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Chemical composition and in vitro antibacterial effects of vapours of essential oils from plants recommended by the European Medicines Agency against respiratory infections

DOCTORAL THESIS

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CERTIFICATION

I, Julien Antih, submitted this dissertation for Ph.D. degree at the Czech University of Life Sciences Prague, Faculty of Tropical AgriSciences, declare that this dissertation is my own work unless otherwise referenced or acknowledged.

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ABSTRACT

Often used as a synonym for pneumonia, lower respiratory tract infections remain among the leading causes of morbidity and mortality worldwide. Antibiotic inhalation therapy, a method involving the precise delivery of aerosolized particles directly into the lungs, offers distinct advantages over systemic antibiotics. However, challenges in executing effective nebulization techniques, coupled with the lack of robust clinical data, have constrained its broader application, especially in cases of lower respiratory tract infections in children under five. In this context, the volatile constituents of essential oils (EOs) appear as a promising alternative in the development of novel inhaled antibiotic therapy. The purpose of this study was first to determine the *in vitro* antibacterial activity of five EOs recommended by the European Medicines Agency (EMA) against cough and common cold. The EOs obtained from three commercial samples of *Eucalyptus globulus*, *Foeniculum vulgare*, *Mentha* x *piperita*, *Pimpinella anisum*, and *Thymus vulgaris* were assessed using broth microdilution volatilization (BMV) method against three standard bacterial strains associated with lower tract respiratory infections, including *Staphylococcus aureus*, *Streptococcus pyogenes* and *Haemophilus influenzae*. With the aim to optimize a protocol for characterizing EO vapours, we initially determined the chemical profile of the most active EO (*T. vulgaris*) using dual column/dual detector gas chromatography system. Subsequently, a thorough characterization of the vapour composition was conducted through headspace analyses above a liquid matrix (LM) of broth and *T. vulgaris* EO. This was achieved by employing both Solid Phase MicroExtraction (HS-SPME) and Gas Tight Syringe (HS-GTS) sampling techniques. Finally, in our efforts to enhance the antimicrobial activity of *T. vulgaris* EO vapour, we profiled its composition using a solid matrix (SM) composed of cellulose. The results showed that, out of these 5 EOs, only *T. vulgaris* possessed a significant activity against the studied respiratory pathogens with MICs comprised in between to 512 and 1024 µg/mL in both liquid and vapour phase. Furthermore, *H. influenzae* displayed the highest susceptibility to thyme EO, with a MIC of 128 µg/mL. In addition, GC-MS analysis results showed a different distribution of volatile compounds in the headspace. Notably, thymol prevalence was surprisingly low with peak percentage area inferior to 5.27 % (HS-SPME) and 0.60 % (HS-GTS) in the headspace above the LM, as opposed to the EOs (max. 48.65%). Furthermore, oxygenated monoterpenoids were significantly more prevalent in the headspace above the SM – e.g. max. thymol MICs = 43.76 % with HS-SPME and

4.87 % with HS-GTS – than above the LM, whereas hydrocarbon monoterpenes experienced a decrease in abundance. Multiple factors could explain these results such as parameters related to the use of a fibre coating assembly, but also the mechanisms governing the release of volatile compounds into the headspace and their interactions with chosen matrices as well as the degradation reactions occurring in between the volatile substances. Overall, this study holds great promise in providing invaluable insights that could lead to both innovative patents and advancements in pharmaceutical product development. Looking ahead, achieving control over the stability and release of EO volatile substances in vapour presents a formidable challenge in designing a potential antimicrobial vapour inhalation device. However, further investigations, including cytotoxicity studies and clinical aromatherapy trials, are necessary to evaluate the efficacy, tolerability, and safety of EO vapour in treating respiratory diseases.

ABSTRAKT

Infekce dolních cest dýchacích, sousloví často používané jako synonymum pro zápal plic, zůstávají celosvětově jednou z hlavních příčin nemocnosti a úmrtnosti. Inhalační antibiotická terapie, metoda zahrnující přesné podávání aerosolových částic přímo do plic, nabízí oproti systémovým antibiotikům výrazné výhody. Problémy při zavádění účinných nebulizačních technik spolu s nedostatkem spolehlivých klinických údajů však omezují její širší použití, zejména v případech infekcí dolních cest dýchacích u dětí mladších pěti let. V této souvislosti se těkavé složky esenciálních olejů (EO) jeví jako slibná alternativa při vývoji nové inhalační antibiotické terapie. Cílem této studie bylo nejprve stanovit *in vitro* antibakteriální aktivitu pěti EO doporučených Evropskou agenturou pro léčivé přípravky (EMA) proti kašli a nachlazení. EOs získané ze tří komerčních vzorků *Eucalyptus globulus*, *Foeniculum vulgare*, *Mentha* x *piperita*, *Pimpinella anisum* a *Thymus vulgaris* byly hodnoceny pomocí mikrodiluční bujónové volatilizační metody (BMV) proti třem standardním bakteriálním kmenům spojovaným s infekcemi dolních cest dýchacích: *Staphylococcus aureus*, *Streptococcus pyogenes* a *Haemophilus influenzae*. S cílem optimalizovat protokol pro charakterizaci výparů EO jsme nejprve stanovili chemický profil nejaktivnějšího EO (*T. vulgaris)* pomocí systému plynové chromatografie s dvojitou kolonou a dvojitým detektorem. Následně byla provedena důkladná charakterizace složení výparů pomocí analýz v prostoru nad kapalnou matricí (LM) bujónu a EO *T. vulgaris.* Toho bylo dosaženo použitím technik mikroextrakce na pevné fázi (HS-SPME) a odběru vzorků pomocí plynové stříkačky (HS-GTS). V neposlední řadě jsme v rámci snahy o zvýšení antimikrobiální aktivity výparů EO *T. vulgaris* profilovali jeho složení pomocí pevné matrice (SM) složené z celulózy. Výsledky ukázaly, že z těchto 5 EO pouze *T. vulgaris* vykazuje významnou aktivitu proti studovaným respiračním patogenům s minimální inhibiční koncentrací (MIC) v rozmezí 512 až 1024 μg/ml v kapalné i plynné fázi. Kromě toho *H. influenzae* vykazoval nejvyšší citlivost k tymiánovému EO s MIC 128 μg/ml. Výsledky analýzy GC-MS navíc ukázaly odlišné rozložení těkavých látek v headspace. Zejména převaha thymolu byla překvapivě nízká s procentuální plochou píku nižší než 5,27 % (HS-SPME) a 0,60 % (HS-GTS) v headspace nad LM, na rozdíl od EO (max. 48,65 %). Kromě toho kyslíkaté monoterpenoidy výrazně převažovaly v headspace nad SM - např. max. MIC thymolu = 43,76 % u HS-SPME a 4,87 % u HS-GTS - než nad LM, zatímco u uhlovodíkových monoterpenů došlo k poklesu jejich množství. Tyto výsledky by mohlo vysvětlit

více faktorů, jako jsou parametry související s použitím sestavy potahových vláken, ale také mechanismy, kterými se řídí uvolňování těkavých látek do headspace a jejich interakce s vybranými matricemi, jakož i rozkladné reakce probíhající mezi těkavými látkami. Celkově si tato studie klade za cíl poskytnout neocenitelné poznatky, které by mohly vést jak k inovativním patentům, tak k pokroku ve vývoji farmaceutických výrobků. Do budoucna představuje dosažení kontroly nad stabilitou a uvolňováním těkavých látek EO ve výparech obrovskou výzvu při navrhování potenciálního antimikrobiálního zařízení pro inhalaci výparů. K vyhodnocení účinnosti, snášenlivosti a bezpečnosti výparů EO při léčbě respiračních onemocnění je však zapotřebí dalšího výzkumu, včetně studií cytotoxicity a klinických aromaterapeutických zkoušek.

Klíčová slova: antimikrobiální aktivita; respirační infekce; esenciální olej; Thymus vulgaris; těkavé sloučeniny; headspace analýza; kapalná a pevná matrice.

RÉSUMÉ

Souvent utilisé comme synonyme de pneumonie, les infections des voies aériennes inférieures demeurent parmi les principales causes de morbidité et de mortalité à l'échelle mondiale. L'administration d'antibiotiques par voie inhalée, méthode consistant à administrer des particules solides sous forme d'aérosol directement dans les poumons, offre des avantages certains par rapport aux antibiotiques oraux. Cependant, des difficultés dans la mise en œuvre de techniques de nébulisation ont restreint leurs utilisations, particulièrement en pédiatrie. Dans ce contexte, les huiles essentielles (HE) et leurs composés volatils émergent comme une alternative prometteuse dans le développement de nouvelles thérapies inhalées contre les agents bactériens des voies respiratoires. L'objectif de cette étude a tout d'abord été de déterminer l'activité antibactérienne *in vitro* de cinq HE recommandées par l'Agence européenne des médicaments (EMA) contre les infections respiratoires. Les huiles essentielles extraites de trois échantillons commerciaux de *Pimpinella anisum*, *Eucalyptus globulus*, *Thymus vulgaris*, *Mentha x piperita* et *Foeniculum vulgare* ont été testées au moyen d'une méthode de microdilution en bouillon permettant de déterminer la concentration minimale inhibitrice (CMI) des échantillons d'HEs à différentes concentrations simultanément sous forme liquide et vapeur. Les cinq échantillons d'HEs ont été testés contre trois souches bactériennes standard associées aux infections des voies respiratoires inférieures, à savoir *Staphylococcus aureus*, *Streptococcus pyogenes* et *Haemophilus influenzae*. À la lumière des résultats micorbiologiques, le profil chimique de l'HE la plus active (*T. vulgaris*) a initialement été déterminé grâce à un système de chromatographie en phase gazeuse à double colonne et double détecteur. La poursuite de l'étude s'est axée sur l'optimisation d'un protocole visant à améliorer la caractérisation des vapeurs d'HE par chromatographie en phase gazeuse associée à la spectrométrie de masse (GC-MS). La caractérisation de la composition de la vapeur a été réalisée en analysant le headspace au-dessus d'une matrice liquide composée d'un mélange de bouillon de Mueller-Hinton et d'HE de *T. vulgaris*, reproduisant ainsi les conditions des essais *in vitro*. Cette étape a été réalisée en recourant à 2 méthodes d'échantillonnage différentes : par micro-extraction en phase solide (HS-SPME) et par seringue hermétique (HS-GTS), avant d'être analysé par GC-MS. Enfin, la dernière phase de cette étude a impliqué une transition d'une matrice liquide (LM) à une matrice solide (SM) composée de cellulose, avec pour but de comprendre et améliorer le profil chimique de la vapeur d'huile essentielle de *T. vulgaris* dans le headspace. Les

résultats ont montré que, parmi ces 5 échantillons d'HE, seule *T. vulgaris* possédait une activité significative contre les agents pathogènes respiratoires étudiés, avec des CMI comprises entre 512 et 1024 µg/mL à la fois en phase liquide et vapeur. De plus, *H. influenzae* a affiché la plus grande sensibilité à l'HE de thym, avec une CMI de 128 µg/mL. Les résultats de l'analyse GC ont révélé une distribution différente des composés volatils dans le headspace. Étonnamment, le thymol a été trouvé en moindre abondance, avec une aire de pic inférieure à 5,27 % (HS-SPME) et 0,60 % (HS-GTS) au-dessus de la LM, comparé à sa prévalence maximale de 48,65 % enregistrée dans l'extrait n-hexane de l'HE. Par ailleurs, les monoterpénoïdes oxygénés ont été significativement plus abondants dans le headspace au-dessus de la SM – par exemple, une prévalence maximale de thymol de 43,76 % avec HS-SPME et 4,87 % avec HS-GTS – qu'au-dessus de la LM. Au contraire, les monoterpènes hydrocarbonés, eux, ont subi une diminution d'abondance en passant d'une matrice à l'autre. En résumé, cette étude prometteuse apporte des données importantes qui pourraient conduire à des avancées innovantes dans le domaine du développement phytopharmaceutique, potentiellement menant à des brevets. À l'avenir, il sera essentiel de maîtriser la stabilité et l'évaporation des composés volatils des HEs, ce qui représente un défi significatif pour la conception de dispositifs d'inhalation pour lutter contre les infections bactériennes respiratoires. Cependant, des recherches supplémentaires, telles que des études de cytotoxicité et essais d'aromathérapie clinique, seront nécessaires pour évaluer l'efficacité et la sécurité de l'utilisation des vapeurs d'HE.

Mots-clés : activité antimicrobienne ; infections respiratoires ; huile essentielle ; *Thymus vulgaris*; composés volatils ; headspace analysis ; matrice liquide et solide.

LIST OF ABBREVIATIONS

ATCC   American Type Culture Collection

BHI   Brain Heart Infusion medium

BMV   Broth Microdilution Volatilisation

CA-MRSA   Community-acquired MRSA

CAP   Community Acquired Pneumonia

CF   Cystic fibrosis

CT   Chemotype

D-HS   Dynamic headspace

DV   Disc volatilisation

EMA   European Medicines Agency

EO   Essential Oil

ESCOP   European Scientific Cooperative on Phytotherapy

FDA   Food and Drug Administration

FID   Flame ionization detector

GAS   Group A Streptococcus

GBD   Global Burden of Diseases, Injuries, and Risk Factors Study

GC   Gas chromatography

GLV   Green leaf volatile

GTS   Gas tight syringe

HAP   Hospital acquired pneumonia

Hib   *H. influenzae* type b

HIV   Human Immunodeficiency Virus

HPLC   High performance liquid chromatography

HMPC   Herbal Medicinal Product Committee

HS   Headspace

IV   Intravenous

LM   Liquid matrix

LRTI   Lower Respiratory Tract Infection

m/z   mass-to-charge ratio

MH	Mueller-Hinton medium
MIC	Minimum Inhibitory Concentration
MID	Minimum Inhibitory Dose
MRSA	Methicillin-Resistant *Staphylococcus aureus*
MS	Mass spectrometer
NAD	Nicotinamide Adenine Dinucleotide
NTHi	Non-Typeable *Haemophilus influenzae*
Ph.Eur.	European Pharmacopoeia
RI	Retention index
RSV	Respiratory Syncytial Virus
S-HS	Static headspace
SFE	Supercritical fluid extraction
SM	Solid matrix
SPME	Solid phase microextraction
URTI	Upper Respiratory Tract Infection
VAP	Ventilator-associated pneumonia
VC	Volatile compounds
WHO	World Health Organization

1. Introduction

Lower respiratory tract infections (LRTI) refer to the infections of the airways that affect trachea, bronchi, bronchioles as well as alveolar sacs, causing symptoms such as cough, fever, difficulty breathing, and chest pain. Often used as a synonym for pneumonia, acute LRTIs also encompass other types of infections such as bronchiolitis, and remain among the leading causes of morbidity and mortality worldwide [1]. This is particularly significant in mid to low-income countries where vulnerable populations and mostly children under five years old are at higher risk. Causative pathogens of such diseases can be viral or bacterial. While viruses are mostly responsible for acute infections, bacteria on the other hand, cause most of pneumonia cases and are involved in bacterial bronchitis in children [2–4]. Common bacterial pathogens causing LRTIs include Gram-positive strains such as *Streptococcus pneumonia, Staphylococcus aureus*, or *Streptococcus pyogenes* as well as Gram-negative bacteria like *Haemophilus influenzae* [1,5].

To reduce bacterial LRTIs fatality, the recommended treatment is the administration of a full course of appropriate antibiotics. Compared to systemic antibiotics, inhalation therapy of aerosolised compounds offers significant advantages [6]. It allows the delivery of higher drug concentrations directly into the lungs, but also a more efficient and faster action while reducing the risk of side effects. However, the widespread implementation of inhalation therapy protocols to other aetiologies has been limited by the lack of robust clinical data [7–9]. This is mainly attributed to patients' physical ability to perform an appropriate breathing manoeuvre when receiving the treatment, but also to drug particle sizes and their aerodynamic behaviour entering the lungs [10,11].

In this context, essential oils could be considered as an interesting alternative for the development of novel antimicrobial volatile agents. Since ancient times, EOs have been used in various traditional medicines around the globe to treat various respiratory tract infections such as pharyngitis, bronchitis or sinusitis [12,13]. Their main physicochemical property is to be highly volatile at room temperature and atmospheric pressure. This unique feature used in inhalation therapy would allow a uniform distribution of volatile compounds down to the lower section of the lungs, at significant concentrations and, without requiring any specific breathing techniques [14]. Furthermore, EOs are complex mixtures containing a broad spectrum of chemically diverse compounds that may work in synergy to provide their antibacterial activity [15,16]. Various studies

also emphasised that it is more difficult for bacteria to develop resistance to multi-compounds EOs than it is to single molecular antibiotics [17]. Oxygenated terpenoids such as phenolic terpenes and phenylpropanoids are the main classes of secondary metabolites associated with the antibacterial activity of EOs [18,19]. Although their working principle has not been fully elucidated yet, it is assumed that these volatile compounds have similar modes of action than other phytochemicals including the disruption of bacterial cell membranes, the inhibition of oxygen uptake or the suppression of bacterial biofilm development [20–22].

In the European Union, EO inhalations for paediatric population are already recommended in inhalation for moderate respiratory infections by the Herbal Medicinal Product Committee (HMPC) of the European Medicine Agency (EMA) [23]. Amongst the nearly 30 EOs approved by the HMPC, *Pimpinella anisum* L. seeds, *Eucalyptus globulus* Labill. leaves, *Thymus vulgaris* L. aerial parts, *Mentha×piperita* L. leaves and *Foeniculum vulgare* Mill. seeds belong to the most important plant sources of EOs used traditionally to treat respiratory disorders. This collection of essential oils is particularly noteworthy as the EMA also recommends their use in inhalation. The EO vapour may be delivered using different volatilization matrices, for instance, using water as a liquid matrix through steam inhalation or opt for a cellulose wadding as a solid matrix when using a stick inhaler [24,25]. Moreover, both their antimicrobial activities and chemical compositions are already well-studied [13,23,26]. Despite the number of recent studies reporting that vapours of EOs show better antimicrobial activity than their liquid forms [27–29], there is still a lack of available data on the efficacy of EO recommended by HMPC for respiratory infections against bacteria causing LTRIs in vapour phase. Additionally, several publications focused on characterization of chemical profiles of their vapour is also limited, especially in the context of volatilization matrices.

Considering the above-mentioned facts and figures, we investigated the chemical composition and antibacterial activity of EOs approved by the HMPC for the treatment of infectious cough and cold in pediatric population. This dissertation reports the *in vitro* growth-inhibitory effects of *P. anisum* seed, *E. globulus* leaf, *T. vulgaris* aerial parts, *Mentha×piperita* leaf, and *F. vulgare* seed EOs in liquid and vapour phase against pneumonia causing bacterial pathogens including *H. influenzae*, *S. aureus*, and *S. pyogenes*. In addition, we concentrated on developing a procedure for the chemical characterization of EO vapours using *T. vulgaris* EO due to the low MICs it produced.

2. Literature review

2.1 Lower respiratory tract infections in paediatric population

Respiratory disorders rank among the top causes of mortality and morbidity worldwide. According to the World Health Organization (WHO), more than 1 billion people suffer from either chronic or acute respiratory disorders. Chronic respiratory conditions cause 4 million premature deaths each year, with new-borns and young children being the most vulnerable. The most common chronic paediatric condition, asthma, affects nearly 340 million young individuals globally. As for acute disorders, respiratory infectious diseases ranked second among the top ten causes of burden in children after neonatal disorders worldwide in 2019 [1,30].

Amongst all illnesses affecting the lungs, infectious diseases pose the highest risk to children under five years of age [31]. They are classed as either upper respiratory tract infections (URTI), touching the airway above the vocal cords, or as lower respiratory tract infections involving the respiratory tract below the level of the larynx. According to epidemiological data LRTIs like pneumonia or bronchiolitis, are significantly more deadly than URTIs, killing each year more than HIV, tuberculosis and malaria combined [1,32]. In children under-5s, the most frequent pathogens involved are both viruses, with mainly the respiratory syncytial virus (RSV), and bacteria leading by *Hemophilus influenzae* and *Streptococcus pneumoniae* [33–35].

2.1.1 Epidemiology

According to the Global Burden of Diseases, Injuries, and Risk Factors Study (GBD) in *The Lancet Infectious Diseases*, which provides a systematic scientific assessment of published data on disease and injury mortality worldwide, pneumonia is responsible for the vast majority of all LRTI episodes [35]. This infection arises from microorganisms invasion and overgrowth in the lung parenchyma, resulting in both an inflammation of lung tissues and intra alveolar exudate [36]. Its typical symptoms are a high fever accompanied with chills, tachycardia, and an increase in respiratory frequency. Often a stabbing chest pain and shortness of breath are observed as well as a productive cough with a yellow to green purulent sputum that may contain blood [37,38].

As mentioned previously, pneumonia is recognised globally as a public health priority, and its widespread occurrence affects disproportionately children under 5 years of age [34]. The highest

risks for getting or succumbing to pneumonia generally originate from low-income homes with limited access to proper nutrition, resulting in child wasting [1]. Main causes include both the lack of breast-feeding and inadequate Zinc, Selenium and Vitamin A and D intakes [39]. Similarly, infants with an immunocompromising condition or suffering from other infectious diseases like HIV or measles are more likely to develop pneumonia. Environmental factors such as living in a crowded house, the use of unclean cooking fuels, passive tobacco smoke exposure, or ambient air pollution also play a critical role [40].

In 2019, the GBD study estimated that there were over 60 million episodes among children younger than 5 years worldwide, equivalent to an incidence of 0.11 cases per child/year [32,41]. Pneumonia principally occurs in developing countries with episodes reported in sub-Saharan Africa, south Asia, and southeast Asia representing three quarter of all cases worldwide [40]. Likewise, the highest number of episodes was recorded in the South Asian region with 18.7 million cases among under-5s. Furthermore, pneumonia remains the deadliest communicable disease having killed approximately 700,000 young individuals in 2019, and accounts for 15% of all under-5s death each year [42]. The extended GBD study on LRTIs of 2016 estimated that the highest rates for pneumonia mortality among children were in Africa including the Central African Republic with 460 deaths per 100,000 people and Chad (425 per 100,000 people). Similarly, due to their larger population, a third of all under-5 years deaths occurred in India with nearly 150,000 fatalities yearly and Nigeria (around 60,000 fatalities per year). Eventually, bacterial infections represented two thirds of all pneumonia cases worldwide with nearly 400,000 deaths while viral infections only accounted for 50,000 deaths in infants [35].

Since the last two decades, substantial improvements in reducing respiratory infections mortality in under-5 children age group were observed (23% decrease in between 1990 and 2016) thanks to the implementation of actions like the deployment of both antipneumococcal conjugated and *H. influenzae* vaccines, the promotion of breastfeeding to end wasting and the reduction of household air pollution [43]. However, until recently only 60% of children with pneumonia symptoms have had access to an appropriate care and only a third received antibiotics [30,40,41].

2.1.2   *Classification*

To begin a suitable therapy and achieve a better outcome, physicians differentiate various types of pneumonia based on several characteristics, encompassing the location acquired, the aetiology of infections, clinical features and the severity of the disease [44].

Community-acquired pneumonia (CAP) is defined as respiratory infection contracted outside hospitals or other healthcare setting. It is transmitted from person-to-person through small droplets of nasal or oral secretions that may contain a variety of microorganisms found in the community [45]. CAP is further divided in typical and atypical pneumonia:

- Typical pneumonia usually begins with non-specific symptoms such as fever, cough, shortness of breath, sputum production and chest pain. In children under-5 the spectrum of bacterial pathogens that causes typical CAP includes predominantly gram-positive strains like *S. pneumoniae* or *S. aureus* but also gram-negative bacteria like *H. influenzae* [46]. Moreover, although *S. pyogenes* is a less common cause, its relative incidence increased since the introduction of antipneumococcal conjugated vaccines. Following vaccination, reduced *S. pneumoniae* colonisation in the nasopharynx may favour non-vaccine pneumococcal colonisation, including by *S. pyogenes* [47]. Such infections were observed in late-onset neonatal pneumonia and secondary to viral infections involving the respiratory syncytial virus, influenza or varicella viruses [48–50].

- Atypical pneumonia however, is caused by bacteria that are not detectable on Gram stain and cannot be cultured using standard methods [51]. Such CAP shows characteristic features that are described as a mixture of upper and lower respiratory infections. It comprises a low-grade fever (< 38 degrees), a persistent dry cough, and more pronounced headaches and myalgia [44,52]. In children under-5s, the most common bacterial pathogens include *Mycoplasma pneumoniae* and *Chlamydia pneumoniae* [45,53]. Neonates are also at risk with organisms located near the birth canal such as *Escherichia coli* and *Listeria monocytogenes* [54].

In developed countries, the gold standard for CAP diagnosis is by radiographic method. Together with clinical features, chest X-ray allows physicians to distinguish the type of CAP they

must treat. In typical pneumonia X-ray will show consolidations in small airways (i.e. the airspace is filled with body fluids creating a cloud-like silhouette) whereas in atypical pneumonia radiographic features present patchy reticular or reticulonodular opacities [52]. In developing countries however, difficulties to access to radiography and laboratory tests have forced health authorities to take a different approach, assuring more children to receive an appropriate treatment [56].

This led the WHO to generate a childhood pneumonia control strategy that is easy to implement in countries with limited resources and constrained healthcare systems. The approach consists of classifying the severity of the infection in children aged 2-59 months using simple clinical features such as fast-breathing or chest indrawing movements before applying the recommended examinations and treatments [57]. Followed by the vast majority of low to middle-income countries, the WHO management guideline uses a two-categories classification [58,59]:

- Non-severe pneumonia clinical features include cough with fast breathing – ≥ 50 breaths/min in a child aged 2-11 months and ≥ 40 breaths/min in a child aged 1-5 years – lower chest wall indrawing, fever, and specific auscultation signs such as coarse crackles (loud sounds caused by constricted larger airways) or bronchial breath sounds. In this situation, patients stay at home, and receive a full course of benzylpenicillin as a first-line antibiotic therapy. Advice to parents is provided in case of further aggravations.

- Severe pneumonia threshold is crossed when life threatening symptoms arise. It comprises central cyanosis, severe respiratory distress like grunting, impossibility of breastfeeding or drink, unconsciousness, and convulsions. These signs present an emergency, and the child is immediately hospitalized. In addition of intravenous (IV) antibiotic therapy (ampicillin and gentamicin IV as first-line treatment), oxygen is also given if blood saturation is below 90%. The child should be constantly checked by nurses and physicians.

Likewise, a chest X-ray is recommended if available and so is, ideally, the isolation of the responsible microorganisms. Many investigations, however, showed that current laboratory techniques lack the sensitivity to identify all relevant bacteria. As a result, when there is a CAP detected, guidelines assume that it could be cause by all likely pathogens [56].

Another common cluster of LRTIs in children is hospital-acquired pneumonia (HAP) also known as nosocomial pneumonia. This group of respiratory infections is defined as occurring at or beyond 48 hours after admission to hospital ward or intensive care unit [43]. In children under five years, HAP is both the most common and fatal nosocomial infection, accounting for 10 to 15% of all paediatric infections in hospital with a mortality higher than 20% [60]. Major risk factors for HAP in developing countries are a prolonged hospitalization and underlying chronic illnesses like malnutrition and immunodeficiency. Furthermore, multiple studies emphasised that other important sources of contamination such as the presence of gastric contents in the trachea due to intubation, colonization of ventilator tubing as well as nebulizers causing the inhalation of aerosolized bacteria [61,62]. In paediatric HAP, gram-negative bacilli such as *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* are the most common pathogens incriminated with *P. aeruginosa* being predominant in intensive care unit [60]. When it comes to gram-positive bacteria, *S. aureus* is by far the most common pathogen identified in both general ward and intensive care unit. In addition, although representing only 3% of all HAP cases, *S. pneumoniae* and *H. influenzae* infections still occurs, particularly within 5 days after hospitalization [62].

Given the afore-mentioned classification, this work examines three most common types of bacterial pathogens infecting children under-5s occurring in both community and hospital settings.

2.1.3 *Respiratory bacterial pathogens in paediatric population*

In order to invade the lower respiratory tract and cause pneumonia, colonisation of the nasopharynx is a critical step for respiratory pathogens such as *H. influenzae*, *S. aureus* and *S. pyogenes* [63]. In most cases, colonisation of the upper airways results in only an asymptomatic carriage followed by the dissemination to other individuals through the environment. In contrast, infections occur when the balance between the pathogenic agents, the host commensal bacteria, and its mucosal immune mechanism is disrupted [64]. Immature and weak immune systems in children, as well as inflammations caused by viruses or environmental factors allow bacteria to migrate and settle into the lungs causing severe infections [65].

2.1.3.1 H. influenzae

Haemophilus influenzae was first isolated by Richard Pfeiffer in 1892. The German bacteriologist described it as a small rod-shaped bacterium and mistakenly believed to be responsible for the flu. Although the discovery of the influenza virus during the 1918 flu pandemic demonstrated that Pfeiffer observations were invalid, *H. influenzae* kept its species name [66,67]. Nowadays, it is widely admitted that the bacterium is a small (i.e., 0.3 μm to 1 μm in width), pleomorphic as it can take various shapes, nonmotile, gram-negative organism also known as coccobacillus. As facultative anaerobe, it has both aerobic and anaerobic metabolisms. It is also defined as capnophilic for its ability to thrive in a carbon dioxide environment. To grow, it requires either or both of the following components: nicotinamide adenine dinucleotide (NAD or factor V) and haemin (factor X) found in erythrocytes. Hence the genus name Haemophilus which translates as "blood loving". Bacteria using exclusively haemin are typically cultured on blood agar whereas the strains necessitating NAD are grown on heat-treated red blood media as this factor is a by-product of red-blood cell haemolysis [68]. Despite its occurrence in academic literature since the beginning of the 20th century, it was not until the 1930s when American bacteriologist Margaret Pittman first classified the species based on the presence of a polysaccharide layer located outside the bacteria cell envelope: the capsule. Until now, *H. influenzae* strains are still broadly categorized into encapsulated and non-encapsulated types. According to its capsular composition, encapsulated strains are further subdivided into 6 groups designated as serotypes a, b, c, d, e, and f. They are traditionally identified based on a precipitation reaction with a serum specific to them [69]. On the other hand, non-encapsulated strains that cannot be serotyped are designated as Non-Typeable *Haemophilus influenzae* (NTHi).

Every serotype, but notably *Haemophilus influenzae* type b (Hib) is a frequent cause of lower respiratory tract infections that mainly affect children under 2 years of age. Additionally, they are capable of causing a wide range of other severe illnesses, including meningitis, epiglottitis, and bacteraemia [70]. As mentioned at the beginning of this section, like other respiratory pathogens, *H. influenzae* must first colonise the host nasopharynx and avoid a range of challenges: from penetrating the mucous barrier and obtaining nutrients to resisting the nasal mucociliary clearance and evading the immune system. To successfully do so, *H. influenzae* developed several virulence mechanisms [71].

One of the most important strategies is the development of the polysaccharide capsule. Its primary role is to help encapsulated strains to avoid being destroyed by the host complement-mediated system as well as to resist phagocytosis [68,72]. Furthermore, both strain types possess a wide variety of exceptionally long and multifunctional proteins known as adhesins and autotransporters which are often incorporated in the bacteria outer membrane [73]. The main objective of such structures is to facilitate the attachment to the host respiratory epithelium, preventing them to be swept away by the mucociliary clearance system [74]. Eventually, another important stratagem is the release of a protease enzyme capable of cleaving mucosal antibodies such as immunoglobulin A1, thus allowing *H. influenzae* to evade the host immune system [75].

Children are considered a reservoir for *H. influenzae* since the bacteria is commonly present in their respiratory tracts. Although most individuals are asymptomatic carriers, there is a particular vulnerability with children aged from two months to three years as they tend to develop severe infections due to their immature immune system [70]. Until the early 1990s, more than 95% of all invasive infections accountable to *Haemophilus* species in children under-5s were estimated to have been caused by *H. influenzae* type b (Hib) [76]. The introduction of the Hib conjugated vaccines resulted in a dramatical decrease in the incidence of Hib respiratory infections worldwide in both vaccinated and unvaccinated children due to the herd effect [77]. A possible explanation is that the high concentration of anticapsular antibodies produced after immunization would first restrict Hib nasopharyngeal colonisation, thus reducing person-to-person transmission from one child to another [78]. In the present post-Hib vaccine era, *H. influenzae* respiratory infections continue to occur with Non-Typeable *Haemophilus influenzae* (NTHi) strains emerging as the most common cause in the paediatric population. For instance, in the South American region, the WHO network identified that 49% of all pneumonia cases in patients under 5 years of age where cause by NTHi in the 2017-2018 period [70]. Although the number of NTHi cases is increasing, mechanisms contributing to its rise are still poorly understood. Several academic studies, however, describe the following facts as potential causes of this surge, including vaccine-mediated strain replacement, improved bacterial detection and serotyping and a potential increased virulence of NTHi strains [71,79].

