

## Mémoire, Partim B

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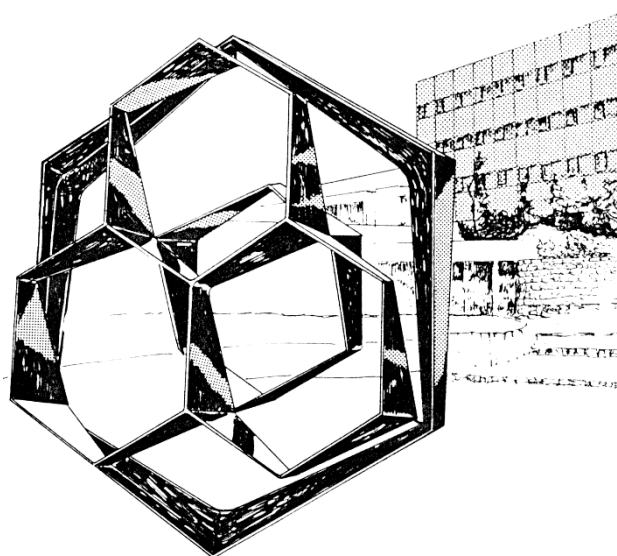
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**Method development and optimization for  
exhaled breath metabolites monitoring**



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## List of abbreviations

ATIS: Adsorbent tube injection system

EIC: Extracted ion chromatogram

E-nose: Electronic nose

GC: Gas chromatography

GCxGC: Comprehensive two-dimensional gas chromatography

GR/Car B: Tenax® GR/Carbopack™ B

HRTOFMS: High resolution time of flight mass spectrometer

IMS: Ion mobility spectrometry

IABR: International association of breath research

IUPAC: International union of pure and applied chemistry

PTR-MS: Proton transfer reaction mass spectrometry

QC: Quality control

R<sup>2</sup>: Coefficient of linear regression

%RSD: Relative standard deviation percentage

SIFT-MS: Selected ion flow tube mass spectrometry

SOP: Standardized operational procedure

TA/Carb B : Tenax® TA/Carbopack™ B

TD: Thermal desorption

TD-GCxGC-HRTOFMS: Thermal desorption unit linked to a comprehensive two-dimensional gas chromatography coupled to a high-resolution time-of-flight mass spectrometer

TD-GCxGC-qMS/VUV: Thermal desorption unit linked to a comprehensive two-dimensional gas chromatography coupled to quadrupole mass spectrometer and to a vacuum UV detector

TIC: Total ion chromatogram

TOFMS: Time of flight mass spectrometer

VOCs: Volatile organic compounds

## I. INTRODUCTION

### *I.1. Exhaled breath*

According to the US EPA, volatile organic compounds (VOCs) are “*organic chemical compounds whose composition makes it possible for them to evaporate under normal indoor atmospheric conditions of temperature and pressure*” [1]. VOCs are widely examined for specific analyses such as: cadaveric decomposition [2], smog environment control and air quality [3], food [4], [5], medical field as organ specific VOC profile [6] or exhaled breath [7]. All the VOCs of a sample form the volatilome, which is specific to their source.

Exhaled breath consists in more than thousands of VOCs. These VOCs are the by-products of metabolic activity in the human body and are transported by the blood to the pulmonary alveoli. The pulmonary alveoli make the transfer between blood and breath to deliver the VOCs in the exhaled breath. The VOCs defined the volatilome of the subject, linked to the metabolome and thus the health conditions. Some VOCs are specific to some diseases. Therefore, the exhaled breath VOCs can be used for diseases monitoring [8].

However, breath-based disease diagnosis is not obvious. Indeed, the exhaled breath is a complex matrix because of numerous confounding factors impacting the volatilome. There are two classes of breath VOCs: the exogenous and the endogenous. The endogenous VOCs are generated inside the body and results from metabolism processes in cells or tissues. They are the VOCs of interest, described previously. The exogenous VOCs are a result of environmental factors on the subject. The gender, the age, the body mass index, the exercise, the time of the day, the diet, the drugs taken, and the smoking habit are the most common confounding factors [8]–[10].

Despite of the matrix complexity, the use of exhaled breath analysis for disease diagnosis is increasing. Wislon and Baietto reported a list of diseases associated to their specific smell in exhaled breath. For example, the smell of ammonia can be related to diabetes or uremia, and liver failure can be characterized by a fish smell decayed or fishy smell [11]. In the past decades, different studies revealed the potential of breath analyses to diagnose a wide range of diseases. N. Zetola *et al.* showed that VOC pattern could diagnose tuberculosis pneumonia [12]. Schleich *et al.*

demonstrated that VOCs are able to discriminate asthma phenotypes [13]. Adiguzel *et al.* brought out the diagnostic of lung cancer thanks to breath sensor [14].

Thanks to these scientific findings, exhaled breath analysis is becoming a promising medical research area. It could complement blood or urine tests for specific disease. Indeed, the breath tests are easy to conduct, not invasive and patient compliant. However, compared to blood or urine tests, the breath matrix is less stable because of the environment, the patient diet and lifestyle, and the sampling itself that could induce variations in the exhaled breath profile.

There are different technologies to sample and to analyze the exhaled breath. As the trained dogs tracking narcotic or dead body, there are trained dogs detecting some disease such as cancer [15], Alzheimer [16], Parkinson, epilepsy, narcolepsy and diabetes [17]. Dog's smell is extremely developed and can smell emitted VOCs from the body, sometimes characteristic of disease. A biomimetic technology to sample breath is inspired from the nose of trained dog, the electronic nose (E-Nose). The E-nose is composed of several sensors, specific to their disease detection. Such technology avoids the dog presence in hospital. Because the exhaled breath is directly analyzed, the results are obtained in real time.

Selected ion flow tube mass spectrometry (SIFT-MS) and proton transfer reaction mass spectrometry (PTR-MS) are other exhaled breath analysis technologies that provide the results in real time with the direct analysis of VOCs present in exhaled breath. SIFT-MS is based on the ionization of the VOCs by collision with previously selected ions. Then, the ionized VOCs are detected by a quadrupole MS [18]. The PTR-MS ionizes the VOCs by proton transfer ( $H^+$ ). The ionized VOCs are detected by a time of flight mass spectrometer (TOFMS) [19].

Ion mobility spectrometry (IMS), gas chromatography (GC) and two-dimensional gas chromatography (GC×GC) are other instruments used for exhaled breath analyses. For the IMS, the VOCs are ionized and separated according to their mobility (influenced by bulk and the charge) under an electric field [20]. The GC and GC×GC separates the VOCs according to their physical and chemical properties [21]. For the IMS, the GC and the GC×GC technology, the exhaled breath need to be collected in a collection device, such as Tedlar<sup>®</sup> bags, and then concentrated onto thermal desorption (TD) tubes.

Lots of other studies on breath analysis have been conducted today but none of them standardized and compared the different analytical techniques with each other, neither the different sampling methods of breath. Standardization and comparison are essential tools to progress in new findings to ensure inter-laboratory reproducibility.

## *1.2. Peppermint Initiative*

The peppermint Initiative was created to address these essential points of standardization and comparison of analytical methods for exhaled breath analysis. This project is an initiative of the international association of breath research (IABR). It is inspired by the Metabolomics Standard Initiative, who addresses the problem of standardization in metabolomics research. The peppermint Initiative aims to establish an inter-laboratory screening of breath analysis results between research groups. The obtained results should represent the variability range between laboratories and different types of instrumentation. This is essential to reach the standardization of exhaled breath analysis [22], [23].

This benchmarking study has been developed by Dr Charles L. Paul Thomas group at the University of Loughborough (UK) who established the standardized protocols and is centralizing the results [22]. All the participating laboratories have to follow an identical standardized workflow (Figure 1): sampling the exhaled breath of at least ten different individuals at different given time points after the ingestion of a peppermint oil capsule. Afterward, the exhaled breath samples are analyzed by the instrument specific to the analytical platforms. The signals obtained have a profile of washout curve, representing the evolution of peppermint oil metabolites in the exhaled breath, over time [22].

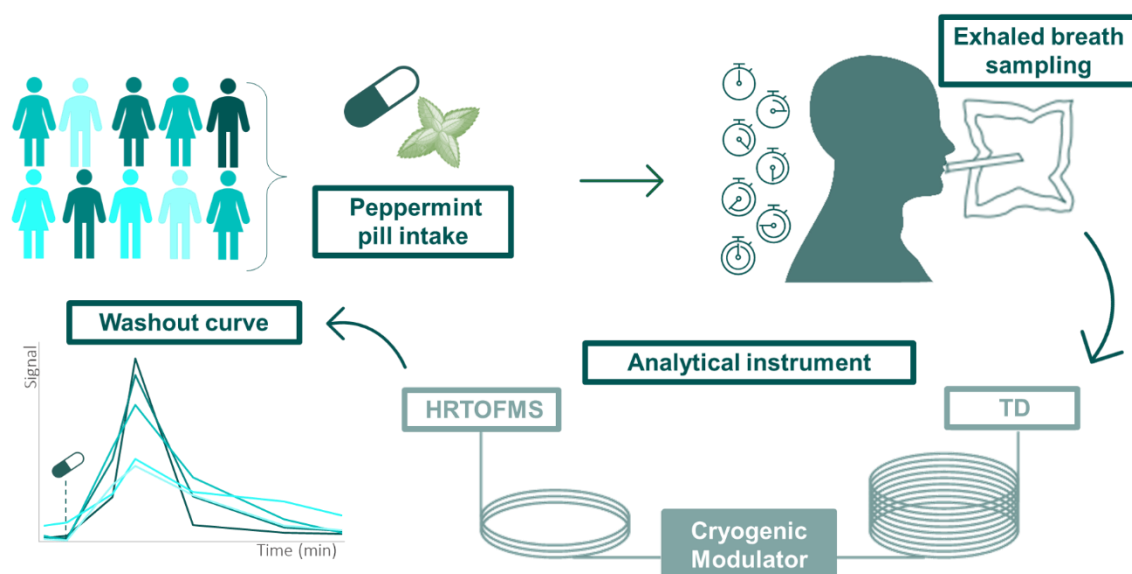


Figure 1: Graphical abstract of the peppermint Initiative applied in the OBiAChem laboratory

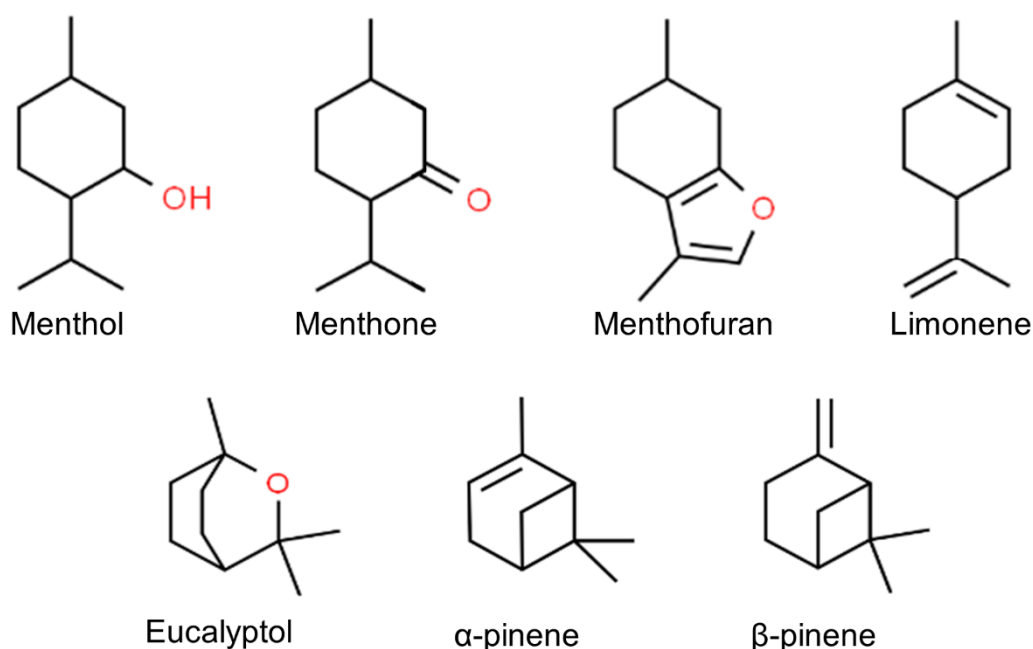
Indeed, the ingestion of peppermint oil induces variations in the exhaled breath profile because of the metabolization of the pill [8]. After the pill intake, the gelatin wrapper is dissolved in the stomach. The contained peppermint oil is released. The peppermint metabolites are reabsorbed along the digestive system by the alimentary canal cells before joining the blood system. When the metabolites reach the pulmonary alveoli, they are transferred from the blood to the breath and are eliminated from the body by the exhaled breath, where they can be detected as VOCs [8].

The standardized workflow is simple but could be affected by numerous factors [22]. There are factors associated to the tested individuals, factors associated to the pills, and factors associated to the laboratories. First, regarding the factors associated to the tested individuals, we can particularly cite the exogenous factors such as the environment, the time of the day, and endogenous factors such as the lifestyle, the diet of the subject, and the digestive system. Concerning the pills, the thickness of wrapper could influence the liberation time of the peppermint metabolites in the organism and the concentration by pill could be different. Finally, for the factors linked to the laboratories, we can cite the differences in instrumentation used both for the sampling and for the analysis [22].

This interlaboratory study aims to compare standardized protocols based on the results obtained from different laboratories using different instrumentation [24]. Consequently, no limitation of these laboratory variations will be done. However, to limit the variation of the breath profile, the concentration of peppermint components must be as similar

as possible and thus, the peppermint capsules used from all the laboratories are coming from the same industrial batch. This latter limits the variation of the wrapper's thickness. The variation of the breath profile due to exogenous factors cannot be limited and must be kept in mind when data are treated [24].

Before the creation of the peppermint Initiative, P. Thomas *et al.* studied the peppermint oil washout curve in their laboratory by PTR-MS and GC-MS [24]. They found several terpene compounds in the peppermint oil and analyzed the peppermint oil metabolites in the exhaled breath. Among those compounds, seven were found in the exhaled breath, with a sufficient concentration: the menthol, the menthone, the menthofuran, the limonene, the eucalyptol, the  $\alpha$ -pinene and the  $\beta$ -pinene (Figure 2). They were defined as the compounds of interest for the peppermint Initiative [24].

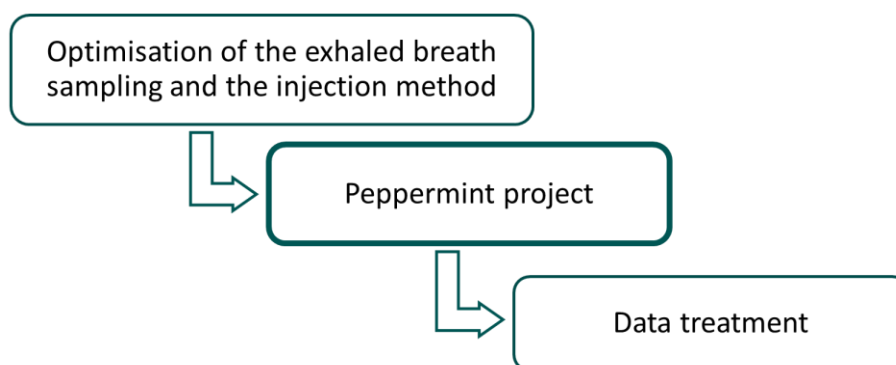


*Figure 2: Chemical structure of the compounds of interest of the peppermint Initiative<sup>1</sup>*  
*See Table 2 on page 28 for more information about these metabolites (boiling point, retention index, exact mass, ...)*

<sup>1</sup> Chemical structure images come from the chemical base ChemSpider

## II. OBJECTIVES

The peppermint Initiative was carried out in the OBiAChem Laboratory. Within the OBiAChem laboratory, the peppermint Initiative was called the “peppermint project” and will be referred to as such in the rest of the master thesis. The peppermint project was the main objective of this master thesis. The laboratory is specialized in gas chromatography and especially comprehensive gas chromatography coupled to mass spectrometry. The instrument used to complete successfully this project consisted of a thermal desorption unit linked to a comprehensive two-dimensional gas chromatography coupled to a high-resolution time-of-flight mass spectrometer, the TD-GC×GC-HRTOFMS. The instrument is described in the section *III.1. Materials: TD-GC×GC-HRTOFMS* on page 15.



*Figure 3: Structuration of the master thesis*

As resumed in the Figure 3, the master thesis project was separated in three objectives. The peppermint project, introduced earlier and developed in the sub section “Peppermint project” on page 52, was the central objective. However, before carrying out the peppermint project, optimization of the instrumental method and the exhaled breath sampling were required. The optimization is an essential part of this project and is resumed in the sub section “Exhaled breath sampling and method optimization” on page 29. The last aim concerned the data handling and data treatment of the peppermint project results to evaluate the procedure and instrumental reproducibility for exhaled breath analysis and to determine the most suitable compounds to monitor the exhaled breath for the peppermint project.

### III. MATERIALS AND METHODS

#### *III.1. Materials: TD-GC×GC-HRTOFMS*

TD-GC×GC-HRTOFMS is a highly powerful analytical instrument for complex volatile molecules analysis [25]. Indeed, the use of TD provides high capacity and reliable trapping of the VOCs on trap tubes. The GC×GC allows separating the numerous compounds of the exhaled breath according to their physical and chemical properties in a more resolved way than classical GC. The mass spectrometer supports the identification of the eluted molecules according to their mass and their specific fragmentation. Therefore, TD-GC×GC-HRTOFMS is the powerful instrument for breath analysis [25].

##### III.1.A. Injection: thermal desorption (TD)

###### *i. Sampling onto TD tubes:*

The complexity of the exhaled breath matrix and the low concentrations of most of the VOCs make exhaled breath analysis challenging. Fortunately, TD allows injecting the entire sample in the GC×GC and minimized the sample loss. Moreover, it allows to detected trace level analytes by a pre-concentration of the analytes. Therefore, TD turns into the preferred solution for the extraction. Concerning the complexity, the GC×GC-HRTOFMS has high separation and detection power.

To sample the exhaled breath, the subjects blow normally into a bag to fill it. When the bag is fully filled, it is connected to a TD tube itself connected to a pump (Figure 4). The pump sucks the exhaled breath contained in the bag at a flow of 185 mL/min and the present VOCs are concentrated onto the TD tube. The standardized operational procedure (SOP) of the exhaled breath sampling can be found in the Annex 1.



*Figure 4: Concentration of the exhaled breath, contained into a Tedlar® bag, onto a TD tube*

The TD tubes are stainless steel tubes filled with different adsorbents [26]. In this study, we used TD tubes containing a mixture of Tenax<sup>®</sup> GR and Carbopack<sup>™</sup> B. The trapping of the VOCs concentrates the VOCs onto the TD tubes, remaining representative to the sample composition. A wide range of sorbents having specific properties are available. In general, the sorbents must be inert and not interact chemically with the sample. Moreover, the sorbents must cover a wide range of physico-chemical affinities with VOCs in order to catch as many of them as possible and therefore must have a given sorbent strength. However, the sorbent strength must be appropriate to permit the complete desorption of the target VOCs (*from the TD tubes to the GC column*). Finally, the sorbent should be hydrophobic. The retention of water vapor in the TD tubes could cause damages to the GC columns [26].

