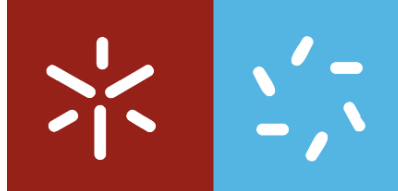


Universidade do Minho

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Cátia Isabel Barbosa Sampaio

**Production and characterization of
nanoencapsulated essential oils: study of its
stability and antimicrobial activity**



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Dissertação de Mestrado

Mestrado em Técnicas de Caracterização e Análise
Química

Trabalho efectuado sob a orientação de

Doutora Alice Maria Esteves Dias

e do

Doutor Miguel Ângelo Ribeiro Cerqueira

outubro de 2019

DIREITOS DE AUTOR E CONDIÇÕES DE UTILIZAÇÃO DO TRABALHO POR TERCEIROS

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AGRADECIMENTOS

Penso que, em qualquer área de trabalho, as relações que temos com os outros são determinantes para o nosso próprio sucesso, uma vez que são as partilhas e ajudas que nos fazem crescer tanto como pessoas como profissionalmente. Por isso, quero aqui aproveitar para agradecer a todos os que tornaram possível este trabalho e que de algum modo me acompanharam e ajudaram nesta fase importante do meu percurso académico e científico.

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STATEMENT OF INTEGRITY

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Produção e caracterização de óleos essenciais nanoencapsulados: estudo da sua estabilidade e atividade antimicrobiana

RESUMO

Atualmente, os consumidores têm-se mostrado mais preocupados com a sua saúde e sustentabilidade e alertados para as consequências do uso de químicos sintéticos. Devido a esta crescente consciencialização, os óleos essenciais têm vindo a ganhar notoriedade como potenciais agentes antimicrobianos na conservação de alimentos. Contudo, os óleos essenciais são bastante instáveis e necessitam de um sistema que os proteja, tal como as nanoemulsões.

O trabalho desenvolvido nesta tese resultou de uma colaboração da *Earth Essences*, uma empresa de produção de plantas aromáticas e medicinais na Póvoa de Lanhoso, com o Departamento de Química da Universidade do Minho e com o *Food Processing Group* do *International Iberian Nanotechnology Laboratory*. Os objetivos principais deste trabalho consistiram em caracterizar quimicamente óleos essenciais de tomilho e cidreira, produzir nanoemulsões por métodos de baixa energia para a sua encapsulação e avaliar a sua estabilidade e atividade antimicrobiana. Com o auxílio de técnicas complementares como cromatografia gasosa acoplada a espetrometria de massa, ressonância magnética nuclear e espectroscopia de infravermelho com transformada de Fourier, foi observado que o óleo essencial de tomilho apresentava três constituintes maioritários, *p*-cimeno, timol e γ -terpineno, enquanto que o óleo de cidreira era essencialmente composto por β -cariofileno. Foi mostrada a degradação dos óleos quando submetidos a um procedimento de alta energia e a importância de usar métodos de baixa energia para a sua encapsulação. De uma série de agentes emulsionantes avaliados, o Tween 80 mostrou ser o único capaz de obter nanoemulsões pelo método de baixa energia. As formulações com uma razão surfactante-óleo igual a 2, revelaram ser estáveis por vários meses quando armazenadas em refrigeração. Nanoemulsões com óleo de tomilho apresentaram elevada atividade antioxidante, que mostrou estar relacionada com a concentração do óleo. Já as nanoemulsões com óleo essencial de cidreira não exibiram atividade antioxidante significativa, contudo observou-se que a sua encapsulação potenciou esta propriedade. Formulações com ambos os óleos essenciais foram eficazes nos ensaios antimicrobianos em *S. aureus*, mas não contra *E. coli* e revelaram ser tóxicas em células Caco-2 a partir de 100 $\mu\text{g/mL}$. Assim, este trabalho permitiu desenvolver um sistema reprodutível e estável, capaz de encapsular os óleos essenciais e cujas formulações mostraram ter um elevado potencial para ser usadas no desenvolvimento de novos produtos na indústria alimentar.

Palavras chave: Baixa energia Cidreira Nanoemulsões Óleo essencial Tomilho

Production and characterization of nanoencapsulated essential oils: study of its stability and antimicrobial activity

ABSTRACT

Consumers are now more concerned about their health and sustainability and are more alerted to the consequences of using synthetic chemicals. Due to this growing awareness, essential oils have gained notoriety and are considered potential antimicrobial agents in food preservation. However, essential oils are quite unstable and require a system to protect them, such as nanoemulsions.

The work developed in this thesis resulted from a collaboration of Earth Essences, a company that produces aromatic and medicinal plants in Póvoa de Lanhoso, with the Department of Chemistry of the University of Minho and with the Food Processing Group of the International Iberian Nanotechnology Laboratory. The main objectives of this work were to chemically characterize thyme and lemon balm essential oils, to produce nanoemulsions by low energy methods for their encapsulation and to evaluate their stability and antimicrobial activity. With the aid of complementary techniques such as gas chromatography coupled to mass spectrometry, nuclear magnetic resonance and Fourier transform infrared spectroscopy, it was observed that thyme essential oil had three major constituents, *p*-cymene, thymol and γ -terpinene, while lemon balm oil was essentially composed of β -caryophyllene. It was shown the degradation of oils when subjected to a high energy procedure and therefore the importance of using low energy encapsulation methods. Among several emulsifying agents evaluated, Tween 80 showed to be the only one capable of obtaining nanoemulsions by low energy methods. Formulations with a surfactant-to-oil ratio of 2 have been found to be stable for several months when stored under refrigeration. Nanoemulsions with thyme oil presented high antioxidant activity, which was shown to be related to oil concentration. On the contrary, nanoemulsions with lemon balm essential oil did not show significant antioxidant activity, however it was observed that their encapsulation increased this property. Formulations with both essential oils were effective in antimicrobial assays in *S. aureus* but not against *E. coli* and were found to be toxic in Caco-2 cells for 100 $\mu\text{g/mL}$ and higher concentrations. Thus, this work allowed to develop a reproducible and stable system, capable of encapsulating essential oils and whose formulations have shown to have high potential to be used in the development of new products in food industry.

Keywords: Essential oil Lemon balm Low energy Nanoemulsions Thyme

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LIST OF ABBREVIATURES AND ACRONYMS

^1H	– Hydrogen nuclei (proton)
^{13}C	– Carbon 13 nuclei
2D	– bidimensional
ABTS	– 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
ANOVA	– Analysis of variance
ATR	– Attenuated Total Reflectance
B	– Baseline
br s	– broad singlet
$^{\circ}\text{C}$	– Celsius
C	– Carbon
CC	– Compound concentration
CDCl_3	– Deuterated chloroform
cm	– Centimeter
CMC	– Carboxymethylcellulose
CME	– Carvacrol methyl ether
cP	– Centipoise
d	– Duplet
dd	– Double duplet
ddd	– Double double duplet
D_h	– Hydrodynamic diameter
D_m	– Particle diffusion coefficient
DEPT	– Distortionless Enhancement by Polarization Transfer
DLS	– Dynamic light scattering
$\text{DMSO-}d_6$	– Deuterated dimethylsulfoxide
DPPH	– 2,2-diphenyl-1-picrylhydrazyl
E	– Electric field strength
EA	– Ethyl acetate
ESCAP	– European Scientific Cooperative on Phytotherapy
EPI	– Emulsion Phase Inversion
eV	– Electron volt
f	– Instrument constant

$f(\kappa\alpha)$ – Henry's function
 FTIR – Fourier-transform Infrared Spectroscopy
 g – Grams
 g/cm^3 – Grams per centimeter cubic
 g/L – Grams per liter
 g/mL – Grams per milliliter
 g/mol – Grams per mols
 GC – Gas Chromatography
 GC-MS – Gas Chromatography coupled with Mass Spectroscopy
 GRAS – Generally Recognized as Safe
 $G(\tau)$ – Measured amplitude autocorrelation function
 h – Hour
 H – Hydrogen
 HMBC – Heteronuclear Multiple Bond Correlation
 HSQC – Heteronuclear Single Quantum Correlation
 Hz – Hertz
 IS – Internal standard
 J – Coupling constant
 k_B – Boltzmann's constant
 kV – Kilovolts
 L – Liter
 LB – Lemon balm essential oil
 LCT – Long chain triglycerides
 LRI – Linear Retention Index
 m – Meters
 m/z – Mass/charge ratio
 MCT – Medium chain triglycerides
 mg – Milligrams
 mg/L – Milligram per liter
 MHz – Megahertz
 min - Minute
 mL – Milliliter

mL/kg – Milliliters per kilogram
 mL/min – Milliliters per minute
 mm – Millimeter
 mM - Millimolar
 mM TE/mL – Millimolar of Trolox equivalent per milliliter of nanoemulsion
 ms – Milliseconds
 MS – Mass Spectroscopy
 MTS – 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
 MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
 mult. – Multiplet
 mV – Millivolts
 n - Refractive index of the solvent
 NE – Nanoemulsion
 NE_1.5 – Nanoemulsion with 10 % of sunflower oil and surfactant-to-oil ratio of 1.5
 NE_1.5_Tx – Nanoemulsion with “x” % thyme essential oil and surfactant-to-oil ratio of 1.5
 NE_1.5_LBx – Nanoemulsion with “x” % lemon balm essential oil and surfactant-to-oil ratio of 1.5
 NE_2 – Nanoemulsion with 10 % of sunflower oil and surfactant-to-oil ratio of 2
 NE_2_Tx – Nanoemulsion with “x” % thyme essential oil and surfactant-to-oil ratio of 2
 NE_2_LBx – Nanoemulsion with “x” % lemon balm essential oil and surfactant-to-oil ratio of 2
 nm – Nanometers
 NMR – Nuclear Magnetic Resonance
 OH – hydroxyl group
 OSA – Octenyl succinic anhydride
 o/w – Oil-in-water emulsion
 PE – Petroleum ether
 PCS – Photon Correlation Spectroscopy
 PDI – Polydispersity index
 PIC – Phase Inversion Composition
 PIT – Phase Inversion Temperature
 PP – Potassium persulfate
 ppm – Parts per million
 q – Scattering vector

qC – Compound quantity
 QELS – Quasi-elastic Light Scattering
 quart. – Quartet
 rpm – Rotations per minute
 s – Singlet
 SE – Spontaneous Emulsification
 sec. – Second
 sept – Septet
 SOR – Surfactant-to-oil ratio
 sp³ – Typical hybridization of carbons attached do 3 hydrogens
 subsp. – Subspecie
 v – Volume
 v/w – Volume-to-weight ratio
 w/o – Water-in-oil emulsion
 w/o/w – water-in-oil-in-water multiple emulsion
 wt – Weight total
 t – Triplet
 T – Thyme essential oil
 TE – Trolox Equivalent
 TEAC – Trolox Equivalent Antioxidant Capacity
 TEM – Transmission Electron Microscopy
 Temp. – Temperature
 THF – Tetrahydrofuran
 TLC – Thin Layer Chromatography
 TME – Thymol methyl ether
 U – Electrophoretic mobility
 USA – United States of America
 ν_s – frequency shift
 ZP – Zeta potential
 δ – chemical shift
 ϵ – Solvent dielectric constant
 η - Viscosity

λ - Wavelength of the laser light

$\mu\text{g oil/mL emulsion}$ – microgram of oil per milliliter of emulsion

μm – Micrometer

μM – Micromolar

θ – Scattering angle

ρ – Density

τ – Delay time

ζ – Zeta potential

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CHAPTER I – MOTIVATION, OBJECTIVES AND OUTLINE

1.1. Motivation

The use of essential oils based products have been increasing in the pharmaceutical, food, cosmetic and perfume industries (Swamy et al., 2016). This demand is mainly due to the fact that consumers are currently more concerned about sustainability and their health and, therefore, the use of products using synthetic chemicals is avoided (Acevedo-Fani et. al, 2017; Amiri, 2012; de Oliveira et al., 2013; Donsi et al., 2011; Lee et al., 2019; Pandey et al., 2017; Salvia-Trujillo et al., 2017). Moreover, food related diseases are growing worldwide, and new efficient strategies for its mitigation are needed (Hyltdgaard et al., 2012). Some synthetic antimicrobials currently available cannot inhibit many pathogenic microbes, and besides that, the usage of synthetic chemicals is limited because of their toxicity, carcinogenic effects and potential environmental risk (Rai et al., 2017; Swamy et al., 2016; Zhang, Vriesekoop, Yuan, & Liang, 2014).

Essential oils and plant extracts have antioxidant, antifungal, antibacterial and antifungal properties against a wide range of pathogenic agents transmitted by food and/or microorganisms responsible for food spoilage (Chouhan et al., 2017; Mith et al., 2014). Thus, they are globally considered potential antimicrobial agents for the food preservation, being more ecologic and/or safe alternatives to the treatment of infectious diseases (Chouhan et al., 2017; Pandey et al., 2017). Several studies showed the potential use of essential oils as antimicrobials in foods and as flavoring agents, contributing to improve the quality, safety and shelf-life of food products (Mith et al., 2014). The European Union Commission approved the use of essential oils in foods on the Regulation (EC) No 1334/2008 of the European Parliament and of the Council of 16 of December of 2008 (European Commission, 2008). Likewise, Food and Drug Administration recognize essential oils as safe substances (GRAS - Generally Recognized as Safe) (Pandey et al., 2017; Stefanakis et al., 2013). Therefore, due to their properties, essential oils can be a powerful tool to reduce the bacterial resistance in both food and medicinal areas, giving alternatives to the conventional bactericides and fungicides (Chouhan et al., 2017; Pandey et al., 2017).

However, essential oils are commonly unstable and oxidize easily, limiting their use in the food industry. Moreover, their usage in some foods and beverages is sometimes limited due to their relatively low solubility in water and strong flavor/odor. To solve these problems, encapsulation, through nanostructures, has been pointed out as a solution for essential oils that are sensitive to pH, oxygen, light, heat and temperature, which can also be used as a carrier agent and stabilizer according with the

intended applications (Acevedo-Fani et al., 2017; Asprea et al., 2017; Benavides et al., 2016; Chang et al., 2012; Gonçalves et al., 2017; Pathakoti et al., 2017; Sotelo-Boyás et al., 2017; Zhang et al., 2014).

This thesis is a result of a collaboration between three entities: University of Minho, International Iberian Nanotechnology Laboratory and Earth Essences. The company Earth Essences is dedicated to the production of aromatic and medicinal plants in Póvoa de Lanhoso (Portugal) and wanted to study possible applications for its products, namely essential oils, giving the motivation for this study. The experimental work was performed in the two research institutes.

1.2. Objectives

The main objective of this thesis was the encapsulation of essential oils. With this main goal, four specific objectives were defined, namely: (1) the chemical characterization of two essential oils – thyme and lemon balm; (2) production and characterization of edible nanostructures for their encapsulation; (3) assess their stability over time in different storage conditions; and (4) study its antimicrobial activity. Another important analyses performed during the thesis were the toxicity and antioxidant activity of the developed nanostructures, that were used to complement the specific objective number 4.

1.3. Outline

This thesis is structured in five chapters. This is the first chapter and here it is described the contextualization of this work, partners involved, objectives and the outline of the thesis. Chapters II to V are the following:

CHAPTER II - “Introduction”: In this chapter is presented a review regarding the state-of-art on the topic.

CHAPTER III – “Essential oils”: Englobes both results and discussion of essential oils characterization and materials and methods used for these analyses.

CHAPTER IV – “Nanoencapsulation”: This chapter presents the results and discussion of the development of nanoemulsions loaded with essential oils, their characterization and properties. Materials and methods used in these tests are also described.

CHAPTER V – “General Conclusions”: In this last chapter are presented overall conclusions of this work and some suggestions for future studies.

CHAPTER II – INTRODUCTION

2.1. Essential oils

Essential oils are aromatic, oily, volatile and hydrophobic. These liquids are synthesized by plants, can have more than 45 constituents and their components are secondary metabolites (Hylgaard et al., 2012; Pandey et al., 2017; Sumalan et al., 2013).

2.1.1. Physical and chemical properties

Essential oils are inflammable and soluble in alcohol, ether and organic solvents, but insoluble in water. They have a characteristic odor and are commonly liquid and colorless at room temperature. Usually they have densities smaller than one (smaller than the density of the water) and have high refractive index and optical activity (Dhifi et al., 2016; Pandey et al., 2017). These oils have bioactive compounds and are responsible for the different aromas that plants emit.

Plants produce essential oils because they play an important role in their defense. Besides having antimicrobial properties, they control or regulate their environment. This ecological role is played through the attraction of pollinator insects, the repellent action to predators, the inhibition of seeds germination and the communication between plants (signs of emission that chemically signalize the presence of herbivores, for instance) (Chouhan et al., 2017; Dhifi et al., 2016; Hylgaard et al., 2012).

Essential oils are very complex natural mixtures, since they can have about 20 to 60 organic volatile constituents in very different concentrations (Chouhan et al., 2017; Swamy et al., 2016). Usually, their components are molecules with low molecular weight (below 300 g/mol) (Dhifi et al., 2016; Sumalan et al., 2013). However, essential oils are commonly characterized by two or three main compounds, present in higher concentrations (20-70%) (Chouhan et al., 2017; Swamy et al., 2016). The aromatic nature of essential oils, obtained from aromatic medicinal plants, is due to a mixture of various chemical substances that belongs to different chemical families, including terpenes, alcohols, aldehydes, phenols, esters, ethers, ketones, amines and amides (Dhifi et al., 2016; Swamy et al., 2016). The main components (about 90%) are monoterpenes and both the aroma and other bioactive properties of an essential oil depend on its chemical constituents (Swamy et al., 2016). The compounds belonging to the family of terpenes that were identified in essential oils, occur in the form of functionalized derivatives of alcohols, ketones, aldehydes, esters and phenols (Dhifi et al., 2016).

Essential oils are located in the cytoplasm and are produced in several organs of the plant, like trichomes, epidermal cells and internal secretory cells (Dhifi et al., 2016; Swamy et al., 2016). They can

be obtained from very different parts of the plant, such as: leaves, flowers, fruits, seeds, buds, roots, herbs, twigs, woods and barks (Chouhan et al., 2017; Dhifi et al., 2016; Hyldgaard et al., 2012; Mith et al., 2014; Vasile et al., 2017). The composition varies according to the different parts of the plants, but also according with the species of plants and even within the same specie (Chouhan et al., 2017; Swamy et al., 2016). This high variability in composition, both qualitative and quantitative, depends on various factors. They can be grouped in two categories: intrinsic factors, related to the plant and its interaction with the environment (soil, climate, etc.), and the maturity of the plant and time of harvest; and extrinsic factors, related to the extraction method and the environment. In some cases, it is hard to isolate factors, once they are interrelated and influence mutually each other (Dhifi et al., 2016; Swamy et al., 2016). The stereochemical properties of essential oils can also vary and are strongly influenced by the extraction method, affecting their biological activity (Swamy et al., 2016).

Essential oils can be obtained through various methodologies, like expression (for example, cold pressing), solvent extraction, enfleurage or, mostly used in commercial essential oils, steam or hydro distillation (Chouhan et al., 2017; Mith et al., 2014; Vasile et al., 2017).

2.1.2. Applications

Essential oils, as referred above, play an important role in the preservation of species in nature. Besides that, they have also of great interest to our society, since they possess a wide range of properties that can be useful in various applications. Essential oils have properties like antioxidant, antimicrobial, antibacterial, anti-viral, insecticidal, antifungal, antiparasitic, anthelmintic, antibiotic, antiseptic, anti-inflammatory, cytotoxic, anesthetic, antinociceptive, repellent, ovicidal, molluscicidal and immunomodulatory (Chouhan et al., 2017; Dhifi et al., 2016; Hyldgaard et al., 2012; Pandey et al., 2017; Vasile et al., 2017).

Their therapeutic properties are used in medicine since the XIX century and, because of their aroma and flavor, they started to be also used in perfumery, cosmetics and as condiments in the form of spices or herbs. Almost 3000 different essential oils are known and about 300 are commercialized in the "market" of flavor and perfumes. Food industry uses predominantly essential oils as flavoring agents (Hyldgaard et al., 2012). However, they have been increasingly used in the preservation of foods and in the crop's protection. This is happening since essential oils have low toxicity and are effective against agents that affect stored products, like bugs, pathogenic human fungi and bacteria (Hyldgaard et al., 2012; Pandey et al., 2017; Sumalan et al., 2013). They are widely used in perfumery and also in aromatherapy through massages, inhalations and baths (Dhifi et al., 2016). Also noteworthy are their

applications in the pharmaceutical, nutraceutical, agronomic and sanitary industries. In the pharmaceutical industry they are important to the treatment of cardiovascular diseases, diabetes, Alzheimer and cancer (Swamy et al., 2016). Essential oils have a relevant importance in dermatology since, at least 90 essential oils are recommended for dermatologic use in about 1500 combinations. In this area, they are mostly used for the treatment of infections caused by bacteria, fungi or virus (62%); for the treatment of skin inflammatory diseases (20%), like dermatitis, eczema and lupus; and in the general manutention of the skin (18%), like wrinkles, scars and scabs (Orchard & Van Vuuren, 2017).

2.2. Thyme

The genus *Thymus*, from the family Lamiaceae, has about 400 species of herbaceous perennial aromatic plants (evergreen and semi-evergreen) with numerous subspecies, varieties, subvarieties and forms (Borugă et al., 2014). Thyme, *Thymus vulgaris* L., (**Figure 2.1**) is a small perennial subshrub, originating from the south of Europe and the Mediterranean region (Fachini-Queiroz et al., 2012; Reddy V, 2014; Satyal et al., 2016) and exist 9 subspecies and varieties of *T. vulgaris* (Satyal et al., 2016). The plant grows until about 15-30 cm of height and 40 cm of width. Their stalks become ligneous with the age and their leaves are small, ovals to rectangular, fleshy and hairy, may having white borders. Thyme grows well in a mild climate, dried, quite sunny and where there is not much shade. Very rainy climates are harmful and lead to plant rot. Light soils, well drained and with a pH between 5.0 and 8.0 favor their growth (Reddy et al., 2014). Thyme is a plant widely used as a flavoring and medicinal herb and is a chemically variable specie (Fachini-Queiroz et al., 2012; Reddy et al., 2014; Satyal et al., 2016).



Figure 2.1 – Plant of thyme and details of its leaves and flowers.

2.2.1. Chemical composition

According to European Pharmacopoeia 5.0, the minimum amount of essential oil in thyme is 12 mL/kg (Borugă et al., 2014). As previously referred, the chemical composition of essential oils suffers

several variations and it is dependent of many factors like the geographic distribution and the process of harvesting plant material (Borugă et al., 2014; Reddy et al., 2014), leading to the existence of innumerable varieties of *T. vulgaris* (Satyal et al., 2016).

Thyme essential oil consist in a mixture of monoterpenes, being thymol frequently mentioned as the major constituent (Fachini-Queiroz et al., 2012). Usually, the main components are thymol (23%-60%), γ -terpinene (18%-50%), *p*-cymene (8%-44%), carvacrol (2%-8%) and linalool (3%-4%) (Satyal et al., 2016). However, 20 different chemotypes of thyme exist, according to the predominant monoterpene in the essential oil (Satyal et al., 2016). The most frequent chemotypes are thymol, carvacrol, linalool, *p*-cymene, γ -terpinene, sabinene hydrate, geraniol and borneol (Borugă et al., 2014; Fachini-Queiroz et al., 2012; Satyal et al., 2016). Additionally, there are some chemotypes with multiple components, such as, *p*-cymene/thymol, sabinene hydrate/terpinen-4-ol and cyclocitral/verbenol, and other more complex mixtures. Essential oils, besides this variability, can also have diverse enantiomeric distributions (Satyal et al., 2016).

Table 2.1 show the compounds that are frequently found in an amount higher than 1% in thyme essential oils, alongside their physical characteristics and properties. Additionally, thyme has other terpenes, flavonoids in the aglycone form, glycosidic flavonoids and phenolic acids (Fachini-Queiroz et al., 2012).

2.2.2. Biological activity

Thyme essential oil has several important biologic activities, like antimicrobial, antibacterial and antifungal properties (Borugă et al., 2014; Fachini-Queiroz et al., 2012; Satyal et al., 2016), that are largely associated to their constituents thymol and its phenolic isomer carvacrol (Fachini-Queiroz et al., 2012).

2.2.3. Other applications

Besides biological activities, thyme is known for its gastronomical applications, being used since ancient times as an aromatic culinary ingredient. It started being used as flavor of liqueurs, cheeses and meats, like rabbit, wild boar and lamb. Nowadays it is also used as a component of *bouquet garni* and herbs of Provence (Fachini-Queiroz et al., 2012; Satyal et al., 2016). The flavoring properties of thyme are determined by the active compounds that constitute it (Borugă et al., 2014).

Alongside being interesting for culinary, thyme has been very used in folk medicine in various applications. These include the treatment of alopecia, dental plate, dermatophytic infections, bronchitis,

cough, inflammatory skin disorders and gastrointestinal disorders (Satyal et al., 2016). Thyme is also used for expectorant, antispasmodic, anthelmintic, carminative, diuretic, diaphoretic and sedative properties (Borugă et al., 2014; Fachini-Queiroz et al., 2012). Generally, it is administered as an infusion or externally used in baths, to cure rheumatic and cutaneous diseases (Fachini-Queiroz et al., 2012).

2.2.4. Thyme essential oil applications

Thyme has also anti-inflammatory properties, mainly associated to its essential oil (thymol and, principally, carvacrol), that result from the inhibition of the inflammatory edema and the leucocytes migration (Borugă et al., 2014; Fachini-Queiroz et al., 2012; Satyal et al., 2016). Furthermore, thyme essential oil is a strong antioxidant and preservative, being very used in cosmetic and food preservation (Borugă et al., 2014; Fachini-Queiroz et al., 2012).

Thyme essential oil, known for its antimicrobial and antioxidant properties, has been increasingly pointed out as a strong candidate in pharmaceutical applications and as a natural antiseptic in the food industry (Benavides et al., 2016). Studies have shown the potentiality of its use in various scopes, like preservative in bakery products (Debonne et al., 2018; Gonçalves et al., 2017), active packaging (antimicrobial) (Lin, Zhu, & Cui, 2018), in the disinfection of archived documents (Pietrzak et al., 2017), in the antimicrobial protection of natural textiles (Walentowska & Foksowicz-Flaczyk, 2013), as lettuce washing agent (Siroli et al., 2017) and in the preservation of refrigerated lettuces (as antioxidant and antimicrobial) (Viacava et al., 2018). Thyme proved to be an efficient fungicide in tomato plantations (Ben-Jabeur, Ghabri, Myriam, & Hamada, 2015) and in organic bananas during the post-harvesting period, without negatively affecting their physicochemical and sensorial quality (Vilaplana, Pazmiño, & Valencia-Chamorro, 2018). It has also potential as synergist of insecticides (Faraone, Hillier, & Cutler, 2015). The majority of these applications was tested with the encapsulated oil, due to the problems previously referred.

Table 2.1 – Physical and biological properties of compounds that are frequently found in *Thymus vulgaris* essential oil

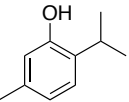
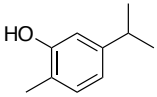
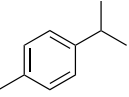
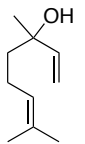
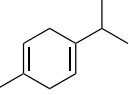
Compound	Structure	Boiling point (°C)	Molecular weight (g/mol)	Density (g/cm ³)	Solubility	log P	Property(ies)	Reference(s) supporting the property(ies)
Thymol		232-233	150.221	0.965	Ethanol Chloroform	3.30	Antioxidant, antimicrobial, antibacterial, antifungal, antitussive, antispasmodic, expectorant, anti-inflammatory, prevents platelet aggregation, analgesic	(Al-Asmari et al., 2017; Asprea et al., 2017; Deng et al., 2016; Fachini-Queiroz et al., 2012; Fancello et al., 2016; Quiroga et al., 2014; Shabnum & Wagay, 2011; Uritu et al., 2018)
Carvacrol		236-238	150.221	0.976	Ethanol Ether Acetone	3.49	Antioxidant, antimicrobial, antibacterial, antifungal, antitussive, antispasmodic, expectorant, larvicidal	(Asprea et al., 2017; Fachini-Queiroz et al., 2012; Fancello et al., 2016; Govindarajan et al., 2016; Quiroga et al., 2014; Shabnum & Wagay, 2011)
<i>p</i> -cymene		176-178	134.222	0.857	Ethanol Acetone	4.10	Antimicrobial, antifungal, antioxidant	(Al-Asmari et al., 2017; Shabnum & Wagay, 2011)
Linalool		198-200	154.253	0.865	Ethanol Ether	2.97	Antibacterial, antifungal, antioxidant	(Chouhan et al., 2017; Mith et al., 2014; Shabnum & Wagay, 2011)
γ -terpinene		182-183	136.238	0.853	Water	4.50	Antimicrobial, antioxidant, antitrypanosomal	(Al-Asmari et al., 2017; Baldissera et al., 2016; Mezza et al., 2018)

Table 2.1 (cont.) – Physical and biological properties of compounds that are frequently found in *Thymus vulgaris* essential oil

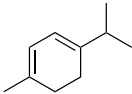
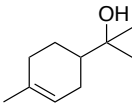
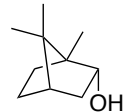
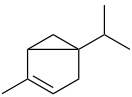
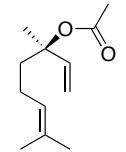
Compound	Structure	Boiling point (°C)	Molecular weight (g/mol)	Density (g/cm ³)	Solubility	log P	Property(ies)	Reference(s) supporting the property(ies)
α -terpinene		173-175	136.238	0.837	Ethanol	4.25	Antioxidant, antitrypanosomal	(Baldissera et al., 2016; Mezza et al., 2018)
α -terpineol		218-221	154.253	0.935	Ethanol Ether Acetone Water	2.98	Antimicrobial, antibacterial, antioxidant	(Al-Asmari et al., 2017; Chouhan et al., 2017; Mezza et al., 2018)
α -pinene		155-156	136.238	0.858	Ethanol Ether Chloroform	4.44	Antibacterial, antifungal, antioxidant, anticarcinogenic, anti-inflammatory, chondroprotective, repellent of mosquitoes and ticks, anti-osteoarthritic	(Al-Asmari et al., 2017; Aydin et al., 2013; Chen et al., 2015; da Silva et al., 2012; Jiang et al., 2011; Nerio et al., 2010; Rufino et al., 2014; Sieniawska et al., 2013)
Borneol		210-212	154.253	1.010	Ethanol Ether	2.69	Antibacterial, antioxidant	(Mezza et al., 2018; Zaouali, Bouzaine, & Boussaid, 2010)
α -thujene		151-152	136.238	Approx. 0.9	Ethanol	4.02	Antioxidant	(Zaouali et al., 2010)
Linalyl acetate		220-221	196.290	0.895	Ethanol Ether	3.93	Antioxidant	(Zaouali et al., 2010)

Table 2.1 (cont.) – Physical and biological properties of compounds that are frequently found in *Thymus vulgaris* essential oil

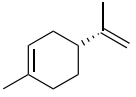
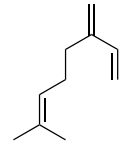
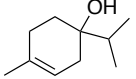
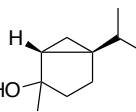
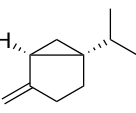
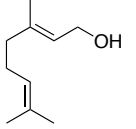
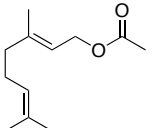
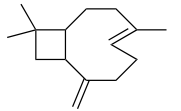
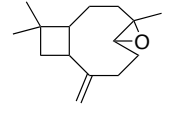
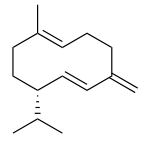
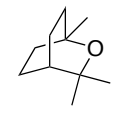
Compound	Structure	Boiling point (°C)	Molecular weight (g/mol)	Density (g/cm ³)	Solubility	log P	Property(ies)	Reference(s) supporting the property(ies)
Limonene		177-178	136.238	0.841	Ethanol	4.57	Antifungal, antibacterial, insecticidal, acaricidal, antitumoral, repellent of mosquitoes, anti-inflammatory, gastroprotective	(Cardoso-Teixeira et al., 2018; Carvalho et al., 2018)
Myrcene		167	136.238	0.794	Ethanol Ether Chloroform	4.33	Antioxidant, anti-ulcer, anticarcinogenic	(Al-Asmari et al., 2017)
Terpinen-4-ol		209	154.253	0.926	Water	3.26	Antimicrobial, antifungal, antibacterial, anti-tumoral, antioxidant, sedative, anti-inflammatory, antitrypanosomal, anesthetic, vascular relaxant, larvicidal	(Baldiçsera et al., 2016; Brilhante et al., 2016; Calcabrini et al., 2004; Govindarajan et al., 2016; Maia-Joca et al., 2014; Mezza et al., 2018; Shabnum & Wagay, 2011; Souza et al., 2018)
Sabinene hydrate		200-201	154.253	1.030	Ethanol	2.35	Antioxidant	(Mezza et al., 2018; Quiroga et al., 2014)
Sabinene		163-164	136.238	0.844	Ethanol	3.10	Antifungal, antioxidant	(Kohzaki et al., 2009; Mezza et al., 2018)
Camphene		158-161	136.238	0.839	Ether	4.22	Hypolipidemic, hepatoprotective, anticarcinogenic, insecticidal	(Al-Asmari et al., 2017; Benelli et al., 2018; Girola et al., 2015)

Table 2.1 (cont.) – Physical and biological properties of compounds that are frequently found in *Thymus vulgaris* essential oil

Compound	Structure	Boiling point (°C)	Molecular weight (g/mol)	Density (g/cm ³)	Solubility	log P	Property(ies)	Reference(s) supporting the property(ies)
Geraniol		229-230	154.253	0.889	Ethanol Acetone Chloroform	3.56	Antimicrobial, antibacterial, antifungal, antioxidant, anti-inflammatory, repellent of tick, insecticidal, anti-ulcer, hypolipidemic, anti-helminthic, cytoprotective, anti-tumoral	(Bhattamisra et al., 2019; W. Chen & Viljoen, 2010; Nerio et al., 2010; Sharma, Khan, & Manzoor, 2016; Siddique et al., 2016; Wang et al., 2016)
Geranyl acetate		242	196.290	0.907	Ethanol	4.04	Anti-inflammatory, antimicrobial, antifungal, antioxidant	(M. J. Gonçalves et al., 2012; Quintans-Júnior et al., 2013)
β-caryophyllene		262-264	204.357	0.905	Ethanol	6.30	Anti-inflammatory, antimicrobial, antioxidant, anticarcinogenic, repellent of mosquitoes, local anesthetic, analgesic	(Al-Asmari et al., 2017; Dahham et al., 2015; Fidy, Fiedorowicz, Strzdała, & Szumny, 2016; Mezza et al., 2018; Nerio et al., 2010)
Caryophyllene oxide		279	220.356	Approx. 0.9	Ethanol	3.62	Anticarcinogenic, anti-inflammatory, antileishmaniotic, antifungal, antiviral, antioxidant, analgesic, prevents platelet aggregation	(Fidy et al., 2016; W. Y. Lin et al., 2003; Ngamprasertsith, Menwa, & Sawangkeaw, 2018)
Germacrene-D		279-280	204.357	Approx. 0.9	Ethanol	6.57	Antimicrobial, antibacterial, antifungal, insecticidal	(Ravi Kiran & Sita Devi, 2007; Sitarek et al., 2017; Zarai et al., 2011)
1.8-cineole (Eucalyptol)		176	154.253	0.927	Ethanol Water Ether Chloroform	2.74	Antibacterial, antifungal, antioxidant, anti-inflammatory, anti-tumoral, analgesic	(Aparicio, Alcalde, Dávila, García, & Leal, 2007; Brown, Garver, & Orlando, 2017; Chouhan et al., 2017; Dhifi et al., 2016; Jiang et al., 2011; Juergens, 2014; Moteki et al., 2002)

2.3. Lemon balm

Lemon balm (**Figure 2.2**), *Melissa officinalis* L., like thyme, is an aromatic perennial subshrub that belongs to the Lamiaceae family (Avci & Giachino, 2016; Miraj et al., 2017; Mokhtarzadeh et al., 2017; Saeb & Gholamrezaee, 2012; Turhan, 2006). This medicinal plant has its origin in the South of Europe and the Mediterranean region, and is now widely distributed throughout all Europe, North America, Central and Western Asia, North Africa and New Zealand (Allahverdiyev et al., 2004; Boneza & Niemeyer, 2018; Dastmalchi et al., 2008; Jandaghi et al., 2016; Miraj et al., 2017; Miron et al., 2013; Pérez-Sánchez et al., 2016; Turhan, 2006). There are three subspecies of *M. officinalis* L.: subsp. *officinalis*, subsp. *inodora* and subsp. *altissima*. However, only the subspecies *officinalis* has commercial value and the typical citric odor of lemon balm (Avci & Giachino, 2016; Turhan, 2006). Its morphological characteristics are dependent of various factors such as the genotype and environmental conditions. This plant normally grows up to about 0.6-1.0 m in height, being able to reach 1.5 m, by 0.5-1.0 m of width. Its leaves in egg or heart shape measure 2-8 cm of length and are soft, hairy, very venous, perfumed and with jagged or notched edges. The small flowers (0.5-1.5 cm) are white or pale pink (Mokhtarzadeh et al., 2017; Saeb & Gholamrezaee, 2012; Seidler-Łożykowska et al., 2013; Turhan, 2006). Lemon balm is a quite resistant plant and has a radicular system with numerous lateral roots, making it more adaptable to different environmental conditions. It prefers fertile sandy and clayey soils, well drained and with pH between 5.0 and 7.0. It grows well in full sun or partial shade, with temperatures of 15 to 35°C and a precipitation of 500 to 600 mm (Miraj et al., 2017; Seidler-Łożykowska et al., 2013; Turhan, 2006). Lemon balm is chemically very variable and largely used for its flavoring and therapeutic properties (Argyropoulos & Müllera, 2011).



Figure 2.2 – Plant of lemon balm: details of its leaves and flower. The second photography is an image of the specie *Melissa officinalis* subsp. *altissima* by Don Loarie from Jardim Botânico UTAD, Flora Digital de Portugal.

2.3.1. Chemical composition

Literature is not very consistent relatively to lemon balm composition. The total content of the essential oil was found to range between 0.01–0.39%, which is below the usual values for other members of the family Lamiaceae. This small amount leads to the increase of lemon balm production cost and therefore its commercial price (Argyropoulos & Müllera, 2011; Avci & Giachino, 2016; Bogdanovic et al., 2016; Saeb & Gholamrezaee, 2012; Turhan, 2006). The dry leaves are said to usually contain at least 0.05% (v/w) of essential oil (Allahverdiyev et al., 2004; Patora, Majda, Góra, & Klimek, 2003) and a 0.15 L cup of lemon balm tea is reported to have approximately 10 mg/L of the oil (Chung, Cho, Bhuiyan, Kim, & Lee, 2010; Jun et al., 2012).