2.1.3.2   S. aureus

Staphylococci were first identified in 1881 by Scottish physician Sir Alexander Ogston during a surgical procedure he was performing on a knee joint abscess. The surgeon described the bacteria he observed in pus as a "grape-cluster berry" (from *staphyle* and *kokkos* in Greek) and named the new bacterial genus accordingly [80]. *Staphylococcus aureus* is described today as a facultative anaerobic, nonmotile, spherical-shaped (0.5 - 1.5 µm diameter), gram-positive coccus that forms large, golden-yellow colonies when observed under the microscope [81].

In human population, *S. aureus* is both a skin and mucosa commensal microorganism and an opportunistic pathogen. It frequently causes infections in skin and soft tissues as well as severe diseases such as meningitis, septicemia, and pneumonia [82]. One of the most important biological properties of *S. aureus* is its ability to asymptomatically colonise healthy individuals. Although the bacteria can be naturally found on different body locations (i.e., the skin and both the urogenital and gastrointestinal tracts), the anterior nares remain its main reservoir [83]. Furthermore, the asymptomatic nasal colonisation of *S. aureus,* occurring in nearly 30% of the entire human population, is not only a predisposition to develop a serious infection but is also presumed to play a key role in the transmission among individuals [84,85].

The most characteristic symptom of staphylococcal disease is a rapid and intense inflammatory response. This inflammation is triggered by the bacterium's numerous virulent factors including its adherence to host cells, internalization as well as the direct contact between *S. aureus* secreted toxins, and the airways mucosa [86]. The attachment of *Staphylococcus aureus* to the host cells is made possible by a class of surface adhesins known as microbial surface component recognizing adhesive matrix molecules (MSCRAMMs). Their main function is to recognise particular extracellular matrix components such as fibrinogen or collagens and strongly anchor the bacterium [87]. Subsequently, the bonds created between MSCRAMMs and their ligands allow *S. aureus* active internalization into the host cells [88]. Once internalized, it may either remain locally, escaping the host immune system and antimicrobial therapies or alternatively, it may multiply and secrete cytolytic toxins that lyse the host cell allowing bacteria to further disseminate [89]. Multiple specific virulence factors such as the staphylococcal protein A, a MSCRAMMs, and pore-forming toxins (i.e., α-toxin, β-toxin, and PVL toxin) are of a significant importance in *S. aureus* pneumonia since they are implicated in the aggravation of the inflammatory response [90,91].

Until recently, *S. aureus* was considered an uncommon cause of respiratory infection in paediatric: community-acquired staphylococcal pneumonia was observed primarily, yet very rarely in adults recovering from influenza-like episodes [92]. In contrast, HAP cases, although important in elderly patients, did not demonstrate such a frequent occurrence [93]. In the past two decades, however, rates of childhood staphylococcal pneumonia dramatically increased, and in 2019, cases in children under five years of age accounted over 1.40 million hospital admissions worldwide (approximately 6% of all pneumonia episodes requiring hospitalisation). This phenomenon can be partially explained by both the change in health-seeking behaviour and improved access to healthcare in developing countries, but also by the increasing number of under-5s individuals [94]. Nowadays, *Staphylococcus aureus* is recognised as an important cause of HAP in both paediatric and neonatal hospitalised population [95]. According to a 2020 study, 10% of paediatric patients placed under mechanical ventilation in intensive care units developed a ventilated-associate pneumonia, representing 63 episodes per day for 1000 ventilators [96,97]. Furthermore, the 1960s saw the apparition of antibiotics resistant strain: the methicillin-resistant *S. aureus* (MRSA). Today, it is estimated that 20 to 40% of all hospital-associated pneumonia episodes are caused by MRSA [98]. Similarly, MRSA infections existed solely within the healthcare settings and predominantly limited to skin and soft tissue. During the 2000s, however, the emergence of a new MRSA pulmonary strain has been increasingly identified in young patients contaminated outside the healthcare system, marking the outbreak of community-acquired MRSA (CA-MRSA) pneumonia. Early 2000s studies in both France and the US [99,100] described CA-MRSA pneumonia as affecting mainly healthy children with a preceding influenza-like episode (approximately 70% of all cases in both studies) and characterized by very severe respiratory symptoms (around 60% of all episodes required intubation with nearly 30% mortality rate in both studies). Eventually, CA-MRSA in the paediatric population has risen significantly – from 3% in 2008 to 20% in 2019. This could be potentially explained by the recent increase in both the use and misuse of antibiotics in community settings [101].

2.1.3.3 S. pyogenes

The first identification of *Streptococcus* genus is attributed to the Austrian surgeon, Theodor Billroth who in 1874 described it as a "small organism found isolated or sometimes in chains of four to twenty or more links" [102]. Bacteria of this genus share the same characteristics, they are

small (0.5 – 1 µm diameter), facultative anaerobic, non-motile and non-spore-forming gram-positive cocci arranged in chains of up to 30 cells [81]. They have a thick peptidoglycan cell wall providing them with rigidity, and a capsule protecting them from the host's immune system. Their classification is based on three criteria including the morphology of their colonies, their haemolytic ability, and their specific serology with the latter two being the most distinctive. Regarding their haemolytic ability, three groups of streptococci are distinguished: α-haemolytic (greenish decolouration of the agar around colonies, incomplete haemolysis), β-haemolytic – when grown on blood agar, colonies are surrounded by a clear zone showing a complete lysis of red blood cells – and γ-haemolytic (no haemolysis). Their serologic grouping however, is based on the composition of their cell wall, which include the presence of both specific carbohydrate antigens (Lancefield groups A to V) and surface proteins [103].

Streptococcus pyogenes is a group A β-haemolytic streptococcus (GAS). Although it forms a significant part of the normal human nasopharyngeal flora, the bacterium is also an opportunistic pathogen commonly associated with skin and throat infections such as pharyngitis (strep throat), tonsillitis, impetigo, and scarlet fever [81]. On occasions, however, it is also capable of causing life-threatening invasive infections like necrotizing fasciitis, streptococcal toxic shock syndrome and severe pneumonia [104].

As with other pathogens, adherence to epithelial cells is an essential first step in initiating infection. In the nasopharynx, two important cell wall components work together in the attachment of GAS to the mucosal lining: the M protein and lipoteichoic acids. In this phase, lipoteichoic acids, considered as adhesins, bind fibronectin molecules on the epithelial cells' extracellular matrix. This is made possible thanks to the help of M proteins that work as scaffolds supporting lipoteichoic acids [105]. Beside their role in adhesion, M proteins are also involved in two other virulence mechanisms. They first play a key role in the process of intracellular internalisation allowing GAS to invade the host's cells and thus hide from its immune system. Similarly, together with the hyaluronic acid capsule, they confer the bacteria with antiphagocytic properties [106,107]. Eventually, some GAS strains are able to secrete enzymes (i.e., streptokinase) and pore-forming toxins (streptococcal pyrogenic exotoxins) which trigger the host inflammatory mechanism, leading to tissue damage and lung failure in severe haemorrhagic pneumonia [108].

Since the past three decades, the incidence of group A streptococcus invasive diseases have continuously increased until reaching a maximum in mid-2010s [47]. Today, *S. pyogenes* is the

third most common bacterium involved in respiratory diseases and an important cause of morbidity and mortality [109]. On a global scale, this represents over 650,000 invasive infections annually – with pneumonia accounting for 7 to 10% of them – and leading to nearly 160,000 deaths each year [110,111]. Although it may affect any age groups, GAS infections prevalence is higher in children under 5 years of age. This is mainly explained by their multiple exposures to bacteria in community, for instance, in school and nurseries but also due to their weaker immune systems [112]. Previous review showed that children infected by GAS developed more severe respiratory disease comparing to those affected by *S. pneumoniae*, representing up to 20% of all severe paediatric pneumonia episodes [49]. Furthermore, the incidence of invasive *S. pyogenes* infections also varies according to the geographic regions. In industrialized countries, the incidence in children under 5 years is estimated to be relatively low, around 0.12 to 3.1 episodes per 100,000 individuals per year [113]. In temperate climates, GAS pneumonia are often associated with viral respiratory coinfections such as seasonal influenza and respiratory syncytial virus, following their seasonal pattern [111,112]. In September 2022, for instance, several European countries including France, Ireland, the Netherlands and, Sweden have reported an increase in GAS respiratory infection episodes among children under 10 years of age, which according to the European Centre for Disease Prevention and Control, is attributed to the return of pre-pandemic circulation level of respiratory viruses [114]. By contrast, in developing countries where 95% of all GAS invasive infections occurs, data on paediatric pneumonia are still limited. Few recent studies, however estimated that rates of GAS pneumonia in children are higher in less developed regions, with the highest rates being observed in Sub-Saharan Africa (i.e., Kenya with 29 cases/100,000 person-years among children <5 years) [109], and in the Pacific Islands (Fiji with 26 cases/100,000 person-years among children <5 years) [115]. Epidemiologists explain this significant difference in incidence by the natural geographical variation of *S. pyogenes* strains, but also by the non-white population's susceptibility to the bacterium. For example, considerably higher rates were observed in Australia and New Zealand within the aboriginal population (83 cases /100,000 person-years) compared to white European descents (12 cases /100,000 person-years) [112].

2.1.4   *Antibiotic treatments*

In practice, the first step in treating a child with pneumonia is to determine the severity of the infection, and whether it can be safely handled within the community or if referral to a hospital is

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necessary. This initial and thorough examination usually occurs in primary care setting and establishes decisions to initiate the antibiotic treatment, its duration, and the level of medical and nursing care required in case of hospitalisation [116].

2.1.4.1   Oral and parenteral antibiotics

When antibiotic therapy is required, physicians should select the antimicrobial agent based on several criteria including the aetiology and the presence of risk factors for atypical or resistant bacteria [117]. In children, however, isolating and identifying the responsible microorganism is often complicated as sputum samples are difficult to obtain and are frequently contaminated with the upper bacterial flora [118,119]. Furthermore, viral pneumonia involving mainly influenza and respiratory syncytial viruses are very common in younger children and their clinical features cannot be distinguished from infections caused by bacteria. For instance, it has been reported that viral aetiology represents up to 70% of all CAP cases from which nearly 50% are viral-bacterial co-infections [120]. As a result, to prevent unnecessary fatalities, antibiotics are almost always prescribed for all children with a strongly suspected or confirmed diagnosis of pneumonia, particularly if the clinical signs and symptoms are severe. In the last 10 years, many guidelines on treatments for paediatric pneumonia were published with the aim to optimise prescriptions and avoid antibiotic overuse [119]. In industrialized countries, these national and international guidelines differ significantly, and clinical signs remain the major criteria when deciding to start antibiotics. Furthermore, given the age-related importance of bacterial pathogens in determining CAP, affected children can be divided into 2 categories: neonates and between 3 months to 5 years of age [121].

In the first two months of life, the treatment of choice is a combination of parenteral drugs including a *β*-lactam antibiotics from the penicillin group, ampicillin, together with an aminoglycoside (i.e., gentamicin IV), as Group B streptococci and gram-negative bacteria such as *H. influenzae* are the most common pathogens [119]. In cases of allergy to penicillin or treatment failure, a potential alternative includes the administration of a broad-spectrum agent like a third-generation cephalosporin (i.e., ceftriaxone or cefotaxime). Eventually, in very severe cases, *S. aureus* infection should be treated using either a *β*-lactamase-resistant penicillin like cloxacillin or an intravenous glycopeptide antibiotic such as vancomycin, the latter also remaining one of the

principal agent of choice in the treatment of MRSA [121,122]. Regarding the posology, doses in parenteral administration depend on the child's weight and gestational age.

Between 3 months and 5 years of age, most guidelines recommend the use of amoxicillin as the first-line therapy for non-severe pneumonia as it is effective against the majority of frequent pathogens including *S. pneumonia*, *H. influenzae,* and *S. pyogenes*. In contrast to neonates, however, oral therapy is preferred to parenteral administration at a daily dosage of 50–90 mg/kg in 2 to 3 courses for 7–10 days in children able to swallow [121]. In case of clinical failure, oral amoxicillin–clavulanate at the same daily dosage or a third-generation cephalosporin (i.e., cefuroxime axetile at 30 mg/kg/days in 2 divided doses for 7-10 days) are the recommended alternatives. Furthermore, atypical bacteria such as *Mycoplasma pneumoniae* are increasingly important microorganisms in children under 5 years. Despite the lack of significant evidence of its benefits, the supported antibiotic treatment is a combination therapy including both *β*-lactamase-resistant penicillin and a macrolide such as oral azithromycin (10 mg/kg/day in 1 dose for 3 days) or clarithromycin (15 mg/kg/day in 2 divided doses for 10-14 days) [123]. Ultimately, in critically ill patients, *S. aureus* pneumonia should be suspected and parenteral antistaphylococcal antibiotics (i.e., cloxacillin or vancomycin) should be administered [124].

On the other hand, socio-economic factors such as the availability of antibiotics drug, cost limitations and healthcare accessibility often leads developing countries to adopt a different strategy, having to reduce the mortality rate with scarce financial resources [122]. Although the WHO guideline recommends the same antibiotic therapy for neonates as developed countries, discrepancies occur in the treatment of children older than 2 months. For non-severe pneumonia, the use of a combination of antibiotic medication called co-trimoxazole (trimethoprim and sulfamethoxazole) as an alternative to amoxicillin is prescribed [125]. Furthermore, the recommended first-line drug for severe episodes consists of the administration of parenteral benzylpenicillin (200,000 units/kg/day in 4–6 doses for 3 days) complemented with oral amoxicillin (50–90 mg/kg/day for a further 5 days). If the child does not improve within 48 hours or deteriorates, the alternative is intravenous chloramphenicol at a dosage of 75 mg/kg/day for 10 days [124]. Similarly, in case of severe *S. aureus* pneumonia, the standard treatment is a combination of cloxacillin IV and gentamicin IV for a total course of 3 weeks or a third generation cephalosporin (i.e., ceftriaxone 50 mg/kg once a day) instead of vancomycin IV [59,125].

Despite this antibiotic arsenal and treatment strategies, pneumonia remains a challenging condition to treat. The sequestration of bacterial pathogens deep within the lower regions of the respiratory tract as well as the poor penetration into the lung parenchyma of certain drugs such as aminoglycosides and β-lactams reduces the effectiveness of oral and intravenous administration routes [126,127]. For instance, studies have shown that amoxicillin concentration in the lungs can be as low as 50% compared to its plasmatic concentration as the alveolar-capillary membrane has low permeability to such compounds [128]. Furthermore, a vast majority of systemic antibiotics recommended in guidelines are associated to undesirable and potentially severe adverse effects, leading to treatment discontinuation. For example, important numbers of hypersensitivity reactions to β-lactams are reported each year with an incidence close to 10%. Similarly, nephrotoxicity and ototoxicity may occur in nearly 20% in patients treated with aminoglycosides [129]. Ultimately, the widespread misuse and overuse of oral and intravenous antibiotics in monotherapy have contributed to the development of drug-resistant bacteria [130]. Today, it is estimated that 90 to 95 % of all *S. aureus* strains have already developed a resistant to penicillin. Likewise, a study from Egypt reported that 17% of all tested gram-negative isolates were resistant to aminoglycosides [131]. As an alternative, delivering antibiotics directly to the site of infection via inhalation could overcome such obstacles.

2.1.4.2 *Inhalation therapies*

The concept of delivering medicine directly to the respiratory tract has been around for thousands of years. In ancient Greece, for instance, the fathers of pharmacy Dioscorides and Galen already recommended the inhalation of sulfuric vapours for treating respiratory diseases. But it was not until the 1940s that the first attempts to aerosolize synthetic antibiotics such as neomycin, polymyxin, and even penicillin G for patients with pneumococcal pneumonia occurred [132]. Today, nebulised antibiotic treatments are included in hospital care protocols, in particular against *Pseudomonas aeruginosa* superinfections in cystic fibrosis (CF) [133].

Compared to systemic therapies, inhaled antibiotics possess undoubted advantages. Besides being directly and rapidly delivered to the site of infection, this local administration route prevents the degradation of active compounds by both the gastrointestinal tract and the first-pass metabolism in the liver [134]. This allows the delivery of higher drug concentrations in the sputum compared to conventional administration routes, inducing a rapid clinical response. In addition,

inhaled antibiotics have a low risk of adverse effects due to their minimal systematic absorption, lowering body exposure to drug toxicity. Eventually, achieving high concentrations in lungs is also important in the treatment of infections caused by polyresistant strains as it reduces the abusive use of oral or intravenous antibiotics, preventing the emergence of new resistance [130].

To be effective and make full use of the above-mentioned benefits, inhalation therapies must meet three essential criteria: an appropriate active compound, an adequate aerosol formulation, and last but not least, a delivery device engineered to dispatch an accurate dose of antibiotic at a consistent flow rate [135]. When it comes to active compounds, the ideal inhaled antibiotic should be of a lipophilic nature, have a net-positive charge and high molecular mass. According to available studies, such characteristics would reduce the drug systemic absorption allowing a high drug concentration for a longer period on the site of infection [136]. Furthermore, the efficacy of an inhaled antibiotic also depends on the amount of drug deposited into the patient's lungs. This is mainly influenced by the aerosol particle characteristics. In fact, it has been demonstrated that in order to properly penetrate the lower lung regions with a therapeutic efficiency superior to 90%, aerosolized particles should ideally be in between 1 and 5 μm in size [137]. Particles smaller than 1 μm may be breathed away during exhalation whereas those larger than 5 μm may either be deposited in the oropharynx or caught into the nebulizer circuit before reaching the upper airways [138]. Eventually, the ability to achieve an optimal drug delivery lies in the choice of the administration device. Among all modern apparatus, jet, vibrating mesh, or ultrasonic soft-mist nebulizers are the preferred delivery equipment as they equally allow for slow and deep inhalations, facilitating particles to deeply reach the lower section of lungs and the alveolar space. Their main principle is to transform a water-based solution or a suspension into an aerosol containing the antibiotic drug particles dispersed in fine droplets. These are consequently inhaled and deposited in the lung parenchyma [139]. Inhalers such as pressurized metered-dose inhalers, and dry-powder inhalers are also considered an alternative in the treatment of respiratory infections. Compared to nebulizers, their main advantage lies in reducing patients' burden of having to go through long-term treatments. They allow the drug to be delivered in the form of a dry powder that conserves better, they can also be used on an outpatient basis as they are smaller and portable, and finally they require less to no maintenance after each administration [140]. Such devices are, however, less utilised: not only do they allow for the delivery of smaller drug quantities (less than 1 mg per inhalation), but also they require for patients to provide both an

inspiratory effort and a breathing manoeuvre. These can be difficult to perform, especially for children under-5 [10,11].

Currently, only three antibiotics are formulated for inhalation and approved by either the US Food and Drug Administration (FDA) or the EMA in the treatment of *Pseudomonas aeruginosa* in patients older than 7 years with cystic fibrosis [135]. Aminoglycosides aztreonam (solution form) and tobramycin (solution and dry powder form) are the most effective and the safest inhaled antibiotics developed to date [141]. While inhaled tobramycin is mostly active against gram-negative strains, aztreonam, on the other hand, is particularly efficient against the gram-positive flora and resistant to most β-lactamase enzymes [142]. Similarly, colistin (colistimethate sodium), an antibiotic belonging to the polymyxin class, is also mainly active against gram-negative microorganisms. At present, colistin is only approved in its dry powder form by the EMA, since reports of acute respiratory distress syndrome in patients after administration have led the FDA to reject its use in the US [134].

Despite all assets, difficulties with implementing optimal inhalation techniques as well as the lack of robust clinical data have limited the widespread adoption of inhaled antibiotic treatments against respiratory infections, particularly in children under 5 years [7,8]. Among other reasons, this can be mostly attributed to both particle-related and patient-related factors. For instance, efficient delivery of aerosolised antibiotic particles in the lower parts of the lungs depends on their aerodynamic behaviour. As mentioned earlier, larger particles tend to accumulate in the oropharyngeal area, while smaller one deposit in the lower respiratory tract. As a consequence, this limits the efficiency of drug delivery in the lungs as small particles carry fewer active substances [139]. This is particularly true in children under 5 years, who see a large majority of the aerosol drug deposited in their oropharynx due to a smaller airway morphology and variable air-flow dynamics compared to adults [143]. Furthermore, drug delivery depends on patients' breathing frequencies: rapid and forceful inspirations will see an increased deposition of drug in the upper airways, whereas slow and deep inspirations deposit particles in the lower part of the respiratory system [10]. Compared to adults, paediatric patients have a rapid, an often variable breathing frequencies and a high resistance due to the small diameter of the airways, resulting in greater drug loss in the upper airways. Poor drug delivery in under-5s can also be attributed to behavioural aspects. Indeed, infants and toddlers will poorly tolerate a mouth-piece equipment or an aerosol mask, limiting the proper conduct of the treatment [143].

As a result, the patient's age, its morphology, the severity of the respiratory disease, or even the physical capability to perform a correct inspiration affect the quality of drug delivery and consequently the efficacy of inhalation therapy [11]. Experimental data conducted on patients under inhalation therapy during spontaneous, unassisted breathing have demonstrated that only 3 % of the aerosolized drug is delivered to the lungs of neonates, nearly 5 % of children under-5s whereas in adult, full doses reach the lower airways in 58 % of the patients [143].

Ultimately, the limited number of available antibiotics for inhalation, along with the increasing incidence of severe respiratory infections among children under five years, have led physicians of using intravenous formulation for inhaled administration [144]. However, physical properties of such products (i.e., viscosity, high osmolarity, pH and, preservatives) are not designed for this route of administration and often cause severe complications. For instance, the arginine contained in the aztreonam IV solution has been associated with declining lung function in young patients with CF [135]. Developing new antimicrobial volatile agents suitable for children under 5 years of age is therefore urgently needed.

In this context, plant-derived preparations and more particularly essential oils could provide an ideal alternative. Already studied for their antimicrobial activities, their unique physicochemical properties used in inhalation therapy would allow for a better distribution of volatile compounds down to the lower section of the lungs, at significant concentrations, and without requiring any specific breathing techniques [145].

2.2   Essential oils

Vascular plants have the ability to synthesise a vast diversity of chemicals, selected throughout their evolutionary history as a response to specific needs [146]. Although found in very small quantities compared to the plant total weight – e.g., rose petals EO represent only ~0.02–0.03% – essential oils play a significant role in the ecological functioning of plants, assuring their survival in nature. For instance, EOs attract pollinators and seed dispersers for reproduction, protect the plant against herbivores and insects as well as against environmental stressors. Eventually, they may also be used as means of communication with the surrounding vegetation [147].

2.2.1 The botany of aromatic plants

Plant bearing EOs also known as aromatic plants are a particular botanical group that possesses specialized biological features allowing them to biosynthesise, store, and secrete larger quantities of EOs [14]. Until today, 1500 aromatic species belonging to over 90 botanical families have been identified. From these plants, 3000 EOs can be extracted but only 300 of them are of commercial value, especially in the flavour and fragrance industries [148]. As a consequence of their economic importance, their cultivation is widely practiced in many parts of the world, principally in warm and temperate areas such as in the Mediterranean rim as well as in tropical regions. Significant source of aromatic plants and therefore EOs mainly belong to the following botanical families [149]:

- Apiaceae counts more than 3800 species in about 446 genera adapted to temperate climates and are generally found in the northern hemisphere. They include plants species like fennel (*Foeniculum vulgare*) or anise (*Pimpinella anisum*) for example. EOs are mainly found in their roots, leaves or fruits [150].

- Asteraceae is the largest family in the plant kingdom that consist in 32,000 known species in over 1900 genera. Their distribution is rather unusual, since they are found on all continents and climates. Species from this large and diverse family are generally herbs or shrubs such as chamomile (*Matricaria chamomilla*) or yarrow (*Achillea millefollium*) for example [150].

- Cupressaceae is a family with a worldwide distribution. It consists of nearly 140 species of 27 to 30 genera, composed mostly of trees and shrubs such as cypresses (*Cyprus* spp), junipers (*Juniperus* spp), and redwoods (*Sequoia* spp). EOs of these plants comes mainly from their resins [150].

- Lamiaceae is composed of approximately 2700 species, growing in warm and temperate climate. Plants from this group are mainly herbs and shrubs including species such as thyme (*Thymus vulgaris*), peppermint (*Mentha x piperita*) or Lavender (*Lavandula angustifolia*) [151].

- Lauraceae is also an important botanical family in terms of numbers (~3000 species over 45 genera). Many are evergreen trees or shrubs found predominantly in tropical areas, including cinnamon (*Cinnamomum verum*) and ravintsara (*Camphora officinarum*) [150].

- Myrtaceae family includes no less than 5900 species with about 130 genera. With a preference for warm regions, species from this family are generally evergreen trees, shrubs or even bushes such as eucalyptus (*E. globulus*) and tea tree (*Melaleuca alternifolia*) [150].

- With around 780 genera and around 12,000 different species, Poaceae is a large family of herbaceous plants commonly known as grasses. Mainly found in tropical countries, aromatic grasses include species such as lemongrass (*Cymbopogon citratus*) with its essential oil extracted from the leaves [150].

- Pinaceae or pine family are conifer trees and shrubs preferring temperate climates. The family encompass nearly 250 species distributed in 11 genera such as firs (*Abies* spp), pines (*Pinus* spp), and cedars (*Cedrus* spp). Their EO is generally found in both the needles and the resin of their trunks [152].

- Rutaceae also known as citrus family comprises around 2000 species, divided into 150 different genera. Most species are trees or shrubs growing preferably in tropical and subtropical regions such as bitter orange (*Citrus × aurantium* var. *amara*) or grapefruit (*Citrus × paradisi*), whose EO derived from the fruit skin [153].

- Zingiberaceae is a smaller family made up of about 50 genera with a total of about 1600 known species. These aromatic herbs represented by spices like ginger (*Zingiber officinale*), turmeric (*Curcuma longa*), and cardamom (*Elettaria cardamomum*). EOs from this family, localized within their rhizomes, are generally difficult to extract, making them rare and precious [150].

Although EOs are mostly produced in plants' aerial parts – i.e., leaves, bark, wood, stems, buds, flowers, fruits, and seeds – many are also found belowground in organs such as roots, rhizomes

and bulbs depending on the genera [148]. In occasion, different organs of the same plant may produce different EOs. For instance, bitter orange (*C. aurantium* var. *amara*) synthetises three distinctive oils: petitgrain bigarade EO from both stems and leaves, neroli EO from the flower's petals, and bitter orange EO from fruits. At a microscopic level, however, essential oils are commonly found in special secretory structures either on the surface of the plant or within the plant tissues. The type of structure is specific to the family, genera, or species. Glandular trichomes, for example, are hair-like epidermal structures found covering leaves, stems, and parts of flowers of about 30% of all vascular plants, and more particularly in species of the Lamiaceae [154]. Alternatively, other types of secretory structures have evolved to collect larger quantities of oil. These include secretory cells found in rhizome and cortex of Zingiberaceae, secretory cavities present in fruits and leaves of plants from the Citrus family (Rutaceae), and secretory duct common in all species of the Apiaceae, Asteraceae, and Pinaceae families [155].

In our global economy, essential oils have become highly valued and large quantities are produced worldwide to fuel various industries. In the European Union, market's demand mostly comes from the food and beverage industry (35%), followed by cosmetics and perfumery (27%), household cleaning products (16%), pharmaceutical (15%), agricultural (5%) and ultimately aromatherapy (2%) [156,157].

Besides their typical use as fragrance in perfumes, soaps, lotions, or ointments, EOs are extensively employed in the food sector as flavouring agents. For instance, the soft drink industry relies heavily on citrus fruits EOs, especially *C. limon* and *C.* x *aurantiifolia*, which are fundamental for the production of cola drinks [22]. Other examples involve synthetic volatile compounds – e.g., carvone, geranyl acetate or limonene – used by the household cleaning industry to disguise the unpleasant odour of other ingredients, including in air fresheners, disinfectants, laundry and dish detergents, or surface cleaners [158].

Furthermore, thanks to their antimicrobial and antioxidant activity, EOs are also used to preserve food from spoilage and pathogenic microorganisms. As an illustration, plant-based preservatives such as DMC Base Natural containing a mixture of *Rosmarinus officinalis*, *Salvia officinalis* and *Citrus* spp. EOs are already available and marketed as shelf life enhancers for industrialised ingredients including sauces, dressings, juices, ready-to-eat meals, and bakery [157]. Similarly, the agricultural industry developed and commercialised bioinsecticides using EOs as

principal constituents. The best example of such products is ECOTEC, a broad-spectrum insecticide, containing *R. officinalis* (10 %) and *M.* x *piperita* (2 %) as active ingredients [159].

Eventually, for human health, EOs are also used for their antibacterial activity in many herbal medicinal products. Commonly available in different forms such as capsules, tablets, lozenges, tinctures, or syrups, they are often sold over the counter in pharmacies. For the treatment of respiratory conditions, however, nasal sprays and drops as well as aromastick inhalers are preferred as they allow the fast delivery of the active volatile compounds directly to infection site [160]. Multiple products exist on the market, in Czechia in particular, Pinio-Nasal is a spray indicated in the treatment of rhinitis and nasopharyngeal inflammations. It is composed of a blend of 3 EOs – i.e., *Pinus sylvestris*, *M.* x *piperita*, and *E. globulus* – as well as thymol, the main volatile compounds of *T. vulgaris* [161]. Likewise, GeloMyrtol® recommended in acute and chronic airways infections such as rhinosinusitis, bronchitis, and chronic obstructive pulmonary disease, is made of capsules filled in with 4 EOs including *E. globulus*, *C. sinensis*, *Myrtus communis*, and *C. limon* enriched in limonene, 1,8-cineole, and α-pinene [162].

Nevertheless, before EOs can be included in the different products and marketed, they have first to be extracted from the plant matrix and chemically analysed [163].

2.2.2   Chemistry

2.2.2.1   Extraction methods

Essential oils are complex mixtures containing a broad spectrum of chemically diverse aromatic and volatile secondary metabolites [164]. Besides various factors including both physiological variations and environmental conditions during the plant growth, the composition of an EO is largely affected by the extraction method used. It is therefore crucial to select the most appropriate and convenient technique to collect the targeted active compounds into the EO [165]. Various traditional methods can be employed for this purpose, including solvent extraction, maceration, or more commonly, cold expression and distillation.

Cold expression is an antique and traditional process used long before the discovery of distillation [166]. This technique is mainly employed to extract essentials oils contained within the peel of citrus fruits due to their relative thermal instability. In order to break the secretory cavities, a mechanical pressure in cold conditions is applied so that the EO is ejected and washed away with

water. While this method has the advantage of generating a minor heat during the extraction, it only allows to collect small quantities of EOs often contaminated with other plant materials such as pigments [14].

Another traditional way to extract volatile compounds as EOs from the plant matrix is distillation. During distillation, the plant material, generally fragmented, is either completely immersed into boiling water (i.e., hydrodistillation) or only exposed to steam (i.e., steam distillation). In both techniques, EO is extracted through evaporation: as the water evaporate, the steam vaporises and carries off the volatile compounds. Subsequently, indirect water-cooling condenses this vapour mixture back into 2 immiscible phases: the essential oil and the hydrosol, which is water containing some of the most polar volatiles. EOs are usually less dense than water and therefore present as the upper part of the distillate [167]. Eventually, aromatic plant material typically yields 1–2% by weight of EO on distillation, depending on diverse criteria such as the quality of the plant material, the distillation time, and both the temperature and operating pressure [14]. Among all techniques, steam distillation is the most employed for obtaining EO's at large scale as it is a fast, cost-effective process, suitable for field operation. However, the elevated temperature and the prolonged extraction time can often cause chemical degradation of EOs' heat-sensitive compounds as well as loss of the most volatile metabolites.

As a consequence of the traditional methods disadvantages as well as the increasing cost of energy and the drive to reduce carbon footprints, several new techniques for EOs extraction have recently received a lot of attention [163]. These emergent methods include microwave-assisted extraction, ultrasound-assisted extraction, and most importantly supercritical fluid extraction (SFE). SFE utilizes the property of some gases to behave as fluids when subjected to temperature and pressure conditions above their critical points, making them effective solvents. In the case of EOs, carbon dioxide is a supercritical solvent of choice since it is neither toxic, nor carcinogenic and allows the use of a relatively low pressure at room temperature, thus preserving the original composition of volatiles [168].

Once extracted, EOs are usually liquid, but a few are solid (e.g., *Iris germanica*) or semi-solid (*Guaiacum officinale*) at room temperature. The majority are colorless or pale yellow, although a few are deeply colored, for instance, in blue (*M. chamomilla*), orange (*C. × sinensis*), or green (*Valeriana officinalis*) [169]. Furthermore, their vapour pressure at room temperature is sufficiently high so that they are partly found in vapour state. Ultimately, EOs are highly sensitive

extracts as they may easily degrade when exposed to air, heat, or light. It is therefore highly recommended to store them in a cool, dark and dry place [170].