Tenax<sup>®</sup> sorbent is a polymer resin of 2,6-diphenylene-oxide and Carbopack<sup>™</sup> B is graphitized carbon [27], [28]. The combination of these two sorbents in one TD tube is widely used because of the numerous properties: high thermal stability, high efficiency for the concentration of the target compounds, inert, weak affinity with water vapor, wide range of affinity and complementarity [27], [28].

In a recent study of our group, Franchina *et al.*, exposed that Tenax<sup>®</sup>/Carbopack<sup>™</sup> B tubes were adapted for breath analysis [29]. The mix of these two sorbents helped to catch the largest range of VOCs while providing highly reproducible results.

*ii. Extraction and injection of the sample:*

The TD tubes (Figure 5) containing the VOCs are first flushed at low helium flow and low temperature (40°C) to remove any water that could have been trap. Then, tubes are heated under helium flow to desorb the analytes on a cold trap at -10°C, which enables a refocusing of the analytes. The trap is heated to high temperature to release the VOCs and the gas flow blows them into the GC column.



*Figure 5: TD Tubes (A) without caps, (B) with semi-hermetic caps for the analysis and (C) with storage caps*

For concentrated samples, an injection split can be applied. The whole VOCs adsorbed on the TD tubes are vaporized in the vector gas but only a portion is injected in the column. The remaining part is either recollected on the tube or flushed to waste.

### III.1.B. Separation: Comprehensive two-dimensional gas chromatography (GC×GC)

#### i. Gas chromatography principles

The IUPAC defines gas chromatography (GC) as “a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary (the stationary phase) while the other (the mobile phase) moves in a definite direction and is a gas” [21]. This technique is used to separate the VOCs according to their affinity with the stationary phase or their volatility. A GC system is composed of an injector, a column placed in an oven and a detector. First, the sample is injected and heated to obtain volatile molecules. Then, these molecules are mixed to a vector gas, which carried them through the column. After, the molecules are separated inside the column depending on their affinity with the stationary phase and finally analyzed in the detector. The results are usually represented in a chromatogram.

The obtained chromatogram must be well resolved to treat the data. The resolution between two signals is illustrated in the Figure 6 and defined by the following equation.

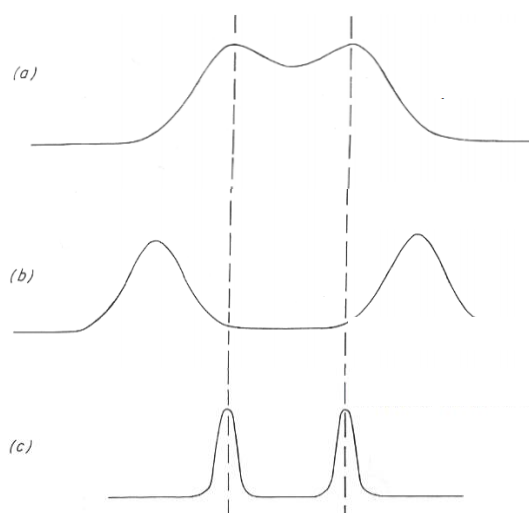


Figure 6: (a) partially resolved peak, (b)(c) well resolved peaks [30]

$$R = \frac{2 \cdot [t_r(B) - t_r(A)]}{W(A) + W(B)}$$

Where  $t_r$  correspond to the retention time and  $W$  to the base peak width. The two peaks are considered as well-resolved when  $R$  is lower than 1,5.

Well-resolved data depend also of the peak capacity of the column. Capacity depends on several characteristics: the sphere's radius of the stationary phase ( $R_s$ ) and the number of theoretical plateaux ( $N$ ). Those parameters are linked according to the formula:

$$\text{Capacity of pic} = \frac{\sqrt[2]{N}}{4 \cdot R_s}$$

The number of theoretical plateaux is related to the length of the column and the height of the theoretical plateau as defined by this equation.

$$\text{HEPT} = \frac{L}{N} \leftrightarrow N = \frac{L}{\text{HEPT}}$$

Because  $N$  is also relied with the length of the column and the height of the theoretical plateau, the capacity of peaks in the chromatogram depends as well on these two parameters.

The peak capacity increases for a high length of column, a low height of theoretical plateau and a small sphere's radius of the stationary phase. Better the peak capacity of the column, more peaks could contain the chromatogram.

For complex mixture, separation could be limited by the peak capacity. To improve this separation, a second column can be added. The principle is described in the next part.

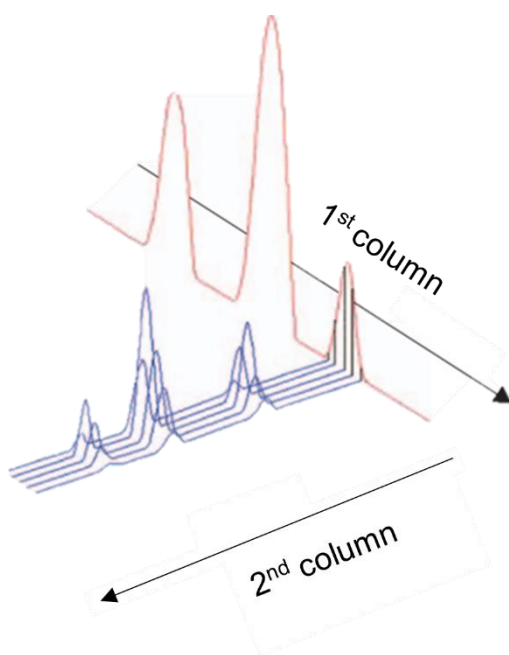
## *ii. The comprehensive principle*

The comprehensive principle tends to increase the peak capacity thanks to the addition of a second column (bringing the second dimension to the chromatography). For the normal orthogonality approach, the compounds are separated according to their volatility in the first column, whereas in the second one, they are separated according to their polarity. In our case, the GCxGC column set was established as the standard set for VOCs analysis; a middle polar column for the first dimension and a polar column for the second dimension. The second dimension brings a better resolution of the coeluting compounds of the first dimension [31].

The comprehensive principle works only when some criteria are considered.

- The stationary phase of the two columns must be composed differently; otherwise, it is just a longer column added and coeluting compounds would not be separated.
- The first dimension must refine at best the separation to separate the remaining overlapped compound in the second dimension.
- The second dimension must be operated in fast conditions compare to the first in order to maintain the separation achieved in the first dimension in the second dimension.

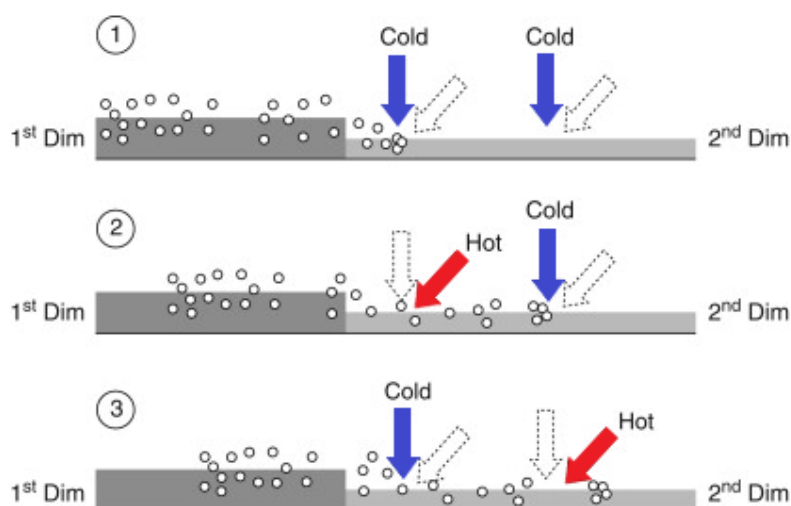
To explain this last criterion, the comprehensive chromatography principle must be detailed. The Figure 7 shows that the obtained peaks of the first dimension are divided in four other chromatograms in the second dimension. The two dimensions are spatially separated; therefore, the acquisition of the second dimension must be quicker than the first, to keep the separation of the first dimension [31].



*Figure 7: Comprehensive principle [31]*

To build the second dimension, the two columns are linked by a modulator. It aims to trap the compounds coming out of the first column during the separation process and reinject them on the second column. In this work, the modulator is a quad-jet dual-stage modulator (Figure 8). This modulator is a combination of a cryogenic gas, to trap the VOCs, and pulses of hot air jet, to reinject the trapped VOCs in the 2<sup>nd</sup> dimension [31].

In concrete terms, the modulator works as follows. First, a cold jet of liquid nitrogen condensates the eluted compounds and trapped them into the cold jet. Then, the cold jet stops and, due to a hot jet of nitrogen, the compounds are delivered in the delay tube. This step delays the injection of the VOCs in the second column to keep the separation obtained in the first dimension. When the delay time is over, VOCs reach a second cold jet of liquid nitrogen which traps and refocuses the compounds. Finally, a second hot jet volatilizes the trapped VOCs in a narrow pulse and focalized them before their injection in the second column [31].



*Figure 8: Working of GCxGC cryogenic modulator [31]*

This kind of modulator is particularly sensitive, perfectly adapted for exhaled breath VOCs but rather expensive due to large amount of cryogen fluid required [31].

### III.1.C. Detection: Mass spectrometry (HRTOFMS)

Because of the powerful separation resulting from the GCxGC, the detector must be appropriate and identify all the eluted compounds. Among all the detectors, mass spectrometer is the most qualified detector. It allows identifying and quantifying the eluted compounds unlike flame ionization detector which is a purely quantitative method [32]. Moreover, the detector must have a high data acquisition rate. During the injection, eluted compounds, coming from the GCxGC, reach perpetually the detector. To keep the separation done in the 2<sup>nd</sup> column, the detector must be able to directly analyze the eluted compounds and in a short time. Because of this limitation, mass spectrometer with low acquisition frequency, such as quadrupole or ion trap, are not fast enough to be by coupled with GCxGC, since coeluting compounds would not be resolved [33]. Indeed, the quadrupole, such as the ion trap, scans and separates in

time the mass of the fragmented ions. A spatial separation of the  $m/z$  fragments is preferred [34].

Practically, at the end of the second dimension, the eluted compounds are fragmented by electronic ionization [34]. The obtained fragments are charged, separated according to their  $m/z$  and finally guided towards the detector of the mass spectrometer. The  $m/z$  separation is created thanks to the analyzer, the TOFMS. Ionized fragments with approximatively the same kinetic energy are accelerated toward the detector among a specific distance. Due to the kinetic law, they will reach the detector more or less fast according to the  $m/z$  [34].

$$E_{\text{kinetic}} = \frac{1}{2} m \cdot v^2, \text{ where } m \text{ is the mass and } v \text{ is the velocity}$$

The acquisition time of the spectra is extremely short and allows obtaining mass spectra for each eluted compound in the GCxGC.

Reflectron could be placed to increase the distance to travel and thus improve the mass separation and the resolution. The high-resolution TOFMS (HRTOFMS) has more than one reflectron whereas classical TOFMS has only one. However, with a longer ion flow path distance, the heavy fragments may be lost in the reflectron, the number of heavy fragments decrease and thus the sensibility as well [34].

The resolution in mass spectrometry is based on the same principle as in GC; the ability to discriminate 2 masses ( $m_1$  and  $m_2$ ), separated by a  $\Delta m$  defined by the equation:

$$R = \frac{m}{\Delta m}$$

The higher the resolution of the MS is, the better close masses are separated, and the purer the MS signals are. High resolution can be accompanied by high accuracy when proper MS calibration is carried out. This allows the production of high accuracy MS signals that, when parent ions are preserved, can be used to determine molecular formulae and even allows the identification of the compound [34]. This is typically the case for VOCs when the HRTOFMS offers an accuracy below 1 ppm.

### III.1.D. TD-GC×GC-HRTOFMS: instrumentation for exhaled breath analyses

TD-GC×GC-HRTOFMS gives a three-dimensional<sup>2</sup> chromatogram with an accurate mass spectrum for each peak. Therefore, complete information about the sample is obtained. The instruments were chosen to be adapted to exhaled breath analysis and are advanced technologies.

The TD unit allowed injecting the totality of the sample without any loss. Moreover, the sorbent of the TD tubes was chosen for an optimal sorption of the exhaled breath VOCs.

The GC×GC unit was used for an optimal separation of the complicated exhaled breath matrix and their VOCs. The cryogenic modulator re-focalizes the analytes before injecting them into the second column which give thinner and more intense compounds. This type of modulator gives a better resolution of the peaks.

Finally, the HRTOFMS contributed to a high-resolution quantification and a qualification of the exhaled breath VOCs.

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<sup>2</sup> The retention time in the first column, the retention time in the second column and the intensity measured

### III.2. Experimental parameters

Breath analysis is carried out frequently in the OBiAChem laboratory [13], [29], [35]–[37]. This is the reason why the experimental parameters, used for this study, were already developed, and optimized. These parameters are described in the following table.

Table 1: Experimental parameters used for the master thesis

#### SAMPLING

<i>Pump</i>	MARKES International <sup>TM</sup> - Acti VOC Low-Flow Pump
<i>Pump flow</i>	180-185 mL/min
<i>Bag</i>	Tedlar <sup>®</sup> Bags with push lock valve
<i>Tube</i>	Tenax <sup>®</sup> GR/Carbopack <sup>TM</sup> B

#### TD

<i>Instrument type</i>	MARKES International <sup>TM</sup> TD100-xr
<i>Tube desorption</i>	<i>Temperature: 290°C</i> <i>Duration: 5 minutes</i>
<i>Trap desorption</i>	<i>Temperature: 300°C</i> <i>Duration: 3 minutes</i>
<i>Split</i>	20 (residual portion recollected on the tube)

#### GC×GC

<i>Instrument type</i>	Agilent Technologies <sup>TM</sup> 7890B GC system (LECO)
<i>Helium flow</i>	1 mL/min
<i>First column</i>	Mid polar - 30m×250µm×1.4µm – Rxi624-SilMS <i>Initial temperature: 40°C</i> <i>Duration: 3 minutes</i> <i>Ramp temperature: 5°C/min</i> <i>Duration: 32minutes</i> <i>Ramp temperature: 10°C/min</i> <i>Duration: 4minutes</i> <i>Final temperature 240°C</i> <i>Duration: 3 minutes</i>

<i>Second column</i>	Polar - 0.1m×250µm×0.5µm - Stabilwax <i>Initial temperature: 45°C</i> <i>Duration: 3 minutes</i> <i>Ramp temperature: 5°C/min</i> <i>Duration: 32minutes</i> <i>Ramp temperature: 10°C/min</i> <i>Duration: 4minutes</i> <i>Final temperature 245°C</i> <i>Duration: 3 minutes</i>
<i>Modulator type</i>	Quad-jet dual-stage cryogenic modulator
<i>Modulation time</i>	4s
<i>Hot pulse time</i>	0.8s
<i>Cool time between stage</i>	1.2s

## **HRTOFMS**

<i>Instrument type</i>	Pegasus™ GC-HRT 4D (LECO)
<i>Ion source temperature</i>	250°C
<i>Electron energy</i>	70eV
<i>Acquisition delay</i>	210s
<i>Acquisition rate</i>	200 spectra/second
<i>Mass range</i>	29 to 450 mu

## **DATA TREATMENT**

<i>LECO ChromaTOF™</i>	Target m/z method
<i>Microsoft excel</i>	Data treatment and data visualization

## IV. RESULTS AND DISCUSSION

### IV.1. Determination of the peppermint VOCs of interest

The preparatory phase of the peppermint project was the identification of the compounds of interest for the peppermint project.

First of all, the leaders of the peppermint Initiative gave a sample consisting of the peppermint standards to each analytical platform, to perform the identification within the peppermint consortium. To realize these peppermint standards samples, a scotty gas cylinder<sup>3</sup> containing the identified compounds of the peppermint oil's pill was used. It allowed to directly concentrate the standards compounds onto the device/equipment specific to the different platform, a TD tube for this study.

This peppermint standards' TD tube was injected in the TD-GC×GC-HRTOFMS to identify the target compounds defined by the peppermint Initiative (Figure 9).

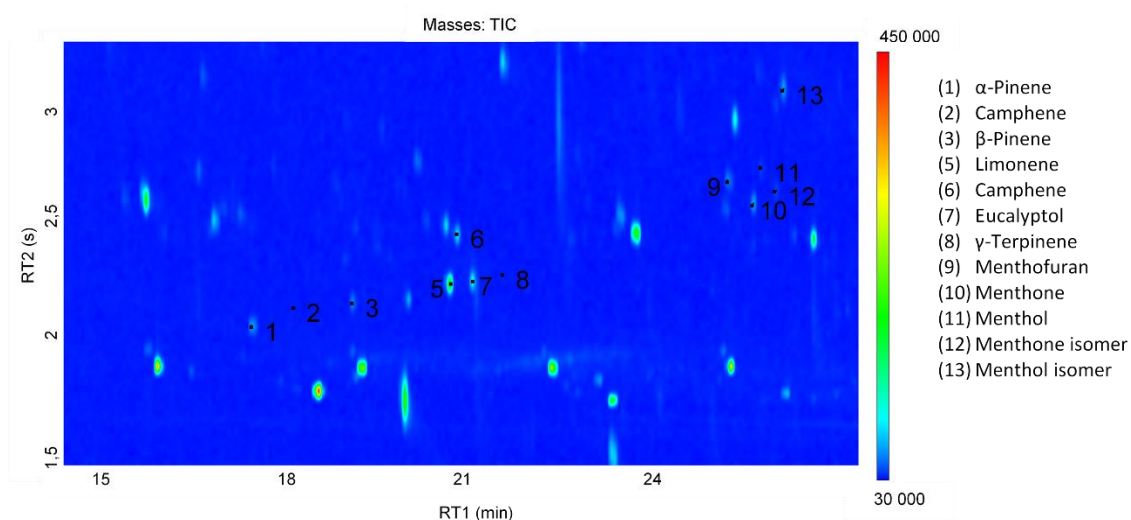


Figure 9: GCxGC chromatogram zoomed on the area of the 13 target compounds of the peppermint experiment

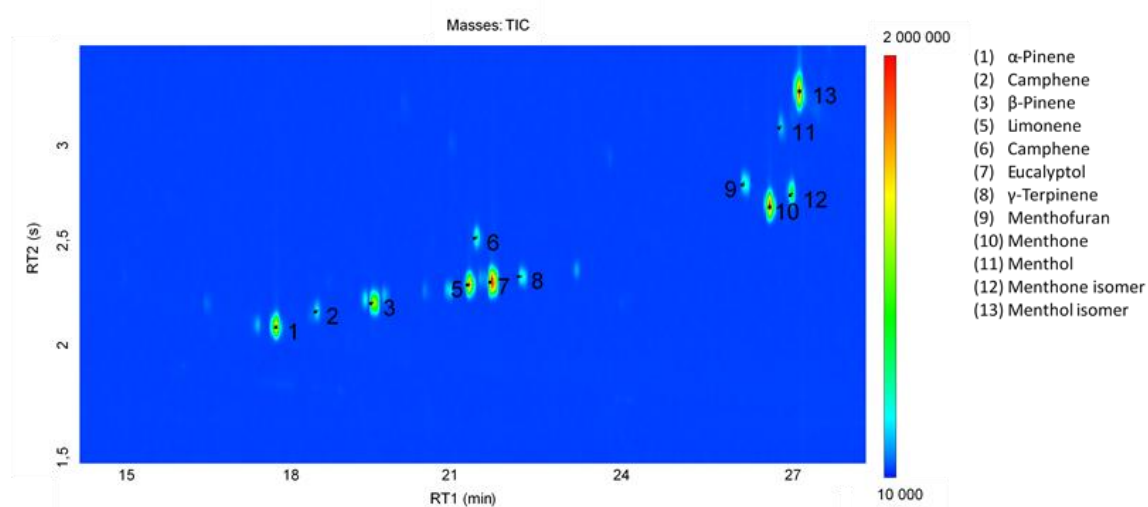
To determine the peppermint target compounds, Michaela Malásková *et al.* analyzed the composition of the peppermint oil and the composition of the exhaled breath after peppermint pill intake [24]. Since the instrument used for this master thesis project is

<sup>3</sup> It is a cylinder containing mix of gaseous standards

different from the one used by the peppermint Initiative leaders, the main analyses were reproduced on the TD-GC×GC-HRTOFMS.