Lemon balm essential oil contains many compounds and an enormous variability, its rate and chemical composition are affected by various factors such as harvest period, nutrients, temperature, light and age (Argyropoulos & Müller, 2014; Turhan, 2006; Yadegari, 2017). With such inconsistency it is difficult to select the main constituents. Nevertheless, the ones mostly pointed are neral, geranial, citronellal, geraniol, linalool, β -caryophyllene, β -caryophyllene oxide and germacrene-D (Argyropoulos & Müller, 2014; Avci & Giachino, 2016; Bogdanovic et al., 2016). Despite the variability, in **Table 2.2** are listed some of the compounds that are often found in an amount higher than 1% in lemon balm essential oils, and their physical characteristics and properties. Lemon balm also possesses other terpenes, terpenoids, tannins, phenolic acids, flavonoids and bitter principles (Allahverdiyev et al., 2004; Bogdanovic et al., 2016; Pourghanbari et al., 2016).

2.3.2. Biological activity

Lemon balm essential oil has several biological properties such as: antimicrobial, antifungal, antiviral and antibacterial properties. These activities are related with the main constituents of the essential oil, such as, citral (geranial and neral), citronellal and β -caryophyllene (Allahverdiyev et al., 2004; Argyropoulos & Müller, 2014; Avci & Giachino, 2016; Boneza & Niemeyer, 2018; Khorshidian et al., 2017; Mimica-Dukic et al., 2004; Miraj et al., 2017).

2.3.3. Other applications

Lemon balm has been cultivated during many years for its culinary and medicinal properties and there are records of its use for about 2000 years (Jun et al., 2012; Kennedy et al., 2002; Turhan, 2006). It is very used in teas and as food ingredient for salads, sandwiches, pasta, marinades, sauces, soups,

egg and meat dishes, roasted chicken, compotes, vinegars, among others. It is also used in desserts (cheesecake), biscuits and some alcoholic beverages, such as liqueurs and wines (Chung et al., 2010; Mimica-Dukic et al., 2004; Turhan, 2006). Moreover, lemon balm has applications in the perfume and cosmetic industry, due to its aromatic essential oil, besides ornamental uses (Chung et al., 2010; Pérez-Sánchez et al., 2016; Turhan, 2006).

Alongside its culinary qualities, lemon balm has been used in traditional medicine to treat depression, nervousness, anxiety, insomnia, psychosis, hysteria, bronchitis, asthma, menstrual (amenorrhea) and gastrointestinal problems, flatulence (carminative), hypertension, migraines and headaches, vertigo, fever, influenza, snakebite, eczema, gout, (Allahverdiyev et al., 2004; Bogdanovic et al., 2016; Boneza & Niemeyer, 2018; Dastmalchi et al., 2008; Jandaghi et al., 2016; Joukar et al., 2016; J. T. Lin et al., 2012; Mimica-Dukic et al., 2004; Miraj et al., 2017; Miron et al., 2013; Seidler-Łożykowska et al., 2013; Turhan, 2006) indigestion, cramps, nausea, syncope, anemia, heart failure, arrhythmia, epilepsy, rheumatism, acne, ulcers and wounds (Allahverdiyev et al., 2004; Dastmalchi et al., 2008; Jandaghi et al., 2016; Jun et al., 2012; Miraj et al., 2017; Miroliaei et al., 2011; Turhan, 2006). It has also been used in the treatment of toothache, ear pain and high blood pressure, and as diaphoretic, antihyperlipidemic (hypolipidemic) and memory enhancer (Avci & Giachino, 2016; Bogdanovic et al., 2016; Bounihi et al., 2013; Jun et al., 2012; J. T. Lin et al., 2012; Miraj et al., 2017). Moreover, it has hepato and neuroprotective effects. Lemon balm was approved by German Commission E and recommended by ESCOP (European Scientific Cooperative on Phytotherapy) for the treatment of neurological, sleep and gastrointestinal disorders (Bogdanovic et al., 2016; Joukar et al., 2016). The antitumoral activity of the oil is strongly associated to its main constituent, citral, which has the capability of inducing cellular death in cells of breast cancer and leukemia (Armijos et al., 2018). Associated to its essential oil, lemon balm has also antioxidant (due to the components citronellal and neral), antispasmodic (due to citral), anti-inflammatory and antihyperglycemic activities (Avci & Giachino, 2016; Bounihi et al., 2013; Chung et al., 2010; J. T. Lin et al., 2012; Miraj et al., 2017).

2.3.4. Lemon balm essential oil applications

Although some possible applications for lemon balm essential oil have already been reported, there are still few bibliographical references that do so, most of which refer to the extract. In addition to being used in natural cosmetics (Argyropoulos & Müller, 2014), lemon balm essential oil offers a safe and effective treatment of agitation in people with severe dementia, also improving the main parameters of quality of life (Ballard et al., 2002). It has good potential for antioxidant activity and can be used in

foods containing lipids. The oil may still be suitable in the treatment of herpetic infections since it has shown antiviral activity against *H. simplex* type 1 (labial) and type 2 (genital). It would be an important alternative to conventional antiviral treatments, as viruses have become increasingly resistant to these drugs and they may be toxic (Allahverdiyev et al., 2004; Miraj et al., 2017; Schnitzler et al., 2008). It could also be used in the treatment of other microbial infections in humans or plants, as well as in food preservation (Abdellatif et al., 2014). Lemon balm essential oil also appears to be a potential natural antimicrobial in the treatment of infections whose pathogens have developed resistance to antibiotics (Jalal et al., 2015) and it can be used as an antimicrobial (mainly antifungal) preservative in cheeses (Khorshidian et al., 2017). It has been shown to be effective against several human cancer cell lines, inhibiting the viability of A 549 (lung), HL-60 and K562 (leukemia), MCF-7 (breast) and Caco-2 (colon) (Sousa et al., 2004). Thus, another possible application would be its use as an antitumor agent in the treatment or prevention of cancer (Saeb & Gholamrezaee, 2012). Lemon balm essential oil and citral were individually effective against glioblastoma multiforme, reducing its viability and inducing apoptosis in cancer cells. Therefore, the oil has a potential interest in the treatment of this disease, whose current therapies are not effective (De Queiroz et al., 2014).

Table 2.2 – Physical and biological properties of compounds that are frequently found in *Melissa officinalis* essential oil

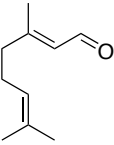
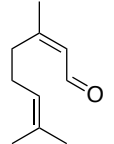
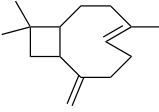
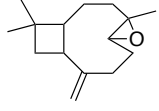
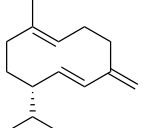
Compound	Structure	Boiling point (°C)	Molecular weight (g/mol)	Density (g/cm ³)	Solubility	log P	Property(ies)	Reference(s) supporting the property(ies)
Geranial (Citral or <i>trans</i> -citral)		226-229	152.237	0.891-0.897	Ethanol	3.45	Antimicrobial, antifungal, antibacterial, anti-tumoral, anti-inflammatory, acaricidal, insecticidal, anti-corrosive, antioxidant, antispasmodic, sedative, relaxant,	(Carvalho et al., 2018; De Queiroz et al., 2014; Giteru et al., 2015; Goldbeck et al., 2014; Petrović et al., 2017; Ce Shi et al., 2016; Chao Shi et al., 2016)
Neral (Citral B or <i>cis</i> -citral)		226-229	152.237	0.891-0.897	Ethanol	3.45	Antimicrobial, antifungal, antibacterial, anti-tumoral, anti-inflammatory, acaricidal, insecticidal, anti-corrosive, antioxidant, antispasmodic, sedative, relaxant	(Carvalho et al., 2018; De Queiroz et al., 2014; Giteru et al., 2015; Goldbeck et al., 2014; Petrović et al., 2017; Ce Shi et al., 2016; Chao Shi et al., 2016)
β-caryophyllene		262-264	204.357	0.905	Ethanol	6.30	Anti-inflammatory, antimicrobial, antioxidant, anticarcinogenic, repellent of mosquitoes, local anesthetic, analgesic	(Al-Asmari et al., 2017; Dahham et al., 2015; Fidyt et al., 2016; Mezza et al., 2018; Nerio et al., 2010)
Caryophyllene oxide		279	220.356	Approx. 0.9	Ethanol	3.62	Anticarcinogenic, anti-inflammatory, antileishmaniotic, antifungal, antiviral, antioxidant, analgesic, prevents platelet aggregation	(Fidyt et al., 2016; W. Y. Lin et al., 2003; Ngamprasertsith et al., 2018)
Germacrene-D		279-280	204.357	Approx. 0.9	Ethanol	6.57	Antimicrobial, antibacterial, antifungal, insecticidal	(Ravi Kiran & Sita Devi, 2007; Sitarek et al., 2017; Zarai et al., 2011)

Table 2.2 (cont.) – Physical and biological properties of compounds that are frequently found in *Melissa officinalis* essential oil

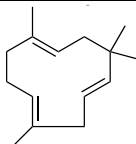
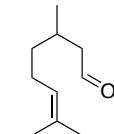
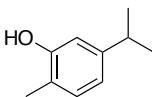
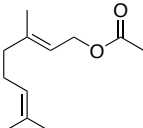
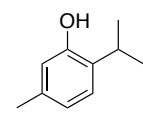
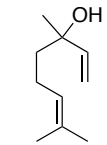
Compound	Structure	Boiling point (°C)	Molecular weight (g/mol)	Density (g/cm ³)	Solubility	log P	Property(ies)	Reference(s) supporting the property(ies)
α -caryophyllene (Humulene)		166-168	204.357	0.886	Ethanol	6.59	Anticarcinogenic, larvicidal, insecticidal	(Al-Asmari et al., 2017; Benelli et al., 2018; Fidy et al., 2016)
Citronellal		205	154.253	0.853	Ethanol	3.53	Antifungal, antibacterial, repellent of mosquitoes, antioxidant	(Goldbeck et al., 2014; Miraj et al., 2017; Nerio et al., 2010; Wu, OuYang, & Tao, 2016)
Carvacrol		236-238	150.221	0.976	Ethanol Ether Acetone	3.49	Antioxidant, antimicrobial, antibacterial, antifungal, antitussive, antispasmodic, expectorant, larvicidal	(Asprea et al., 2017; Fachini-Queiroz et al., 2012; Fancello et al., 2016; Govindarajan et al., 2016; Quiroga et al., 2014; Shabnum & Wagay, 2011)
Geranyl acetate		242	196.290	0.907	Ethanol	4.04	Anti-inflammatory, antimicrobial, antifungal, antioxidant	(M. J. Gonçalves et al., 2012; Quintans-Júnior et al., 2013)
Thymol		232-233	150.221	0.965	Ethanol Chloroform	3.30	Antioxidant, antimicrobial, antibacterial, antifungal, antitussive, antispasmodic, expectorant, anti-inflammatory, prevents platelet aggregation, analgesic	(Al-Asmari et al., 2017; Asprea et al., 2017; Deng et al., 2016; Fachini-Queiroz et al., 2012; Fancello et al., 2016; Quiroga et al., 2014; Shabnum & Wagay, 2011; Uritu et al., 2018)
Linalool		198-200	154.253	0.865	Ethanol Ether	2.97	Antibacterial, antifungal, antioxidant	(Chouhan et al., 2017; Mith et al., 2014; Shabnum & Wagay, 2011)

Table 2.2 (cont.) – Physical and biological properties of compounds that are frequently found in *Melissa officinalis* essential oil

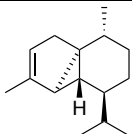
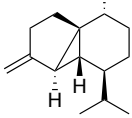
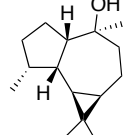
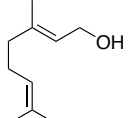
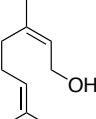
Compound	Structure	Boiling point (°C)	Molecular weight (g/mol)	Density (g/cm ³)	Solubility	log P	Property(ies)	Reference(s) supporting the property(ies)
α -cubebene		245-246	204.357	Approx. 0.9	Ethanol	6.26	Anti-inflammatory, antioxidant, antiviral, anti-septic, neuroprotective	(Choi et al., 2009; S. K. Lee et al., 2012; Park et al., 2013)
β -cubebene		283-285	204.357	Approx. 0.9	Ethanol	6.14	—————	—————
Viridiflorol		293-294	222.372	Approx. 1.0	Ethanol	4.84	Anti-inflammatory, antioxidant, anti-mycobacterial	(Trevizan et al., 2016)
β -pinene		165-166	136.238	0.860	Ethanol Chloroform	4.16	Antimicrobial, antifungal, antibacterial	(da Silva et al., 2012)
Geraniol		229-230	154.253	0.889	Ethanol Acetone Chloroform	3.56	Antimicrobial, antibacterial, antifungal, antioxidant, repellent of ticks, insecticidal, anti-inflammatory, anti-ulcer, hypolipidemic, anti-helminthic, cytoprotective, anti-tumoral	(Bhattamisra et al., 2019; W. Chen & Viljoen, 2010; Nerio et al., 2010; Sharma et al., 2016; Siddique et al., 2016; Wang et al., 2016)
Nerol		225	154.253	0.881	Ethanol Ether Chloroform	3.47	Antimicrobial, antibacterial, antifungal, antioxidant, anti-inflammatory, anti-helminthic, antidepressant, anxiolytic, antinociceptive. anticonvulsive	(Coêlho et al., 2016; Gochev, Dobрева, Girova, & Stoyanova, 2010; Jirovetz et al., 2007; Mihai & Popa, 2015; Tian et al., 2017, 2013)

Table 2.2 (cont.) – Physical and biological properties of compounds that are frequently found in *Melissa officinalis* essential oil

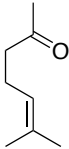
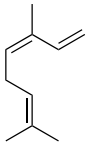
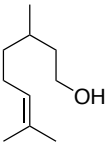
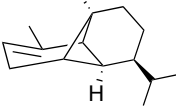
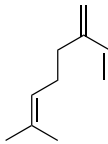
Compound	Structure	Boiling point (°C)	Molecular weight (g/mol)	Density (g/cm ³)	Solubility	log P	Property(ies)	Reference(s) supporting the property(ies)
6-methyl-5-hepten-2-one (Sulcatone)		173	126.199	0.855	Ethanol Ether	1.95	Insecticidal (larvicidal)	(Emara, 2004)
Ocimene		176-178	136.238	Approx. 0.8	Ethanol	4.19	_____	_____
Citronellol		224	156.269	0.855	Ethanol Ether	3.91	Anti-inflammatory, antioxidant, reducer of appetite and cholesterol, antidiabetic, vasodilator, repellent of mosquito and tick, cardioprotective, anti-hypertensive, anticarcinogenic	(Ferreira et al. 2017; Gochev et al., 2010; Ribeiro-Filho et al., 2016; Srinivasan & Muruganathan, 2016)
α-copaene		246-251	204.357	0.910	Ethanol	5.71	Antioxidant	(Türkez, Çelik, & Toğar, 2014)
Myrcene		167	136.238	0.794	Ethanol Ether Chloroform	4.33	Antioxidant, anti-ulcer, anticarcinogenic	(Al-Asmari et al., 2017)

Table 2.2 (cont.) – Physical and biological properties of compounds that are frequently found in *Melissa officinalis* essential oil

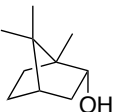
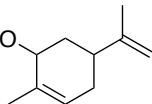
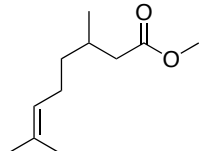
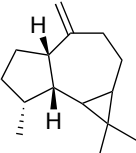
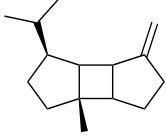
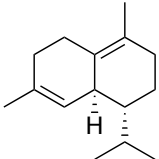
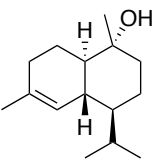
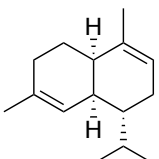
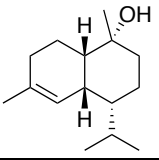
Compound	Structure	Boiling point (°C)	Molecular weight (g/mol)	Density (g/cm ³)	Solubility	log P	Property(ies)	Reference(s) supporting the property(ies)
Borneol		210-212	154.253	1.011	Ethanol Ether	2.69	Antibacterial, antioxidant	(Mezza et al., 2018; Zaouali et al., 2010)
Carveol		226-227	152.237	0.958	Ethanol	3.12	Repellent, vasorelaxant, anticarcinogenic	(Cardoso-Teixeira et al., 2018; Nerio et al., 2010)
Methyl citronellate		216	184.279	—	Ethanol	3.60	—	—
Allo- aromadendrene		257-258	204.357	0.923	Ethanol	6.41	Antioxidant, antibacterial	(de Lima et al., 2016; Yu et al., 2014)
β-bourbonene		255-256	204.357	0.899-0.908	Ethanol	6.13	—	—

Table 2.2 (cont.) – Physical and biological properties of compounds that are frequently found in *Melissa officinalis* essential oil

Compound	Structure	Boiling point (°C)	Molecular weight (g/mol)	Density (g/cm ³)	Solubility	log P	Property(ies)	Reference(s) supporting the property(ies)
δ -cadinene		279-280	204.357	0.916	Ethanol	6.27	Antibacterial	(Pérez-López et al., 2011)
α -cadinol		302-304	222.372	Approx.0.9	Ethanol	4.90	Antioxidant, antifungal. Hepatoprotective, acaricidal, antitumoral	(S.-T. Chang, Chen, Wang, & Wu, 2001; Cheng, Wu, Chang, Kao, & Chang, 2004; He et al., 1997; H. Li et al., 2017; Sylvestre, Pichette, Longtin, Nagau, & Legault, 2006; Tung et al., 2011)
α -muurolene		271-272	204.357	0.911-0.917	Ethanol	6.56	—————	—————
α -muurolol		301-303	222.372	—————	Ethanol	4.95	—————	—————

2.4. Antimicrobial activity of essential oils compounds

Thyme and lemon balm essential oils have activities against numerous human pathogenic microorganisms. Both are effective, for example, against *E. coli*, *L. monocytogenes*, *S. aureus* and *S. sonnei*. Thyme has also activity against *C. botulinum*, *C. perfringens*, *S. lutea*, *M. avus*, *B. thermosphacta*, *L. innocua*, *S. enterica* ser. Typhimurium, *S. putrefaciens*, *P. putida*, *A. niger* and *A. flavus*. Lemon balm, on the other hand, has activity against the bacteria *P. aeruginosa*, *S. enterica* ser. Enteritidis, *S. enterica* ser. Typhi, *S. lutea*, *M. flavus*, *S. epidermidis*, *B. subtilis* and *B. cereus* and against the fungi *T. mentagrophytes*, *T. rubrum*, *T. tonsurans*, *M. canis*, *E. floccosum* and *C. albicans* (Khorshidian et al., 2017; Mimica-Dukic et al., 2004; Swamy et al., 2016; Viuda-Martos et al., 2007). Usually, Gram-negative bacteria are less susceptible to essential oils than Gram-positive bacteria, since the outer membrane surrounding the cell wall of the Gram-negative contains hydrophilic lipopolysaccharides. Thereby, they provide greater tolerance of Gram-negative bacteria to hydrophobic antimicrobial compounds, such as those found in essential oils, by acting as a barrier to macromolecules and hydrophobic compounds and restricting its diffusion (Dhifi et al., 2016; Hyldgaard et al., 2012; Khorshidian et al., 2017; Pandey et al., 2017).

Analyzing individually the activities of essential oils constituents, it was found that some of them are not efficient antimicrobials. For example, β -pinene, *p*-cymene, β -myrcene, β -caryophyllene, limonene and γ -terpinene have little or no activity against *E. coli*, *S. aureus* and *B. cereus*. Likewise, *p*-cymene and γ -terpinene are not good fungicides against *S. cerevisiae*. These *in vitro* studies indicate that unsubstituted terpenes are generally not effective as antimicrobials when applied individually (Hyldgaard et al., 2012). However, *p*-cymene, although not individually active, exhibited the ability to potentiate the activity of other compounds such as carvacrol. *p*-cymene, besides being hydrophobic and causing turgescence of the cytoplasmic membrane, affects the synthesis of proteins in *E. coli* cells (Chouhan et al., 2017; Gutierrez, Barry-Ryan, & Bourke, 2008; Hyldgaard et al., 2012).

The compounds carvacrol and thymol inhibit efficiently pathogenic agents such as *E. coli*, *P. aeruginosa*, *S. aureus*, *S. enterica*, *L. monocytogenes*, *B. cereus*, *H. pylori*, *L. sake* and *V. vulnificus*, among others (Chouhan et al., 2017; Dhifi et al., 2016; Hyldgaard et al., 2012; Swamy et al., 2016). They are also effective against the fungi *Cladosporium* spp., *Aspergillus* spp., *F. oxysporum*, *B. cinerea*, *Penicillium* spp., *A. alternate* and *R. oryzae*, being dependent on the concentration used (Vasile et al., 2017). Additionally, thymol is effective against *B. subtilis* (Kavoosi, Dadfar, & Purfard, 2013). It was shown that thymol and carvacrol ruptured vesicles and cell membranes and compromised ergosterol biosynthesis in *Candida* strains, affecting the integrity of the cell membrane, once ergosterol regulates

the fluidity and asymmetry of the membrane, similarly to the cholesterol in animal cells. Likewise, Sharma *et al.* (2016), shown that geraniol has the ability to inhibit *T. rubrum* through the inhibition of ergosterol synthesis and, consequently, of the disturbance of the cell membrane integrity. In another study, Bennis *et al.* (2004), thymol induced cell lysis and only altered the structure of proliferating *S. cerevisiae* cells, indicating that the effect of thymol would depend on cell proliferation (Bennis, et al., 2004; Sharma et al., 2016). Geraniol is considered a fast-acting antimicrobial, like carvacrol, once it inactivates *E. coli* and *Salmonella* in only about 5 minutes (Chouhan et al., 2017). The compounds geraniol, nerol and citronellol inhibit *B. cereus*, *S. aureus*, *S. epidermidis*, *S. abony*, *P. aeruginosa*, *P. fluorescens*, *P. putida*, *E. coli*, *C. albicans* e *C. tropicalis* (Gochev et al., 2010). Geraniol also inhibits *H. pylori* and *C. glabrata* and nerol inhibits *A. niger*, *A. ochraceus* and *A. flavus* (Bhattamisra et al., 2019; Mihai & Popa, 2015; Sharma et al., 2016; Tian et al., 2013). Citral has activity against *L. innocua*, *L. monocytogenes*, *S. aureus*, *L. monocytogenes*, *E. coli* and *S. cerevisiae*, (Armijos et al., 2018; Chouhan et al., 2017; Espina et al., 2017; Gupta et al., 2017), affects the cellular membrane of *C. sakazakii* (Chao Shi et al., 2016) and inhibits mycelial growth of *C. albicans* (Armijos et al., 2018). Citral and citronellal have activity against *S. enterica* ser. Typhimurium, *S. aureus*, *S. dysenteriae*, *L. monocytogenes*, *E. coli* and *P. fluorescens* (Goldbeck et al., 2014). Citronellal is also an antifungal against *P. digitatum* (Wu et al., 2016) and limonene inhibits *Fusarium* sp. (Sumalan et al., 2013). Linalool has activity against *P. aeruginosa*, *L. monocytogenes*, *E. aerogenes*, *S. enterica* ser. Typhimurium, *P. fluorescens* and *L. amazonensis* (Baldiisera et al., 2016; Hyldgaard et al., 2012; Mith et al., 2014). Like α -pinene and α -terpineol, linalool is also effective against the bacteria *A. hydrophila*, *E. coli*, *B. thermosphacta* and *P. fragi* (Chouhan et al., 2017). α -pinene and 1,8-cineol inhibit *S. aureus*, *S. epidermidis*, *A. niger*, *P. aeruginosa*, *P. vulgaris*, *B. subtilis* and *C. albicans*, this last is also inhibited by β -pinene. The compounds α -pinene and β -pinene have the ability to inhibit *C. neoformans* and *R. oryzae* (Silva et al., 2012; Jiang et al., 2011). α -pinene has also activity against *F. oxysporum*, *F. proliferatum* and *S. sclerotiorum* (Pitarokili, Tzakou, & Loukis, 2008).

2.5. Methods of separation and characterization of essential oils

Essential oils, as previously referred, are complex mixtures of compounds. The main method used in their characterization is gas chromatography associated to mass spectroscopy (GC-MS). This technique allows to separate the oil constituents and their individual characterization. In this study, nuclear magnetic resonance (NMR) was also used. NMR requires previous oil fractionalization to facilitate the interpretation of the spectra, once this method has not the capability of separate different oil fractions.

Both methods can provide qualitative characterization or quantitative determinations if an internal standard is used.

2.5.1. Gas Chromatography - Mass Spectrometry (GC-MS)

The combination of gas chromatography (GC), for the separation, and mass spectroscopy (MS), for the detection and identification of the components of a mixture of compounds, is a powerful technique that is widely used in analytical laboratories at research and industrial level (Figure 2.3).

The GC system is a particularly useful analytical tool for separating and analyzing mixtures of volatile substances. The mixtures to be analyzed are injected into an inert gas flow, called the mobile phase, which passes into a tube containing the stationary phase (chromatographic column), where the separation of the mixture takes place. One disadvantage of this technique is its limitation to volatile compounds, as they pass in the column in their gaseous form. For most GC detectors, the identification is based on the retention time in the column. Though, there are different compounds that have the same retention time, which can cause doubts about the nature of the compounds separated by the detector. The MS method involves the ionization of the sample and the separation of the formed ions according to their mass/charge (m/z), thereby allowing them to be detected qualitatively and quantitatively by their respective m/z through a detector.

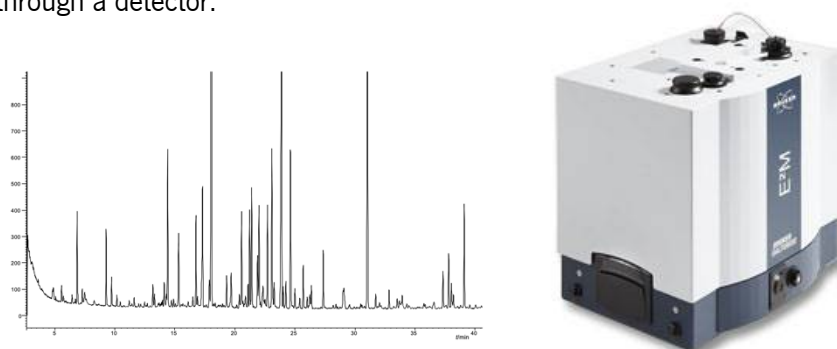


Figure 2.3 – Example of a gas chromatography spectrum and a GC equipment with mass spectrometer. Image of the equipment was taken from Bruker.

The combination of the two techniques in a single GC-MS system, forms an instrument capable of separating mixtures in its components. The mass spectrometer must be adjusted and calibrated prior to analysis. The data obtained requires a computer with a large storage capacity for data processing and identification of components. The main disadvantages of the system are its high cost and time required. Further, determining the molecular structure of a compound by interpreting a mass fragmentation spectrum is a work that requires a highly trained expert (McMaster, 2008). The GC-MS is a common method used to separate and identify essential oil components from a low volume sample (0.1 μ L). The

constituents are identified by comparison with different fragmentation patterns, where the spectra obtained in the analysis are related to data banks (or libraries) of compounds (Daferera et al., 2000; Safaei-Ghomi et al., 2009).

Nuclear Magnetic Resonance (NMR) (**Figure 2.4**) is a technique fairly used to identify components, as it allows to infer about the structure of carbon and hydrogen compounds. Nuclei with spin $1/2$ or $-1/2$ as ^1H and ^{13}C can be studied by NMR, since they are sensitive to the presence of an external magnetic field and behave like small magnetic bars, aligning themselves in favor of this, corresponding to the state of spin α (more stable), or against, being in the state of spin β . Once electromagnetic radiation of a given frequency is applied, it is absorbed by the nuclei that move from the less energetic spin state (α) to the more energetic (β) spin state. When the nuclei relax, they return to the spin α state and release energy, which is the signal detected by NMR. For a nuclear magnetic resonance analysis, a deuterated solvent is used, in order to not interfere with the obtained spectrum (Solomons & Fryhle, 2011). The identification of compounds in mixtures may become somewhat difficult using this technique, however, prior fractionation of the extracts by liquid-liquid extraction, for example, or the use of standards may make this task more accessible.

Figure 2.4 – Example of a nuclear magnetic resonance spectrum and an NMR equipment. Image of the equipment was taken from Bruker.

The infrared spectroscopy measures the frequencies of vibration of molecular bonds. In this technique, infrared radiation passes through the sample and part of this radiation is absorbed and another part is transmitted. There are some absorption bands that appear typically at certain frequencies, independently of the rest of the molecule, that allow to identify functional groups. The overall resulting spectrum is characteristic of each sample or molecule and this is why this spectroscopy is so useful

(Boughendjioua & Boughendjioua, 2017; Grumezescu, 2017; Rohman, 2017; Sim, Lee, Lu, & Samling, 2014; Smith, 2011). The intensity of the bands can be presented in transmittance or absorbance.

The Fourier-transform Infrared (FTIR) technique is the most utilized infrared spectroscopy. These equipments (**Figure 2.5**) have an extra optical component called interferometer that modulates the wavelength from the radiation source. The interferometer has a divisor of the radiation beam that splits it into two equal beams. One of them will be reflected in a fixed mirror and the other is reflected in a mobile mirror, that changes the wavelength of this last one. Then, these two beams are combined, interfering with each other and making a single beam that passes through the sample. The obtained interferogram is decoded into a FTIR interpretable spectrum through a mathematical operation called Fourier transform (Grumezescu, 2017; Skoog, Holler, & Crouch, 2018; Smith, 2011). FTIR analysis is a rapid, easy, non-invasive and non-destructive technique (Rohman, 2017), that allows high-resolutions and the use of very small amount of sample (Skoog et al., 2018).

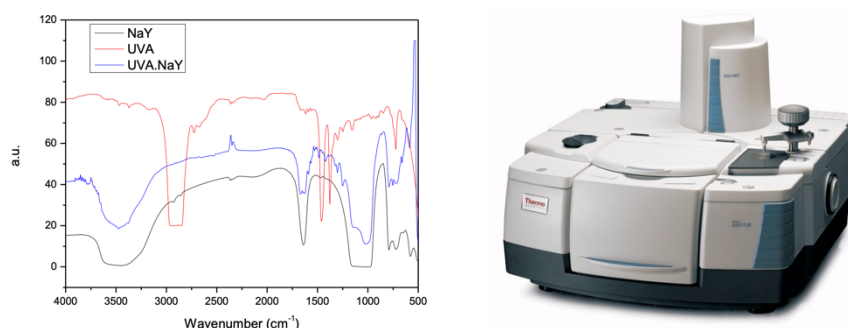


Figure 2.7 – Example of Fourier Transform Infrared spectra and an FTIR equipment. Image of the equipment was taken from ThermoFisher.

2.6. Encapsulation of essential oils in nanostructures

Essential oils are being presented as a strong alternative of some synthetic compounds that, due to their relation with multidrug resistance and toxicological issues, are being avoided by food industry. However, the use of essential oils in foods has several limitations, since they have low stability and are sensitive to some physicochemical factors that difficult their incorporation into food products. The combination of oxygen and light can lead to the oxidation of unsaturated compounds, originating free radicals, and high temperature potentiates the loss of its volatile constituents and can provoke thermal degradations. Essential oils are also sensitive to variations in pH that can lead to the decomposition of some components (Benjemaa et al., 2018; Dima & Dima, 2015; Ferreira & Nunes, 2019; Guerra-Rosas et al., 2017; Majeed et al., 2015). Furthermore, they have intense aroma and flavor, which can alter the organoleptic characteristics of the product (Guerra-Rosas et al., 2016; Moraes-Lovison et al., 2017;

Sugumar et al., 2016). Other aspects that limit their usage are their potential toxicity at high doses (Salvia-Trujillo, Rojas-Graü, Soliva-Fortuny, & Martín-Belloso, 2014) and low water solubility (Chuesiang et al., 2019; Salvia-Trujillo et al., 2013).

In order to overcome these limitations, it is crucial to develop methodologies that preserve the components of the essential oils from undergoing reactions that compromise the effectiveness of their activity (e.g. antimicrobial). Therefore, the use of encapsulation at nanoscale can be a solution, since the nanoencapsulation are able to control stability, improve solubility, bioavailability and control the release of bioactive compounds (Keivani Nahr et al., 2018; Rodríguez et al., 2016). Nanoencapsulation involves the incorporation, absorption or dispersion of bioactive compounds into structures at nanoscale. The encapsulation process is selected according to the intended use of the final formulation, which may vary depending on the size, shape or nature of the encapsulated compounds. Examples of nanostructures used in the food industry include nanofibers, nanocapsules and nanoemulsions (Weiss, Takhistov, & McClements, 2006). Therefore, the limitations of the essential oils can be overcome by their incorporation into nanostructures, in order to increase their stability and promote the controlled release of the essential oils, and thus increasing their antimicrobial potential (Chouhan et al., 2017).

2.7. Nanoemulsions

The term emulsion refers to a colloidal dispersion of two immiscible liquids, usually water and oil, where one of them is dispersed as droplets within the other. The liquid that is inside the droplets is called the dispersed phase, and the liquid that surrounds the droplets is referred to as the continuous phase. They are composed by oil, water and at least one emulsifying agent that is used as stabilizer, protecting droplets against aggregation. Emulsions can be classified into two types: if the oil is dispersed in the water phase, it is named as an oil-in-water (o/w) emulsion, as it can be seen represented in **Figure 2.6**; on the contrary, if the water phase is dispersed in the oil phase, the emulsion is called water-in-oil (w/o) (Gulotta, Saberi, Nicoli, & McClements, 2014; Komaiko & McClements, 2016; McClements, 2011; McClements & Rao, 2011). Nanoemulsions are a class of emulsions, where the emulsions present a diameter at the nanoscale, being one of the nanometric encapsulation systems most used in the food industry. They are also often referred in the literature as mini-emulsions, ultra-fine emulsions and submicron emulsions. Nanoemulsions have diameters between 20-200 nm and, depending on its droplet size, they can be transparent or milky white. (Acevedo-Fani et al., 2017; Borrin et al., 2016; Fathi et al., 2012; Gulotta et al., 2014; Gupta et al., 2016; Kotta et al., 2015; McClements, 2011; McClements & Rao, 2011; Salvia-Trujillo et al., 2014).

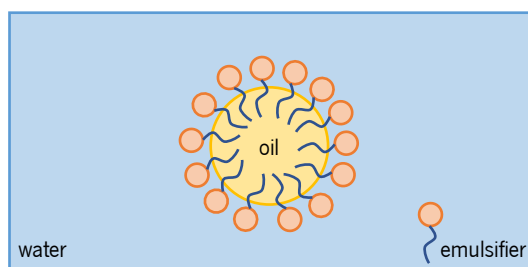


Figure 2.10 – Scheme of a general oil-in-water emulsion.

Nanoemulsions are kinetically stable over long periods of time. However, they are thermodynamically unstable, since the isolated oil and water phases have a lower free energy than their respective emulsified phases. The small droplets of nanoemulsions allow them to have high stability against gravitational separation, since the Brownian motion effects dominate the gravitational forces. These systems also tend to be highly stable to particle aggregation and robust to physical and chemical changes. Other important feature is that they are metastable and so can be diluted in water without changes in the droplet size distribution. Despite having better stability than emulsions, some destabilization can occur if the preparation method, composition and components of the system are not appropriately selected, or even during food processing, storage and transportation under undesirable conditions (Acevedo-Fani et al., 2017; Fathi et al., 2012; Guerra-Rosas et al., 2016; Gupta et al., 2016; Lee & McClements, 2010; McClements, 2011; McClements & Rao, 2011; Ostertag et al., 2012; Öztürk, 2017; Silva et al., 2011). **Figure 2.7** presents the possible disruption mechanisms of nanoemulsions, illustrating the diverse processes that lead to the separation of the formulation into different phases. Coalescence occurs when the droplets merge into each other, due to the weak repulsion between them, leading to bigger drops. In flocculation, the particles attach to each other by attractive forces and move as a single droplet, but do not combine, as it happens in coalescence. Ostwald ripening occurs due to the diffusion of droplets driven by the difference in chemical potential of the solute in particles of different sizes. The chemical potential is higher in smaller droplets, compelling them to move towards the larger ones. Then, mass transfer from the small to the big happens, leading to the grow of the largest droplets consuming the smaller ones and increasing the mean droplet size. Finally, all these three processes end in a last one called creaming. In creaming, the thermal fluctuations are dominated by the buoyant force, leading to the rise of the particles and phase separation occurs (Guerra-Rosas et al., 2016; Gupta et al., 2016). Due to their small particle size, nanoemulsions are more stable to gravitational separation, coalescence and flocculation than the conventional emulsions, but they are more susceptible to Ostwald ripening (McClements & Rao, 2011; Salvia-Trujillo et al., 2017; Walker, Gumus, Decker, & McClements, 2017).

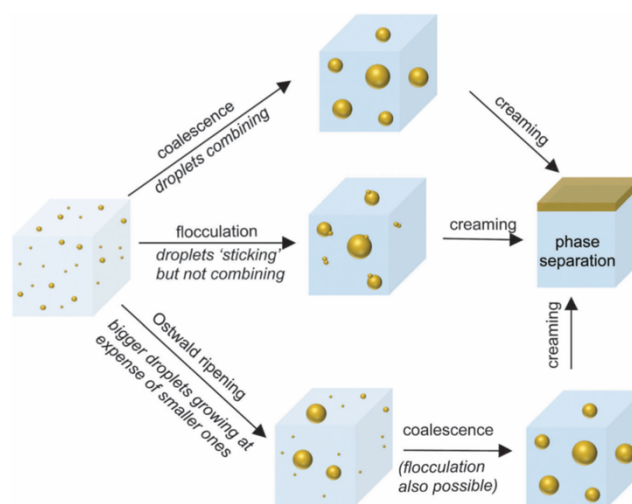


Figure 2.11 – Scheme of the different mechanisms of destabilization of nanoemulsions and their evolution. A. Gupta, H. B. Eral, T. A. Hatton and P. S. Doyle, *Soft Matter*, 2016, 12, 2826 - Published by The Royal Society of Chemistry.

Oil-in-water nanoemulsions are suitable for the encapsulation of hydrophobic bioactive compounds, as they can improve the bioavailability of the drug, because of their small droplet size and high surface area (Artiga-Artigas, Acevedo-Fani, & Martín-Belloso, 2017; Y. Chang & McClements, 2014; Lee & McClements, 2010; Silva et al., 2011). These systems have been very used in the food, supplement and pharmaceutical industries to encapsulate, protect and control the release of bioactive agents (Komaiko & McClements, 2015; Walker et al., 2017). Once the compounds are encapsulated, they are protected from the air, light and adverse environment. So, nanoemulsions protect the drugs from oxidation and hydrolysis in addition to the improvement of their bioavailability and solubility (Zhang et al., 2014)

2.7.1. Emulsifiers

Emulsifiers are surface active amphiphilic molecules, being composed by a hydrophilic and a lipophilic part, allowing them to form a protective coating around the droplets (Salvia-Trujillo et al., 2017; Silva et al., 2011). Emulsifiers are used to stabilize emulsions through their adsorption at the interface between the disperse and the continuous phases, keeping the two liquids dispersed into one another, preventing their separation into two immiscible phases and allowing the small size of droplets. The addition of an emulsifier in the formulation leads to the reduction of the interfacial tension (the surface energy per unit of area) in the border of the particles, reducing the free energy penalty related to the droplet formation. It is possible to form a nanoemulsion without these molecules, however the formulation would be highly unstable, therefore the system would rapidly breakdown through the various mechanisms that were presented before. Thus, emulsifiers are used to help nanoemulsion formation and to guarantee

its kinetic stability over time (McClements, 2012; McClements & Rao, 2011). These active molecules also prevent or slow down particle aggregation, through the increase of repulsive electrostatic interactions and steric hindrance between them (Gupta et al., 2016; Komaiko & McClements, 2016; Öztürk, 2017; Silva et al., 2011).

