the detection of asthma, COPD (chronic obstructive pulmonary disease), lung cancer, diabetes and many more diseases (22). Several compounds, mainly long alkanes, are significantly associated with asthma inflammation (23). In a cross-sectional study, they discovered that predominantly benzene derivatives, alkanes, and alkane derivatives, discriminated between patients with and without lung cancer (24).

The current golden standard for breathomics is gas chromatography mass spectrometry (GC-MS). It uses a capillary column for gas analysis and identification of VOCs. Molecules are divided and identified based on their chemical properties and their relative affinity for the stationary phase of the column. It has some disadvantages, such as a necessary sample preparation step, a long analysis time and a need for trained personnel. In table 1, the GC-MS is compared with an alternative technique: **selected ion flow tube mass spectrometry** (SIFT-MS), which has some advantages compared to GC-MS: firstly, SIFT-MS is easier to use and requires only minimal training. Secondly, SIFT-MS has reduced maintenance, because of the elimination of chromatographic columns, a very clean ion source and detection systems, and a gas-only analysis. And thirdly, SIFT-MS can be used for real-time quantification.

Disadvantages of the SIFT-MS are that it is not ideal for broad profiling, and that it can only identify and quantify VOCs in the library. Additionally, GC-MS is better at identifying VOCs, because it makes use of a chromatography step (25, 26).

Table 1: comparison of gas chromatography mass spectrometry (GC-MS) with selected ion flow tube mass spectrometry (SIFT-MS) (25, 26).

	GC-MS	**SIFT-MS**
Sample preparation	Yes (preparation and/or pre-concentration)	No
Suitable matrices	Gas and liquid	Gas
Analysis time	Long (10-45 minutes)	Short (seconds to minutes)
Calibration	Regularly (using a set of dilutions of known concentrations)	Infrequently (gas standard)
Validation	Spiked samples and blanks	Automated online analysis of gas standard
Quantitation	Very high (chromatography)	High (multiple reagent ions)
Identification of new components	Yes	No (only VOCs in the library)
Maintenance	Often (frequent fouling of ion source and column)	Reduced (primarily vacuum pumps)
Analysis	Offline (no real-time findings)	Online and offline
Use	Hard (trained personnel)	Easy (minimal training)

The principle of SIFT-MS is described in figure 3. First, reagent ions (H_3O^+, NO^+ and O_2^+) are formed by microwave discharge via moist air. These reagent ions are used because they do not significantly react with the major components of air (oxygen, nitrogen and carbon dioxide), but they do react with many trace-elements in the breath sample. Next, one of the reagent ions will be selected by a quadrupole mass filter. The reagent ion is injected into the flow tube and excess energy is removed via collision with a carrier gas (usually helium). The sample is introduced into the flow tube at a controlled rate and the reactive compounds in this sample are ionized by the reagent ion, resulting in the formation of product ions. Thirdly, the product ions and unreacted reagent ions flow into the mass spectrometer chamber which contains a

second quadrupole mass filter and an electron multiplier detector. These are used to separate the ions by their mass-to-charge ratio (m/z) and to measure the count rates (counts per second (c/s)) of the ions in the desired m/z range (27). Absolute concentrations of the trace gases can be determined down to the parts per billion (ppb) levels, with the use of a reaction coefficient (k), branching ratios and the instrument calibration factor (ICF) (28).

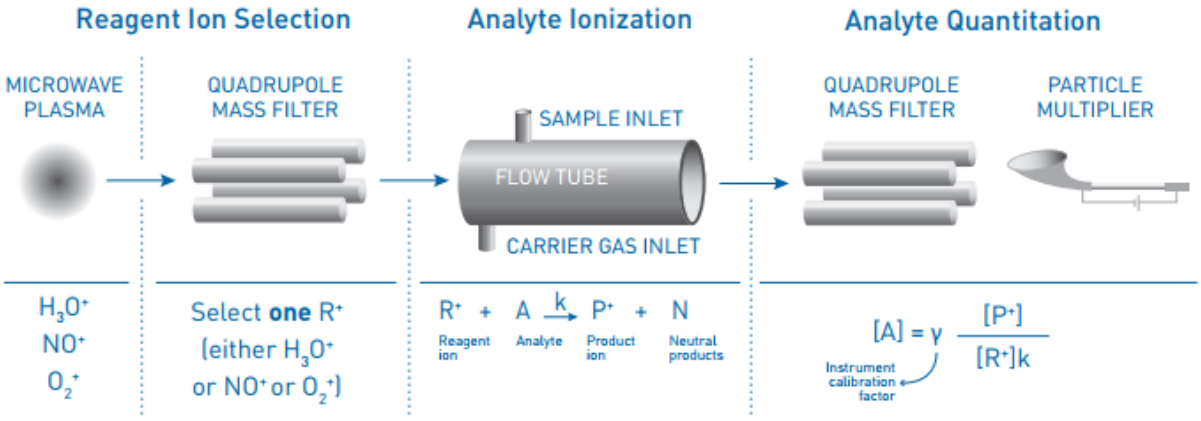

Figure 3: a selected ion flow tube mass spectrometer (SIFT-MS) (27).

1.4 Influence of pathogens

Wheezing, rattling and asthma exacerbations are often caused by respiratory **pathogens**. These pathogens can be either viral or bacterial in nature. Johnston *et al.* found that two thirds of the viral infections that cause asthma exacerbations were picornaviruses (29). Shilts *et al.* stated that a respiratory viral infection during infancy has been linked to the development of childhood wheezing and asthma later on in life (30). The most common bacterial pathogens in the nose and nasopharynx of children are *Streptococcus pneumoniae*, *Moraxella catarrhalis* and *Haemophilus influenzae*. They may increase the risk of developing a wheezing illness (31).

It is also important to note that these pathogens could influence the VOC profiles. Bacterial strains have a typical metabolism, which results in the production of bacteria-specific VOCs, such as hydrogen sulfide, ammonia, formaldehyde, ethanol, etcetera (32). Pathogen detection is used to identify these different pathogens. It would be interesting to find a difference between pathogens that cause wheezing and pathogens that cause rattling.

1.5 Hypothesis and objectives

Based on the different underlying mechanisms and different therapy needs we **hypothesize** that there is a molecular difference between rattlers and wheezers in exhaled breath. To prove this hypothesis, we first need to select a method for exhaled breath analysis: SIFT-MS. A

second hypothesis is that the SIFT-MS is equal to or better than the golden standard of breathomics, GC-MS.

This project can be divided into 3 objectives: firstly, the exhaled breath collection method will be optimized; SIFT-MS will be validated for different VOCs including peppermint related VOCs; and different analysis techniques (SIFT-MS and GC-MS) are compared in a peppermint washout experiment.

2. Material and methods

2.1 Efficiency clean air supply for exhaled breath collection

The CASPER® clean air supply (Owlstone medical, Cambridge, United Kingdom) scrubs the ambient air and provides clean air filtered from exogenous VOCs to the breath sampler. The standard flow for adults, 40 L/min, was compared to a lower flow of 10 and 15 L/min. The SIFT-MS Voice 200 (Syft technologies, Middleton, New Zealand) was used with the following settings: full scan mode, m/z ratios between 15+ and 200+, 100 milliseconds (ms) dwell time, 10 repeats, count limit of 10.000 and 60 seconds scan time. Each condition was measured 10 times spread over several days. The raw data on counts per second was multiplied by the ICF in order to correct for differences between days.

2.2 Validation SIFT-MS

2.2.1 Selection volatile organic compounds

Based on previously generated results, p-xylene was chosen to test whether the previously determined validation parameters were still valid at higher concentrations. A stock solution of 800 ppb was made with a glass Pasteur pipette and a balance. From this stock solution the dilutions of 400 and 200 ppb were made. The previously generated data on p-xylene for the 5 lower concentrations were provided by a colleague who had already performed the experiment.

Two µL of the dilution was injected into a 10L Teflon sampling bag while it was filled up to 9L with nitrogen gas. The bags were placed in an oven at 70°C for 5 minutes, making it possible for the dilution to evaporate into the gas. The bags were measured with the SIFT-MS Voice 200. Each concentration was measured 7 times on 5 different days with the following settings: a selected ion method (SIM) scan, a count limit of 10.000 counts, a dwell time of 100 ms, a settle time of 3 seconds, and a scan time of 60 seconds.

2.2.2 Validation parameters

To calibrate the instrument, the branching ratios and rate constants or k-values were adapted relative to the values included in the software. The branching ratios were adapted based on the measured c/s, which were corrected for ICF on different days. Based on the concentrations measured with the adapted branching ratios, the k-values were calculated.

The adapted branching ratios and k-values were used by the software to calculate the measured concentration. These calculated or measured concentrations were used to

determine different validation parameters of the SIFT-MS: trueness, repeatability, reproducibility, limit of detection, limit of quantification, and linearity (table 2).