Therefore, two additional experiments were done to be able to confirm the identity of the target metabolites from the peppermint pill to be monitored in the exhaled breath, for our analytical platform.

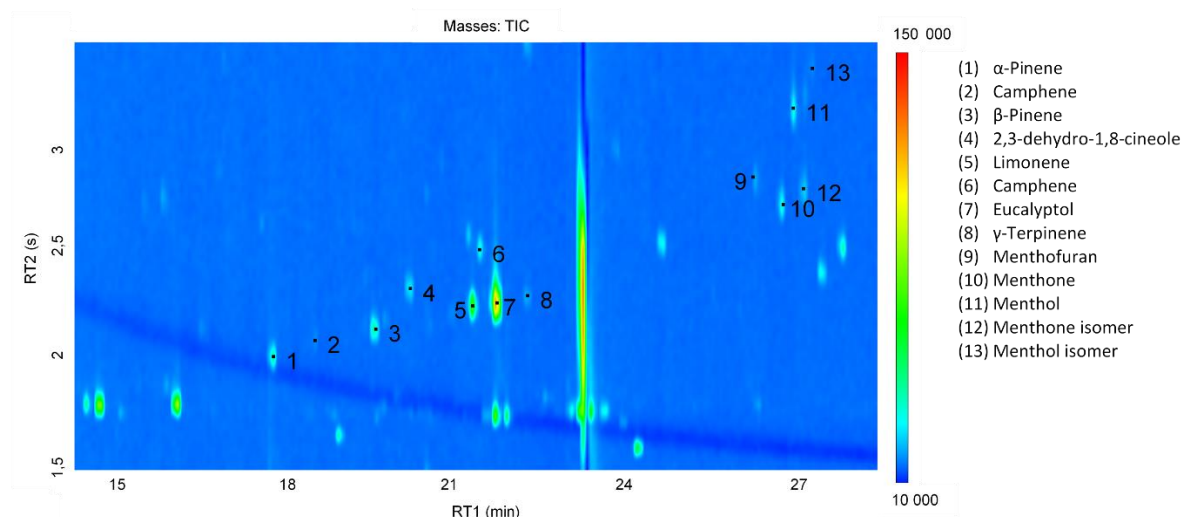
The first experiment consisted of analyzing the peppermint oil based on peppermint Initiative leaders' workflow [38]. The peppermint oil contained in a pill was extracted with a syringe. A total of 400 µL of peppermint oil was collected, corresponding to almost the totality of what is contained in the gelatin envelope. The 400 µL of peppermint oil were transferred in a 20 mL headspace vial. After 5 minutes of equilibrium, the septum was pierced by two hypodermic needles, with one connected to a TD tube, itself connected to a pump. The head space mixture was pumped for 3 minutes, under a flow of 180 mL/min (Annex 4). The result of this experiment can be seen on the Figure 10.



*Figure 10: GC×GC chromatogram zoomed on the peppermint oil compounds area*

The peppermint compounds found in this experiment were also found onto the peppermint standard TD tube (Figure 9). However, there is an additional compound onto the peppermint standard TD tube: the citronellal. The manner to extract the VOCs peppermint oil was slightly different between the two studies. In our study, we used directly a TD tube whereas the reference work used a needle trap device to extract the peppermint oil volatiles. Thus, the sorbent used were different, which may have led to a difference in VOC trapping.

The second experiment focused on the analysis of the peppermint metabolites in the exhaled breath. A first peppermint experiment was realized to monitor the peppermint oil components in exhaled breath over time (Figure 11). The peppermint experiment was the main experiment realized in this master thesis. It refers to the analyses of the sampled exhaled breath by TD-GC×GC-HRTOFMS at several time points (before the pill intake, when the pill is swallowed, and 1h, 1h15, 1h30, 2h45, 4h45 and 6h after the pill intake).

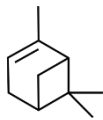
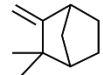
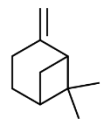
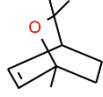
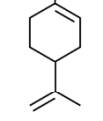
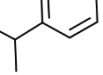
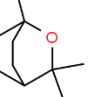
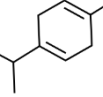
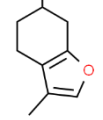
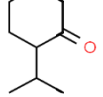
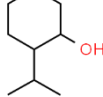


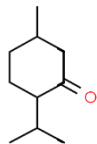
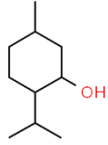
*Figure 11: GCxGC chromatogram zoomed on the peppermint metabolites area found in the exhaled breath 90 min after the peppermint pill intake*

The second experiment results allowed defining the peppermint compounds detected in a sufficient amount in the exhaled breath to be monitored. All these compounds were designated as target peppermint's compounds for this master thesis work and are listed in the Table 2 on page 28. The target peppermint's compound defined by the peppermint Initiative are the compounds\* 1, 3, 5, 7, 10, 11, 13 (indicated by a \* in Table 2). In the "Data treatment" part, on page 54, an extensive analysis was led to define among those thirteen compounds, the most suitable to monitor the exhaled breath with the TD-GC×GC-HRTOFMS, and compared them to the ones defined by the peppermint Initiative.

Table 2: List of the 13 identified metabolites of the peppermint oil found in exhaled breath

(<sup>1</sup>) Not able to identify with certitude, (<sup>2</sup>) Chemical structure images come from the chemical base ChemSpider, \* indicates the target compounds defined by the peppermint Initiative

Name	Chemical structure <sup>(2)</sup>	Formula	Cas-number	<sup>1</sup> t <sub>R</sub> (min)	<sup>2</sup> t <sub>R</sub> (s)	Boiling point (°C)	Exact mass
1 <b>α-Pinene</b> *		C <sub>10</sub> H <sub>16</sub>	2437-95-8	17,8	2,1	155	136,1252
2 <b>Camphene</b>		C <sub>10</sub> H <sub>16</sub>	79-92-5	18,6	2,1	159	136,1252
3 <b>β-Pinene</b> *		C <sub>10</sub> H <sub>16</sub>	127-91-3	19,6	2,2	167	136,1252
4 <b>2,3-dehydro-1,8-cineole</b>		C <sub>10</sub> H <sub>16</sub> O	92760-25-3	20,2	2,4	180	152,1201
5 <b>Limonene</b> *		C <sub>10</sub> H <sub>16</sub>	138-86-3	21,3	2,3	176	136,1252
6 <b>Cymene</b>		C <sub>10</sub> H <sub>12</sub>	1195-32-0	21,4	2,6	177	132,0939
7 <b>Eucalyptol</b> *		C <sub>10</sub> H <sub>18</sub> O	470-82-6	21,7	2,3	176	154,1358
8 <b>γ-Terpinene</b>		C <sub>10</sub> H <sub>16</sub>	99-85-4	22,2	2,4	182	136,1252
9 <b>Menthofuran</b> *		C <sub>10</sub> H <sub>14</sub> O	494-90-6	26,2	2,9	211	150,1045
10 <b>Menthone</b> *		C <sub>10</sub> H <sub>18</sub> O	89-80-5	26,7	2,8	207	154,1358
11 <b>Menthol</b>		C <sub>10</sub> H <sub>20</sub> O	2216-51-5	26,9	3,3	212	156,1514

12	<b>Menthone isomer<sup>(1)</sup></b>		C <sub>10</sub> H <sub>18</sub> O	<sup>(1)</sup>	27,0	2,9	207	154,1358
13	<b>Menthol isomer<sup>*(1)</sup></b>		C <sub>10</sub> H <sub>20</sub> O	<sup>(1)</sup>	27,2	3,5	212	156,1514

It is interesting to note that, in exhaled breath, there is an additional compound which is not found in the peppermint oil, the 2,3-dehydro-1,8-cineole (number 4 in Table 2). This compound is probably a (by-)product of the peppermint pill metabolism. This hypothesis is strengthened by the absence of the compound in the exhaled breath before the pill intake and in the extensive analysis in section “Data treatment - Metabolization pathways and by-product” on page 63.

#### IV.2. *Exhaled breath sampling and method optimization*

This section was realized before the peppermint project. It includes all the optimizations' steps of the peppermint procedure specific to the laboratory realized within the framework of this master thesis. The optimization's steps are applied to the TD tubes, the cleanliness of the bags, the injection parameters, and the type of bags to be used for exhaled breath sampling.

Since the exhaled breath is a complex matrix, it was necessary to control the variation caused by the instruments or the sampling materials by injecting an external standard to each set of analyses. The standard components of exhaled breath were already defined in the laboratory because it is well-developed in exhaled breath analyses. Thus, the standard solution was easy to product.

This standard solution is composed of 19 standards with distinctive physical and chemical properties, covering a wide range of physico-chemical properties. The injection of the 19 standards on a TD tube gave a chromatogram (Figure 12) with standards signals covering the largest volatility range. Each standard signal was defined as a reference point, enabling the identification of compounds with similar properties. The 19 standards are notified in the Table 3 and indexed in the Figure 12

Table 3: List of the 19 exhaled breath standards used

<sup>(1)</sup> Not detected on the chromatogram

Name	Formulae	Cas-number	Concentration (ppm)	<sup>1</sup> t <sub>R</sub> (min)	<sup>2</sup> t <sub>R</sub> (s)	Boiling point (°C)	Exact mass
Methanol	CH <sub>4</sub> O	67-56-1	Solvent			64	32,026215
2,3-Butanediol <sup>(1)</sup>	C <sub>4</sub> H <sub>10</sub> O <sub>2</sub>	6982-25-8	53,0			183	90,06808
1-Propanol	C <sub>3</sub> H <sub>8</sub> O	106-42-3	40,2	5,8	1,2	138	60,057514
2-methyl-2-butanol	C <sub>5</sub> H <sub>12</sub> O	75-85-4	40,2	8,1	3,4	102	88,088145
1-ethyl-3-methyl-cyclopentane	C <sub>18</sub> H <sub>16</sub>	3726-47-4	41,1	12,2	1,9	121	112,1252
2-Hexanone	C <sub>6</sub> H <sub>12</sub> O	591-78-6	40,6	13,5	2,8	127	100,08882
p-Xylene	C <sub>8</sub> H <sub>10</sub>	71-23-8	43,0	16,0	2,8	97	106,07825
Decane	C <sub>10</sub> H <sub>22</sub>	124-18-5	28,0	19,8	1,8	174	142,17215
Undecane	C <sub>11</sub> H <sub>24</sub>	1120-21-4	29,0	23,2	1,8	196	156,1878
1-Octanol	C <sub>8</sub> H <sub>18</sub> O	111-87-5	36,0	23,6	3,7	196	130,13577
Nonanal	C <sub>9</sub> H <sub>18</sub> O	124-19-6	40,0	24,6	2,6	93	142,13577
2-Ethylhexanoic acid	C <sub>8</sub> H <sub>16</sub> O	149-57-5	38,0	26,0	1,0	228	144,11503
2,6-Dimethylphenol	C <sub>8</sub> H <sub>10</sub> O	576-26-1	32,0	26,1	0,0	203	122,07317
2,6-Dimethylaniline	C <sub>8</sub> H <sub>11</sub> N	87-62-7	32,0	27,5	1,8	214	121,08915
Methyl caprate	C <sub>11</sub> H <sub>22</sub> O <sub>2</sub>	110-42-9	42,0	30,6	2,5	108	186,16198
1-Tetradecene	C <sub>14</sub> H <sub>28</sub>	1120-36-1	41,8	31,9	2,0	251	196,2191
Methyl undecanoate	C <sub>12</sub> H <sub>24</sub> O <sub>2</sub>	1731-86-8	42,0	33,4	2,6	246	200,17763
Dicyclohexylamine	C <sub>12</sub> H <sub>23</sub> N	101-83-7	31,0	33,8	2,6	117	181,18305
1-Pentadecene	C <sub>15</sub> H <sub>30</sub>	13360-61-7	41,0	34,6	2,1	268	210,23475
Methyl laurate	C <sub>13</sub> H <sub>26</sub> O <sub>2</sub>	111-82-0	41,0	35,9	2,6	262	214,19328

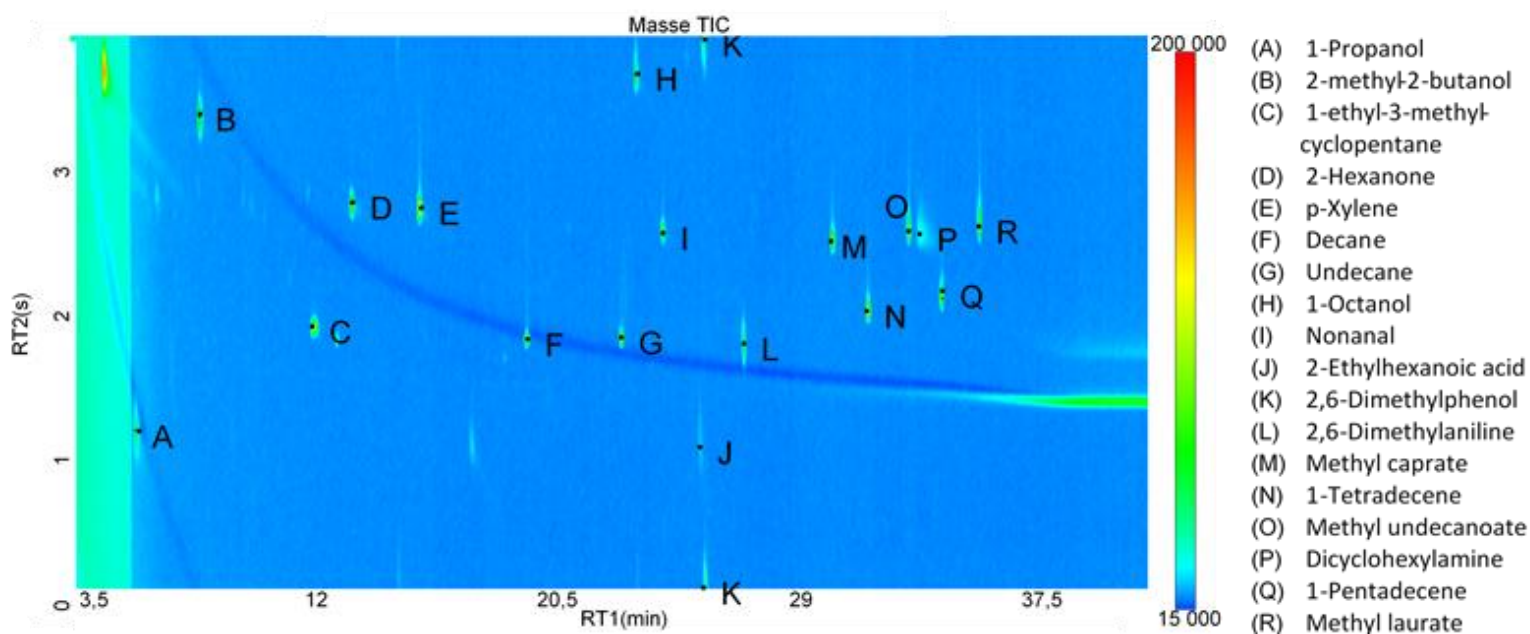


Figure 12: GCxGC chromatogram of 19 exhaled breath standards used

## Verification of the compatibility of the TD tubes' adsorbent to sample exhaled breath

When this project was firstly imagined, it was decided to carry it out with TD tubes made of Tenax<sup>®</sup> TA/Carbopack<sup>™</sup> B, according to the results of a previous study realized in the laboratory [29]. However, TD tubes with similar properties were ordered, the Tenax<sup>®</sup> GR/Carbopack<sup>™</sup> B. Those TD tubes were not analyzed in the previous project [29]. Since the two types of TD tubes were available in the laboratory, a benchmarking study was completed to ensure that Tenax<sup>®</sup> GR and Tenax<sup>®</sup> TA shared similar properties, in terms of selectivity towards the analytes and reproducibility.

This benchmarking study has been conducted with the Adsorbent Tube Injection System (ATIS). The ATIS enables to flash vaporize a specific volume of standard in a bag filled with exhaled breath at 80% full bag's capacity. In concrete terms, nitrogen is sent through a carbon filter to get extra pure nitrogen with a flow set at 100 mL/min. Then, 2 µL of our solution of 19 standards (from 28 to 53 ppm) is flash vaporized at 120 °C for 2 min with a nitrogen flow of 100 mL/min. The full bag, containing the exhaled breath spiked with the mixture of standards, is desorbed onto the TD tubes [29]. This experiment has been repeated 3 times on each type of TD tubes; Tenax<sup>®</sup> TA/Carbopack<sup>™</sup> B and Tenax<sup>®</sup> GR/Carbopack<sup>™</sup> B. The SOP of the ATIS experiment can be found in the Annex 2.

The results of the benchmarking study between Tenax<sup>®</sup> TA/Carbopack<sup>™</sup> B (TA/Carb B) and Tenax<sup>®</sup> GR/Carbopack<sup>™</sup> B (GR/Car B) are presented in the following Table. For the tubes' types, the abbreviations in parenthesis will be used throughout the text.