An emulsifier should have three important characteristics: it should rapidly adsorb to the surface of the new droplets, cause a drastically reduction in the interfacial tension and form a protective membrane that prevents droplet aggregation during emulsion processing, storage and utilization (Silva et al., 2015; Yalçınöz & Erçelebi, 2018). The emulsifiers allowed to be used in food applications are restricted by some factors such as safety issues, costs and practical questions like matrix compatibility and processing conditions (McClements, 2011; Öztürk, 2017). There are many types of emulsifiers like high molecular biopolymers (amphiphilic proteins and polysaccharides), small molecule surfactants (cationic, anionic and non-ionic), phospholipids and sucrose esters. Among these molecules, natural emulsifiers like proteins and polysaccharides, have great potential to be used in the food industry since nowadays consumers are demanding more “label-friendly” products. Thus, there is a growing interest in nanoemulsions produced with them (McClements & Rao, 2011; Öztürk, 2017; Silva et al., 2011).

2.7.2. Nanoemulsions in food applications

The incorporation of many compounds like vitamins, antimicrobials, antioxidants, flavorings and colorants into food matrices is a challenge since they have low stability and, in many cases, poor water solubility. These compounds degrade when exposed to adverse external conditions like pH, temperature or light. A possible way to overcome this problem, and a widely used one, is through nanoemulsions that are systems able to encapsulate, protect and release these sensitive compounds (Artiga-Artigas et al., 2017; Benjemaa et al., 2018; McClements, 2012; Salvia-Trujillo et al., 2017).

Nanoemulsions are able to improve the physical and thermal stability of the active ingredients, as well as their solubility and absorption (Fathi et al., 2012; Gupta et al., 2016; Hategekimana et al., 2015; Silva et al., 2015). These encapsulation systems can help to increase the concentration of antimicrobial agents in the part of food where the microorganisms are mostly situated, such as liquid-solid interfaces and water-rich phases (Donsì et al., 2011; Zhang et al., 2014). Due to their small droplet sizes and high surface area, nanoemulsions activate passive mechanisms of cell absorption, reducing the resistance to mass transfer and, consequently, improving the transport of active compounds through biological membranes. With this, they enhance once more the availability of bioactive compounds and the bactericidal activity of antimicrobials or nutraceuticals (Acevedo-Fani et al., 2017; Donsì et al., 2012;

Moraes-Lovison et al., 2017; Salvia-Trujillo et al., 2015; Severino et al., 2015; Sugumar et al., 2016). Moreover, nanoemulsions have greater long-term stability than conventional emulsions and high optical clarity, which are important for several applications into food and beverage products (Li et al., 2013; McClements, 2011, 2012; Moghimi et al., 2016).

The majority of the commercial applications of nanoemulsions is within the liquid food products, being functionalized with lipophilic active ingredients such as flavors, colors, antioxidants and vitamins (Acevedo-Fani et al., 2017; S. J. Lee & McClements, 2010; McClements, 2011). However, it has been a growing interest in applications in edible coatings of solid foods and food packaging (Acevedo-Fani et al., 2017). Nanoemulsions have been used by Unilever to produce low fat ice-cream with a significant reduction of the fat content from 16% to 1%. The company Aquanova AG sells beverages with natural colorants, such as chlorophyll, β -carotene, lutein and curcumin, or other compounds like various vitamins and omega-3 fatty acids. They also have a product named *NovaSOL sustain* that is functionalized with coenzyme Q10, for fat reduction, and α -lipoic acid, for satiety. NutraLease and Jamba Juice are other companies that produce fortified beverages with active compounds encapsulated into nanoemulsions. There are other examples of functionalized products in the food market, for example: a chocolate with antioxidant ingredients that also increase hydration and reduce lactic acid during exercise (RBC Life Sciences, Inc); supplements that increase wetness and absorption of nutrients (RBC Life Sciences, Inc); and a canola active oil enriched with phytosterols (Shemen) (Salvia-Trujillo et al., 2017; Yalçınöz & Erçelebi, 2018). As it can be seen, nanoemulsions are versatile and are already been used in some commercial products. The use of nanoemulsions is growing within the food and nutraceutical industries, due to their characteristics, advantages over emulsions, possibility of being produced with natural ingredients and possibility to scale-up their production (Donsì et al., 2011; McClements, 2011).

2.7.3. Nanoemulsions with essential oils

Nanoemulsions are one of the more suitable and most promising encapsulation systems for the incorporation of essential oils in food applications. These colloidal delivery systems can be produced with edible ingredients and incorporated into some foods and beverages (Chuesiang et al., 2019; Donsì et al., 2012; Moghimi et al., 2016). Essential oils encapsulation improves their water solubility, stability and bioavailability (Benjemaa et al., 2018; Majeed et al., 2015). Similar to what happens for other hydrophobic compounds, nanoemulsions loaded with essential oils, usually increase their antimicrobial activity (Acevedo-Fani et al., 2015; Donsì et al., 2012; Li et al., 2013; Moghimi et al., 2016; Moraes-

Lovison et al., 2017). This allows to reduce the concentration of essential oils used, diminishing the risk of toxicity caused by high doses (Salvia-Trujillo et al., 2014; R. Severino et al., 2015).

Various studies have shown that nanoemulsions loaded with essential oils have great activity against several food borne pathogens (Moghimi et al., 2016). In these works, different possible applications were evaluated, being efficient in food coatings and as additives to beverages, for example. A nanoemulsion coating with lemongrass essential oil proved to be an effective post-harvesting technology to reduce microbial growth and deterioration of grape berries during storage (Oh et al., 2017). In another study, the same essential oil was used for nanoemulsions coatings in fresh-cut apples, and they appeared to have potential use in improving the safety and quality of these fruits, due to its antimicrobial properties (Salvia-Trujillo et al., 2015). There are some other examples of studies of using bioactive coatings with encapsulated essential oils or their constituents on foods, such as: the incorporation of carvacrol nanoemulsions in coatings showed effective antibacterial activity in green beans (Severino et al., 2015); thymol nanoemulsions in coatings had a protective effect against spoilage fungi and increased the shelf life of refrigerated strawberries in 4 days (Robledo et al., 2018); coatings based on nanoemulsions with ginger essential oil were able to prolong the shelf life of chicken breast fillets (Noori et al., 2018); and nanoemulsion-based coatings with lemon essential oil extended the shelf life of rucola leaves in 3-7 days (Sessa, Ferrari, & Donsi, 2015). Moreover, films with nanoemulsions with clove bud and oregano essential oils, were more effective at prolonging the shelf life of sliced bread than the commercial antifungal agent used (Otoni et al., 2014). Oregano essential oil encapsulated in nanoemulsions showed potential to be incorporated into chicken pâté as an antibacterial additive (Moraes-Lovison et al., 2017), and nanoemulsions with *Melaleuca alternifolia* and D-limonene were able to increase the shelf life of pear and orange juices (Donsi et al., 2011). There are other examples where nanoemulsions loaded with essential oils showed to be effective antimicrobials in food products, such as milk, fresh lettuce and plums (Majeed et al., 2015; Nirmala & Nagarajan, 2017). Despite showing activity against food born microorganisms and having all these potential benefits and applications, the majority of this data was obtained using simple model systems. These systems are not able to consider the complexity of many commercial food products and consumers are still apprehensive about the usage of nano food ingredients (Nirmala & Nagarajan, 2017; Salvia-Trujillo et al., 2017). Much work is needed to validate the efficiency and safety of these systems and to produce economic and scalable products (Salvia-Trujillo et al., 2017; Xue & Zhong, 2014). Nevertheless, nanoemulsions loaded with essential oils, due to their properties and easy production, could constitute great antimicrobial additives to protect liquid foods or coatings in minimally processed foods (Acevedo-Fani et al., 2015; Echeverría & Albuquerque, 2019).

2.8. Methods for the production of nanoemulsions

Nanoemulsions are non-equilibrium systems which cannot be created spontaneously, for this reason, it is required energy for their formation (Borrin et al., 2016; Solè et al., 2010). There are two types of approaches to produce nanoemulsions: high and low energy (Fathi et al., 2012; Komaiko & McClements, 2016). High energy methods rely on equipments that apply high energy to disturb and blend the oil and water phases, leading to the formation of small droplets (Qian & McClements, 2011; Silva et al., 2015). This energy can be obtained using devices such as high-pressure homogenizers and microfluidizers, that provide high pressure or high shear conditions (Borrin et al., 2016). Low energy methods are based on the chemical characteristics of the components used for their formulation and their capacity to form an interface (spontaneous) between phases and therefore small droplets (Borrin et al., 2016). The homogenization technique that can be used in the production of nanoemulsions is highly dependent on the type of emulsifier that is utilized (McClements & Rao, 2011). In spite of the need for specialized instruments, nanoemulsions are generally produced by high energy methods, but both can be used to prepare nanoemulsions loaded with essential oils (Chuesiang et al., 2019; María Inés Guerra-Rosas et al., 2016; Ostertag et al., 2012).

2.8.1. High energy methods

The production of nanoemulsions through high energy methods use specialized equipment, called homogenizers, that create strong forces capable of disturb and blend the oil and aqueous phases. This leads to the breakup of the dispersed phase, forming small droplets along the continuous phase. For this, high pressure homogenizers, sonicators and microfluidizers are the mostly used (Barzegar et al., 2018; Chuesiang et al., 2019; S. J. Lee & McClements, 2010; Mayer et al., 2013; McClements, 2011; Saberi et al., 2013; Silva et al., 2011; Yildirim et al., 2017). There are two steps in the process of production of nanoemulsions with high energy methods. The first one involves the formation of a coarse emulsion through the mixture of the organic and aqueous phases. Then, in the second step, this pre-emulsion is subjected to a homogenization method that disrupt the big drops previously formed, obtaining the small droplets of the nanoemulsion (Acevedo-Fani et al., 2017; Öztürk, 2017).

In the food industry, nanoemulsions are mostly produced by high energy methods since they are well-established techniques with devices widely available and used, they allow large scale production, they require low quantities of emulsifier and they are very versatile in the starting materials that can be used (McClements & Rao, 2011; Salvia-Trujillo et al., 2017). These approaches also allow the utilization of natural ingredients and the conditions of production are easy to control (McClements & Rao, 2011;

Öztürk, 2017). However, these equipments require a great investment, since they are very expensive, and have high operational expenses, with elevated maintenance costs due to potential mechanical problems (Chuesiang et al., 2019; Mayer, Weiss, & McClements, 2013b). Alongside these monetary issues, high energy methods require high power and can present certain challenges to the usage of some types of food ingredients, such as slowly adsorbing emulsifiers and extremely viscous oils (Yang et al., 2012). These methods turn out to be cost-inefficient once only about 0.1% of the energy produced is actually used for the emulsification, with the majority of it being dissipated as heat. Therefore, some compounds can be degraded or even destroyed during the process of production of nanoemulsions through high energy methods (Gohtani & Prasert, 2014; Silva et al., 2011).

2.8.2. Low energy methods

Low energy methods are based on the spontaneous production of small droplets in mixtures of oil, water and emulsifier, when the system composition or environmental conditions are changed (Chuesiang et al., 2019; Komaiko & McClements, 2015; McClements, 2011; Silva et al., 2011). These methods use the inner chemical energy of the system to form the intended nanoemulsion, taking advantage of the interfacial properties of its constituents that are critical for the phase transitions that occur during the process (Barzegar et al., 2018; Silva et al., 2015; Solè et al., 2010; Yildirim et al., 2017).

Low energy methods are classified according to the physicochemical principle that they use to form the emulsion. They are named transitional phase inversion methods if they rely on the change of formulation parameters, like the physicochemical characteristics of the water or oil phases or the surfactant, by modifications in the temperature, pH or ionic strength. On the other hand, these techniques are called catastrophic phase inversion methods when they rely on alterations in a composition parameter, such as the water-to-oil ratio (McClements & Rao, 2011; Ostertag et al., 2012). Low energy approaches are also divided in two other categories according to the approach used to induce the phase transition. If the composition of the system is changed and the temperature is kept constant during the production of the nanoemulsion, the method is called isothermal. Examples of isothermal processes are spontaneous emulsification (SE), emulsion phase inversion (EPI) and phase inversion composition (PIC). On the other hand, if the composition remains unchanged and the temperature is altered, the process is classified as thermal. A thermal method is phase inversion temperature (PIT) (Komaiko & McClements, 2015; Silva et al., 2011; Solè et al., 2010).

Producing nanoemulsions with low energy methods has several advantages. These techniques do not require any special devices and a simple batch stirrer could be enough to produce nanoemulsions

with satisfactory characteristics. These approaches require less energy input and then they are more energy efficient than the other methods. Besides not requiring expensive equipments, they have reduced operating costs and they are easy to perform and implement (Chuesiang et al., 2019; Gupta et al., 2016; Li et al., 2013; Mayer et al., 2013a, 2013b; Silva et al., 2011; Yang et al., 2012). In some situations, low energy methods are more effective at producing tiny droplets, with their size being controllable by adjusting the surfactant to oil ratio (SOR), and they can be easier to scale-up (Anton & Vandamme, 2011; Chang & McClements, 2014; McClements & Rao, 2011; Saberi et al., 2013).

Moreover, as these methods do not use high amounts of energy (i.e. heat), do not influence the active agents that are being encapsulated (Zhang et al., 2014). For all these advantages, there has been an increase in the interest of low energy methods and researchers have already been studying their development, design, improvement and application for many years (Borrin et al., 2016; Komaiko & McClements, 2015; Mayer et al., 2013b). Besides all the advantages of low energy methods, they are not commonly used in the food industry, once the factors that affects their performance are not yet well understood (Saberi et al., 2013). Moreover, these methods have three major disadvantages that also limit their usage in food applications. One is the need of high concentrations of emulsifier, that could restrict their use due to taste, safety and economic motives. This sometimes could be overcome by the dilution of the nanoemulsions. Other drawback that often restrict their application is the limitation of the types of oils that can be used. It is difficult to form small droplets and stable nanoemulsions using long chain triglycerides (LCT), and medium chain triglycerides (MCT) are the best type of oil for being used in these methods. The third disadvantage is the limitation on the type of emulsifier that can be used (Chang & McClements, 2014; J. S. Komaiko & McClements, 2016; Mayer et al., 2013a; McClements & Rao, 2011; Moraes-Lovison et al., 2017). Small surfactants and phospholipids are the most effective at producing nanoemulsions through both high and low energy techniques (Komaiko & McClements, 2016; McClements & Rao, 2011). On the contrary, polysaccharides and proteins are not commonly suitable for producing small droplets with low energy approaches (McClements & Rao, 2011). Until now, and because of the nature of the physicochemical mechanisms involved in these methods, it was only possible to form nanoemulsions by low-energy methods using synthetic surfactants. Researchers are currently trying to use natural emulsifiers, which would make low energy methods much more appealing and it would also allow their use in more applications (Komaiko & McClements, 2016).

The approach used to form nanoemulsions in this study was the emulsion phase inversion method (EPI). It was chosen an isothermal low energy technique since essential oils are sensitive to high temperatures as previously referred. This method relies on a catastrophic phase inversion that occurs

with the titration of the aqueous phase over the organic phase (**Figure 2.8**). First, the oil and a hydrophilic surfactant are mixed together to form the organic phase. Then, with the addition of the aqueous phase, it is formed an unstable water-in-oil (w/o) emulsion. After adding more water, a viscous liquid crystalline phase or bicontinuous system is created, and this is believed to be an important intermediate in the process. With the continuous aqueous phase titration and stirring, the system changes to a water-in-oil-in-water (w/o/w) multiple emulsion and its viscosity decreases. This w/o/w emulsions are also thought to be a critical step to create the small oil droplets in the final nanoemulsions. More aqueous phase is added and, when a certain water content is exceeded, the system reaches a catastrophic phase inversion point where the emulsion is transformed into an oil-in water (o/w) emulsion (Borrin et al., 2016; Gupta et al., 2016; Komaiko & McClements, 2015; Komaiko & McClements, 2016; Mayer et al., 2013a; McClements, 2011; McClements & Rao, 2011; Ostertag et al., 2012; Öztürk, 2017).

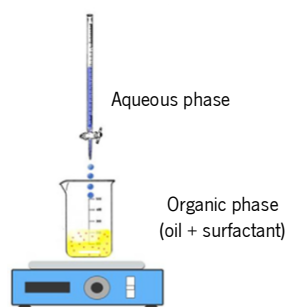


Figure 2.13 – Schematic representation of the setting used to produce nanoemulsions through the emulsion phase inversion method. An aqueous phase is titrated into an organic phase (oil + surfactant), under continuous stirring. Adapted from the scheme published in Journal of Colloid and Interface Science, 388, Ostertag, F., Weiss, J., McClements, D., Low-energy formation of edible nanoemulsions: Factors influencing droplet size produced by emulsion phase inversion, 95-102, Copyright Elsevier (2012), license number 4675540941974.

This method requires low energy to form small droplets once, at the inversion point, the interfacial tension of the oil-water boundary is very low (Gupta et al., 2016). After finishing the water addition, the system is further stirred in order to ensure the breakdown of any bigger structures and the homogeneity of the final formulation (Komaiko & McClements, 2016). The critical water concentration at which the phase inversion occurs, and the size of the droplets, are dependent on various factors: stirring type and speed, flow rate of the water addition and surfactant type and concentration (Borrin et al., 2016; McClements, 2011; McClements & Rao, 2011). The emulsifier is typically limited to small molecule surfactants in methods based on catastrophic phase inversion, like EPI. This is due to the need to stabilize both w/o and o/w emulsions (McClements, 2011; McClements & Rao, 2011).

2.9. Characterization of nanoemulsions

Nanoemulsions can be characterized in terms of size, homogeneity and charge with the dynamic light scattering technique. Another powerful and frequently used method is transmission electron microscopy that allows to confirm their morphology and size.

2.9.1. Dynamic Light Scattering (DLS)

Dynamic light scattering (DLS), also known as Photon Correlation Spectroscopy (PCS) and Quasi-elastic Light Scattering (QELS), is a non-invasive technique used to evaluate the mean particle size, polydispersity index (PDI), that describes the distribution width, and zeta potential (ZP), which is the surface electrical charge of particles (Grumezescu, 2017; Kim et al., 2018; Severino et al., 2012; Stetefeld et al., 2016). **Figure 2.9** shows an optical diagram of the equipment used for particle size and zeta potential analysis.

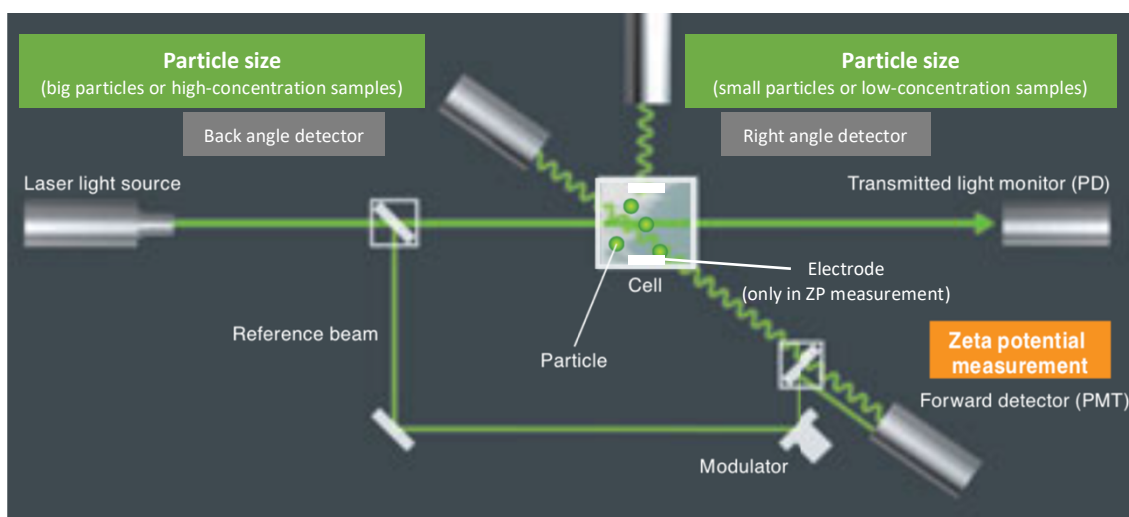


Figure 2.14 – Optical diagram of the equipment used for particle size and zeta potential analysis. Image adapted from Horiba Scientific.

In this method, the illumination of the sample with a monochromatic laser beam causes the Brownian motion of particles in suspension, and this movement is related to their size (diameter). When the laser hits the sample, light scatters in all directions as a function of the size and shape of particles. The movement of the particles is dependent of their size, temperature and solvent viscosity, and causes random changes in the intensity of scattered light (Stetefeld et al., 2016). The signal is collected either at 90 degree (right angle) or 173 degree (back angle), depending on the size or concentration of the sample and it can be interpreted using an autocorrelation function. This has an exponential decay and, with the subtraction of the baseline, the diffusion coefficient can be calculated using **Eq. 2.1**, where $G(\tau)$

is the measured amplitude autocorrelation function, B is the baseline, f is the instrument constant, D_m is the particle diffusion coefficient, q is the scattering vector (with $q = (4\pi n/\lambda) \sin(\theta/2)$, where n is the refractive index of the solvent), λ is the wavelength of the laser light, θ is the scattering angle and τ is the delay time. Then, particle size (diameter) is calculated from the diffusion coefficient using the Stokes-Einstein equation that is described in **Eq. 2.2**, where D_h is the particle hydrodynamic diameter, k_B is the Boltzmann's constant, $Temp$ is the temperature and η is the viscosity (Horiba Scientific, n.d., 2012).

$$G(\tau) = B + Bf \exp(-2 D_m q^2 \tau) \quad \text{Eq. 2.1}$$

$$D_h = k_B Temp / (3\pi\eta D_m) \quad \text{Eq. 2.2}$$

The stability and characterization of nanoemulsions is deeply related with their particle size and polydispersity index (PDI), which indicates the homogeneity of the size distribution. For being considered nanoemulsions, droplets in the emulsion must have mean diameters below 200 nm. A PDI with a value below 0.2 means that an emulsion has a monodisperse size distribution, having uniformity among its droplets and better stability. On the other hand, if the PDI is close to 1, the emulsion has a heterogeneous or multimodal distribution. When both parameters remain unchanged over time, nanoemulsions are considered physically stable (Guerra-Rosas et al., 2016; Komaiko & McClements, 2015; Komaiko & McClements, 2016; Mittal & Matsko, 2012; Yildirim et al., 2017).

The other analysis that is frequently made with DLS is the zeta potential of the particles (ZP). ZP quantifies the charge on a particle surface in a specific liquid medium. This charge influences the ionic environment in the region close to the particle surface. The ionic environment of a charged particle is characterized by a double layer model: the Stern layer that is adjacent to the particle surface with ions strongly attached, and the following diffuse layer that is still under the influence of the particle and thus the ions move with it. Zeta potential is the potential measured at the shear plane, that corresponds to the frontier between this electric double layer and the ions in equilibrium in the solution, as illustrated in **Figure 2.10**.

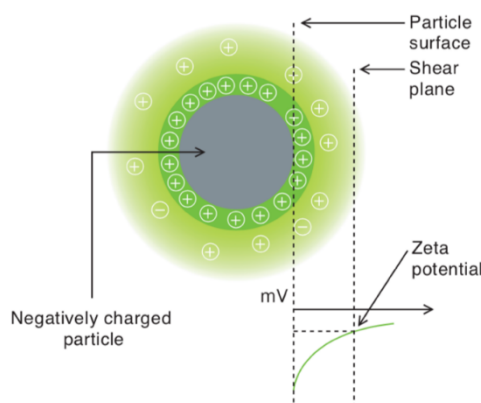


Figure 2.16 – Representation of the shear plane, where Zeta potential is measured. Image from Horiba Scientific.

In DLS, the sample is placed into a cell containing two electrodes that are used to create a magnetic field. When an electric field is applied, the charged particles interact with it and acquire movement. They move toward either the anode or cathode and this direction indicates if the surfaces are positive or negative charged. The velocity of this motion is proportional to the magnitude of the charge. Like in size analysis, samples are illuminated with a laser and particles scatter light. As shown in **Figure 2.9**, a second beam (the reference beam) is mixed with the scattered beam in order to obtain the Doppler (frequency) shift in the scattered light. And then, this is used to find the particle velocity and the electrophoretic mobility (U), this last being calculated by **Eq. 2.3**, where λ is the wavelength of the laser light, ν_d is the measured frequency shift, E is the electric field strength, n is the refractive index of the medium and θ is the scattered light angle. Finally, Zeta potential (ζ) is calculated using **Eq. 2.4**, where η is the solvent viscosity, ε is the solvent dielectric constant and $f(\kappa a)$ is the Henry's function describing the ratio of the particle radius to the double layer (Horiba Scientific, n.d., 2012).

$$U = \lambda \nu_d / [2En \sin(\theta/2)] \quad \text{Eq. 2.3}$$

$$\zeta = U\eta / [\varepsilon f(\kappa a)] \quad \text{Eq. 2.4}$$

ZP is related with the stability of the nanoemulsions and influences their polydispersity index. Particles must have positive or negative potentials bigger than 30 mV, in order to achieve high repulsion forces, leading to an electrostatic stabilization. Thus, nanoemulsions with ZP near neutral values are prone to aggregate and those with high ZP do not, maintaining their monodispersity (Grumezescu, 2017; Ohshima & Makino, 2014).

The DLS software makes all the calculations to obtain these parameters and this is a useful tool to analyze size and charge of nanoemulsions. However, for size analysis, DLS consider that all particles are perfect spheres, even when they are non-spherical. For this reason, complementary techniques must be used to assemble the morphology of the particles in study (Horiba Scientific, 2012; McClements, 2011).

2.9.2. Transmission Electron Microscopy (TEM)

Transmission electron microscopy (TEM) is an important technique to assess morphology and size. In this method, a beam of electrons passes through a thin sample to produce a magnified image or an electron-diffraction pattern on a viewing screen or camera system. The image magnification is typically between 10^3 and 10^6 . **Figure 2.11** represents schematically the principal components of a TEM microscope, vertically lined up on a column.

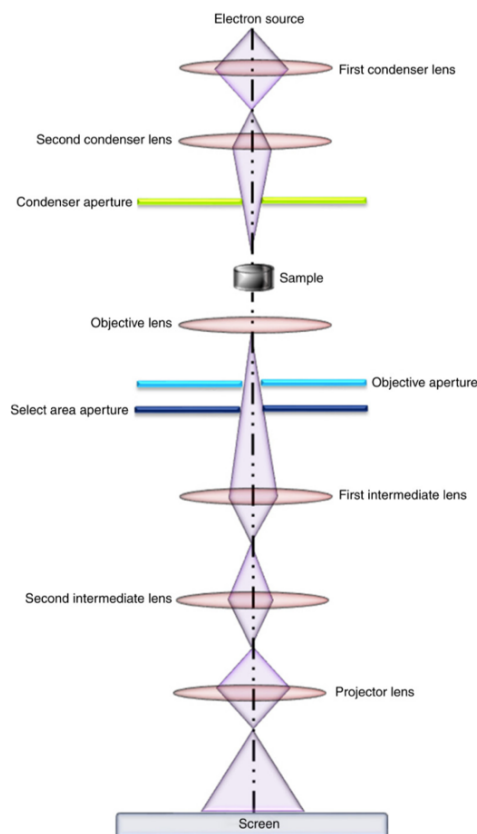


Figure 2.19 – Schematic illustration of the principal components of a TEM microscope. Image from Grumezescu, 2017.

The electron gun is where the beam of electrons is formed and encompasses the electron source and a chamber to accelerate it. The electron source is the cathode and has a negative potential. The cathode has a coverage called Wehnelt cylinder that has a more negative potential than that of the electron source and a small hole to allow electrons to pass through. This bias potential has a repulsive effect on the emitted electrons, focusing them near the anode. The bias potential creates a repulsion between the hole and the electron beam that leads to its condensation. The anode consists of a metal plate with a central hole fixed at ground potential, making the cathode negative relatively to the anode. After being emitted from the cathode, due to the potential difference between cathode and anode, electrons are accelerated towards the anode. The majority of these electrons hits the anode, being absorbed and does not participate in the image formation from the microscope. Some electrons pass through the orifice in the center of the anode and originate an electron column that is focused by electromagnetic lenses to form a narrow beam. After the formation of a stable electron beam, it has to be focused or condensed in order to reduce dispersion. For this purpose, at least two electromagnetic lenses are used. These lenses use electric energy in a coil to form a magnetic field that is able to concentrate the electron beam reducing its diameter. The first condenser lens has high potency with fixed current or with a reduced number of adjustable positions that condense the beam in different diameters. The second condenser lens is less

powerful and is with this one that the beam intensity is controlled to optimize the image conditions. Alongside the condenser lenses, there are some apertures to help to control the beam. They are made from metal and have a small hole, whose function is to limit the electron beam diameter or to eliminate electrons that are deviated from the center. Condenser lenses also have a stigmator to correct for lens residual astigmatism. The objective lens is one of the most important and complex in a TEM, because it is the last lens from which electrons pass through before forming the image. Thus, any imperfection introduced by the objective lens would be impossible to correct. Like condenser lenses, the objective lens has a set of apertures that can be used in order to improve the obtained image. They are called contrast apertures and are placed between the sample and the inferior part of the objective lens. Their function is to adsorb the majority of the electrons that are non-elastic scattered (strongly deviated) and some of the elastic scattered electrons (slightly deviated), leading to an improvement of the final image. The sample is placed in the objective lens and its position, inclination and rotation can be adjusted. The sample holder is inserted in the objective plan of the lens through a chamber that allows to isolate the interior of the microscope from the exterior environment in the laboratory. This permits to keep the high vacuum needed to the microscope operation. Some intermediate lenses allow to change the magnification, by the alteration of the focal length, or to produce an electron diffraction pattern on the viewing screen. Then, there is the projector lens that permits to produce the image or diffraction pattern in the TEM fluorescent screen. The image can also be recorded digitally with an electronic camera system, allowing to do some later image processing. As referred, to produce images, TEM uses electrons instead of visible radiation, thus any color related information is lost. Colors are a result of image treatment that can help the visualization but are not indicative of the real nature of the sample.

In TEM analysis, sample preparation is a critical step. The image of the sample is dependent of its thickness and composition. It has to be thin, because with a thick sample, electrons suffer great dispersion during their passage through the material, resulting in a decrease of the resolution power. The composition is important since certain zones are too transparent to the electrons of the beam for having a few heavy atoms. For this reason, it is used chemical doping in some samples with heavy atoms, creating differential dispersion and an image with good contrast. Moreover, samples need to be dried and this is also an important factor because an incorrect drying could introduce artifacts like shrinkage, collapse, distortion, rupture or wrinkle. Despite the time-consuming process of sample preparation, TEM is a powerful tool to analyze the size and morphology of nanoemulsions (Egerton, 2005; Grumezescu, 2017; Williams & Carter, 2009).

CHAPTER III – ESSENTIAL OILS

3.1. Results and discussion

3.1.1. Essential oils characterization

Essential oils of thyme and lemon balm were characterized by Nuclear Magnetic Resonance (NMR), Gas Chromatography coupled with Mass Spectrometry (GC-MS) and Fourier Transform Infrared Spectroscopy (FTIR). The first two methodologies were used for both qualitative and quantitative analysis, producing results with some similarities. The characterization by NMR required previous fractionation of the oils by column chromatography in order to simplify the spectra. The results of NMR and GC-MS showed that thyme essential oil has as its main compounds thymol, *p*-cymene and γ -terpinene, while the major constituents of lemon balm essential oil are β -caryophyllene, δ -cadinene and caryophyllene oxide. As essential oils are complex mixtures, FTIR only allowed to confirm the presence of the functional groups belonging to the previously identified main compounds.

3.1.1.1. Nuclear Magnetic Resonance

The characterization of essential oils by nuclear magnetic resonance (NMR) was divided into two types of analysis: qualitative and quantitative. Due to the overlap of signals in the NMR spectra of the essential oils, they were previously fractionated by column chromatography, using silica gel as adsorbent and eluents with different polarities. The obtained fractions of both oils were further analyzed by these techniques, in order to make a more complete qualitative analysis possible.

3.1.1.1.1. Qualitative analysis

The analysis of the essential oils demonstrated that they are complex mixtures of compounds, resulting in very complicated Nuclear Magnetic Resonance (NMR) spectra. In order to make their qualitative characterization possible by this technique, essential oils were fractionated by column chromatography accordingly to the polarity of their components. The fractions were evaporated at reduced pressure to eliminate the solvents, and residues were then analyzed by ^1H , ^{13}C and 2D NMR techniques. The most relevant spectra for the identification of the compounds are presented in **Annexes - Group A**.

In this work, the structural elucidation of the essential oils components was made taking into account the chemical shifts of the proton and carbon signals and, the multiplicity and the coupling constants of the proton signals. Likewise, the carbon signals were assigned on the basis of DEPT 135

(Distortionless Enhancement by Polarization Transfer) and bidimensional analysis of heteronuclear correlation techniques, which included HSQC (Heteronuclear Single Quantum Correlation) and HMBC (Heteronuclear Multiple Bond Correlation). Compounds were definitely identified by comparison of the obtained spectral data with data reported in the literature.

According to the NMR data, thymol is the main component of the thyme essential oil analyzed. This compound was identified comparing the signals observed in the ^1H and ^{13}C NMR spectra (**Table 3.1**) with the data found in the literature. The information obtained by DEPT 135 and correlations observed in the HSQC and HMBC spectra confirmed the assignment of the structure. In ^1H NMR spectra, the presence of the 1,2,5-trisubstituted aromatic ring was confirmed by the three signals observed at δ 7.17 ppm (d, J = 8.0 Hz), δ 6.81 ppm (d, J = 8.0 Hz) and δ 6.65 ppm (d, J = 0.8 Hz). The signal of the proton attached to the tertiary carbon (H-8) was easily identified by its septet at δ 3.29 ppm with a coupling constant of J = 6.8 Hz. This same constant was detected for the six equivalent protons (d, J = 6.8 Hz) at δ 1.33 ppm, which were assigned to the two methyl groups attached to the same carbon (H-9 and H-10). All the ^{13}C and DEPT 135 carbon signals were identified and HMQC and HMBC correlations supported the assignments. The carbon attached to the hydroxyl group (C-1) with a chemical shift of δ 152.7 ppm is one of the most characteristic signals, supporting the presence of the phenol ring.

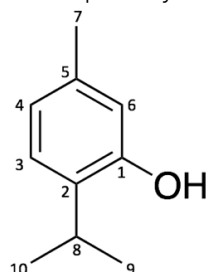
Another important constituent of this oil is *p*-cymene, that was also characterized by ^1H , ^{13}C and 2D NMR (**Table 3.2**). In the ^1H NMR spectrum, signals of the aromatic 1,4-disubstituted ring were well identified with two doublets at δ 7.12 ppm and δ 7.15 ppm, integrating for two protons with the same coupling constant (J = 8.4 Hz). This compound presented also a characteristic septet signal at δ 2.89 ppm (J = 6.8 Hz), belonging to the H-8 proton from the tertiary carbon. All the carbon signals were identified and, once more the DEPT 135 and bidimensional techniques were a very important support to properly assign the data.

The third main constituent of thyme essential oil is γ -terpinene (**Table 3.3**). The cyclic unit present in this compound is not an aromatic ring, so it does not show any signal in the δ 6-7 ppm region in the ^1H NMR spectrum. The signals of the protons belonging to the aliphatic ring appeared at δ 5.46 ppm, for the hydrogens attached to carbons with the double bonds, and δ 2.62 ppm, for the other protons. With the help of ^{13}C and 2D NMR, all the signals of γ -terpinene carbons were assigned. Here, the signals of the quaternary carbons can be detected at high chemical shifts: δ 131.3 ppm (C-1) and δ 140.6 ppm (C-4).

Linalool (**Table 3.4**) was not fully characterized by ^1H NMR, but it was easily identified due to its characteristic signals belonging to the terminal double bond: three double doublets at δ 5.26 ppm (J =

17.2; 1.2 Hz), δ 5.103 ppm (J = 10.8; 1.2 Hz) and δ 5.95 ppm (J = 17.6; 10.8 Hz). DEPT 135 and bidimensional techniques allowed not only the full carbon characterization of linalool, but also gave additional data for proton identification. The signals attributed to linalool could be confirmed by comparing them to the literature data.

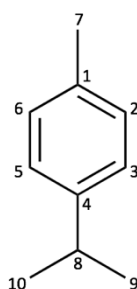
Table 3.1 – ^1H and ^{13}C NMR data experimentally obtained for the compound thymol (^1H NMR: 400 MHz CDCl_3 ; ^{13}C NMR: 100 MHz CDCl_3)



Thymol ^1H NMR				
δ (ppm)	integration	Multiplicity	J (Hz)	Assignment
7.17	1	d	8.0	H-3
6.81	1	d	8.0	H-4
6.65	1	d	0.8	H-6
2.34	3	s	—	H-7
3.29	1	sept	6.8	H-8
1.33	6	d	6.8	H-9, H-10
5.55	1	br s	—	OH
Thymol ^{13}C NMR				
δ (ppm)	Assignment			
152.7	C-1			
131.5	C-2			
126.1	C-3			
121.4	C-4			
136.3	C-5			
116.0	C-6			
20.7	C-7			
26.5	C-8			
22.6	C-9, C-10			

s - singlet; br s - broad singlet; d - duplet; sept - septet.

Table 3.2 – ^1H and ^{13}C NMR data experimentally obtained for the compound *p*-cymene (^1H NMR: 400 MHz CDCl_3 ; ^{13}C NMR: 100 MHz CDCl_3)

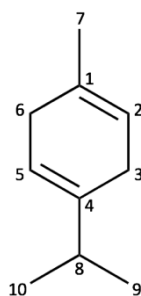


<i>p</i> -cymene ^1H NMR				
δ (ppm)	integration	Multiplicity	J (Hz)	Assignment
7.12	2	d	8.4	H-2, H-6
7.15	2	d	8.4	H-3, H-5
2.34	3	s	—	H-7
2.89	1	sept	6.8	H-8
1.27	6	d	7.2	H-9, H-10

<i>p</i> -cymene ^{13}C NMR	
δ (ppm)	Assignment
135.1	C-1
126.3	C-2, C-6
129.0	C-3, C-5
145.9	C-4
21.0	C-7
33.7	C-8
24.1	C-9, C-10

s - singlet; d - duplet; sept - septet.

Table 3.3 – ^1H and ^{13}C NMR data experimentally obtained for the compound γ -terpinene (^1H NMR: 400 MHz CDCl_3 ; ^{13}C NMR: 100 MHz CDCl_3)

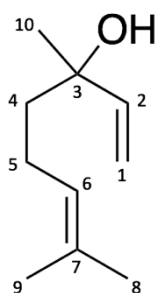


γ -terpinene ^1H NMR				
δ (ppm)	integration	Multiplicity	J (Hz)	Assignment
5.46	2	br s	—	H-2, H-5
2.62	2	br s	—	H-3, H-6
1.70	3	s	—	H-7
2.22	1	sept	—	H-8
1.04	6	d	6.8	H-9, H-10

γ -terpinene ^{13}C NMR	
δ (ppm)	Assignment
131.3	C-1
118.9	C-2
27.5	C-3
140.6	C-4
116.0	C-5
31.6	C-6
23.0	C-7
34.5	C-8
21.3	C-9, C-10

s - singlet; br s - broad singlet; d - duplet; sept - septet.