Table 2: The validation parameters and their experimental setup, based on the CMA/6/A procedure.

Parameter	Experiment
Trueness	5 analyses of the same concentrations under conditions of intra reproducibility
Repeatability	5 analyses of two concentration levels separated with at least a factor 10
Reproducibility	5 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	7 different concentration levels

To calculate these parameters, p-xylene was brought into a Teflon bag of 10L. This bag was measured directly onto the SIFT-MS.

2.3 Gas chromatography mass spectrometry

The SIFT-MS was compared to the current golden standard for breath analysis, GC-MS. The GC-MS combines gas chromatography in the form of a capillary column with a mass spectrometer. The output is a chromatogram and a mass spectrum. The GC-MS consisted of a TD-100 desorber (Markes International, Llantrisant, United Kingdom), a Rxi-5Sil-ms column (Restek, Bellefonte, PA, USA), and an Agilent GC-MS detector (Agilent Technologies, Santa Clara, CA, USA).

Since trained personnel is necessary for operating the GC-MS, the results were provided by a colleague at VITO (Flemish Institute for Technological Research). The settings of the GC-MS were: desorb temperature of 300°C, desorb flow of 25 ml/min, desorb time of 10 min, cryo trap of 10-350°C maximum heating rate, trap heating time of 5 min, and flow rate of 1.5 ml/min, using helium as a carrier gas.

2.3.1 Preconcentration using sorbent tubes and thermal desorption

One liter of the breath sample collected in Teflon bags was pumped over carbograp 1TD/Carbopack X sorbent tubes (Markes International) with the Gilian personal air sampler (Sensidyne, St. Petersburg, FL, USA) combined with a Low Flow module (Sensidyne). These sorbent tubes were desorbed with the use of the UNITY thermal desorption device (Markes

international). This technology uses heat to increase the volatility of compounds to remove them from the solid matrix inside the sorbent tubes. This was done prior to analysis with the GC-MS, as a pre-concentration step.

2.4 Peppermint washout experiment

2.4.1 Set-up

The peppermint washout experiment was a benchmark study to test the rapid and reproducible analysis of breath biomarkers. It was a collaboration between multiple institutes. Five volunteers ingested a peppermint capsule and at set time points, the peppermint VOCs were measured in their breath sample. All subjects were informed before recruitment and were required to sign an informed consent. The results and information of the individuals were coded and cannot be traced back to the individual. The subjects were also requested to fill out a questionnaire on factors that can influence the quality of the breath samples.

The five healthy individuals were between 18 and 50 years old, without a history of chronic or recent acute diseases. Exclusion criteria were persons with bad allergies, persons that smoke or vape, pregnant women or persons that regularly take anti-inflammatory medication.

One breath collection was made from the same individual prior to ingestion to provide a baseline concentration for the VOCs of interest. Then, after ingestion, 3 breath samples were taken with different methods at five different time points: 60, 90, 165, 285, and 360 min, see table 3. After consumption of the peppermint capsule, there should be a large increase compared to the baseline measurement for the following VOCs: menthol, menthone, gamma-terpinene, eucalyptol and menthofuran. An ambient air sample was taken with both the participant and researcher present.

During this experiment, 3 different sampling techniques were compared: firstly, collecting exhaled breath into a Teflon bag and measuring this bag directly with the SIFT-MS. Secondly, tidal breathing into the Hex sampling arm on the SIFT-MS itself. Thirdly, sampling exhaled breath with the bag and using the Gilian personal air sampler to pump the VOCs over a sorbent tube. This tube was measured with a combination of thermal desorption and GC-MS.

Table 3: The schedule of the peppermint washout experiment.

Experiment schedule

1. Participant preparation
    a. Provide information sheet and obtain informed consent.
    b. -24 hr from capsule ingestion. Excluding peppermint and peppermint associated products from diet and personal care routines until completion of peppermint washout.
2. Peppermint Washout experiment.

Breath samples/measurements are to be taken at 60, 90, 165, 285, and 360 min.

- a. -0.5 hr from capsule ingestion.
    - i. Baseline sample measurement.
- b. 0 hr
    - i. Capsule is ingested and 150 cm^3 water is swallowed as quickly as is comfortable.
- c. 60 min
    - i. Washout sample 1
- d. 90 min
    - i. Washout sample 2
- e. 165 min
    - i. Washout sample 3
- f. 285 min
    - i. Washout sample 4
- g. 360 min
    - i. Washout sample 5

3. Environmental/ambient air sample.
    a. Select at random one of the sample points t = 60 min, 90 min, 165 min, 285 min and 360 min.
    b. On completion of the breath sampling of that chosen sample point, collect an environmental/ambient air sample, or make a measurement.
    c. Use the same sample/measurement volume as the breath samples/measurements while the researcher and participant are still present in the room.
4. Collect an air supply/instrument blank sample after completion of a breath sampling.

2.4.2 Selection volatile organic compounds

Multiple dilutions were made of the peppermint VOCs of interest: eucalyptol, gamma-terpinene, menthofuran, menthol and menthone. Stock solutions were made and diluted with glass Pasteur pipettes and a balance. From the stock solution of 200 ppb, the dilutions of 100, 50, 25, 10 and 1 ppb were made. The exception was menthol: from the stock solution of 70 ppb, dilutions of 50, 25, 10 and 1 ppb were made.

The same procedure as with the previous validation of the SIFT-MS was followed for the filling of the Teflon bags. Each concentration was measured 7 times on 5 different days with the following settings: a SIM scan, a count limit of 10.000 counts, a dwell time of 10 ms, a settle time of 3 seconds, and a scan time of 60 seconds.

2.4.3 Validation of peppermint compounds

The same validation parameters were determined as before (see 2.2.2: validation parameters), after adaptation of the branching ratios and k-values.

2.5 Statistical analysis

The statistical program R (version 3.3.2) was used for data analysis of the results of the CASPER® efficiency. The assumptions of normality were tested for all data: equal variance with the Bartlett test and normality with the Shapiro-Wilk test. If the assumptions were met, an ANOVA and pairwise t-test were performed to compare the results of the scrubbed air and the ambient air. Otherwise the results were analyzed with a Kruskal and a Wilcoxon test. The proportion of ions (m/z) significantly lower in scrubbed air than ambient air was calculated for all tested flow rates of the CASPER®.

3. Results

3.1 Efficiency clean air supply

The CASPER® worked equally well with a flow volume of 15 L/min (84.24%, $p<0.05$), as with 40 L/min (the standard for adults) (83.30%, $p<0.05$). A flow of 10 L/min was inadequate (63.60%, $p>0.05$), as can be seen on figure 4.

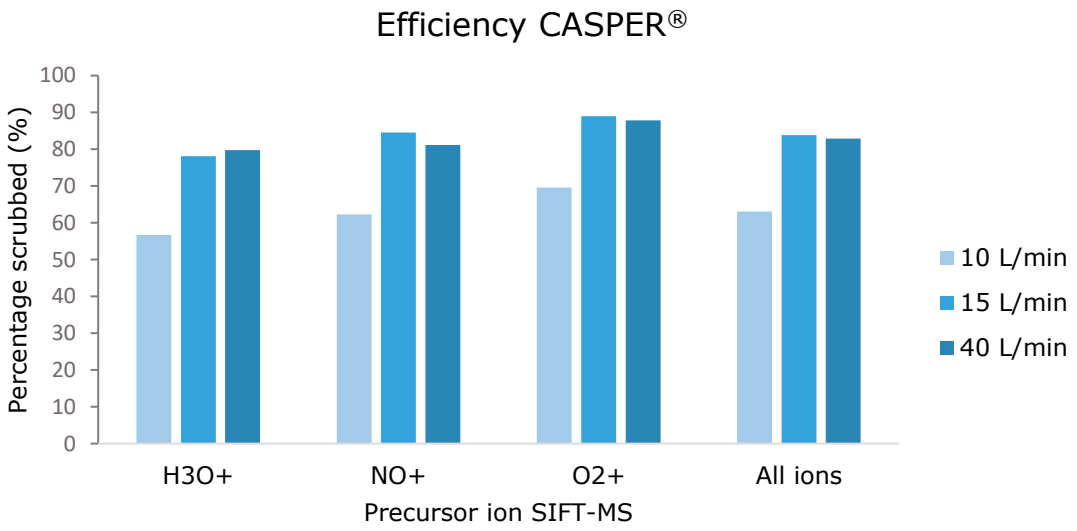

Figure 4: The efficiency of the CASPER® clean air supply. The percentage scrubbed is plot against the precursor ion used on the SIFT-MS.