2.2.2.2   Chemical composition

As mentioned in the previous section, EOs are composed of lipophilic and highly volatile secondary metabolites, usually with a molecular weight below 300 g/mol. To date, more than 1700 volatile compounds, often structurally related, have been identified. Typically, each oil can be composed of 20 to 100 substances, varying in concentrations, with 2 or 3 major VCs representing up to 70 % of its entire content [171]. Based on their biosynthetic origin, this huge diversity of molecules can be classified into several major groups: terpenes and terpenoids, phenylpropanoids and fatty acid derivatives [14]. The constituents of these groups contain a basic frame of carbons and hydrogens to which may be attached functional groups [172].

Terpenes are defined as substances made up of isoprene molecules (C_5 units) assembled together via either the mevalonic acid or methyleruthirol phosphate biosynthetic pathways. The resulting VCs encountered in EOs are predominantly monoterpenes (C_{10}) followed by sesquiterpenes (C_{15}) [173]. Monoterpenes are light molecules that evaporate quickly. Structurally, they can either be aliphatic such as ocimene and β-myrcene or cyclic like α-pinene, limonene or camphene. Sesquiterpenes, on the other hand, are less volatile but have a stronger and more persistent scent. Similarly, they can present an aliphatic structure like α-farnesene or a cyclic one such as β-caryophyllene, α-bisabolene or chamazulene [174]. Eventually, diterpenes (C_{20}) and triterpenes (C_{30}) may also be detected in EOs, however in negligeable quantities because of their low volatility [170].

Although possessing similar hydrocarbon structures – same biosynthetic pathways – terpenoids possess additional functional groups conferring them their biological activities [175]. Depending on these moieties, they can be subcategorised in various chemical classes including alcohols, ketones, aldehydes, phenols, esters, or oxides. For instance, well-known terpenoids include linalool (aliphatic monoterpenoid alcohol), thymol (cyclic monoterpenoid phenol), 1,8-cineol (cyclic monoterpenoid oxide) nerolidol (aliphatic sesquiterpenoid alcohol) and bisabolol (cyclic sesquiterpenoid alcohol). Together, terpenes and terpenoids are, by far, the most important groups of VCs found in essential oils, representing more 80,000 compounds [176].

Even though phenylpropanoids constitute a relatively small part of EOs, they remain the second largest group of plant VCs. Synthesized from an amino acid precursor (i.e., phenylalanine) via the shikimic acid pathway, their basic structure (C_6-C_3) is composed of an aromatic phenol group (C_6) linked to a three-carbon tail (C_3) which is often unsaturated and oxygenated [174]. Examples of extensively studied phenylpropanoids include anethol found in fennel (*F. vulgare*), cinnamaldehyde in *C. verum* or eugenol from *S. aromaticum* buds.

Fatty acid derivatives constitute the third major class of VCs. Although, there are three main paths by which essential oil's VCs are formed in this family of metabolites, the degradation of lipids also known as the lipoxygenase pathway is the predominant one [14]. This metabolic route involves the oxidation and cleavage of long chain molecules like linoleic or linolenic acids, resulting in C_6 / C_9 aldehydes, alcohols, or esters. These VCs, known as green leaf volatiles, have been reported to serve as messengers, communicating with neighbouring plants in case of imminent danger such as the presence of predators or pathogens [177]. Significant metabolites include, for instance, aliphatic alcohols such as 1-octanol in lavender EO (*L. angustifolia*), (E)-4-decenal (aldehyde) in cardamom (*Elettaria cardamomum*), or isobutyl angelate (ester) in roman chamomile flowers (*C. nobilis*) [178].

Eventually, sulphur-containing compounds, may occasionally be found in essential oils of plants with high concentration of polysulfides. Of particular interest, garlic (*Allium sativum)* essential oil, characterized by its abundance of sulphur-containing allyl compounds, specifically allicin and ajoene. These compounds are derived from the enzymatic conversion of alliin (or S-allyl-l-cysteine sulfoxide), leading to the formation of fractions with underexploited antimicrobial properties[179,180]. Additionally, sulphur-containing compounds like allyl isothiocyanate are naturally generated through the presence of allyl glucosinolate found in mustard (*Bracica juncea*) oil and other members of the Brassicaceae family such as cabbage (*Brassica oleracea* var. *capitata*),cauliflower (*Brassica oleracea* var. *botrytis*) and horseradish (*Armoracia rusticana*) [181,182]

In most cases, the percentage of an EO constituents is prone to variations depending on various elements. For instance, the terpinen-4-ol content of tea tree (*M. alternifolia*) oil normally fluctuate between 30% and 55%, but it is rarely found in commercially produced tea tree oils [169]. Furthermore, the factors affecting the EO chemical composition can be extrinsic such as the geographical location, weather conditions, soil types and the use of fertilizer. In Europe, for

example, studies have demonstrated that *T. vulgaris* chemical composition of two similar plants grown in the same conditions in both France and Serbia presented different profiles. EOs from the French specimen was dominated by linalool (~76 %) whereas in Serbia geraniol (~ 60 %) was the main VC [183]. Intrinsic factors also significantly influence the EO constituents' percentage, including the plant age or the harvest time of the year. Great seasonal variations in 1,8-cineole content of Moroccan *E. globulus* oil can be seen, with lows (~ 62%) when harvested in May and highs (~80%) in July [184].

Ultimately, minor genetic modifications can lead to significant changes in the chemical profile of individual plants belonging to the same species. The variations of proportions of these compounds are defined as the plant chemotype, often named after the most abundant component present in the EO. *T. vulgaris*, for example, counts seven different chemotypes, depending on whether the dominant VC of the essential oil is thymol, carvacrol, linalool, geraniol, thujanol, α-terpineol, or 1,8-cineole [185].

2.2.2.3 *Vapour analytical methods: headspace analysis using GC-MS*

The need for systematic analysis and the necessity to detect adulterations in EOs can be traced back in the 19th century when the industrial production was established to meet the growing demand in flavourings and fragrances [186]. Rapid market changes and the increasing competition often led producers to adulterate their products by adding lower-value compounds or develop more sophisticated techniques to reconstitute commercially valuable oils [14]. Simultaneously, the growing scientific curiosity surrounding the chemistry and biochemistry of plant volatiles resulted in the creation of various analytical methods for their study. Unraveling the chemistry of EOs, however, has always been a challenging task. Many compounds are only present in small quantities and therefore are hard to detect. Some are very similar to each other and are difficult to distinguish (e.g., isomers), other are simply complex to identify.

Nowadays, modern analytical methods routinely use high resolution chromatography techniques with low detection limits such as gas chromatography (GC), which is the most commonly employed instrument for liquid EOs characterization. In GC, the separation of EOs' compounds is based on their partition between a stationary phase and a mobile gaseous phase (i.e., inert gas such as He, H_2 or N_2). When injected into the chromatograph, the sample is heated, vaporised, and passed into the carrier gas stream that transports the compounds through the GC

column. In principle, volatiles` that have strong interactions with the stationary phase will be more actively retained, and elute later, while components that have weaker interactions, will travel faster through the column, and elute earlier. The partitioning quality depends on several factors, including the volatile compounds chemical properties (e.g., molecular weight, vapour pressure, polarity), the column properties (e.g., temperature, length, diameter) as well as the stationary phase polarity and the mobile phase flow rate. Over the years, GC has undergone several advancements, transitioning from packed and capillary column to multidimensional techniques. Since the late 1990s, the emergence of two-dimensional GC (GC-GC) allowed for simultaneous analysis of a sample through two columns usually differing in polarity, enabling the separation of complex EOs with closely eluting compounds [187]. A recent study, describing the use of a simultaneous GC dual-column/dual-detector system, reported a reduction in compounds overlapping in EO analysis, leading to a better identification [188]. This technique can reveal multiple identifiable peaks that may have been detected as a single peak on older GC equipment [169].

When it comes to EO vapour, however, the first stage of the analytical process is to extract VCs from the gaseous sample. As physicochemical characteristics of the different compounds in essential oils vary greatly, the effectiveness with which these volatiles are extracted will be highly dependent on the sampling techniques used [187]. There are two basic approaches that include a more traditional solvent-based extraction – i.e., solvent-assisted flavour evaporation (SAFE), a high-vacuum distillation technique that can isolate volatile compounds from complex matrices [189] – and headspace extraction (HS). The latter, and maybe the most commonly employed in laboratories, is a simple, rapid, and solventless technique that allows the analysis of highly volatile analytes directly from the air [190]. These methods are usually divided into two types: static headspace (S-HS) and dynamic (D-HS) headspace sampling. In S-HS, an equilibrium is established between VCs contained in the liquid EO sample and the headspace above it. When the equilibrium is reached, an aliquot of the vapour phase is collected from the vial and directly analyzed using a chromatographic system. As an example, several studies have already explored the chemical composition of *T. vulgaris*' volatile agents using different S-HS techniques such as solid-phase microextraction (HS-SPME) or single drop microextraction [191–193]. In D-HS, however, a constant flow of a carrier gas is passed through the liquid EO, carrying away VCs into an adsorbent or cryogenic trap. When the gas extraction is complete, the trap is heated, and the volatiles are released and transferred into the chromatographic system for further analysis –

– e.g., Purge and Trap. Several other specialized headspace sampling techniques are available, including multi-step headspace extraction, a discontinuous process designed to determine the total compound amount in a sample. The total vaporization technique is employed for samples with minimal analyte transfer to the headspace phase. Additionally, headspace-mass spectrometry stands out as a non-separative approach, directly linking a mass spectrometer with headspace extraction for swift sample classification [194].HS-SPME is currently the most developed and preferred technique to examine complex, volatile mixtures in laboratories [195]. This method uses a fused-silica fibre coated with an adsorbent material that is directly exposed to the sample headspace. After adsorption of the volatile compounds to the fibre coating, the sample is transferred into a chromatographic injection port for thermal desorption [190]. HS-SPME sampling is not only a sensitive technique due to the concentration achieved by the fibre but also a selective one thanks to the different coating material available. Repeatability is also one of its assets when used with an autosampler [196,197]. However, using fibre coating suffers limitations. For instance, the coating material used is often not uniformly sensitive. As a result, competitivity between VCs may be observed because of the limited number of active sites. Similarly, selectivity will be different depending on the coating polymer used. Consequently, chromatograph's peak areas might not reflect the exact compounds' composition and proportion in the headspace [198,199]. That is why other approaches can be considered for analysing the EO vapour profile, including gas tight syringe headspace sampling (HS-GTS). This technique is the most convenient and inexpensive way to sample VCs from the headspace of a closed vial [200]. The gas syringe with a pressure-lock valve is inserted into the headspace, and a fraction of its volume is removed. The gas sample, locked into the syringe, is then transferred, and injected into the chromatographic system.

The full analytical power of GC was achieved when mass spectrometry detectors were made available at an affordable price for laboratories [167]. Nowadays, MS detector is the most used analytical instrument coupled with GC (GC-MS) for VCs detection and identification [201]. In practice, after elution, volatile compounds are ionized and fragmented. The resulting ions are then separated according to their mass-to-charge ratios (m/z) by passing through an electromagnetic field. Subsequently, they reach a detector that produces a signal proportional to their abundance. As a result, for each substance, a mass spectrum is generated, laying out a graphic that displays the correlation between the relative abundance of the ions versus their m/z ratios [14]. Eventually,

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the most frequent and simple identification procedure in GC-MS consists of a comparison of the acquired mass spectra with those contained in mass spectral libraries such as the NIST standard reference database [202]. Compounds such as isomers, however, can be incorrectly identified, especially when analyses EOs. Indeed, as already seen in a previous section, EOs compositions are mainly composed of terpenes/terpenoids known for their isomerism, which often generate similar mass spectra [187]. As a consequence, to enhance the confidence in identifying volatiles analytes, a superior approach is to combine MS library search with retention indices (RI). This component provides a measure of a compound's retention time, on a determined chromatographic column and experimental conditions, relative to a set of reference standards (i.e., C_8 to C_{32} aliphatic hydrocarbons). For example, Adams RIs data list were reported using a DB-5MS column at a programmed temperature ranging from 60 to 240 °C at 3 °C/min [188]. The use of RIs in GC-MS studies is already well established and analysts adopt such procedures in their routine analysis to confirm VCs identities.

When discussing EO vapour analysis, it is crucial to consider the matrices they are inserted into and their capacity to release volatile constituents into the headspace. This challenge becomes particularly pronounced due to their physico-chemical properties. For example, because of their hydrophobic nature, EOs do not dissolve well in water-based solutions. This may result in their uneven distribution within the matrix, even when using appropriate solubilizing agents such as DMSO, Tween 20/80 or Span 20/80. This, in turn, may affect VCs evaporation and dispersion in the headspace [203]. To overcome this limitation, intensive research has already been conducted to explore the potential of solid matrices. These biopolymer materials have demonstrated their effectiveness in entrapping various EOs and active compounds, serving both medical and food-related purposes such as fruit preservation or combating foodborne pathogens [204–207]. In addition, cellulose-based materials are interesting polymers given several recognized properties. As they derived from various plant sources, they are renewable, abundantly available, biodegradable, and nontoxic for both human health and the environment [208]. As an example, Sánchez-González et al.[209] developed a series of antimicrobial food packaging by incorporating EOs from *Citrus bergamia*, *Citrus limon*, and *Melaleuca alternifolia* into matrices made of chitosan and hydroxypropylmethylcellulose. This was tested on three pathogens, including *E. coli*, *L. monocytogenes* and *S. aureus*. In the majority of cases in a complete microbial growth inhibition observed for EO tested and in all film matrices.

2.2.3 *Susceptibility testing methods for the antimicrobial assessment of EOs vapour phase.*

When it comes to EO, antimicrobial susceptibility testing assays can be conducted either by direct contact with the pathogen or through the vapour phase. Unlike the well-established methods in solid (agar disc diffusion) and in liquid (broth microdilution) media, there is still a lack of standardized assays to determine the antimicrobial effect of volatile compounds in vapour phase. In recent years, however, despite the absence of standardized methods from organizations such as the Clinical and Laboratory Standards Institute (CLSI) or the European Committee on Antimicrobial Susceptibility Testing (EUCAST), numerous techniques have emerged for evaluating the antimicrobial properties of volatile compounds [210,211]. This absence of standardization has resulted in challenges not only in the interpretation but also in the comparison of antimicrobial growth results. For instance, in studies investigating the antimicrobial activity of T. vulgaris EO vapour, it is common to encounter results expressed in diverse formats, such as unit volume of air [27], or even varying definitions of minimum inhibitory concentrations [212].

Below, we outline the principles of some of these methods, which have been categorized into two groups: solid and liquid matrix volatilization methods. This classification is based on whether the tested volatiles were applied to solid matrices (e.g., paper disc) or were in liquid form (e.g., pure compounds or dissolved in broth or solvents):

2.2.4.1 *Solid matrix volatilization methods*

Disc volatilization assay et derived methods

Also known as the inverted Petri plate method, disc volatilization assay is a direct modification of the standardized disc diffusion method. Widely employed for assessing the antimicrobial effects of volatiles in the vapour phase, its procedure involves inoculating Petri dishes with an appropriate solidified medium containing the microorganism to be tested. Filter paper discs are subsequently impregnated with the EO sample at a desired concentration and placed on the cover of the Petri dishes. Then, the plates are inverted and sealed with parafilm or adhesive tape to prevent any leakage of the active component's vapours into the atmosphere. The incubation of these sealed Petri dishes, carried out under suitable conditions based on the microbial pathogens tested, allows antimicrobial agents to diffuse from the disc to the atmosphere inside the plates and, therefore, to

the agar. This diffusion process inhibits the growth of the test microorganism [191]. The assessment of antimicrobial activity is based on the diameters of inhibition zones, with interpretations categorized into weak activity (inhibition zone ≤ 12 mm), moderate activity (12 mm < inhibition zone < 20 mm), and strong activity (inhibition zone ≤ 20 mm). However, it is crucial to note that results obtained through this method can exhibit significant variability. Various factors, such as disc size (with diameters ranging from 3 to 10 mm), the quantity of the applied compound on the disc (volume varying from 10 to 260 µL), and the characteristics of the agar medium and its volume, contribute to the observed variations [213].

Nedorostova et al. [212] introduced a modification to this method by implementing agar sealing with warm medium poured into both the Petri dish and its cover. This adaptation aimed to prevent the adsorption of EOs onto the plastic material of the Petri dish cover. Additionally, when microorganisms are seeded on both agar parts, this assay combines principles from standard disc diffusion and disc volatilization assay techniques, allowing for the evaluation of two different inhibition zones.

Eventually, Kloucek et al. [214] introduced an innovative modification to the disc volatilization assay through the development of a multi-screening method in Petri dishes divided into four sections. Each section, including the lid, is filled with warm agar. Following solidification, three sections of the dish are inoculated with different microorganisms, leaving the fourth section as a purity control. The EO sample is then applied to a larger, round sterile filter paper disc (85 mm in diameter), strategically positioned on the walls that divide the sections of the Petri dish. Finally, the Petri dish is hermetically sealed with the lid containing solidified medium. This method offers increased output compared to the above-mentioned methods, where various microorganisms can be seeded on a single agar plate within a single Petri dish.

In vitro dressing model

An alternative to the disc volatilization assay developed by Edwards-Jones et al. [215] introduced a more targeted alteration to the disc volatilization method by modifying the matrix from which the tested compound evaporates. The experiment is conducted in a Petri dish covered

with four layers composed of different materials commonly used in the treatment of skin infections – i.e., Gamgee, gauze, Flamazine™, and Telfa Clear™ or Jelonet™. Initially, the agar plate is inoculated with a bacterial suspension and covered with the four-layer dressings, which include a Gamgee or gauze layers containing the volatile agent. Following an incubation period, inhibition zones on the agar surface are measured. This model finds application in the development of new wound healing preparations in the field of medicine.

Airtight apparatus disc volatilization methods

Inouye et al. [27] enhanced the disc volatilization assay method by introducing an airtight box, where petri dishes are securely placed. In this method, a Petri dish with inoculated medium is placed in a 1.3 L airtight box covered with aluminum foil. Nine centimeters paper discs, impregnated with the sample solution, are inserted into the top of the airtight box, separated from the petri dish with the inoculated medium. Boxes are then incubated under the required conditions. While this method offers the advantage of assessing various inoculated materials and larger objects for surface decontamination, experiments with multiple boxes to evaluate the antimicrobial potential of EOs in different concentrations demand considerable space. Furthermore, the location of the paper disc at the top of the airtight box and the distribution of vapors from top to bottom raise some questions.

In order to simultaneously evaluate the antimicrobial effect of volatile compounds at different concentrations, Seo et al. [216] developed a specialized airtight experimental apparatus. It comprises an upper chamber with seven wells containing agar medium, inoculated with bacteria for testing. The lower chamber, however, features seven wells containing paper discs impregnated with twofold serially diluted EO samples. To prevent vapour leakage, O-rings are placed at the junction of the upper and lower well rims as well as around the entire set of wells. Additionally, the four corners and center of the apparatus are securely sealed using nuts and bolts. Following incubation, the growth inhibitory effect is determined by observing the colour change in the agar, indicating a growth of microorganisms. The subsequent MIC is then determined. While this method is expected to facilitate the assessment of the antimicrobial impact of EO vapour, allowing for the simultaneous testing of multiple concentrations, it requires specialized equipment that may not be easily accessible in a standard laboratory setting.

2.2.4.2   Liquid matrix volatilization methods

Microplate patch volatilization assay

Feyaerts et al. [217] introduced an innovative assay method also known as vapour-phase-mediated patch assay. This approach employs U-shaped, 96-well microtiter plates, defining a patch as the set of wells within a square area around one or more test wells. The procedure involves adding microbial inoculum to all wells, followed by introducing the desired volume of the EO sample into the center of a squared patch, comprising either 9 or 36 wells. After covering the microtiter plate with its lid, it is incubated under the specified conditions, and the results are assessed via an optical density scan of each well using a spectrophotometer. While this layout is effective in revealing false-positive results caused by vapours, it allows for testing only one or two samples in a single microtiter plate. Furthermore, the lack of quantitative precision and does not provide precise values for evaluating the level of antimicrobial potential in the vapour phase.

Agar plug-based vapour phase assay

Amat et al. [218] introduced a comprehensive assay method capable of delivering both qualitative and quantitative results on the antimicrobial activities of volatile substances vapour phase. This method involves the use of two distinct agar plates. In the initial step, the first plate is inoculated with the pathogen and allowed to incubate for 1 hour. Subsequently, agar plugs (13 mm in diameter) are extracted from this pathogen-seeded plate. Moving to the second plate, four sections of agar are removed (10 mm in diameter), and sterile caps from 1.5 mL disposable/conical microtubes, containing the volatile compounds, are inserted. The agar plugs prepared from the first plate are then placed on top of these caps. After 24 hours of incubation, visual examination is conducted to assess bacterial growth. Eventually, for a quantitative evaluation of antibacterial activity, the agar plugs containing bacterial cells are immersed in broth and subsequently plated on agar to determine cell viability.

Compared to assays conducted in petri dishes based on inhibition zone measurements, the agar plug-based vapour phase assay yields more precise data on reduced growth potential. This approach allows for the evaluation of whether the EO or volatile substance antimicrobial effect is

biostatic or biocidal. Notably, this model enables the simultaneous testing of several sample replicates against a single bacterium or one volatile compound against different bacterial strains on a single agar plate, accommodating a range of concentrations. However, it is acknowledged that the preparation of agar plugs may involve labour and time-intensive procedures.

Today, disc volatilisation assay is the most frequently used method for the evaluation of antimicrobial effects of EO vapour phase [219]. While various modifications to these methods have already been developed, it is important to note that these useful techniques do possess aa number of limitations. Typically, they are not designed for high-throughput screening, some restrict the evaluation to a single concentration per sample, and they generally yield qualitative rather than quantitative data [218]. In consequence, their main drawbacks include relatively high material and time consumption as well as labour requirements.

That is why, the broth microdilution volatilization method (BMV) introduced by Houdkova et al. [220] is a powerful alternative to the previously developed techniques.

2.2.4.3   Broth microdilution volatilization method

Based on the combine principles of broth microdilution and disc volatilization methods. The BMV assay is conceived to evaluate the EOs' in vitro growth-inhibitory effect in both liquid and vapour phases simultaneously and at different concentrations. The experiments are conducted using standard 96-well microplates, equipped with tight-fitting lids featuring flanges designed to minimize evaporation. In the initial phase, agar is carefully pipetted into each flange of the lid and inoculated with a bacterial suspension. Subsequently, in the microplate's wells, seven twofold serially dilutions of EO samples are prepared and inoculated with the same bacterial suspensions. In order to fasten tightly the microplate and lid together, clamps as well as handmade wooden pads are used for an improved fixation. Microplates are then incubated under specific conditions, and MICs are determined through visual assessment of bacterial growth. This assessment is conducted after staining metabolically active bacterial colonies with thiazolyl blue tetrazolium bromide dye, and the interface of colour changes from yellow to purple is observed in both the broth and agar.

In contrast to the aforementioned techniques, the BMV assay presents several advantages that address limitations observed in other methods. Firstly, it stands out for its cost and labour efficiency [221]. Microplates, being standard laboratory equipment, are widely accessible, unlike specialized airtight experimental apparatus used in certain methods, as seen in Seo et al. [216]. Moreover, microplates can be seamlessly integrated into fully automated workstations, a feature lacking in DV assays that employ Petri dishes and, consequently, face repeatability challenges [222]. While other studies have employed microplates for detecting the antimicrobial activity of volatile substances, such as the vapour phase-mediated patch assay by Feyaerts et al. [217], their designs primarily enable the determination of relative microbial inhibition values, with a notable limitation of providing qualitative results[218]. An additional strength of the BMV assay lies in its adaptability, allowing for easy modifications to suit new applications. For example, Netopilova et al. [223] made modifications to the test to investigate the combined effects of volatile substances through a chequerboard design. This modification allows for the determination of fractional inhibitory concentration indices, a capability not achievable with other methods.

In summary, these features make our method well-suited for high-throughput screening, rendering it a simple, fast, and reliable assay while delivering reproducible and quantitative results [220]. However, it's essential to acknowledge that despite its numerous advantages, serially produced microplates are not specifically designed for testing volatile substances, contributing to weaknesses shown by the assay.

2.2.4 Antimicrobial activities of EO and their compounds

As with all natural product extracts, EOs vapour composition plays a determinant role in their antimicrobial activity [157]. Until today, there is still a lack of available data regarding vapours antimicrobial potential, compared to the significant amount of evidence collected on EOs liquid phase. A first series of investigations published in the early 2010's, however, demonstrated that EO vapours are more effective than their liquid form [224]. These studies suggested that, when mixed with an aqueous phase, essential oil compounds gather to form micelles, reducing their availability to interact with bacterial pathogens. By contrast, VC in vapour do not have this constraint, thus remain fully available to bond with their target sites [225]. This was observed, for instance, in EOs such as *T. vulgaris* [193], *E. globulus*, *M. alternifolia* [226,227], *F. vulgare* and

L. angustifolia [228]. In addition, although EOs antibacterial properties are believed to be the result of complex interactions between the different classes of volatile compounds, in most cases, it is accepted to attribute them to the predominant compounds. For example, the antimicrobial activity of *Thymus* species is usually associated to the presence of phenolic terpenoids such as thymol and carvacrol while the efficacy of *S. aromaticum* and *C. verum* EOs are related to their content in phenylpropanoids: eugenol and cinnamaldehyde, respectively [229,230].

Predictions about the EO antimicrobial effectiveness remain, nevertheless, difficult and require a thorough investigation of the volatile compounds target sites as well as their modes of action [175]. As mentioned in a previous section, the antimicrobial activity of most terpenoids is linked to their moieties. Since the late 1990's, multiple studies demonstrated that the hydroxyl group of oxygenated terpenoids are able to inhibit two biochemical processes essential to bacterial survival: the oxygen uptake and the oxidative phosphorylation, both responsible for bacterial cellular energy production. As an example, the substitution of the hydroxyl group of carvacrol with methyl ether was described to affect the VC interaction with bacterial pathogens, reducing its antimicrobial activity as a consequence [231]. Other research focused on the antibacterial properties of oxygenated monoterpenoids, such as thymol, menthol, and geraniol, showed significant effect against both Gram-positive and negative strains, including *S. aureus* and *E. coli* [19]. Likewise, oxygenated sesquiterpenoids including farnesol, nerolidol and bisabolol have been described to inhibit the development of biofilm of staphylococci including *S. aureus* [232].

On the other hand, although terpenes were shown to permeabilise bacteria cell membrane, this group of compounds do not represent a good source of antimicrobials when used alone [175]. For instance, p-cymene, one of the major constituents in *T. vulgaris*, do not show any antimicrobial activity against several Gram-negative pathogens even at high concentration [233]. Similarly, another study reported low activity of α-pinene, β-myrcene, β-caryophyllene, and limonene, against several Gram-positive bacteria including *S. aureus* [234]. However, when used in combination therapy, such compounds may work in synergy to enhance the effect of existing antibiotic treatments. For example, a 2019 study reported that limonene exhibited synergistic modulation effects when used together with gentamicin against *S. aureus* and *E.coli* [235]. Likewise, it was recently reported that sesquiterpenes are potent enhancers of growth-inhibitory action against Gram-negative bacteria when combined with antibiotics such as penicillin or vancomycin [236].

As for phenylpropanoids, their antibacterial properties cannot be attributed to a single mode of action. According to investigations, these mechanisms will mainly depend on the type and the number of functional groups present on the compound aromatic ring [237,238]. For instance, it has been suggested that phenylpropanoids containing a phenolic hydroxyl group, such as eugenol, showed a high antimicrobial activity when tested against 25 different bacterial strains [239]. Such studies also demonstrated that eugenol increases the bacteria cell membrane permeability, inducing both a potassium and ATP leakage out of the cells [240–242]. In addition, the presence of a methoxy group and a double bond in the side chain in molecules such as eugenol, estragole and anethole was also considered important for their antimicrobial properties [175]. Phenylpropanoids aldehydes like cinnamaldehyde or vanillin, however, have a different mode of action. The aldehyde groups have the ability to interfere with the normal functioning of the cell by bonding with the bacterial DNA and proteins [243]. For instance, early 2000's research demonstrated that, at low concentration, cinnamaldehyde inhibits different enzymes involved in the cytoplasmic division during meiosis and mitosis, whereas at higher, but lethal doses, it acted as an enzymatic inhibitor and a cell-membrane disruptor [244,245].

Eventually, although the antibacterial mechanism of volatile fatty acids is still poorly understood, it is believed that like terpenoids, these VCs mainly interfere with the cellular energy production (i.e., oxidative phosphorylation). Their other modes of action may include both the impairment of nutrient uptake and the direct lysis of bacterial cells [246]. In Roman chamomile (*C. nobile*) for example, a recent study found that its main fatty acid constituents, including isobutyl angelate, 2-meth-ylbutyl acetate and 2-methylbutyl 2-methylbutyrate, showed medium to high antimicrobial effects against all strains of Gram-positive and Gram-negative bacteria tested including *S. aureus* [247].

2.3 EOs recommended against cough and cold in paediatric population.

Following the 2004 Herbal Directive (Regulation (EC) No 726/2004), the EMA established the Herbal Medicinal Products Committee (HMPC) to evaluate the safety and efficacy of herbal medicines. One of the HMPC's responsibilities is to create monographs for the European Union providing all information necessary for the use of a medicinal product containing a specific herbal substance or preparation, including essential oils [248]. These EMA herbal monographs provide information on the substance's medicinal uses, pharmacological effects, and suggested dosages. Amongst the nearly 30 EO monographs recommended by the HMPC, EOs of *T. vulgaris* aerial parts, *E. globulus* leaves*, , M. × piperita* leaves*, F. vulgare* seeds, and *P. anisum* seeds remain the most frequently used in inhalation for the treatment of respiratory tract infections, including cold and cough within the paediatric population [249].

2.3.1 *Thymi aetheroleum*

According to its HMPC monograph, thyme oil is obtained via steam distillation of the dried flowering aerial parts of *T. vulgaris*, *T. zygis* or a mixture of both species. The dried herbal material should contain up to 2.5 % of essential oil. After extraction, the EO collected is a clear, yellow, or very dark reddish-brown liquid with a characteristic aromatic fragrance reminiscent of thymol. As mentioned in a previous section, there are at least 7 chemotypes (CT) of *T. vulgaris* depending on the dominant VC – i.e., thymol, carvacrol, linalool, geraniol, thujanol, α-terpineol, and 1,8-cineole – however, only the thymol-type complies with the definition of the European Pharmacopoeia [185,250]. Eventually, its composition made by GC-MS must include mainly thymol (percentage content of 37.0 to 55.0 %) and carvacrol (0.5 to 5.5 %) as well as p-cymene (14.0% to 28.0%), γ-terpinene (4.0 to 12.0 %), linalool (1.5 to 6.5 %) , β-myrcene (1.0 to 3.0 %) and, terpinen-4-ol (0.1 to 2.5 %) [249].

Regarding its *in vitro* growth-inhibitory activity, thyme oil has been extensively studied since the late 1970s and numerous studies demonstrated significant antibacterial effect against a wide range of Gram-positive and Gram-negative bacteria [26,251,252]. Other research also reported that *T. vulgaris* EO vapour was effective against respiratory tract pathogens. For example, the study of Inouye et al. [27] showed that its growth-inhibitory effect against *H. influenzae, S. pyogenes* and *S. aureus* with minimum inhibitory doses (MIDs) of 3.13, 6.25 and 12.50 mg/L of

air, respectively. Multiple investigations proved that higher percentage in thymol and carvacrol in the thyme EO contribute to its greater antibacterial activity [253]. As an illustration, Inouye et al. [27] study showed that against different strains of *S. pneumoniae*, the sample of thyme EO containing 80 % of carvacrol was more active (MID = 3.13 mg/L air) than the one containing only 25.5 % of thymol (MID = 6.25 mg/L air). Ultimately, in addition to its antibacterial properties, *T. vulgaris* EO has been found to have expectorant properties, making their inhalation useful to alleviate congestion and clear the respiratory tract when treating respiratory infections. In addition, besides thymol, its isomer: carvacrol contained in *T. vulgaris* EO has been reported to produce strong antimicrobial effect. As biosynthetic precursors, p-cymene and γ-terpinene, often found amongst the major compound in the EO composition, have also been studied and recognized for their antimicrobial activities. Furthermore, *T. vulgaris* EO also contains bioactive sesquiterpenes such as β-caryophyllene being mainly active against Gram-positive bacteria. Furthermore, depending on the environmental conditions, antimicrobial compounds such as geraniol and 1,8 cineol can be found in *T, vulgaris* EO. Although linalool and borneol – generally found in *T. vulgaris* EO in minor quantity – are not as potent as the aforementioned substances, they still exhibit antimicrobial properties, working in synergy with other with them to contributes to the overall efficacy of EO.

In children under 5 years, indirect inhalations of *T. vulgaris* EO are traditionally preferred to treat respiratory tract conditions. For instance, the EMA report recommends a 10–20-minute bath every day with *T. vulgaris* EO as bath additive – e.g., children 3-6 years use 1.7 to 8.2 g of EO per 100 litres – for no longer than a week. Nevertheless, thyme EO in children should be used with caution, as excessive doses may cause adverse effects such as mucosa and skin irritation, nausea, and headaches [250].