Table 3.4 – ^1H and ^{13}C NMR data experimentally obtained for the compound linalool (^1H NMR: 400 MHz CDCl_3 ; ^{13}C NMR: 100 MHz CDCl_3)



Linalool ^1H NMR				
δ (ppm)	integration	Multiplicity	J (Hz)	Assignment
5.28	1	dd	17.2; 1.2	H-1a
5.10	1	dd	10.8; 1.2	H-1b
5.95	1	dd	17.6; 10.8	H-2
1.61	2	t	4.0	H-4
5.15	1	mult.	—	H-6
1.71	3	s	—	H-8
1.63	3	s	—	H-9
1.31	3	s	—	H-10

Linalool ^{13}C NMR	
δ (ppm)	Assignment
111.7	C-1
144.7	C-2
73.7	C-3
41.9	C-4
22.7	C-5
124.2	C-6
131.8	C-7
24.9	C-8
17.5	C-9
27.5	C-10

s - singlet; dd - double duplet; t - triplet; mult. - multiplet.

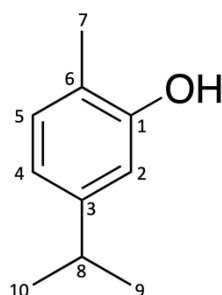
The fractionation of the thyme oil by column chromatography allowed to identify other minor compounds. Three proton signals allowed the identification of carvacrol (**Table 3.5**): one aromatic proton at δ 7.05 ppm (d, J = 8.0 Hz), the proton of the tertiary carbon at δ 2.90 ppm (sept, J = 6.8 Hz), and a singlet at δ 2.30 ppm assigned to the methyl group directly attached to the aromatic ring. The other two compounds identified in the obtained fractions of the oil were assigned to derivatives of thymol and carvacrol, namely thymol methyl ether (TME) and carvacrol methyl ether (CME), respectively (**Tables 3.6** and **3.7**). The two compounds showed one septet at δ 3.39 ppm (TME) and at δ 2.97 ppm (CME), both with coupling constants of J = 6.8 Hz, revealing the presence of the CH protons from the isopropyl groups. Singlets at δ 2.42 ppm, in the case of TME, and at δ 2.29 ppm, for CME, were assigned to the methyl group directly connected to the aromatic ring. The presence of one methoxyl group, observed as a singlet signal at δ 3.90 ppm for TME and at δ 3.92 ppm for CME, was determinant in the assignment of both structures. Additionally, despite the low concentrations of these two components, HMQC and HMBC correlations of these methoxyl signals also allowed the identification of the carbons present at one and two bonds for each compound.

Myrcene (**Table 3.8**) was identified by its double duplet at δ 6.40 ppm (J = 17.6; 10.8 Hz) from $\text{RCH}=\text{CH}_2$ of the terminal alkene. This proton signal had a correlation at 2 bonds (HMBC) with a carbon at δ 115.3 ppm, corresponding to the carbon $\text{RCH}=\text{CH}_2$. This assignment was confirmed by DEPT, which showed that this signal was a CH_2 group.

Lemon balm essential oil is a much more complex mixture of compounds. Due to the presence of many and long alkyl chains, the region of δ 3.0-0.5 ppm was extremely complicated. Likewise, the characteristic region of, for example, double bonds at δ 6.0-4.5 ppm showed a great overlap of signals. For these reasons, only a few constituents of lemon balm oil were characterized by NMR.

Geranial (**Table 3.9**) and its isomer neral (**Table 3.10**) were easily identified by their proton signals from the aldehyde, with duplets at δ 9.99 ppm (J = 8.0 Hz) and δ 9.90 ppm (J = 8.4 Hz), respectively. Through the help of 2D NMR techniques, it was possible to identify not only the carbon signals from the aldehyde group at δ 191.2 ppm for geranial, and at δ 190.7 ppm for neral, but also the carbon of the adjacent double bond at δ 127.3 ppm and δ 128.6 ppm, respectively. Geranial was also identified in thyme essential oil and it was possible to assign the proton from the alkene adjacent to the aldehyde group (H-2), with a signal detected at δ 5.88 ppm (d, J = 7.6 Hz).

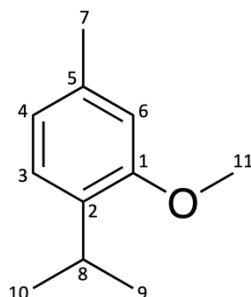
Table 3.5 – ^1H NMR data experimentally obtained for the compound carvacrol (400 MHz CDCl_3)



Carvacrol ^1H NMR				
δ (ppm)	integration	Multiplicity	J (Hz)	Assignment
7.05	1	d	8.0	H-5
2.30	3	s	—	H-7
2.90	1	sept	6.8	H-8

s - singlet; d - duplet; sept - septet.

Table 3.6 – ^1H and ^{13}C NMR data experimentally obtained for the compound thymol methyl ether (^1H NMR: 400 MHz CDCl_3 ; ^{13}C NMR: 100 MHz CDCl_3)

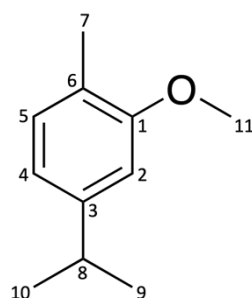


Thymol methyl ether ^1H NMR				
δ (ppm)	integration	Multiplicity	J (Hz)	Assignment
2.42	3	s	—	H-7
3.39	1	sept	6.8	H-8
3.90	3	s	—	H-11

Thymol methyl ether ^{13}C NMR	
δ (ppm)	Assignment
156.5	C-1
55.3	C-11

s - singlet; sept - septet.

Table 3.7 – ^1H and ^{13}C NMR data experimentally obtained for the compound carvacrol methyl ether (^1H NMR: 400 MHz CDCl_3 ; ^{13}C NMR: 100 MHz CDCl_3)

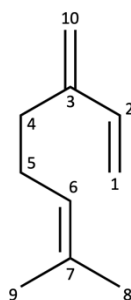


Carvacrol methyl ether ^1H NMR				
δ (ppm)	integration	Multiplicity	J (Hz)	Assignment
2.29	3	dd	—	H-7
2.97	1	sept	6.8	H-8
3.92	3	s	—	H-11

Carvacrol methyl ether ^{13}C NMR	
δ (ppm)	Assignment
157.5	C-1
55.2	C-11

s - singlet; dd - double duplet; sept - septet.

Table 3.8 – ^1H and ^{13}C NMR data experimentally obtained for the compound carvacrol methyl ether (^1H NMR: 400 MHz CDCl_3 ; ^{13}C NMR: 100 MHz CDCl_3)

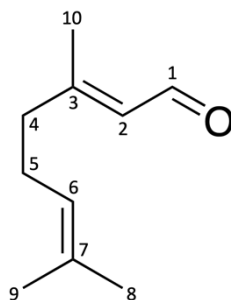


Myrcene ^1H NMR				
δ (ppm)	integration	Multiplicity	J (Hz)	Assignment
6.40	1	dd	17.6; 10.8	H-2

Myrcene ^{13}C NMR	
δ (ppm)	Assignment
115.3	C-1

dd - double duplet.

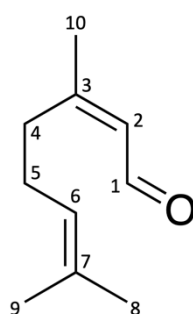
Table 3.9 – ^1H and ^{13}C NMR data experimentally obtained for the compound geranial (^1H NMR: 400 MHz CDCl_3 ; ^{13}C NMR: 100 MHz CDCl_3)



Geranial ^1H NMR				
δ (ppm)	integration	Multiplicity	J (Hz)	Assignment
9.99	1	d	8.0	H-1
5.88	1	d	8.0	H-2
2.16	3	d	1.2	H-10
Geranial ^{13}C NMR				
δ (ppm)	Assignment			
191.2	C-1			
127.3	C-2			
163.8	C-3			

d - duplet.

Table 3.10 – ^1H and ^{13}C NMR data experimentally obtained for the compound neral (^1H NMR: 400 MHz CDCl_3 ; ^{13}C NMR: 100 MHz CDCl_3)



Neral ^1H NMR				
δ (ppm)	integration	Multiplicity	J (Hz)	Assignment
9.90	1	d	8.4	H-1
1.98	3	d	1.6	H-10
Neral ^{13}C NMR				
δ (ppm)	Assignment			
190.7	C-1			
128.6	C-2			
163.8	C-3			

d – duplet.

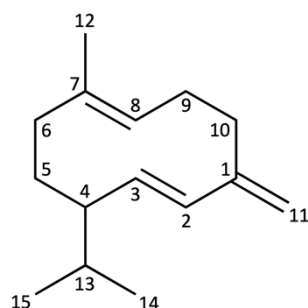
Characterization of the oil fractions enabled to identify the presence of germacrene-D (**Table 3.11**) due to the observation of an isolated duplet at δ 5.81 ppm (J = 15.6 Hz) in one of the analyzed spectra. This key proton signal showed two and three bonds correlations with the quaternary carbon near the double bond at δ 148.8 ppm and terminal alkene at δ 109.0 ppm.

β -caryophyllene (**Table 3.12**) could be only identified and characterized due to the fractionation of the oil by column chromatography. In the ^1H NMR spectrum, two prominent broad singlets stand out at δ 4.85 ppm and δ 4.97 ppm that were assigned to the two protons of the terminal double bond. Correlations of these two signals in the bidimensional spectra led to the full carbon characterization. In particular, the characterization of the carbons of the two double bonds was well established: C-1 at δ 135.4 ppm, C-8 at δ 154.6 ppm, C-11 at δ 124.3 ppm, and C-15 at δ 112.1 ppm.

The identification and characterization of caryophyllene oxide (**Table 3.13**) was also possible due to the fractionation of the oil. Like the previous constituent, this one showed two prominent broad singlets at δ 4.84 ppm and δ 4.96 ppm, assigned to the two protons from the terminal double bond. Once more, these two signals allowed the identification of the others by bidimensional techniques. Full carbon

characterization was also achieved. The characteristic carbon signals from epoxides could be detected at δ 59.8 ppm (C-1) and δ 63.7 ppm (C-11), and alkenes appeared at δ 151.7 ppm (C-8) and δ 112.7 ppm (C-15). This compound was also found in thyme essential oil. Since this oil has less complicated spectra, it was possible to complete a little more the NMR data of caryophyllene oxide, assigning the three signals from its methyl groups: H-12 at δ 1.20 ppm (s), H-13 at δ 1.10 ppm (s) and H-14 at δ 1.08 ppm (s).

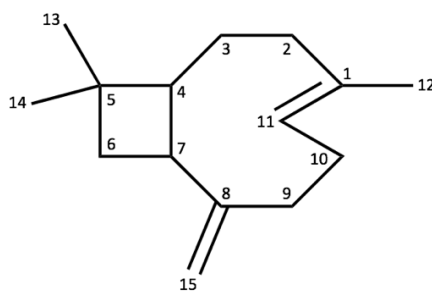
Table 3.11 – ^1H and ^{13}C NMR data experimentally obtained for the compound germacrene-D (^1H NMR: 400 MHz CDCl_3 ; ^{13}C NMR: 100 MHz CDCl_3)



Germacrene-D ^1H NMR				
δ (ppm)	integration	Multiplicity	J (Hz)	Assignment
5.81	1	d	15.6	H-2
Germacrene-D ^{13}C NMR				
δ (ppm)	Assignment			
148.8	C-1			
135.4	C-2			
52.9	C-4			
34.5	C-10			
109.0	C-11			

d - duplet.

Table 3.12 – ^1H and ^{13}C NMR data experimentally obtained for the compound β -caryophyllene (^1H NMR: 400 MHz CDCl_3 ; ^{13}C NMR: 100 MHz CDCl_3)

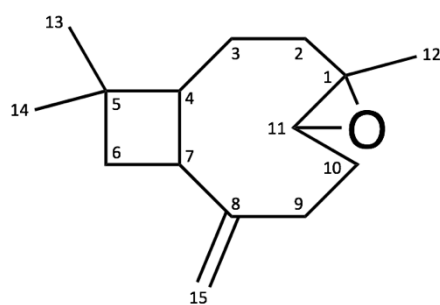


β -caryophyllene ^1H NMR				
δ (ppm)	integration	Multiplicity	J (Hz)	Assignment
2.35	1	quart.	9.2	H-7
2.23	2	mult.	—	H-9
5.34	1	mult.	—	H-11
4.85	1	br s	—	H-15a
4.97	1	br s	—	H-15b

β -caryophyllene ^{13}C NMR	
δ (ppm)	Assignment
135.4	C-1
40.0	C-2
29.4	C-3
53.6	C-4
33.0	C-5
40.4	C-6
48.5	C-7
154.6	C-8
34.9	C-9
28.4	C-10
124.3	C-11
16.3	C-12
30.1	C-13
22.6	C-14
112.1	C-15

br s - broad singlet; quart. - quartet; mult. - multiplet.

Table 3.13 – ^1H and ^{13}C NMR data experimentally obtained for the compound caryophyllene oxide (^1H NMR: 400 MHz CDCl_3 ; ^{13}C NMR: 100 MHz CDCl_3)



Caryophyllene oxide ^1H NMR				
δ (ppm)	integration	Multiplicity	J (Hz)	Assignment
2.59	1	mult.	—	H-7
2.86	1	dd	10.8; 4.4	H-11
1.20	3	s	—	H-12
1.10	3	s	—	H-13
1.08	3	s	—	H-14
4.84	1	br s	—	H-15a
4.96	1	br s	—	H-15b
Caryophyllene oxide ^{13}C NMR				
δ (ppm)	Assignment			
59.8	C-1			
39.1	C-2			
27.1	C-3			
50.7	C-4			
33.9	C-5			
39.7	C-6			
48.7	C-7			
151.7	C-8			
30.0	C-9			
32.5	C-10			
63.7	C-11			
16.9	C-12			

br s - broad singlet; dd - double duplet; mult. - multiplet.

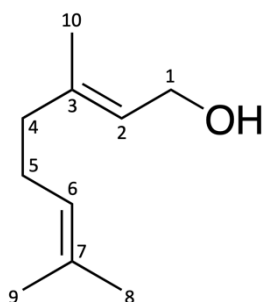
Table 3.13 (cont.) – ^1H and ^{13}C NMR data experimentally obtained for the compound caryophyllene oxide (^1H NMR: 400 MHz CDCl_3 ; ^{13}C NMR: 100 MHz CDCl_3)

Caryophyllene oxide ^{13}C NMR	
δ (ppm)	Assignment
29.8	C-13
21.5	C-14
112.7	C-15

The fractionation of the lemon balm oil also allowed the identification of some other compounds. Geraniol (**Table 3.14**) and nerol (**Table 3.15**) were identified by their duplets at δ 4.14 ppm ($J = 7.2$ Hz) and at δ 4.08 ppm ($J = 6.8$ Hz), respectively, assigned to the CH_2 near the hydroxyl group (H-1). These methylenic carbons (CH_2) appeared as signals with chemical shifts at δ 59.3 ppm (geraniol) and at δ 58.9 ppm (nerol). By HMBC and DEPT135 data, it was possible to assign the signals from the carbon in the nearest alkene (C-2) at δ 124.2 ppm, for geraniol, and δ 125.5 ppm, for nerol. Likewise, its quaternary carbon was identified at δ 139.5 ppm and δ 141.3 ppm, following the same order. A signal observed at δ 9.75 ppm (t, $J = 2.4$ Hz) suggested the presence of the aldehyde of citronellal but, since it was at such low concentration, none correlation was established in bidimensional techniques.

Ocimene was also identified with different signals for its *cis* and *trans* isomers (**Tables 3.16** and **3.17**, respectively). The proton from the terminal alkene (H-2) has a double double duplet at δ 6.82 ppm ($J = 17.2$; 10.8; 0.8 Hz), for *cis*-ocimene, and a double duplet at δ 6.38 ppm ($J = 17.2$; 10.8 Hz), in the case of *trans*-ocimene. Likewise, the carbons attached to these hydrogens (C-2) showed significantly different signals: δ 133.4 ppm for *cis*-ocimene and δ 141.5 ppm for *trans*-ocimene. Moreover, the signals of the nearest methyl group (C-10) showed differences in its chemical shifts depending on the isomer analyzed: δ 19.7 ppm (*cis*-ocimene) and δ 11.6 ppm (*trans*-ocimene).

Table 3.14 – ^1H and ^{13}C NMR data experimentally obtained for the compound geraniol (^1H NMR: 400 MHz CDCl_3 ; ^{13}C NMR: 100 MHz CDCl_3)

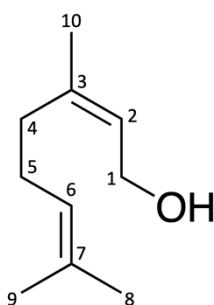


Geraniol ^1H NMR				
δ (ppm)	integration	Multiplicity	J (Hz)	Assignment
4.14	2	d	7.2	H-1

Geraniol ^{13}C NMR	
δ (ppm)	Assignment
59.3	C-1
124.2	C-2
139.5	C-3

d - duplet.

Table 3.15 – ^1H and ^{13}C NMR data experimentally obtained for the compound nerol (^1H NMR: 400 MHz CDCl_3 ; ^{13}C NMR: 100 MHz CDCl_3)

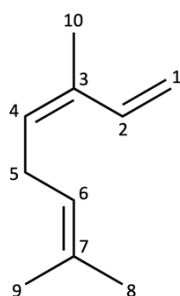


Nerol ^1H NMR				
δ (ppm)	integration	Multiplicity	J (Hz)	Assignment
4.08	2	d	6.8	H-1

Nerol ^{13}C NMR	
δ (ppm)	Assignment
58.9	C-1
125.5	C-2
141.3	C-3

d - duplet.

Table 3.16 – ^1H and ^{13}C NMR data experimentally obtained for the compound *cis*-ocimene (^1H NMR: 400 MHz CDCl_3 ; ^{13}C NMR: 100 MHz CDCl_3)

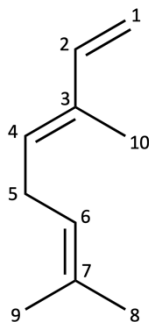


<i>cis</i> -ocimene ^1H NMR				
δ (ppm)	integration	Multiplicity	J (Hz)	Assignment
6.82	1	ddd	17.2; 10.8; 0.8	H-2

<i>cis</i> -ocimene ^{13}C NMR	
δ (ppm)	Assignment
133.4	C-2
131.7	C-4
19.7	C-10

ddd - double double duplet.

Table 3.17 – ^1H and ^{13}C NMR data experimentally obtained for the compound *trans*-ocimene (^1H NMR: 400 MHz CDCl_3 ; ^{13}C NMR: 100 MHz CDCl_3)



<i>trans</i> -ocimene ^1H NMR				
δ (ppm)	integration	Multiplicity	J (Hz)	Assignment
6.38	1	dd	17.2; 10.8	H-2

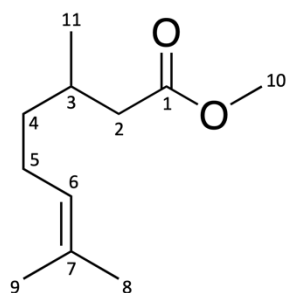
<i>trans</i> -ocimene ^{13}C NMR	
δ (ppm)	Assignment
141.5	C-2
131.7	C-4
11.6	C-10

dd - double duplet.

Additionally, there are four prominent isolated signals in lemon balm essential oil spectrum that were not definitely identified. The two proton signals at δ 3.68 ppm and δ 3.66 ppm were initially identified as a duplet, since they have similar 2D NMR spectra. However, they showed correlations to different carbons at δ 175-165 ppm, revealing that they must be associated with two individual signals, which may be probably due to two quite similar structures, like enantiomers. From the list of components obtained by GC-MS analysis, the only compound that could produce these signals was methyl citronellate (**Table 3.18**). However, this suggestion could only explain one of the singlets at δ 3.7-3.6 ppm.

Other unidentified signals involved a triplet at δ 4.21 ppm ($J = 5.6$ Hz), apparently originated from an OCH_2 group, and two double duplets at δ 7.70 ppm and δ 7.52 ppm ($J = 5.6; 3.2$ Hz) suggesting the presence of two aromatic protons. HMBC data showed that these three signals correlate to the same carbon at δ 167.7 ppm, suggesting that they should be originated from structural unities incorporated into the same molecule. This constituent was not reported in the GC-MS analysis, since none of the identified components could exhibit such NMR spectrum. Then, a literature search was carried out looking for components of reported essential oils that could fit these signals and all their corresponding correlations. Any results were found, suggesting that this unknown compound should be quite unusual. Although proton (δ 7.5-7.7 ppm) and carbon signals (approximately δ 130 ppm) were typical of an aromatic ring, the coupling constants of the double duplets ($J = 5.6; 3.2$ Hz) did not fit with a benzene ring and led to suspect of the presence of a heterocyclic ring. These characteristic coupling constants fit well with coupling constants of a thiophene ring, making thiophenes strong candidates to explain these signals. Additionally, the triplet signal at δ 4.21 ppm ($J = 5.6$ Hz) showed correlations with carbon signals with low chemical shifts (δ 20-40 ppm). Thus, all these data suggested that this compound should incorporate a thiophene ring, an alkyl chain and a carbonyl group that is close to the thiophene and to a CH_2 . ^1H and ^{13}C chemical shifts data of this molecule are shown in **Table 3.18** and were associated with a proposal of a structural fragment that fits all signals. Additionally, parts of the ^1H NMR spectra corresponding to the proposed assignments are shown in **Figure 3.1**.

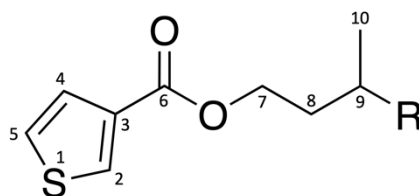
Table 3.18 – ^1H and ^{13}C NMR data experimentally obtained for the compound methyl citronellate (^1H NMR: 400 MHz CDCl_3 ; ^{13}C NMR: 100 MHz CDCl_3)



Methyl citronellate ^1H NMR				
δ (ppm)	integration	Multiplicity	J (Hz)	Assignment
3.66	3	s	—	H-10
Methyl citronellate ^{13}C NMR				
δ (ppm)	Assignment			
173.7	C-1			
40.3	C-2			
29.5	C-3			
50.7	C-10			

s - singlet.

Table 3.19 – ^1H and ^{13}C NMR data identified for the unknown compound and the structural fragment proposed (^1H NMR: 400 MHz CDCl_3 ; ^{13}C NMR: 100 MHz CDCl_3)



Thiophene derivative ^1H NMR				
δ (ppm)	integration	Multiplicity	J (Hz)	Assignment
7.70	1	dd	5.6; 3.2	H-4
7.52	1	dd	5.6; 3.2	H-5
4.21	2	t	5.6	H-7

Thiophene derivative ^{13}C NMR		Correlations
δ (ppm)	Assignment	HMBC
167.7	C-6	H-4, H-7
132.8	C-3	H-4, H-5
128.7	C-4	H-5
130.8	C-5	H-4
68.0	C-7	—
38.7	C-8	H-7
30.0	C-9	H-7
23.7	C-10	H-7

dd - double duplet; t - triplet.

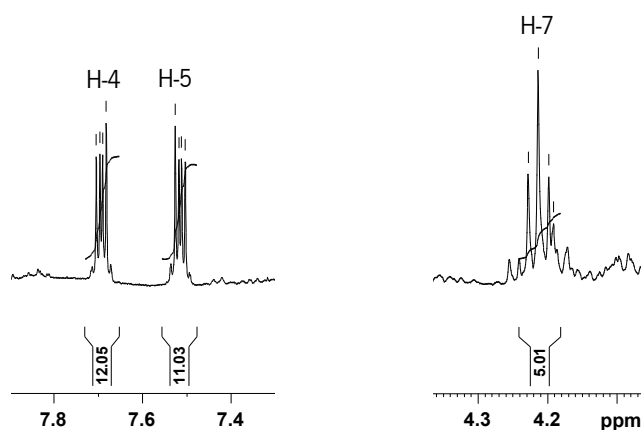


Figure 3.1 – Isolated signals in the ^1H NMR spectra of a fraction extracted with 50% petroleum ether - 50% ethyl acetate, and their assignments to the proposed structure. Do not consider these integrations, once these signals here presented are from different expansions.

NMR is a powerful method for structural characterization of natural products, but it does not possess the capability of separation. However, separation could be achieved by complementary separation techniques, like column chromatography. This technique is quite time-consuming, but it has to be done only once to identify the signals which appeared isolated in the total spectra and these data may be used later for qualitative or quantitative routine analysis. In **Table 3.20** is listed the eluotropic series used in the column, with crescent polarity, and the compounds identified in each fraction. With this data, it was also possible to assess the polarity of the compounds.

Table 3.20 – List of the eluotropic series used in the fractionation of essential oils by column chromatography and the compounds identified in each fraction

Eluotropic series	Identified compounds in essential oils	
	Thyme	Lemon balm
100 % PE	<i>p</i> -cymene γ -terpinene Myrcene β -caryophyllene	β -caryophyllene <i>cis</i> -ocimene <i>trans</i> -ocimene Germacrene-D
90 % PE – 10 % EA	<i>p</i> -cymene	<i>cis</i> -ocimene <i>trans</i> -ocimene
75 % PE – 25 % EA	Thymol Carvacrol Thymol methyl ether Carvacrol methyl ether Caryophyllene oxide	—
50 % PE – 50 % EA	Thymol Linalool Carvacrol	Caryophyllene oxide Geranial Neral Citronellal Methyl citronellate Thiophene derivative Geraniol Nerol
25 % PE – 75 % EA	Thymol Linalool Carvacrol Geranial	Geranial Neral
10 % PE – 90 % EA	—	—
100 % EA	—	—

PE - petroleum ether; EA - ethyl acetate;

Despite the complexity of essential oils and consequent overlap of signals, this previous fractionation of the oils allowed the identification of 11 constituents of thymol essential oil and 10 constituents of lemon balm oil, showing that NMR could be a useful technique to a quick assessment of their composition.

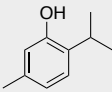
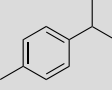
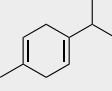
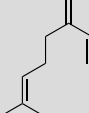
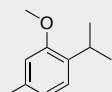
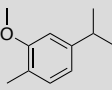
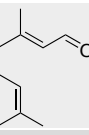
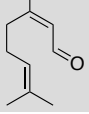
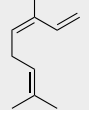
3.1.1.1.2. Quantitative analysis

In this study, a qualitative analysis of the composition of the essential oils was carried out combining NMR characterization and separation of a series of minor components of essential oils by column chromatography, allowing to achieve a key set of data for quantitative characterization.

The quantitative ^1H NMR technique using anthracene as internal standard and deuterated dimethylsulfoxide as solvent, enabled to access the quantitative composition of all the major components and also some of the minor components present in thyme essential oil. On the contrary, in lemon balm oil, the main constituents were not quantified due to the overlap of signals. Likewise, due to the complexity of the total spectrum of lemon balm, only a few compounds were able to be measured in this oil. **Table 3.21** show a list of the compounds quantified by ^1H NMR, values of their respective concentration and notes giving a comparison to GC-MS results were also incorporated. The most significant spectra obtained in this analysis are presented in **Annexes - Group B**.

These results demonstrate that the quantitative characterization of essential oils by ^1H NMR technique may be used for essential oils with moderate complexity, like thyme, as an alternative to the commonly used GC-MS analysis. Although it is not a methodology that gives a complete list of components, quantitative NMR demonstrated its potentiality to be a useful tool to a quick assess of the quality of essential oils, since their commercial value is evaluated according to their main compounds.

Table 3.21 – Quantitative analysis of thyme and balm essential oils by ^1H NMR using the internal standard method ^x

^1H NMR quantitative			
	Name	Structure	Concentration (g/L)
Thyme	Thymol		510.51 ± 136.21^A
	p-cymene		441.06 ± 131.17^B
	γ -terpinene		154.54 ± 50.87^B
	Myrcene		35.62 ± 15.88^A
	Thymol methyl ether		9.82 ± 1.95^B
	Carvacrol methyl ether		6.55 ± 1.30^B
Lemon balm	Geranial		47.18 ± 10.25^A
	Neral		29.39 ± 4.19^B
	<i>trans</i> -ocimene		27.55 ± 8.01^A

^x anthracene was used as internal standard and $\text{DMSO-}d_6$ was used as solvent

^A no significant differences between the concentrations obtained by NMR and GC-MS ($p > 0.05$, one-way ANOVA test).

^B concentrations obtained by NMR and GC-MS are statistically different ($p < 0.05$, one-way ANOVA test).

3.1.1.2. Gas Chromatography – Mass Spectroscopy

The analysis of the essential oils by GC-MS, confirmed that they are complex mixtures of compounds. The identification was performed comparing mass spectra and retention indices with those of pure standard compounds. In some cases, constituents were identified by using commercial libraries and consulting published data. In thyme essential oil (**Table 3.22** and **Graph C.1** in Annex), 39 constituents were identified, and 2 uncommon compounds are believed to be thymol and carvacrol derivatives, due to their mass spectra. In lemon balm essential oil (**Table 3.23** and **Graph C.2** in Annex) 49 compounds were identified. In both oils, several minor constituents were not identified, since their spectra were not similar to none of the available standards nor were found in published data or libraries.

Like NMR, this technique showed that thymol, *p*-cymene and γ -terpinene are the main compounds of thyme oil. However, here *p*-cymene is found in higher quantities than thymol, contrary to quantitative NMR results. Carvacrol, borneol, linalool, myrcene, α -thujene, limonene and 1,8-cineole are examples of other constituents of this oil according to GC-MS analysis. Initially, thymol methyl ether and carvacrol methyl ether were not identified by this technique. Their mass spectra gave some clues, but they were not assigned with certainty to any compounds. Their presence was firstly noticed by NMR analysis, providing a very important clue for their identification by GC-MS. The knowledge of their structures and quantification of their NMR signals, allowed the association of these data with the two previously unknown GC-MS peaks.

Lemon balm essential oil is also a mixture of compounds, even more complex than thyme oil, as it was suggested by NMR. Contrary to thyme, lemon balm only has one main constituent that is β -caryophyllene. GC-MS analysis showed that other important components of this oil are δ -cadinene, caryophyllene oxide, citronellol, geranial, α -copaene and methyl citronellate.

Analysis of these results revealed some limitations of the method, in particular the fact that at least some compounds were overestimated, mostly in thyme. This could be noticed since the sum of the concentration of the identified constituents was higher than the density of the oil and even not all compounds were considered. To avoid errors, more analysis should be made, adjusting the concentration of the internal standard (higher concentration) and performing new calibrations for each compound.

Table 3.22 – Quantitative analysis of thyme essential oil by GC-MS, and their corresponding concentration and Linear Retention Index (LRI) on a column Sapiens-Was MS

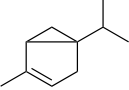
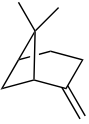
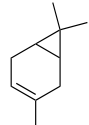
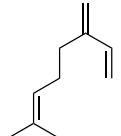
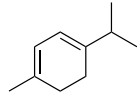
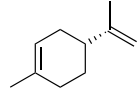
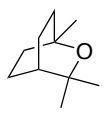
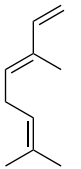
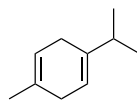
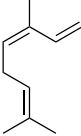
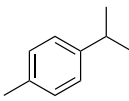
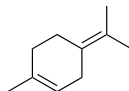
	Name	Structure	LRI	C (g/L)
1	α -pinene		1022	11.77 ± 2.50
2	α -thujene		1033	22.93 ± 3.15
3	camphene		1070	10.12 ± 1.76
4	β -pinene		1108	6.04 ± 0.97
5	δ -3-carene		1145	2.29 ± 0.63
6	myrcene		1160	37.98 ± 7.53
7	α -terpinene		1176	7.97 ± 1.67
8	limonene		1195	10.47 ± 1.96
9	1,8-cineole		1202	21.85 ± 4.24
10	<i>cis</i> -ocimene		1228	0.23 ± 0.12
11	γ -terpinene		1239	201.01 ± 40.71
12	<i>trans</i> -ocimene		1244	0.70 ± 0.32
13	<i>p</i> -cymene		1261	617.82 ± 137.64
14	terpinolene		1273	1.35 ± 0.29

Table 3.22 (cont.) – Quantitative analysis of thyme essential oil by GC-MS, and their corresponding concentration and Linear Retention Index (LRI) on a column Sapiens-Was MS

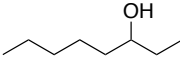
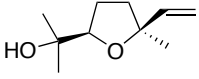
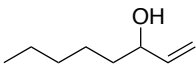
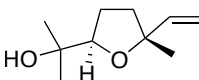
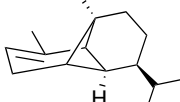
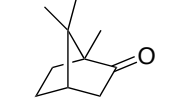
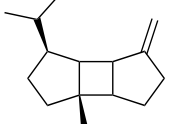
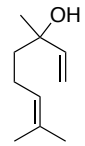
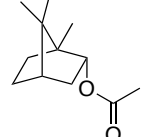
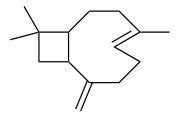
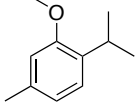
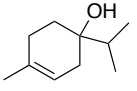
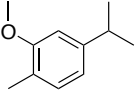
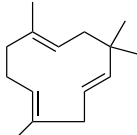
	Name	Structure	LRI	C (g/L)
15	3-octanol		1387	1.85 ± 0.75
16	furan linalool oxide I		1430	1.98 ± 0.32
17	1-octen-3-ol		1443	14.24 ± 2.19
18	furan linalool oxide II		1458	1.38 ± 0.37
19	α-copaene		1489	0.94 ± 0.21
20	camphor		1490	7.42 ± 6.34
21	β-bourbonene		1502	1.92 ± 0.45
22	linalool		1540	52.71 ± 10.29
23	bornyl acetate		1562	3.56 ± 0.55
24	β-caryophyllene		1575	10.53 ± 1.28
25	thymol methyl ether		1576	28.16 ± 4.37
26	terpinen-4-ol		1584	18.74 ± 3.14
27	carvacrol methyl ether		1591	17.13 ± 2.75
28	α-humulene		1644	0.33 ± 0.13

Table 3.22 (cont.) – Quantitative analysis of thyme essential oil by GC-MS, and their corresponding concentration and Linear Retention Index (LRI) on a column Sapiens-Was MS

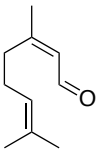
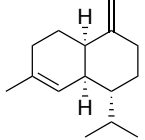
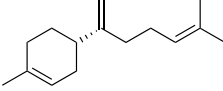
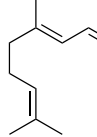
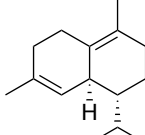
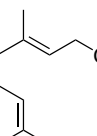
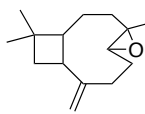
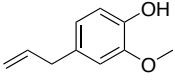
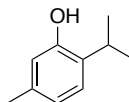
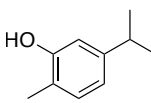
	Name	Structure	LRI	C (g/L)
29	neral		1663	0.41 ± 0.09
30	γ-muurolene		1667	3.41 ± 0.38
31	borneol		1680	29.66 ± 4.12
32	β-bisabolene		1711	1.33 ± 0.10
33	geranial		1713	0.71 ± 0.13
34	δ-cadinene		1735	4.74 ± 1.18
35	geraniol		1833	1.68 ± 0.11
36	caryophyllene oxide		1943	30.34 ± 3.68
37	eugenol		2132	0.46 ± 0.04
38	thymol derivative	?	2144	6.36 ± 0.93
39	thymol		2161	606.61 ± 107.02
40	carvacrol derivative	?	2178	6.73 ± 1.17
41	carvacrol		2186	41.92 ± 9.96

Table 3.23 – Quantitative analysis of lemon balm essential oil by GC-MS, and their corresponding concentration and Linear Retention Index (LRI) on a column Sapiens-Was MS

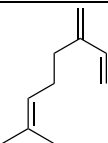
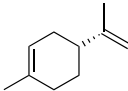
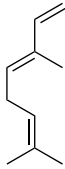
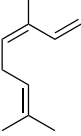
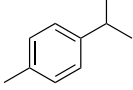
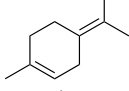
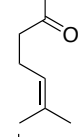
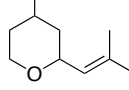
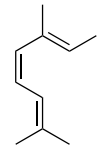
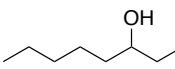
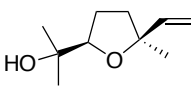
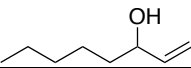
	Name	Structure	LRI	C (g/L)
1	myrcene		1160	1.75 ± 0.23
2	limonene		1195	4.72 ± 0.79
3	1,8-cineole		1202	0.67 ± 0.19
4	<i>cis</i> -ocimene		1228	2.23 ± 0.34
5	<i>trans</i> -ocimene		1244	24.59 ± 2.78
6	<i>p</i> -cymene		1261	4.34 ± 0.53
7	terpinolene		1273	0.35 ± 0.07
8	6-methyl-5-hepten-2-one		1330	20.33 ± 3.71
9	rose oxide I		1342	7.81 ± 1.04
10	rose oxide II	?	1355	3.05 ± 0.35
11	allocymene I		1364	1.43 ± 0.16
12	3-octanol		1387	2.02 ± 0.21
13	furan linalool oxide I		1430	0.79 ± 0.23
14	1-octen-3-ol		1443	6.98 ± 0.88

Table 3.23 (cont.) – Quantitative analysis of lemon balm essential oil by GC-MS, and their corresponding concentration and Linear Retention Index (LRI) on a column Sapiens-Was MS

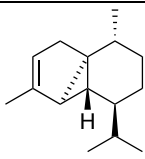
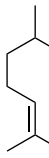
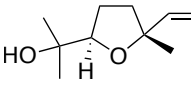
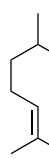
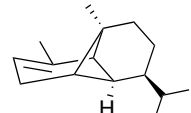
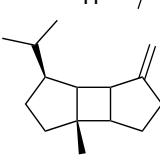
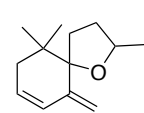
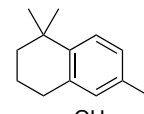
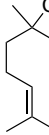
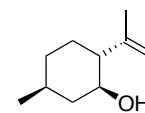
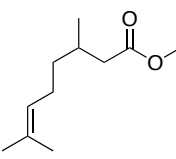
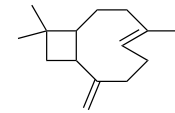
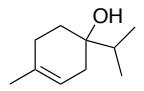
	Name	Structure	LRI	C (g/L)
15	α -cubebene		1447	3.78 ± 0.38
16	6-methyl-5-hepten-2-ol		1456	0.51 ± 0.07
17	furan linalool oxide II		1458	0.50 ± 0.09
18	citronellal		1468	4.60 ± 0.43
19	α -copaene		1489	43.36 ± 9.09
20	β -bourbonene		1502	19.96 ± 4.05
21	vitispirane		1512	0.28 ± 0.09
22	α -ionene		1534	2.78 ± 0.55
23	linalool		1540	26.52 ± 4.34
24	isopulegol I		1543	2.74 ± 0.21
25	methyl citronellate		1556	36.11 ± 3.00
26	β -caryophyllene		1575	357.86 ± 74.25
27	terpinen-4-ol		1584	0.70 ± 0.11

Table 3.23 (cont.) – Quantitative analysis of lemon balm essential oil by GC-MS, and their corresponding concentration and Linear Retention Index (LRI) on a column Sapiens-Was MS

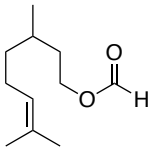
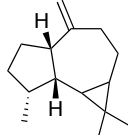
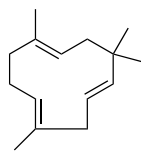
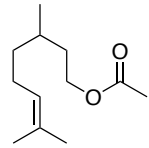
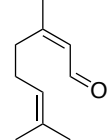
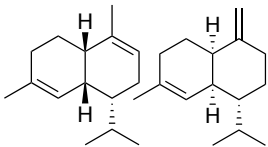
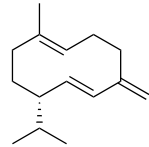
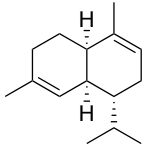
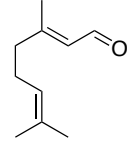
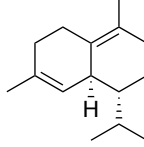
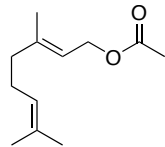
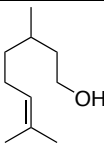
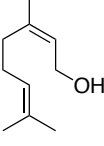
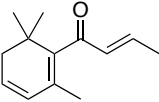
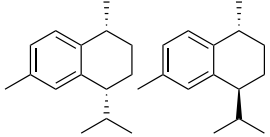
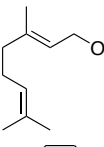
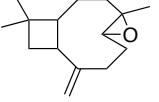
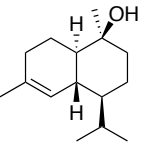
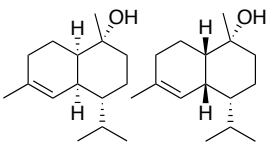
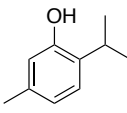
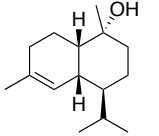
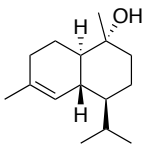
	Name	Structure	LRI	C (g/L)
28	citronellyl formate		1610	5.23 ± 0.37
29	allo-aromadendrene		1621	10.66 ± 1.87
30	α-humulene		1644	26.11 ± 4.98
31	citronellyl acetate		1651	2.40 ± 0.55
32	neral		1663	25.17 ± 4.79
33	α-amorphene/ γ-muurolene		1664	31.52 ± 5.68
34	germacrene D		1684	22.07 ± 3.56
35	α-muurolene		1703	27.76 ± 5.83
36	geranial		1713	43.98 ± 5.35
37	δ-cadinene		1735	89.59 ± 8.39
38	geranyl acetate		1746	7.54 ± 1.34

Table 3.23 (cont.) – Quantitative analysis of lemon balm essential oil by GC-MS, and their corresponding concentration and Linear Retention Index (LRI) on a column Sapiens-Was MS

	Name	Structure	LRI	C (g/L)
39	citronellol		1755	48.85 ± 5.48
40	nerol		1785	13.01 ± 2.05
41	β-damascenone		1795	3.13 ± 0.39
42	<i>cis-/trans</i> -calamenene		1803	5.93 ± 0.76
43	geraniol		1833	17.50 ± 3.05
44	caryophyllene oxide		1943	70.78 ± 17.58
45	τ-cadinol		2146	9.11 ± 1.84
46	τ-/α-muurolol		2155	13.88 ± 3.36
47	thymol		2161	5.48 ± 0.30
48	δ-cadinol		2168	3.05 ± 0.39
49	α-cadinol		2197	25.6 ± 6.12

3.1.1.3. Comparison between NMR and GC-MS

In this work, thyme and lemon balm essential oils were quantitatively analyzed by NMR and GC-MS. GC-MS showed more complete results due to its sensitivity and ability to separate the constituents. The statistical test One-Way ANOVA (for 95% of probability) was performed to compare the concentrations obtained by NMR and GC-MS (see superscript letters in **Table 3.21**). For thyme essential oil, the results were statistically different in 2/3 of the compounds quantified by both techniques. In the case of lemon balm oil, they were statistically different in 1/3 of the components. Various studies of essential oils or some of their constituents, showed that these two methods provided equal results (AbouZid, 2016; Cerceau et al., 2016, 2016b; Pieri et al., 2012). However, in the analysis of essential oils from different species of *Ocimum*, Freitas *et al.* (2018) obtained experimental results where NMR gave higher, lower and equal values than the ones obtained by gas chromatography, showing that the variability found in this study is not unique. However, it could be said that these differences were already expected, since at least some compounds were overestimated in the GC-MS analysis. In fact, in the majority of the cases where NMR and GC-MS did not give the same results, the concentrations from GC-MS were much higher than the ones found in NMR. Thus, it could be said that NMR gave reliable results, once they were equal or lower than the ones obtained from GC-MS.