3.2 Validation parameters

All validation parameters for p-xylene were within range (table 4). Trueness, or the degree of similarity between the mean of a range of measurements and the true value, was 98.79%. Repeatability or when the experiment is performed multiple times and the variables between measurements are kept as low as possible, was 2.74%. The reproducibility is again a repeated experiment, but with as many variables as possible; it was 5.40%. The limit of detection was 1.58 ppb and the limit of quantification was 3.15 ppb. And finally, the measurement uncertainty or the dispersion of values attributed to a measured quantity, was 12.33%. Measurement uncertainty was calculated using the absolute bias and relative reproducibility.

Table 4: Summary of the validation parameters of p-xylene. All parameters were within range.

Compound	**p-xylene**	**Range**		
		Very good	Acceptable	Not acceptable
Trueness	98.79%	105-95%	110-90%	Outside
Relative repeatability	2.74%	<5%	<10%	>10%
Relative reproducibility	5.40%	<10%	<20%	>20%
Limit of detection (ppb)	1.58	<5	<10	>10
Limit of quantification (ppb)	3.15	<5	<10	>10
Measurement uncertainty	12.33%	<30%	<50%	>50%

These parameters were calculated for high and low concentrations. In table 5, the trueness, relative repeatability and reproducibility, linearity and measurement uncertainty are compared between the high (800, 400 and 200 ppb) and low (100, 75, 50, 25 and 1 ppb) concentrations. The results of both are closely related. The results of the low concentrations were provided by previously done experiments, however, the adjusted k-values and branching ratios were taken into account.

Table 5: Comparison of the validation parameters of the SIFT-MS for high and low concentrations of VOCs.

Ppb	**Trueness**	**Relative repeatability**	**Relative reproducibility**	**Linearity (R^2)**	**Measurement uncertainty**
High (800, 400 and 200)	98,79%	2,74%	5,40%	0,999	12%
Low (100, 75, 50, 25 and 1)	94.27%	0.89%	5.77%	0.995	17%

3.3 Peppermint washout experiment

3.3.1 Explorative tests

First, some explorative tests were performed to investigate whether the set-up of this experiment was correct or if adjustments needed to be made. One individual was used for this test. Before the experiment, a breath sample was taken with the use of the Hex sampling arm to ensure the baseline concentrations of the VOCs of interest. At time point 0, the peppermint capsule was ingested. Subsequently, at time points 30, 60, 90, and 120 minutes, the washout samples were taken. An average of the five peppermint VOCs of interest was made. These VOCs were eucalyptol, gamma-terpinene, menthofuran, menthol and menthone. The graph

(figure 5) shows a steady decrease of these peppermint VOCs in the exhaled breath of the test subject over time.

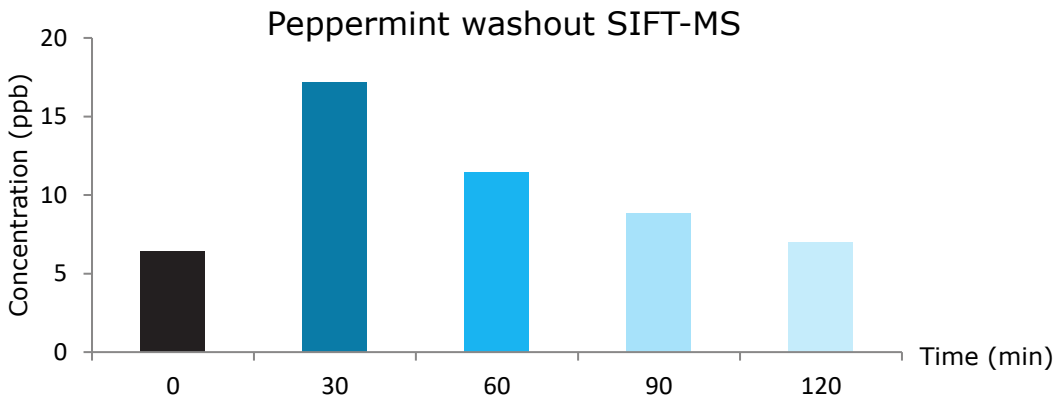

Figure 5: An explorative test of the peppermint washout experiment. These results are the average of 5 peppermint VOCs found in the exhaled breath of one individual. Black is the baseline measurement. The concentration in ppb is plot against the time in minutes. The sampling was done with the use of the Hex sampling arm.

During the explorative tests, the difference between the use of the Hex sampling arm of the SIFT-MS and a Teflon bag was investigated. Both are used for direct measurements. The results are shown in figure 6: the concentration measured with the sampling arm was much higher than the concentration measured with the bag.

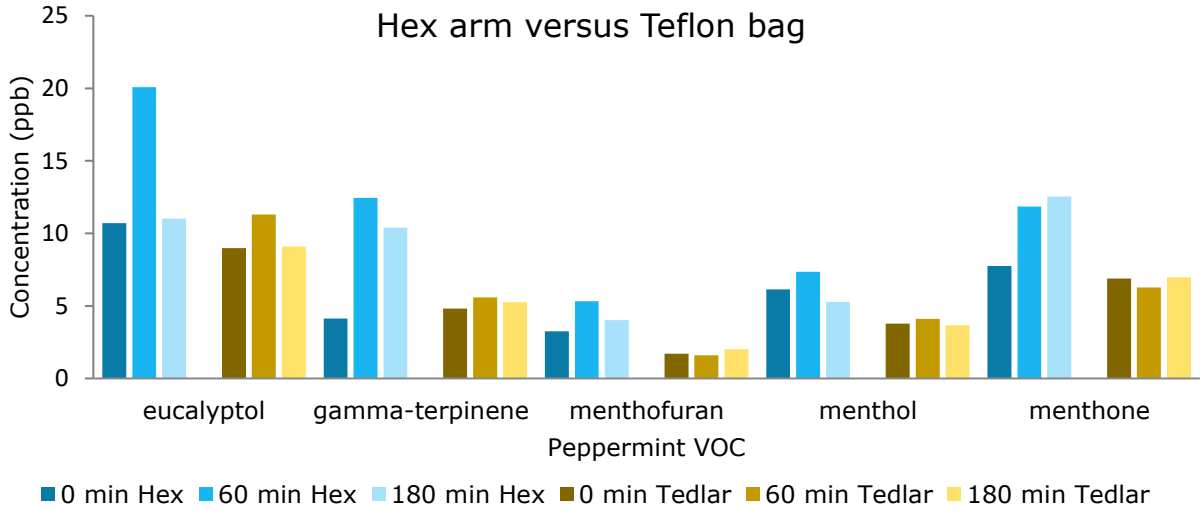

Figure 6: A comparison of direct measurement on the SIFT-MS by using the Hex sampling arm (blue), or by using a Teflon bag (yellow). On the y-axis is the concentration in ppb and on the x-axis are the different measurements. Each peppermint VOC was measured on 3 timepoints (0, 60 and 180 min).

3.3.2 Validation experiment

During the validation experiment, measurements of the five VOCs of interest were done on five different days. The branching ratios and k-values were adjusted relative to the values included in the software. In table 6, the expected concentration was compared with both the results directly from the SIFT-MS and the results after these adaptations. The results of the SIFT-MS with the use of library parameters were not as expected. For example, an expected concentration of 200 ppb, gave as result 35.45 ppb for gamma-terpinene, while after adjusting the parameters, this was 190.70 ppb. A much better result. A range from 1 to 200 ppb was chosen for the validation, because during the explorative test the SIFT-MS showed peaks up till 140 ppb, even if the average stayed lower.

Table 6: Calculated concentration of gamma-terpinene. Comparison of the expected ppb, the measured ppb with the SIFT-MS library parameters and the measured ppb after adjusting the parameters. The parameters were adjusted for the ICF, branching ratio and k-value. Additionally, the trueness was calculated in percentage. These are the results of 1 day of measurements.

Expected concentration (ppb)	**SIFT-MS library parameters (ppb)**	**Adjusted parameters (ppb)**	**Trueness (%)**
200	35.45	190.70	91.53
100	18.48	104.64	88.87
50	9.57	49.90	99.52
25	6.50	29.53	116.61
10	4.55	19.39	184.10
5	3.62	13.30	265.43
1	3.55	21.63	1324.31

The same validation parameters, as were done earlier, were calculated for these peppermint VOCs. The results are found in table 7. Menthol was omitted due to very bad results and consequently unmeasurable validation parameters.

Table 7: Summary of the validation parameters of the peppermint VOCs: eucalyptol, gamma-terpinene, menthofuran, and menthone.

Compound	Eucalyptol	Gamma-terpinene	Menthofuran	Menthone
Bias	-17.15%	-32.92%	-2.56%	-9.91%
Trueness	82.85%	67.08%	97.44%	90.09%
Relative repeatability	7.93%	4.93%	7.24%	6.54%
Relative reproducibility	7.99%	18.35%	2.19%	3.20%
Limit of detection (ppb)	9.92	7.23	10.39	30.89
Limit of quantification (ppb)	19.84	14.46	20.79	61.78
Measurement uncertainty	33.12%	69.62%	6.95%	16.31%

The trueness was determined by calculating the difference between the measured and expected concentration; this is the bias. The average absolute bias of the four components was 15.64% (sd: ±11.23%). The average trueness was 84.37% (±11.23%) at the concentration of 100 ppb; this was always taken as the reference concentration in the calculations. The highest absolute bias was from gamma-terpinene (-32.92%) and the lowest was from menthofuran (2.56%).