Table 4: Results of the benchmarking study between sorbent TD tubes (Tenax® TA/Carbopack™ B and Tenax® GR/Carbopack™ B)

. <sup>(1)</sup> Undefined because the target compound was not detected on the three replicates (the average area values and the %RSD are based on triplicates)

<sup>(2)</sup> These values are not taking into account the data of the compound 10 and 16

Compounds		TA/Car B		GR/Car B		p-value
		Average area	%RSD	Average area	%RSD	
1	1-Propanol	1 799 940	5%	1 673 298	2%	0.09
2	2-methyl-2-butanol	1 056 559	1%	1 011 253	8%	0.38
3	1-ethyl-3-methyl-cyclopentane	973 597	3%	945 002	4%	0.40
4	2-Hexanone	1 414 012	9%	1 398 124	1%	0.83
5	p-Xylene	2 087 495	5%	2 082 024	2%	0.93
6	Decane	678 930	2%	689 413	3%	0.52
7	Undecane	965 304	12%	1 158 671	18%	0.23
8	1-Octanol	191 987	6%	267 791	5%	$2.10^{-3}$
9	Nonanal	163 950	14%	191 462	5%	0.13
10	2-Ethylhexanoic acid	Undefined <sup>(1)</sup>		13 771	28%	
11	2,6-Dimethylphenol	185 481	8%	169 311	4%	0.16
12	2,6-Dimethylaniline	55 008	19%	77 741	17%	0.08
13	Methyl caprate	186 465	2%	279 568	5%	$4.10^{-4}$
14	1-Tetradecene	100 685	9%	131 402	7%	0.02
15	Methyl undecanoate	77 461	12%	113 554	8%	0.01
16	Dicyclohexylamine	Undefined <sup>(1)</sup>		14 143	66%	
17	1-Pentadecene	31 385	12%	42 038	7%	0.02
18	Methyl laurate	50 156	13%	53 149	12%	0.61
Average			8%	11%   7% <sup>(2)</sup>		0.27
Median			8%	6%   5% <sup>(2)</sup>		0.15
Range of values			1-19%	1-66%   1-18% <sup>(2)</sup>		

First, by analyzing the average value of the areas of the compounds measured by the extracted ion chromatogram (EIC), we noticed that the most polar and most volatile compounds, i.e., from compound 1 to compound 4, Table 4, had a higher signal with TA/Carb B TD tubes than with GR/Carb B TD tubes. But for the non-polar compounds, the average value was higher with GR/Carb B TD tubes. The difference between the tube types was significant for eight compounds, and particularly for the non-polar

compounds. This probably revealed a slightly better adsorption of the non-polar compounds with the GR/Carb B TD tubes. However, the global average p-value was not significant enough to discriminate the use of one type of tube comparing to the other.

Concerning the relative standard deviation percentage (%RSD), the %RSD of the GR/Car B tubes is generally slightly lower or similar than when using the TA/CarB, indicating a slightly better reproducibility when using GR/Carb B tubes. However, since the global %RSD averages are similar<sup>4</sup>, it proves that the two types of tube stay suitable for exhaled breath analyses.

Then, if we focus on the compound's detection in the triplicates, it is important to notice that the dicyclohexylamine and the 2-ethylhexanoic acid (compound 10 and 16, Table 4) are not detected in each TA/Carb B tubes triplicates. Additional GR/Car B values (in orange in the Table 4) have been calculated without considering those two compounds to see the impact of them and to get a more representative idea of the other compounds' statistical comportment between the two sorbents.

Considering the compounds 10 and 16, Table 4, the range of %RSD values is higher for the GR/Carb B tubes. Nonetheless, the GR/Carb B range of %RSD values would be reduced between 1 to 18% if these two compounds were not considered, which is similar to the GR/Car B's. Because of the two undetected compounds (10 and 16, Table 4), the GR/Car B global %RSD (11%) average value is higher than the TA/Car B's (8%) but would be lowered to 7% if they were not considered. Moreover, since the median GR/Car B %RSD value is slightly lower, the distribution of GR/Car B %RSD values is more important for the lower percentage. The lower is the %RSD values, the better is the reproducibility. To conclude, without considering the compounds 10 and 16, Table 4, as they are close to the signal to noise ratio, the reproducibility of the two sorts of adsorbents is similar since their respective global average and median values are comparable.

Figure 13 demonstrates one more time that the trapping efficiency of the two types of tubes is comparable.

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<sup>4</sup> If we consider the orange %RSD value for the GR tubes, which did not include the dicyclohexylamine and the 2-ethylhexanoic acid. The reason is detailed in the following paragraph.

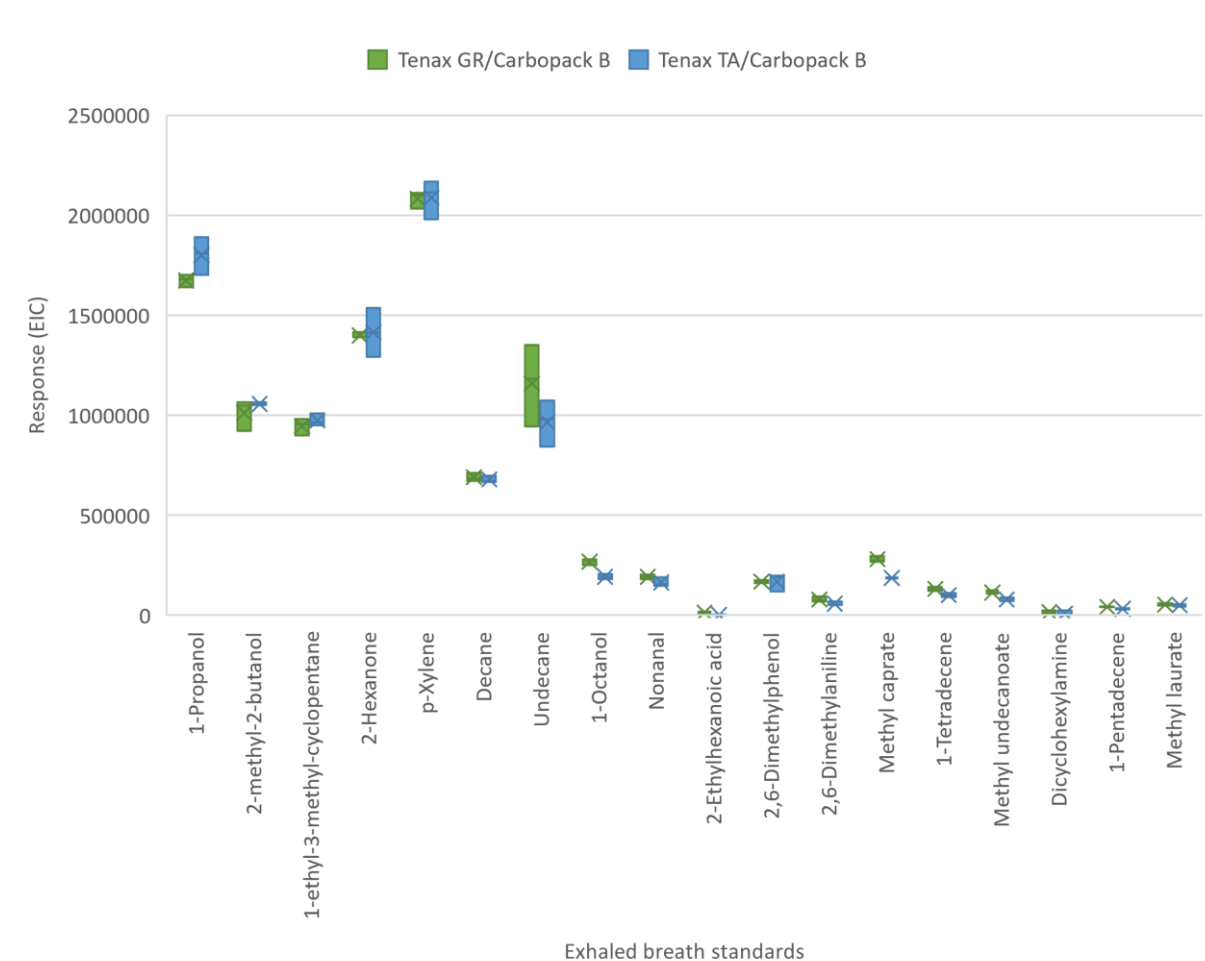


Figure 13: Box plot of Tenax® TA/Carbopack™ B and Tenax® GR/Carbopack™ B for sorbent TD tubes evaluation

Since the two types of TD tubes led to similar extraction efficiency and reproducibility, the two types of sorbent materials are suitable for exhaled breath analysis. Therefore, the brand-new GR/Carb B tubes were used for the following part of the project.

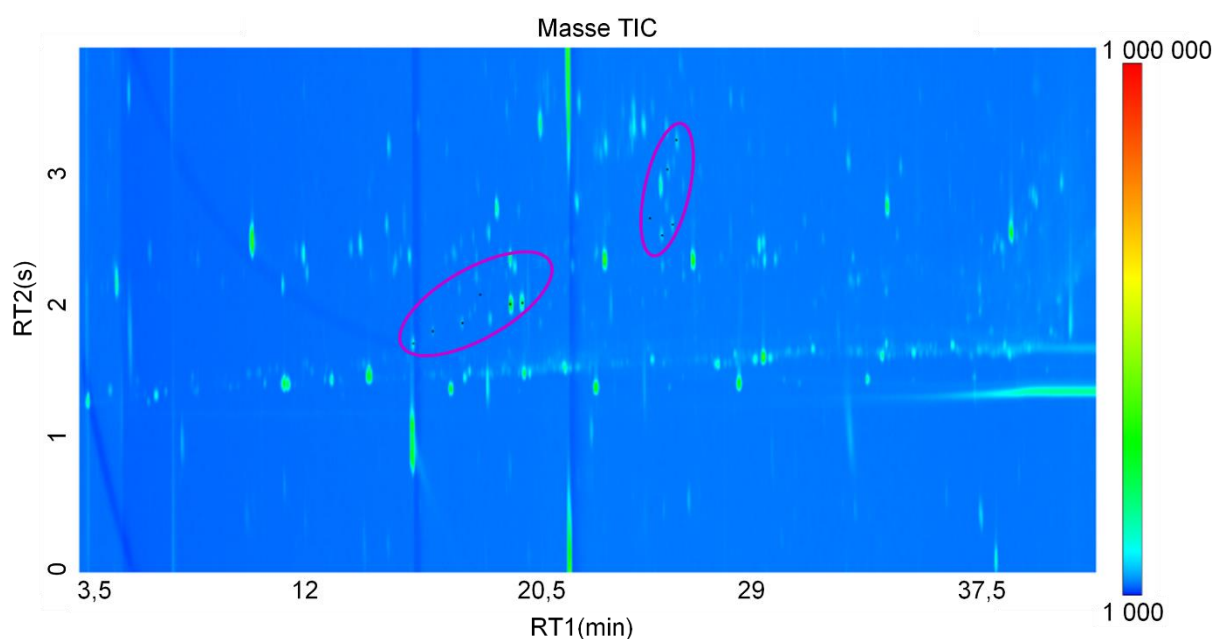
### Cleaning procedure of the Tedlar® bags

After that, the efficiency of the cleaning procedure of the Tedlar® bags was verified. The experiment was based on the comparison of the peppermint target compounds' signals before and after the cleaning step. This allowed obtaining the remaining fraction of the compounds of interest after the cleaning step.

In practice, once the sampling bag has been used for the sampling of exhaled breath under the peppermint pill intake, the bag is cleaned following the manufacturer's recommendation. After this cleaning step, the clean bag was filled with nitrogen and then, the gaseous mixture, contained in the bag, was discharged onto the TD tube

similarly as the exhaled breath (Annex 1). For more facilities, we will call this type of experiment a bag blank experiment.

The resulting chromatogram (Figure 14) displays the presence of peppermint metabolites in the bag blank experiment. The residual fraction of target compounds was not negligible enough to insure the efficiency of the cleaning procedure. The cleanliness of the TD tube was dismissed by a tube blank analysis. Therefore, those metabolites could either come from the bag, or from the connector tube between the bag and the TD tube. The tube connector is not reconditioned between the samples, thus could bring a contamination.



*Figure 14: GCxGC chromatogram of a clean bag reconditioned before reconditioning procedure optimization*

*The target compounds areas are circled in purple*

An additional experiment of peppermint breath sampling was conducted on a brand-new bag<sup>5</sup> and an older bag with brand new connectors to be compared to the signals after reconditioning. After reconditioning, a bag blank experiment was done, and peppermint metabolites were present in the two bags (Table 5).

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<sup>5</sup> A brand-new bag was filled with exhaled breath, then reconditioned and a control experiment was done on it.

*Table 5: Contamination test results*

*(<sup>1</sup>) % remaining = Area after conditioning / Area exhaled breath sampling*

Peppermint metabolites	% of remaining metabolites after conditioning <sup>(1)</sup>		
	Brand-new bag	Old bag	New bag with higher volume cleaning
a-Pinene	4.2%	4.0%	1.8%
Camphene	6.2%	5.1%	1.9%
β-Pinene	0.0%	0.0%	1.2%
Cineole	0.2%	0.4%	0.0%
D-limonene	6.3%	5.6%	2.8%
Cymene	10.9%	9.2%	2.1%
Eucalyptol	4.1%	3.5%	0.0%
γ-Terpinene	5.7%	9.4%	0.0%
Menthofuran	1.3%	0.3%	0.1%
Menthone	7.2%	8.8%	0.9%
Menthol	2.5%	0.7%	0.6%
Menthone isomer	1.9%	1.8%	0.4%
Menthol isomer	38.1%	56.1%	5.0%

These results prove that contamination comes from the bag and its reconditioning step, and not from the connector. Additional search in the literature revealed that the volume used to recondition the bag was too low [39]. Indeed, the optimal washing volume is 80% whereas the bags were filled less than 50% of their full volume.

Therefore, the bags were reconditioned at 80% of their capacity and an additional cleanliness control experiment has been done. As presented in the last column of the Table 5, the higher volume of nitrogen used for the conditioning allows to get rid of the contamination. These last results ensure the efficiency of the cleaning procedure and solve the contamination issue coming from the reconditioning step. The SOP of the reconditioning step can be consulted in Annex 3.C.

#### *Extra step of the cleaning procedure for the COVID-19*

Because of the COVID-19 pandemic, additional measures were thought and taken to limit the contact and the potential contamination; since the coronavirus is highly transmissible by air and that the peppermint project involves the sampling of exhaled breath. The national recommendations were obviously applied, but additional

precautions were taken in the experimental way to protect the volunteers and the researcher(s).

To limit the direct contact between the patient's mouth and the bag, individual joins<sup>6</sup> with single use only, for each sampling were placed on the bag's valve. The outside of the bag valve was cleaned with ethanol at the end of the reconditioning process.

The reconditioning procedure of the Tedlar<sup>®</sup> bags was improved in order to limit the potential contamination of the peppermint project volunteers. Since the bags were re-used between the volunteers, a disinfection step was added to the reconditioning procedure (Annex 3.B). Straight after the exhaled breath sampling step, ethanol 70% was sprayed on the bags right before placing them in a UV hood for two cycles of 25 minutes, one cycle for each side of the bag [40]–[42]. The next steps were the same as the usual protocol. Moreover, this protocol also included to let the bag in the oven at 50°C for 30 minutes, which is also an operation that can have a destructive effect on viruses [43].

A previous bag blank experiment on a bag treated under UV rays was done to ensure that the UV rays did not damage the Tedlar<sup>®</sup> bags and its properties.

By comparing the bag blank experiment chromatograms before and after UV rays (Figure 15), the Tedlar<sup>®</sup> VOC pattern is unchanged despite the UV treatment. This prove that the UV treatment extra step does not impact the signal and the Tedlar<sup>®</sup> bag properties. Therefore, the extra step has been added to the procedure during the COVID-19 pandemic for sanitary security reasons, with no risk on the data validity.

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<sup>6</sup> The individual single-use joins were treated in the same way, cut on the same length and are from the same silicon tube

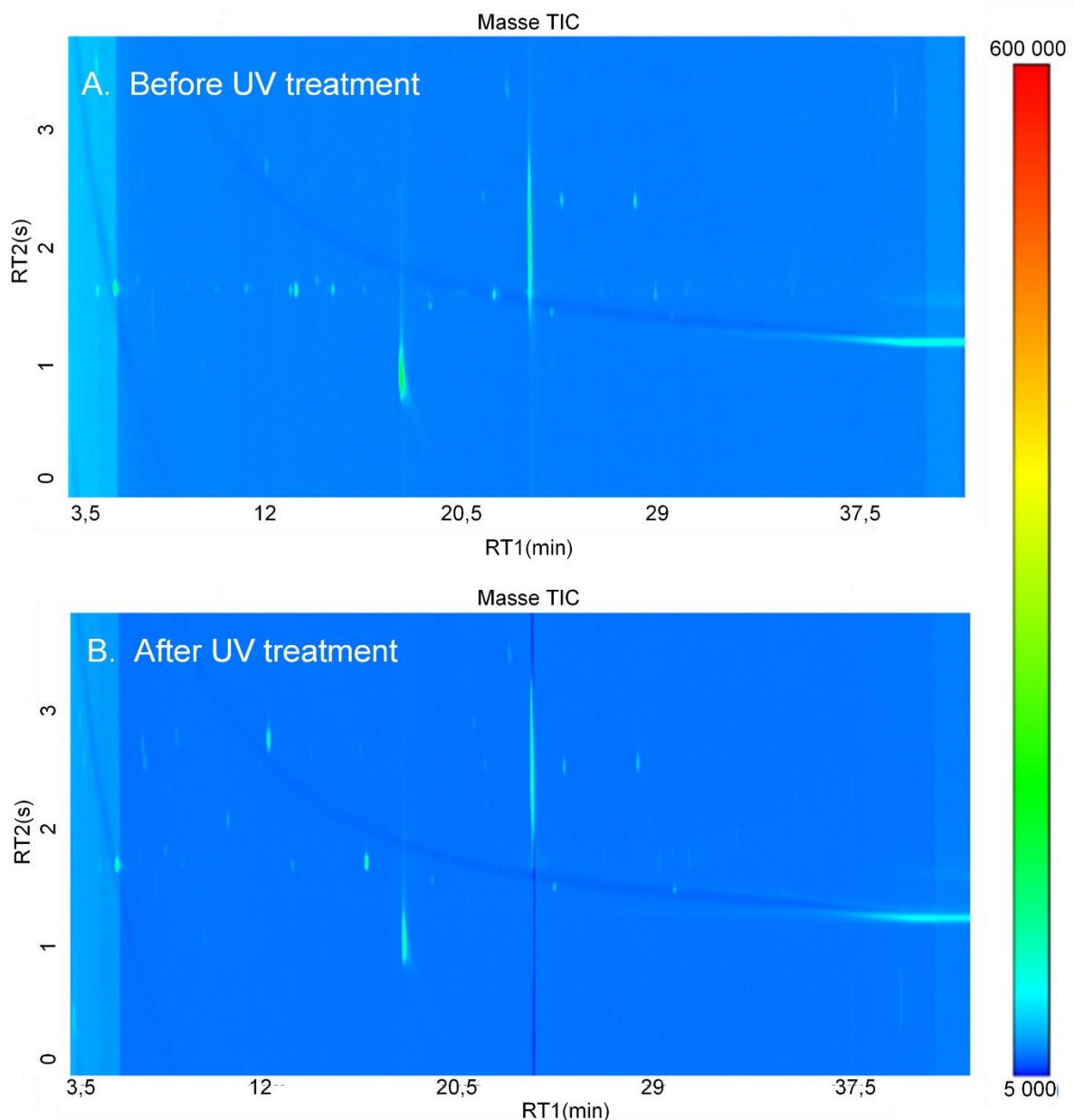


Figure 15: Comparison of the Tedlar® bag chromatogram pattern before (A) and after (B) the UV ray's treatment

### Washout curve profiles

Before the beginning of the peppermint project, at least one trial peppermint experiment should be done to ensure that the curves obtained have a washout profile.

A washout profile can be explained as follow. Before and at the pill ingestion, the peppermint metabolites are extremely low concentrated, even not detected, because there are, or are not, naturally present in the human body or following food intake. Once the pill digestion starts, peppermint metabolites are gradually released in the

blood, and then in the exhaled breath through the pulmonary alveoli. The quantity of each peppermint metabolite in exhaled breath increases proportionally to the delivered quantity in blood, and thus to the digestion of the pill, until reaching a maximum. When the pill is totally digested, peppermint metabolites are progressively eliminated from the body and their quantity in exhaled breath decreases.