The long list of compounds identified in this work, even the trace components, demonstrated that chromatography is really a powerful technique as it allows to identify several constituents of essential oils in the same analysis regardless their complexity. However, this method is highly time consuming and its results are not always perfect, since here it gave concentrations higher than the real ones. On the other hand, NMR is an easy and quick technique that produced reliable results and could be quite useful for quality control. For these reasons, quantitative NMR seems to be a good approach for routine analysis of essential oils of low to moderate complexity.

3.1.1.4. Fourier-transform infrared spectroscopy

Essential oils were also analyzed by Fourier Transform Infrared Spectroscopy (FTIR). This technique allowed to confirm the presence of the functional groups belonging to some of the main compounds previously identified and the results are represented in **Figure 3.3**. For both oils, the OH band of alcohols is well visible at 3600-3100 cm^{-1} . The stretching region of the sp^3 CH (2960-2860 cm^{-1}) had intense and broad bands and the signals of the carbonyls are also present at 1720-1710 cm^{-1} . Thyme oil showed a pair of bands at 1618 and 1581 cm^{-1} that were assigned to the C=C bond vibration in aromatic

alkenes, and since its two main compounds have aromatic rings, these peaks are well visible. The other signals found in the fingerprint region correspond to the bending vibrations of the main constituents of thyme oil: *p*-cymene, thymol and γ -terpinene. Here it could be highlighted the strong signal at 813 cm^{-1} from the overlap of the out-of-plane C-H wagging vibration of *p*-cymene and thymol. In the spectrum of lemon balm oil, characteristic bands of its main compound – β -caryophyllene are well visible. At $1450\text{--}1360\text{ cm}^{-1}$ bands related to CH_2 deformation modes were observed and a signal from the wagging vibration of the alkene terminal is visible at 885 cm^{-1} .

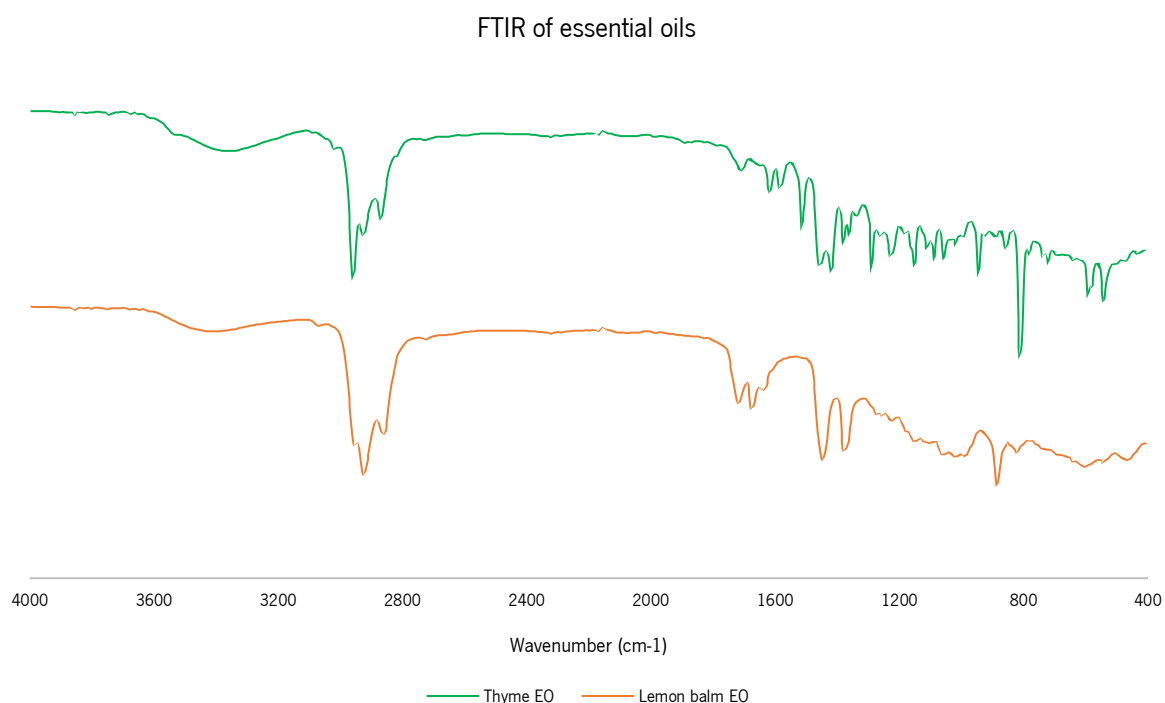


Figure 3.2 – FTIR spectra of thyme and lemon balm essential oils.

3.2. Materials and Methods

3.2.1. Raw Materials

Pure essential oils of thyme (*Thymus vulgaris*) and lemon balm (*Melissa officinalis*) were supplied by the company Earth Essences (Portugal). Petroleum ether (CAS 64742-49-0), ethyl acetate (CAS 141-78-6), Acetone (CAS 67-64-1) and hexane (CAS 92112-69-1) were acquired from Fisher Scientific, USA. Toluene (131745) and tetrahydrofuran (133537) were obtained from Panreac, Spain. Deuterated chloroform (D007H) was from Eurisotop, France. Deuterated dimethylsulfoxide (CAS 2206-27-1) and anthracene (99%, CAS 120-12-7) were from Acros Organics, USA. Dichloromethane (ref. 1.06054) was purchased from Merck (Germany), 2-octanol (74860) from Sigma-Aldrich (USA), and helium GHE4x from Praxair (USA). It was used silica gel 60 of granulometry 0.063-0.200 mm from Merck (Germany), and silica gel plates in aluminum support with ultraviolet fluorescence indicator ALUGRAM Xtra SIL G/UV₂₅₄ from Macherey-Nagel, Germany. The Sapiens-WaxMS column (30 m x 0.15 mm; 0.15 µm film thickness) was purchased from Teknokroma, Spain.

3.2.2. Essential oils fractionation - Column Chromatography

Fractionation of the essential oils was performed by column chromatography in a column (2x22 cm) prepared with Silica gel 60 (granulometry 0.063-0.200 mm). For thyme essential oil fractionation, the sequence of eluents used was: 100% petroleum ether (PE); 90% PE-10% ethyl acetate (EA); 75% PE - 25% EA; 50% PE - 50% EA; 25% PE - 75% EA; 100% EA. In the fractionation of the lemon balm essential the sequence of eluents used was: 100% PE; 90% PE - 10% EA; 75% PE - 25% EA; 50% PE - 50% EA; 25% PE - 75% EA; 10% PE - 90% EA; 100% AE; 100% Acetone. The introduction of eluents into the column was monitored by TLC analysis of the eluted fractions and similar fractions were combined. The fractions or set of fractions were then concentrated on the rotary evaporator, removing the maximum of solvent that was possible under reduced pressure using the equipment BUCHI Rotavapor R-114 (Switzerland). The temperature was maintained below 40 °C in a water bath.

3.2.3. Thin Layer Chromatography

Essential oils and their fractions were analyzed by TLC using silica gel plates in aluminum support with ultraviolet fluorescence indicator. Different eluent systems were essayed for TLC: a mixture of hexane and ethyl acetate (50:50) was firstly tested, but the separation was not efficient. Then, two systems described in the literature for the analysis of essential oils were used: (a) mixture of toluene and ethyl

acetate (93:7) (Wagner & Bladt, 2009); (b) hexane and ethyl acetate (75:25) (Purcell et al., 2016). This latter eluent had been selected since a very satisfactory stain separation was observed. Samples of total oil were diluted in tetrahydrofuran (THF) before application on the silica plates. Fractions obtained from separation in the column were analyzed directly. After evaporation of the solvent in the rotary evaporator under reduced pressure, aliquots of the resulting oily residue were diluted in THF and analyzed by TLC in order to compare their composition before and after evaporation. Chromatograms were visualized in ultraviolet lamps at 254 nm.

3.2.4. Nuclear Magnetic Resonance

Samples for ^1H and ^{13}C NMR analysis were obtained by dissolving the total essential oils or the previously fractionated oils in 650 μL of deuterated chloroform (CDCl_3). These studies were carried out in a Bruker Avance 3400 (USA) at 400 MHz for ^1H NMR and 100 MHz for ^{13}C NMR.

Solutions of the total oils were also analyzed in deuterated dimethylsulfoxide ($\text{DMSO}-d_6$) for quantitative analysis. Quantitative ^1H NMR was obtained analyzing $\text{DMSO}-d_6$ solutions of the same oils in the presence of anthracene as internal standard (Pieri et al., 2012), according to the following procedures. For the thyme characterization, 5 mg of anthracene was dissolved in 1 mL of $\text{DMSO}-d_6$. Then, 650 μL of the solution were added to 1 drop of thyme essential oil accurately weighed. For the characterization of lemon balm essential oil, the same procedure was performed, only modifying the quantity of internal standard merely to 1 mg. To calculate the concentration and percentage of each constituent, the following procedure was used: firstly, density and volume of the oil used in the analysis had to be calculated (Eqs. 3.1 and 3.2, respectively); then, quantification of each component (qC) was obtained by Eq. 3.3 (Cerceanu et al., 2016); finally, their concentration (CC) in the essential oil was given by Eq. 3.4.

$$\rho \text{ (density of the oil)} = \frac{\text{mass of oil in } 10 \mu\text{L (mg)}}{10 (\mu\text{L})} = \text{g/mL} \quad \text{Eq. 3.1}$$

$$v \text{ (volume of the oil)} = \frac{\text{mass used in NMR (g)}}{\rho \text{ (g/mL)} \times 1000} = L \quad \text{Eq. 3.2}$$

$$qC \text{ (mg)} = \frac{\text{integration of the compound}}{\text{integration of anthracene (1000)}} \times \frac{\text{number of H in the signal of anthracene}}{\text{number of H in the signal of the compound}} \times \frac{\text{molecular mass of the compound (g/mol)}}{\text{molecular mass of anthracene (g/mol)}} \times \text{mass of anthracene (mg)} \quad \text{Eq. 3.3}$$

$$CC \text{ (concentration of the compound)} = \frac{qC \text{ (mg)}}{v \text{ (L)} \times 1000} = \text{g/L} \quad \text{Eq. 3.4}$$

3.2.5. Gas Chromatography – Mass Spectroscopy

Essential oils (thyme and lemon balm) were firstly dissolved in solvent – 10 μL of the essential oil in 5 mL of dichloromethane. Then, before GC-MS analysis, 0.5 mL of this solution was diluted to a final volume of 5 mL and 2.5068 μg of 2-octanol (internal standard; 100 μL x 25.068 mg/L) were added.

Diluted essential oils were analyzed in a gas chromatograph Varian 3800 (USA) equipped with a 1079 injector and an ion-trap mass spectrometer Varian Saturn 2000 (USA). Each 1 μL injection was made in splitless mode (30 sec.) in a Sapiens-WaxMS column (30 m x 0.15 mm; 0.15 μm film thickness). The carrier gas was helium at a constant flow of 1.3 mL min^{-1} . The detector was set to electronic impact mode with an ionization energy of 70 eV, a mass acquisition range (m/z) from 35 to 300 and 610 ms acquisition interval. The oven temperature was initially set to 60 $^{\circ}\text{C}$ for 2 min and then raised to 234 $^{\circ}\text{C}$ at a rate of 3 $^{\circ}\text{C min}^{-1}$, raised again to 260 $^{\circ}\text{C}$ at 5 $^{\circ}\text{C min}^{-1}$ and finally maintained at 260 $^{\circ}\text{C}$ for 10 min. Injector temperature was set to 250 $^{\circ}\text{C}$ with a 30 mL min^{-1} split flow and transfer line was maintained at 250 $^{\circ}\text{C}$. Compounds were identified using MS Workstation version 7.0.1 (Varian, USA) software, by comparing mass spectra and retention indices with those of pure standard compounds. In some cases, compounds were identified by using commercial libraries (NIST14 and Wiley6, USA) and consulting published data. Quantification was performed as 2-octanol equivalents.

3.2.6. Fourier-transform infrared spectroscopy

FTIR spectra of the oils were recorded with a Bruker FT-IR VERTEX 80/80v (USA) in Attenuated Total Reflectance mode (ATR) in the wavenumber range: 4000-400 cm^{-1} , using 64 scans at a resolution of 4 cm^{-1} . Before analysis, an open bean background spectrum was recorded as a blank.

CHAPTER IV - Nanoencapsulation

4.1. Results and Discussion

4.1.1. The effect of high energy methods on essential oils

One of the reasons for using low energy methods for the production of nanoemulsions against high energy methods is the effect of high shear and temperature (a consequence of high energy methods) on the chemical structure of the encapsulated compounds. The same can happen for the essential oils and, due to this fact, the effect of a high energy method (ultrasounds) on the quality of essential oils was assessed. The assessment was performed by visual and olfactory evaluation and by FTIR. For this comparison, three samples of free oils were used: sunflower oil (used as a control), a mixture of sunflower and thyme oil (8:2) and a mixture of sunflower and lemon balm oil (8:2). After processed with ultra-turrax and ultrasounds, all these samples changed significantly their odor. These results indicate that their composition changed during the high energy process and there were visible some modifications in FTIR spectra, showing that oils actually suffered some chemical changes. FTIR results obtained for thyme and lemon balm essential oils are presented in **Figures 4.1** and **4.2**, respectively.

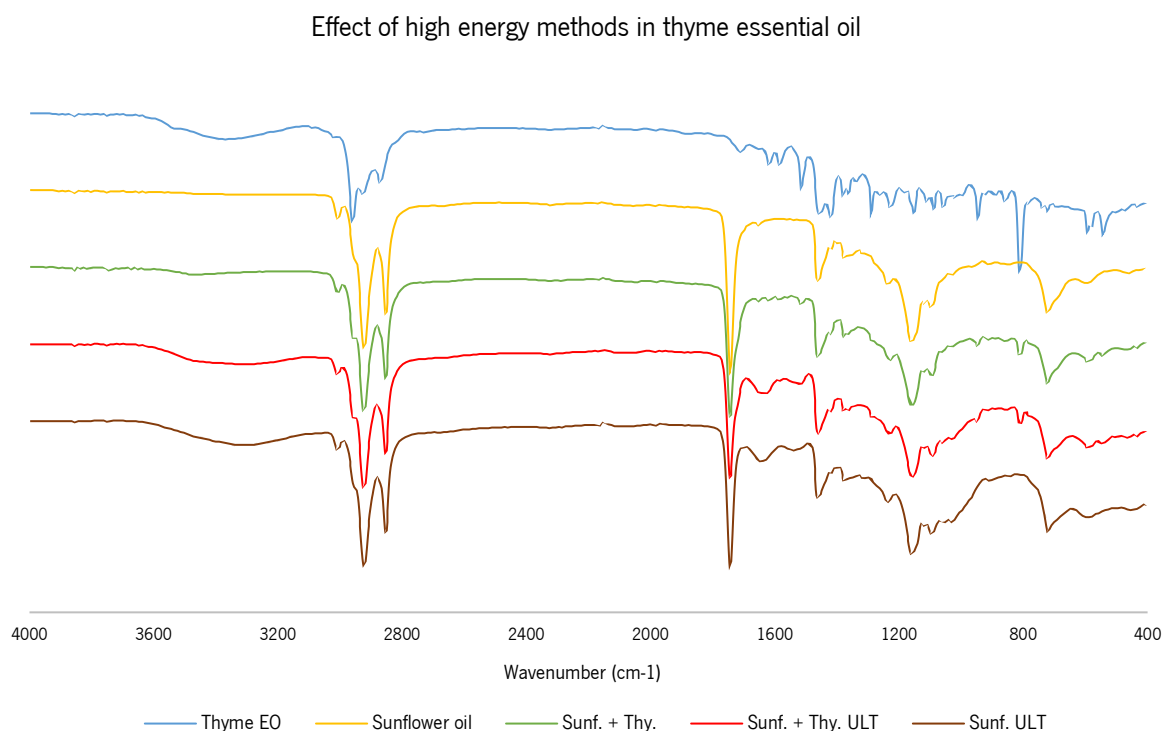


Figure 4.1 – FTIR study on the effect of high energy approaches, namely ultra-turrax and ultrasounds, on the quality of thyme essential oil.

Effect of high energy methods in lemon balm essential oil

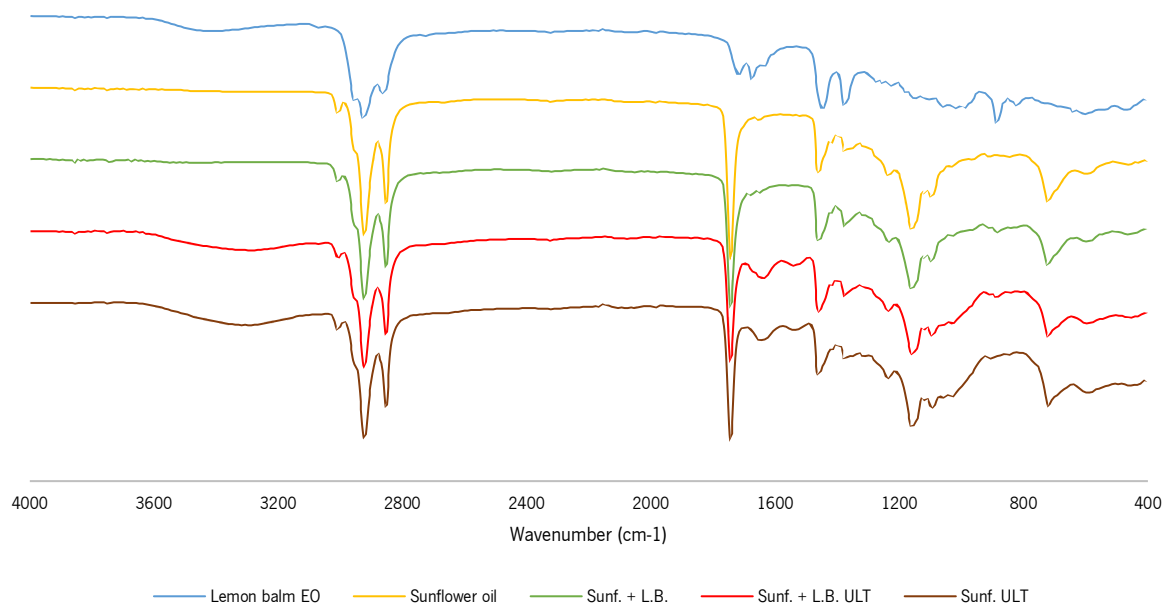


Figure 4.2 – FTIR study on of the effect of high energy approaches, namely ultra-turrax and ultrasonounds, on the quality of lemon balm essential oil.

The ripening inhibitor itself (sunflower oil) was degraded by this high energy approach (yellow vs brown line). This was already expected since even this oil changed extremely its odor after submitted to high energy treatment. It is also possible to see some chemical modifications in mixtures of sunflower oil and essential oil (green vs red lines). In both graphs, there are three regions were these changes are clear, namely in the bands 3650-3050 cm^{-1} , 1700-1500 cm^{-1} and 1020-945 cm^{-1} . The OH broad band that appeared at 3650-3050 cm^{-1} can be explained, for example, by the allylic oxidation that is quite frequent among terpenes. This thermal degradation results in alcohol formation through the oxidation of the carbon adjacent to the carbon-carbon double bond of some compounds. Alterations observed in the 1700-1500 cm^{-1} region could be due to the cleavage of some double bonds. Other thermal degradation that could justify modifications in this zone is the dehydrogenation of terpenes with one or two double bonds in 6 membered rings, leading to the formation of aromatic compounds (McGraw et al., 1999). Then, modifications in the range 1020-945 cm^{-1} are consequence of these degradations, with changes in the stretching vibrations of alkenes and aromatics.

This assessment showed that high energy methods effectively degrade the oils used in this analysis. Therefore, in a future work, it would be interesting to complement this study with the evaluation of oils previously encapsulated, assessing if this behavior is maintained during the encapsulation process.

4.1.2. Emulsification by the EPI method

For the production of nanoemulsions by the EPI method different emulsifying agents were tested.

Table 4.1 presents the emulsifiers used and their main characteristics.

Table 4.1 – Emulsifiers tested in the production of nanoemulsions with the emulsion phase inversion method

Name of the emulsifier	Definition	Information given by the supplier	Supplier
Rhamnolipids	Biosurfactant isolated from microorganisms using fermentation processes (Bai & McClements, 2016)	- From <i>Pseudomonas aeruginosa</i> - Glycolipids containing L-rhamnose and β -hydroxyl fatty acids - Critical micellar concentration: 5-380 mg/L	Sigma-Aldrich
Octenyl succinic anhydride (OSA) modified starch	Biopolymer obtained from the derivatization of starch molecules by their hydrophobic modification with OSA (Chivero et al., 2016)	- Very low viscosity profile - Used to replace gum Arabic in flavor encapsulation and flavor emulsions	Cargill
Sodium carboxymethylcellulose (CMC)	Cellulose ether produced by the reaction of alkali cellulose with sodium monochloroacetate (Arancibia et al., 2016)	- Viscosity: 1500-3000 cP (1% H ₂ O 25 °C) - Solubility in H ₂ O: 10 mg/mL	Sigma-Aldrich
Tween 80	Polyoxyethylene (20) sorbitan monooleate, also called Polysorbate 80, synthetic, non-ionic	- Viscosity: 400-620 cP (25 °C) - Density: 1.064 g/cm ³ - Critical micellar concentration: 13-15 mg/L - Hydrophilic Lipophilic Balance: 15 - Composition: $\geq$ 58% oleic acid	Sigma-Aldrich

4.1.2.1. Rhamnolipids

In the case of rhamnolipids different conditions were tested. These conditions are listed in **Table 4.2**. However, a phase separation, as presented in **Figure 4.3**, always occurred. Also, Haba *et al.* (2014) reported this instability of emulsions produced with rhamnolipids by a low energy method. During the addition of water, the mixture was constantly liquid, without forming any intermediate gel phase as reported in literature. These samples were not analyzed in DLS.

Table 4.2 – Conditions tested for the production of nanoemulsions using rhamnolipids

SOR	Rhamnolipids dissolved in	Time of mixture (h)	Aqueous flux rate (mL/min)
0.11	Oil	0.5	4
0.5	Oil	1	4
1	Oil	2	4
1	Oil	2	2
1	Oil	2	8
1	Water	0.5	4

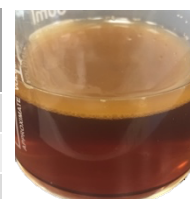


Figure 4.3 – Example of the phase separation that always occurred with rhamnolipids.

4.1.2.2. OSA modified starch

Octenyl succinic anhydride (OSA) modified starch proved to be a better emulsifier than rhamnolipids using the EPI method, even though the results obtained showed that is not possible to obtain nanoemulsions. **Table 4.3** shows the tested conditions in studies with this surfactant and their respective DLS results (after eye inspection some samples were not evaluated by DLS). The results showed that when a SOR of 0.5 is used big drops of oil appeared on the top and a phase separation occurred. Then, for higher SORs, it was not possible to mix OSA starch with the oil using the magnetic bar and a spatula was used. For samples using a SOR of 1, 1.5 and 2, some small drops of oil were visible on the top, alongside some phase separation, being these three samples similar when evaluated by eye inspection. The sample with SOR 1 was analyzed in DLS, having PDI and size values higher than 1 and 2 μm , respectively. In all these tests, a strong, compact and filamentous structure was formed once the water was added to the emulsifier/oil mixture and then it disappeared with the continuous water addition.

Various publications showed that OSA modified starch is usually dissolved in water, instead of oil (Charoen et al., 2012, 2011; Chivero et al., 2016; Liang et al., 2013; Liu et al., 2018; Zhang, Bing, & Reineccius, 2016; Zhong, Wang, & Qin, 2018). For this reason, some tests were carried out by dissolving OSA modified starch in water. In these studies, the mixture remained liquid during the entire procedure, neither forming a gel or other stiff structure. After the addition of the water/surfactant to the oil, many opaque droplets were visible throughout the mixture. In the end, many drops of oil appeared on the top and great phase separation occurred, with the top layer having a yellow tonality. First, a SOR of 1 was tested and it did not form stable emulsions. Secondly, SOR 2 was tried, but only 30 minutes were not enough to dissolve the OSA starch in water, so it was mixed overnight to ensure its full dissolution. Once more, it did not produce satisfactory emulsions. Then, in some publications the OSA starch was dissolved in water at high temperatures (Dokić, Krstonošić, & Nikolić, 2012; Hategekimana et al., 2014; Jafari, He, & Bhandari, 2007; Krstonošić et al., 2015; Li et al., 2018), so the dissolution was tested at 50 and 70 $^{\circ}\text{C}$, however they led to similar results.

Ostertag *et al.* (2012), showed that using the EPI method, the initial surfactant (Tween 80) location has influence on nanoemulsions. Their results revealed that, to have small sizes, the initial quantity of the surfactant in the aqueous phase should not be higher than 50% (Ostertag et al., 2012). For this reason, it was carried out a test with 50% of OSA starch dissolved in oil and 50% dissolved in water. However, once more it was not efficient. OSA modified starch dissolved only in oil at 50 $^{\circ}\text{C}$ was then tested. This sample was not stable but had less drops of oil on the top than the tests with dissolution

in water. These last two samples were analyzed in DLS, having both an enormous PDI (>1) and size ($>8\ \mu\text{m}$). In all these samples great phase separation occurred.

Borrin *et al.* (2016) pointed out that the stirring type has influence on the EPI results. For this reason, the agitation plate was substituted by a metal 4-blade mixer in tests using SOR between 0.5 and 2.5. Note that with SOR 1 or higher, it is difficult to mix OSA with the oil, ever with this stirrer, because in volume there was much more powder than liquid. In all these samples, with the addition of the water, a strong stiff structure was formed, then turning liquid with continuous water addition. It was notorious an improvement with the use of this stirrer and a change with the increase of the SOR. The visual aspect of these emulsions is presented in **Figure 4.4** and DLS results are shown in **Table 4.3**. With a SOR of 0.5, a tenuous phase separation occurred, and big drops of oil were seen on the top. For samples using a SOR of 1, 1.5 and 2, it was observed that after stopping the stirring, the emulsion was homogeneous with foam on the top. This foam disappeared with time and drops of oil appeared on the top. For samples using a SOR of 2.5, the emulsion was homogeneous and creamy. Despite the visual appearance being good, it presents high PDI (1.0 ± 0.6) and size ($2.5 \pm 0.7\ \mu\text{m}$). Samples with a SOR of 1 with a 20 wt% of oil was also tested but no good results were obtained.

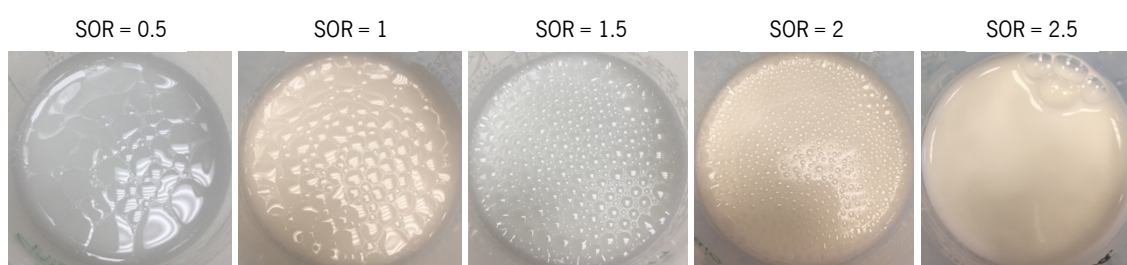


Figure 4.4 – Effect of the alteration of the SOR in the aspect of emulsions made with the 4-blade mixer.

After DLS analysis, some samples were stirred for at least more 16 hours, to see the impact of the second mixture time duration. Both PDI and size values remained enormous: >1 and $>8\ \mu\text{m}$, respectively. For this reason, only 1 hour for the second mixture was maintained in the next studies.

Table 4.3 – Conditions tested for the production of nanoemulsions using OSA modified starch

wt% of oil	SOR	OSA starch dissolved in	1 st mixture	2 nd mixture	PDI	Size (z-average) (µm)
10	0.5	Oil	30 min Agitation plate	1 h Agitation plate	—	—
10	1	Oil	— Spatula	1 h Agitation plate	>1	>8
10	1.5	Oil	— Spatula	1 h Agitation plate	—	—
10	2	Oil	— Spatula	1 h Agitation plate	—	—
10	1	Oil	— Spatula	overnight Agitation plate	>1	>8
10	1	Water	30 min Agitation plate	1 h Agitation plate	—	—
10	2	Water	overnight Agitation plate	1 h Agitation plate	—	—
10	1	Water at 50°C	30 min Agitation plate	1 h Agitation plate	—	—
10	1	Water at 70°C	30 min Agitation plate	1 h Agitation plate	—	—
10	1	50% in oil 50% in water	24 h (1 h at 50°C) Agitation plate	1 h Agitation plate	>1	>8
10	1	Oil at 50°C	45 min Agitation plate	1 h Agitation plate	>1	>8
10	0.5	Oil	30 min 4-blade mixer	1 h 4-blade mixer	>1	>8
10	1	Oil	30 min 4-blade mixer	1 h 4-blade mixer	>1	>8
10	1.5	Oil	30 min 4-blade mixer	1 h 4-blade mixer	>1	>8
10	2	Oil	30 min 4-blade mixer	1 h 4-blade mixer	>1	3.3 ± 0.2
10	2.5	Oil	30 min 4-blade mixer	1 h 4-blade mixer	1.0 ± 0.6	2.5 ± 0.7
20	1	Oil	— Spatula	1 h 4-blade mixer	>1	>8

4.1.2.3. OSA modified starch + CMC

As tests with only OSA modified starch did not produce any nanoemulsions, some mixtures of OSA starch and sodium carboxymethylcellulose (CMC) were tested, as it is shown in **Table 4.4**. CMC made the formulations very thick, even in concentrations as low as 0.5 wt% of the total mass, not being possible to analyze it in DLS. Higher quantities of CMC made it impossible to finish the procedure. A SOR of 1 with 0.1 wt% of CMC, was worst in terms of aspect and DLS results than the sample made only with OSA

starch. For this, CMC was abandoned.

Table 4.4 – Conditions varied in the trials using mixtures of OSA modified starch and CMC

SOR	Wt% total of CMC	Mixer	PDI	Size (z-average) (µm)
1	3	Agitation plate	—	—
1	2	Agitation plate	—	—
1	0.1	Agitation plate	>1	>8
2.5	0.5	4-blade mixer	—	—

4.1.2.4. OSA modified starch + Tween 80

After the unsuccessful tests with the previous formulations, mixtures of OSA modified starch and Tween 80 were carried out. Based on the best tests using only OSA modified starch, these trials were made with SOR 1 using the agitation plate and SOR 2.5 with the 4-blade mixer. The conditions used and DLS results are listed in **Table 4.5**.

Table 4.5 – Conditions tested using as surfactant mixtures OSA modified starch and Tween 80 and their approximated DLS results

SOR	Wt% of Tween 80 in the surfactant mixture	Wt% of OSA starch in the surfactant mixture	Mixer	PDI	Size (z-average) (µm)
1	20	80	Agitation plate	>1	>8
1	30	70	Agitation plate	>1	>8
1	40	60	Agitation plate	>1	>8
1	50	50	Agitation plate	>1	>8
2.5	0.1	99.9	4-blade mixer	>1	>8
2.5	1	99	4-blade mixer	>1	>8
2.5	10	90	4-blade mixer	>1	>8
2.5	20	80	4-blade mixer	>1	5.7 ± 2.0
2.5	30	70	4-blade mixer	>1	7.0 ± 1.8
2.5	40	60	4-blade mixer	>1	>8

In these tests, there was not a clear tendency of the results. In samples made with the agitation plate, there were not any signs of oil drops on the top. With time, phase separation into two layers occurred in all these samples, with the top being white and the bottom translucent. In all samples made with the 4-blade mixer, the emulsions were creamy with foam that, with resting, had different behaviors. In the sample with 0.1% of Tween 80, a yellow layer appeared on the top and, with more time, oiling-off occurred. The sample with 1% of Tween 80 was separated into three layers: the top that presented a white color, the middle that presented a tenuous yellow tonality and the bottom that was translucent. Then, many white flakes appeared throughout all sample in a phenomenon called flocculation. Samples with

10% and 20% of Tween 80, were similar in aspect: the top layer was white and the bottom was translucent. The one with 20% also presented flocculation. Samples with 30% and 40% were visually similar and they had three layers: the top presented a white color, the middle presented a yellow tonality and the bottom was translucent. These three layers are shown in **Figure 4.5**. With more time, the top layer was a thick creaming and in the bottom flocculation was visible.

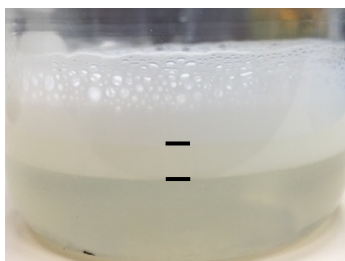


Figure 4.5 – Example of phase separation that occurred in tests with mixtures of OSA modified starch and Tween 80. In this image there are represented the three layers that were seen in some samples made with the 4-blade mixer.

With the addition of the water, in all samples a strong, stiff and filamentous structure was formed, then turning liquid with the continuous water addition, like in tests with only OSA starch. However, with the addition of tween 80, this intermediate structure suffered some changes. With percentages of 20, 30 and 40 wt% of Tween 80 in the surfactant mixture, this structure became a little more like a gel.

All these samples were not nanoemulsions nor stable. Jafari *et al.* (2007), tested mixtures of OSA starch and Tween 20 in the production of nanoemulsions with the microfluidization method. They also reported that their samples were very unstable, and the continuous and dispersed phases separated after a short time. This could be due to the competition between small surfactants (Tween) and big biopolymers (OSA modified starch). They also referred that this instability can lead to flocculation and subsequently to re-coalescence, caused by free surfactant molecules and non-adsorbed biopolymers (Jafari et al., 2007). In this work, flocculation and phase separation occurred and therefore mixtures of OSA starch and Tween 80 were also abandoned.