The average relative repeatability and average relative reproducibility were 6.66% (±1.11%) and 7.93% (±6.40%), respectively. The highest and lowest relative repeatability were 7.93% for eucalyptol and 4.93% for gamma-terpinene. The highest and lowest relative reproducibility were 18.35% for gamma-terpinene and 2.19% for menthofuran.

The limit of detection and limit of quantification were based on the standard deviation of 30 measurements of zero gas (bag filled with nitrogen gas). The average limit of detection and limit of quantification were 14.61 (±9.48) ppb and 29.22 (±18.95) ppb, respectively. The lowest limit of detection and quantification were 7.23 and 14.46 ppb for gamma-terpinene. The highest limit of detection and quantification were 30.89 and 61.78 ppb for menthone.

From the bias and relative reproducibility, the measurement uncertainty was calculated. The average measurement uncertainty was 31.5% (±23.92%). The lowest measurement uncertainty was 6.95% for menthofuran and the highest was 69.62% for gamma-terpinene.

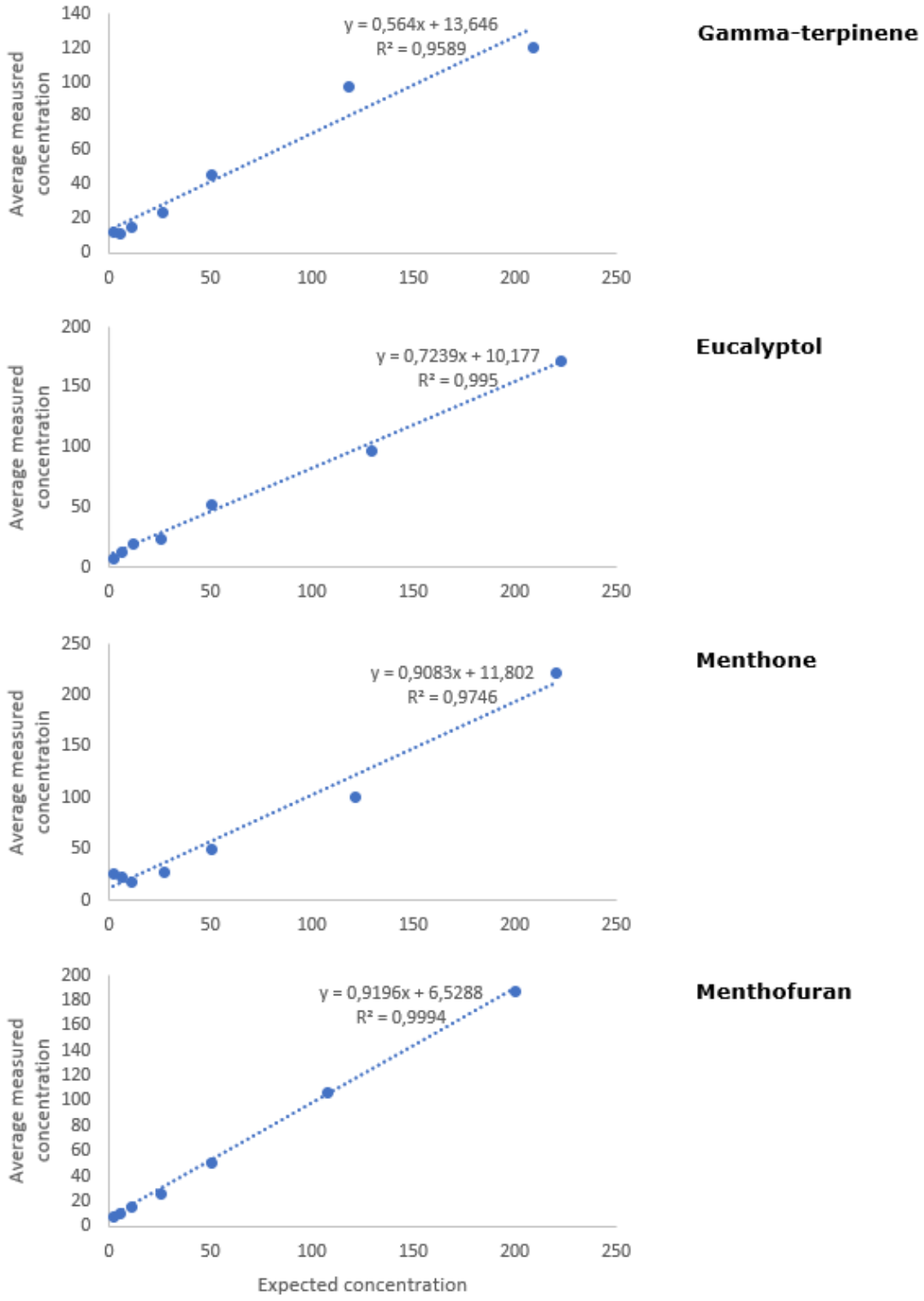

Figure 7: Linearity of 4 peppermint components: gamma-terpinene, eucalyptol, menthone and menthofuran. The average measured concentration is plot against the expected concentration.

The linearity for the four volatile compounds is shown in figure 7. To be as linear as possible, the R^2 should be close to 1. For gamma-terpinene, this was 0.9589 and for eucalyptol, 0.9950. The R^2 of menthone was 0.9746 and of menthofuran was 0.9994.

3.3.3 Benchmark study

The same setup as in the explorative tests was used, only now with 5 individuals and washout samples were taken at time points 60, 90, 165, 285 and 360 minutes. The baseline measurement was taken 30 minutes before ingestion of the peppermint capsule.

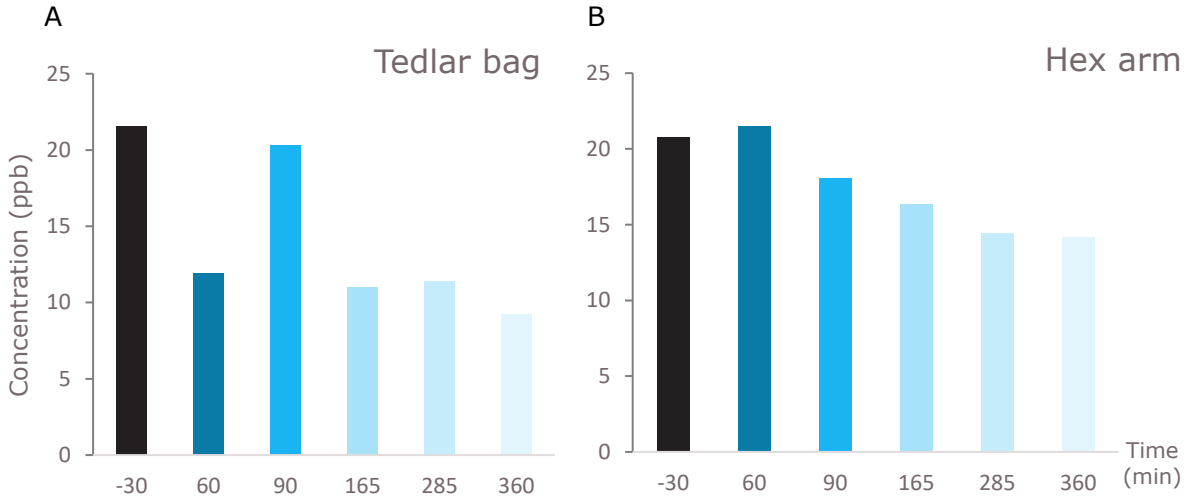

Figure 8: The peppermint washout experiment with the use of a Teflon bag (A) or the Hex sampling arm (B). These results are the average of 3 peppermint VOCs found in the exhaled breath of five individuals: eucalyptol, gamma-terpinene and menthofuran. Black is the baseline measurement. The concentration in ppb is plot against the time in minutes.

In figure 8, the average of three peppermint VOCs for the five individuals together can be seen. It is remarkable that the baseline measurement (in black) was so high, for the Teflon bag even the highest concentration of all timepoints. The results of the Hex sampling arm were closest to what was expected. The main difference between the Teflon bag and Hex sampling arm was the time point of 60 minutes: the concentration measured with the Teflon bag was much lower than with the Hex sampling arm.