The full curves in the Figure 16 show that the washout curve profile of the tested patient is not a washout curve profile. Indeed, the highest maximum is at time point 165 min but there is a second local maximum at time point 60-75 min, depending on the target compounds. According to the feelings of the tested patient, whose washout curve is displayed in Figure 16, the peppermint's flavors were maximal for the time points 75 and 90 min rather than for the time points 165 min, which is not fitting with the washout curve profile presented. Moreover, compared to another tested patient's<sup>7</sup> exhaled breath, the maximum signal was around 60 min, therefore the maximum release of the peppermint metabolites was expected around this time.

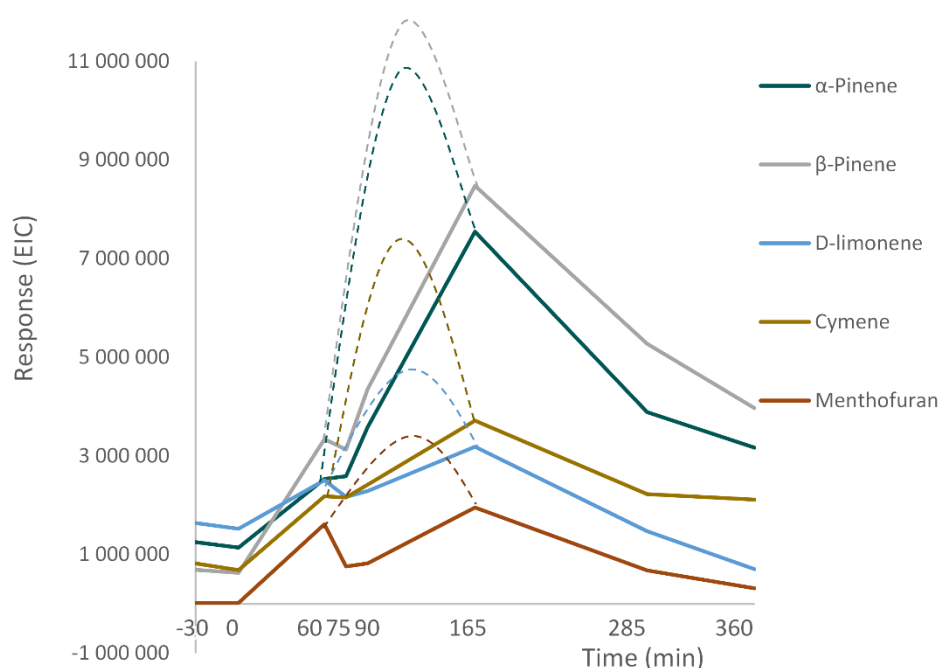
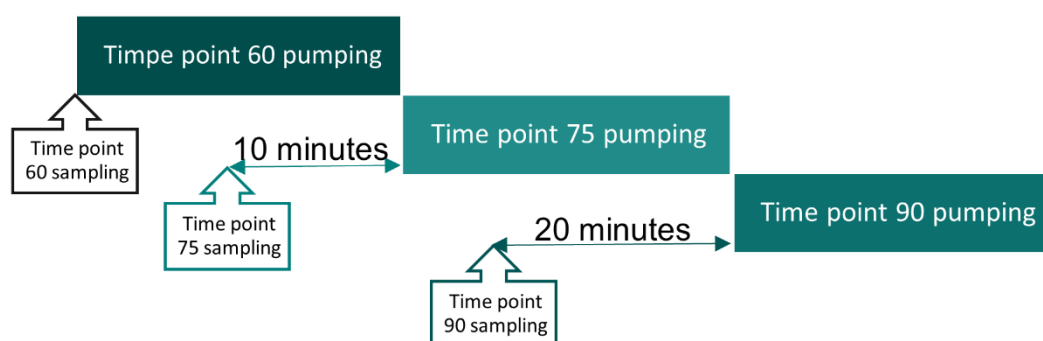


Figure 16: Experimental washout curve

*Dotted curves represented the theoretical washout profile whereas the full curves correspond to the measured signal*

<sup>7</sup> The time point 75 was not sampled for this patient.

Thinking about this inconsistency, the solution to the issue appeared. Since the pump was calibrated at 200mL/min, the time duration to pump the 5 L of exhaled breath from the bag was 25 minutes. However, when the time point 75 was sampled, the sample 60 was still being concentrated onto the TD tube and similarly when the time point 90 min was collected, the sample 75 was also still being concentrated onto the tube (Figure 17). Thus, the exhaled breath stayed 10 minutes in the bag for the time point 75 and 20 minutes for the time point 90 min.



*Figure 17: Pumping overlap for time point 60-, 75- and 90-min sampling*

The composition of the exhaled breath was not anymore the same compared to the other sampling points because the Tedlar® bags are somewhat permeable [37]. This permeability leads to a gaseous exchange between the exhaled breath and the ambient air, and thus, a decrease of target compounds' intensity in the exhaled breath, which migrate to the ambient air. The longer the exchange time is, the more the composition of the exhaled breath could change, and thus, the intensity of the target compounds too. Therefore, the decrease of intensity for the time point 75 min should be lower than for the time point 90 min. The dotted curves in Figure 16 represents the hypothetical washout curve profile, assuming that the bags were not permeable and therefore that no gaseous exchange occurred. This hypothetical curve seemed to fit with the patient feeling and explained the biased washout curve presenting two local maxima.

By deleting the time point 75 min, the sampled breath was directly concentrated onto the TD tube and thus no more gaseous exchange could occur. Indeed, the washout profile performed without sampling the exhaled breath at 75 min had a normal look, as shown in Figure 18. Moreover, the suppression of this time point is not problematic regarding the peppermint Initiative, because this time point was specially added in our

laboratory. This time point was added because the first tested individual had his maximum between time point 60 min and time point 90 min.

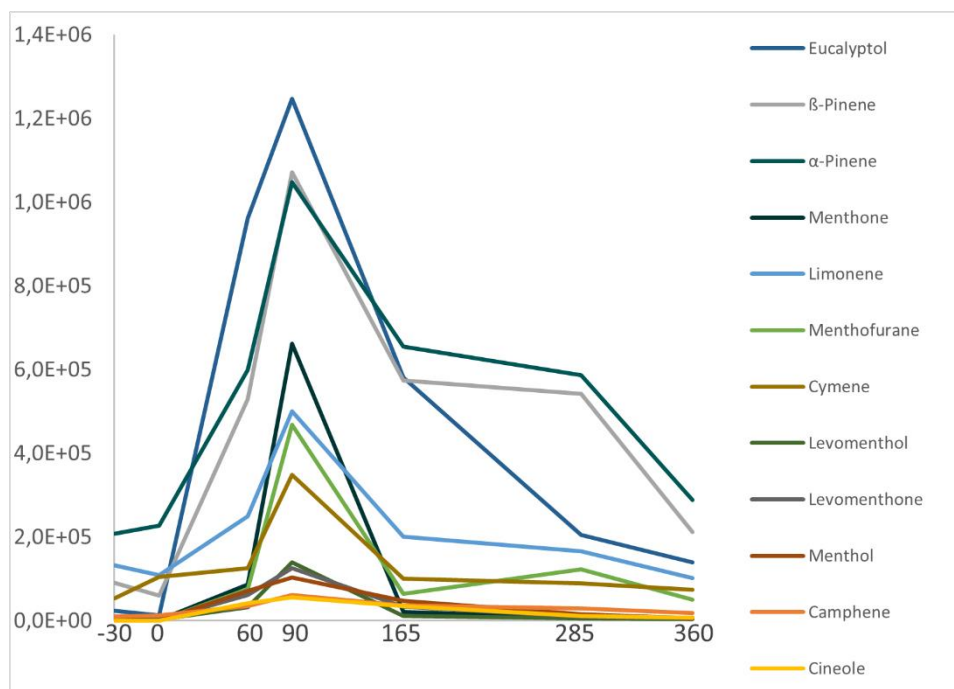


Figure 18 : Washout profile obtained without the time point 75 sampling

### Optimization of the injection procedures

The preliminary experimental washout curves allowed verifying if the actual parameters of injections were optimal. For example, that the sample's concentration was in the linearity domain of the instrument, and that there was no saturation of the signal. The optimization of the method concerning the column set, injection parameters, etc. were realized prior to the master thesis.

The analysis of the results of trial peppermint experiment allowed the optimization of the injections. Indeed, the metabolites curves had a similar profile except for the eucalyptol. The chromatogram analysis of the eucalyptol (Figure 19) shows that the peak is splitting into two peaks when the concentration is too high. The area of the peak is erroneous, smaller than the expected value. Indeed, when a compound's concentration is too high, the detector can be switched off during a microsecond to secure the instrument. Most of the time, this split takes place at the critical time point of 165 minutes. The look of eucalyptol's washout is thus not worrisome but for next injections, the split used for 5 L samples was increased at 50 instead of 20, which eliminates the split peak.

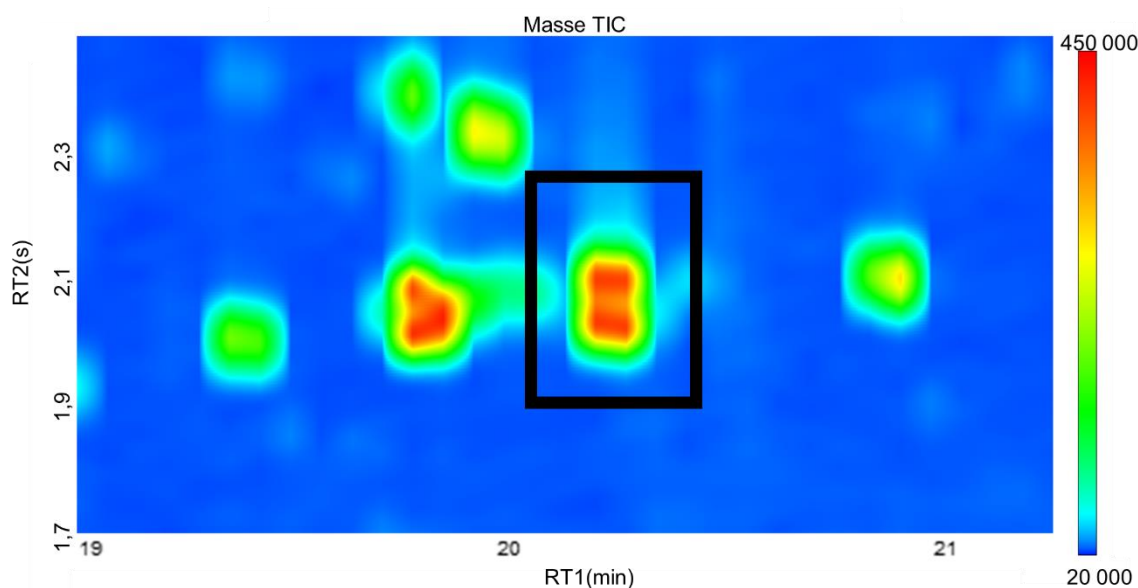


Figure 19: GCxGC chromatogram zoomed on the eucalyptol' signal splitting into two peaks

After the split change, it was necessary to ensure that the low-concentrated metabolites were still detectable and in sufficient quantity to observe a washout profile, and that the eucalyptol's signal were not splitting into two peaks anymore. These two requirements were met after the split change and thus the split for 5 L injection was settled to split 50.

#### Determination of the optimal bags to sample exhaled breath

A benchmarking of different sampling bags was done to identify the optimal volume and the optimal material of the sample bags. The two different materials studied were Tedlar® and multifoil and the two different volumes were 5 L and 1 L (Figure 20). The classic Tedlar® bags are well-known to release volatile plasticizers in the sample, which is sometimes a nuisance for the data treatment. Testing the multifoil bags in comparison to classic bags could prove to be more effective for exhaled breath test. Concerning the volume, reducing the sampling volume to 1 L would make it more comfortable for the patients, and time efficient for the clinician. The section "Comparison of the volumes of sampling bags" on page 49 would reveal if it can be envisaged.



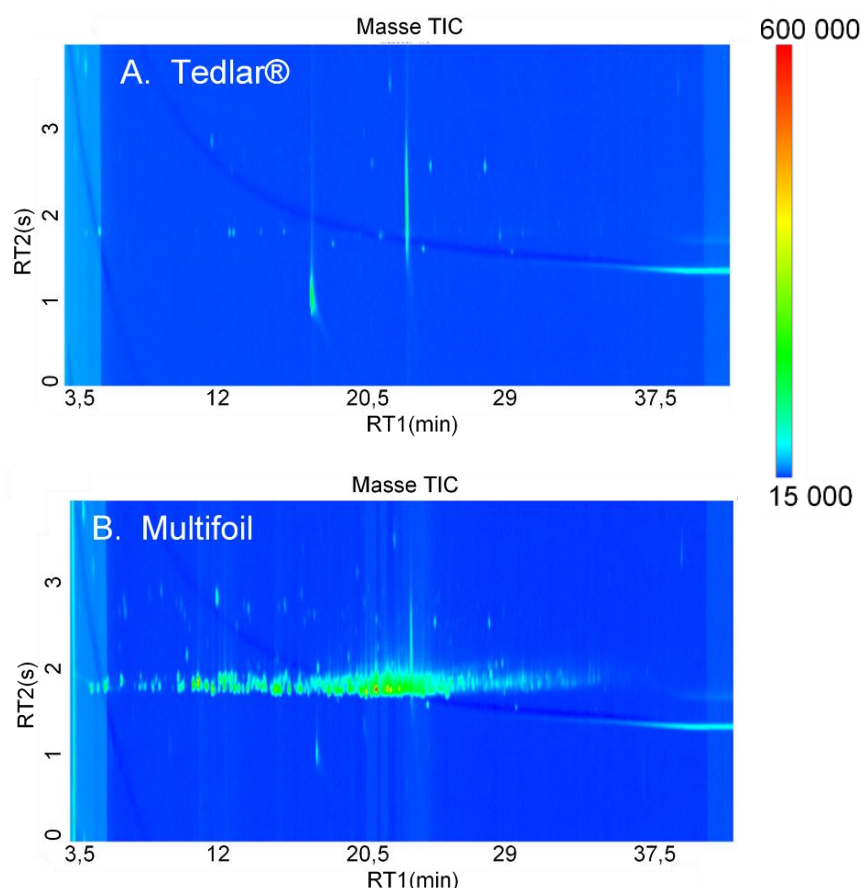
*Figure 20: The four bags tested for the benchmarking study: (A) classic Tedlar® bag of 1 L, (B) classic Tedlar® bag of 5 L, (C) multifoil bag of 1 L and (D) multifoil bag of 5 L*

The determination of the optimal bag to sample exhaled breath was done by comparing the complete washout curves profiles with the different types of bag. Proceeding in this way allowed taking into consideration the low concentrate analytes or time points. The first comparison was between the two types of materials, Tedlar® and multifoil, and the second comparison was between two volumes, 5 L and 1 L.

#### *Comparison of the materials sampling bags*

To determine the optimal materials, bag blank experiments and peppermint experiments on Tedlar® bags and multifoil bags were made on two different days.

The bag blank experiment allowed getting the pattern of the two bag materials: Tedlar® and multifoil (Figure 21). These results are stunning because the two chromatograms seem to come from totally different analysis. The Tedlar® pattern was already known since Tedlar® bags are currently used in the laboratory unlike the multifoil bags. The multifoil pattern was very bulky, characterized by intense emissions of hydrocarbons, a crucial area in the 2D chromatogram for breath analysis.



*Figure 21 : GCxGC chromatograms of bag blank experiments on Tedlar® (A) and multifoil (B) sampling bags*

An additional experiment was realized to check if it was possible to decrease this background noise signal. This experiment involves several bag blanks measure after each reconditioning cycle. A total of five reconditioning cycles on the same bag have been realized to stay in line with the ease factor to decrease the background noise. This experiment took place for one week. The bag blank experiment was realized every day, followed by a reconditioning cycle to favor an identical equilibrium time with ambient air. The resulting chromatograms are presented in Figure 22.

The background noise decrease is very slight after the five reconditioning cycles, the contamination is still clearly present. This brings us to the conclusion that it is not easy to eliminate the background noise of the multifoil bags and this promotes the use of Tedlar® bags instead of multifoil bags for exhaled breath analysis. Indeed, the metabolites' concentrations are in trace level in exhaled breath and could be taking over by the background noise contaminant.