It was not possible to form nanoemulsions using the EPI method with the above emulsifiers. These results are in agreement with Mayer *et al.* (2013) that also tested some natural surfactants (Quillaja saponin, whey protein isolate, casein and sucrose monopalmitate) using the EPI method and they concluded that none of them was able to produce nanoemulsions (with diameter less than 200 nm). Their size results were between 10 and 150 μm , depending on the emulsifier, and they referred that their systems had poor stability to gravitational separation. This could be due to the lack of molecular or physicochemical characteristics of these surfactants needed to spontaneously form nanoemulsions (Mayer et al., 2013b). Moreover, in a review, it is said that usually in EPI method surfactants must be

small molecules that are able to stabilize both W/O and O/W emulsions (McClements, 2011). Thus, the next step was to produce nanoemulsions with Tween 80.

4.1.2.5. Tween 80

Tween 80 was used to produce nanoemulsions by EPI, and different conditions were evaluated. **Table 4.6** presents the conditions tested and the obtained values of size, PDI and Zeta potential for the samples. Results showed that for the three different SOR tested (1, 1.5 and 2), using the 4-blade mixer, all the samples present a diameter below 200 nm. The PDI decreased for higher SOR values, however only samples with a SOR of 1.5 and 2 present PDI values ≤ 0.200 . These results are in disagreement to Mayer *et al.* (2013), which evaluated the influence of SOR on PDI of nanoemulsions produced by the EPI method and, in their results, higher SOR values led to an increase on the PDI (Mayer et al., 2013b). However, as referred in several publications, the intermediate o/w/o multiple emulsion is essential for the formation of small droplets in the final emulsion and these smaller droplets have larger surface area, requiring larger quantities of surfactant to stabilize them. So, if there is not sufficient surfactant to cover all the interfacial areas of all the droplets formed, there is not an efficient decrease in the interfacial tension of such large areas. Consequently, some of the droplets would coalesce when colliding with each other (Komaiko & McClements, 2015, 2016; Mayer et al., 2013b; Ostertag et al., 2012). Finally, if only some droplets grow, even slightly, there is an increase on the PDI. So, using high surfactant-to-oil ratios this can be avoided, and it could explain why higher concentrations of surfactant produced nanoemulsions with lower PDI results.

In these samples with only Tween 80 as emulsifier, with the addition of water, a gel-like intermediate structure was formed. This gel phase is said to be critical to be able to produce nanoemulsions using the EPI method (Komaiko & McClements, 2016; Mayer et al., 2013b). As this structure was not formed when using the other surfactants tested, this could explain why nanoemulsions were only achieved using Tween 80.

Different mixers (D-shaped and plate) were tried using a SOR of 2. Results showed that the sizes are maintained around 200 nm but the PDI is much higher than 0.2. The plastic D-shaped stirrer tool was small and flexible and the magnetic bar in the agitation plate stopped to stir since it was stuck in the gel phase. Therefore, these two mixers had not enough strength to overcome the resistance of the stiff intermediate structure, not being able to properly stir the formulation and to produce nanoemulsions.

Table 4.6 – Conditions tested with Tween 80 as emulsifier and their respective DLS results

SOR	Time of the 1 st mixture (min)	Mixer	PDI	Size (mean) (nm)	Zeta Potential (mV)
1	30	4-blade mixer	0.324 ± 0.058	196.0 ± 11.2	—
1.5	30	4-blade mixer	0.204 ± 0.029	192.5 ± 8.3	-47.3 ± 1.4
2	30	4-blade mixer	0.131 ± 0.003	182.6 ± 4.9	-47.9 ± 2.0
2	30	D-shaped (plastic)	0.638 ± 0.095	208.7 ± 30.3	—
2	30	Agitation plate	0.595 ± 0.020	197.6 ± 4.7	—

These results can be compared to other studies that also produced nanoemulsions by the EPI method using Tween 80. Mayer *et al.* (2013b) used 10 wt% medium chain triglycerides (MCT) with a SOR of 1 and 2 and obtained nanoemulsions with sizes of 90 nm and 40 nm, respectively. Despite having low sizes, these formulations present high PDI values, reaching to value of 0.21 (SOR 1) and 0.47 (SOR 2) (Mayer et al., 2013b). In another study, a nanoemulsion with 4 wt% D-limonene and using a SOR of 1.5 presented sizes of 40 nm, but when the oil phase was changed to have 85 % D-limonene and 15 % of sunflower oil, the droplet size increased to 120 nm (Li et al., 2013). In 2016, Borrin *et al.* produced nanoemulsions using soybean oil and a SOR 1 and they presented sizes of 295 nm with a bimodal distribution (Borrin et al., 2016). In another study, formulations with MCT and a SOR 2 presented sizes of 100 nm but when the MCT were replaced by long chain triglycerides (LCT) and high SOR (2.5) was used the sizes increase to 600 nm, showing that the type of oil have a great influence on the nanoemulsions size (Ostertag et al., 2012).

Results show that when MCTs are generally used, the nanoemulsions produced present a small size if compared with LCTs. Despite this behavior, the results obtained showed that is possible to achieve nanoemulsions with good size and PDI values using a LCT. The nanoemulsions here presented had sizes below 200 nm and a monomodal distribution. The use of refined sunflower oil presents several advantages since is 100% vegetal, cheap, easy to acquire and it is well used within the food industry and is highly unsaturated (Nirmala & Nagarajan, 2017).

Nanoemulsions with SOR 1.5 and 2 produced with the 4-blade mixer were selected and used for further studies, being named NE_1.5 and NE_2, respectively.

The Zeta Potential obtained for all samples was higher than -30 mV, being a sign of stability (Grumezescu, 2017; Ohshima & Makino, 2014). Tween 80 is a non-ionic surfactant and so droplets stabilized by this emulsifier should have no surface charge. However, these nanoemulsions had a negative charge that can be explained by the presence of free fatty acids or other ionic impurities or ions from the water phase (McClements, 2011; Salvia-Trujillo et al., 2017).

4.1.3. Production of nanoemulsions containing essential oils

Both formulations, with SOR 1.5 and 2, were able to produce nanoemulsions with quantities of essential oil between 0.5 and 2 wt%. However, samples with SOR 1.5 were unstable at the third day of storage, showing visible creaming. Higher quantities of essential oil (3 and 4 wt%), did not allowed to achieve emulsions with satisfactory PDI, size or Zeta Potential. This can be explained by the hydrophilicity of the essential oils which can be partially soluble in water. Thus, nanoemulsions loaded with EOs have tendency to suffer Oswald ripening (Chang, McLandsborough, & McClements, 2015). Chang *et al.* (2012), evaluated nanoemulsions with pure thyme essential oil as oil phase and Tween 80 using a high-pressure homogenizer. They reported that the resulting emulsion had enormous sizes and was highly unstable to droplet growth, creaming and oiling off, as a result of Ostwald ripening (Chang et al., 2012). So, the combination effect of essential concentration and SOR are important for the development of the nanoemulsion and their stability over time.

4.1.3.1. Nanoemulsions with a SOR of 1.5

Table 4.7 shows the DLS results of the nanoemulsions with the lowest content of Tween 80, for the different concentrations of essential oil (both thyme and lemon balm essential oil). All samples were homogeneous with a visual aspect like the ones produced without the addition of essential oil.

Table 4.7 – DLS results (polydispersity, size and Zeta potential) of nanoemulsions with a SOR of 1.5 and different concentrations (0.5, 1 and 2 wt%) of thyme or lemon balm essential oil. The same superscript letters mean that there was no significant difference between results within the same column and essential oil ($p < 0.05$, one-way ANOVA test)

	Sample	Wt% of essential oil	Wt% of sunflower oil	PDI	Size (mean) (nm)	Zeta potential (mV)
Thyme	NE_1_T0.5	0.5	9.5	0.092 ± 0.033^a	188.7 ± 4.3^a	-47.0 ± 1.2^a
	NE_1_T1	1	9	$0.124 \pm 0.011^{a,b}$	196.6 ± 5.1^a	-44.4 ± 1.3^a
	NE_1_T2	2	8	$0.153 \pm 0.019^{a,b}$	196.8 ± 3.7^a	-44.9 ± 3.0^a
Lemon balm	NE_1_LB0.5	0.5	9.5	0.126 ± 0.031^a	186.8 ± 5.5^a	-44.4 ± 0.2^a
	NE_1_LB1	1	9	0.123 ± 0.024^a	191.2 ± 1.7^a	-42.6 ± 2.3^a
	NE_1_LB2	2	8	0.146 ± 0.003^a	196.7 ± 6.5^a	-40.3 ± 5.9^a

All the conditions produced nanoemulsions with good PDI (i.e. < 0.2), being the size in the nanometric range and the values of Zeta potential higher than ± 30 . However, these formulations were unstable during storage, as it will be showed in section 3.1.3.1. For this reason, higher concentrations of essential oil were not tested.

4.1.3.2. Nanoemulsions with a SOR of 2

The same determination was performed to nanoemulsions with a SOR 2, however in this case higher concentration of essential oils were used. DLS results of these nanoemulsions with thyme or lemon balm essential oil are presented in **Table 4.8**. During the production of these samples, it was observed that the intermediate gel phase for formulations with a concentration of 3 and 4 wt% of thyme essential oil was different than normal in samples, being transparent rather than opaque. Nevertheless, the final samples look homogeneous like the ones produced without the addition of essential oil and for a lower SOR.

Results showed that the PDI and size values of the nanoemulsions tend to increase for higher concentrations of essential oil. However, there are some studies with low energy methods and MCT that showed a different tendency. Lou *et al.* (2017) produced nanoemulsions using MCT with essential oil of *Citrus medica* and Tween 80 (SOR 2) by spontaneous emulsification (SE). They showed that sizes decrease for higher concentrations of essential oil in the lipid phase, being 165 nm for samples with 20 % essential oil and 73 nm with 50 %. Then, the size increased to 95 nm in formulations with 60 % essential oil (Lou et al., 2017). Likewise, samples with MCT, orange oil and Tween 80 (SOR 2, SE method) had a decrease in droplet size with the increase of orange oil in the lipid phase up to 50 %, and then it suffered a huge increase (Chang & McClements, 2014). The same behavior was presented by Chang *et al.* (2013), when nanoemulsions with MCT, carvacrol and Tween 80 (SOR 1, SE method) decreased their size from 160 nm to 60 nm when 25 % of carvacrol oil was added, and then increased to 800 nm when the concentration reached the 60 % of carvacrol (Chang, McLandsborough, & McClements, 2013). None of these works made reference to PDI and therefore is not clear if the size values presented are resultant from a monomodal or bimodal population. A study of nanoemulsions with cinnamon oil, MCT and Tween 80 (SOR 2), produced by the PIT method, showed that intermediate concentrations of essential oil (30-40 % in the oil phase) produced the formulations with lower PDI (0.17) and sizes (100-107 nm). And large droplets were created at lower (0-20 %) and higher (60-100 %) concentrations, with broad multimodal size distributions (Chuesiang et al., 2018). The same happened in formulations with cinnamon oil, coconut oil (MCT) and Tween 80 (SOR 1), produced by the SE method (Yildirim et al., 2017). In a study of nanoemulsions produced by the phase inversion temperature method (PIT) with oregano essential oil, sunflower oil, Cremophor RH 40 and Span 80, it was observed an increase of the PDI with higher quantities of essential oil in the formulation: 0.08 (5 % essential oil) to 0.16 (7 % essential oil) (Moraes-Lovison et al., 2017) that is in agreement with the results presented in this study.

Table 4.8 – DLS results (polydispersity, size and Zeta potential) of nanoemulsions with a SOR of 2 and different concentrations (0.5, 1, 2, 3 and 4 wt%) of thyme or lemon balm essential oil. The same superscript letters mean that there was no significant difference between results within the same column and essential oil ($p < 0.05$, one-way ANOVA test)

	Sample	Wt% of essential oil	Wt% of sunflower oil	PDI	Size (mean) (nm)	Zeta potential (mV)
Thyme	NE_2_T0.5	0.5	9.5	0.110 ± 0.006^a	188.4 ± 1.9^a	-45.0 ± 5.6^a
	NE_2_T1	1	9	0.142 ± 0.037^a	$191.4 \pm 4.2^{a,b}$	-47.3 ± 2.8^a
	NE_2_T2	2	8	$0.154 \pm 0.035^{a,b}$	$200.8 \pm 4.6^{b,c}$	-44.3 ± 1.7^a
	NE_2_T3	3	7	0.256 ± 0.067^b	208.4 ± 6.1^c	-48.0 ± 1.3^a
	NE_2_T4	4	6	0.499 ± 0.019^c	242.6 ± 2.6^d	-2.3 ± 1.0^b
Lemon balm	NE_2_LB0.5	0.5	9.5	0.103 ± 0.016^a	187.8 ± 8.5^a	$-43.3 \pm 5.7^{a,b}$
	NE_2_LB1	1	9	0.125 ± 0.025^a	$193.1 \pm 6.0^{a,b}$	-48.6 ± 1.4^a
	NE_2_LB2	2	8	0.132 ± 0.039^a	$194.8 \pm 3.9^{a,b}$	$-41.5 \pm 2.2^{a,b}$
	NE_2_LB3	3	7	0.203 ± 0.015^b	$202.6 \pm 2.4^{a,b}$	-38.7 ± 0.9^b
	NE_2_LB4	4	6	0.279 ± 0.015^c	207.9 ± 6.4^b	-28.7 ± 1.6^c

Nanoemulsions with 0.5, 1 and 2 wt% of essential oil present a good PDI (<0.2), however for higher quantities of essential oil (3 and 4 wt%) the nanoemulsions produced present not satisfactory values of PDI (>0.2) and Zeta Potential ($<+/-30$). For these reasons, these samples were not used in any further studies.

4.1.4. Morphological analysis – TEM

The morphological analysis of nanoemulsions with Transmission Electron Microscopy (TEM) allowed to confirm that both formulations, with and without essential oil, have a spherical shape, as it can be seen in **Figure 4.6**. One interesting feature of TEM analysis, is that it allowed to observe the core-shell structure of the particles of these samples (**Figure 4.6A**), that is formed by the layer of Tween 80 that is establishing the oil-water interface. This shell layer was visible in various droplets of the nanoemulsions analyzed and had a thickness of 9.6 ± 2.6 nm. In terms of volume, this shell layer corresponds to approximately 34.6 ± 7.6 % of the total volume of the droplets.

The size of the droplets observed by TEM were determined and presented in **Table 4.9** and are compared to the ones obtained with DLS. They do not present any statistical differences ($p < 0.05$) being the results obtained by TEM in good agreement with the results obtained by DLS. The same test revealed that the oil phase composition had no statistically significant influence on droplet size. So, both the shell layer and the lipophilic core of the droplets were not significantly influenced by this parameter.

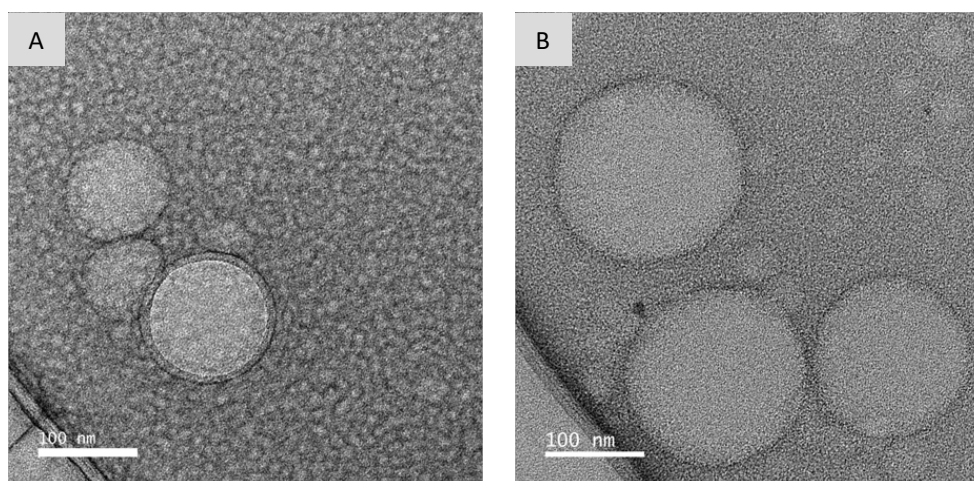


Figure 4.6 – TEM images of nanoemulsions produced with SOR 2. **A** - sample NE_2: 10 wt% sunflower oil, 20 wt% Tween 80 and 70 wt% Milli-Q water. **B** - sample NE_2_LB2: 2 wt% lemon balm essential oil, 8 wt% sunflower oil, 20 wt% Tween 80 and 70 wt% Milli-Q water. Conditions: 200 kV, 100x and with UranylLess as contrasting agent.

Table 4.9 – Mean sizes obtained by transmission electron microscopy and dynamic light scattering for 3 nanoemulsions. The same superscript letters mean that there was no significant difference between results ($p < 0.05$, one-way ANOVA test)

Sample	Size given by TEM (nm)	Size given by DLS (nm)
NE_2	168.7 ± 32.6^a	182.6 ± 4.9^a
NE_2_T2	153.9 ± 39.1^a	200.8 ± 4.6^a
NE_2_LB2	170.5 ± 22.5^a	194.8 ± 3.9^a

4.1.5. Stability of nanoemulsions

The selected nanoemulsions were evaluated in terms of size, PDI, zeta potential and creaming during storage at 4 and 25 °C. Also, some accelerated kinetic stability tests, like centrifugation and a cycle of heating and cooling were carried out. All of them resisted to the centrifugation, but they collapsed during the cycle.

4.1.5.1. Storage

Figure 4.7 and **4.8** present the size and PDI for nanoemulsions without essential oil for the two SOR tested (NE_1.5 and NE_2). NE_1.5 stored at 20 °C showed three significant variations in size, relatively to the day 1 (**Figure 4.7**). These main variations were observed at the 3rd day, 4 months and 6 months. The PDI was only considered different at 6 months of storage. At 4 °C the nanoemulsions' size and PDI was constant during 6 months of storage. **Figure 4.8** shows that NE_2 was stable during the entire storage time, excepting for when it was stored at 20 °C, having a significant size reduction at 4 months. These results showed that both formulations were stable for half a year when stored at 4 °C.

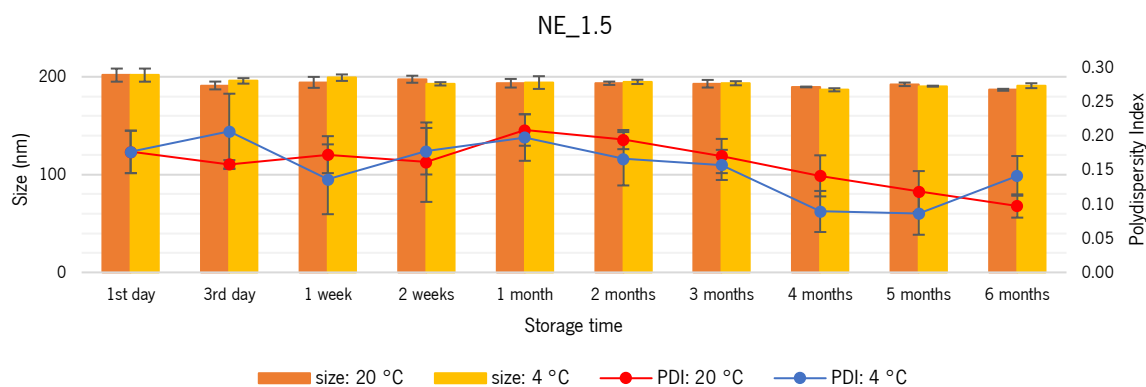


Figure 4.7 – Size and polydispersity index of NE_1.5 (SOR 1.5, 10 wt% sunflower oil) during 6 months at two storage conditions, 20 °C and 4 °C.

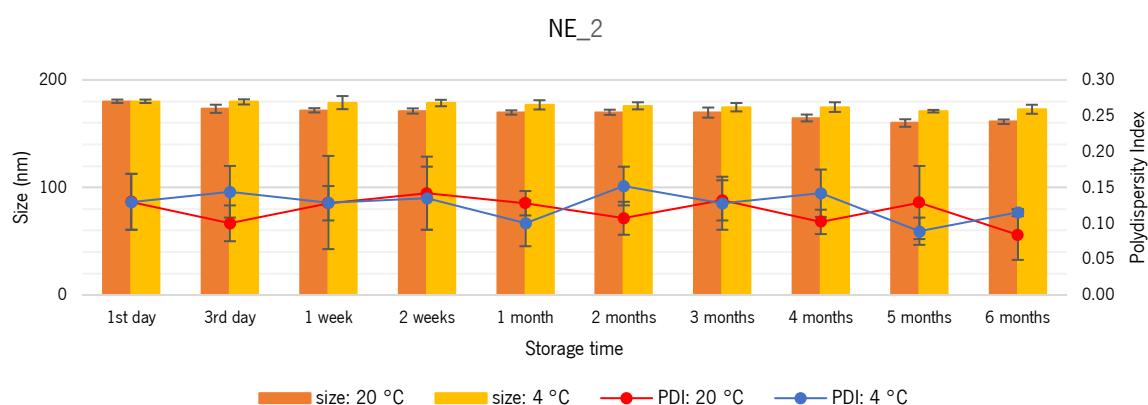


Figure 4.8 – Size and polydispersity index of NE_2 (SOR 2, 10 wt% sunflower oil), during 6 months at two storage conditions, 20 °C and 4 °C.

Nanoemulsions with a SOR of 1.5 with essential oils were monitored only for one month, since at the third day of storage they showed destabilization through the appearance of creaming. This phenomenon was higher for nanoemulsions loaded with lemon balm essential oil. Nanoemulsions with 0.5 and 1 wt% thyme oil, namely NE_1.5_T0.5 (**Figure 4.9**) and NE_1.5_T1 (**Figure 4.10**), did not show any significant differences in size during the storage time, both at 20 °C and 4 °C. The PDI values of NE_1.5_T0.5 increased at 1 month, when stored at 20 °C and 4 °C. The nanoemulsion with 2 wt% of thyme essential oil, NE_1.5_T2 (**Figure 4.11**), had a variation in size at 1 month of storage and its PDI remained always statistically unchanged, for both storage conditions.

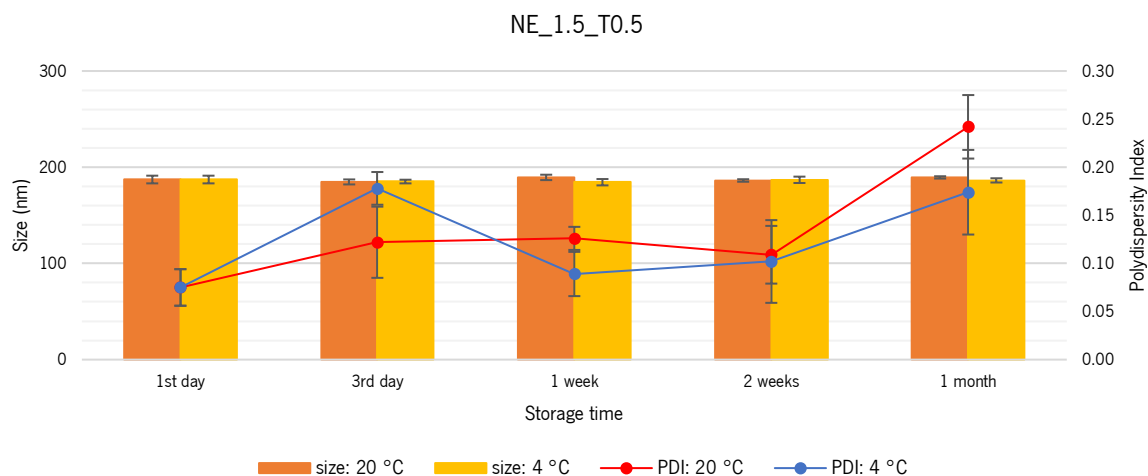


Figure 4.9 – Size and polydispersity index of NE_1.5_T0.5 (SOR 1.5, 0.5 wt% thyme essential oil and 9.5 wt% sunflower oil), during 1 month at two storage conditions, 20 °C and 4 °C.

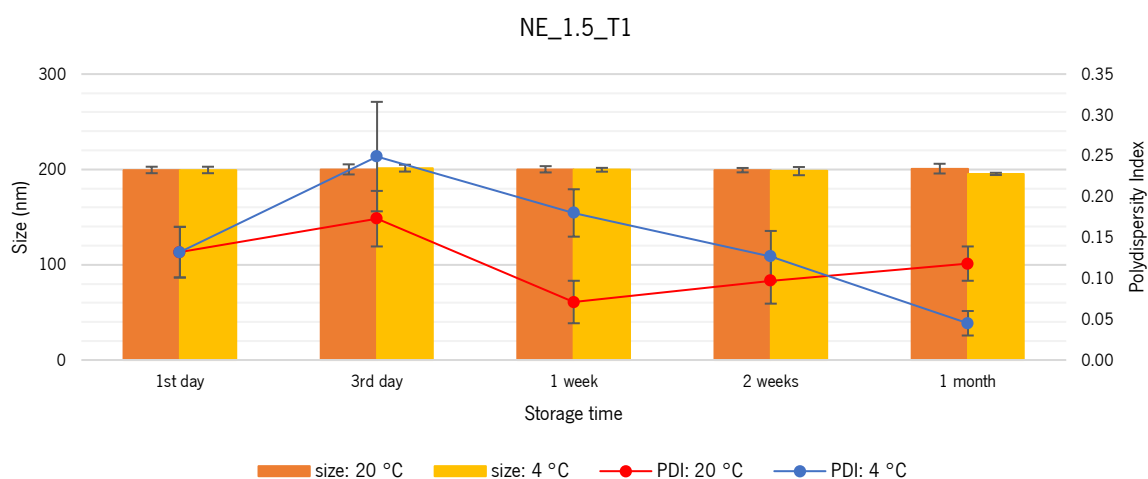


Figure 4.10 – Size and polydispersity index of NE_1.5_T1 (SOR 1.5, 1 wt% thyme essential oil and 9 wt% sunflower oil), during 1 month at two storage conditions, 20 °C and 4 °C.

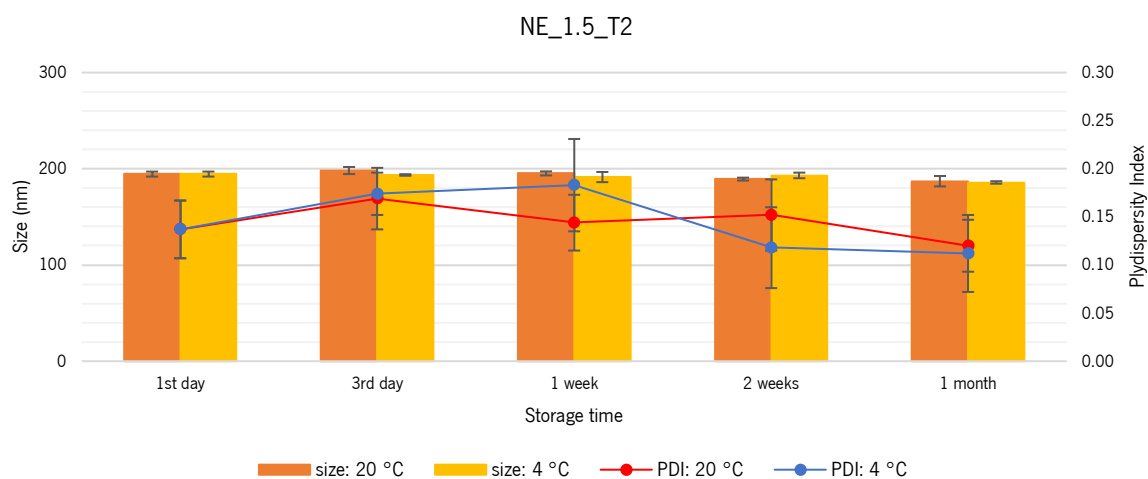


Figure 4.11 – Size and polydispersity index of NE_1.5_T2 (SOR 1.5, 2 wt% thyme essential oil and 8 wt% sunflower oil), during 1 month at two storage conditions, 20 °C and 4 °C.

The three nanoemulsions loaded with lemon balm oil, NE_1.5_LB0.5 (Figure 4.12), NE_1.5_LB0.5 (Figure 4.13) and NE_1.5_LB2 (Figure 4.14), showed a significant reduction in size at the 3rd day of storage. This coincides with the day when a great creaming layer was noticed in all formulations. This creaming could have occurred since these samples have droplets with different sizes, but within the same range since the distribution is monomodal, and the larger droplets tend to migrate fast to the top (creaming) (Silva et al., 2015). Also, some coalescence could have occurred, contributing to the appearance of creaming. Thus, the diminution of sizes can be explained by the migration of the larger droplets, leaving the smaller in the nanoemulsion and showing that the two phenomena are related (creaming and smaller sizes). This migration of droplets usually occurs some hours after the emulsification (Guerra-Rosas et al., 2016), explaining why it was noticed at the third day of storage and not in any day later. Relatively to the PDI of these nanoemulsions, NE_1.5_LB0.5 and NE_1.5_LB2 showed no significant variations.

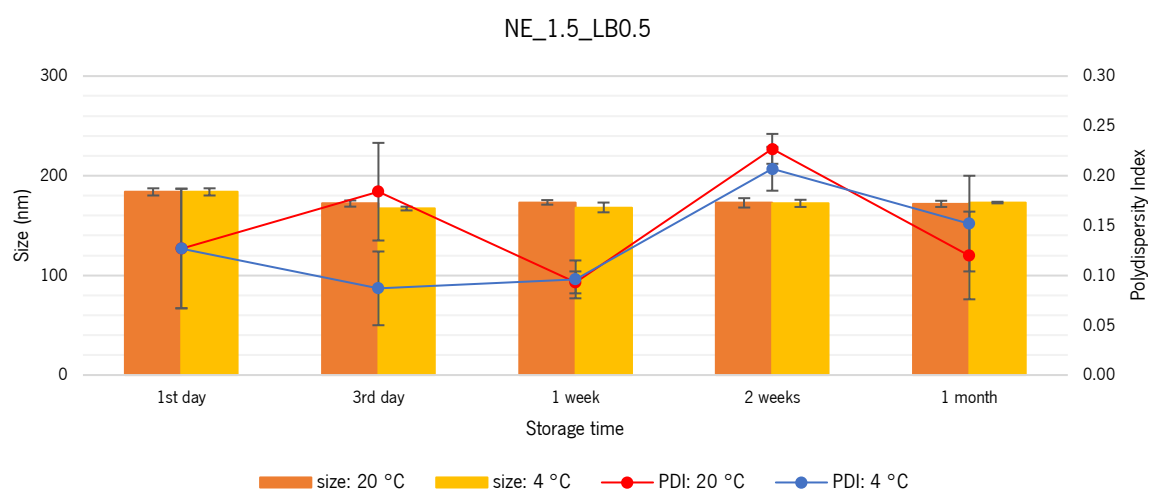


Figure 4.12 – Size and polydispersity index of NE_1.5_LB0.5 (SOR 1.5, 0.5 wt% lemon balm essential oil and 9.5 wt% sunflower oil), during 1 month at two storage conditions, 20 °C and 4 °C.

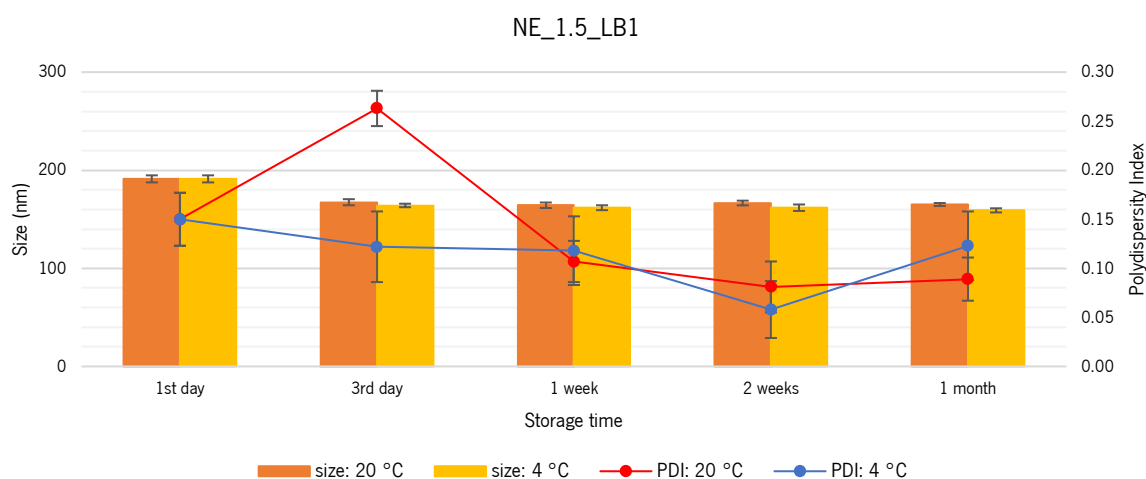


Figure 4.13 – Size and polydispersity index of NE_1.5_LB1 (SOR 1.5, 1 wt% lemon balm essential oil and 9 wt% sunflower oil), during 1 month at two storage conditions, 20 °C and 4 °C

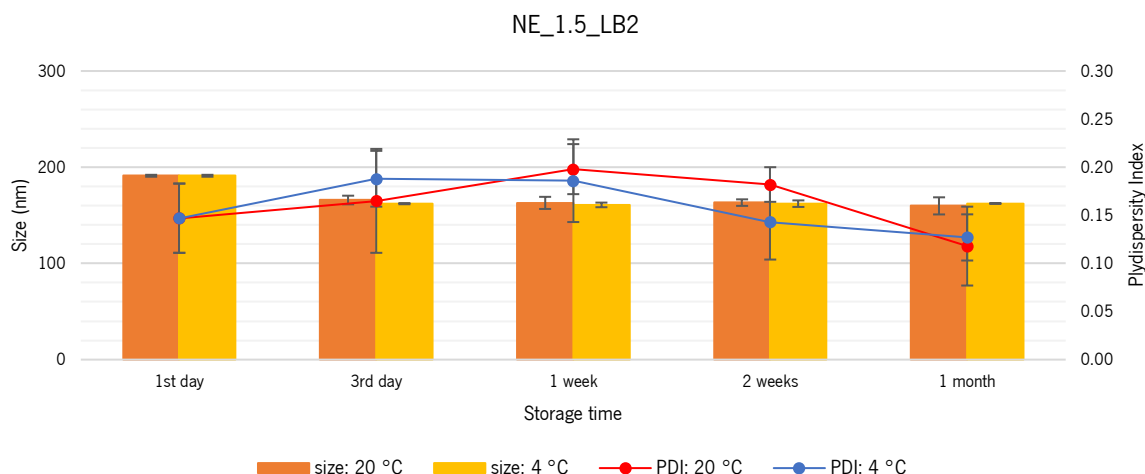


Figure 4.14 – Size and polydispersity index of NE_1.5_LB2 (SOR 1.5, 2 wt% lemon balm essential oil and 8 wt% sunflower oil), during 1 month at two storage conditions, 20 °C and 4 °C.

Nanoemulsions with a SOR 2 loaded with essential oils did not show evident creaming and, for this, they were followed for three months. Nanoemulsions with 0.5 wt% and 1 wt% of thyme essential oil, NE_2_T0.5 (**Figure 4.15**) and NE_2_T1 (**Figure 4.16**), respectively, did not show any significant differences in size during the entire storage time, for both temperatures tested. The nanoemulsion with 2 wt% of thyme oil (NE_2_T2, **Figure 4.17**) had a size variation in the 2nd month at 20 °C and none at 4 °C. At 20 °C, NE_2_T0.5 only had a PDI variation at 1 month and returned to be similar to the initial value at 2 months, while the PDI of NE_2_T1 remained unchanged the PDI of NE_2_T2 had a significant change in PDI in the 3rd month. The three formulations had no significant variations in PDI when stored at 4 °C.

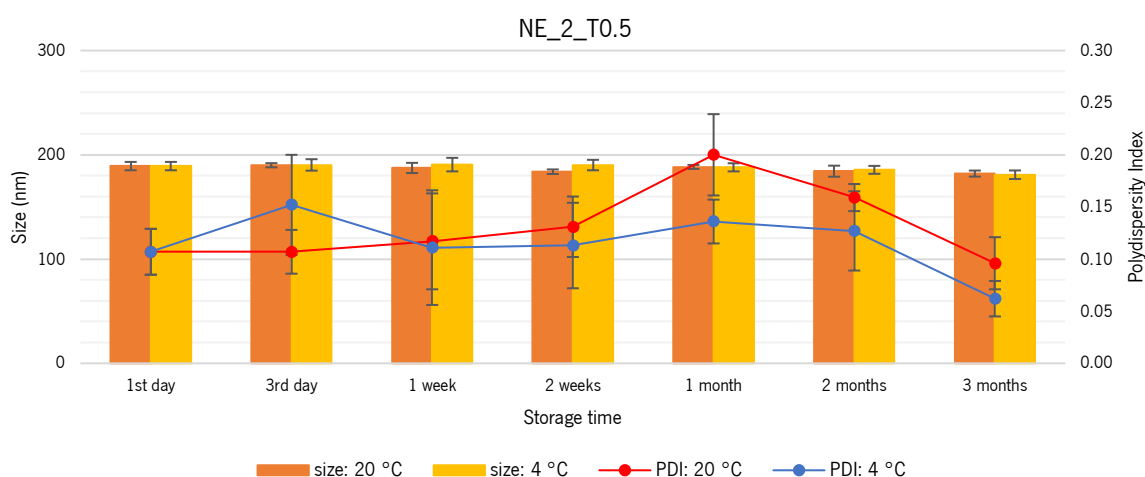


Figure 4.15 – Size and polydispersity index of NE_2_T0.5 (SOR 2, 0.5 wt% thyme essential oil and 9.5 wt% sunflower oil), during 3 months at two storage conditions, 20 °C and 4 °C.

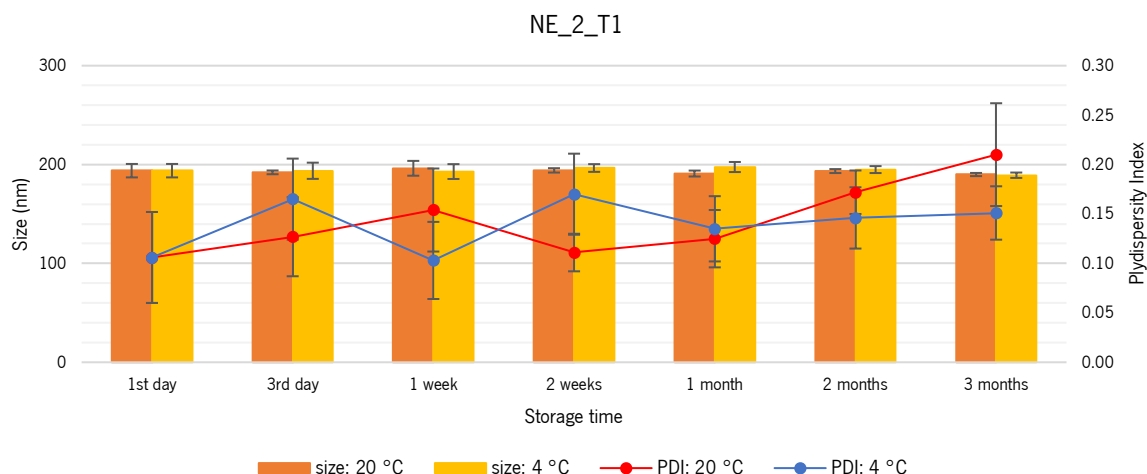


Figure 4.16 – Size and polydispersity index of NE_2_T1 (SOR 2, 1 wt% thyme essential oil and 9 wt% sunflower oil), during 3 months at two storage conditions, 20 °C and 4 °C.