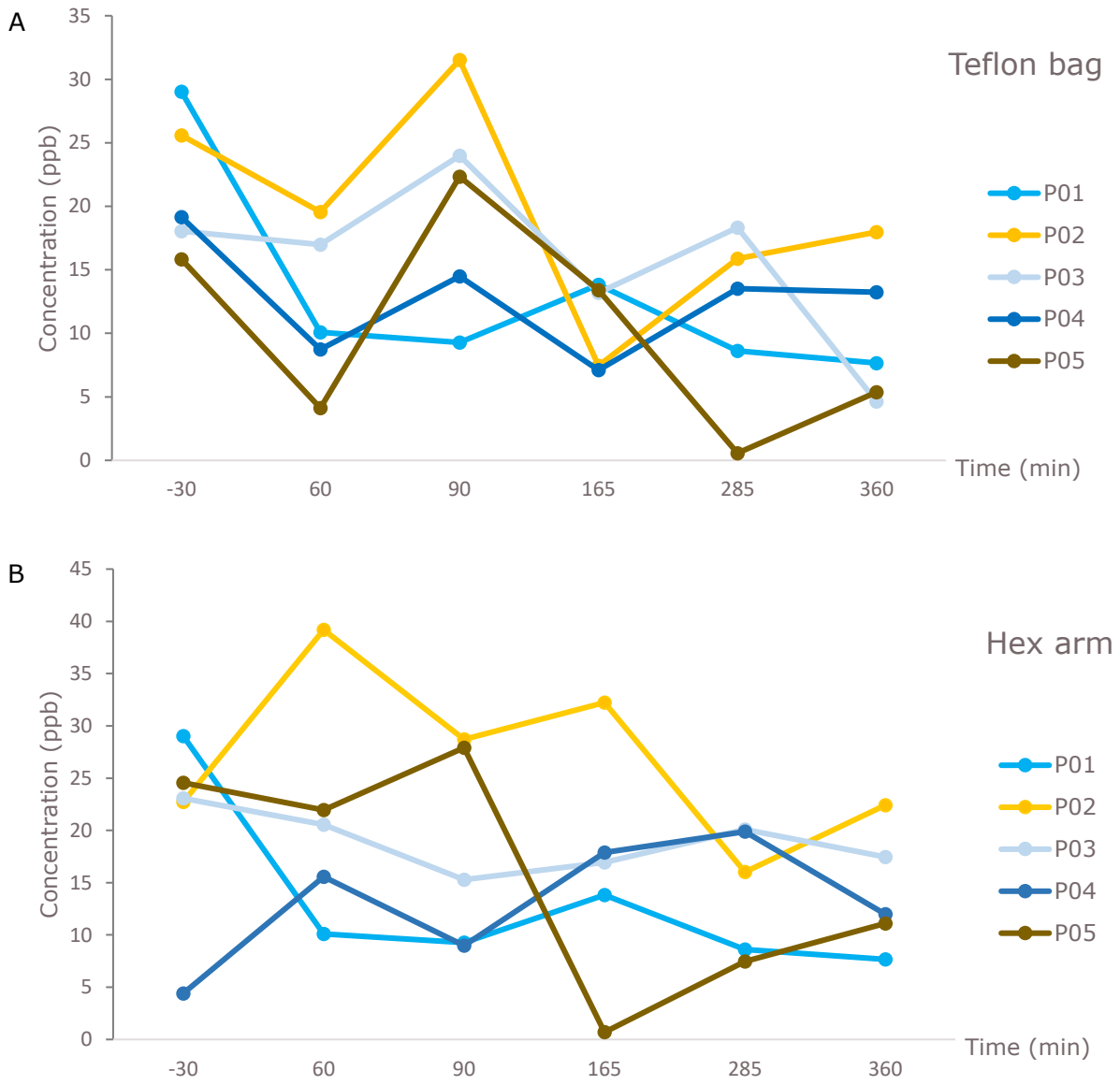

Figure 9: The peppermint washout of five different individuals, with the use of a Teflon bag and the Hex sampling arm. The average of three peppermint VOCs was taken: eucalyptol, gamma-terpinene and menthofuran. The concentration in ppb is plot against the time in minutes.

Next, the average of the three peppermint VOCs for each individual separately was plot in a graph. The course of the graphs (figure 9) is very chaotic and not what was expected. Normally, the baseline measurement (timepoint -30 minutes) should have the lowest concentration. At 60 minutes, the concentration should be the highest, but for the Teflon bag, this was not the case. As time progresses, the concentration should decrease and eventually become equal to the baseline measurement.

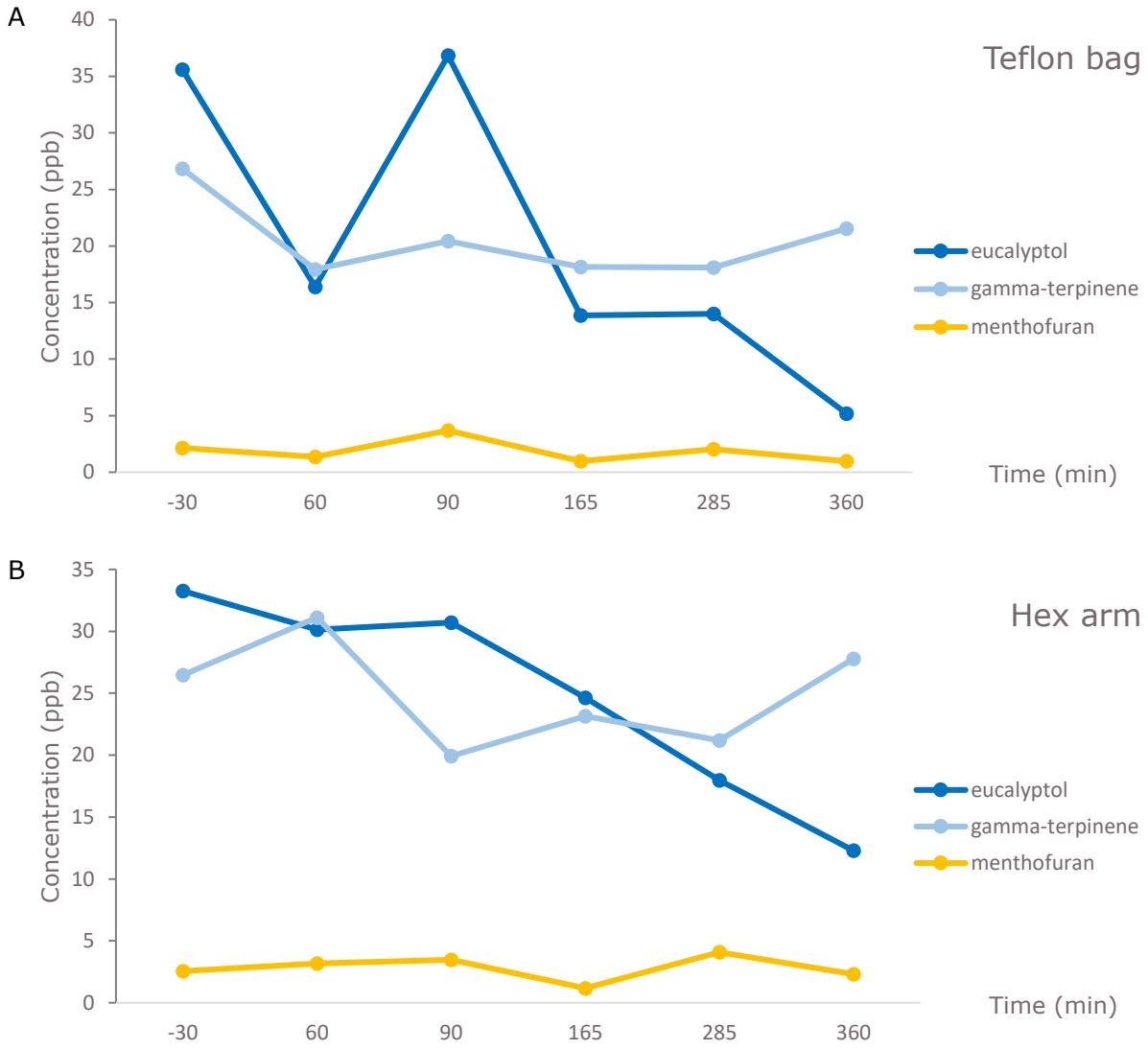

Figure 10: The peppermint washout of the average of the five individuals for the three different VOCs (eucalyptol, gamma-terpinene and menthofuran), with the use of a Teflon bag (A) and the Hex arm (B). The concentration in ppb is plot against the time in minutes.

In figure 10, the opposite of the previous graph is done. Here, the three different VOCs are compared while the average of the individuals is used. In the graph, it becomes clear that menthofuran stayed constant over the different timepoints, both with the use of a Teflon bag and the Hex sampling arm. Eucalyptol seemed to change the most over time.