2.3.2   *Eucalypti aetheroleum*

Likewise, eucalyptus essential oil is obtained by steam distillation from the fresh or partially dried leaves as well as the fresh terminal branchlets of species rich in 1,8 cineole. The most frequently used species include *E. globulus, E. polybractea,* and *E smithii*. To be considered of good quality, the plant material distilled must provide in between 0.5 and 3.5 % of a clear colourless or pale-yellow liquid with a strong and characteristic camphoraceous fragrance [254]. While younger leaves tend to produce a greater volume of oil, mature ones on the other hand, have

a higher content in 1,8 cineole, which amount varies along the season [255]. The EU herbal monograph specifies that the composition in GC-MS must include predominantly 1,8 cineole (not less than 70.0 %), but also minor compounds such as α-pinene (0.05 to 10.0 %), β-pinene (0.05 to 1.5 %), sabinene (not more than 0.3%), α-phellandrene (0.05 to 1.5 %), limonene (0.05 to 15.0 %) and camphor (maximum 0.1 %) [249].

Numerous *in vitro* studies have been conducted to investigate the antimicrobial activity of *E. globulus* oil. While some research have reported broad-spectrum activity against various bacterial strains, other works have demonstrated only modest activity [256,257]. As an example, these investigations determined *E. globulus* EO's minimum inhibitory concentration (MIC) to range from 1.25 µl/ml to 50.0 µl/ml against the most important respiratory tract pathogens, including *S. pyogenes*, *S. pneumoniae*, *S. aureus*, and *H. influenzae* [258]. Although this activity is often attributed to 1,8-cineole, other research observed that crude eucalyptus oil exhibited greater efficacy against respiratory tract strains grown in suspensions compared to pure 1,8-cineole. For instance against *S. aureus*, *E. globulus* EO registered a MIC of 4.0 mg/mL while 1,8 cineole was active from 16.0 mg/mL [259]. Eventually, eucalyptus EO and its volatile compounds is also known for its bronchodilator [14], mucolytic [260], anti-inflammatory [261], ciliary transport promotion and lung function improvement [254], making it a potent remedy for respiratory infections.

The EMA report notes that *E. globulus* EO has a number of potential therapeutic benefits, including its ability to relieve respiratory symptoms such as coughs, colds, and bronchitis. For children older than 30 months, inhalation of 2 to 4 drops of EO per 250 ml of boiling water is recommended 3 times daily for 5 to 7 days. Similarly, eucalyptus EO can be added to the bath water at 0.5 to 3.0 g of EO per 100 litres of water with a treatment of 10 to 20 minutes, 3 times a week. Besides that eucalyptus EO should be used with caution as it may potentially cause mucosa and skin irritation, the oil is contraindicated in children under 30 months of age as there is high risk of laryngospasm induced by 1,8-cineole [254].

2.3.3   *Menthae piperitae aetheroleum*

Peppermint essential oil is equally obtained through the steam distillation of *Mentha* x *piperita* fresh leaves. According to the European Pharmacopoeia, the plant material used should yield 1.2 to 3.0 % of a colourless or pale greenish-yellow liquid with a distinct fragrance and taste that

induces a cooling sensation [262]. The resulting EO when analysed with GC-MS must not only contain menthol (30.0 to 55.0 %) and menthone (14.0 to 32.0 %) as major compounds, but also isomenthone (1.5 to 10.0 %), menthyl acetate (2.8 to 10.0 %), menthofuran (1.0 to 9.0 %), 1,8-cineole (3.5 to 14.0 %), limonene (1.0 to 5.0 %), not more than 3 % of pulegone and not more than 1 % of carvone [249].

Even though *M.* x *piperita* EO is mostly known for its effect on the gastrointestinal tract, research showed that it also possesses significant antimicrobial activity against both Gram-positive strains such as staphylococci and Gram-negative bacteria like *Pseudomonas aeruginosa, Klebsiella pneumoniae,* and *E. coli* [203,263,264]. As for the microorganisms commonly associated with respiratory tract infections, studies demonstrated that peppermint oil vapour also exhibited inhibitory effects. For instance, Inouye et. al [27] *in vitro* study employing an airtight apparatus with disc volatilization methods, reported that this EO demonstrated activity against *H. influenzae* with a MID of 12.5 mg/L air. Additionally, it exhibited activity against *S. aureus* and *S. pyogenes* with an MID of 25 mg/L air, suggesting its potential as a promising antimicrobial agent against these three pathogens. Furthermore, this antimicrobial activity is mainly be credited to peppermint EO's major compounds: menthol and menthone. Amongst all studies, İşcan et al. [263] showed a moderate inhibition of *S. aureus* when using an EO sample, menthol, and menthone – i.e., MIC values of 0.625 mg/mL for all 3. In addition, besides its antimicrobial activity, other arguments in favour of using peppermint EO for respiratory tract infections include its antitussive activity at low concentration as well as its ability to provide a decongestion effect on the nasal airways [265].

For the relief of symptoms of cough and cold in children older than 2 years of age, 3 to 4 drops of EO can be added to 250 mL of hot water and the vapour can be inhaled up to three times daily. Alternatively, the EO can be included in an oral preparations such as lozenges or oral sprays and administered to older children [262]. However, *M.* x *piperita* EO is contraindicated in children under 2 years of age, as the direct application of preparation containing menthol to the nasal area or chest may induce reflex apnoea and laryngospasms [249].

2.3.4   *Foeniculi amari fructus aetheroleum*

Bitter-fennel fruit EO is a clear, colourless, or pale-yellow liquid collected from the dried, ripe fruit of *Foeniculum vulgare* via steam or hydrodistillation [249]. There are at least 3 chemotypes

of *F. vulgare* depending on the main VC – i.e., estragole CT, estragole/anethole CT and anethole CT – however, only the anethole-type complies with the Ph.Eur. definition, which specifies that the most abundant compounds in the EO should include trans-anethole (55.0 to 75.0 %), fenchone (12.0 to 25.0 %) and estragole (maximum 6.0 %) when analysed using GC-MS [266]. Other volatiles such as α-pinene (1.0 to 10.0 %), limonene (0.9 to 5.0 %), and up to 0.5 % of cis-anethole are regularly found in its composition [267].

In vitro studies have demonstrated the antimicrobial potential of *F. vulgare* fruit EO against various microorganisms, including bacteria and fungi [268–270]. For instance, Roby *et al*. [271] showed that *F. vulgare* EO inhibited the growth of *E. coli* (MIC: 12.5 μg/mL), *Salmonella typhimurium* (MIC: 12.5 μg/mL), *Bacillus cereus* (MIC: 12.5 μg/mL), and *Candida albicans* (MIC: 10 μg/mL) using broth microdilution susceptibility assay. In regard to common respiratory tract pathogens, however, most of the studies only focused on the growth-inhibitory activity of fennel EO against *S. aureus* [272–274]. In a recent study by Ghasemian *et al*. [275], three *F. vulgare* EOs originated from 3 different locations exhibited remarkable antimicrobial effects against *S. aureus* with MIC values ranging from 64 μg/mL to 128 μg/mL using a similar method Similarly, Kazemi *et al.* [276] studied antistaphylococcal effect and found a significantly higher activity of *F. vulgare* CT trans-anethole against human pathogenic *S. aureus* with MIC value of 0.5 μg/mL than trans-anethole alone (MIC: 4.0 μg/mL), indicating that other less predominant components most likely contribute to its antibacterial properties.

Ultimately, bitter fennel fruit EO is a traditional herbal medicinal product commonly used as an expectorant in coughs associated with the common cold. In children over 1 year of age, the EO is traditionally administered in the form of syrup at a posology 3 to 3.25 mg 2-3 times daily or 7.0 mg in one single dose [267]. However, the use of fennel EO products in children and adolescents under 18 years of age is not recommended due to insufficient data on the presence of estragole, a suspected genotoxic and carcinogenetic agent as indicated by a European Union Committee on Herbal Medicinal Products report [277].

2.3.5   *Anisi aetheroleum*

Anise seeds essential oil is obtained by steam distillation of green anise (*P. anisum*) dried ripe fruits. According to the HMPC monograph, the distilled plant material must yield between 2.0 and 6.0 % of a clear, colourless or pale-yellow liquid with a sweet, warm, and spicy odour reminiscent

of anethole [249]. When analysed with GC-MS, major volatile compounds include trans-anethole (percentage content of 87.0 to 94.0 %) and its isomer, estragole (0.5 to 5.0 %). In addition, the EO contains other noteworthy constituents in much smaller amounts such as pseudoisoeugenyl-2-methylbutyrate (0.3 to 2.0 %), up to 1.4 % of anisaldehyde, and cis-anethole (0.1 to 0.4 %). However, in contrast to fennel EO, it does not contain appreciable amounts of fenchone (maximum 0.01 %) [278].

When it comes to its bioactivity, *P. anisum* seeds EO possesses strong *in vitro* inhibitory effects against the growth of a wide spectrum of microorganisms known to be pathogenic for man [270,279–281]. When assayed using the agar dilution method, anise seeds EO exhibited promising activity against several Gram-positive bacteria such as *S. aureus* (MIC: 0.25 % v/v) as well as Gram-negative strains including *E. coli* and *S. typhimurium* with MIC values of 0.5 and 2.0 %, respectively [282]. Only few research, however, focused on the antibacterial activity of *P. anisum* EO on common respiratory pathogens. For instance, only Abdel-Reheem and Oraby [283] reported that anise seeds EO displayed remarkable activity against *S. aureus, P. aeruginosa*, and *S. pyogenes* with MIC values of 3.0 μg/mL, 3.0 μg/mL and 4.0 μg/mL respectively, using broth microdilution method. These results are in line with antimicrobial activity of trans-anethole recorded by Kazemi *et al.* [276] against similar respiratory microorganisms. For instance, using the same method, MIC values for *S. aureus* and *P. aeruginosa* were 4.0 μg/mL and 1.5 μg/mL, respectively, suggesting that trans-anethole is a volatile compound very active in inhibiting the growth of pathogenic respiratory bacteria.

Anise seeds EO has been traditionally used to treat respiratory complaints, mainly as an expectorant in cough associated with common cold [249]. In adult and adolescent older than 12 years of age, the EMA herbal monograph suggested dosage is ranging from 0.05 to 0.4 ml of anise EO (1 to 8 drops) to be taken orally, maximum three time daily. In children, however, due to the presence of compounds that do not have a clear toxicological profile such as estragole – potential genotoxic and carcinogenetic – anise oil is not recommended [278].

3. Hypothesis

Lower respiratory tract infections, including pneumonia, continue to pose a substantial global health challenge, with children under five years old being particularly vulnerable:

i. Due to the difficulties associated with treating these infections using conventional antibiotics, we suppose that the vapour emitted by EOs recommended by the EMA for paediatric cough and cold may exhibit a significant antibacterial activity. This could become a promising alternative to current antibiotic treatments against respiratory bacterial pathogens.

ii. As the antibacterial activity of EO is closely linked to their chemical composition, we suppose that EO chemical profile may provide key elements contributing to EO effectiveness. Furthermore, we expect that employing a dual column/dual detector GC-MS system will significantly improve the characterization of *T. vulgaris* EO allowing for a more detailed identification of major constituents and their relative abundance.

iii. Eventually, EO vapour profile may also be affected by extraction techniques used. While HS-SPME is the most commonly utilized method for EO vapour analysis, it is subject to certain limitations, and we presume that optimising the headspace analysis methodology might reflect more accurately the exact volatile compounds' composition and proportion in the headspace, providing a foundation for future studies exploring the efficacy of EO vapour phase. Additionally, we hypothesize that the matrix in which the EO is inserted could play a significant role in influencing the rate of evaporation. Different matrices may possess varying physical and chemical properties that can either enhance or inhibit the release of EO vapour, potentially altering the composition of the headspace.

4.   Research questions

i.   Does the chosen set of five EOs recommended by the EMA for paediatric cough and cold demonstrate a significant *in vitro* antimicrobial activity against pathogens causing pneumonia in both liquid and vapour phases?

ii.   How does the utilization of a dual column/dual detector GC-MS system enhance the characterization of chemical profiles, major constituents, and their abundance in the most active *T. vuglaris* EO samples?

iii. What differences exist in the chemical composition of EO vapours when sampled with HS-SPME and HS-GTS sampling techniques? Is the evaporation of the EO influenced by the matrix in which it is inserted?

5. Objectives

The aim of this study was to assess the antibacterial activity of the vapour of five EOs hydrodistilled from aromatic plants species approved by HMPC of the EMA for the treatment respiratory infections and analyse their chemical compositions.

The specific objectives of the study are as follow:

i.   Evaluation of the *in vitro* growth-inhibitory effect of EOs against bacterial pathogens causing pneumonia in liquid and vapour phase using broth microdilution volatilization method.

ii.   Characterization of the chemical composition of *T. vulgaris* EO obtained from 3 different commercial suppliers using dual column/dual detector GC-MS.

iii. A comparative headspace-GC-MS analysis of chemical composition of samples obtained by HS-SPME and HS-GTS techniques from *T. vulgaris* EO vapours released from liquid and solid matrices.

6. Materials and Methods

6.1 Plant material

Seeds of *Pimpinella anisum* L. and *Foeniculum vulgare* Mill., dried leaves of *Eucalyptus globulus* Labill. and *Mentha×piperita* L. and dried aerial parts of *Thymus vulgaris* L. were purchased from three distinct suppliers located in Czech Republic (supplier A = Království chuti s.r.o., Prague; supplier B = Byliny Mikeš s.r.o., Číčenice; supplier C = Lbros s.r.o., Vrchlabí). Subsequently, plant materials were ground and homogenised using a Grindomix apparatus (GM 100 Retsch, Haan, Germany). The residual moisture content was evaluated gravimetrically at 130 °C by Scaltec SMO 01 Analyzer (Scaltec Instruments, Gottingen, Germany) according to the Official Methods of Analysis of the Association of Official Agricultural Chemists [284] in triplicates and results were expressed as arithmetic average.

6.2 EO extraction

EOs were extracted by hydrodistillation of the ground material following the indication provided by the European Pharmacopoeia [285]: 100 g of ground plant materials was added to 1 L of distilled water and processed during 3 h using a Clevenger-type apparatus (Merci, Brno, Czech Republic). Since hydrodistillation is one of the commonly used methods for commercial production of the five selected EOs, the sample properties prepared in this study is considered similar to those commercially available. The extracted EOs were stored in sealed glass vials at 4 °C until further handling. The EO yield was determined by considering the dry mass of the plant material, which was obtained by subtracting the moisture content from the initial mass.

6.3 Microorganisms and Media

In this study, the following standard strains of the American Type Culture Collection (ATCC) were used: *H. influenzae* ATCC 49247, *S. aureus* ATCC 29213, and *S. pyogenes* ATCC 19615. The cultivation and assay media (both broth and agar) for the specified bacteria were prepared using tailored growth mediums. Mueller-Hinton (MH), supplemented with Haemophilus Test Medium and defibrinated horse blood was used for cultivation of *H. influenzae*. For *S. aureus*, the cultivation medium was MH only. Eventually, when working with *S. pyogenes*, Brain Heart Infusion (BHI) medium was utilized. Additionally, the pH of all broths was equilibrated to a final

value of 7.6 using Trizma base (Sigma-Aldrich, Prague, Czech Republic). All microbial strains, growth media, and additions were purchased from Oxoid (Basingstoke, Hampshire, UK).

Bacterial strains' stock cultures were incubated (Memmert GmbH & Co. KG, Buchenbach, Germany) in suitable media at 37 °C for 24 hours before conducting the tests. The turbidity of the bacterial suspension was adjusted to a 0.5 McFarland standard using Densi-La-Meter II (Lachema, Brno, Czech Republic), resulting in a final concentration of 10^7 CFU/mL. Ultimately, ampicillin, oxacillin, and amoxicillin were acquired from Sigma-Aldrich (Prague, Czech Republic) and utilized as positive antibiotic controls [210].

6.4   Broth microdilution volatilisation assay.

The *in vitro* growth-inhibitory effect of EOs was assessed using the broth microdilution volatilisation (BMV) assay, which allows the simultaneous evaluation of EOs' antibacterial activities in both liquid and vapour phases [220,286]. Standard 96-well microtiter plates (well volume of 400 µL) covered by lids with flanges were employed (SPL Life Sciences, Naechon-Myeon, Korea).

Initially, 30 µL of agar was pipetted into every flange except the outermost ones, and after solidification, inoculated with 5 µL of bacterial suspension. In the meantime, EO samples were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Prague, Czech Republic) at a maximum concentration of 1% of the solvent and diluted in the appropriate broth medium. Seven two-fold serial dilutions were then prepared directly in the plate, resulting in a final mixture volume of 100 µL per well with concentrations of EOs ranging from 1024 µg/mL to 8 µg/mL. Bacterial suspension was subsequently added into the wells using a 96-pin multi-blot replicator (National Institute of Public Health, Prague, Czech Republic). Eventually, control wells for growth and purity were simultaneously prepared with inoculated and non-inoculated broth. In broth media, oxacillin, ampicillin and amoxicillin were used as positive antibiotic controls for susceptibility confirmation of *S. aureus, S. pyogenes* and *H. influenzae*, respectively. Empty outermost wells were used to prevent the edge effect. The plate layout is shown in Figure 1a and b. Ultimately, microtiter plates were clamped (Emil Lux GmbH & Co. KG, Wermelskirchen, Germany) together with lids using handmade wooden pads and incubated at 37°C for 24 hours (Figure 2).

The minimum inhibitory concentrations (MICs) were determined by visually assessing bacterial growth after staining metabolically active bacterial colonies with thiazolyl blue tetrazolium

bromide dye (MTT) at a concentration of 600 µg/mL (Sigma-Aldrich, Prague, Czech Republic). The interface of colour changes from yellow to purple, relative to that of control wells, was recorded in both broth and agar (Figure 3a and b.). The MIC values were determined as the lowest concentrations of EOs that inhibited bacterial growth compared to the compound-free control and were expressed in µg/mL (1024, 512, 256, 128, 64, 32, 16, and 8 µg/mL). In the determination of MIC values for the vapour phase, consideration was given to estimate the distribution of volatile compounds within the well. The initial concentration of the EO sample in the liquid phase occupied a volume of 100 µL in a total well volume of 400 µL. Given the uncertainty regarding the extent of volatile compounds evaporation, the assumption was made that volatile compounds were uniformly distributed throughout the entire well volume, encompassing both liquid and vapour phases. To maintain consistency in reporting, a dividing factor of 4 was applied, representing the ratio of the total well volume to the initial volume of the EO sample. As a result, MIC values for the vapour phase were expressed as weight of volatile agent per volume unit of a well, and reported as 256, 128, 64, 32, 16, 8, 4, and 2 µg/cm³ for 1024; 512; 256; 128; 64; 32; 16 and 8 µg/mL, respectively. This approach accommodates the variability introduced by potential evaporation of volatile compounds, providing a practical and consistent representation of MIC values under the experimental conditions.

DMSO at a concentration of 1% was used as the negative control, and it did not inhibit any of the tested strains in either broth or agar media. All experiments were conducted in triplicates in three independent measurements, and the results were expressed as median/modal MIC values. In cases where triplicate endpoints fell within the two- and three-dilution ranges, the mode and median were used for the final value calculation, following the widely accepted norm in MIC testing.
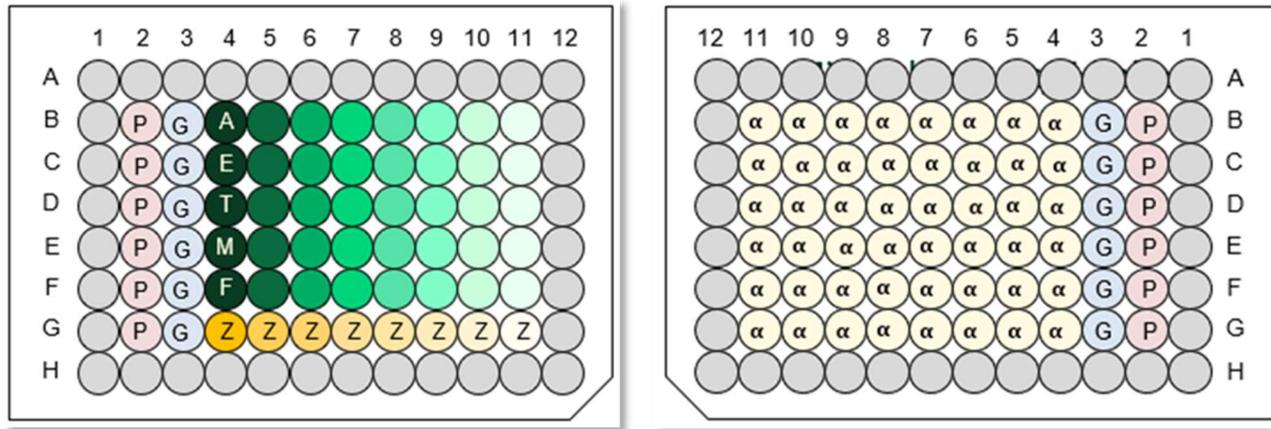
Figure 1: Microtiter plate (a) and lid (b) layouts of broth microdilution volatilisation assay

P: Sterility control (non-infected medium control; 0% growth of bacteria); G: Growth control (infected medium control; 100% growth of bacteria); Z: Positive antibiotic control in two-fold dilutions; A: *P. anisum* EO sample, E: *E. globulus* EO sample, T: *T. vulgaris* EO sample, M: *M.* x *piperita* EO sample, F: *F. vulgare* EO sample in two-fold dilutions (starting at 1024 µg/mL); α: Inoculated agar
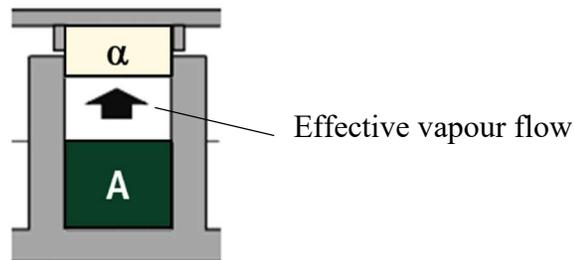
Figure 2 Schematic cross-sectional view of a closed well during the assay.

A: EO sample inserted into broth medium inoculated with bacterial suspension; α: inoculated agar

Figure 3. Broth microdilution volatilization method (a) microplate's wells, seven twofold serially dilutions of EO samples prepared before inoculation of bacterial suspensions; (b) pipetting of agar in the lid's flanges: 30 μl pipetted into every flange of lid and inoculated with 5 μl of bacterial suspension; (c) microplate and lid fastened together with clamps and handmade wooden pads before incubation for 24 hours at 37 °C.
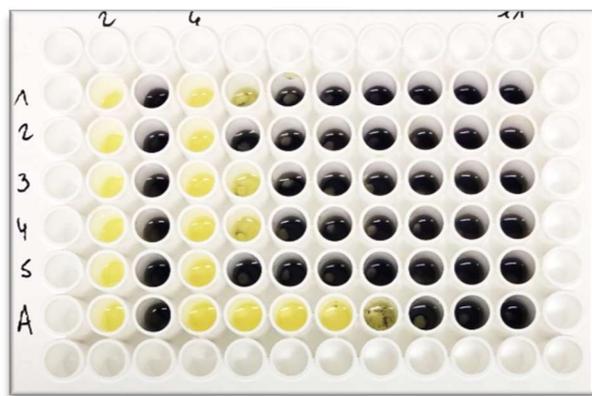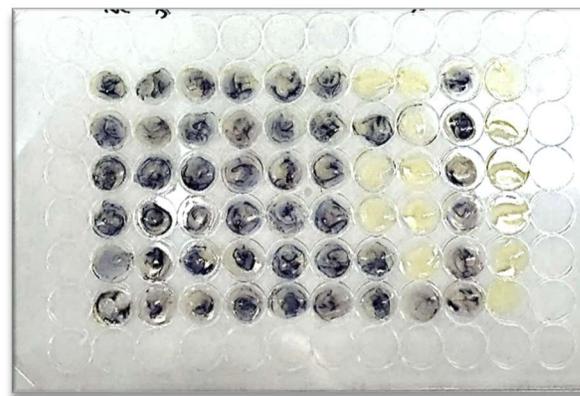
Figure 4 Evaluation of living bacterial colonies with thiazolyl blue tetrazolium bromide dye on lid (a.) and in plate (b.)

Purple wells and flanges: medium with bacterial growth; yellow wells and flanges: medium with bacterial growth inhibition; white wells and flanges: not used.

6.5 Gas chromatography-mass spectrometry analysis (GC-MS)

For EO sample characterisation, GC-MS analysis was performed using a dual column/dual detector gas chromatograph Agilent GC-7890B system (Agilent Technologies, Santa Clara, CA, USA) equipped with autosampler Agilent 7693, two columns, a fused silica HP-5MS column (30m × 0.25 mm, film thickness 0.25 μm, Agilent 19091s-433) and a DB-HeavyWAX (30 m × 0.25 mm, film thickness 0.25 μm, Agilent 122–7132), and a flame ionisation detector (FID) coupled with single quadrupole mass selective detector Agilent MSD-5977B. The operational parameters were the following: helium as carrier gas at 1 mL/min, injector temperature 250 °C for both columns. The oven temperature was raised for both columns after 3 min from 50 to 280 °C. Initially, the heating velocity was 3 °C/min until the system reached a temperature of 120 °C. Subsequently, the velocity increased to 5 °C/min until a temperature of 250 °C, and after 5 min holding time, the heating speed reached 15 °C/min until obtaining a temperature of 280 °C. Heating was followed by an isothermic period of 20 min. The EO samples were diluted in n-hexane for GC-MS (Merck KGaA, Darmstadt, Germany) at the concentration 20 μL/mL. One microliter of the solution was injected in split mode at a split ratio of 1:30. The mass detector was set to the following conditions: ionisation energy 70 eV, ion source temperature 230 °C, scan time 1 s, mass range 40–600 m/z.

The identification of EO constituents was performed by comparing their retention indices (RIs), retention times (RT), and mass spectra with the National Institute of Standards and Technology Library ver. 2.0.f as well as with authentic standards of the main volatile substances identified, namely thymol, carvacrol and *p*-cymene [214]. RIs were calculated for isolated compounds using a n-alkanes series ranging from C_8 to C_{40} (Sigma-Aldrich, Prague, Czech Republic) on an HP-5MS column. The final number of compounds in each EO was determined by adding the components identified using both columns and the constituents identified exclusively by the DB-HeavyWAX column. Quantitative data were expressed as the relative percentage content of constituents determined by the FID.

6.6 Headspace analysis using SPME and GTS sampling techniques.

6.6.1 Volatile compounds extraction when using a liquid matrix.

The analysis of the chemical composition of the headspace above a liquid matrix (LM) of MH broth and *T. vulgaris* EO (suppliers A, B and C) at a concentration of 512 µg/mL (i.e., the lowest MIC value obtained from the BMV assay) was performed using two different sampling techniques: HS-SPME and HS-GTS. For the 10 experiments conducted (1 experiment for supplier A and B, 3 experiments for supplier C per sampling method), a set of five samples were prepared, and a volume of 2 mL of the above-mentioned mixture was introduced into a 4 mL glass vial. With the exception of the first sample (t = 0 h), all EO samples were placed into an oven set at a temperature of 37 °C for incubation until their analysis at 3, 6, 9, and 12 h (Figure 4) [287]. In HS-SPME, the headspace sampling was achieved using a fibre assembly coated with a 50/30 µm mixed layer of divinylbenzene/carboxen/polydimehylsiloxane (DVB/CAR/PDMS – Supelco, Bellefonte, PA, USA). Subsequently, the needle of the HS-SPME holder was inserted into the vial, and the coated fibre was exposed to the headspace for 15 min for adsorption of the volatile compounds using Vihanova et al. [288] methodology as a guiding reference. (Figure 5a). The needle was subsequently removed, inserted into the GC injector port, and set in splitless mode, where the desorption of analytes occurred. The injector temperature was set at 250 °C, and the fibre was left into the injector for the whole analysis until the next measure.

As for HS-GTS, however, the sampling technique was carried out using a 2.5 mL SampleLock gas tight syringe (Hamilton Bonaduz AG, Bonaduz, Switzerland), including a twist valve lock and a positive rear plunger stop to prevent sample loss. At equilibrium, with the valve of the syringe closed, the needle was passed through the vial septum and inserted until reaching the centre of the headspace (Figure 5b). The valve was then opened, and a 2.5 mL sample was collected. Afterwards, the valve was closed again, the syringe removed from the vial and inserted into the GC injector at a similar temperature of 250 °C equally set in splitless mode. Eventually, the valve was opened one last time in order to inject the headspace aliquot, and the syringe was immediately removed.

For both sampling methods, measurements were repeated every 3 h during a 12-h incubation period. Eventually, analyses were performed on the HP-5MS column with similar operational parameters as described in Section 4.5 for GC-MS analyses.
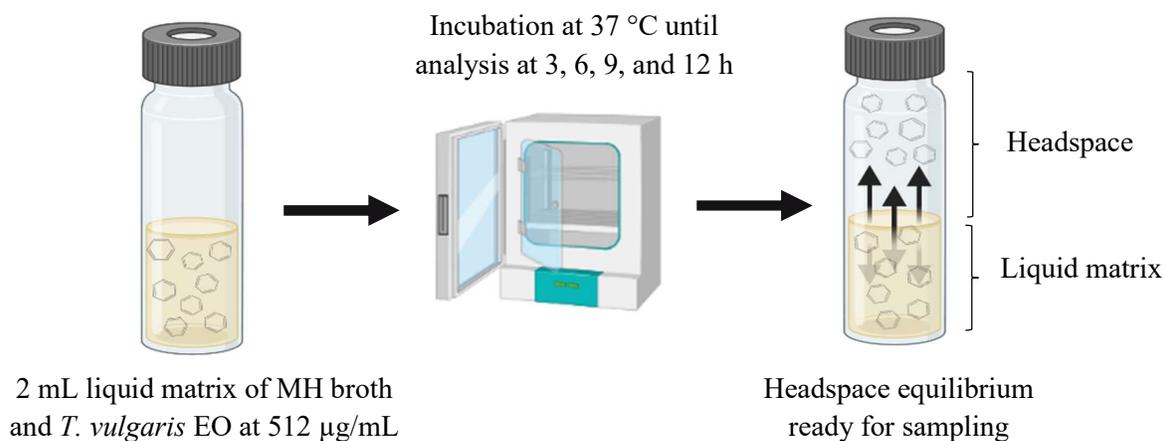
Figure 5. Liquid matrix sample preparation for headspace analysis

a

b
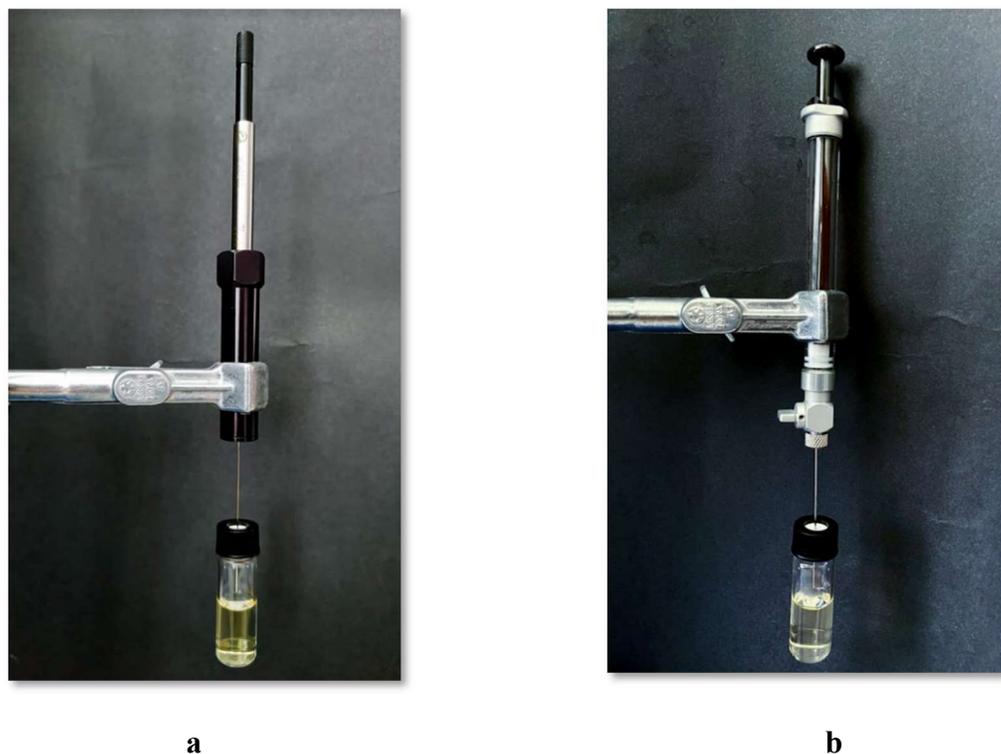
Figure 6. Headspace sampling techniques HS-SPME (a) and HS-GTS (b) above a liquid matrix

a.: HS-SPME sampling technique using a fibre assembly coated with a 50/30 μm mixed layer of divinylbenzene/carboxen/polydimethylsiloxane; **b.:** HS-GTS sampling technique carried out using a 2.5 mL SampleLock gas tight syringe.