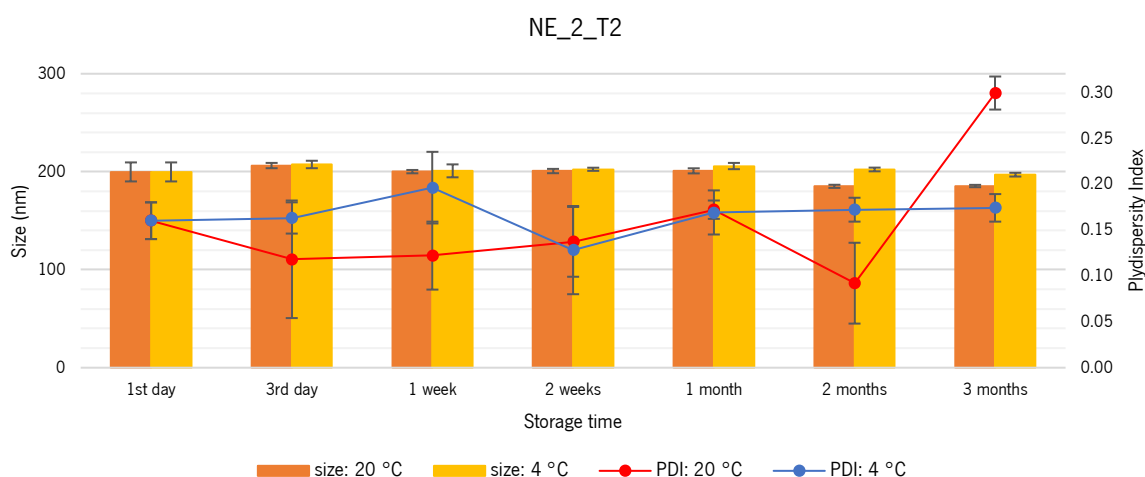


Figure 4.17 – Size and polydispersity index of NE_2_T2 (SOR 2, 2 wt% thyme essential oil and 8 wt% sunflower oil), during 3 months at two storage conditions, 20 °C and 4 °C.

Nanoemulsions with 0.5 wt% (NE_2_LB0.5, **Figure 4.18**) and 1 wt% (NE_2_LB1, **Figure 4.19**) of lemon balm essential oil, showed a significant size change at the 3rd month at 20 °C. The same happened for the nanoemulsion with 2 wt% of lemon balm oil (NE_2_LB2, **Figure 4.20**) at 4 °C. The nanoemulsions NE_2_LB0.5 and NE_2_LB1 stored at 4 °C had no changes in size during, while NE_2_LB2 had a significant size reduction in the 3rd day of storage when maintained at 20 °C. The PDI of these formulations had no significant variations for the 3 months of storage at both temperatures tested, with the exception of NE_2_LB2. This nanoemulsion, when stored at 20 °C, showed a variation of the PDI values (lower values) in the 2nd week and 2nd month, but then changed again for values similar to the ones obtained in the 1st day.

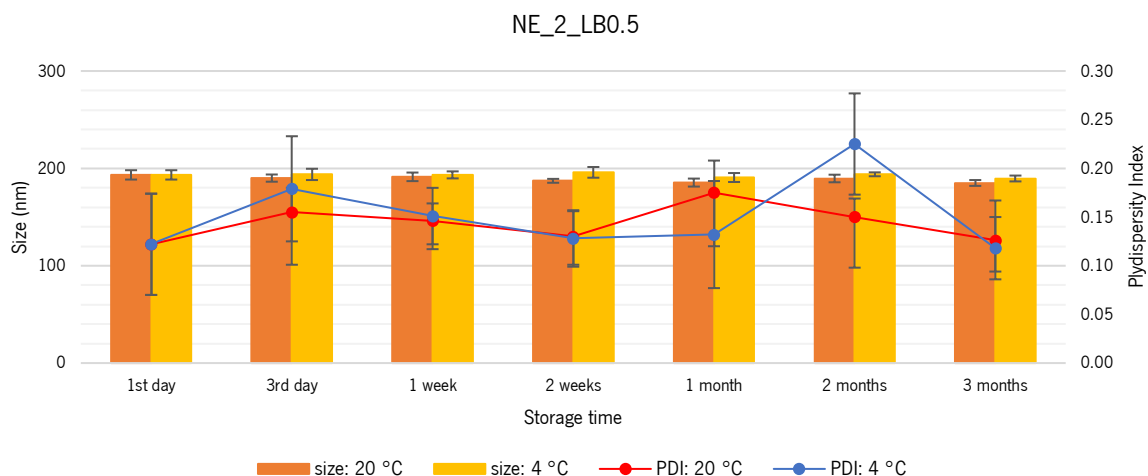


Figure 4.18 – Size and polydispersity index of NE_2_LB0.5 (SOR 2, 0.5 wt% lemon balm essential oil and 9.5 wt% sunflower oil), during 3 months at two storage conditions, 20 °C and 4 °C.

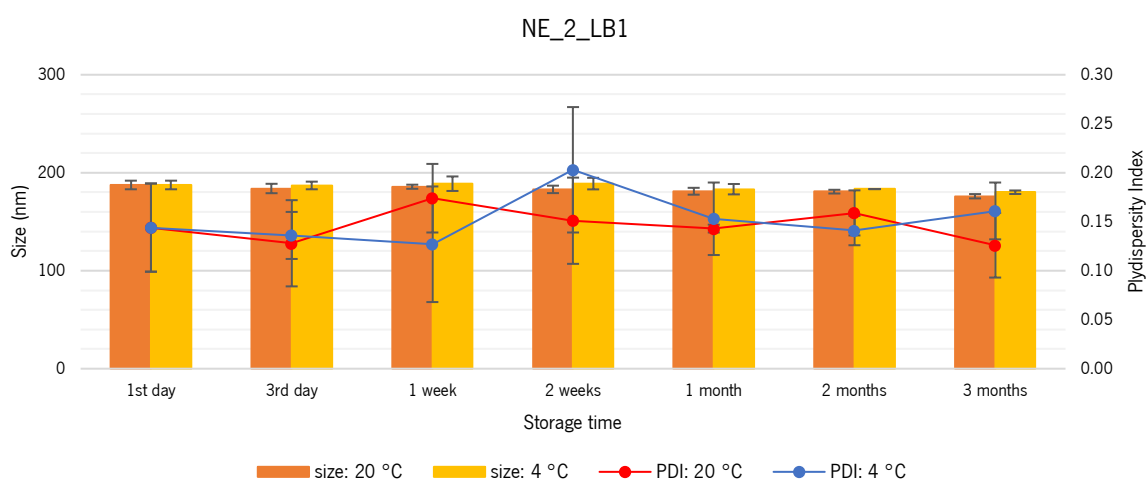


Figure 4.19 – Size and polydispersity index of NE_2_LB1 (SOR 2, 1 wt% lemon balm essential oil and 9 wt% sunflower oil), during 3 months at two storage conditions, 20 °C and 4 °C.

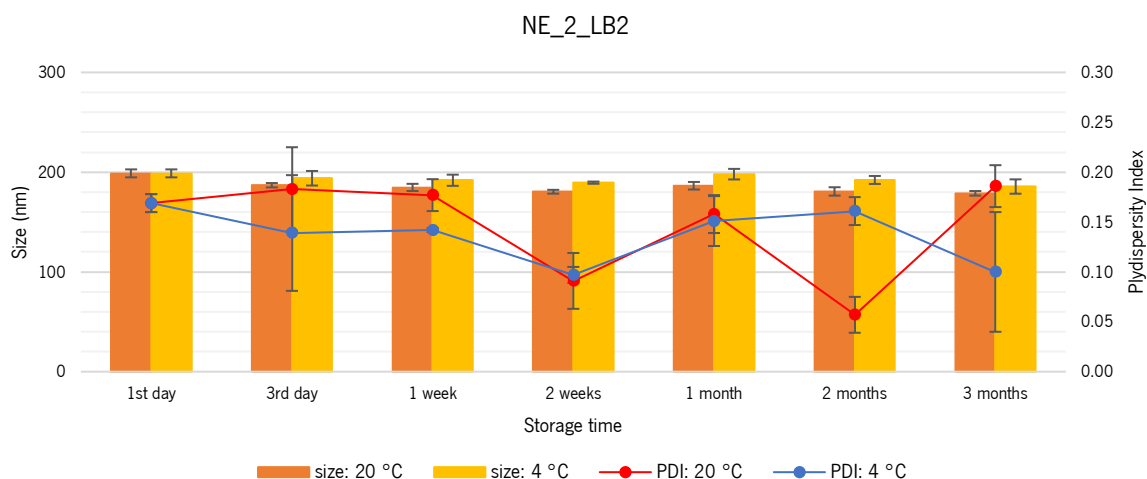


Figure 4.20 – Size and polydispersity index of NE_2_LB2 (SOR 2, 2 wt% lemon balm essential oil and 8 wt% sunflower oil), during 3 months at two storage conditions, 20 °C and 4 °C.

In this work, the droplet sizes of nanoemulsions had tendency to decrease with time and were more stable under refrigeration. In another study, nanoemulsions with D-limonene and Tween 80 (SOR 1.5), produced by the EPI method, increased their size when stored during 12 days and were more stable at 28 °C than at 4 °C. The initial droplet size of that sample was 40 nm, changing to 169 nm, when stored at 4 °C, and only to 108 nm, at 28 °C (Li et al., 2013). Formulations with soybean oil and Tween 80 (SOR 1), produced by the same method, also increased droplet size after 15 days of storage, and a significant change in the distribution curve occurred, increasing its PDI (Borrin et al., 2016). The size of nanoemulsions with essential oil of *Ocimum basilicum* and Tween 80 (SOR 1, EPI method) increased during 30 days of storage and their PDI diminished but remaining a polymodal distribution (Sundararajan et al., 2018). In another study, using high energy method (high pressure homogenization), nanoemulsions with 3 wt% thyme essential oil, 7 wt % corn oil and Tween 80 (SOR 0.1), were stable during 30 days, without changes in particle size and PDI (Chang et al., 2012). In the present work, it can be said that the results were comparable to the ones obtained by high energy methods once the PDI and size remained stable, even during 6 months.

Zeta potential was also evaluated for all these formulations at both temperature conditions (see Figures in **Annexes – Group D**). The values of Zeta potential obtained ranged between - 53 mV and -35 mV during storage. This is an indicator of droplets electrostatic stabilization, since they had negative potentials bigger than 30 mV (Grumezescu, 2017; Ohshima & Makino, 2014).

Results showed that nanoemulsions with SOR 1.5 were not stable, with the appearance of a huge creaming layer at the 3rd day and having significant size and PDI variations. On the other hand, formulations with SOR 2, revealed to be stable, mainly when refrigerated, regardless the amount of essential oil loaded. Due to this, samples with SOR 1.5 were abandoned and the following tests were carried out using formulations with SOR 2.

4.1.5.2. Accelerated kinetic stability tests

Two kinetic stability tests, centrifugation and cycle of heating-cooling, were used to evaluate the stability of nanoemulsions. Based on the previous results (stability during storage) three nanoemulsions with a SOR of 2 with different oil phases were chosen: NE_2 (10 wt% sunflower oil), NE_2_T1 (9 wt% sunflower oil + 1 wt% thyme essential oil) and NE_2_LB1 (9 wt% sunflower oil + 1 wt% lemon balm essential oil).

None of the samples showed any phase separation after the centrifugation test. All of them remained homogeneous and without showing any oiling-off. Therefore, they were further tested in the

cycle of heating-cooling cycle test.

After the second cycle, of the cycle of heating-cooling test, all samples showed a significant creaming. Formulations made with 10 wt% of sunflower oil were the ones with the lowest layer (12.72% of the total volume). Nanoemulsions with essential oil had creaming with approximately twice the size of the one existing when only sunflower oil was used. Formulations with 1 wt% of lemon balm essential oil presented a creaming of 25.45% of their total volume and samples with 1 wt% of thyme essential oil showed a creaming of 29.59% of their total volume. Despite the instability seen, the cycle of heating-cooling was carried on, checking if the creaming would be changed. Until the end of the sixth cycle, the layers of creaming remained unaltered.

4.1.6. Antioxidant activity

The antioxidant activity of the selected nanoemulsions (NE_2, NE_2_T2 and NE_LB2) was evaluated using two different methodologies: the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging and the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) tests. Both methodologies showed an increase of the antioxidant activity with higher quantities of thyme essential oil loaded. In the case of lemon balm oil, it was shown that its activity has been enhanced with its nanoencapsulation. On the other hand, nanoemulsions with thyme oil showed similar values (if evaluated using DPPH) or lower (if evaluated using ABTS) when compared with the free essential oil.

Table 4.10 presents the results obtained by both methods, expressed as Trolox Equivalent Antioxidant Capacity (TEAC). According to the DPPH assay, all nanoemulsions with lemon balm oil (NE_2_LBx) showed no significant differences in comparison to the formulation without any essential oil (NE_2). Likewise, no significant variations were observed in nanoemulsions loaded with different concentrations of lemon balm essential oil. On the other hand, there is a clear tendency of the antioxidant capacity to increase for nanoemulsions with higher concentrations of thyme essential oil. The unencapsulated lemon balm oil (LB_free) had lower activity than the equivalent nanoemulsion (NE_2_LB2). This shows that there was an improvement of the antioxidant capacity (TEAC) of lemon balm essential oil, when it was incorporated into nanoemulsions. The results for unencapsulated (T_free) and encapsulated (NE_2_T2) thyme essential oil were not significantly different ($p>0.05$). So, according to this method, the encapsulation of thyme oil offered no advantage in terms of antioxidant activity.

Table 4.10 – Trolox Equivalent Antioxidant Capacity of nanoemulsions or free essential oil, obtained by the DPPH or ABTS free scavenging tests. The same superscript letters mean that there was no significant difference between results within the same column ($p < 0.05$, one-way ANOVA test)

Sample		TEAC (Trolox Equivalent Antioxidant Capacity) (mM/mL)	
		DPPH	ABTS
Control	NE_2	4.68 ± 1.30 ^a	16.21 ± 1.94 ^{f,k}
Thyme	NE_2_T0.5	15.42 ± 1.50 ^b	74.17 ± 9.77 ^g
	NE_2_T1	25.04 ± 2.26 ^c	203.16 ± 14.63 ^h
	NE_2_T2	37.40 ± 4.53 ^d	338.90 ± 12.32 ⁱ
	T_free	35.95 ± 0.79 ^d	395.92 ± 5.53 ^j
Lemon balm	NE_2_LB0.5	5.11 ± 2.37 ^{a,e}	16.05 ± 5.34 ^{f,k}
	NE_2_LB1	5.70 ± 2.99 ^{a,e}	17.47 ± 1.81 ^f
	NE_2_LB2	7.24 ± 1.30 ^a	19.91 ± 1.73 ^f
	LB_free	3.74 ± 1.08 ^e	11.49 ± 6.81 ^k

The ABTS test provided higher values of TEAC, in comparison to the DPPH method. Seibert *et al.* (2018), studied the antioxidant activity of low energy nanoemulsions loaded with an essential oil, by both DPPH and ABTS methods. They also studied the activity of the free essential oil. Their inhibition results were higher for the ABTS method and they justified this situation by the higher sensitivity of this test. They referred that ABTS is more versatile since its working solution is soluble in both aqueous and organic solvents, being able to evaluate the activity in polar and non-polar samples (Seibert et al., 2019). Despite these differences in values, in this work, both methods showed the same tendency.

Relatively to lemon balm oil, the results from ABTS are in agreement to the ones obtained by DPPH. Nanoemulsions with this essential oil showed no significant difference from formulations without essential oil and no changes were observed in the antioxidant capacity for formulations with different quantities of lemon balm oil. In the same way as for DPPH tests, the encapsulation proved to be advantageous since LB_2_LB2 had higher activity than LB_free. Nanoemulsions with thyme essential oil showed once more a strong tendency of the increase in antioxidant capacity with higher quantities of this essential oil. The only difference between DPPH and ABTS results, is that this last show a significant reduction in antioxidant activity when thyme essential oil is encapsulated (T_free vs NE_2_T2).

Studies showed that the DPPH radical scavenging activity of different essential oils (*Cymbopogon densiflorus*, *Citrus medica* and *Ocimum basilicum*) was higher when they were encapsulated into nanoemulsions produced by EPI and SE methods (Lou et al., 2017; Seibert et al., 2019; Sundararajan et al., 2018). The nanoencapsulation of *Cymbopogon densiflorus* essential oil showed also higher ABTS

radical scavenging activity than the unencapsulated essential oil (Seibert et al., 2019). At the present work, this was only observed in the case of lemon balm essential oil.

4.1.7. Antimicrobial activity

The antimicrobial activity tests of the produced nanoemulsions were carried out using nanoemulsions with a SOR of 2. **Table 4.11** shows the obtained results for nanoemulsions with thyme or lemon balm essential oil against *E. coli* and *S. aureus*, using the agar diffusion method. Nanoemulsions with only sunflower oil offered no inhibition to *S. aureus*, showing that the inhibition of this bacterium was due exclusively to essential oils. However, the same formulation inhibited *E. coli*, and this means that the nanoemulsion itself has antimicrobial activity against this bacterium, that can be explained by the surfactant and/or oil used in the production of the nanoemulsion (Nielsen et al., 2016; Yoon et al., 2018).

Table 4.11 – Antimicrobial activity against *E. coli* and *S. aureus* of nanoemulsions with different quantities of thyme essential oil, using the agar diffusion method with paper disks. The same superscript letters mean that there was no significant difference between results within the same column ($p < 0.05$, one-way ANOVA test)

Antimicrobial activity of nanoencapsulated essential oils					
Sample		E. coli		S. aureus	
		Inhibition diameter (cm)	Inhibition	Inhibition diameter (cm)	Inhibition
Control	Blank	0 ± 0^a	—	0 ± 0^a	—
	NE_2	1.10 ± 0.43^b	+	0 ± 0^a	—
Thyme	NE_2_T0.5	0.95 ± 0.18^b	+	0.73 ± 0.14^b	+
	NE_2_T1	1.00 ± 0.25^b	+	$0.95 \pm 0.18^{b,c}$	+
	NE_2_T2	0.90 ± 0.00^b	+	$1.30 \pm 0.02^{b,c}$	+
Lemon balm	NE_2_LB0.5	0.97 ± 0.14^b	+	$0.90 \pm 0.43^{b,c}$	+
	NE_2_LB1	0.97 ± 0.38^b	+	$0.90 \pm 0.50^{b,c}$	+
	NE_2_LB2	0.90 ± 0.35^b	+	1.35 ± 0.53^c	+

Results showed that nanoemulsions, with or without essential oils, present similar ($p > 0.05$) antimicrobial activity against *E. coli*. However, when compared with the control a difference ($p < 0.05$) was observed, showing that the nanoemulsion has antimicrobial activity. As mentioned before this can be explained by the surfactant and oleic acid presented in sunflower oil used for the NE production. Nevertheless, it was expected to observe an effect of the presence of the essential oils once these oils are reported to possess activity against this bacterium (Chapter II, section 2.4). However, is also known that Gram-negative bacteria, such as *E. coli*, are less susceptible to essential oils than Gram-positive bacteria (Dhifi et al., 2016; Hyldgaard et al., 2012; Khorshidian et al., 2017; Pandey et al., 2017). Thus,

according to these results, can be concluded that the concentrations used of thyme and lemon balm oils were not sufficient to improve the inhibition of *E. coli*.

For *S. aureus* it was observed that the NE without essential oils and blank did not inhibit this bacterium, while nanoemulsions loaded with thyme and lemon balm oils inhibited. Lou *et al.* (2017) tested the antimicrobial activity of nanoemulsions loaded with *Citrus medica* essential oil (carrier oil: MCT; surfactant: tween 80; SOR: 2; method: SE) against *E. coli* and *S. aureus*. Their formulations possessed activity against both bacteria but having higher influence on *S. aureus* (Lou et al., 2017). In this work, despite not affecting *E. coli*, nanoemulsions with essential thyme and lemon balm oils reduced the growth of the bacterium that they were mostly likely to inhibit - *S. aureus*. However, contrary to the work of Lou *et al.* (2017), the inhibition of *S. aureus* did not show statistical differences ($p > 0.05$) for higher amounts of both essential oils. With this in mind, it could be said that formulations with the lowest concentration on both oils can be used if the goal is the microbial inhibition.

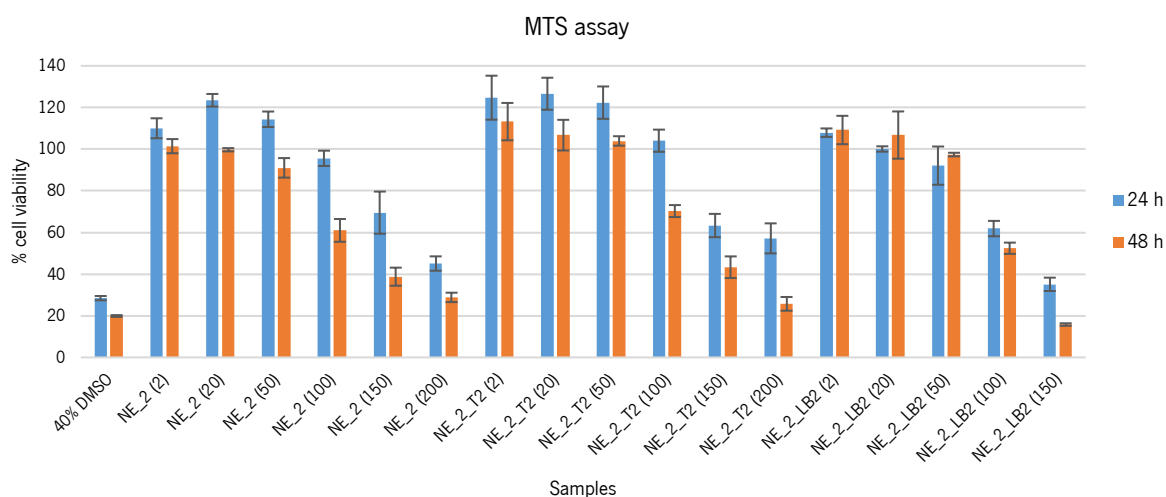
4.1.8. Toxicity of nanoemulsions – cell viability assessment

The toxicity of the selected nanoemulsions (NE_2, NE_2_T2 and Ne_2_LB2) was evaluated by two different methods of cell viability assessment: MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) and resazurin assays. The results were expressed as percentage of cell viability and samples presenting values below 70% were considered toxic to the cells. The results showed that the formulation with thyme essential oil (NE_2_T2) has similar toxicity to the one with only surfactant and carrier oil (NE_2). On the contrary, nanoemulsions loaded with lemon balm oil revealed to be more toxic.

Figure 4.21 shows the results of the MTS assay in Caco-2 cells at 24 h and 48 h of sample exposure. As it can be seen for each formulation, there is a clear tendency of the reduction of cell viability as its concentration increases. At the 24 h, NE_2 and NE_2_T2 revealed to be toxic at 150 µg oil/mL emulsion and NE_2_LB2 was toxic at 100 µg oil/mL emulsion (cell viability < 70%). This shows that the ingredients of the formulation themselves (Tween 80 and/or sunflower oil) have some toxicity to Caco-2 cells. Thyme essential oil did not alter the concentration at which nanoemulsions are toxic, and lemon balm oil provided higher toxicity to the formulation. At 48 h of exposure, all three samples were toxic at 100 µg oil/mL emulsion. Sousa *et al.* (2004), tested the cytotoxicity of lemon balm essential oil through a MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay at 48 h and their results showed that this oil was toxic at 90-100 µg/mL, which in good agreement to the results presented in this work (Sousa et al., 2004). Another study evaluated the toxicity of thymol, one of the main compounds of

thyme essential oil. Their results revealed that thymol was toxic to Caco-2 cells at concentrations higher than 100 µg/mL in a lactate dehydrogenase assay at 24 h of exposure (Putala, Nurminen, & Tiihonen, 2017).

Figure 4.21 – Effect of emulsions on the viability of Caco-2 cells after incubation for 24 and 48 h. The cell viability was determined by measuring absorbance using the MTS assay ($\lambda = 450$). DMSO and culture medium were used as positive and negative (100% cell viability) controls, respectively.



The results of the resazurin assay in Caco-2 cells at 24 h and 48 h of sample exposure are represented in **Figure 4.22**. Like in the MTS assay, the cell viability is decreased with the increase of the concentration of the sample and lemon balm oil showed higher toxicity than thyme oil. At 24 h of exposure, NE_2 and NE_2_T2 were toxic at 200 µg oil/mL emulsion and NE_2_LB2 was toxic at 150 µg oil/mL emulsion (cell viability < 70 %). At the 48 h time point, neither NE_2 nor NE_2_LB2 altered their toxic concentration in Caco-2 cells, and the formulation with thyme essential oil, NE_2_T2, was toxic at 150 µg oil/mL emulsion. The obtained toxic concentrations through this method are higher than the ones obtained in the MTS assay.

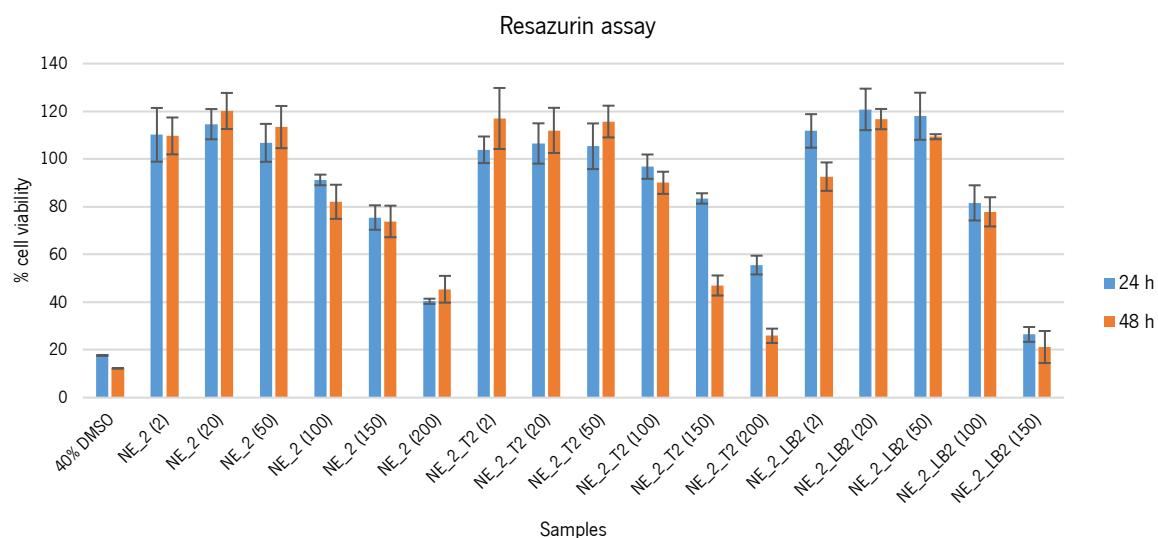


Figure 4.22 – Effect of emulsions on the viability of Caco-2 cells after incubation for 24 and 48 h. The cell viability was determined by measuring fluorescence using the resazurin reduction assay ($\lambda_{\text{ex}}=560$ nm, $\lambda_{\text{em}}=590$). DMSO and culture medium were used as positive and negative (100% cell viability) controls, respectively.

4.2. Materials and Methods

4.2.1. Raw Materials

For the development of the nanoemulsions were used refined sunflower oil (3ás, Fula, Portugal), rhamnolipids (R90, AGAE Technologies, USA), octenyl succinic anhydride modified starch (C*EmCap, Cargill, USA), sodium carboxymethylcellulose (C5013, Sigma-Aldrich, USA), Tween 80 (P1754, Sigma-Aldrich, USA) and ultrapure water (Milli-Q, USA). There were used pure oils of thyme (*Thymus vulgaris*) and lemon balm (*Melissa officinalis*), supplied by the company Earth Essences (Portugal). TEM grids (ultra-thin carbon film on Lacey carbon support film, 400 mesh, Copper, ref. 01824) were acquired from Ted Pella Inc. (USA), and UranyLess (22409) from Electron Microscopy Sciences, USA. DPPH (D9132), Trolox (23881), ABTS (A1888) and Plate Count Agar (PCA) plates were provided from Sigma-Aldrich, USA. Ethanol 99% was purchased from Honeywell (USA), and 96-well microplates (611F96) were from Thermo-Fisher, USA.

4.2.2. Comparison between high and low energy methods

In order to verify if there was any advantage in using low energy methods instead of high energy, free oils were submitted to a high energy procedure. For this, mixtures of essential oil and sunflower oil (2:8) and only sunflower oil as control were sheared in an ultra-turrax (T18 digital, IKA, Germany) for 2 minutes at 5000 rpm. Then, samples were submitted to ultrasounds (Digital Sonifier 450, Branson, USA) for 15 minutes with an amplitude of 50%, with 30 seconds on and 30 seconds off, making a 30 minutes procedure. After this, samples were analyzed for their sensorial (odor) and chemical (FTIR) properties. FTIR analysis was performed as described in section 3.2.6.

4.2.3. Nanoemulsions production by the EPI method

Nanoemulsions were produced by the Emulsion Phase Inversion (EPI) method. The procedure was adapted from Ostertag *et al.* (2012). The organic phase was prepared by mixing the surfactant and the oil (10 wt%), or mixture of oils, at 750 rpm for 30 minutes. It was used ultrapure water (Milli-Q) as the aqueous phase. The titration of the water into the organic phase was made with a syringe pump (NE-1000, New Era Pump Systems, USA) at a flow rate of 4 mL/min, with agitation (750 rpm) for 60 minutes. Different surfactants, surfactant-to-oil ratios, essential oil concentrations and agitation type and duration were evaluated. The surfactants used were rhamnolipids, octenyl succinic anhydride modified starch, sodium carboxymethylcellulose and Tween 80. The agitation was carried out with a Heidolph (Germany)

agitation plate MR Hei-Tec or with a VWR (USA) overhead stirrer VOS 14 S40 equipped with a metal 4-blade or plastic D-shaped stirrer tools. To each sample, it was given a code accordingly to their SOR, essential oil used and its quantity.

4.2.4. Dynamic Light Scattering

Dynamic Light Scattering analysis were performed in a Horiba SZ-100 (Japan). Polydispersity and size measurements (mean sizes in intensity) were carried out in a disposable cell with four openings, and for Zeta Potential evaluation it was used a carbon electrode cell. Temperature was set to 25 °C and measurements were made with a fixed angle of 90°. Before analysis, 10 µL of sample were diluted in Milli-Q water up to 5 mL (J. Komaiko & McClements, 2014). The equipment used is specified only for sizes between 0.3 nm and 8.0 µm.

4.2.5. Transmission electron microscopy

Transmission electron microscopy was used to assess the morphology and size of the produced nanoemulsions. The sample preparation was carried out in the day before the analysis. First, TEM grids were previously cleaned in a 1020 Plasma Cleaner (Fischione, USA) for 3 minutes to remove any contaminants. Then, the grid was immersed on a drop of sample and it was removed the excess with a filter paper. Next, the grid was negatively stained through the contact with a drop of contrasting agent, UranylLess, and again the excess was removed with filter paper. The time of contact with each of the drops was for about 1 minute. After this, samples were air-dried before analysis. The samples were observed using a JEM-2100 transmission electron microscope (JEOL, Japan) operating at a 200 kV accelerating voltage.

The volume of the shell layer of droplets was assessed in this analysis. First, the volume of the lipophilic core (v_1) and the total volume of the droplets (v_2) were calculated according to the equation of the volume of a sphere (Eq. 4.1), where r is the radius of the sphere. Then, the volume of the shell layer (v_{shell}) was obtained by Eq. 4.2 and its corresponding percentage (% v_{shell}) relatively to the total volume of the droplet was assessed by Eq. 4.3.

$$v_1 \text{ or } v_2 = \frac{4\pi r^3}{3} \quad \text{Eq. 4.1}$$

$$v_{shell} = v_2 - v_1 \quad \text{Eq. 4.2}$$

$$\% v_{shell} = \frac{v_{shell} \times 100}{v_2} \quad \text{Eq. 4.3}$$

4.2.6. Storage stability

The physical stability of formulations under storage was assessed by both DLS analysis and visual inspection for any creaming at two temperatures: 20 °C and 4°C. Samples without essential oil were followed during 6 months, while samples with essential oils were monitored during 3 months, whenever possible.

4.2.7. Accelerated kinetic stability tests

These tests were performed to samples that presented a high storage stability. Were selected samples with a SOR 2 with different oil phases: 10 wt% sunflower oil, 9 wt% sunflower oil + 1 wt% thyme essential oil and 9 wt% sunflower oil + 1 wt% lemon balm essential oil. Each formulation was analyzed in duplicate, following the procedure of Shafiq-un-Nabi *et al.* (2007).

4.2.7.1. Centrifugation

Samples were centrifuged at 5000 *g* for 30 min in a VWR Micro Star 17R centrifuge (USA). If the formulations did not show any phase separation, they proceeded to the heating-cooling cycle test.

4.2.7.2. Cycle of heating-cooling

During this heating-cooling cycle, samples were exposed to six cycles between 4 °C and 50 °C for more than 48 h at each temperature, in a Memmert oven (Germany).

4.2.8. Antioxidant activity

The antioxidant activity of the produced nanoemulsions with and without essential oil and also of the free oils were assessed by two methodologies: the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging and the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) methods. Free essential oils were used in a concentration equal to the one found in NE_2_T2, in the case of thyme oil, or in NE_2_LB2, for lemon balm oil.

4.2.8.1. DPPH assay

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging test was carried out using the method described by Ballesteros *et al.* (2015), with some modifications. First, it was prepared a solution of 150 µM DPPH in ethanol 99% and a stock solution of 1000 µM Trolox in the same solvent. Then,

various Trolox standards for the calibration curve were prepared with concentrations of 750, 500, 300, 200, 100, 80, 40 and 20 μM and the samples to be analyzed were diluted in ethanol 99% at the proportion of 1:20. The absorbance at 515 nm of 225 μL of the DPPH solution was adjusted to be 0.70 ± 0.02 through the dilution of the solution with ethanol. During all the procedure the solutions were kept away from the light. Finally, in a 96-well microplate, it was put 25 μL of the standard, sample or ethanol (blank) and to each of these pools it was added 200 μL of DPPH solution. These produced solutions were left to react for 1 h, protected from the light. Then their absorbance at 515 nm was measured in a spectrophotometric microplate reader (Synergy H1 Hybrid Multi-mode Reader, BioTek, USA). The percentage of inhibition and Trolox Equivalent Antioxidant Capacity (TEAC) were calculated by Eq. 4.4 and Eq. 4.5, respectively. TEAC values were expressed as mM of Trolox equivalent (TE) per mL of nanoemulsion or free oil (mM TE/mL).

$$\% \text{ inhibition} = \left(1 - \frac{\text{Absorbance of sample}}{\text{Absorbance of blank}}\right) \times 100 \quad \text{Eq. 4.4}$$

$$\text{TEAC} = \text{concentration given by calibration curve} \times \frac{\text{volume of solvent used in the dilution}}{\text{volume of sample used}} \quad \text{Eq. 4.5}$$

4.2.8.2. ABTS assay

The 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) method was executed following the procedure of Re *et al.* (1999), with modifications. In the day before the analysis, it was prepared a solution of 7 mM ABTS in Milli-Q water and a solution of 2.45 mM potassium persulfate (PP) also in Milli-Q water. These two solutions were then mixed at 1:1 and left reacting for 12-16 h, under agitation and protected from the light. In the day of the analysis, it was prepared a stock solution of 1000 μM Trolox in ethanol 60% and various Trolox standards for the calibration curve with concentrations of 700, 650, 600, 500, 300, 200, 100, 80, 50, 30 and 15 μM . All the samples were diluted in ethanol 99%, with different proportions (1:20, 1:40 or 1:80) in order to make sure each of them had values in the calibration curve. The absorbance at 734 nm of 210 μL of the ABTS:PP solution was adjusted to be 0.70 ± 0.02 through the dilution of the solution with Milli-Q water. During all the procedure the solutions were kept away from the light. Finally, it was put 10 μL of the standard, sample or Milli-Q water (blank) in a multiplate and it was added 200 μL of ABTS:PP solution to each of the wells. These mixtures were left to rest for 6 minutes protected from the light and then their absorbance at 734 nm was measured. The percentage of inhibition and Trolox Equivalent Antioxidant Capacity were calculated using the same equations as in the DPPH assay. TEAC values were expressed as mM of Trolox equivalent (TE) per mL of nanoemulsion or free oil (mM TE/mL).

4.2.9. Antimicrobial activity

The antibacterial activity was tested against two bacterial strains: *Staphylococcus aureus* CECT 240 (Gram-positive) and *Escherichia coli* CECT 516 (Gram-negative) (Spain) by the disc agar diffusion test. Plate Count Agar (PCA) was prepared based on the respective instructions. The bacteria culture was grown in Nutrient broth medium at 37 °C during 24 h and 0.1 mL was inoculated in PCA plates. Sterile paper discs were immersed in 50 µL of different nanoemulsion solutions and placed on surface of each inoculated plate. As the essential oil compounds are volatile, each sample was used in an individual plate. The agar plates were incubated for 24 h at 37 °C and diameters of the inhibitory zone of clearance (cm) surrounding the discs were measured to estimate the antimicrobial activity. It was used a sample with only sunflower oil and samples of nanoemulsions with different quantities of thyme or lemon balm essential oil (0.5, 1 and 2 wt%). Sterile paper discs were used as control.

4.2.10. Toxicity of nanoemulsions - cell viability assessment

The toxicity of nanoemulsions with and without essential oil were evaluated by two methods of cell viability assessment: MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) and resazurin assays.

4.2.10.1. Cell Culture

The cell viability assessment was performed using MTS or resazurin assays with Caco-2 cells (passage 26-30), human colon epithelial cancer cells, obtained from American Type Culture Collection (USA). Caco-2 cells were cultured in minimum essential medium, supplemented with 20% fetal bovine serum, 1% non-essential amino acids, 1% sodium pyruvate and 1% penicillin/streptomycin. The cells were kept at 37°C and 5% CO₂ in 75 cm² flasks. For the *in vitro* assays, confluent cells were detached using 0.25% trypsin-EDTA solution, then precipitated by centrifugation at 1080 rpm for 5 min and resuspended in fresh medium at a concentration of 1×10⁵ cells/mL. Cells were seeded onto 96-wells plates at a density of 1×10⁴ cells (100 µL of cellular suspension) per well and left adhering overnight in a humidified atmosphere of 5% CO₂ in air at 37°C.

4.2.10.2. MTS assay

After overnight adhesion, the culture medium was removed and replaced by 100 µL of fresh medium. Samples (nanoemulsions at different concentrations – 2, 20, 50, 100, 150, 200 µg oil/mL

emulsion) were added to each well and incubated for 24 or 48 h. A negative control was performed using the cells growing in the culture medium (considered as 100% of cell viability) or 40% (v/v) DMSO was used as a positive control (cell death). At each time point, both culture medium and sample were collected, leaving only the viable cells. Then, in each well, it was added 100 µL of a solution of 5% MTS in culture medium and incubated at 37 °C for 3h. The absorbance was read at 450 nm using a Microplate Reader (Synergy H1, Biotek, USA). The cell viability was expressed in percentage of absorbance (460 nm) in treated cells in relation to the absorbance of cells growing in the cell culture medium (negative control) as described in **Eq. 4.6**:

$$\% \text{ cell viability} = \frac{Abs_{TC}}{Abs_C} \times 100 \quad \text{Eq. 4.6}$$

where Abs_{TC} is the absorbance of treated cells and Abs_C is the absorbance of cells growing in the culture medium.