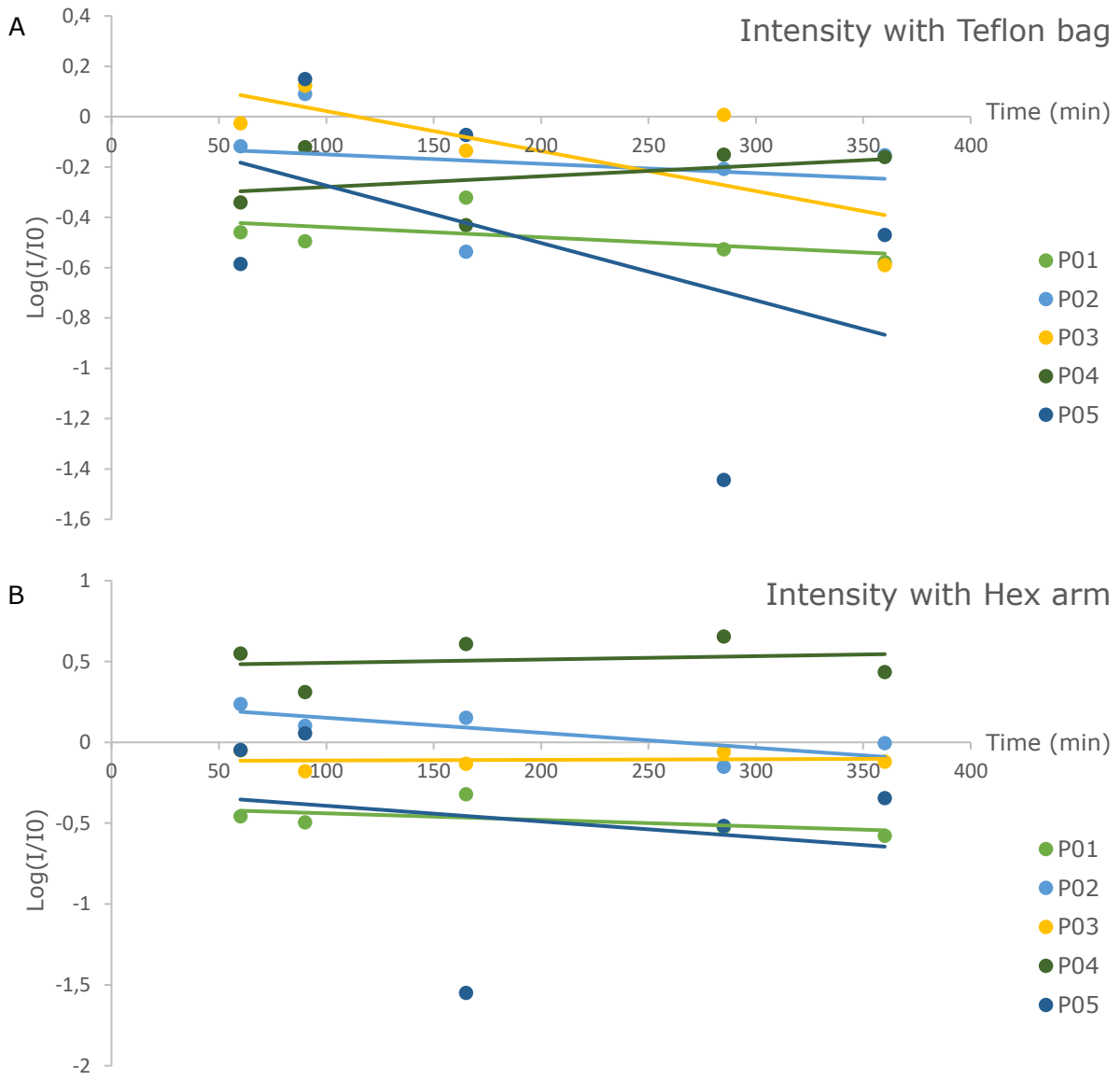

Figure 11: The intensity of the average of the three peppermint VOCS (eucalyptol, gamma-terpinene and menthofuran) for five different individuals. The variation log of the ratio of the intensity of the VOCs at different timepoints is plot against the intensity at baseline. This is shown for both the Teflon bag (A) and the Hex sampling arm (B).

Finally, the intensity was calculated (figure 11). A decrease of the intensity over time was expected and this was reached by every individual, except for P04, with the use of the Teflon bag. Compared with the Hex sampling arm, P04 again showed a slight increase, and P03 seemed to be invariable.

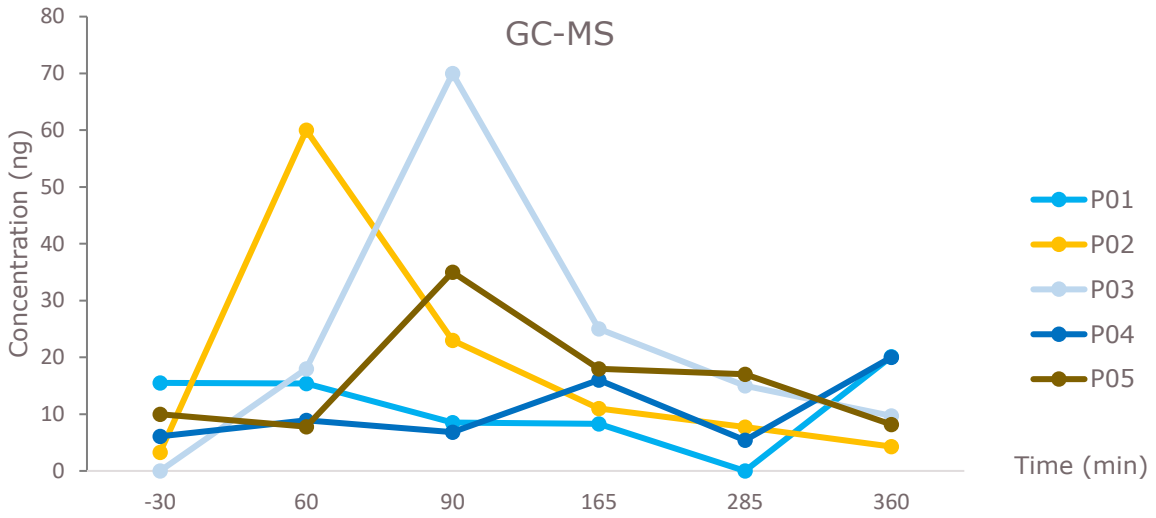

Figure 12: The peppermint washout of five different individuals, with the use of sorbent tubes and measured with the GC-MS. The VOC measured here was eucalyptol. The concentration in ng is plot against the time in minutes.

The same samples were measured with the GC-MS, with the use of sorbent tubes. Figures 12 and 13 show a much lower baseline measurement, than the results of the SIFT-MS. 60 and 90 minutes showed the highest concentration, depending on the individual and after 90 minutes, the concentration of eucalyptol decreased and became baseline again. Remarkable in figure 12 is the near constant concentration of P01 and P04.

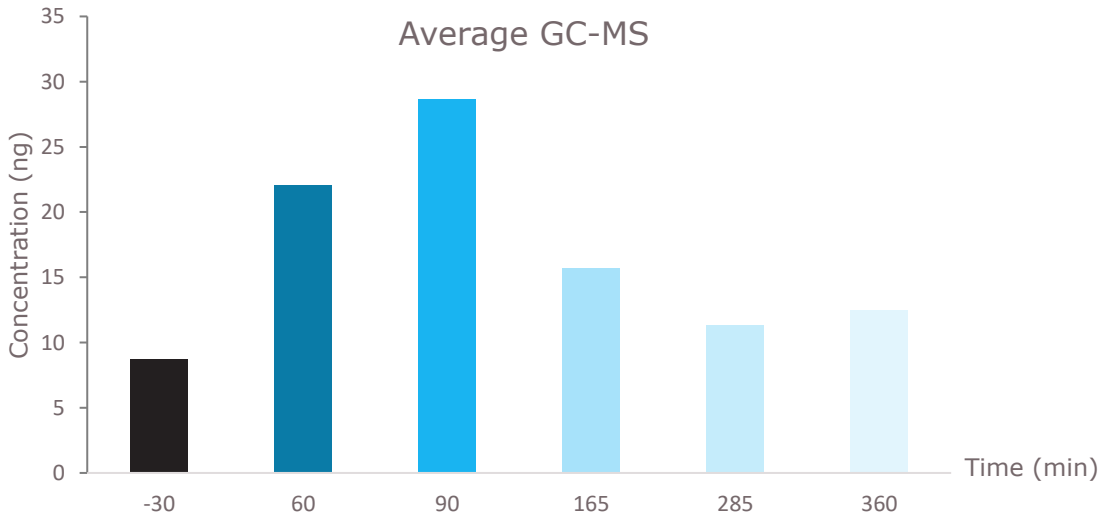

Figure 13: The peppermint washout experiment with the use of sorbent tubes and measured with the GC-MS. These are the results of eucalyptol found in the exhaled breath of five individuals. Black is the baseline measurement. The concentration in ppb is plot against the time in minutes.