6.6.2 Volatile compounds extraction using a solid matrix.

The analysis of the chemical composition of the headspace above a solid matrix (SM) was carried out using 20 mm cellulose filter disc (BÜCHI Labortechnik AG, Flawil, Switzerland) cut out at a diameter of 10 mm and impregnated with 91 µL of MH broth and *T. vulgaris* EO (supplier C) mixture at a concentration of 512 µg/mL. For the 6 experiments conducted (3 experiments on supplier C's EO per sampling method), a set of five disc-samples were prepared, and inserted into a 4 mL glass vial (Figure 6). Except for the first sample (t = 0 h), all EO samples were placed into an oven set at a temperature of 37 °C for incubation until their analysis at 3, 6, 9, and 12 h (Figure 7).

Similarly, both HS-SPME and HS-GTS sampling techniques were performed following the same workflow as in Section 5.6.1 (Figure 8a and b). Eventually, the chemical analyses of the headspace above the SM were carried out with similar operational parameters as described in Section 4.5 for GC-MS analyses.

Figure 7. Solid matrix samples for headspace analysis

6.6.3 Statistical analysis

The chemical analysis of *T. vulgaris* EO sample from supplier C (the most active EO based on the results of the antimicrobial assay) was performed in triplicate, including the chromatographic analysis of its liquid-phase and the headspace analysis using both extraction techniques (HS-SPME and HS-GTS). Relative peak area percentages were expressed as mean average of these three measurements ± standard deviation. For all EO samples' chemical profiles to be compared with one another, chemical analysis of EO samples from suppliers A and B was carried out in one replication only.

Incubation at 37 °C until
analysis at 3, 6, 9, and 12 h

Headspace

Solid matrix

Paper disc with 91 µL of MH broth
and *T. vulgaris* EO at 64 µg/mL

Headspace equilibrium
ready for sampling
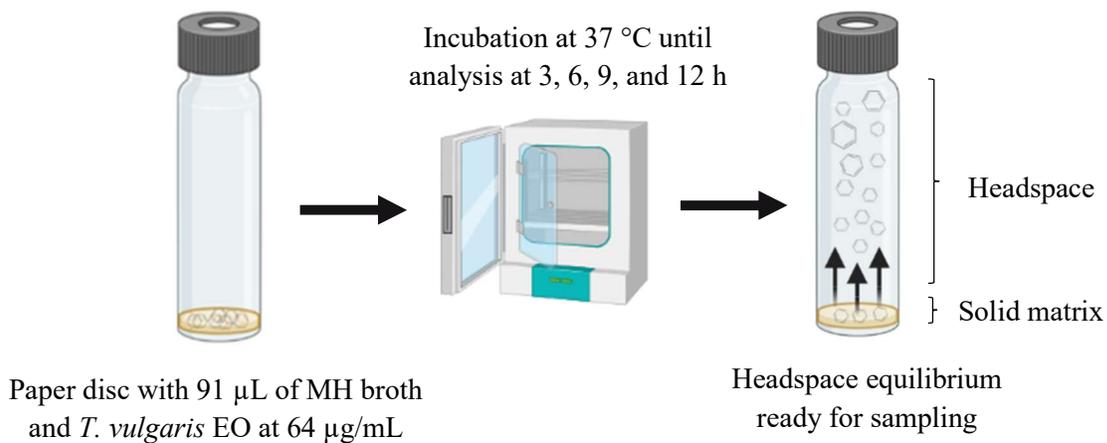
Figure 8. Solid matrix sample preparation for headspace analysis

a.   b.
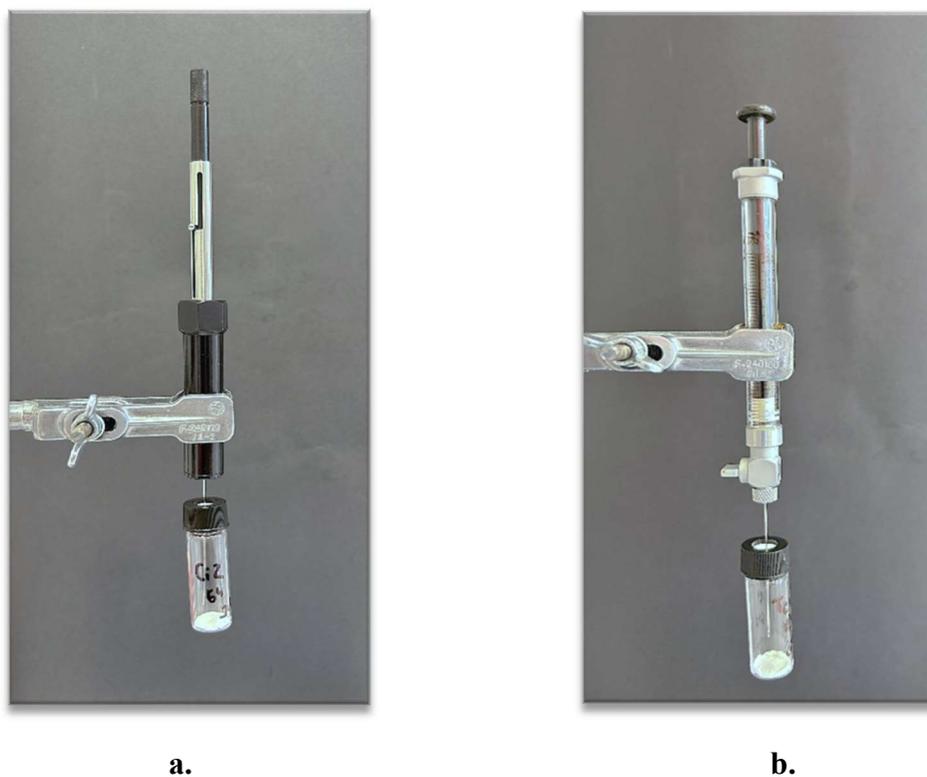
Figure 9. Headspace sampling techniques HS-SPME (a) and HS-GTS (b) above a solid matrix

7. Results and Discussion

7.1 Physico-chemical properties of EOs

In this study, dried plant material from 5 plant species, namely *Eucalyptus globulus, Foeniculum vulgare*, *Mentha × piperita, Pimpinella anisum*, and *Thymus vulgaris*, and were purchased from 3 different commercial suppliers and processed by hydrodistillation to obtain 15 EOs. The yields exhibited significant variations among the different plant species and suppliers (Table 1). For instance, *P. anisum* and *F. vulgare* demonstrated yields ranging from 1.56 % to 2.17 % and 0.11 % to 3.59% respectively – i.e., 13.1 to 19.8 mL/kg and 1.1 to 35.9 mL/kg of anhydrous fruits – while *E. globulus* (0.82 % to 0.91 % equivalent of 8.2 to 9.1 mL/kg) and *M. × piperita* EOs yielded lower amounts (0.59 % to 0.80 % equivalent of 5.9 to 7.9 mL/kg of cut dried leaves). Compared to the recommendations provided by the European Pharmacopoeia [289] while *P. anisum* and *F. vulgare* EO yields barely fell within the recommended range (not lower than 20 mL/kg and 40mL/kg, respectively of anhydrous fruits), *E. globulus* and *M. × piperita*, on the contrary, exhibited yields below the lower limit accepted (not lower 20 mL/kg of whole or cut dried leaves and 9 mL/kg of cut dried leaves, respectively). As reported by Telci et al. [290], the yield of EO is significantly influenced by environmental factors. Despite their high adaptability, aromatic species thrive in specific conditions, requiring a warm climate and extended vegetation periods to achieve high yields. Consequently, the observed disparities can be attributed to a range of factors, including geographical location, weather conditions, and soil composition. Additionally, accounting for the age of the plants at harvest and their stage within the growth cycle is also an interesting parameter to consider [14,169]. Similarly, *T. vulgaris* (0.22 % to 1.38 % equivalent of 3.4 to 21.9 mL/kg), showed a wider array of yields and which was in accordance with their Ph.Eur.'s upper limits of 12 mL/kg of cut dried leaves aerial parts.

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Plant species	Plant part used	Essential oil					
		Supplier A		Supplier B		Supplier C	
		Yield (%)	Colour	Yield (%)	Colour	Yield (%)	Colour
Pimpinella anisum	seeds	1.98	translucent	1.95	translucent	1.56	light yellow
Eucalyptus globulus	leaves	0.91	yellow	0.82	orange	0.87	yellow
Thymus vulgaris	aerial parts	1.38	orange	0.34	dark orange	0.22	orange
Mentha × piperita	leaves	0.74	light yellow	0.59	light yellow	0.80	light yellow
Foeniculum vulgare	seeds	2.17	translucent	3.59	translucent	0.11	translucent

Table 1. EO yields and colours obtained from dried plant samples of 3 commercial suppliers.

7.2   Antibacterial effect of EOs against respiratory pathogens

In the following stage of this study, determination of the *in vitro* antimicrobial activity assays of all EOs against three bacterial strains associated with lower respiratory infections in children under 5 were performed. The strains tested encompassed two Gram-positive varieties (*S. aureus* and *S. pyogenes*) as well as a Gram-negative bacterium, *H. influenzae*. The comprehensive MIC values, acquired through broth microdilution volatilization method, are presented in the Table 2. The results of this screening revealed that the 15 EOs assessed demonstrated variable degrees of antibacterial activity against the tested bacteria.

Among the selected plant species, the EO of *Thymus vulgaris* aerial parts was the most active against all bacteria strains. The EOs from supplier A, B and C presented a certain degree of antibacterial efficacy ranging from 512 to 1024 µg/mL in both liquid and vapour phases. Supplier C's EO was the most active, with the lowest MICs value of 512 µg/mL in both liquid and vapour phases for the three bacteria strains. Similarly, *S. pyogenes* and *H. influenzae* growth were more affected by the EO of supplier B than *S. aureus* with MICs at 512 µg/mL and 1024 µg/mL in both

58

broth and agar, respectively. On the contrary, the least effective EO source was from supplier A: results showed mild efficacy against *H. influenzae* (512 µg/mL), whereas a weaker inhibitory effect of 1024 µg/mL was observed against both *S. aureus* and *S. pyogenes*. Likewise, each EO affected the growth of *S. pyogenes*, *H. influenzae*, and *S. aureus* similarly in both liquid and vapour phases. No discrepancy between broth and agar results was observed on the tested strains. *H. influenzae* was the most susceptible bacterial strain (MICs = 512 µg/mL for all EOs tested) followed by *S. pyogenes* (MICs = 512 µg/mL supplier B and C and MIC = 1024 µg/mL for supplier C), while *S. aureus* was the least sensitive (MIC = 512 µg/mL supplier C and MICs = 1024 µg/mL for supplier A and B). As reported by Houdková and Kokoska [203], several assays have previously been developed for the evaluation of the antibacterial activity of volatile plant compounds in the vapour phase. However, there is still a lack of standardised methods, something that makes any interpretation and comparison difficult. For instance, Ács et al. [291] evaluated the antibacterial activity of thyme EO against *H. influenzae* (MIC = 110 µg/mL in liquid phase, MIC = 0.025 µg/cm^3 of air in vapour phase) and *S. pyogenes* (MIC = 430 µg/mL in liquid phase, MIC = 0.125 µg/cm^3 of air in vapour phase) in 2 separate experiments – i.e., broth microdilution and vapour phase tests – confirming the same inhibitory trend on the Gram-negative bacteria. Furthermore, as already mentioned in section 2.3.1, a study conducted by Inouye et al. [27] assessed the *in vitro* growth-inhibitory effects of *T. vulgaris* EO vapour against *H. influenzae, S. pyogenes,* and *S. aureus* using an airtight device. Introducing the minimum inhibitory dose expressed as amount of the volatile agent per unit of air, they determined that the vapour of carvacrol chemotype EOs were more active against Gram-negative *H. influenzae* than against Gram-positive strains such as *S. pyogenes* and *S. aureus* – MIDs of 3.1, 6.2, and 12.5 µg/cm^3 air, respectively. In this antimicrobial screening, we reported similar results: *H. influenzae* was more susceptible – i.e., MIC = 512 µg/mL or 128 µg/cm^3 considering the volume of the entire well – to the vapour of our thymol chemotype EOs than the *S. pyogenes*, and *S. aureus* – MICs comprised in between 1024–512 µg/mL or 256–128 µg/cm^3 for both bacteria strains.

Table 2 *In vitro* growth-inhibitory effect of essential oils from *Pimpinella anisum, Eucalyptus globulus, Thymus vulgaris, Mentha* x *piperita* and *Foeniculum vulgare* in liquid and vapour phase against respiratory infection bacteria.

Plant species	Supplier	Staphylococcus aureus Broth μg/mL	Agar μg/mL	Agar μg/cm³	Streptococcus pyogenes Broth μg/mL	Agar μg/mL	Agar μg/cm³	Haemophilus influenzae Broth μg/mL	Agar μg/mL	Agar μg/cm³
P.anisum	A	NA	NA	NA	NA	NA	NA	NA	NA	NA
	B	NA	NA	NA	NA	NA	NA	NA	NA	NA
	C	NA	NA	NA	NA	NA	NA	NA	NA	NA
E. globulus	A	NA	NA	NA	NA	NA	NA	NA	NA	NA
	B	1024	1024	256	NA	NA	NA	1024	1024	256
	C	NA	NA	NA	256	1024	256	1024	1024	256
T.vulgaris	A	1024	1024	256	1024	1024	256	512	512	128
	B	1024	1024	256	512	512	128	512	512	128
	C	512	512	128	512	512	128	512	512	128
M. × piperita	A	NA	NA	NA	NA	NA	NA	NA	NA	NA
	B	NA	NA	NA	NA	NA	NA	NA	NA	NA
	C	NA	NA	NA	NA	NA	NA	NA	NA	NA
F. vulgare	A	NA	NA	NA	NA	NA	NA	NA	NA	NA
	B	NA	NA	NA	NA	NA	NA	NA	NA	NA
	C	NA	NA	NA	NA	NA	NA	NA	NA	NA
Oxacilin		0.25	>2	>0.5	ND	ND	ND	ND	ND	ND
Amoxicilin		ND	ND	ND	0.06	>2	>0.5	ND	ND	ND
Ampicilin		ND	ND	ND	ND	ND	ND	0.5	>16	>4

NA: not active at highest concentration tested (minimum inhibitory concentration >1024), ND: not determined.

It is largely admitted that Gram-negative bacteria are more resistant to EOs than Gram-positive ones. This weak antibacterial activity was attributed to the presence of hydrophilic polysaccharide chains in the outer membrane structure, preventing hydrophobic EOs from reaching the bacteria cell membrane [292,293]. One reason that could explain the higher susceptibility of *H. influenzae* to EOs would be the more hydrophobic nature of its outer membrane composed of oligosaccharide shorter chains [27]. This was confirmed by Reyes-Jurado et al. [294], who demonstrated that compounds including *p*-cymene, linalool, and thymol were able to disintegrate such outer membrane structures. Eventually, *T. vulgaris* EO antibacterial activity could largely be ascribed to its content in phenolic compounds as already reported by Teixeira et al. [295], who demonstrated that an elevated concentration of thymol and carvacrol in thyme EO enhances its antibacterial potency.

In addition, various studies have subsequently assessed the growth-inhibitory effects of *T. vulgaris* EO vapour using vapour diffusion assay developed by Lopez et al. [191]. Nedorostova et al. [212] reported a MIC of 17 µL/L (15.5 µg/cm^3) against *S. aureus,* which is, according to the author, equivalent to the result of Inouye et al. [27] against the same bacterium (MID = 12.5 µg/cm^3 of air). Similarly, Kloucek et al. [214] have observed that the vapour of *T. vulgaris* EO with different chemotypes had MICs comprised between 125 µL/L (115 µg/cm^3) and 250 µL/L (230 µg/cm^3) against *S. aureus* as well. In contrast to these findings, MIC values recorded in our study were usually higher – i.e., 1024–512 µg/mL or 256–128 µg/cm3 considering the volume of the entire well. This discrepancy in results could firstly be explained by the quality of the EO samples used and their chemical compositions [183,184], but also by both the disparate utilization of solvents and strains of bacteria used in the different antimicrobial assays [296,297]. Most importantly, this variation may also be attributed to the diverse methods used to explore the antimicrobial effect of *T. vulgaris* EO vapour, allowing various interpretations of the MIC [191,214,220].

That is why the BMV assay presented here is a powerful alternative to the previously developed techniques. While their designs only enable the assessment of EO vapour at a single concentration [214], the BMV assay is conceived to evaluate the EOs' in vitro growth inhibitory effect in liquid and gaseous phases simultaneously and at different concentrations, something that allows fast comparison of MIC values in both liquid and solid media. Based on broth microdilution [298] and disc volatilisation (DV) assays [219], the BMV experiments are conducted on standard 96-well

immune plates, which offer several advantages such as cost and labour efficiency [214,221]: microplates are standard laboratory equipment that are commonly available compared, for example, to the special airtight experimental apparatus used in certain methods such as in Seo et al. [216]. Furthermore, microplates can also be employed in fully automated workstations, unlike Petri dishes used in DV assays which therefore suffer from a lack of repeatability [222]. Other studies developed assays using microplates for detecting volatile substances antimicrobial activity, such as the vapour phase-mediated patch assay of Feyaerts et al. [217]. However, contrary to the BMV assay, their designs only allow to determine relative microbial inhibition values, and their main limitation lies in only providing qualitative results [218]. Eventually, another asset of our assay is that modifications can be easily implemented for new applications. For instance, Netopilova et al. [29] modified the test to explore the combinatory effects of volatile substances using a chequerboard design and thus determine fractional inhibitory concentration indices, something not possible with other methods. Overall, these features allow our method to be suitable for high-throughput screening and thus be a simple, fast, and reliable assay as well as providing reproducible and quantitative results [299]. Nevertheless, despite its numerous advantages, serially produced microplates are not designed for volatile substance testing, something that contributes to weaknesses shown by our assay. For example, only a limited volume of agar can be pipetted into each flange of the lid; this could impact the bacterial growth and thus affect the results. Most importantly, when testing EO vapours, the BMV assay also faces specific problems linked to the physico-chemical nature of EOs and, more particularly, their high volatility, viscosity, and hydrophobicity [203]. Firstly, volatility allows a loss of active substances by evaporation that can happen at different steps of the protocol, including during sample handling and experiment preparation, complications that are shared by all tests assessing EO properties [300,301]. More specifically to volatilisation assays, the level of vapour transition from the matrix into which the EO is included and its distribution into the inner atmosphere of the well during the experiment are two critical factors that may affect the results. For instance, the matrix influences both the intensity and the speed of the evaporation [220]. In our assay, a broth medium was used, which according to Orchard and van Vuuren's [213] observations, would slow the level of vapour transition during the experiment and thus affect the bacterial growth. Similarly, as described by Reyes-Jurado et al. [163], the hydrophobicity and viscosity of the EO may also cause its uneven distribution through the broth medium, something that could also alter the distribution of the volatile agents into the

well's atmosphere. That is why the concentrations in the vapour phase used in our experiment should only be considered as indicative values.

As for the EOs extracted from *E. globulus* leaves, they all produced moderate or weak antibacterial activity on the three bacteria strains with MIC values starting from 256 µg/ml but mostly exceeding 1024 µg/mL in both liquid and vapour phases. Like for *T. vulgaris* EO, supplier C's EO was the most active, with the lowest MICs value of 256 µg/mL against *S. pyogenes* in liquid phase. In contrast, the least effective EO source was from supplier A: results showed low efficacy against all three bacterial strains, with MIC values consistently greater than 1024 µg/mL in liquid phases and greater than 256 µg/cm³ in vapour phase. In like manner, *H. influenzae* was more susceptible to this EO (MICs = 1024 µg/mL for supplier B and C EOs in both liquid and solid media) than *S. aureus* and *S. pyogenes*. Interestingly, the result of our study follows the inhibitory trend of previous studies carried out on *E. globulus* EO antimicrobial activity against respiratory pathogens. Some of these investigations have reported extensive activity, such as those by Mulyaningsih et al. [302] who recorded MIC values of 250 and 60 µg/mL using broth microdilution assay against *S. aureus* and *S. pyogenes,* respectively or Cermelli et al. [258] who evaluated, with the same method, the activity on *H. influenzae* (MIC = 1.25 µg/mL in liquid phase) *S. aureus*, and *S. pyogenes* (both with MICs = 50 µg/mL in broth medium). Other studies, however, have found either no activity or only a minimal effect including, for instance, those by Bosnic et al. [257] and Hendry et al. [259] who both assessed the antimicrobial activity of *E. globulus* EO on *S. aureus* strains obtaining MIC values of 380 and 4000 µg/mL, respectively, using broth microdilution assay. Furthermore, to the best of our knowledge, only the research Ács et al. [291] investigated the antimicrobial activity of *E. globulus* vapour phase, reporting mild to low antimicrobial activity in both liquid and solid media against *H. influenzae* (MIC = 1410 µg/mL in liquid phase, MIC = 0.125 µg/cm³ of air in vapour phase) and *S. pyogenes* (MIC = 2820 µg/mL in liquid phase, MIC > 1.5 µg/cm³ of air in vapour phase).

Eventually, EOs of *M. × piperita* leaves, *P. anisum* seeds, and *F. vulgare* seeds were not active at concentrations tested. No substantial differences were observed between suppliers or bacteria strains, with consistently high MIC values, exceeding 1024 µg/mL in broth medium and >256 µg/cm³ in agar medium. Contrary to expectations, our results diverge from prior research studies

that have documented convincing antimicrobial activity against respiratory bacteria strains in both types of media.

Regarding *M. × piperita*, Ács et al. [291] found that the EO was active against *H. influenzae* (MIC = 210 µg/mL in liquid phase, MIC = 0.05 µg/cm^3 of air in vapour phase) and *S. pyogenes* (MIC = 350 µg/mL in liquid phase, MIC 0.25 µg/cm^3 of air in vapour phase). Likewise, two studies conducted by İşcan et al. [263] and Tyagi and Malik [264] revealed a moderate inhibition on *S. aureus*, registering MIC values of 625 and 1130 µg/mL, respectively. In addition, Inouye et al. [26] study showed that peppermint EO vapour also exhibited a mild activity against *H. influenzae*, *S. aureus* and *S. pyogenes*, evidenced by MIDs of 12.5, 25 and 25 µg/cm^3 air, respectively.

As for *P. anisum* EO, research on the antibacterial activity against pathogens causing respiratory infections are until today relatively limited. One example of such research can be found in the study by Abdel-Reheem and Oraby [283], who documented that anise seeds EO presented important activity against *S. aureus* (MIC = 3.0 µg/mL) and *S. pyogenes* (MIC = 4.0 µg/mL) as determined by the broth microdilution method. Furthermore, Kazemi et al. [276] observed a similar activity when testing trans-anethol alone against *S. aureus* (MIC = 4.0 µg/mL) using the same method. This observation reinforces that trans-anethole, as a volatile compound, holds a considerable role in the inhibition of pathogenic bacteria associated with respiratory infections.

Concerning *F. vulgare* EO, most of the research focused on the *in vitro* growth-inhibitory properties against *S. aureus*. A recent investigation by Ghasemian et al. [275] revealed that three bitter fennel EOs, derived from three distinct locations, displayed substantial antimicrobial effect against *S. aureus*. The MIC values were found to range between 64 µg/mL and 128 µg/mL, utilizing a method comparable to that used in the above-mentioned studies. Like for *P. anisum* EO, Kazemi et al. [276] examined the antistaphylococcal effect of *F. vulgare* EO, discovering a notably high activity against pathogenic *S. aureus*, with an MIC value of 0.5 µg/mL. This was significantly more effective than trans-anethole alone, which had an MIC of 4.0 µg/mL. These findings suggest that other less prevalent components within the EO might also be contributing to its antibacterial activity.

Overall, the discrepancies seen in the results between the existing literature and the samples of *E. globulus*, *M.* x *piperita*, *P. anisum*, and *F. vulgare* EOs evaluated in this study could stem from a variety of factors. According to Reyes-Jurado review on EO antimicrobial testing [163], the significant variations seen across different publications may largely be attributed to the quality

of the plant material. This quality is influenced by various environmental factors such as the plant species' geographical origin, its exposure to light, temperature, weather conditions, as well as the quantity and quality of water and nutrients received. Research showed that these elements, related to the plant's growing process, can modify the composition of the plant EO. Since the compounds found in an EO are responsible for its antimicrobial activity, any alteration could influence this biological function [183,184,303,304]. Additionally, variations in methodology, from handling the plant samples to evaluating the antimicrobial activity of their EOs, could have played a significant role. This encompasses the specific drying and processing techniques used by suppliers, the extraction methods and the chosen antimicrobial assays, including both solvents and growth media employed for sample preparation as well as the microbial strains selected in studies [296,297].

7.3 Chemical composition of *T. vulgaris*

7.3.1 Chemical analysis of EO

To characterize the EO of *T. vulgaris*, a new series of hydrodistillation were conducted using dried plants from suppliers A, B, and C from the same batch. The respective yield values for these extractions were 0.73%, 1.23%, and 1.25%. All EOs presented a strong herbaceous fragrance while being of different shades of orange colour. The complete chemical analyses of all samples are provided in Table 4. Using a HP-5MS column, 54 compounds were identified in EOs of suppliers A and B, whereas 62 components were found in supplier C's EO, representing 99.5, 99.6, and 99.6% of their respective total constituents, respectively. Similarly, using the DB-HeavyWAX column, 44, 43, and 36 components were determined, which constituted 99.3, 99.7, and 99.6 % of the volatile oils, respectively. In the three samples analysed, monoterpenoids represented by thymol (phenolic monoterpene) followed by sesquiterpenoids mainly represented by β-caryophyllene were the two dominant groups of volatile agents identified. To a lesser extent, other groups were identified, such as oxygenated phenylpropanoids, oxygenated aliphatics, furanoids, and oxygenated diterpenes.

The antibacterial properties of *T. vulgaris* are mainly attributed to the chemical composition of its EO, which has already been extensively studied. Its major constituents are mainly monoterpenoids, such as carvacrol, thymol, γ-terpinene, and *p*-cymene, but also sesquiterpenoids such as β-caryophyllene. Within the same species, the variation of proportions of these compounds defines the EO chemotype, which is named after the predominant constituent identified [305]. Our chemical analyses showed that thymol was the most abundant constituent within our samples, followed by *p*-cymene and carvacrol (Figure 9 and 10). This characterises our three EOs as thymol chemotypes. These findings are in accordance with several studies previously published. For instance, Schmidt et al. [306], Grosso et al. [307], and Nikolic et al. [308] reported thymol as the major component of their *T. vulgaris* thymol chemotype EOs (peak percentage area of 38.8%, 41.6%, and 49.1%, respectively); *p*-cymene was the second most abundant with 24.0%, 28.9%, and 20.0%, respectively. However, instead of carvacrol, γ-terpinene was detected as the third most abundant constituent (9.5%, 5.1%, and 4.2%, respectively). The variations in yields and concentrations of volatile compounds in our samples could be attributed to several factors occurring at different stages, from the growing conditions of the plants, the harvesting period to the storage conditions of the plant materials by the commercial suppliers [14,164]. For instance, Golparvar et al. [309] showed that *T. vulgaris* harvested in the same location but at different stages of the plant growth resulted in different yields of EOs: the highest oil yield (2.42%) was obtained during the flowering period, whereas the lowest yield (0.63%) corresponded to the fruiting stage. Furthermore, Nezhadali et al. [310], while comparing the antimicrobial activity of Thymus species with variable content in main compounds, highlighted a notable decline in antimicrobial activity. This decline was observed not only with a substantial decrease in thymol concentration (e.g., 63.01% to 38.23% of the total EO content) but also when o-cymene and γ-terpinene concentrations were reduced (e.g., 15.47% to 5.56% and 7.17% to 4.43%, respectively). Given that the differences in concentration of main compounds among the three suppliers in our study fall within a comparable range, these findings strongly indicate that the observed variations in the composition of essential oils from suppliers A, B, and C may account for the variability seen in antimicrobial results. Consequently, we specifically emphasized our focus on the T. vulgaris EO from supplier C for the remainder of our study, due to its superior activity.

Table 3 Chemical composition of *Thymus vulgaris* essential oils obtained from 3 different commercial suppliers.