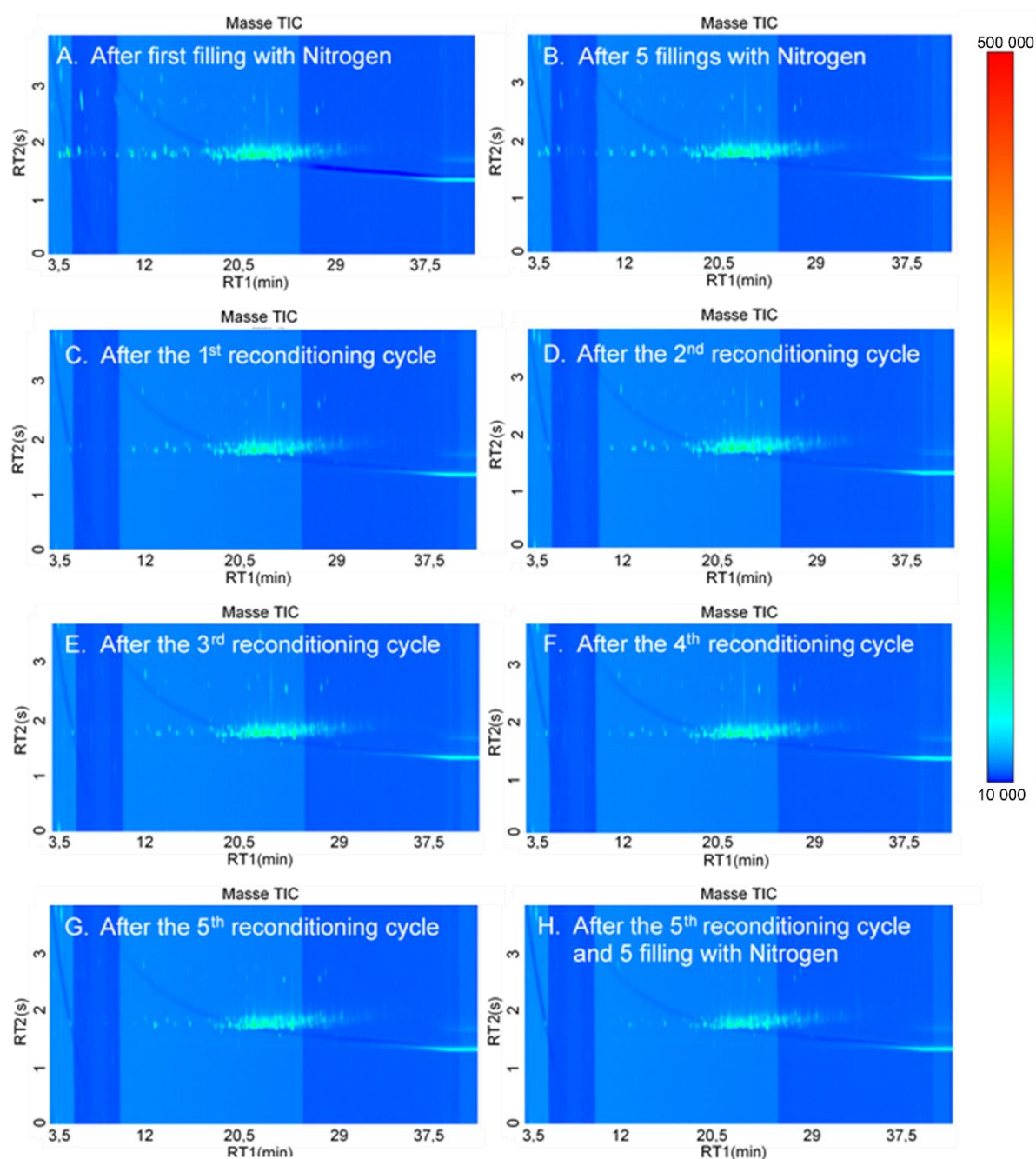
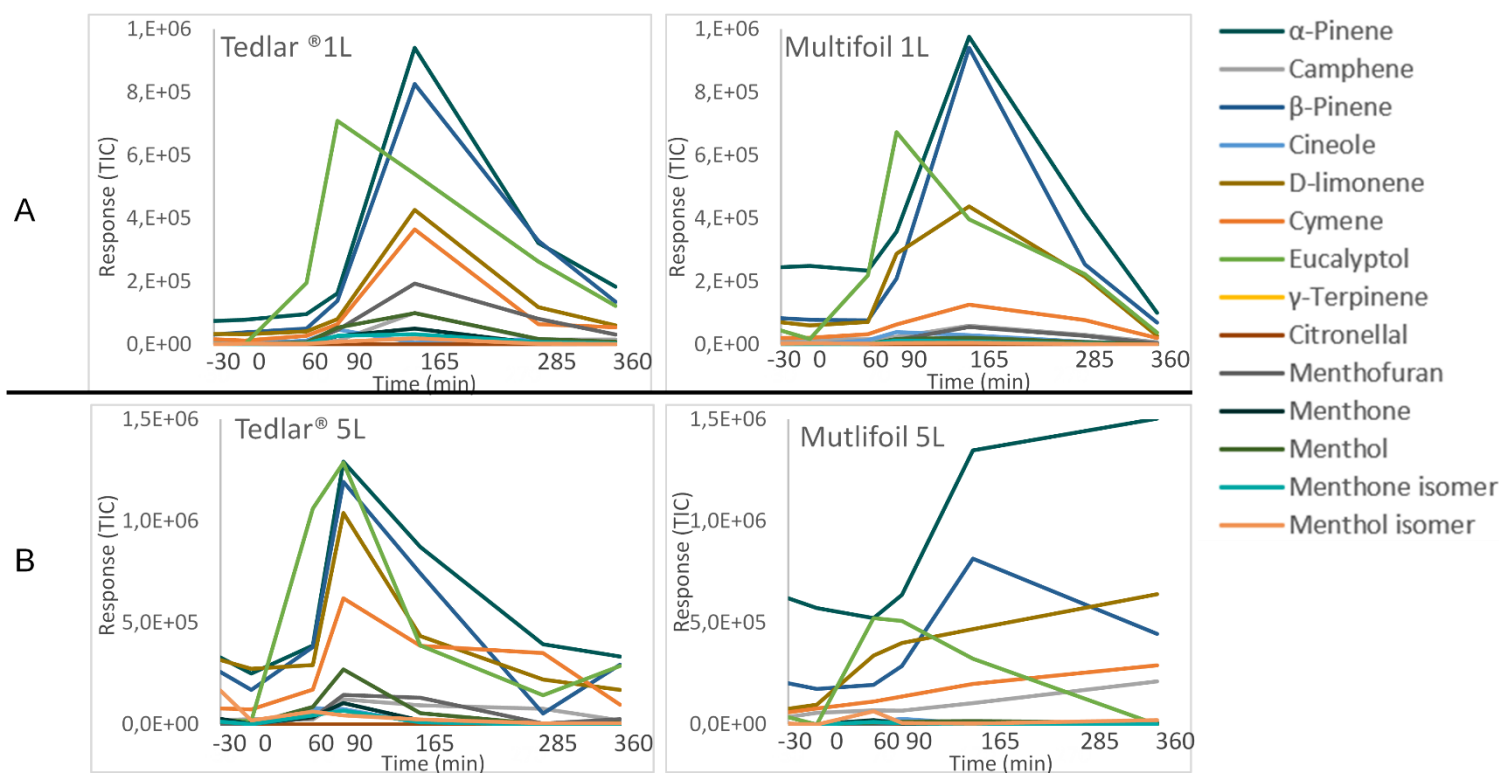


Figure 22: GCxGC chromatograms of bag blank experiments to reflect the evolution of background noise of multifoil bags after several reconditioning cycle

The analysis of the peppermint experiment results was oriented toward the washout curve profile and not the strict comparison of the area response. Indeed, the exhaled breath might be different from one peppermint experiment to the other because of the stability of the exhaled breath matrix in the bag.

Figure 23 proves that, the high contaminant background noise of the multifoil bags, coelutes with the compounds of interest, and thus the signal of interest is erroneous.



*Figure 23: Washout profiles comparison of Tedlar® and multifoil bags in 1 L and 5 L volumes. 1 L volume samplings (A) were conducted simultaneously whereas 5 L volume samplings (B) were done on two different days*

In order to reach beyond the suitability of the multifoil bags for peppermint project, an additional ATIS experiment with the breath standards has been made with 1 L bags. This additional experiment serves to verify that the multifoil bags are not suitable either for general breath analysis, nor for the peppermint project, since in this experiment, breath standards are analyzed. The results are presented in the next table.

*Table 6 : Results of the benchmarking study between materials bags (Tedlar® and multifoil)*

*The average area values and the %RSD are based on triplicates.*

*(<sup>1</sup>) Because of the coelution with the VOCs of the multifoil pattern*

Compounds		Multifoil		Tedlar®		p-value
		Average	%RSD	Average	%RSD	
1	1-Propanol	1 671 947	5%	1 421 149	7%	0.03
2	2-methyl-2-butanol	977 579	6%	825 881	4%	0.02
3	1-ethyl-3-methyl-cyclopentane	920 007	1%	804 935	1%	4.10 <sup>-5</sup>
4	2-Hexanone	1 112 798	6%	1 236 686	1%	0.03
5	p-Xylene	912 668	9%	1 968 208	3%	6.10 <sup>-5</sup>
6	Decane	1 051 924	15%	729 869	4%	0.02
7	Undecane	Undefined <sup>(1)</sup>		901 612	6%	
8	1-Octanol	103 609	12%	230 216	9%	9.10 <sup>-4</sup>
9	Nonanal	99 056	5%	176 465	5%	2.10 <sup>-4</sup>
10	2-Ethylhexanoic acid	9 916	65%	11 745	5%	0.65
11	2,6-Dimethylphenol	57 395	52%	133 709	24%	0.04
12	2,6-Dimethylaniline	40 981	7%	91 029	13%	2.10 <sup>-3</sup>
13	Methyl caprate	74 967	9%	238 275	8%	2.10 <sup>-4</sup>
14	1-Tetradecene	105 143	25%	123 337	8%	0.33
15	Methyl undecanoate	25 589	23%	103 844	8%	2.10 <sup>-4</sup>
16	Dicyclohexylamine	8 684	79%	16 971	48%	0.25
17	1-Pentadecene	15 837	11%	40 975	7%	2.10 <sup>-4</sup>
18	Methyl laurate	6 642	170%	46 775	6%	4.10 <sup>-3</sup>
Average			29%		9%	0.08
Median			11%		7%	3.10 <sup>-3</sup>
Range of values			1-170%		1-48%	

First, the average values of the signal for the lower molecular compounds have a higher signal with multifoil bags than with Tedlar® bags, but for the high molecular compounds, the average value is higher with Tedlar® bags. This trend can be explained by the manufacturer recommendations, mentioning that the multifoil bags are more recommended for low-molecular weight compounds [44].

Then, if we focus on the compound's detection in the triplicates, it is important to notice that the undecane is coeluting with the VOCs of the multifoil pattern, which unable to the analysis of similar compounds.

A statistical t-test was done to verify that the difference between the two bags was significant ( $p\text{-value} < 0,05$ ). The global average  $p\text{-value}$  is close to, but not under, the defined value. The median  $p\text{-value}$  clearly shows that the results of more than the half of the compounds differ significantly between the two bags analysis. Actually, fifteen compounds are concerned, which is more than 80% of the analyzed compounds. These numbers make the discrimination of the use of one type of material bags evident.

A poorer reproducibility is obtained when using the multifoil bags with %RSD ranging from 1% to 170%, while %RSD ranging from 1% to 48% were obtained when using Tedlar® bags for the sampling. Moreover, the multifoil's %RSD are generally higher, such as the global %RSD.

This contrast in %RSD profiles can probably be relied with the amount of contaminants in the multifoil bags, where the coelution of contaminants and target compounds prevents a right measure of the signals. These facts highlight the drawbacks of the multifoil bags for exhaled breath analysis.

According to literature and manufacturer recommendations, the multifoil bags are ideal for low molecular weight compounds. Moreover, the multifoil bags are not recommended for collecting low-level VOCs because the background noise is problematic, including our experiment on the target peppermint VOCs or the standard exhaled breath VOCs [44]. However, the multifoil bags are less permeable than Tedlar® bags, which could be an advantage for samples stored longer. Though, in our experiment and in general ways, the transfer of the exhaled breath onto the TD tubes is immediate.

The results of our experiments revealed that the background noise of the multifoil bags is bulky and persists after reconditioning cycles. It also reduces the possibility of identifying compounds of interest in the background noise area which is really restrictive for exhaled breath experiment.

Due to all their drawbacks, the use of multifoil bags compared to the Tedlar® bags is not justified in this study.

#### *Comparison of the volumes of sampling bags*

To compare the two volumes, peppermint experiments on 1 L and 5 L Tedlar® bags were made simultaneously. Briefly, the 1 L Tedlar® bags were filled at first and concentrated onto TD tubes, followed by the 5 L bags. This set up was chosen because the duration of the concentration of the exhaled breath onto TD tubes is smaller for 1 L than 5 L, and thus, it limits the gap time between the two simultaneous sampling. The comparison of the two volumes will lead to verify the possibility to reduce the sampling volume to 1 L. This will make it more comfortable for the patients, and time efficient for the clinician.

Then, this kind of experiment will not tell the better volume sampling because there are too many variations in experimental parameters. Firstly, the split used was not the same. The split used was 20 for 1 L and 50 for 5 L. The method was adapted to make possible the analysis of 1 L. Indeed, there was less material on the TD tube, thus, by decreasing the split, more material was injected on column. Secondly, there was a gap time between 1 L and 5 L sampling, leading to a slight difference of the exhaled breath composition. Blowing in the two bags directly would not solve this issue because we do not know if the exhaled breath composition would be the same after blowing as much breath. Moreover, as explained in the section about the time point 75 min on page 38, during the short delay where the exhaled breath stays in the bag, an exchange between exhaled breath of the bags and ambient air is taking place, leading also to variation of the exhaled breath composition.

This scheduled experiment has been repeated two times to increase confidence in the obtained results. The resulting washout curves are presented in the Figure 24.



Figure 24: Washout profiles comparison of Tedlar® 1 L and Tedlar® 5 L

There is no significant difference between the washout curves with 1 L bags and with 5 L bags. This demonstrates the reproducibility and robustness of the method, since over one day and over the two volumes, the washout profile and the maximum are the same. This also ensures that the two tested volumes are suitable for this type of experiment and for the peppermint project. A complete comparison of strengths and

weaknesses for each volume will help to consider and to approve the decrease of sampling volume.

First, 1 L of exhaled breath is easy to fill than 5 L, more specially for patients with breathing difficulties or patients suffering from lung diseases such as inflammation, and infections. There is also an unknown parameter about the stability of exhaled breath composition after blowing 5 L. Then, reducing the volume allows lowering the nitrogen quantity used for the cleaning steps, which leads to an economic gain. Moreover, the time saved for the manipulation time, including the sampling time (fill with breath and concentration onto TD tubes), the reconditioning time, is 4 to 5 times lower for the 1 L than the 5 L. This is significant, especially when the experiment must be repeated 7 times to produce the washout curve of one individual and times the 10 individuals tested. Other benefits exist such as the lower cost or the short desorption time, limiting the time of exhaled breath in the bag and thus, the variation of composition due to the permeability of the bag.

However, the bigger volume allows the production of replicates and the use of an instrument with a lower sensitivity.

Nonetheless, as our technology is highly sensitive, and that no replicates will be needed by patient, the 1 L bags seems to be the better option for sampling the exhaled breath of the volunteers. It will also allow, thanks to the time saved, to analyze as many patients as possible to evaluate the effects of diet, patients' lifestyle and so on, in a reliable way.

### IV.3. Peppermint project

This benchmarking study leads to the sampling of at least ten subjects, men or women, aged between 18 and 50 years old [22]. The exhaled breath of the subjects was measured 30 min before the pill intake, when the pill was taken, 1h, 1h30, 2h45, 4h45 and 6h after the pill intake (Figure 25). These specific times have been optimized by the peppermint Initiative [22].

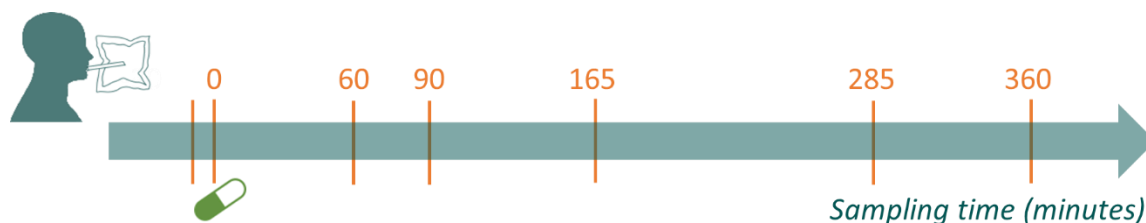


Figure 25: Sampling time points of the peppermint experiment

Before the beginning of the peppermint project, a clean empty tube was injected to ensure the cleanliness of the TD traps (cold trap, purge trap, recollection trap).

The sampling of the subjects was conducted in the same conditions: same room, same hour of ingestion and sampling. The bags and tubes were conditioned in the same way between each patient. These precautions have been taken to be consistent and to limit the variations created by the sampling. As already said before, the exhaled breath matrix is considerably variable between subjects and therefore, it is relevant to limit the external variations.

For each set of peppermint experiment, a blank of the instrument was first performed followed by the 7 exhaled breath samples (i.e., corresponding to the different ingestion time points as can be seen in Figure 25), and closed by a quality control (QC) sample. The blank of the instrument was done to ensure that the instrument was clean and that there was no carry-over from the previous injections. The QC samples, consisting in a spiked TD tube with 2  $\mu$ L of a 28-53 ppm solution of exhaled breath standards, were injected and enabled building a QC chart to monitor the instrumental variations (Annex 5).

Following the injection, a target search was performed to extract the response of the peppermint metabolites. For this search, the first and second retention times together with the exact masses of characteristics ions were selected. The response was then consistently extracted from all the chromatograms using the same  $m/z$  for all targeted

metabolites. The response was then used to build the washout curves of the peppermint experiment. The washout curves reflect the metabolization of each metabolite over the time for each patient.

Once the collected peppermint profiles were normalized, they were sent to the coordinator of the study, C. L. P. Thomas (University of Loughborough). Those data will enable the evaluation of the variations between the results of the different laboratories. Such data treatment approach was not within the framework of this master thesis and will be conducted by the peppermint coordinator.

Moreover, this benchmarking study was also realized in collaboration with another analytical platform in Gembloux which used a TD-GC×GC-qMS/VUV<sup>8</sup>. Among the total of patients, eight were part of the multiplatform analysis, thus were analyzed by TD-GC×GC-HRTOFMS and by TD-GC×GC-qMS/VUV. Additional peppermint experiments will be done, in a later stage, to get enough samples for this multiplatform analysis.

#### Monitored peppermint metabolites by GC×GC-HRTOFMS

During this master thesis project, additional target compounds have been monitored compared to the ones (\*) defined by the peppermint Initiative. The peppermint Initiative used a GC-MS to define the target compounds, which is less sensitive than the GC×GC-MS used for this project. The gain in sensitivity allowed monitoring six supplementary target compounds. Therefore, a total of 13 target compounds had been monitored in the exhaled breath:  $\alpha$ -Pinene\*, camphene,  $\beta$ -pinene\*, 2,3-dehydro-1,8-cineole (cineole), limonene\*, cymene, eucalyptol\*,  $\gamma$ -terpinene, menthofuran\*, menthone\*, menthol, menthone isomer and menthol isomer\*. For the 2,3-dehydro-1,8-cineole, the short name in parenthesis will be used throughout the text.

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<sup>8</sup> A thermal desorption unit linked to a comprehensive two-dimensional gas chromatography coupled to quadrupole mass spectrometer and to a vacuum UV detector

#### *IV.4. Data treatment*

In this project, one of the aims was to evaluate the procedure and instrumental reproducibility for exhaled breath analysis.

Moreover, analyzing the volunteers' metabolites' pattern would enable determining the most suitable metabolites to monitor within the exhaled breath using TD-GCxGC-HRTOFMS.

##### **Data pre-processing**

Since the aim was to compare the data obtained for the 10 volunteers, the normalization of the data was performed. The data normalization aimed to minimize the instrumental variations. The biological variations are important in exhaled breath analyses and should not be influenced by the data normalization.

Four normalization methods were sought: the normalization by median, by sum, by internal standard, and by external standard [45], [46]. These four methods differ from their normalization factor. The respective normalization's factors of the four methods are, respectively: the median of the target signals, the total area of the target compounds peaks, the area of the internal standard peak, and the area of the external standard peak [45], [46].

The median normalization method should be adapted because of the longitudinal aspect of the peppermint experiment. When the experiment is longitudinal, the data ranges vary considerably between the time points. It implicates considerable variations on the median value of the different time point's response. Normalizing each set of data by its median would therefore eliminate the variations which define the washout profile. To maintain the washout profile, the set of measures for one patient must be normalized by the same median value, and thus the median of the set of patient's responses, which is approved by the low instrumental variations observed in the QC charts (Annex 5).

The same reasoning could be applied to the normalization by the sum. For each patient, the normalization factor would be the sum of the total area of the target metabolites' peaks.

The normalization methods eliminate instrumentals variations only when the normalization factor's value of the replicas of the experiment are similar to each other

[46]. For the peppermint project, the replicas are defined as the set of patient's results, and the patients have different responses. By using the sum normalization or the median normalization for this project, the biological variations between the volunteers would be modified overly, which is not wanted for this study.

The internal standard's principle allows monitoring and eliminating the instrumental variations at any time point, while maintaining the biological variations. Since it is injected with the sample and do not depend on the exhaled breath matrix, every instrumental variations are detected (background noise, loss of matter, ...). Unfortunately, no internal standard was injected for this work, but it remains an ideal normalization for the peppermint experiment and exhaled breath monitoring experiment [23].