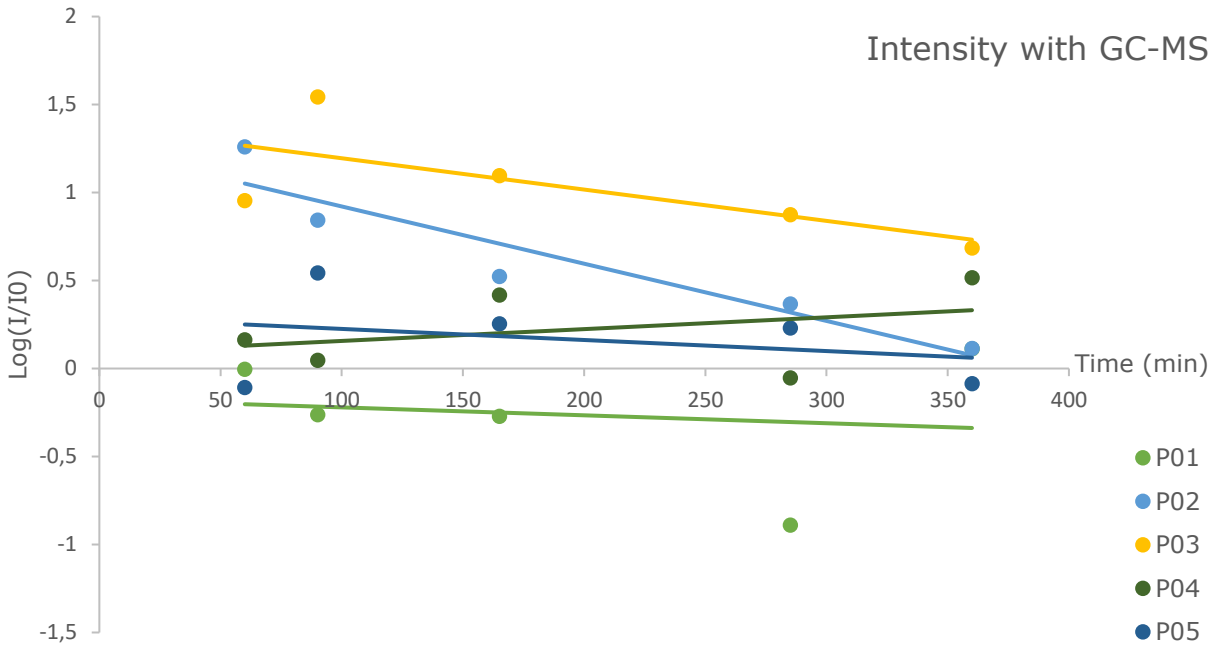

Figure 14: The intensity of the average of eucalyptol for five different individuals. The variation log of the ratio of the intensity of the VOCs at different timepoints is plot against the intensity at baseline.

The intensity with the GC-MS can be seen in figure 14. P04 still had a rising trend. Normally, it is expected to have the same slope for all individuals even when they did not have the same starting point, however, this was not the case. Compared to the intensity with the SIFT-MS, see figure 11, more of the intensities were positive (above zero). This means that the washout samples were higher than the baseline measurement.

4. Discussion

The first hypothesis of this thesis, "there is a molecular difference between rattlers and wheezers in exhaled breath", could not be answered. The second hypothesis however, "the SIFT-MS is equal to or better than the golden standard of breathomics, GC-MS", has been disproved based on the peppermint washout study. The GC-MS showed better results and less hassle than the alternative method.

4.1 Efficiency clean air supply

The CASPER® clean air supply scrubs the ambient air so that the air that the individual breaths in using the ReCIVA (Respiration Collector for In Vitro Analysis) breath sampler is free of exogenous volatile organic compounds that are floating around the air in the room. The CASPER® is efficient when the concentration of VOCs after scrubbing are lower than the concentration of VOCs in the ambient air.

The standard flow volume of 40 L/min is perfect for use with adults, however, since this study revolves around infants and their noisy breathing, a reduced flow volume would be beneficial. The results show that the CASPER® works equally well with a flow volume of 15L/min as with the standard, 40L/min. 84% of the VOCs are scrubbed from the ambient air when using a flow volume of 15 L/min, compared to 83% when using 40 L/min.

Some differences between m/z values were not significant or were even higher after scrubbing than in the ambient air. This contradicting data could be explained by contamination caused by long tubing. VOCs can be released from the tubing when air is streaming through, or VOCs can diffuse from the outside into the tubing. Additionally, the material of the scrubber itself could be a source of contamination. CASPER® uses a scrubber made of airpel 10 (Desotec Ltd, Roeselare, Belgium), made from activated carbon. This type of material is very common in clean air experiments (33).

4.2 Validation parameters

The efficiency of the SIFT-MS was tested using the volatile compound p-xylene. All validation parameters were within range. These parameters were almost equal for high and low concentrations. High concentrations showed only slightly better results, except for relative repeatability: 0.89% for low ppb's and 2.74% for high ppb's. The slightly better results for the high concentrations can be explained by the limit of detection; this was 1.58 ppb, meaning that the lowest concentration of 1 ppb is below the detection limit. This influences the validation parameters in a bad way, whereas the high concentrations are perfectly within the detection range.

4.3 Peppermint washout experiment

4.3.1 Set-up experiment

This study is a collaboration between different research facilities in Europe, with the intention to standardize exhaled breath analysis. Standardization of breathomics could speed up the development of new and even more sensitive tests for threatening diseases. This type of breath analysis can lead to new non-invasive, more comfortable tests that can be used on patients and public health studies; instead of the current tests such as coughing up sputum, blood collection or a wash of the lungs. During this study, a change in the exhaled breath profile was measured. Each subject ingested a peppermint capsule. In the stomach, the capsule is dissolved, and peppermint oil is released. The volatile components in the oil are taken up in the bloodstream and are detectable in the exhaled breath, since VOCs in the bloodstream are exchanged with air in the lung's alveoli. Exhaled breath is thus a useful tool to measure metabolic processes in the body in a non-invasive way.

Over time, the subject will metabolize and eliminate these components, and this will be reflected in the decrease of the concentration of volatile components in exhaled breath. Preliminary data shows that the peppermint washout profile varies between subjects, but the decline in time stayed the same. After six hours, the levels of the target VOCs decreased to baseline levels (34).

4.3.2 Explorative tests

An explorative test was performed on one individual to question the set-up of the experiment. The baseline measurement had the lowest concentration and there was a downward trend over time after ingestion of the capsule until the baseline concentration was again reached after 2 hours. This trend was more pronounced for certain compounds. No clear conclusions can be made because of the number of subjects.

During the explorative test, the Hex sampling arm on the SIFT-MS was compared to the use of Teflon bags; both techniques are used for "online" or direct measurement onto the SIFT-MS. Tedlar bags are the most commonly used breath collection containers in research, which is comparative to Teflon bags; they work the same way, but the latter are more robust (35). Our results show almost a doubling of the concentration measured with the Hex arm compared to the Teflon bag. This could become a problem when the concentration in the bag is below the detection limit of the SIFT-MS.

4.3.3 Validation

The instrument was calibrated by adjusting the branching ratios and k-values relative to the values included in the software. When the precursor ion reacts and collides with the sample VOC, this can lead to the formation of multiple product ions. The branching ratio indicates the distribution of these multiple product ions. The k-value or reaction constant determines how much product is formed during a reaction. When the value is higher, the reaction is faster, leading to the formation of more product ions. The value is determined by the reaction parameters, such as pressure, temperature and flow of the carrier gas, and is constant when these parameters stay the same.

The importance of these parameters is clear: an expected concentration of 100 ppb corresponded with a concentration of 18.48 ppb when using the parameters of the SIFT-MS library. After adjustment of the parameters, the concentration was 104.64 ppb; a much more agreeable result. The fault could lie in how the SIFT-MS software calculates its results: the SIFT-MS uses a tolerance ratio of 20% which means that when the results of the ions are 20% bigger than the lowest concentration of one of the ions, they are not included into the calculation. For example: H_3O^+ gives a concentration of 5.8 ppb, whereas O_2^+ is 98.21 ppb and NO^+ is 103.68 ppb. This would mean that only the concentration of H_3O^+ is used for the calculation of this VOC.

The validation parameters for eucalyptol, gamma-terpinene, menthofuran and menthone were calculated; these validation parameters determine the accuracy of the SIFT-MS for these VOCs. Menthol was excluded due to bad initial results. What immediately stands out is the low trueness (67.08%) and the high relative reproducibility (18.35%) and measurement uncertainty (69.62%) for gamma-terpinene. The maximum accepted measurement uncertainty is 50%. Also, the limit of detection and limit of quantification of menthone is very high. Menthone was excluded from the benchmark study due to these bad results. Eucalyptol, gamma-terpinene and menthofuran were included.