RI [a]		Compound	Cl. [b]	Supplier/Column/Peak area (%)						Identification [e]	
				A		B		C [c]			
Obs.	Lit			HP-5MS	DB-WAX	HP-5MS	DB-WAX	HP-5MS	DB-WAX	HP-5MS	DB-WAX
761	783 [d]	methyl α-methylbutanoate	OA	0.08	-[g]	0.08	-	0.05 ± 0.01	-	GC/MS	-
923	924	α-thujene	MH	0.10	0.08	0.10	0.13	0.26 ± 0.02	0.24 ± 0.06	RI, GC/MS	GC/MS
929	939	α-pinene	MH	0.62	0.38	0.61	0.62	0.43 ± 0.03	0.38 ± 0.07	RI, GC/MS	GC/MS
944	945	camphene	MH	0.44	0.26	0.43	0.43	0.19 ± 0.01	0.18 ± 0.03	RI, GC/MS	GC/MS
972	969	sabinene	MH	0.09	0.05	0.08	0.09	0.10 ± 0.01	0.09 ± 0.02	RI, GC/MS	GC/MS
977	979	1-octen-3-ol	OA	0.31	0.20	0.32	0.30	0.35 ± 0.02	0.35 ± 0.06	RI, GC/MS	GC/MS
989	988	β-myrcene	MH	0.31	0.21	0.31	0.35	0.58 ± 0.04	0.58 ± 0.11	RI, GC/MS	GC/MS
994	996	3-octanol	OA	0.05	-	tr.[f]	-	tr.	-	RI, GC/MS	-
1002	1002	α-phellandrene	MH	0.06	-	0.06	0.05	0.10 ± 0.01	0.08 ± 0.02	RI, GC/MS	GC/MS
1007	1008	3-carene	MH	-	-	-	-	tr.	-	RI, GC/MS	-
1014	1014	α-terpinene	MH	0.62	-	0.61	-	1.07 ± 0.07	-	RI, GC/MS	-
1025	1020	*p*-cymene	AH	**22.15**	**16.43**	**22.03**	**25.35**	**12.75 ± 0.84**	**12.90 ± 2.55**	RI, GC/MS, Std.[i]	GC/MS
1027	1031	D-limonene	MH	-	0.16	-	0.25	0.41 ± 0.03	0.23 ± 0.04	RI, GC/MS	GC/MS
1028	1023	*m*-cymene	AH	0.46	-	0.47	-	-	0.18 ± 0.17	RI, GC/MS	GC/MS
1030	1032	1,8-cineole	OM	0.69	0.51	0.70	0.76	0.39 ± 0.03	0.47 ± 0.10	RI, GC/MS	GC/MS
1058	1054	γ-terpinene	MH	**2.82**	**2.02**	**2.78**	**2.90**	**5.22 ± 0.33**	**5.40 ± 1.11**	RI, GC/MS	GC/MS
1065	1068	*cis*-thujanol	OM	0.17	0.06	0.18	0.07	0.37 ± 0.03	0.14 ± 0.03	RI, GC/MS	GC/MS
1071	1078	*cis*-linalool oxide	F	tr.	-	tr.	-	-	-	RI, GC/MS	-
1087	1086	terpinolene	MH	0.18	-	0.17	-	0.11 ± 0.01	-	RI, GC/MS	-
1096	1102	*trans*-thujanol	OM	-	0.09	-	0.13	tr.	0.37 ± 0.08	RI, GC/MS	GC/MS
1100	1095	linalool	OM	1.80	1.08	1.82	1.77	1.53 ± 0.07	1.38 ± 0.24	RI, GC/MS	GC/MS
1149	1141	camphor	OM	0.32	0.23	0.18	0.34	0.06 ± 0.01	0.16 ± 0.03	RI, GC/MS	GC/MS
1165	1165	endo-borneol	OM	1.14	-	1.15	-	0.63 ± 0.03	-	RI, GC/MS	-
1176	1174	terpinen-4-ol	OM	0.61	-	0.61	-	0.62 ± 0.04	-	RI, GC/MS	-
1186	1180	m-cymen-8-ol	OM	0.05	-	-	-	-	-	RI, GC/MS	-
1190	1183	*p*-cymen-8-ol	OM	0.14	0.11	0.12	0.16	-	-	RI, GC/MS	GC/MS
1196	1189	α-terpineol	OM	0.14	-	0.14	-	0.16 ± 0.02	-	RI, GC/MS	-
1204	1195	*cis*-dihydrocarvone	OM	tr.	-	tr.	-	tr.	-	RI, GC/MS	-
1235	1235	thymol methyl ether	OPM	1.15	**2.11**	1.15	**3.01**	0.76 ± 0.05	**3.17 ± 0.67**	RI, GC/MS	GC/MS
1244	1241	carvacrol methyl ether	OPM	0.91	0.86	0.91	1.35	0.66 ± 0.04	1.09 ± 0.23	RI, GC/MS	GC/MS
1256	1242	carvone	OM	1.39	1.05	1.40	1.38	1.01 ± 0.05	1.05 ± 0.24	RI, GC/MS	GC/MS
1269	1255	geraniol	MH	0.05	-	0.05	0.09	0.05 ± 0.01	tr.	RI, GC/MS	GC/MS
1286	1282	anethole	OPP	0.22	0.21	0.23	0.30	0.11 ± 0.06	0.07 ± 0.02	RI, GC/MS	GC/MS
1304	1290	thymol	PM	**38.42**	**29.52**	**38.93**	**41.31**	**48.09 ± 2.53**	**48.65 ± 10.80**	RI, GC/MS, Std.	GC/MS
1310	1298	carvacrol	PM	**10.61**	**3.84**	**10.85**	**5.59**	**10.92 ± 3.50**	**3.60 ± 0.78**	RI, GC/MS, Std.	GC/MS
1361	1357	estragole	OPP	-	-	0.07	0.05	tr.	0.09 ± 0.02	RI, GC/MS	GC/MS
1379	1374	α-copaene	SH	0.21	-	0.21	-	0.09 ± 0.01	-	RI, GC/MS	-

RI[a]		Compound	Cl.[b]	Supplier/Column/Peak area (%)						Identification[e]	
				A		B		C[c]			
Obs.	Lit.			HP-5MS	DB-WAX	HP-5MS	DB-WAX	HP-5MS	DB-WAX	HP-5MS	DB-WAX
1388	1387	β-bourbonene	SH	0.07	tr.	0.07	-	tr.	-	RI, GC/MS	GC/MS
1424	1418	β-caryophyllene	SH	1.79	-	1.79	-	**2.33 ± 0.13**	-	RI, GC/MS	-
1433	1446[d]	isogermacrene D	SH	-	-	-	-	tr.	-	GC/MS	-
1458	1452	α-humulene	SH	0.09	tr.	0.08	0.07	0.11 ± 0.01	0.12 ± 0.02	RI, GC/MS	GC/MS
1475	1475	geranyl propionate	OM	0.06	0.06	0.10	0.14	0.10 ± 0.01	0.11 ± 0.03	RI, GC/MS	GC/MS
1480	1478	γ-muurolene	SH	0.19	0.15	0.21	0.22	0.21 ± 0.02	0.21 ± 0.04	RI, GC/MS	GC/MS
1483	1493	α-amorphene	SH	-	-	tr.	-	tr.	-	RI, GC/MS	-
1498	1495	valencene	SH	0.09	-	0.09	-	0.11 ± 0.01	-	RI, GC/MS	-
1503	1499	α-muurolene	SH	0.10	-	0.10	-	0.09 ± 0.01	-	RI, GC/MS	-
1510	1509	β-bisabolene	SH	0.06	-	0.06	-	0.06 ± 0.01	-	RI, GC/MS	-
1518	1513	γ-cadinene	SH	0.38	0.43	0.39	0.58	0.33 ± 0.02	0.73 ± 0.16	RI, GC/MS	GC/MS
1527	1524	δ-cadinene	SH	0.48	-	0.48	-	0.50 ± 0.03	-	RI, GC/MS	-
1561	1564	nerolidol	OS	tr.	-	-	-	tr.	-	RI, GC/MS	-
1585	1578	spathulenol	OS	-	-	tr.	-	tr.	-	RI, GC/MS	-
1591	1581	caryophyllene oxide	OS	1.31	0.92	1.32	1.24	0.68 ± 0.03	0.66 ± 0.15	RI, GC/MS	GC/MS
1617	1606	humulene epoxide II	OS	0.06	tr.	0.06	0.05	tr.	-	RI, GC/MS	GC/MS
1621	1627	epicubenol	OS	0.06	tr.	0.06	-	tr.	tr.	RI, GC/MS	GC/MS
1628	1630	γ-eudesmol	OS	0.13	0.10	0.13	0.13	0.13 ± 0.01	0.13 ± 0.03	RI, GC/MS	GC/MS
1634	1642	cubenol	OS	tr.	-	-	0.09	tr.	-	RI, GC/MS	GC/MS
1648	1640	α-epi-cadinol	OS	0.48	tr.	0.49	-	0.36 ± 0.03	0.05 ± 0.05	RI, GC/MS	GC/MS
1652	1645	δ-cadinol	OS	-	0.06	-	-	tr.	tr.	RI, GC/MS	GC/MS
1661	1653	α-cadinol	OS	0.05	-	0.05	-	0.07 ± 0.02	-	RI, GC/MS	-
1682	1677	cadalene	SH	0.12	-	0.14	-	tr.	-	RI, GC/MS	-
[h]	1001	4-carene	MH	-	-	-	0.64	-	1.05 ± 0.23	-	GC/MS
[h]	NA	4-pentenyl butyrate	OM	-	tr.	-	0.06	-	-	-	GC/MS
[h]	1351	α-cubebene	SH	-	0.06	-	0.10	-	0.07 ± 0.01	-	GC/MS
[h]	1290	isobornyl acetate	OM	-	0.08	-	0.11	-	-	-	GC/MS
[h]	1521	calamenene	SH	-	-	-	0.19	-	tr.	-	GC/MS
[h]	1156	isoborneol	OM	-	0.86	-	1.17	-	0.74 ± 0.16	-	GC/MS
[h]	1455	aromandendrene	SH	-	tr.	-	0.05	-	tr.	-	GC/MS
[h]	NA	lavandulyl butyrate	OM	-	0.05	-	0.07	-	tr.	-	GC/MS
[h]	1372	p-cymen-7-ol	OM	-	tr.	-	0.05	-	-	-	GC/MS
[h]	2105	phytol	OD	-	0.07	-	0.23	-	tr.	-	GC/MS
		Total identified (%)		99.55	99.30	99.65	99.68	99.62	99.61		

[a] RI = retention indices; Obs. = retention indices determined relative to a homologous series of *n*-alkanes (C$_8$–C$_{40}$) on an HP-5MS column; Lit. = literature RI values [311,312]; [b] Cl = class; AH — aromatic hydrocarbon, F — furanoid, MH — monoterpene hydrocarbon, OA — oxygenated aliphatic, OD — oxygenated diterpene, OM — oxygenated monoterpene, OPM — oxygenated phenolic monoterpene, OPP — oxygenated phenylpropanoid, PM — phenolic monoterpene, OS — oxygenated sesquiterpene, SH — sesquiterpene hydrocarbon; [c] Relative peak area percentage as mean of three measurements ± standard deviation.; [d] Literature RI values [27]; [e] Identification method: GC/MS = mass spectrum was identical to that of National Institute of Standards and Technology Library (ver. 2.0.f); RI = the retention index was matching literature database; [f] tr. = traces, relative peak area < 0.05%; [g] - = not detected; [h] Retention indices were not calculated for compounds determined by DB-WAX column; [i] Std. authentic standard for identification.

Figure 10 GC chromatograms of *Thymus vulgaris* essential oil from supplier C analysed by HP-5MS (a) and DB-HeavyWAX (b) columns.

a.

b.
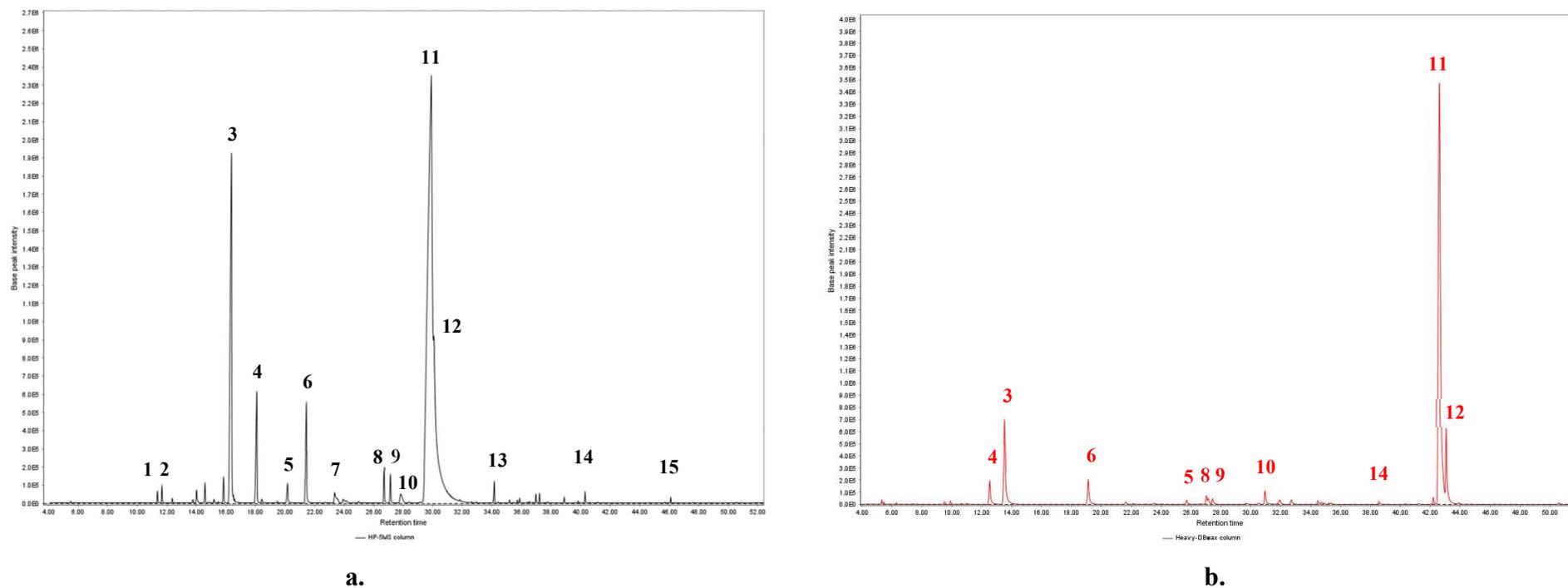
1. α-pinene, **2.** camphene, **3.** *p*-cymene, **4.** γ-terpinene, **5.** linalool, **6.** methyl octanoate (internal standard), **7.** camphor, **8.** thymol methyl ether, **9.** carvacrol methyl ether, **10.** carvone, **11.** thymol, **12.** carvacrol, **13.** β-caryophyllene, **14.** δ-cadinene, **15.** caryophyllene oxide.

Figure 11 Main volatile compound structures found by GC/MS in *Thymus vulgaris* essential oil from supplier C.

3. *p*-cymene   4. γ-terpinene   11. thymol   15. carvacrol
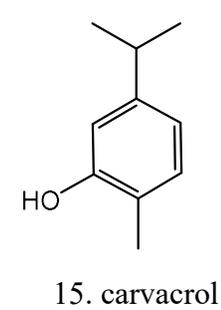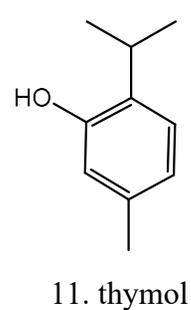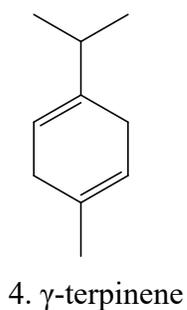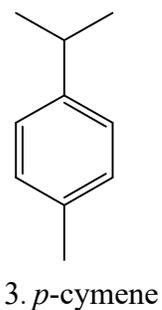
Subsequently, the characterisation of the EOs was carried out by GC/MS using two capillary columns of different polarities. In addressing the raised concerns about the high concentration of the injected sample (20 mg/ml) and potential system overloading, it is essential to consider the context of our methodology in relation to established practices. The decision to use this concentration was influenced by methodologies commonly employed in the analysis of tropical essential oils, exemplified in studies like in Jaramillo-Colorado B.E. et al. [313] and Matulyte I. et al. [314], which achieved successful compound separation under similar chromatographic conditions. Notably, our study focused on the analysis of thyme oil, a more commonplace essential oil, and we acknowledge the observed chromatographic issues. We recognize the need for adaptability in methodological choices for a more suitable application. On the contrary, employing a higher sample concentration of 20 µl/ml was essential in our method, given that a portion is directed towards the Flame Ionization Detector and the rest to the Mass Spectrometer. This approach guarantees optimal detection sensitivity, crucial for effectively capturing a broad range of compounds within the EO, ensuring maximum detection without data loss [167]. Furthermore, the concomitant use of a polar column (DB-HeavyWAX) along with a non-polar (HP-5MS) allows revealing overlapped signal peaks and thus improves the identification of the separated compounds. Hudaib et al. [315], who analysed the chemical profile of *T. vulgaris* EO by GC/MS using the same approach, demonstrated that the polar column helped to enhance the resolution between compounds co-eluting on a non-polar column such as the couples (α-thujene, α-pinene) or (sabinene, β-pinene). This has also been observed in our study with, for instance, the couple (thymol-carvacrol) or (*p*-cymene-limonene) in EO obtained from supplier A. Furthermore, as outlined by Fan et al. [316], although the main constituents of an EO are equally detected on both columns, a portion is exclusively identified by one or the other. In our investigation, we found that 23 out of 75 compounds were exclusively detected using HP-5MS, constituting an average of 5.82% of the compounds identified across the three samples. Additionally, DB-HeavyWAX revealed 10 compounds exclusively (averaging 1.88%). The difference in detection recorded would be the result of the different polarity and material of the columns used [223]. Overall, these results suggest that complementing a non-polar with a polar column provides a more precise picture of the EO analysed than if displayed individually.

7.3.2   *Headspace analysis of the EO vapours above a LM*

In this study, the sampling of the headspace above a mixture of *T. vulgaris* EO and MH broth has been carried out using two different methods of extraction – i.e., HS-SPME and HSGTS extractions. Headspace chemical compositions were measured every 3 h during a 12-h experiment using the HP-5MS column. Complete analyses are provided in Tables 5 and 6, as well as Figures 11 and 12. Using HS-SPME extraction, a total of 40, 38, and 43 volatile compounds were identified in the samples of suppliers A, B, and C, respectively. This represented 99.8, 99.8, and 99.6 % of their respective total constituents at 12 hrs. In contrast, a significantly lower number of compounds was detected when using the HS-GTS extraction method. While 32 constituents were found in EO sample C, only 26 components in samples A and B were found, which accounted for 99.8, 99.9, and 99.7% of their total contents at time 12 h. Regardless of the extraction method used and the three samples analysed, monoterpenoids followed by sesquiterpene hydrocarbons were by far the two most predominant chemical groups of volatile compounds identified in the headspace. Other chemical categories present in minor amounts (oxygenated phenylpropanoids and furanoids) were identified using HS-SPME extraction only. On the other hand, cyclic ethers such as furan derivatives were only present in samples obtained by HS-GTS extraction.

Using the HS-SPME extraction method, the most abundant volatile substance across all *T. vulgaris* was *p*-cymene. Its percentage values during the whole experiment were rather similar in the headspace of samples A, B, and C, ranging from 54.57 to 58.61%, 69.91 to 74.50%, and 58.38 to 67.21%, respectively. Likewise, the second most abundant compound was γ- terpinene. While in sample A the peak percentage area was between 19.74 and 20.45% during the 12 h experiment, these values were lower in both supplier C and B, ranging from 12.37 to 16.18% and 7.07 to 9.30%, respectively. Thymol was the third abounding compound in A, B, and C samples during the entire 12-h period, with percentage values ranging from 2.61 to 3.36%, 2.13 to 5.25%, and 3.72 to 5.27%, respectively. Similarly using HS-GTS extraction, *p*-cymene (47.05 to 50.73% for sample A, 52.28 to 57.41% for sample B, and 44.80 to 49.28% for sample C), as well as γ-terpinene (13.40 to 17.45%, 5.76 to 6.99%, and 9.54 to 11.85% for sample A, B, and C, respectively), were the first two most abundant compounds in the headspace for all EO samples and during the full experiment. However, the third volatile substance detected in a significant amount was α-pinene, with

percentage values over the 12-h experiment between 5.74 and 6.83%, 6.34 and 8.98%, and 6.90 and 7.96% for EO samples A, B, and C, respectively.

Other differences have been observed when comparing the two sampling methods. Firstly, the number of compounds detected using HS-SPME was higher than in HS-GTS extraction – i.e., on average, 41 versus 28 compounds. Then, the chemical analysis showed that when using HS-SPME extraction, a larger number of sesquiterpene hydrocarbons (14 components) were found in amounts lower than 3.09%, 1.59%, and 2.94% in EO samples A, B, and C, respectively. In contrast, using the HS-GTS method, only six compounds were found, including α-copaene, β-bourbonene, β-caryophyllene, γ-muurolene, γ-cadinene, and δ-cadinene, at amounts lower than 0.38% in sample A, 0.28% in sample B, and 0.58% in sample C. Similarly, percentage values of phenolic monoterpenes and derivatives were considerably higher using HS-SPME sampling method (overtime for samples A, B, and C, lower than 3.14%, 5.25%, and 5.27%, respectively) than HS-GTS extraction (values lower than 0.67%, 0.49%, and 0.58%, respectively). Eventually, despite the above-mentioned discrepancies, the headspace analysis of both sampling methods showed that there were no significant changes in the chemical composition in the vapour of the three EO samples of *T. vulgaris* over time.
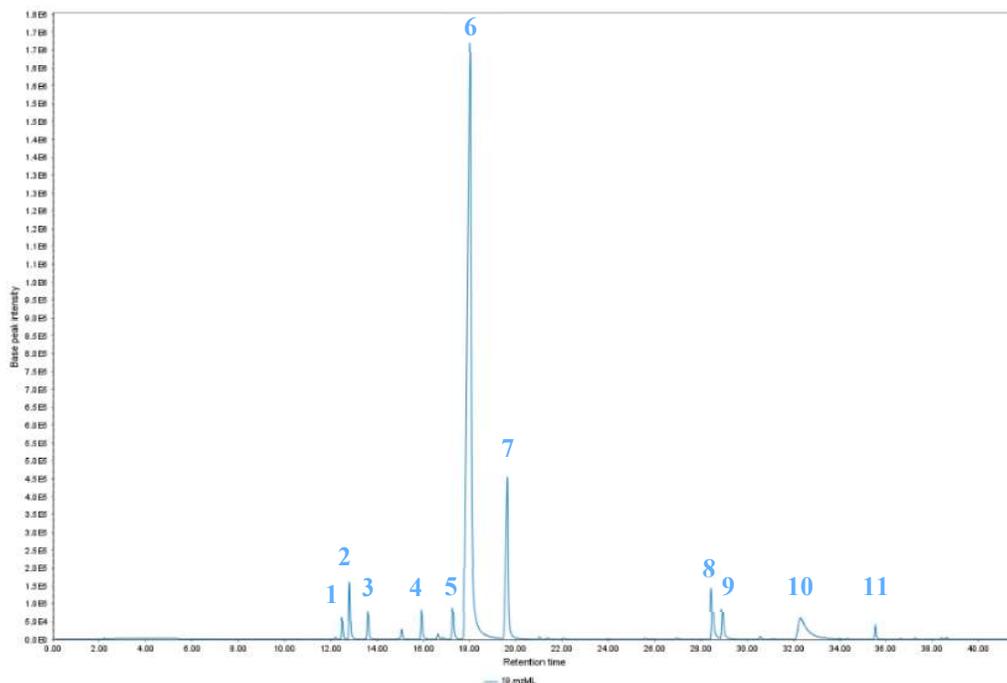
Table 4 Chemical composition of the headspace above a mixture of Mueller–Hinton broth and *Thymus vulgaris* essential oils at a concentration of 512 µg/mL over a 12-hour period using solid-phase microextraction sampling technique.

RI [a]	Lit.	Compounds	Cl. [b]	A					B					C [c]				
Obs.				0	3	6	9	12	0	3	6	9	12	0	3	6	9	12
921	921	Tricyclene	MH	0.05	0.05	0.05	0.05	0.05	tr. [e]	tr.	0.05	0.07	0.07	0.08 ± 0.02	0.08 ± 0.01	0.07 ± 0.02	0.10 ± 0.01	0.08 ± 0.02
927	924	α-Thujene	MH	0.95	0.91	0.83	0.84	0.93	0.97	0.79	0.98	1.10	1.07	1.01 ± 0.05	1.00 ± 0.03	0.93 ± 0.03	0.94 ± 0.13	0.94 ± 0.05
933	939	α-Pinene	MH	**2.37**	**2.25**	**2.18**	**2.11**	**2.37**	**1.97**	**1.60**	**2.23**	**2.45**	**2.41**	**2.73 ± 0.18**	**2.69 ± 0.13**	**2.57 ± 0.26**	**2.82 ± 0.30**	**2.70 ± 0.26**
949	945	Camphene	MH	1.47	1.42	1.37	1.33	1.51	0.85	0.83	1.58	1.69	1.67	1.58 ± 0.56	1.56 ± 0.53	1.52 ± 0.58	2.13 ± 0.16	1.82 ± 0.57
976	969	Sabinene	MH	tr.	tr.	-[f]	tr.	tr.	tr.	tr.	0.05	0.06	0.06	tr.	tr.	tr.	0.07 ± 0.00	0.06 ± 0.02
978	974	β-Pinene	MH	0.49	0.47	0.42	0.45	0.48	0.43	0.40	0.49	0.51	0.53	0.45 ± 0.01	0.49 ± 0.06	0.48 ± 0.07	0.56 ± 0.04	0.51 ± 0.06
991	988	3-Octanone	OA	tr.	tr.	-	tr.	tr.	-	-	tr.	tr.	tr.	-	-	-	-	-
995	988	β-Myrcene	MH	**2.76**	**2.70**	**2.63**	**2.48**	**2.72**	**1.53**	**1.45**	**1.79**	**1.88**	**1.88**	**1.62 ± 0.32**	**1.53 ± 0.18**	**1.51 ± 0.29**	**1.83 ± 0.03**	**1.62 ± 0.26**
1009	1002	α-Phellandrene	MH	0.36	0.35	0.34	0.33	0.35	0.19	0.19	0.20	0.20	0.20	0.26 ± 0.03	0.26 ± 0.02	0.25 ± 0.03	0.28 ± 0.01	0.24 ± 0.03
1013	1008	3-Carene	MH	0.21	0.21	0.21	0.20	0.22	0.09	0.08	0.13	0.14	0.14	0.11 ± 0.04	0.10 ± 0.03	0.10 ± 0.03	0.14 ± 0.01	0.12 ± 0.03
1021	1014	α-Terpinene	MH	**3.76**	**3.72**	**3.61**	**3.58**	**3.72**	1.61	1.58	**2.34**	**2.37**	**2.38**	**2.20 ± 0.66**	**2.17 ± 0.64**	**2.14 ± 0.65**	**2.92 ± 0.05**	**2.46 ± 0.64**
1036	1020	*p*-Cymene	AH	58.61	56.24	55.41	54.57	56.35	74.22	74.50	71.03	71.16	69.91	67.21 ± 7.03	66.38 ± 7.46	65.89 ± 6.38	58.38 ± 0.23	60.62 ± 6.81
1055	1023	*m*-Cymene	AH	tr.	0.05	0.05	tr.	tr.	-	-	-	-	-	-	-	-	tr.	tr.
1067	1054	γ-Terpinene	MH	**19.7**	20.45	20.30	20.23	20.18	7.07	7.23	9.30	9.18	9.17	12.37 ± 2.86	12.51 ± 2.64	12.67 ± 3.02	16.18 ± 0.16	14.14 ± 2.88
1094	1086	Terpinolene	MH	0.31	0.33	0.31	0.32	0.32	0.10	0.11	0.24	0.24	0.24	0.16 ± 0.10	0.16 ± 0.10	0.17 ± 0.10	0.27 ± 0.01	0.21 ± 0.09
1101	1089	*p*-Cymenene	MH	0.20	0.25	0.19	0.21	0.22	0.15	0.14	0.19	0.21	0.20	0.12 ± 0.06	0.10 ± 0.04	0.09 ± 0.02	0.14 ± 0.01	0.11 ± 0.03
1115	1095	Linalool	OM	0.27	0.26	0.25	0.26	0.33	0.13	0.10	0.28	0.34	0.34	0.23 ± 0.21	0.16 ± 0.12	0.16 ± 0.13	0.29 ± 0.03	0.19 ± 0.10
1153	1141	Camphor	OM	0.05	tr.	tr.	0.05	0.05	tr.	tr.	0.06	0.06	0.06	tr.	tr.	0.06 ± 0.04	0.09 ± 0.01	0.08 ± 0.04
1186	1165	Endo-borneol	OM	tr.	tr.	tr.	tr.	tr.	0.08	0.07	tr.	0.05	0.05	0.11 ± 0.02	0.09 ± 0.01	0.08 ± 0.01	0.07 ± 0.01	0.08 ± 0.01
1192	1174	Terpinen-4-ol	OM	0.06	0.08	0.08	0.10	0.10	tr.	tr.	0.08	0.08	0.09	tr.	tr.	0.06 ± 0.05	0.10 ± 0.01	0.09 ± 0.04
1213	1195	Estragole	OPP	tr.	tr.	tr.	tr.	tr.	-	-	-	-	tr.	0.10 ± 0.06	0.13 ± 0.09	0.11 ± 0.04	0.17 ± 0.03	0.18 ± 0.06
1247	1235	Thymol methyl ether	OPM	**1.78**	**2.48**	**2.68**	**2.82**	**2.44**	**3.05**	**3.77**	**2.68**	**2.24**	**2.58**	**1.83 ± 0.13**	**2.41 ± 0.17**	**2.77 ± 0.41**	**2.61 ± 0.17**	**2.91 ± 0.34**
1257	1241	Carvacrol methyl ether	OPM	**1.15**	**1.52**	**1.68**	**1.68**	**1.51**	**1.67**	**2.02**	**1.55**	**1.40**	**1.49**	**1.08 ± 0.12**	**1.37 ± 0.11**	**1.50 ± 0.14**	**1.54 ± 0.03**	**1.69 ± 0.11**
1293	1287	Bornyl acetate	OM	0.17	0.24	0.29	0.33	0.21	0.05	0.08	0.15	0.11	0.14	tr.	0.15 ± 0.08	0.15 ± 0.06	0.25 ± 0.04	0.24 ± 0.11
1306	1284	Anethol	OPP	tr.	tr.	tr.	0.05	tr.	-	-	-	-	-	tr.	tr.	0.08 ± 0.00	0.12 ± 0.02	0.12 ± 0.03
1339	1290	Thymol	PM	**2.61**	**2.80**	**3.14**	**3.36**	**3.12**	**5.25**	**3.50**	**2.13**	**2.64**	**3.15**	**5.27 ± 1.27**	**4.57 ± 0.81**	**4.62 ± 0.84**	**3.72 ± 0.07**	**5.05 ± 0.45**
1368	1298	Carvacrol	PM	tr.	tr.	tr.	tr.	tr.	-	-	-	-	-	-	tr.	tr.	tr.	tr.
1381	1372	*p*-Cymen-7-ol	OM	tr.	tr.	tr.	tr.	tr.	-	-	tr.	tr.	tr.	-	-	-	tr.	tr.

| RI [a] | | Compounds | Cl. [b] | Supplier /Time (h)/Peak area (%) | | | | | | | | | | | | | | |
| Obs. | Lit. | | | A | | | | | B | | | | | C [c] | | | | |
				0	3	6	9	12	0	3	6	9	12	0	3	6	9	12
1386	1374	α-Copaene	SH	0.09	0.13	0.17	0.19	0.12	tr.	tr.	0.11	0.07	0.11	tr.	tr.	0.07 ± 0.08	0.16 ± 0.01	0.19 ± 0.05
1395	1387	β-Bourbonene	SH	tr.	tr.	0.05	0.07	tr.	tr.	tr.	tr.	tr.	0.05	tr.	tr.	tr.	0.09 ± 0.01	0.10 ± 0.01
1434	1418	β-Caryophyllene	SH	1.59	**2.14**	**2.74**	**3.09**	1.94	0.26	0.32	1.59	1.13	1.49	0.77 ± 0.82	1.19 ± 1.26	1.28 ± 1.29	**2.94 ± 0.32**	**2.40 ± 1.59**
1442	1446 d	isogermacrene D	SH	tr.	0.06	0.09	tr.	tr.	-	-	tr.	tr.	tr.	-	tr.	tr.	0.08 ± 0.06	tr.
1469	1452	α-Humulene	SH	tr.	tr.	0.07	0.07	tr.	-	tr.	tr.	tr.	tr.	tr.	tr.	tr.	0.07 ± 0.01	0.06 ± 0.03
1482	1475	Geranyl propionate	OM	tr.	tr.	tr.	tr.	-	-	-	-	-	-	-	-	-	-	tr.
1491	1478	γ-Muurolene	SH	0.06	0.08	0.12	0.14	0.07	tr.	tr.	0.06	tr.	0.06	tr.	0.07 ± 0.04	0.08 ± 0.05	0.12 ± 0.01	0.11 ± 0.05
1509	1480	Germacrene D	SH	tr.	tr.	tr.	tr.	-	-	-	tr.	-	-	-	-	-	tr.	tr.
1512	1491	Valencene	SH	-	tr.	tr.	tr.	-	-	-	-	-	-	-	-	-	tr.	tr.
1515	1499	α-Muurolene	SH	tr.	tr.	tr.	tr.	tr.	-	-	tr.	tr.	tr.	-	-	-	tr.	tr.
1520	1509	β-Bisabolene	SH	tr.	tr.	tr.	tr.	tr.	-	-	tr.	-	tr.	-	-	-	tr.	tr.
1532	1513	γ-Cadinene	SH	0.08	0.08	0.12	0.14	0.07	tr.	tr.	0.07	tr.	0.07	tr.	0.10 ± 0.04	0.08 ± 0.03	0.12 ± 0.02	0.12 ± 0.05
1538	1524	δ-Cadinene	SH	0.1	0.1	0.15	0.17	0.09	tr.	tr.	0.05	tr.	0.05	tr.	0.07 ± 0.04	0.08 ± 0.04	0.12 ± 0.02	0.11 ± 0.05
1541	1521	Calamenene	SH	tr.	tr.	-	0.07	tr.	tr.	0.05	0.05	0.03	0.05	tr.	0.07 ± 0.01	0.06 ± 0.01	0.07 ± 0.01	0.07 ± 0.01
1605	1581	Caryophyllene oxide	OS	tr.	tr.	tr.	tr.	tr.	-	-	-	tr.	tr.	-	-	tr.	tr.	tr.
		Total identified (%)		99.8	99.8	99.9	99.8	99.9	99.9	99.2	99.7	99.6	99.9	99.3	99.9	99.9	99.7	99.6

[a] RI = retention indices; Obs. = retention indices determined relative to a homologous series of *n*-alkanes (C$_8$-C$_{40}$) on an HP-5MS column; Lit. = literature Ri values [311,312]; [b] Cl = class; AH — aromatic hydrocarbon, MH — monoterpene hydrocarbon, OA — oxygenated aliphatic, OM — oxygenated monoterpene, OPM — oxygenated phenolic monoterpene, OPP — oxygenated phenylpropanoid, PM — phenolic monoterpene, OS — oxygenated sesquiterpene, SH — sesquiterpene hydrocarbon; [c] Relative peak area percentage as mean of three measurements ± standard deviation; [d] Literature RI values from [27]; [e] tr. = traces, relative peak area < 0.05%; [f] - = not detected.

Figure 12 GC chromatograms of the headspace above a mixture of Mueller–Hinton broth and *Thymus vulgaris* EO (512 µg/mL) from supplier C at 12-hour time marks using solid-phase microextraction (a) and gas-tight syringe (b) sampling techniques.

a.

b.
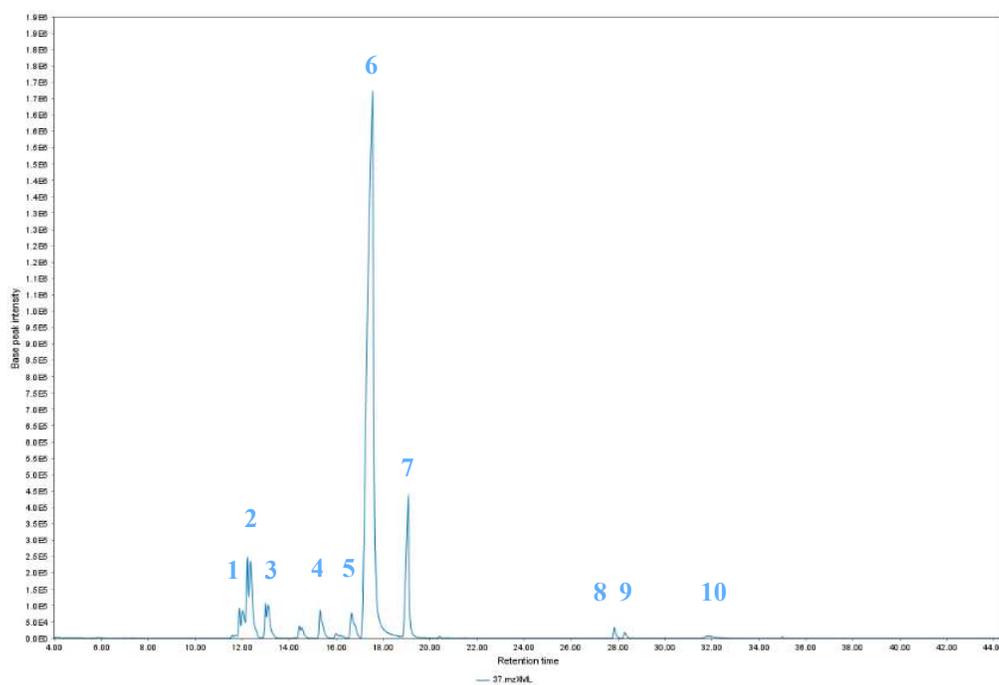
1. α-thujene, 2. α-pinene, 3. camphene, 4. β-myrcene, 5. α-terpinene, 6. *p*-cymene, 7. γ-terpinene, 8. thymol methyl ether, 9. carvacrol methyl ether, 10. thymol, 11. β-caryophyllene

Table 5 Chemical composition of the headspace above a mixture of Mueller–Hinton broth and *Thymus vulgaris* EO (512 µg/mL) over a 12-hour period using gas tight syringe headspace sampling technique.