The normalization by external standard consists of injecting, in the same conditions, an additional TD tube containing the standards, together with the exhaled breath samples. The injected QC samples meet these criteria and thus could be used as external standard. This type of normalization, as well as the internal standard approach, enables to get rid solely of the instrumental variation between the injection sets.

Since the response of the quality controls samples is independent from biological variations of the exhaled breath and is close from each other, conversely to the median and the total area, the normalization by external standard appeared as the best solution for this work. Among the 19 standards of the QC solution (Figure 12 on page 30), the hexanone was well-resolved chromatographically and had the best reproducibility within the QC samples. Therefore, the area of the hexanone of the QC sample was used as normalization factor.

### Data pre-treatment

Several data transformations were done according the data analyses and are detailed in their respective section and resumed by formulae.

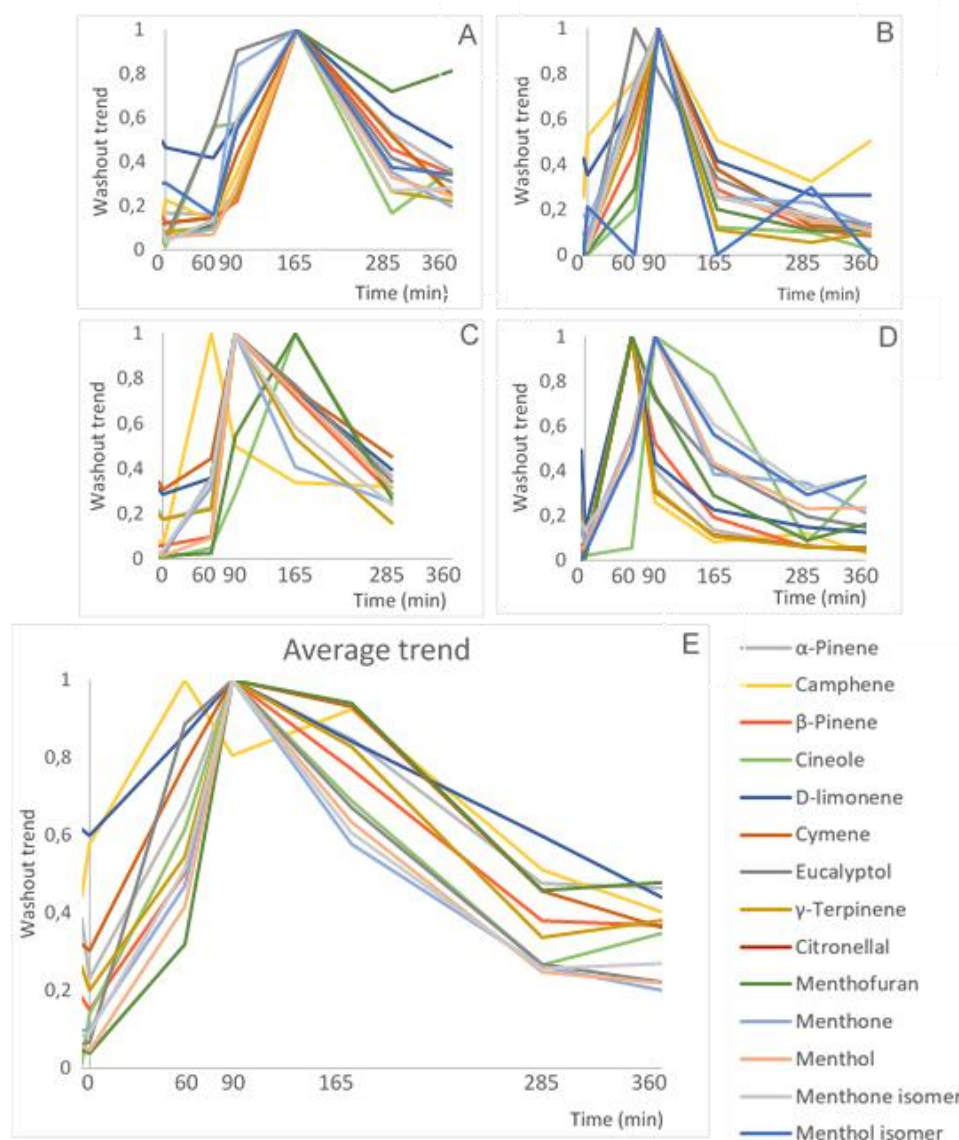
### Washout' profile analysis

The aim of this section was to compare the exhaled breath profiles of the ten tested volunteers, with equalized contribution of the metabolites. Thus, a scaling by maximum intensity of the metabolite was done.

$\tilde{x}_{ij,max} = \frac{x_{ij}}{x_{i,max}}$ , where  $x_{ij}$  corresponds to the peak area of the metabolite of interest

(i) at a given time point (j)

Once the data were pre-processed the global washout trend of the patients was realized for each patient as can be seen on Figure 26. The 10 washout curves of the patient are presented in the Annex 6 with the two main trends presented in the Figure 26. Figure 26.E represents the average response of the peppermint metabolites obtained from all the patients.



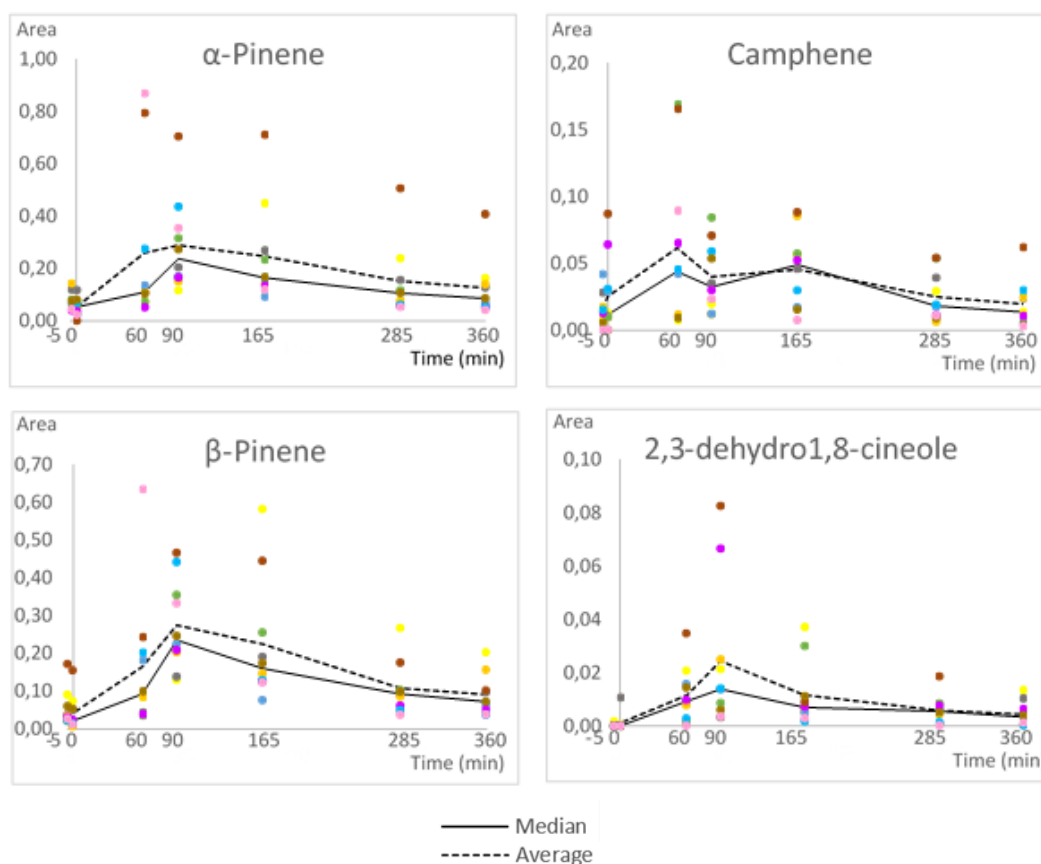
*Figure 26: Volunteers' washout profile trend*

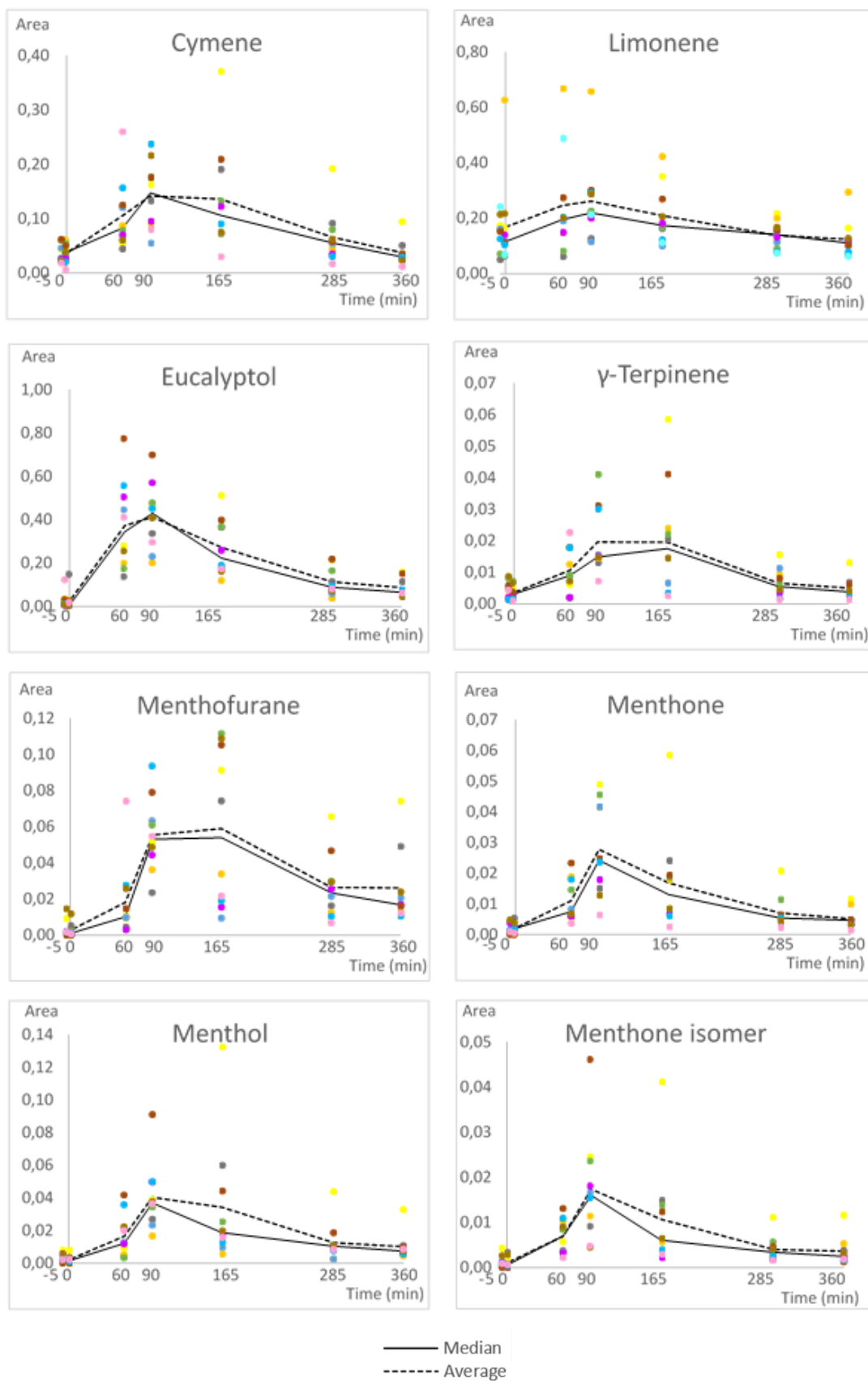
*(A) and (B) are washout profiles with unanimous maximum time point, (C) and (D) are washout profile shared between 2 or 3 maximums, and (E) is the average trend of the 10 volunteers*

Two types of profiles can be identified. For the first type of profile, the washout curve of some patient is really well-defined, such as Figure 26.A and Figure 26.B. Almost the totality of the metabolites had the maximum signal at an identical time (165 min(A) and 90 min(B)). For the other types of profile (Figure 26.C and Figure 26.D), the global maximum is less evident and shared between two or three local maximums. The washout profile is kept but the maximal intensity is variable (from 60 min to 165 min after the pill ingestion) according to the exhaled metabolite monitored.

Then, it can be seen on the average graph (Figure 26.E) that the global maximum of exhalation of the peppermint pill takes place 90 minutes after the pill intake. However, the camphene was characterized by a maximal intensity between time point 60 and 165 min. The compound trend analysis is not obvious on this representation and is developed in the next paragraph.

The analysis of the global trend of the metabolites was based on scatter plot specific to the metabolites. In order to visualize the washout curves of the target compounds obtained from all 10 patients, the median (plain line) and average (dotted line) curves of these scatter plot were added to the scatter plots of each metabolite (Figure 27).





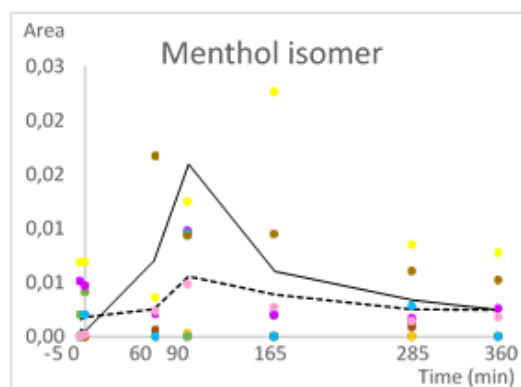


Figure 27: Scatters plots of the 13 compounds of interest for the 10 patients tested  
The color of the different dots represents the patients.

The washout trends are well defined for most of the target metabolites, with a maximum after 90 min. It demonstrates that the peppermint experiment, and thus the monitoring of these metabolites in the exhaled breath is reproducible and robust. However, camphene and menthofurane washout trends are more muddled; the camphene is characterized by two local maxima and the menthofuran's maximum looked like a plateau. The  $\alpha$ -pinene and the  $\gamma$ -terpinene trends are smoothed and their maximum are shifted to a later exhalation time. The limonene curve is also slightly smoothed but it is mainly because of the diet of the volunteers. Despite the slight difference in trend,  $\alpha$ -pinene, limonene, and  $\gamma$ -terpinene stayed in the most interesting for the monitoring of the peppermint pill metabolization. The menthol isomer is closed to the background noise and is detected with difficulties.

To resume, the peppermint experiment is reproducible and robust, and the volunteers' profiles follow a washout profile. Their maximums are shared out between one, two or three time point (60-90-165 min), depending on the volunteers. However, the global average maximum takes place 90 min after the pill intake. Among all the metabolites studied, the  $\alpha$ -pinene\*,  $\beta$ -pinene\*, cineole, limonene\*, cymene, eucalyptol\*,  $\gamma$ -terpinene, menthone\*, menthol and menthone isomer are the compounds ideal to monitor in the peppermint experiment.

## Metabolites' profiles analysis

### *Influence of the times points*

The next analysis provided information on the reproducibility of the compounds at key time points of the experiment. The time corresponding to the maximal intensity of the

exhaled metabolites, which vary between volunteers, and 90 min after the pill intake, which corresponds to a high concentration of the metabolites in breath.

In Figure 28.A, the box plots represented the data normalized by external standard, in Figure 28.B the box plots were scaled by the median.

$$\tilde{x}_{ij,median} = \frac{x_{ij}}{x_{i,median}}, \text{ where } x_{ij} \text{ corresponds to the peak area of the metabolite of interest (i) of a given patient (j)}$$

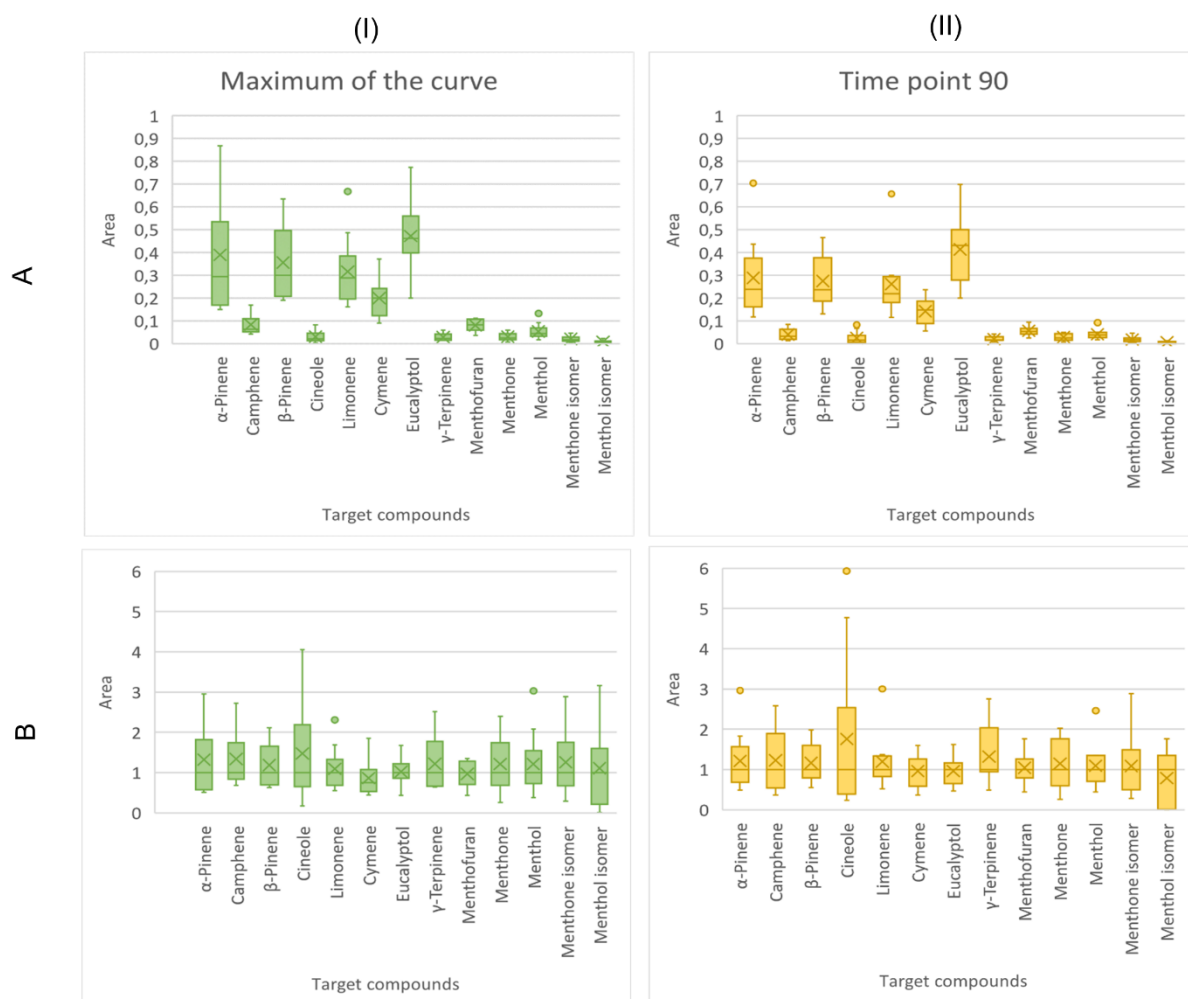


Figure 28: Comparison of the data repartition at different time point. (I) the maximum of the curve, and (II) the time point 90

The median scaled box plot of the maximum value (Figure 28.I.B) is similar to the other median scaled box plot (Figure 28.II.B). However, the variations of the interquartile (Figure 28.I.A) seems to be larger than at 90 time point (Figure 28.II.A). This difference is due to the median scaling, which eliminates the biological variations between the metabolites and displays better the variability of the interquartile. The interquartile box become thus specific to its data distribution. The two representations are relevant to

analyze because they present the results in different ways, one with the biological metabolites variation for a global overview, and the other without for a more detailed analysis.