4.3.4 Benchmark study results

The baseline measurement of the benchmark results is remarkable; it is very high, whilst it should be the lowest concentration. It is unclear why that is the case for the experiment. The first washout sample should have the highest concentration, which is correct for the Hex sampling arm, but the difference between baseline and 60 minutes is negligible. Also, this does not correspond to the measurement with the Hex sampling arm. From 90 minutes onwards, we do see a slight decline or rather a constant between the time points.

There is a lot of variation between the subjects and no pattern can be discerned. The original intention was to include ten individuals, however, due to time constrictions this number was not met. Perhaps a higher population could have led to less variation. Menthofuran stays constant over the different timepoints, showing no effect from the ingestion of the peppermint capsule. Gamma-terpinene is also relatively stable across the different timepoints. Only eucalyptol seems to show great differences over time. This bears the question if menthofuran is taken up into the bloodstream, or the concentration could be under the detection limit, making it impossible to determine correctly. Additionally, when looking at the intensity of the average of the VOCs for each individual, there is a decline for most subjects. However, the slope of all participants should have been parallel, regardless of their starting concentration.

When comparing the SIFT-MS to the GC-MS, the latter seems better based on the acquired results. The baseline measurement with the GC-MS was much lower than the measurements after ingestion of the peppermint capsule, which is what we expected prior to the beginning of the experiments. The washout concentration of eucalyptol is highest at 90 minutes, which could be the timepoint of optimal uptake of peppermint into the bloodstream. After 90 minutes, there is mostly a decrease over time, almost reaching the baseline concentration. Only eucalyptol could be detected using GC-MS. An explanation for this could be the type of sorbent tube used. It could be possible that the sorbent in the sampling tubes does not capture or release the volatile compounds in peppermint. Another explanation is the concentration of the VOCs on the sampling tubes. By pumping a higher volume over the tubes, the concentration will be elevated, making it possible for volatiles with a low concentration to be detectable.

4.3.5 Troubleshooting

It is unethical to ask the subjects to abstain from eating the whole day of the experiment. Consequently, peppermint can be found in the liquids or foods that the subjects ingest, even unbeknownst to them, leading to faulty results. Peppermint is also found in toothpaste, and most subjects were not keen on skipping a day of brushing their teeth.

The subjects were also asked to not consume any dairy products during the course of the experiment, since this can interfere with the release of peppermint oil in the circulatory system. Perhaps this was not respected by all participants. Also, dairy could still be in the stomach of the subjects from their breakfast, again leading to faulty results.

It is possible that something went wrong during the validation of the experiment. Since the parameters of the validation were used to adapt the method that was used for the benchmark study, the fault could be found here. Human inaccuracy could be the cause of faulty results.

Ideal would be to purchase a standard gas cylinder for the validation experiment to rule out any human mistakes. This cylinder would contain known concentrations of a gas. Unfortunately, such standard gasses were not available for the peppermint VOCs. Another way to be sure about the concentration in the Teflon bags is to pump a volume over sorbent tubes and measure this with the GC-MS.

The method that was chosen used 10 ms per m/z instead of 100 ms, what was previously used during experiments. 10 ms is much shorter than 100 ms, and maybe in these 10 ms, the component cannot be found in the breath sample.

The concentration range of the peppermint VOCs is maybe so low that it cannot be detected. The limit of detection is 9.92 ppb for eucalyptol, 7.23 ppb for gamma-terpinene and 10.39 ppb for menthofuran. Any concentration lower than these cannot be measured and gives faulty results.

4.4 Comparison different sampling techniques

4.4.1 Sorbent tubes versus Teflon bag

The advantage of sorbent tubes is that it makes transport of exhaled breath and the VOCs in it possible. Additionally, it leads to the storage of VOCs for multiple days instead of a couple of hours. Disadvantageous is the need for a preconcentration step, making it a longer and more time roving step. However, this is also advantageous because even low concentrations become measurable. Sorbent tubes will not trap every VOC in the exhaled breath. The type of VOC is dependent on the type of sorbent inside the tube. During desorption of the tubes, not every captured VOC will be released (36). However, sorbent tubes can be used on the GC-MS, and Teflon bags not. For this technique, sorbent tubes are a necessity.

Teflon bags can be used directly onto the SIFT-MS; a preconcentration step is not necessary, making it quick and easy. They do not have the tendency to release organic compounds and contaminate the sample. There are also some disadvantages: the VOCs can stick to the wall and even diffuse over the wall of the Teflon bag, resulting in a loss of VOCs. Small VOCs can also diffuse from the outside in, contaminating the sample (33). The filled Teflon bags cannot be stored for long; at most a couple of hours. Evaluation of the Teflon bag showed that the mean recovery of 11 gases was 67.6% and 22.7% after 0.5 and 24 hours sample storage time, respectively (37).

4.4.2 Hex sampling arm versus Teflon bag

The Hex sampling arm is simple because only tidal breathing is needed. The disadvantage: SIFT-MS measures VOCs during the inhale and exhale. This leads to peaks of VOCs (in

concentration), but since the SIFT-MS takes an average along a few minutes, this is not a good representative of the real concentration of the VOCs in the breath. The average of the concentration of the peaks would be more accurate.

With a Teflon bag, there is a continued supply of VOCs in the bag, so there are no peaks, but a flowing line of VOCs (in concentration). The Hex sampling arm is also heated, which is better than a cold Teflon bag, because the cooling of the air inside the bag leads to the VOCs sticking to the wall of the bag and a bad evaporation inside. This can be partially solved by placing the bags inside an oven and using a thermal sleeve; we used this set-up during the benchmark experiment.

Another possibility is using a breath inlet (Syft technologies). This module is a warmed chamber placed upon the SIFT-MS. The chamber is filled with exhaled breath via tidal breathing. The breath is collected and simultaneously send into the SIFT-MS. This solves the problem of the peaks of inhales and exhales. Additionally, what is volatile, remains volatile because the chamber is warmed; this stops condensation.

The biggest advantage of these direct measurements are their possible implications for the medical world: in the future, it would be possible to place a SIFT-MS in the hospital and let patients breathe into the Hex sampling arm, or into a bag when they are not mobile, and be able to predict which disease they have, how the disease is progressing, how they react to medication, etcetera.

4.5 Variation

There are multiple sources of variation in this thesis. Firstly, the production of dilutions: they cannot be measured 100% accurately because of human mistakes and the use of a scale and Pasteur pipettes. The concentration is always a bit higher or lower than intended. The same goes for the injection of the dilution into the Teflon bag. The volume is 2 microliters, but again human mistakes can be made, leading to slightly higher or lower volume in the bag. Additionally, the bags are filled with 9 liters nitrogen, but this can then again be more or less.

The next form of variation is the transport of the Teflon bags. Due to cold weather conditions, taking the bags outside for transport may make the components stick to the wall of the bag. This can even lead to diffusion of the components over the wall, leading to loss of the volume and concentration inside. Finally, since the bags are made of plastic and often reused, slight tears and holes may occur in these Teflon bags. Consequently, the initial volume in the bag will be lower as time goes on since the air can escape through such holes and tears.

5. Conclusion

The results of the peppermint washout experiment do not correspond well to the preliminary data of this multi-institutional study. The reason could be found in multiple directions: the validation, the number of subjects, the sampling, the occurrence of peppermint in daily life, the limit of detection and the SIFT-MS itself.

Peppermint was used in this experiment, but it is something that occurs often in daily life: food, drinks, toothpaste, mouthwash, perfume, cosmetics, etcetera. The intention was to exclude as much peppermint as possible during the experiment and even 24 hours before. However, maybe peppermint was found in resources unbeknownst to the participant.

The second point is the validation of the experiment: this determines the method that is used for calculations of the volatile compounds of peppermint. If something went wrong during the validation, the results during the actual experiment will not be accurate. The source of this faulty validation could be the variation caused by human inaccuracy. This can be checked by testing the concentration of the Teflon bags with the use of sorbent tubes and the GC-MS. An important validation parameter was the measurement uncertainty, which was abnormally high for gamma-terpinene, making the results of this VOC questionable.

The original set-up of the benchmark study asked for ten participants, but due to time constrictions, five people were used. Increasing the number of participants could decrease the large variation that was seen between the different individuals. Maybe then, a clear decrease in time would be seen.

The used sampling methods could be a cause of deviation from the preliminary data. It is unknown which techniques were used to obtain the breath samples in this previous study. Another technique that could possibly be superior are sorbent tubes, it would be interesting to repeat the experiment with the use of these sampling tubes.

Finally, the SIFT-MS seems to be very cumbersome; it was twice defective, causing postponement of the research. More optimization would be beneficious and necessary, before it could ever be implemented into a clinical setting.

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