RI [a] Obs.	Lit.	Compounds	Cl. [b]	A 0	3	6	9	12	B 0	3	6	9	12	C [c] 0	3	6	9	12
-	NA	2-ethyl furan	F	tr. [d]	tr.	tr.	- [e]	tr.	0.09	0.09	0.09	0.07	0.04	0.09 ± 0.05	0.07 ± 0.01	0.08 ± 0.04	0.08 ± 0.02	0.07 ± 0.00
778	NA	methyl α-methylbutyrate	OA	0.08	0.10	0.13	0.08	0.12	0.22	0.17	0.23	0.16	0.11	0.10 ± 0.07	0.08 ± 0.02	0.10 ± 0.04	0.07 ± 0.01	0.09 ± 0.00
912	921	tricyclene	MH	0.11	0.20	0.16	0.12	0.19	0.17	0.15	0.06	0.20	0.18	0.19 ± 0.01	0.17 ± 0.02	0.19 ± 0.01	0.20 ± 0.04	0.24 ± 0.00
918	924	α-thujene	MH	**2.01**	**2.17**	**2.03**	**1.75**	**2.23**	**2.09**	**2.03**	**2.11**	**2.38**	**2.34**	**1.97 ± 0.24**	**1.89 ± 0.07**	**1.95 ± 0.15**	**1.91 ± 0.17**	**2.30 ± 0.00**
924	939	α-pinene	MH	**6.19**	**5.70**	**6.83**	**4.63**	**5.74**	**8.98**	**7.75**	**7.84**	**6.79**	**6.34**	**7.96 ± 2.05**	**6.94 ± 1.11**	**6.90 ± 1.73**	**7.20 ± 0.33**	**7.11 ± 0.00**
939	945	camphene	MH	**3.40**	**3.21**	**3.57**	**2.69**	**3.28**	**4.59**	**4.04**	**4.07**	**3.81**	**3.71**	**4.59 ± 0.91**	**4.22 ± 0.35**	**4.16 ± 0.76**	**4.24 ± 0.03**	**4.45 ± 0.00**
953	969	sabinene	MH	-	-	-	0.07	-	-	-	-	-	-	tr.	tr.	0.05 ± 0.02	0.13 ± 0.19	0.06 ± 0.00
967	980	β-pinene	MH	0.91	0.83	0.85	0.78	0.87	1.07	0.88	0.99	0.89	0.81	0.75 ± 0.37	0.94 ± 0.04	0.91 ± 0.11	0.74 ± 0.25	0.81 ± 0.00
984	988	β-myrcene	MH	**2.88**	**2.80**	**3.14**	**3.13**	**2.88**	**1.88**	**1.89**	**1.98**	**1.90**	**2.06**	**1.82 ± 0.06**	**1.96 ± 0.24**	**1.88 ± 0.08**	**1.63 ± 0.15**	**2.09 ± 0.00**
998	1002	α-phellandrene	MH	0.31	0.35	0.23	0.20	0.35	0.05	0.11	0.11	0.10	0.07	0.13 ± 0.05	0.23 ± 0.06	0.20 ± 0.10	0.19 ± 0.03	0.22 ± 0.00
1002	1008	3-carene	MH	0.28	0.25	0.36	0.13	0.26	0.30	0.12	0.11	0.08	0.05	0.14 ± 0.10	0.10 ± 0.05	0.13 ± 0.08	0.13 ± 0.05	0.16 ± 0.00
1010	1014	α-terpinene	MH	**3.64**	**3.57**	**2.57**	**4.17**	**3.80**	**2.21**	**2.06**	**1.93**	**2.12**	**2.34**	**2.57 ± 0.18**	**2.87 ± 0.50**	**2.77 ± 0.33**	**2.41 ± 0.18**	**2.88 ± 0.00**
1028	1020	*p*-cymene	AH	**45.93**	**47.05**	**47.48**	**50.73**	**48.78**	**52.28**	**54.29**	**56.81**	**57.30**	**57.41**	**44.80 ± 1.03**	**49.19 ± 2.67**	**49.20 ± 3.00**	**46.10 ± 2.10**	**49.28 ± 0.00**
1044	1023	*m*-cymene	AH	tr.	tr.	0.27	0.28	tr.	-	-	-	-	-	0.07 ± 0.07	0.09 ± 0.06	tr.	0.06 ± 0.08	tr.
1058	1054	γ-terpinene	MH	**13.40**	**13.90**	**15.46**	**17.54**	**14.80**	**5.76**	**6.22**	**6.58**	**6.69**	**6.99**	**9.54 ± 1.00**	**11.77 ± 1.52**	**11.82 ± 1.70**	**10.32 ± 0.68**	**11.85 ± 0.00**
1083	1086	terpinolene	MH	0.18	0.19	0.23	0.26	0.20	0.10	0.12	0.14	0.14	0.14	0.13 ± 0.02	0.18 ± 0.04	0.18 ± 0.03	0.14 ± 0.02	0.17 ± 0.00
1089	1089	*p*-cymenene	MH	0.10	0.09	0.13	0.14	0.09	0.08	0.09	0.10	0.09	0.09	0.06 ± 0.00	0.08 ± 0.01	0.07 ± 0.01	0.06 ± 0.02	0.06 ± 0.00
1103	1095	linalool	OM	0.09	0.08	0.15	0.16	0.10	0.08	0.06	0.12	0.11	0.10	0.09 ± 0.03	0.09 ± 0.03	0.09 ± 0.01	0.06 ± 0.03	0.08 ± 0.00
1133	1141	camphor	OM	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.
1171	NA	2-ethyl-5-methylfuran	F	tr.	tr.	tr.	tr.	tr.	tr.	-	tr.	-	tr.	tr.	tr.	tr.	tr.	tr.
1180	1174	terpinen-4-ol	OM	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.
1200	1195	estragole	OPP	-	-	tr.	tr.	-	-	-	-	-	-	tr.	tr.	tr.	tr.	tr.
1234	1235	thymol methyl ether	OPM	0.34	0.34	0.62	0.67	0.43	0.24	0.39	0.45	0.43	0.49	0.31 ± 0.12	0.53 ± 0.15	0.57 ± 0.12	0.43 ± 0.08	0.50 ± 0.14
1244	1241	carvacrol methyl ether	OPM	0.21	0.19	0.38	0.40	0.25	0.13	0.23	0.25	0.25	0.28	0.19 ± 0.08	0.30 ± 0.09	0.33 ± 0.08	0.24 ± 0.05	0.29 ± 0.08

RI [a]		Compounds	Cl. [b]	Supplier /Time (h)/Peak area (%)														
				A					B					C [c]				
Obs.	Lit.			0	3	6	9	12	0	3	6	9	12	0	3	6	9	12
1281	1287	bornyl acetate	OM	tr.	tr.	0.05	0.05	tr.	-	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.	tr.
1328	1290	thymol	PM	0.50	0.27	0.58	0.60	0.28	0.25	0.10	0.28	0.26	0.22	0.46 ± 0.31	0.51 ± 0.36	0.55 ± 0.30	0.25 ± 0.10	0.58 ± 0.37
1372	1374	α-copaene	SH	tr.	tr.	tr.	tr.	tr.	-	tr.	tr.	-	tr.	tr.	tr.	tr.	tr.	tr.
1381	1387	β-bourbonene	SH	-	-	tr.	tr.	-	-	-	-	-	-	-	tr.	tr.	-	tr.
1417	1418	β-caryophyllene	SH	0.17	0.17	0.38	0.34	0.19	0.07	0.11	0.14	0.13	0.15	0.21 ± 0.11	0.37 ± 0.15	0.41 ± 0.00	0.28 ± 0.08	0.39 ± 0.20
1475	1478	γ-muurolene	SH	-	-	tr.	tr.	-	-	-	-	-	tr.	tr.	tr.	tr.	tr.	tr.
1513	1513	γ-cadinene	SH	-	-	tr.	tr.	-	-	-	-	-	-	-	tr.	tr.	-	tr.
1521	1524	δ-cadinene	SH	-	-	tr.	tr.	-	-	-	-	-	-	-	tr.	tr.	-	tr.
		Total identified (%)		99.9	99.8	99.9	99.9	99.9	99.9	99.8	99.9	99.9	99.7	99.87	99.8	99.9	99.9	99.8

[a] RI = retention indices; Obs. = retention indices determined relative to a homologous series of *n*-alkanes (C_8–C_{40}) on an HP-5MS column; Lit. = literature Ri values [311,312];
[b] Cl = Class; AH — aromatic hydrocarbon, MH — monoterpene hydrocarbon, OA — oxygenated aliphatic, OM — oxygenated monoterpene, OPM — oxygenated phenolic monoterpene, OPP — oxygenated phenylpropanoid, PM — phenolic monoterpene, OS — oxygenated sesquiterpene, SH — sesquiterpene hydrocarbon; [c] Relative peak area percentage as mean of three measurements ± standard deviation; [d] tr. = traces, relative peak area < 0.05%; [e] - = not detected.

In recent years, HS-SPME has become the preferred laboratory method for identifying EOs volatile compounds: it is a simple, fast, cost-effective, selective, and sensitive method that provides high-quality results [193,221,317]. With the aim to simulate the conditions of the antimicrobial susceptibility testing experiments performed in this study, the EO samples were prepared identically to the most active EO during the BMV assay (i.e., incubation temperature was at 37 °C and EOs dissolved in Mueller–Hinton (MH) broth medium concentrated at 512 µg/mL). In addition, we used a mixed coating material – divinylbenzene/carboxen/polydimehylsiloxane (DVB/CAR/PDMS) – that gives better extraction yields for both polar and non-polar volatile constituents than simple fibre coatings [192,318]. Furthermore, we considered the incubation times to ensure a comprehensive understanding of the volatile analyte dynamics. Following Cui et al. [287] findings, which highlighted variances in equilibration times based on compound volatility, we implemented incubation periods ranging from 0 to 12 hours. This decision was influenced by the rapid equilibration (10–20 minutes) observed for highly volatile compounds, in contrast to the extended time (>60 minutes) required for less volatile ones. Concerning the time of exposure of the SPME fibre with the headspace, our approach was guided by the methodology of Vihanova et al.[288], who conducted headspace analysis above *Cinnamomum cassia* EO utilizing a DVB/CAR/PDMS SPME fibre and exposure time of 15 minutes. This choice was further substantiated by various other studies, such as Piasenzotto et al. [319] examination of thymol in honey, where a 20-minute exposure time was deemed optimal. Similarly, Prates et al. [320], investigating eugenol as a pest control agent, determined an effective exposure time of 15 minutes using the same SPME fiber coating.

As a result, our investigation revealed that although *p*-cymene and γ-terpinene were abundant in the headspace, the amount of thymol extracted by the coated fibre was unusually low – peak percentage area lower than 5.27% across the three EO samples (Figure 11 and 12). This observation is in contradiction with what was described in previously published research. For instance, Lugo-Estrada et al. [192], Soleimani et al. [321] , and Nezhadali et al. [310], who investigated *T. vulgaris* EO vapour composition, all reported that thymol was the most abundant constituent of the headspace (peak percentage area of 34.28%, 28.50%, and 45.45%, respectively). As described by Adam et al. [318], efficient extractions of EO volatile compounds depend on optimised experimental parameters such as the selection of the fibre coating material and the incubation temperature of the EO sample. The lack of thymol could, therefore, be explained by

several reasons. Firstly, it is the selectivity and sensitivity of the DVB/CAR/PDMS coating. Although it proved to be the most universal assembly for the isolation of compounds with diverse physico-chemical properties [322], Soleimani et al. [321] demonstrated in their comparative study that phenolic monoterpenes and, more particularly, thymol, were better extracted by a simple PDMS fibre than using mixed coating materials. Furthermore, as the transfer rate of volatile agents toward the fibre increases with the incubation temperature of the sample [322], the temperature chosen could have potentially limited the extraction of thymol in our investigation. For example, in their research work, Nezhadali et al. [310] compared the HS-SPME extraction efficiency of *T. vulgaris* EO main volatile agents at 25 °C and 50 °C using a water-based matrix. The result showed that the amount of thymol at higher temperatures is almost twice as high as at lower temperatures (73.09% and 45.45%, respectively). This suggests that different experimental conditions using the HS-SPME technique can yield different distributions of EO volatile compounds, and therefore, the result may not necessarily illustrate the actual chemical composition of the headspace above the EO samples.

In contrast, the HS-GTS technique provided a different perspective on the constituency of the headspace, perhaps closer to the real distribution of volatile compounds in the vial at equilibrium [323]. Despite this, the results showed a peak percentage area for thymol lower than 0.60% across the three EO samples – even below the levels observed with the HS-SPME method. Using identical experimental conditions with both methods has demonstrated unusually low concentration levels of thymol. The possible explanation may therefore lie in the matrix in which the EO was inserted. As previously mentioned, the hydrophobic nature of volatile compounds worsens their solubility in water-based media (here the MH broth medium), which may reduce their dilution capability and result in an unequal distribution throughout the medium, something that may also affect their distribution into the well's atmosphere [163]. The HS-GTS method also yielded interesting results with other components; for instance, the amount of α-pinene obtained with HS-GTS sampling (peak percentage area lower than 7.96% across the three EO samples) was at least three times higher than the amount extracted with HS-SPME – peak percentage area lower than 2.82%. Despite a lack of academic studies examining *T. vulgaris* EO using HS-GTS, these findings are consistent with the data available in other publications. Coleman et al. [322], for instance, compared the distribution of volatile constituents in the headspace above a sample of *Juniperus virginiana* EO using both HS-SPME and HS-GTS techniques. He observed that α-pinene

dominated the headspace with 88.0% of the total composition when using HS-GTS, whereas it only exhibited 32.4% with the HS-SPME technique. According to the author, this difference may be explained by the sorption behaviour of α-pinene over time when using a coated fibre assembly. In fact, he demonstrated that the percentage composition of volatile compounds changes significantly with the fibre exposure time, with α-pinene levels dramatically descending with greater time exposure, while high molecular weight components increased with the sampling time. Eventually, it's crucial to acknowledge that headspace analysis, along with the associated sampling techniques, stands as a pioneering analytical method for EO vapour analysis. Consequently, additional factors related to our methodology may have influenced the chemical profile of the vapour from our T. vulgaris EO samples. Specifically, variations in temperature at the time of sampling or even the temperature of the sampling tools, such as the syringe body, could have played a role in shaping the observed results.

When comparing the chemical profiles of *T. vulgaris* EO in the liquid phase with the headspace analyses, we observed significant differences between both phases. The EO liquid phase contained a greater number of substances with a variable distribution, whereas the headspace composition detected a smaller number of compounds represented mostly by highly volatile substances. This phenomenon is in accordance with what was already described in previous research [323]. Nevertheless, the three EO samples showed identical concentrations of antimicrobial activities during BMV experiments in both liquid and vapour phases. This raises the question about the concentrations of active volatile compounds in the vapour, and more particularly thymol as its main antimicrobial constituent [214]. As a phenolic compound, thymol is very stable, moderately soluble in water and of low volatility, and was detected in a lower amount in the headspace – i.e., peak area percentage lower than 5.27% (HS-SPME) and 0.60% (HS-GTS) – than in the sample of EO diluted in the broth (max. 48.65%). In contrast, α-pinene, which is highly volatile and extremely insoluble in water, followed the opposite trend (peak area percentage lower than 2.82% (HS-SPME), 7.84% (HS-GTS), and 0.62% in the sample of EO diluted in the broth). This was observed in previous studies and is explained by the difference in volatility: when the EO is introduced into a closed environment, volatile compounds start to diffuse at different rates according to their molecular weight until they reach equilibrium [163]. Despite its slower diffusion rate, we could possibly argue that the low levels of thymol detected in the headspace was sufficient to generate the same antimicrobial activity as its amount in the EO concentrated at 512 μg/mL.

Although the correlation between the concentration of essential oil and thymol were not the subject of this study, the results strongly suggest that the effect of antimicrobial constituents of *T. vulgaris* EO (e.g., of thymol) is higher in vapour than in the liquid phase. To further support this hypothesis, an additional investigation focused on thymol behaviour in vapour would be needed. While the analysis of thymol's evaporation provides valuable insights on EO vapour behaviour, it also raises questions about the limitations of using a water-based medium for their characterization, leading us to contemplate how an alternative solid matrix, like a biopolymer, could potentially impact the chemical composition of *T. vulgaris* EO vapours.

7.3.3   *Headspace analysis of the EO vapours above a SM*

In recent investigations, attention has been directed on cellulose-based material for incorporating EO. These matrices already showed promising results, particularly in the food industry where researchers sought to impregnate cellulose, cellulose-acetate or chitosan matrices with EOs such as *Citrus bergamia*, *Citrus limon* and *Melaleuca alternifolia,* with aim to develop industrial application for elimination of microbial pathogens in food products as described by El Fawad et al. [324], Niu et al. [325], or Casalini and Baschetti [326]. Building on this trend, we utilized a cellulose paper disc (SM) impregnated with a mixture of MH broth and *T. vulgaris* EO sourced from supplier C. Complete overtime analysis for both techniques is provided in Table 7 as well as in Figure 13. Using HS-SPME, a total of 45 volatile compounds were identified in the headspace across the various time intervals. The maximum volatile organic compounds detection was observed at both the initial time (t = 0h) and 9-hour incubation mark, with 43 compounds identified, constituting 99.3% and 99.8% of the total volatile constituents, respectively. In contrast, at 12-hour time, the analysis revealed a reduced number of 39 volatile components, which nonetheless accounted for 99.9% of the overall headspace content. Alternatively, employing HS-GTS extraction technique, a cumulative total of 23 volatile organic compounds were found over the different time marks. At time 0 hours, the headspace analysis revealed a minimum of 16 volatile constituents, accounting for 99.2% of the total volatile composition, whereas a maximum was observed at the 12-hour incubation period, with 22 compounds identified representing 98.9% of the headspace constituents.

In HS-SPME method, oxygenated monoterpenes emerged as the most prevalent chemical group, followed by mono and sesquiterpene hydrocarbons (15, 13 and 10 compounds, respectively). Furthermore, the most dominant compound in *T. vulgaris* EO sample was undeniably thymol. Throughout the entire experimental duration, its peak area fluctuated from 29.89 % at 9 hours to 43.76% at 0 hour. Another compound of significance was p-cymene, which exhibited a percentage area between 23.09% at 0 hour and 37.72 % at 9 hours. Likewise, γ-terpinene and linalool, two other key compounds, exhibited values ranging from 2.23% to 7.12% and 3.53% to 5.60%, respectively, across the examined time frame. In addition, γ-terpinene displayed a slightly higher average peak percentage – i.e., 4.58 vs 4.48 % – making it marginally more predominant than linalool.

As regard of HS-GTS technique, monoterpene hydrocarbons were slightly more abundant than oxygenated monoterpenes in the headspace with 9 and 7 compounds, respectively. As opposed to HS-SPME, sesquiterpenes hydrocarbons were notably absent except for β-caryophyllene. Similarly, p-cymene emerged as the primary constituent, with peak percentages comprised between 56.65 % at 0 hours to 72.45 % at 3 hours. γ-terpinene ranked as the second most abundant VC, with peak area percentage in between 4.06 % at 0 hours and 8.84 % at 3 hours. A noteworthy distinction using this extraction technique was the lower prevalence of both thymol and linalool, which exhibited percentage values ranging from 2.42 % to 4.87 % and 0.83 % to 2.43 %, respectively, over the course of the experiment.

Ultimately, besides the greater number of compounds detected in HS-SPME – 42 versus 23 – and the complete absence of sesquiterpenes when using HS-GTS, further significant differences were observed between the two sampling methods. For instance, the chemical analysis revealed that the presence of oxygenated compounds, including aliphatic alcohols, oxygenated mono- and sesquiterpenoids, as well as phenylpropanoids, was less pronounced in the headspace when using HS-GTS compared to HS-SPME. Certain compounds such as 2-hexenal, sabinene hydrate, endo-borneol, isomenthol, estragole, α-terpineol, carvone, carvacrol, and p-cymen-7-ol were entirely undetected, while the other oxygenated VCs, including 1-octen-3-ol, 3-octanol, linalool, camphor, terpinen-4-ol, thymol, its methyl ether derivative, and carvacrol methyl ether, exhibited significantly lower levels of presence compared to HS-SPME.

Table 6 Chemical composition of the headspace above a solid matrix impregnated with a mixture Mueller-Hinton broth and *T. vulgaris* EO (supplier C) at a concentration of 512 µg/mL over a 12-hour period using solid-phase microextraction and gas tight syringe and sampling techniques.

RI[a]		Compounds	C[b]	Extraction method /Time (h)/Content (%)									
				HS-SPME					HS-GTS				
Obs	Lit			0	3	6	9	12	0	3	6	9	12
757	783	methyl α-methylbutanoate	OA	0.32 ± 0.17[f]	0.15 ± 0.09	0.18 ± 0.14	0.11 ± 0.04	0.54 ± 0.42	0.57 ± 0.50	0.26 ± 0.09	0.13 ± 0.23	0.30 ± 0.03	0.27 ± 0.08
842	832[d]	2-hexenal	OA	0.34 ± 0.13	0.12 ± 0.05	0.08 ± 0.00	0.08 ± 0.01	tr.[g]	-[h]	-	-	-	-
918	921	tricyclene	MH	0.14 ± 0.03	0.13 ± 0.11	0.19 ± 0.06	0.24 ± 0.04	0.11 ± 0.04	0.11 ± 0.09	0.21 ± 0.05	0.14 ± 0.16	0.15 ± 0.01	0.32 ± 0.10
924	924	α-thujene	MH	0.31 ± 0.27	1.08 ± 0.53	0.73 ± 0.46	1.12 ± 0.18	0.55 ± 0.16	0.94 ± 0.08	1.50 ± 0.31	1.19 ± 1.40	1.10 ± 0.12	2.85 ± 1.25
939	939	α-pinene	MH	0.32 ± 0.22	0.97 ± 0.49	0.64 ± 0.40	0.98 ± 0.13	0.46 ± 0.13	0.25 ± 0.21	1.22 ± 0.27	0.26 ± 0.20	0.92 ± 0.10	1.09 ± 0.05
945	945	camphene	MH	tr.	tr.	tr.	0.05 ± 0.00	-	-	-	-	-	-
965	969	sabinene	MH	0.07 ± 0.01	0.05 ± 0.07	tr.	tr.	tr.	-	-	-	-	0.22 ± 0.19
967	974	β-pinene	MH	tr.	0.18 ± 0.18	0.09 ± 0.13	0.15 ± 0.06	tr.	0.06 ± 0.05	0.17 ± 0.05	0.12 ± 0.15	0.11 ± 0.01	0.43 ± 0.23
980	978	1-octen-3-ol	OA	**3.10 ± 1.14**	-	1.61 ± 1.46	0.78 ± 0.58	3.64 ± 1.63	0.13 ± 0.12	-	0.33 ± 0.57	0.37 ± 0.19	0.32 ± 0.12
984	988	β-myrcene	MH	0.60 ± 0.52	1.47 ± 0.62	1.03 ± 0.18	1.16 ± 0.15	0.39 ± 0.54	0.11 ± 0.10	0.29 ± 0.37	0.43 ± 0.18	0.36 ± 0.02	0.52 ± 0.10
994	996[c]	3-octanol	OA	0.46 ± 0.13	0.17 ± 0.23	0.23 ± 0.33	-	0.50 ± 0.36	-	0.19 ± 0.33	0.10 ± 0.17	-	-
998	1002	α-phellandrene	MH	0.10 ± 0.09	-	0.20 ± 0.05	0.12 ± 0.01	0.07 ± 0.02	-	-	-	-	-
1010	1004	3-carene	MH	0.59 ± 0.25	0.48 ± 0.56	0.61 ± 0.04	0.74 ± 0.13	0.59 ± 0.25	0.41 ± 0.03	1.46 ± 0.34	0.36 ± 0.38	1.12 ± 0.02	1.22 ± 0.03
1026	1020	p-cymene	AH	**23.09 ± 6.16**	**36.54 ± 6.21**	**28.55 ± 8.10**	**37.72 ± 1.78**	**28.80 ± 6.17**	**56.65 ± 0.96**	**72.45 ± 8.34**	**54.63 ± 13.92**	**63.53 ± 0.95**	**57.34 ± 4.87**
1054	1054	γ-terpinene	MH	**2.55 ± 1.23**	**6.28 ± 2.50**	**4.72 ± 2.73**	**7.12 ± 0.83**	**2.23 ± 1.52**	**4.06 ± 0.21**	**8.84 ± 1.26**	**4.89 ± 2.14**	**7.37 ± 0.30**	**6.63 ± 0.27**
1062	1068	cis-sabinene hydrate	OM	0.35 ± 0.08	0.16 ± 0.03	0.30 ± 0.04	0.32 ± 0.02	0.20 ± 0.21	-	-	-	-	-
1065	1068	trans-sabinene hydrate	OM	0.12 ± 0.00	tr.	-	-	0.12 ± 0.05	-	-	-	-	-
1080	1086	terpinolene	MH	0.10 ± 0.04	0.18 ± 0.04	0.15 ± 0.05	0.19 ± 0.00	0.08 ± 0.04	-	0.09 ± 0.03	tr.	0.05 ± 0.05	tr.
1083	1089	p-cymenene	MH	0.14 ± 0.05	0.24 ± 0.12	0.20 ± 0.11	0.24 ± 0.03	0.06 ± 0.08	-	tr.	-	-	-
1100	1095	linalool	OM	**5.51 ± 1.12**	**3.53 ± 1.12**	**4.36 ± 1.87**	**3.42 ± 0.48**	**5.60 ± 0.29**	0.97 ± 0.31	0.83 ± 0.41	2.43 ± 1.59	1.23 ± 0.55	1.07 ± 0.31
1137	1041	(+)-camphor	OM	0.79 ± 0.10	0.39 ± 0.21	0.45 ± 0.24	0.39 ± 0.07	0.51 ± 0.23	0.33 ± 0.29	0.29 ± 0.27	0.27 ± 0.35	0.35 ± 0.16	0.16 ± 0.26
1165	1165	endo-Borneol	OM	**3.16 ± 0.91**	**1.85 ± 0.48**	**1.95 ± 1.06**	**1.32 ± 0.11**	**3.23 ± 0.19**	-	-	-	-	-
1171	NA	2-ethyl-5-methylfuran	F	-	-	-	-	-	0.17 ± 0.14	0.14 ± 0.33	0.33 ± 0.28	0.28 ± 0.28	0.28 ± 0.30
1173	1174	terpinen-4-ol	OM	1.74 ± 0.33	1.31 ± 0.11	1.28 ± 0.33	1.01 ± 0.16	1.57 ± 0.25	-	0.14 ± 0.02	0.37 ± 0.32	0.15 ± 0.06	0.20 ± 0.11

RI[a]		Compounds	C[e]	Extraction method /Time (h)/Content (%)									
				HS-SPME					HS-GTS				
Obs	Lit			0	3	6	9	12	0	3	6	9	12
1180	1192 b	isomenthol	OM	0.32 ± 0.29	-	0.17 ± 0.24	0.06 ± 0.08	0.65 ± 0.05	-	-	-	-	-
1192	1195	estragole	OPP	0.15 ± 0.12	0.17 ± 0.17	0.08 ± 0.11	0.12 ± 0.05	-	-	-	-	-	-
1198	1189	α-terpineol	OM	0.14 ± 0.05	0.09 ± 0.01	0.10 ± 0.01	-	0.11 ± 0.01	-	-	-	-	-
1230	1235	thymol methyl ether	OPM	**2.45 ± 0.99**	**3.19 ± 1.00**	**2.53 ± 1.48**	**3.69 ± 0.43**	**1.85 ± 0.33**	0.58 ± 0.01	1.29 ± 0.10	1.22 ± 0.85	1.29 ± 0.46	1.18 ± 0.05
1239	1241	carvacrol methyl ether	OPM	1.64 ± 0.58	2.03 ± 0.71	1.78 ± 1.05	2.48 ± 0.25	1.22 ± 0.28	0.35 ± 0.07	0.72 ± 0.05	0.69 ± 0.55	0.63 ± 0.26	0.59 ± 0.06
1264	1242	(+)-carvone	OM	0.14 ± 0.05	0.10 ± 0.06	0.15 ± 0.01	0.13 ± 0.05	0.18 ± 0.04	-	-	-	-	-
1277	1287	bornyl acetate	OM	tr.	-	-	-	-	-	-	0.07 ± 0.06	0.05 ± 0.05	0.10 ± 0.01
1314	1290	thymol	PM	**43.76 ± 9.08**	**31.91 ± 8.49**	**42.12 ± 12.22**	**29.89 ± 2.48**	**43.17 ± 3.58**	**2.42 ± 2.01**	**2.60 ± 2.02**	**4.87 ± 3.81**	**2.84 ± 1.79**	**3.68 ± 2.65**
1363	1298	carvacrol	PM	0.75 ± 0.53	0.50 ± 0.45	0.90 ± 0.62	0.60 ± 0.20	0.75 ± 0.95	-	-	-	-	-
1376	1372	p-cymen-7-ol	OM	0.16 ± 0.14	0.29 ± 0.13	0.21 ± 0.20	0.14 ± 0.10	-	-	-	-	-	-
1379	1387	β-bourbonene	SH	-	0.08 ± 0.11	-	tr.	-	-	-	-	-	-
1416	1418	β-caryophyllene	SH	**2.99 ± 1.29**	**2.60 ± 1.40**	**2.28 ± 1.68**	**3.17 ± 0.60**	**1.20 ± 1.07**	-	0.39 ± 0.33	0.69 ± 0.59	0.83 ± 0.35	1.04 ± 0.15
1420	1455	aromandendrene	SH	0.08 ± 0.02	0.08 ± 0.02	tr.	0.09 ± 0.06	0.06 ± 0.08	-	-	-	-	-
1446	1452	α-humulene	SH	0.11 ± 0.05	tr.	0.06 ± 0.08	0.11 ± 0.01	tr.	-	-	-	-	-
1468	1478	γ-muurolene	SH	0.41 ± 0.14	0.25 ± 0.16	0.24 ± 0.20	0.33 ± 0.04	0.15 ± 0.11	-	-	-	-	-
1485	1491	valencene	SH	0.10 ± 0.03	0.07 ± 0.04	0.05 ± 0.07	0.08 ± 0.01	tr.	-	-	-	-	-
1488	1499	α-muurolene	SH	0.10 ± 0.04	0.07 ± 0.04	0.06 ± 0.08	0.09 ± 0.01	tr.	-	-	-	-	-
1496	1509	β-bisabolene	SH	tr.	-	tr.	tr.	0.16 ± 0.09	-	-	-	-	-
1505	1513	γ-cadinene	SH	0.38 ± 0.12	0.24 ± 0.11	0.27 ± 0.18	0.31 ± 0.04	0.20 ± 0.14	-	-	-	-	-
1513	1524	δ-cadinene	SH	0.54 ± 0.16	0.35 ± 0.13	0.38 ± 0.23	0.44 ± 0.04	-	-	-	-	-	-
1576	1581	caryophyllene oxide	OS	0.05 ± 0.03	tr.	0.08 ± 0.03	0.06 ± 0.02	0.09 ± 0.05	-	-	-	-	-
1634	1645	δ-cadinol	OS	-	tr.	tr.	tr.	tr.	-	-	-	-	-
		Total identified (%)		99.3	99.5	99.8	99.8	99.9	99.2	99.9	99.8	99.1	98.9