The two time points share a similar reproducibility (total interquartile area of 10,83 for time point 90 and of 8,93 for maximum time point). However, for experiment with only one time point analysis, it is not possible to measure at the maximum time point because it is variable between the volunteers, the diet and the lifestyle.

#### *Metabolites metabolism*

The peppermint Initiative defined the ideal decreasing part of washout curve profile as a logarithmic curve (Figure 29) [23]. Therefore, the normalized data were first scaled by the initial intensity ( $I_0, x_{i=0}$ ); corresponding to the measured signal before the pill is taken. Then, the time points and the scaled data were log transformed, to analyze the decreasing part of the washout curves (Figure 30). Linear regressions on the time points of the decreasing part were created to observe the metabolites metabolism.

$\tilde{x}_{ij,log} = \log\left(\frac{x_{ij}}{x_{i,j=0}}\right)$  where  $x_{ij}$  corresponds to the peak area of the metabolite of interest (i) at a given time point (j)

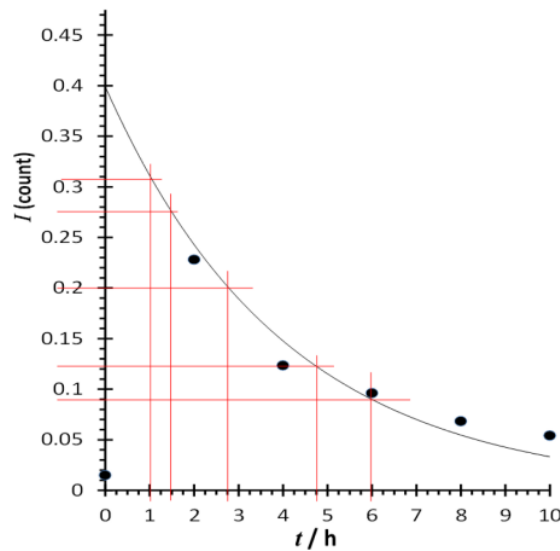


Figure 29: Experimental washout profile obtained by the peppermint Initiative for the menthone [23]

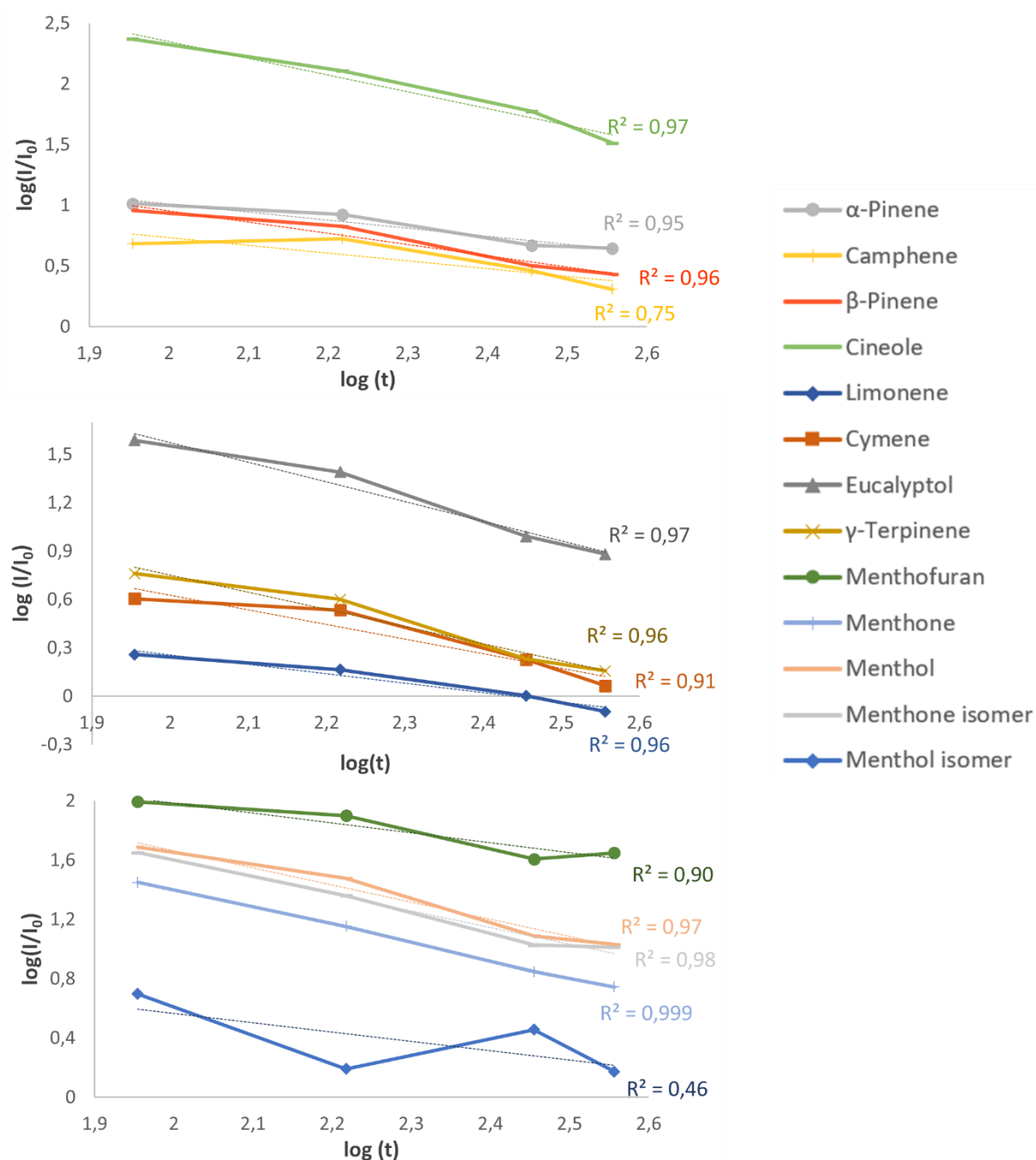


Figure 30: Logarithmic metabolization of the 13 target compounds

The coefficients of the linear regression ( $R^2$ ) values indicate the similarity with the ideal washout decrease. The perfect washout curve has a  $R^2$  equal to 1. Because the presented curves are resulting from the average intensity of the metabolites within the 10 volunteers, the compounds with a  $R^2$  closed to 1 express a good reproducibility in the washout profile. Those compounds are thus ideal to monitor in the exhaled breath.

The  $R^2$  are, for the majority, superior to 0.95. However, the menthol isomer and the camphene are characterized by an  $R^2$  inferior to 0.90, and the menthofuran and the cymene with an  $R^2$  between 0.90 and 0.95.

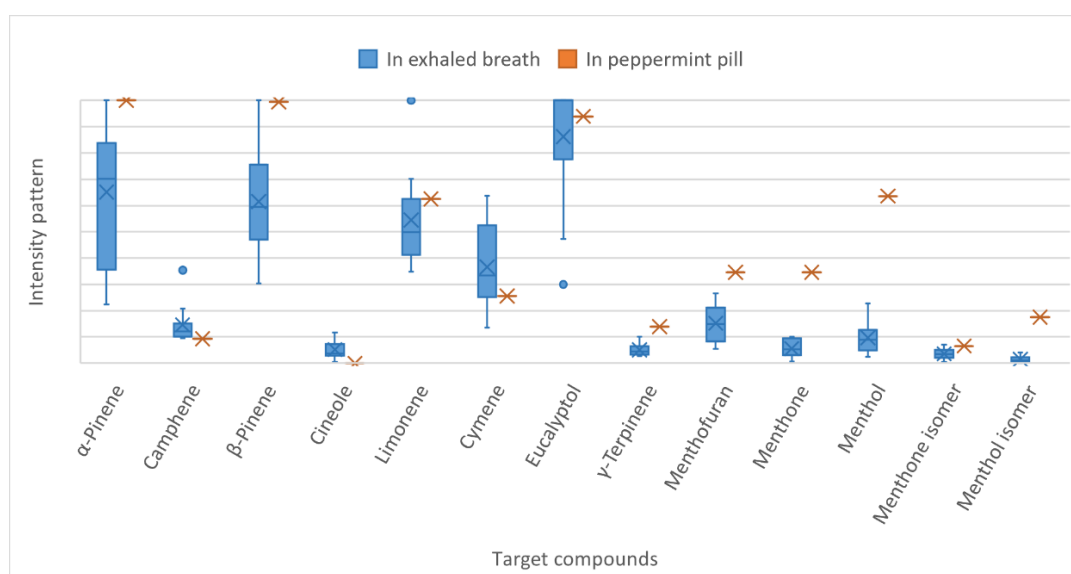
Because of their lower  $R^2$ , the menthol isomer and the camphene did not represent the target compounds of choice to monitor in the exhaled breath.

### *Metabolization pathways and by-product*

The overview of the metabolization pathways can be done by comparing the intensity pattern of the peppermint oil with the pattern measured in the exhaled breath.

The box plots of Figure 31 enabled the comparison of the metabolites present in the peppermint oil and their presence in the exhaled breath resulting from their metabolization. The box plots of the metabolites detected in the exhaled breath were built using the maximal intensity of each metabolites for each patient. The data were scaled by the maximum intensity signal of the patient. The box plot of the peppermint oil was built with the signals collected in the experiment explained on page 26 and scaled by the maximum intensity signal. Such representation enabled the visualization of the different intensities of the metabolites within the 2 matrices.

$\tilde{x}_{ij \max intensity} = \frac{x_{ij \max}}{x_{i \max}}$ , where  $x_{ij \max}$  corresponds to the peak area with the maximum intensity measured for a given patient/in the peppermint oil (i) and for the metabolite of interest (j)



*Figure 31: Box plot comparison of the compounds' intensity patterns in exhaled breath and in peppermint oil*

In the exhaled breath, the eucalyptol is generally the most concentrated metabolite. Some exception occurs for the  $\alpha$ - and  $\beta$ -Pinene, which are the second and third highest compounds found in exhaled breath. The fourth is the limonene. The limonene's outlier point was caused by the orange juice taken as breakfast drink, thus was not considered. Finally, the cymene is also part of the most intense compounds category. The menthol and menthone and their isomers are low concentrated in the exhaled breath, such as the cineol and the  $\gamma$ -terpinene. The camphene and the menthofuran are in between those two categories but closer to the low concentrated one.

The exhaled breath and the peppermint oil pattern are different. The cineole is not detected in the peppermint oil as already explained in the section *Determination of the peppermint VOCs of interest* on page 25. It seems that the cineole could be a by-product of the eucalyptol's metabolization, several facts can approve this hypothesis. First, the structures of the two compounds (Table 2 on page 28) are closed to each other. Then, the eucalyptol is the highest concentrated compounds detected in the exhaled breath, meaning that its by-products metabolites would be more visible. And finally, as observed in the average washout curve graph of Figure 26.E, the eucalyptol compound tends to metabolize slightly earlier than the other compounds. This made possible the apparition of the cineole signal 60 min after the pill intake. Moreover, this hypothesis is strengthened by several study of the eucalyptol metabolization by human body [47], [48].

Another difference is the intensity ratio of the menthol isomer between the exhaled breath and the peppermint pill. The menthol isomer is highly concentrated in the peppermint pill, while being in lower amount in the exhaled breath. Because of the considerable difference between the intensity ratios, it can be assumed that menthol isomer breaks down in a lot of other compounds which are not especially found in the exhaled breath.

The difference in intensity ratio is also visible for the other compounds such as the  $\alpha$ -pinene,  $\beta$ -pinene, the  $\gamma$ -terpinene, the menthofuran, and the menthone and its isomer. This also assumes a metabolization in by-product for these compounds, found or not in the exhaled breath.

The rest of the compounds (camphene, limonene, eucalyptol, and menthol) seem to keep the same intensity ration in the peppermint pill than in the exhaled breath. Those compounds would not break down into by-product in our body.

To conclude, this graph was useful to identify the compounds derived from the peppermint pill metabolization. It also gave an insight of the kind of metabolization pathway. But most importantly, it allowed identifying the category of concentration of the compounds and the different ratio intensity between the metabolites in the exhaled breath. These results are important to consider answering to research question on the metabolites' behavior.

### Monitoring exhaled breath variations

The Table 7 resumes the conclusion of the different statistical analyses.

*Table 7: Summary table of the data analyses*

Target compounds <i>Pages</i>	Washout profiles		Metabolites profiles		
	Volunteers' trend (55)	Metabolites trend (59)	Influence of the time point (59)	Metabolites metabolization (61)	Metabolization pathways (63)
1 <b><math>\alpha</math>-Pinene *</b>	90 min maximum				By-product metabolization
2 <b>Camphene</b>	60 and 165 min maximum				No transformation metabolization
3 <b><math>\beta</math>-Pinene *</b>	90 min maximum				By-product metabolization
4 <b>2,3-dehydro-1,8-cineole</b>	90 min maximum				By-product
5 <b>Limonene *</b>	90 min maximum				No transformation metabolization
6 <b>Cymene</b>	90 min maximum				By-product metabolization
7 <b>Eucalyptol *</b>	60 and 90 min maximum				No transformation metabolization
8 <b><math>\gamma</math>-Terpinene</b>	90 and 165 min maximum				By-product metabolization
9 <b>Menthofuran *</b>	90 and 165 min maximum				By-product metabolization
10 <b>Menthone *</b>	90 min maximum				By-product metabolization
11 <b>Menthol</b>	90 min maximum				No transformation metabolization
12 <b>Menthone isomer</b>	90 min maximum				By-product metabolization
13 <b>Menthol isomer*</b>	90 min maximum				By-product metabolization

The different data analyses demonstrated that the peppermint process, applied in the OBiAChem laboratory is robust and reproducible.

All the analyzed compounds are interesting to monitor in the exhaled breath, but they must be chosen according to the research question. Several compounds are more relevant to study the metabolism mechanism of the exhaled breath, as the metabolization pattern analysis raised.

The low intensity signal of the menthol isomer impacts considerably on the stability of the response. This characteristic can be used to observe the analytical method sensitivity. The camphene, cymene, and menthofuran compounds had variable profiles between the patients. It is relevant to monitor the compounds for stability of the exhaled breath matrix. Likewise, the  $\alpha$ -pinene's,  $\beta$ -pinene's and the  $\gamma$ -terpinene's washout profiles seem to be influenced by exogenous factors. The 2,3-dehydro1,8-cineole had variable responses due to its low intensity but is the only by-products of the pill metabolization monitored. The limonene could be influenced by the diet, a major cofounding factor, as it is present in several fruits and fruity drinks. Regarding the other compounds, they did not seem affected by cofounding factors.

For the peppermint project, among the 13 compounds analyzed, the menthol isomer, the menthofuran, and the camphene are the less suitable.

Among the seven target compounds defined by the peppermint Initiative, the  $\alpha$ -pinene\*,  $\beta$ -pinene\*, limonene\*, eucalyptol\* and menthone\* gave the best results in term of stability, reproducibility, and robustness for the peppermint procedure using GCxGC-HRTOFMS. However, the menthofuran\* and the menthol isomer\* present variable results and are not target compounds of choice after the statistical investigations. In the future of the peppermint project, the multiplatform analysis will enlighten if the variation comes from the metabolites or from the procedure used.

## IV.5. CONCLUSIONS

The goal of this master thesis was to help in the standardization and comparison of analytical methods for exhaled breath analysis by taking part in the peppermint Initiative, using TD-GC×GC-HRTOFMS.

This first part of the master thesis allowed optimizing the procedure for the exhaled breath analysis. First, the reduction of the volume of exhaled breath sampling from 5 L to 1 L enabled saving 20 hours of practical work for the 10 volunteers tested during the peppermint project which. For a clinical study of 250 patients, the time saved would be estimated to 500 hours of practical work. This time is extremely valuable for clinician's ease and the sampling of smaller volume improves the comfort of the patients. Then, benchmarking studies were done on sampling materials: the TD tube adsorbents and the bags materials. Concerning the materials of the sampling bags (Tedlar® or multifoil), the few contaminants of the Tedlar® material was preferred to the bulky, interfering, and persistent background noise of the multifoil material. The profiles of the two TD tubes adsorbent (Tenax® TA/Carbopack™ B and Tenax® GR/Carbopack™ B) were similar, but the Tenax® GR/Carbopack™ B were selected for the peppermint project to enable a future multiplatform analysis with the university analytical chemistry laboratory of Gembloux. The optimization of some sampling and injection parameters allowed to collect and to obtain reproducible washout curve's results. Finally, the reconditioning bags procedure was adapted, which improved the efficiency of the cleaning step, and extra COVID-19 precautions were taken to limit the potential virus contamination.

The peppermint project was the center piece of the master thesis. A total of 13 target compounds were analyzed, including the seven (\*) defined by the peppermint Initiative:  $\alpha$ -pinene\*, camphene,  $\beta$ -pinene\*, 2,3-dehydro-1,8-cineole, limonene\*, cymene, eucalyptol\*,  $\gamma$ -terpinene, menthofuran, menthone\*, menthol, menthone isomer, menthol isomer\*. Thanks to the data analyses, it has been demonstrated that the TD-GC×GC-HRTOFMS was a suitable instrument for exhaled breath analysis and monitoring. This advanced instrument gives a better separation and resolution of the compounds, which had positive repercussions on the reproducibility and robustness of the project. This was confirmed by the similar trends observed between the patient and the time, and the reliable correlation coefficient of the obtained curves. Among the six additional

target compounds analyzed, five revealed to be as suitable to monitor as those defined by the peppermint Initiative\*: the 2,3-dehydro-1,8-cineole, the cymene, the  $\gamma$ -terpinene, the menthol and the menthone isomer. However, the camphene, menthol isomer\* and the menthofuran\*, gave more variable results. The results obtained in this study using GCxGC-HRTOFMS will be compared by the peppermint consortium coordinator to further evaluate the robustness of the developed method compared to other analytical platforms. Then, a standardized approach for exhaled breath analysis will be proposed for each analytical platform, aiming at increasing the inter-laboratory reproducibility for exhaled breath analysis, something virtually not existing at all nowadays.

#### IV.6. FUTURE WORK

The next step of this master project would be to sample additional patient for the multiplatform analysis with the TD-GCxGC-qMS/VUV in Gembloux. In addition, the sampling of the breath of additional patients would enable to confirm and corroborate the obtained results.

Then, for the peppermint project but also for exhaled breath analysis, it would be interesting to do a complementary study with internal and external standard. It would help to visualize the instrumental variation between the injections. The internal standard would be injected onto the TD tubes before the sampling, under gaseous state. The standards used could be brominated, chlorinated or isotopic labelled standards, which are not naturally found in the exhaled breath and are not eluting in the chromatographic area of interest.

Finally, once the peppermint Initiative will be over, it would be interesting to look back at the publication results. They will determine if the variability of the menthofuran and the menthol isomer observed for our procedure was coming from the developed procedure or from the metabolites themselves.

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