[a]RI = retention indices; Obs = retention indices determined relative to a homologous series of *n*-alkanes (C$_8$-C$_{40}$) on a HP-5MS column; Lit = literature RI values [311,312], [b] Sawamura et al. [327], [c]Oliveira et al. [328], [d]Mahmood et al. [329]; [e]C = Class ; AH – aromatic hydrocarbon, MH – monoterpene hydrocarbon, OA – oxygenated aliphatic, OM – oxygenated monoterpene, OPM – oxygenated phenolic monoterpene, OPP – oxygen-ated phenylpropanoid, PM – phenolic monoterpene, OS – oxygenated; [f] relative peak area percentage as mean of three measurements ± deviation standard; [g] tr. = traces, relative peak area < 0.05%; [h] - = not detected.
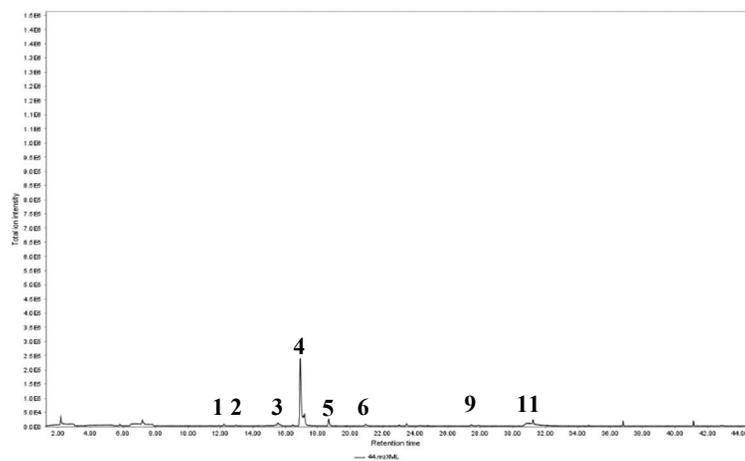
Figure 13 GC chromatogram of the headspace above a solid matrix impregnated with a mixture Mueller-Hinton broth and *T. vulgaris* EO (supplier C) at a concentration of 512 µg/mL using solid phase microextraction (a and b) and gas tight syringe (c and d) sampling techniques at 0-hour (a and c) and 12-hour incubation marks (b. and d.)
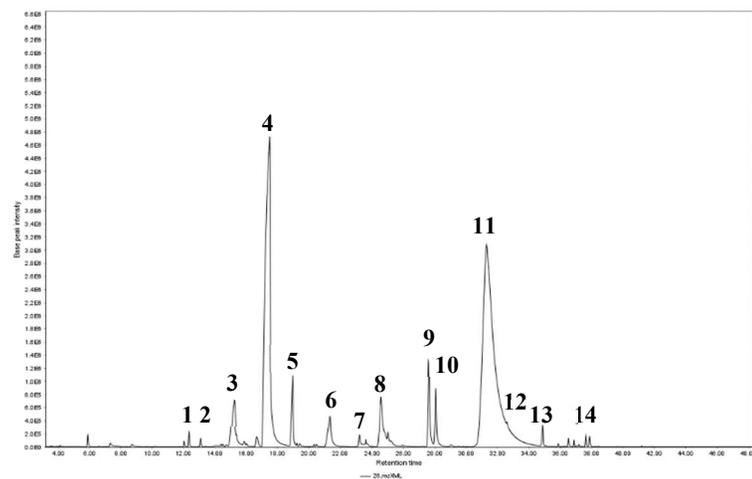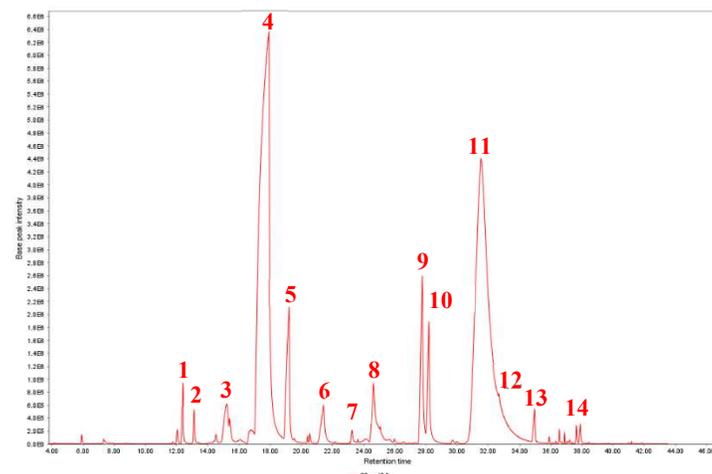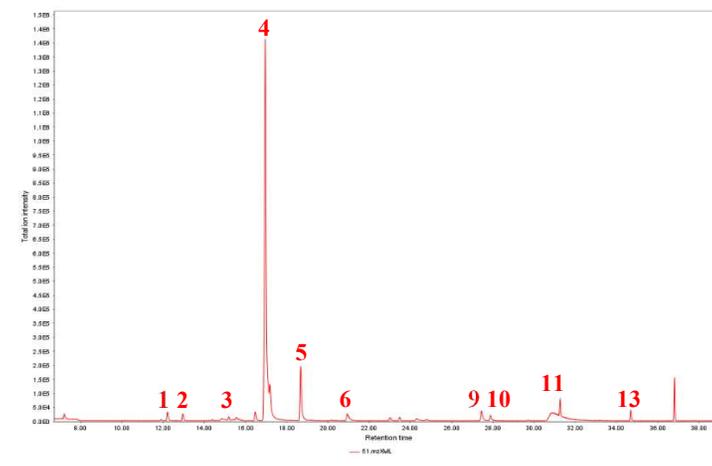

1. α-thujene, **2.** α-pinene, **3.** 1-octen-3-ol, **4.** *p*-cymene, **5.** γ-terpinene, **6.** linalool, **7.** endo-borneol, **8.** terpinen-4-ol, **9.** thymol methyl ether, **10.** carvacrol methyl ether, **11.** thymol, **12.** carvacrol, **13.** β-caryophyllene, **14.** γ- and δ-cadinene

7.3.4 *The effect of volatilization matrix on chemical composition of EO vapours*

Subsequently, to discuss and compare the influence of both LM and SM on the chemical profile of *T. vulgaris* EO vapours, the data for supplier C – originally presented in Tables 5 to 7 – were converted into original heatmaps. These visual representations are depicted in Figures 14 and 15 and allow for better interpretation of the overall results.

Firstly, employing HS-SPME sampling, we observed an average detection of 43 compounds in the headspace above the SM, as opposed to 41 above the liquid one. Likewise, 23 compounds were identified above the SM compared to 26 above the LM using HS-GTS. Both results indicated no substantial differences in compound count between the two matrices.

To continue with HS-SPME sampling technique, findings suggest that oxygenated monoterpenoids were detected in a significantly higher occurrence in the headspace above the SM, in contrast to the LM. For instance, phenolic compounds such as thymol have seen its abundance increased almost 10-fold passing on average from 4.7 % in the LM to 38.2 % in the SM. Such observations align more closely with the above-mentioned studies by Soleimani et al. [321], and Nezhadali et al. [310] who both examined the composition of *T. vulgaris* EO vapour above fine aerial parts powder, comparable to a cellulose SM. Both studies consistently identified thymol as the dominant constituent in the headspace, accounting for 28.50 % and 45.45 %, respectively. Similarly, less prevalent VCs like linalool, borneol, and terpinene-4-ol, which registered only 0.3%, 0.05%, and 0.1% with the LM, exhibited greater prominence with the SM, with peak areas averaging 4.5%, 2.3%, and 1.4% over the 12-hour experimental period. In addition, providing a more accurate representation of the actual VC distribution in the vial as discussed in the previous section, the use of HS-GTS technique confirmed the observed trend. The abundance of thymol, for example, augmented more than five-fold from 0.5 % above the LM to 3.3 % above the SM on average. Likewise, over the 12-hour experimental period, peak percentage areas of linalool and terpinene-4-ol averaged from 0.1 % and 0.05 % above the LM to 1.3 % and 0.2 % above the SM, respectively.

Although specific research comparing EO chemical profiles in the headspace above both LM and SM is scarce in the existing scientific literature, few studies concentrated on the interaction of individual EO volatile compounds with biopolymers like cellulose. Our findings, however, are in contradiction with what was previously described. For instance, Ouédraogo et al. [330] examined

how different type of cellulose-based matrices affected the release of monoterpenoids, including carvone, terpinene-4-ol and α-pinene. They demonstrated that more polar compounds like terpinen-4-ol and carvone were effectively more retained on cellulose than α-pinene, which is a substance of lower polarity. Wicochea-Rodríguez et al. [331] also reported a similar tendency when investigating the evaporation of carvacrol, and limonene on diverse biopolymers including cellulose, starch, pectin and chitosan. As described by Kurek et al. [332], upon adsorption onto a polysaccharidic matrix, volatile compounds polarity play a significant role in their release into the headspace. Cellulose possesses two secondary and one primary alcohol functions per repeating unit, making it a considerably polar macromolecule, proficient to create strong hydrogen bonds with VCs oxygenated functional groups. As a result of this highly hydrophilic nature, it was expected that polar compounds such as thymol, carvacrol, or linalool would show equally low abundance in the headspace above both the SM and LM. Besides the interactions between volatile compounds and the fibre coating material as mentioned in section 6.2.2, other factors could have had a strong influence in the release or retention of these oxygenated substances above the SM, explaining discrepancies in results. First and foremost, it's crucial to recognize that the behaviour of our sample may significantly deviate from that of pure essential oil. Instead, our sample would resemble to an oil-in-water microemulsion, where *T. vulgaris* EO forms droplets inside the MH mixture with the aid of DMSO, a weak surfactant. Described by Pajnik et al. [333] in their study on the evaporation of thymol in distilled water impregnated into several polysaccharidic materials, water molecules swiftly permeated the biopolymer matrix's backbone structure. This process potentially disassembled the water molecule network that had formed around the EO droplets, leading to their breakdown and, therefore, to the increased release of thymol. Similarly, Fernández-Pan et al. [334], reported that a chitosan matrix impregnated with carvacrol-in-water emulsion exhibited a more efficient release of volatile compounds into the headspace compared to the same matrix loaded with carvacrol in olive oil. Cellulose, as an amphiphilic substance, possesses the ability to provide some degree of stability to emulsions by creating a protective physical barrier around absorbed droplets [335]. However, this does not provide an explanation for the observed release of carvacrol into the headspace. The authors suggested that water molecules play a role in weakening the surrounding polymer architecture, which in turn facilitates the release of the VC. In contrast, the unexpectedly low release rate using olive oil was ascribed to its limited penetration into the chitosan matrix, which maintained a closed structure and thus constrained carvacrol

evaporation. Furthermore, using similar experimental conditions, Kurek et al. [332] not only observed this phenomenon but also provided further insights. They proposed that the enhanced release of oxygenated compounds from a polymer matrix would be directly linked to higher levels of humidity and temperature within the vial. In their study, they demonstrated a significant increase in carvacrol evaporation as humidity rose from 0 % to 96 %, and temperature increased from 4°C to 37°C within vials. These conditions would amplify the interaction between the polymer and water, consequently improving the release of the oxygenated volatile compound.

The abundance of the major non-polar volatile compounds, on the other hand, experienced a significant drop in the headspace above the SM regardless of the sampling technique used. With HS-SPME, for example, p-cymene's average peak area percentages were divided twofold, from 63.7 % in the headspace above the LM to 30.9% above the SM during the experimental period. Similarly, the prevalence of other monoterpene hydrocarbons including γ-terpinene, α-pinene, camphene, and, β-myrcene, halved when transitioning from LM to the SM – i.e., from 13.6 %, 2.7 %, 1.7 %, 1.6 % to 4.6 %, 0.6 %, 0.05 %, and 0.9 %, respectively. Another example is α-terpinene which was absent in the headspace above the SM while its average peak area percentage reached 2.4 % above the LM. Regarding HS-GTS sampling technique, apart from p-cymene, which increased in prevalence in the headspace above the SM (60.9%) compared to the LM (47.7%), the abundance's trend of less common VCs confirmed our observations when transitioning from LM to SM. α-Pinene decreased notably, averaging from 7.2 % to 0.7 %, β-myrcene decreased from 1.8 % to 0.3 %, and γ-terpinene decreased from 11.1% to 6.4%. Additionally, compounds like camphene and α-terpinene were completely absent in the headspace above the SM over the 12-hour experimental period.

Despite the limited number of studies addressing the interactions of monoterpene hydrocarbons with carbohydrate polymers such as cellulose, our findings align with previous research in this area. Several potential explanations including the molecular size and diffusion characteristics could provide insights into these results. In their study, Bertolini et al. [336] compared the retention of encapsulated β-pinene, β-myrcene, and limonene on gum arabic. They observed that after being absorbed into a polymer, larger hydrocarbons exhibited reduced diffusion, causing a delay in their migration towards the surface of the matrix. Initially, the authors suggested that the compound with the smallest molar volume would be the least retained. However, this hypothesis did not align with their results, as β-pinene, with a lower molar volume was more retained than β-myrcene and

limonene. They then proposed that the molecular diameter might be the primary factor influencing diffusion through the matrix. Consequently, compounds with smaller molecular diameters, such as β-pinene, tend to diffuse more rapidly.

Yet, this theory doesn't completely elucidate our observations on the increasing abundance of *p*-cymene in the headspace. As highlighted by Turek and Stintzing [170] in their review on EO stability, terpenoids, due to their similar chemical structures, readily convert into one another through processes like oxidation, isomerization, cyclization, or dehydrogenation reactions when exposed to elements like oxygen, light, or elevated temperatures. Moreover, Masotti et al. [337] stability study demonstrated that the decline in hydrocarbon monoterpenes concentration, coincide with an increase in the levels of oxidized metabolic derivatives. This phenomenon is further elucidated by studies such as Poulose and Croteau [338] and Misharina et al. [339], which provided strong evidence regarding the conversion mechanism of γ-terpinene to *p*-cymene and thymol. Specifically, the biosynthetic pathway responsible for the synthesis of thymol involves the aromatization of γ-terpinene, ultimately leading to the formation of p-cymene. Subsequently, thymol is synthesized through the hydroxylation of p-cymene [340]. While our findings in the headspace above the SM do show an increase in thymol and *p*-cymene, as well as a slight decrease in γ-terpinene, offering partial validation for this theory, it is important to recognize that this explanation alone may not entirely account for the observed phenomena.

That is why, to gain a thorough understanding of the processes occurring in the headspace, rigorous calibration studies are imperative. This involves quantifying the main VCs using either internal or external standards and creating calibration curves within the appropriate concentration range [341]. This would not only ensure accurate quantification of biologically active EO components, particularly crucial in pharmaceutical applications, but also would adhere to strict norms set by international regulatory bodies, establishing a robust foundation for reliable analysis in this critical field [342].

Ultimately, this investigation constitutes an initial exploration into headspace analysis in essential oil vapour, highlighting the need for refining techniques to ensure precision and reliability of results. In addressing challenges related to the optimization of both HS-SPME and HS-GTS sampling techniques, key issues include the non-uniform heating of both vial and sampling device after incubation as well as difficulties in sustaining equilibration during vial sampling. In response, a proactive strategy is actively pursued to address these challenges and enhance the overall

efficiency and reliability of both methodologies. For HS-GTS, for instance, the examination of preheating the syringe is underway, aiming to mitigate issues like micro-condensation in the body syringe and potential compound loss. Furthermore, ongoing work involves the assessment of the impact of a reduced sampling volume (1.5mL versus 2.5mL) on the vapour chemical profile. With regard to HS-SPME, the enhancement of adsorption onto the SPME fibre can potentially be achieved by maintaining a consistent post-incubation temperature of 37°C. This can be facilitated through the utilization of an external dry bath block heater during the sampling process. These methodological considerations are essential for advancing the reliability and precision of the research outcomes as the data collected in this study, primarily theoretical, could serve as a basis for practical applications. By adjusting the broth microdilution volatilization method, we could translate this knowledge for the development of a new methods designed to assess the antimicrobial activity of EO vapour inserted on a SM. This will help us maximize the benefits of EO vapour in combatting respiratory infections.

Figure 14 Heatmap comparing the headspace chemical compositions of *T. vulgaris* EO (supplier C) in both liquid and solid matrix using solid phase microextraction sampling technique.
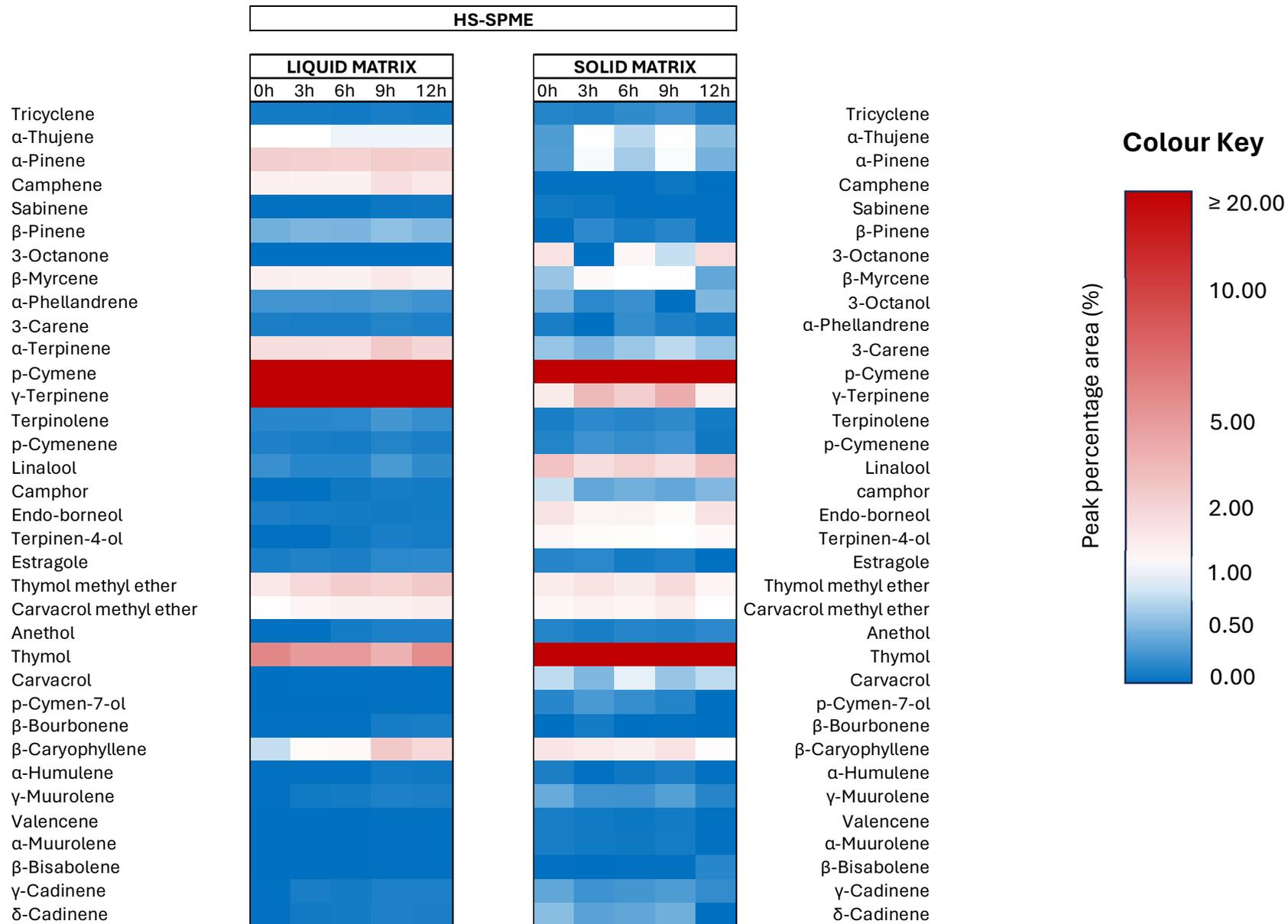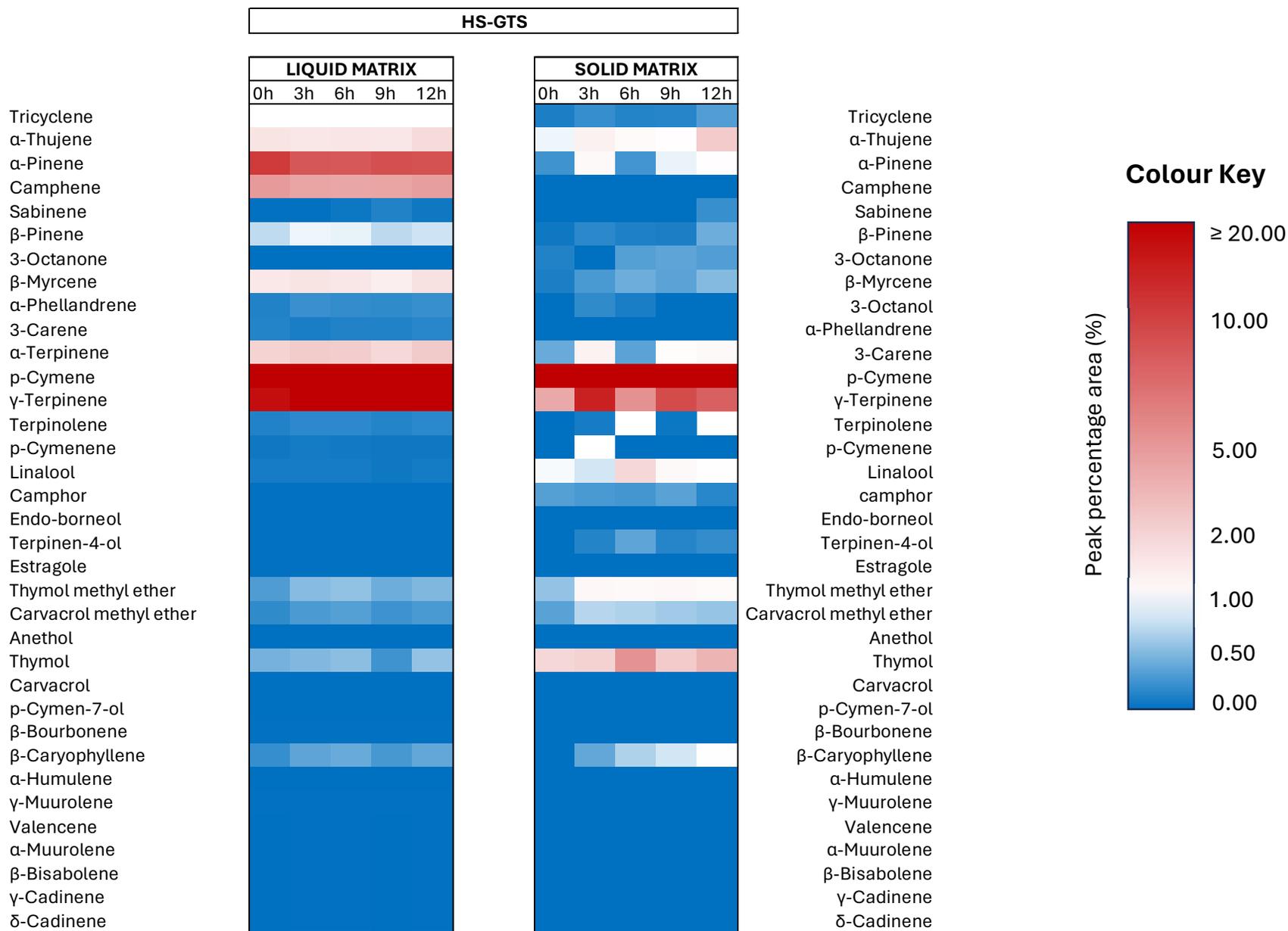
Figure 15 Heatmap comparing the headspace chemical compositions of *T. vulgaris* EO (supplier C) in both in both liquid and solid matrix using gas tight syringe sampling technique.

8.   Conclusion

In this study, we report the antibacterial activity of five EOs recommended by the EMA against cough and common cold in children under 5 years of age. The EOs obtained from three commercial samples of *E. globulus*, *F. vulgare, M.* x *piperita*, *P. anisum* and *T. vulgaris* were assessed using broth microdilution volatilization method against three standard bacterial strains associated with respiratory infections, namely *H. influenzae*, *S. aureus*, and *S. pyogenes*. The results showed that, out of the 5 selected EOs, only the three commercially obtained samples of *T. vulgaris* EO exhibited significant antimicrobial activity against the studied respiratory pathogens in both liquid and vapour phase. Similarly, while all bacterial strains exhibited some level of sensitivity to thyme EO vapours, Gram-negative strain *H. influenzae* displayed the highest susceptibility. Overall, these findings provide experimental background for the development of a novel therapeutic method in treating respiratory infections using EO vapour phase as an alternative to conventional antibiotics, opening new avenues for research and application in paediatric healthcare. Moreover, while our results sheds light on the promising benefits of *T. vulgaris* EO against respiratory pathogens when tested *in vitro*, it is imperative to acknowledge that further comprehensive studies, particularly clinical aromatherapy trials, are necessary to thoroughly evaluate the efficacy, tolerability, and toxicity of EO vapour in treating respiratory tract diseases such as pneumonia in children under five. Currently, clinical aromatherapy trials remain scarce, and those available have certain limitations such as small sample sizes and too short treatment durations, making challenging the safety evaluation of EO vapours.

The following objective of this study was to evaluate the effectiveness of a dual column/dual detector GC-MS system in characterizing the chemical composition of *T. vulgaris* EO. Our findings confirmed a thymol chemotype with major constituents identified as monoterpenoids, including thymol, *p*-cymene, γ-terpinene, and carvacrol. The utilization of a GC-MS equipped with both a non-polar HP-5MS and a semi-polar DB-HeavyWAX columns played a crucial role in improving resolution of analyses. This dual-column approach facilitated a more precise separation of volatile compounds, leading to a more detailed identification of constituents. Additionally, the use of a dual detector system, comprising a FID for quantification and a MS for molecular weight determination, further reinforce our ability to accurately characterize *T. vulgaris* EOs' chemical

profiles. While this study successfully shows the effectiveness of the dual column/dual detector GC-MS system in comprehensive EO analysis, future exploration of stationary phases with even greater polarity differences may yield even more comprehensive results. We believe that this enhanced understanding of EO composition will contribute to the deeper understanding of chemistry of EOs recommended for treatment of respiratory infections. Apart from microbiology assays and traditional GC chemical analysis, an important achievement of this study was the comparison of headspace analyses using both SPME and GTS sampling techniques for *T. vulgaris* EO vapour. Our findings revealed a significant shift in major volatile constituents. Surprisingly, thymol was found in lower abundance, whereas *p*-cymene, γ-terpinene, and α-pinene emerged as the predominant compounds in the headspace. Furthermore, when considering which of the two sampling techniques might be more advantageous for the chemical analysis of EO vapours, it is evident that both methods complement each other and are mutually dependent: HS-SPME, under optimized experimental conditions, may yield more precise results in terms of qualitative aspects, whereas HS-GTS could offer more accurate data reflecting the actual headspace distribution of the EO volatile agents, providing valuable information on the components actually responsible for the antimicrobial activity associated with EO vapours.

As far as the influence of the matrix on *T. vulgaris* EO vapour composition, regardless of the sampling technique used, the outcome of this study showed significant disparities in the headspaces when comparing chemical profiles above both LM and SM. While oxygenated monoterpenoids, such as thymol, carvacrol, and linalool, were significantly more prevalent in the headspace above the SM compared to the LM, hydrocarbon monoterpenes – except for *p*-cymene – experienced a decrease in abundance during this transition. These observed differences in abundance highlight the critical role of the matrix choice in shaping the chemical profile of the EO vapour, and consequently, its antimicrobial efficacy. As a result, these findings call for further research to investigate the antimicrobial efficacy of EO vapour when inserted on a SM. For instance, this could involve the development of new methods utilizing and adapting the broth microdilution volatilization.

Eventually, to the best of our knowledge, this study represents the first attempt to compare the vapour profile of *T. vulgaris* EO using two distinct sampling techniques and matrices. Our research not only contributes to understanding the mechanisms governing the release of volatile compounds

into the headspace and their interactions with chosen matrices but also paves the way for further exploration of the degradation reactions among terpenoids in vapour.

In the future, our work will focus on tackling the challenge of gaining control over the stability and release of EO volatile substances in vapor. By adjusting the broth microdilution volatilization method, we aim to develop new methods designed to assess the antimicrobial activity of EO vapour when inserted a SM. This is crucial for the design of a potential antimicrobial vapour inhalation device. Furthermore, our next tasks not only involve the antimicrobial properties of essential oil vapour phase, but also understand how volatile compounds evaporate and interact with each other on a chosen SM. Therefore, our future research will focus on quantifying the primary VC using either internal or external standards and establishing calibration curves within the appropriate concentration range to acquire a comprehensive insight of the processes occurring in the headspace. This approach will lay a robust foundation for reliable analysis in this critical field as well as it holds the promise of yielding invaluable insights and potentially leading to innovative patents and advancements in pharmaceutical product development. Ultimately, it is imperative that we persist in our efforts to explore and expand our understanding in EO vapour analytical chemistry to better comprehend the therapeutic potential of EO in respiratory care.

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10. APPENDIX

CURRICULUM

Julien Alexandre ANTIH
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EXPERIENCES

2023-2024   Graduate research and teaching assistant 2

Montpellier   *Faculté de Pharmacie, Université de Montpellier*
Laboratoire de Pharmacognosie, Phyto-aromathérapie
Pôle Recherche chimie Balard – IBMM – UMR 5247 – équipe SLB

2022-2023   Graduate research and teaching assistant 1

Paris   *Faculté de Pharmacie, Université Paris-Cité*
Laboratoire *CiTCoM – UMR CNRS 8038 – équipe PNAS*

2015-2018   Drug Master File Specialist

Prague   *Zentiva Group as. – Sanofi Generics*

Evaluation of API related files (ASMF, CEP and TP) for all Sanofi Generics production sites, preparation of technical documents for launching API registrations

2012-2014   Assitant pharmacist

Paris   *Community pharmacies*

Management of teams composed of technicians and other assistant pharmacists, delivering medical prescriptions, management of narcotic drugs registry, and sales.

EDUCATION

2018   **Ph.D candidate – Tropical Agrobiology & Bioresource Management**

Prague   *Czech University of Life Sciences - Faculty of Tropical AgriSciences.*
Laboratory of Ethnobotany and Ethnopharmacology
Supervisor: Pr. L. KOKOSKA.

Ph. D thesis: « *Chemical composition and in vitro antibacterial effects of vapours of essential oils from plants recommended by the European Medicines Agency against cough and cold* »

2014-2015   Master in Research – Drug Discovery

Londres   *University College London – School of Pharmacy*
Centre for Pharmacognosy and Phytotherapy
Supervisor: **Pr. M. HEINRICH**.

Internship: *Universitat de Barcelona – Facultad de Farmacia y Ciencias de la Alimentacion*
Department of Pharmacology, Toxicology and *Therapeutical Chemistry*
Supervisor **Pr. S. CAÑIGUERAL**.

Master thesis: « *Ethnopharmacopoeia and health-seeking strategies of Bolivian community in the Metropolitan Region of Barcelona* »

Degree obtained with Distinction

2006-2012   Diplome de Docteur en Pharmacie

Nancy   *Université de Lorraine – Faculté de Pharmacie*

Professional thesis: « *Medicinal flora of Latin America in European medicine: literature review of a selection of plants described between the 16th and 18th centuries.* »

Degree obtained with mention Très Bien

LIST OF PUBLICATIONS

PUBLICATIONS

P1 – **<u>Antih J.</u>**, Cañigueral S., Heinrich M., Use of medicinal plants by the Bolivian community in the metropolitan region of Barcelona, Revista de Fitoterapia 2015 16(2):141-152.

P2 – **<u>Antih J.</u>**, Houdkova M., Urbanova K., Kokoska L., Antibacterial activity of *Thymus vulgaris* L. essential oil vapours and their GC/MS Analysis using solid phase microextraction and syringe headspace sampling techniques, Molecules 2021, 26(21): 6553.

P3 – Chaure A., Houdkova M., **<u>Antih J.</u>**, Urbanova K., Doskocil I., Kokoska L., Validation of Broth Macrodilution Volatilization Method for Testing of Essential Oils in Liquid and Vapor Phase: Chemical Composition, Cytotoxicity, and Antibacterial Effect of Indian Medicinal Plants against Pneumonia-Causing Pathogens, Molecules 2023, 28(12): 4625.

ORAL COMMUNICATIONS

OC1 – **<u>Antih J.</u>**, Houdkova M., Urbanova K., Kokoska L. Antibacterial activity of essential oil-bearing herbs in vapour phase against respiratory pathogens and headspace analysis optimization of *Thymus vulgaris* sample. 70th International Congress and Annual Meetings of the Society for Medicinal Plant and Natural Product Research, GA 2022 (poster).

OC2 – **<u>Antih J.</u>**, Houdkova M., Urbanova K., Kokoska L., Antimicrobial activity of *Thymus vulgaris* L. essential oil in vapour phase and its headspace GC/MS analysis. ISEO 2021: International Symposium on essential oils (poster).

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