4.2.10.3. Resazurin assay

After adhesion, the culture medium was removed and replaced by culture medium with 0.01mg/mL resazurin. Samples (emulsions at different concentrations – 2, 20, 50, 100, 150, 200 µg oil/mL emulsion) were added to each well and incubated for 24 or 48 h. A negative control was performed using the cells growing in the culture medium (considered as 100% cell viability) and 40% (v/v) DMSO was used as a positive control (cell death). At each time point, both culture medium and sample were collected, leaving only the viable cells. Then, in each well, it was added 100 µL of a solution of 10% resazurin in culture medium and incubated at 37 °C for 3h. The fluorescence intensity that is proportional to the cell viability, was directly measured at each time point (24 or 48h) using a Microplate Fluorescence Reader from BioTek (Synergy H1, USA) at an excitation wavelength of 560 nm and an emission wavelength of 590 nm. The % cell viability was expressed as fluorescence of treated cells compared to the fluorescence of cells growing in the culture medium as follows in **Eq. 4.7**:

$$\% \text{ cell viability} = \frac{F_{TC}}{F_C} \times 100 \quad \text{Eq. 4.7}$$

where F_{TC} is the fluorescence of treated cells and F_C is the fluorescence of cells growing in the culture medium.

4.2.11. Statistical analysis

All the tests were performed at least in triplicate and results were analyzed by the statistical test one-way ANOVA, in the GraphPad Prism software (version 8.2.1, USA) with a confidence interval of 95%.

CHAPTER V – GENERAL CONCLUSIONS

5.1. Conclusions

In this work, essential oils of thyme and lemon balm were characterized and encapsulated into nanoemulsions. First, their chemical characterization revealed that thyme oil was mainly composed by thymol, *p*-cymene and γ -terpinene and that lemon balm essential oil had β -caryophyllene as its main compound. GC-MS analysis of oils identified 39 compounds in thyme and 49 in lemon balm. NMR was not so efficient, since it identified only 11 constituents on thyme essential oil and 10 on lemon balm oil. Despite being limited to signal overlap, requiring in many cases previous oil fractionalization, NMR allowed the identification of 2 compounds that were not previously found in GC-MS analysis of thyme essential oil. The quantification of essential oil components showed some differences according to which technique was used. GC-MS overestimated at least some compounds and, for this reason, its results were equal or higher than the ones found with NMR. Here, it was demonstrated that NMR is a quick technique and produced reliable results. So it could be quite useful for quality control in routine analysis of essential oils of low-moderate complexity.

The second part of this work included the encapsulation of essential oils, characterization of the formulations and study of their activities. Essential oils were submitted to a high energy procedure and it was demonstrated that this methodology effectively degrades them. In the development of nanoemulsions using the emulsion phase inversion method (low energy method), it was not possible to achieve nanoemulsions with none of the natural surfactants tested, namely rhamnolipids, octenyl succinic anhydride modified starch and sodium carboxymethylcellulose. Stable nanoemulsions with surfactant-oil-ratio (SOR) of 1.5 and 2 were produced with the synthetic surfactant Tween 80. These formulations could be loaded with 0.5, 1 and 2 wt% essential oil, maintaining the intended characteristics: sizes < 200 nm, polydispersity index < 0.2 and Zeta potential < - 30 mV. Higher quantities of essential oil destabilized the system and were not able to produce stable nanoemulsions. Samples with SOR 2 were more stable, essentially when stored under refrigeration. TEM analysis showed that 34.6 ± 7.6 % of the total volume of the droplets correspond to a shell layer where Tween 80 is located. DPPH and ABTS antioxidant tests showed that there was an increase of the antioxidant capacity for nanoemulsions loaded with higher amounts of thyme oil; the encapsulation of this oil was not advantageous in terms of this property; and the antioxidant activity of lemon balm essential oil has been enhanced with its nanoencapsulation. These nanoemulsions were not effective against *E. coli* but could inhibit *S. aureus* and this activity was only due to essential oils, since the formulation without them did not inhibit this bacterium. Nanoemulsions with

sunflower oil (without EOs) and with thyme oil revealed to be toxic in Caco-2 cells for 150 µg/mL at 24 h and 100 µg/mL at 48 h. Nanoemulsions loaded with lemon balm oil showed higher toxicity since they were toxic from 100 µg/mL at 24 h and 48 h. Therefore, in this work were developed stable formulations capable of encapsulating essential oils by a non-aggressive method and that possess important biological activities.

5.2. Suggestions for future studies

Although this work has come to an end and the initial objectives were achieved, some improvements could be made in future studies. NMR results could be improved, identifying more compounds and completing the characterization of compounds that were already identified. For this, a more efficient separation in column chromatography could be carried out, using more eluents and/or collecting smaller fractions, in order to try having less different compounds in each. Pure standards should be used to confirm some suspicions or help to identify other compounds. GC-MS results could also be improved, since at least some compounds were overestimated and not all of the constituents were identified. To improve these results, the concentration of the internal standard can be adjusted (higher concentration), making new calibrations for each compound and eventually use more standards to validate some results. The study on the influence of high energy methods on the quality of essential oils could also be complemented with the analysis of nanoemulsions produced by high energy methods and confirm if the high energy also influences the oils during the production method. It is suggested to test again the natural surfactants, or using the same emulsifiers and testing other conditions, or trying some others such as saponins, whey protein and xanthan gum. Finally, since the type of agitation has influence on the produced emulsions, other stirrer tools could be tested like 3-blade, anchor or spiral.

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ANNEXES

Group A – NMR spectra used in the qualitative characterization of essential oils

Thyme essential oil

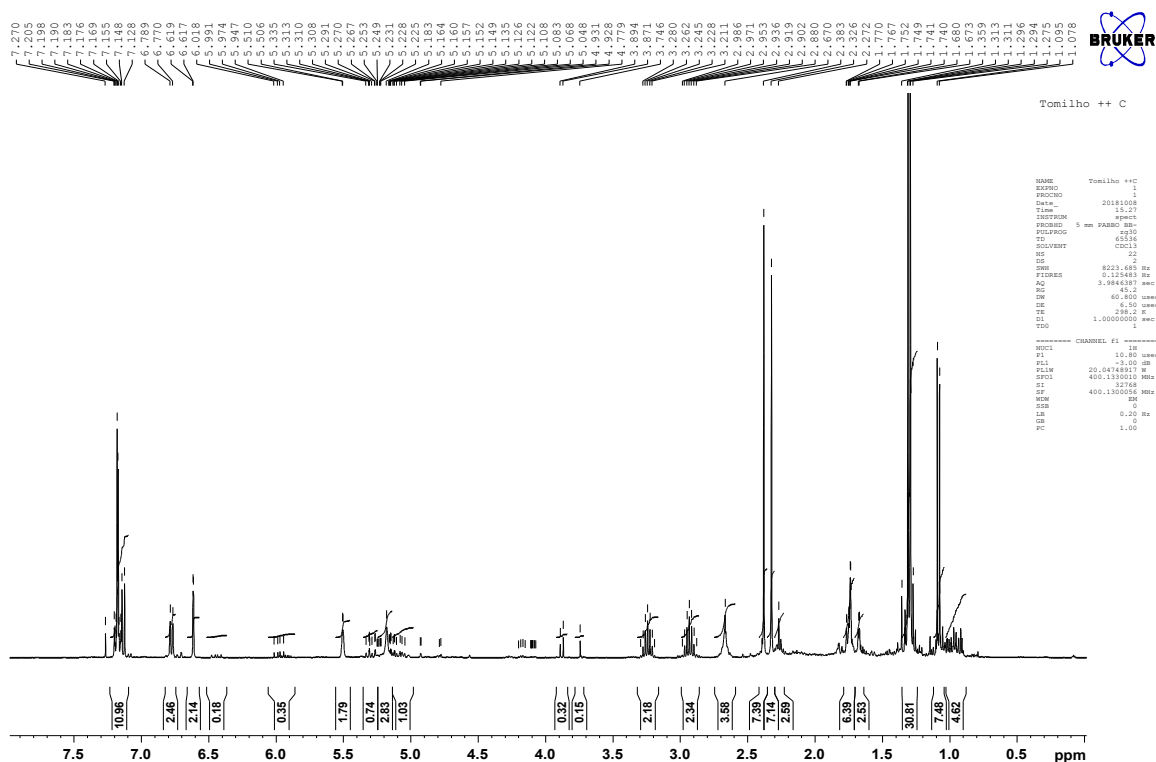


Figure A.1 – ^1H NMR spectrum of thyme essential oil. 400 MHz, CDCl_3 .

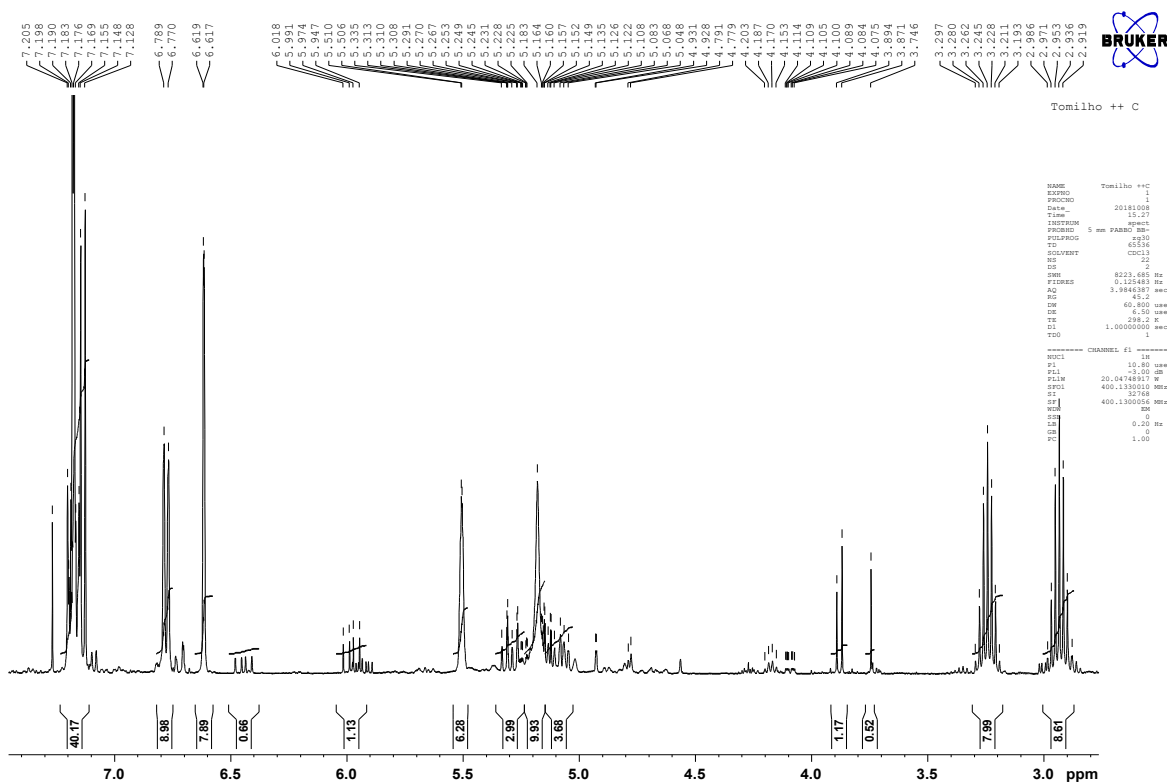


Figure A.1 (expansion 1) – ^1H NMR spectrum of thyme essential oil. 400 MHz, CDCl_3 .

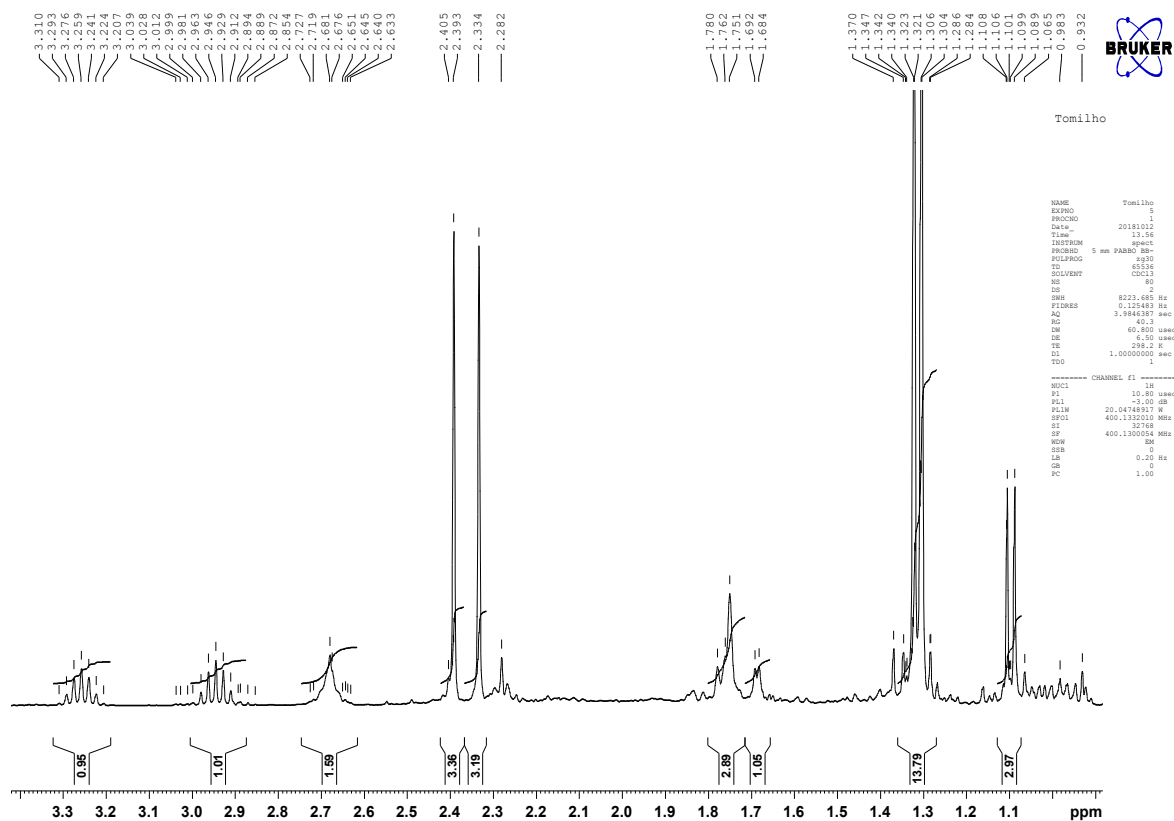


Figure A.1 (expansion 2) – ^1H NMR spectrum of thyme essential oil. 400 MHz, CDCl_3 .

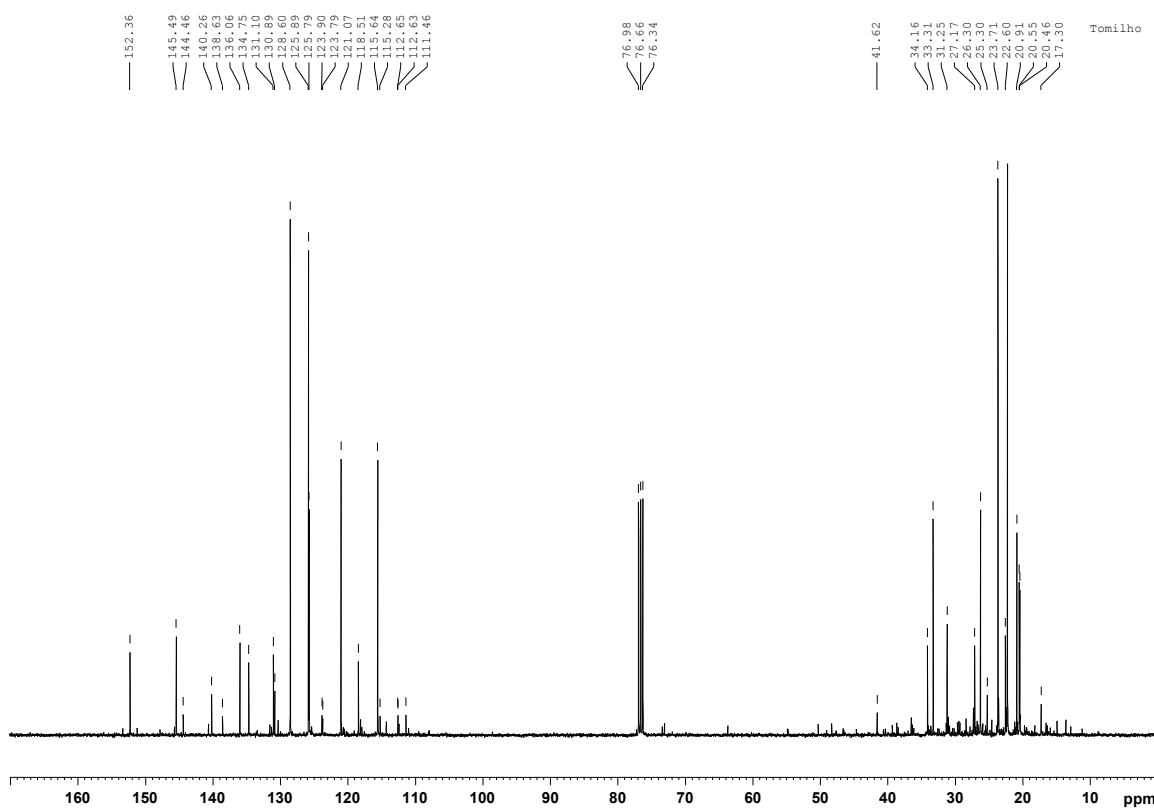


Figure A.2 – ^{13}C NMR spectrum of thyme essential oil. 100 MHz, CDCl_3 .

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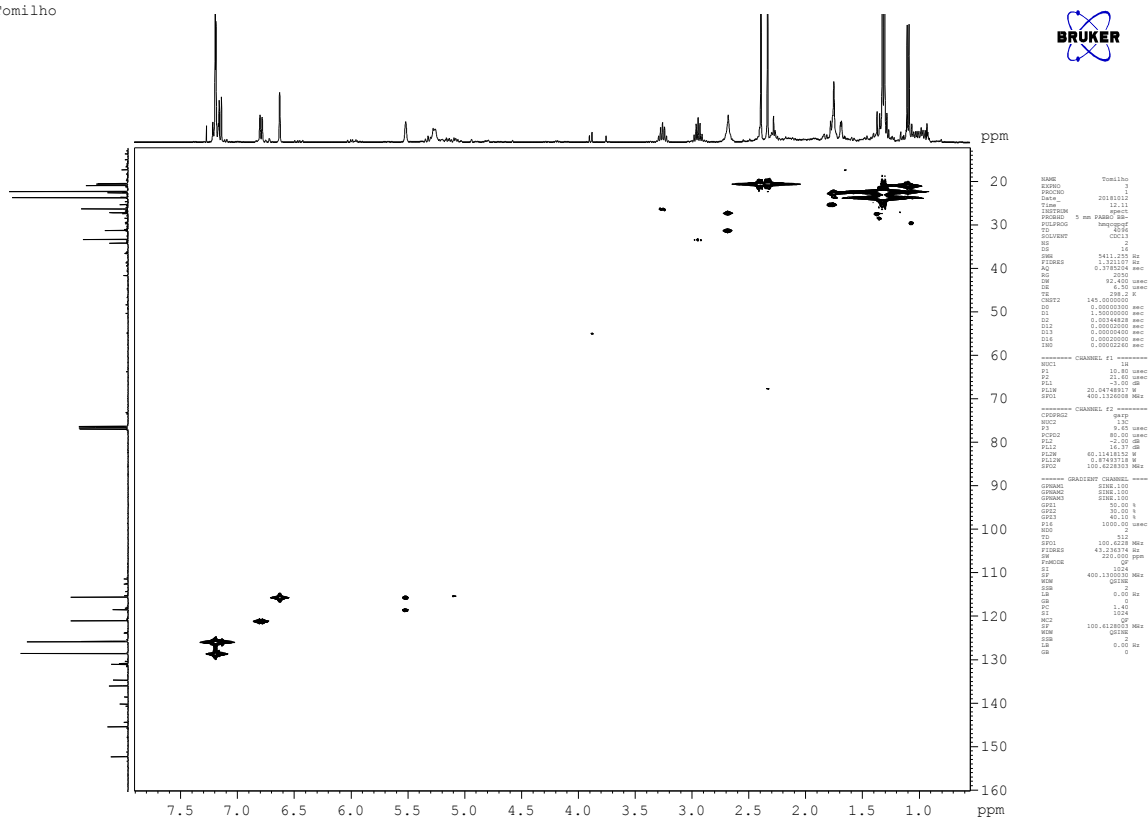


Figure A.3 – HMQC spectrum of thyme essential oil. ^1H - 400 MHz, ^{13}C - 100 MHz, CDCl_3 .

Tomilho

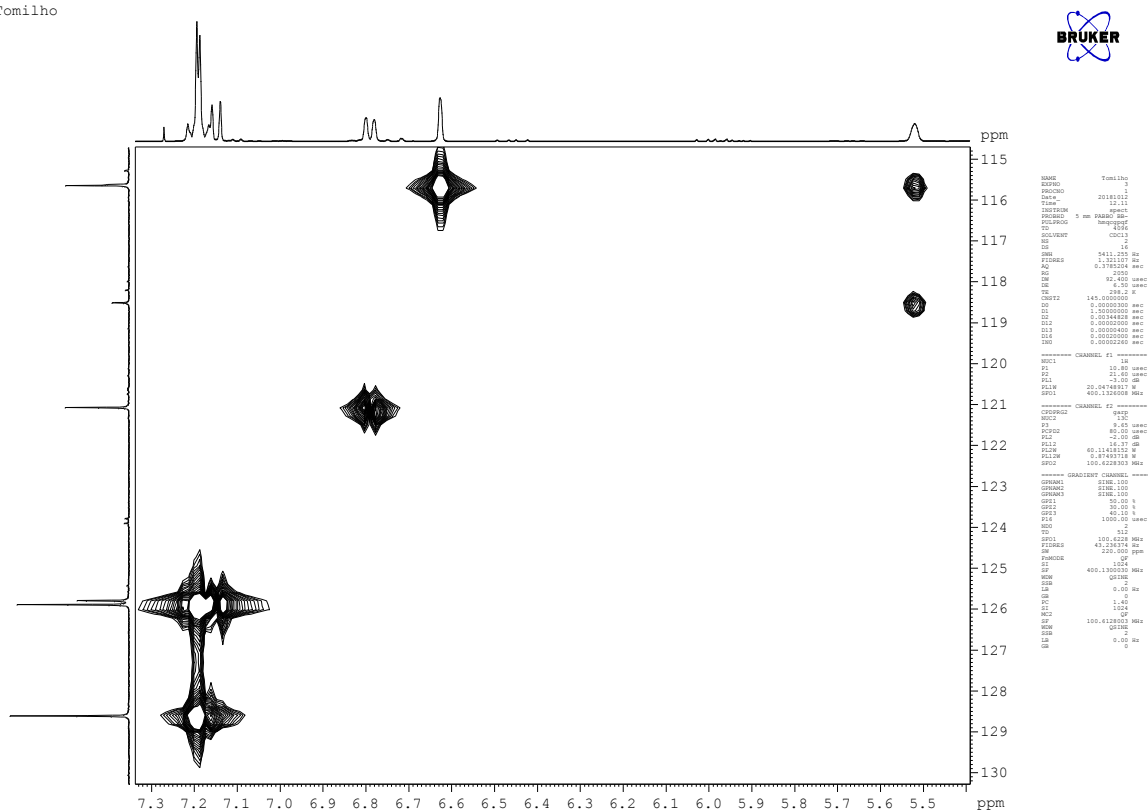
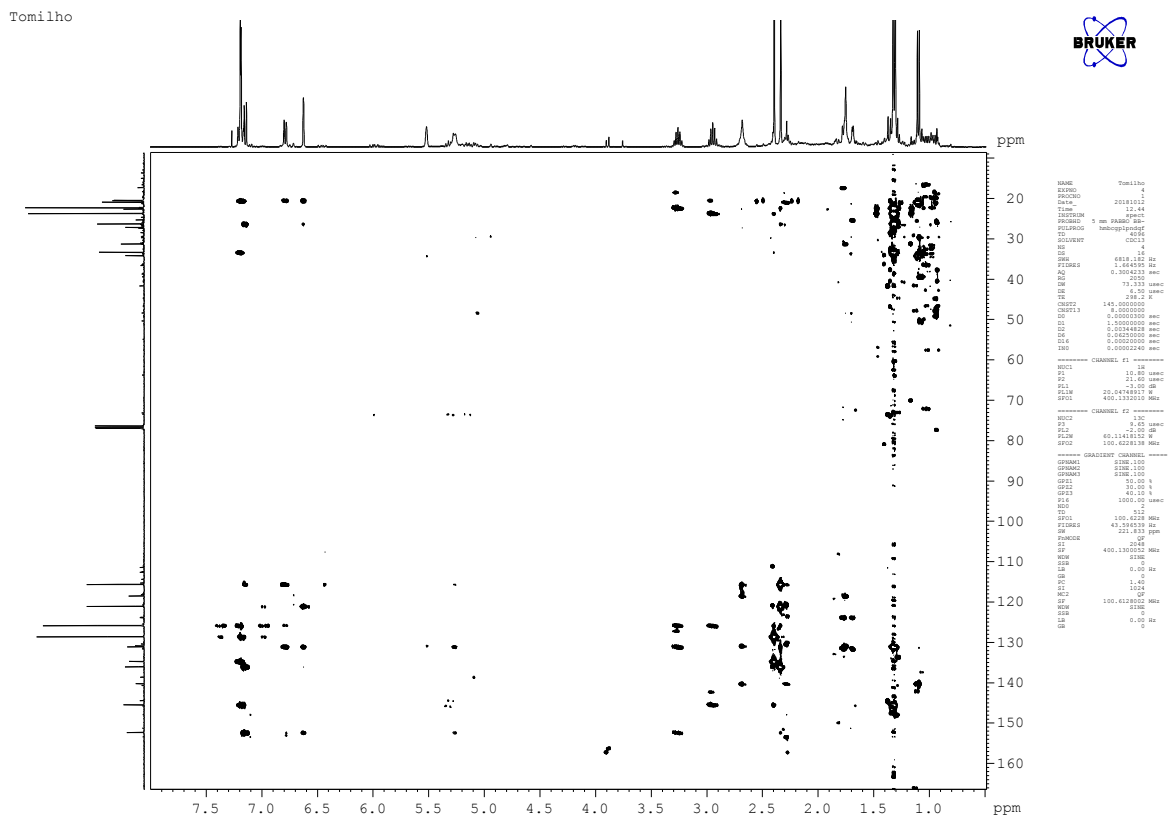
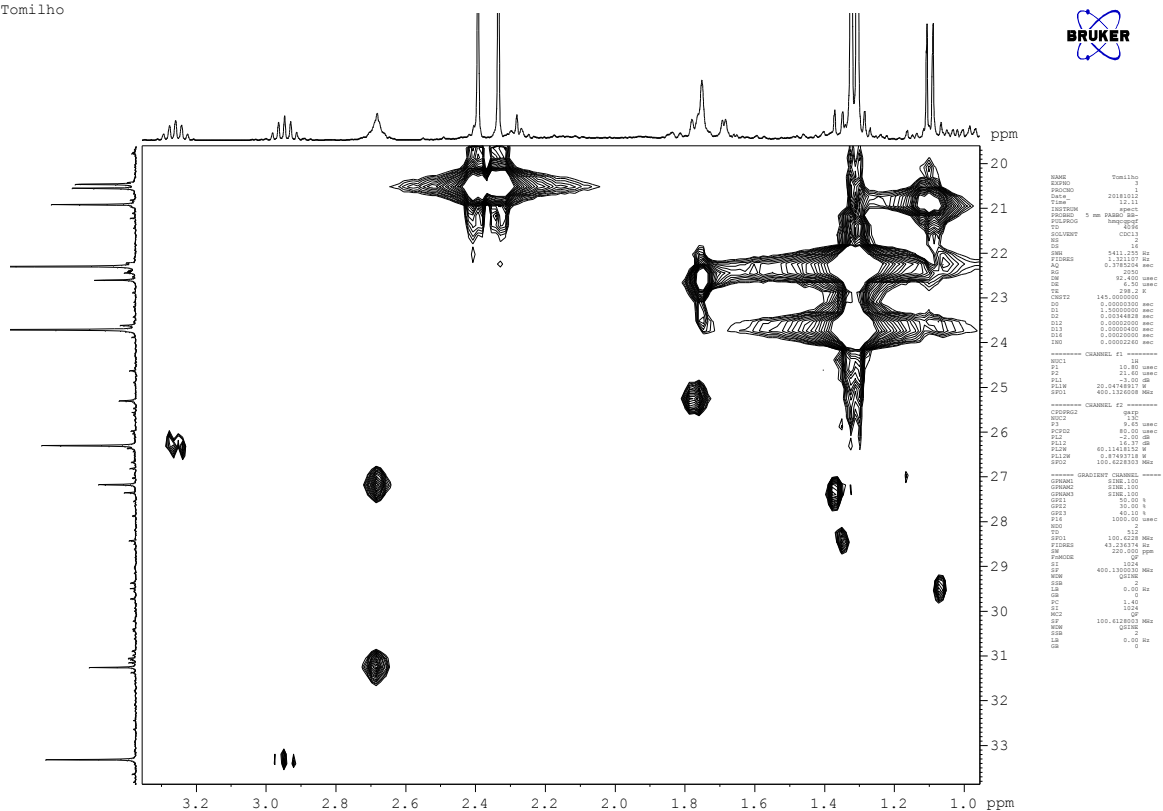


Figure A.3 (expansion 1) – HMQC spectrum of thyme essential oil. ^1H - 400 MHz, ^{13}C - 100 MHz, CDCl_3 .



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Tomilho

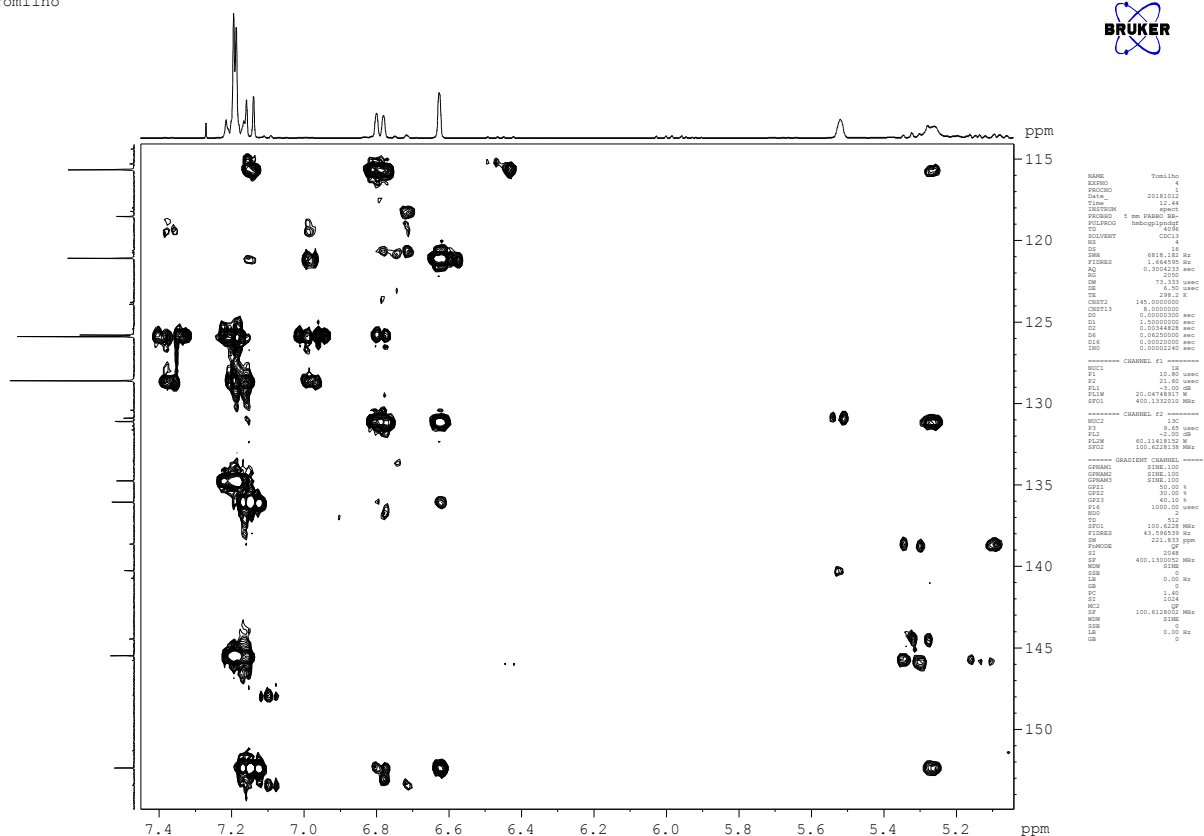


Figure A.4 (expansion) – HMBC spectrum of thyme essential oil. ^1H - 400 MHz, ^{13}C - 100 MHz, CDCl_3 .

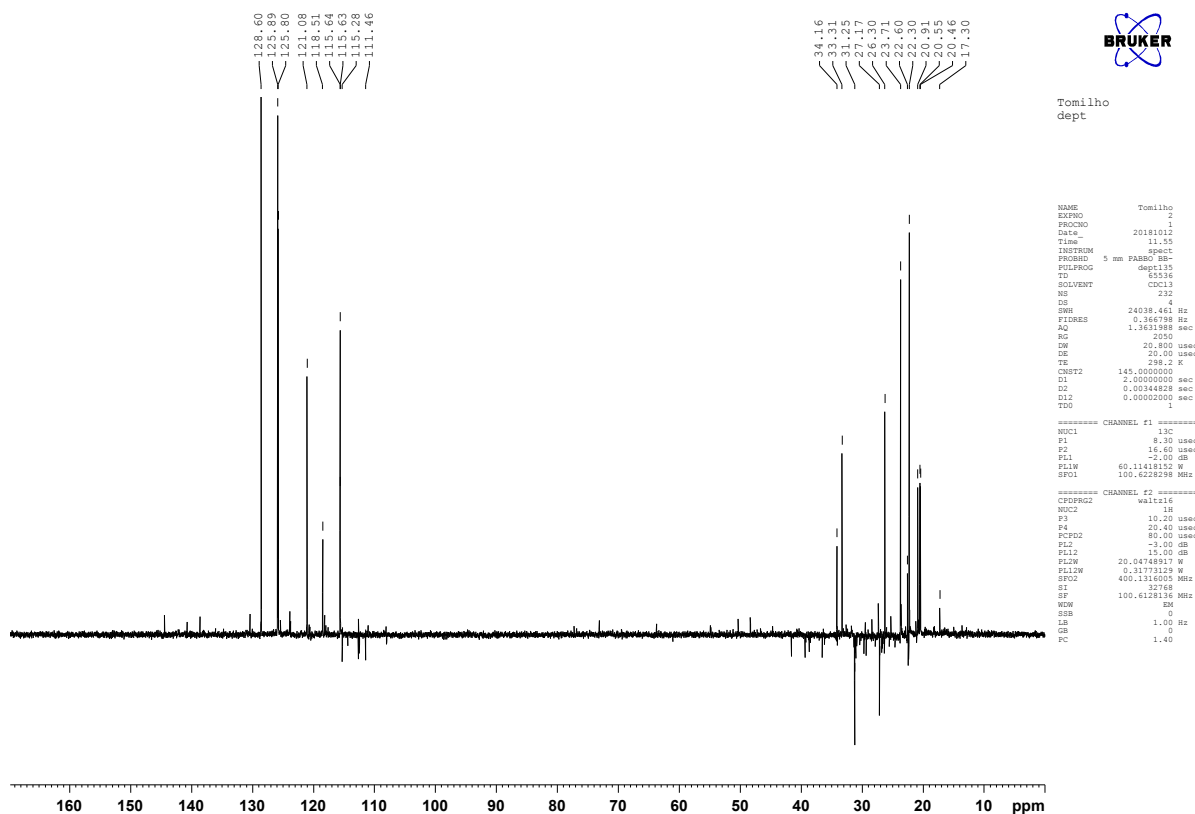


Figure A.5 – DEPT spectrum of thyme essential oil. 100 MHz, CDCl_3 .

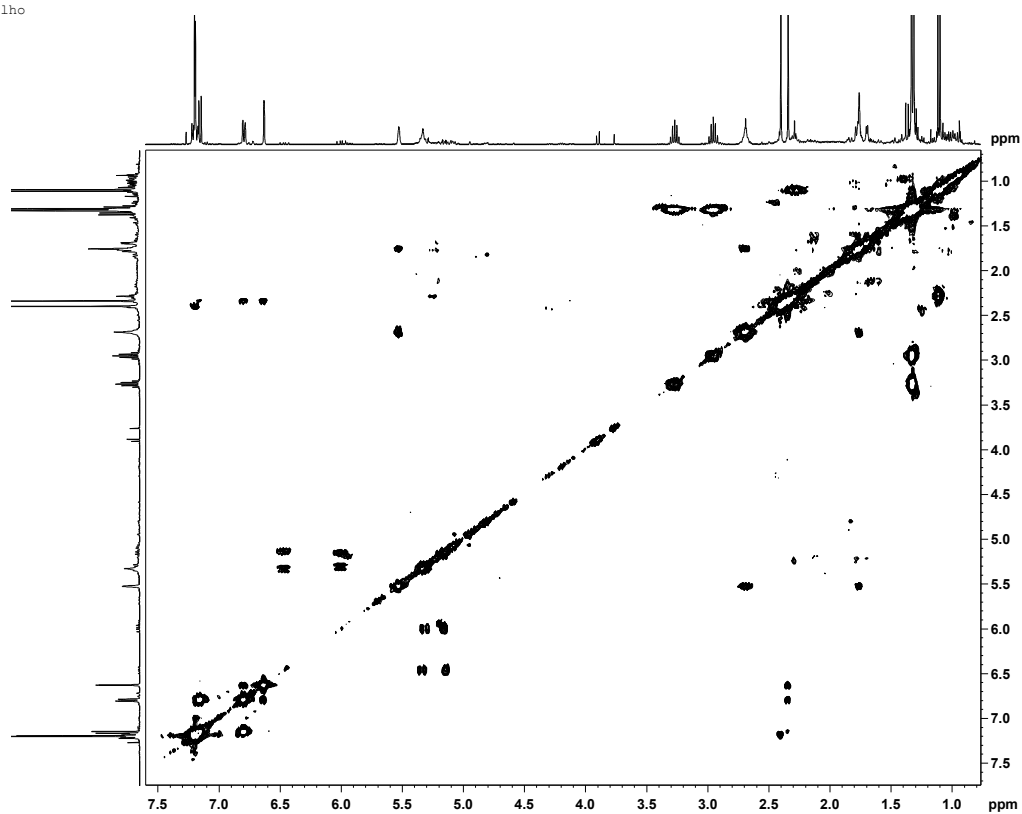


Figure A.6 – COSY spectrum of thyme essential oil. 400 MHz, CDCl₃.

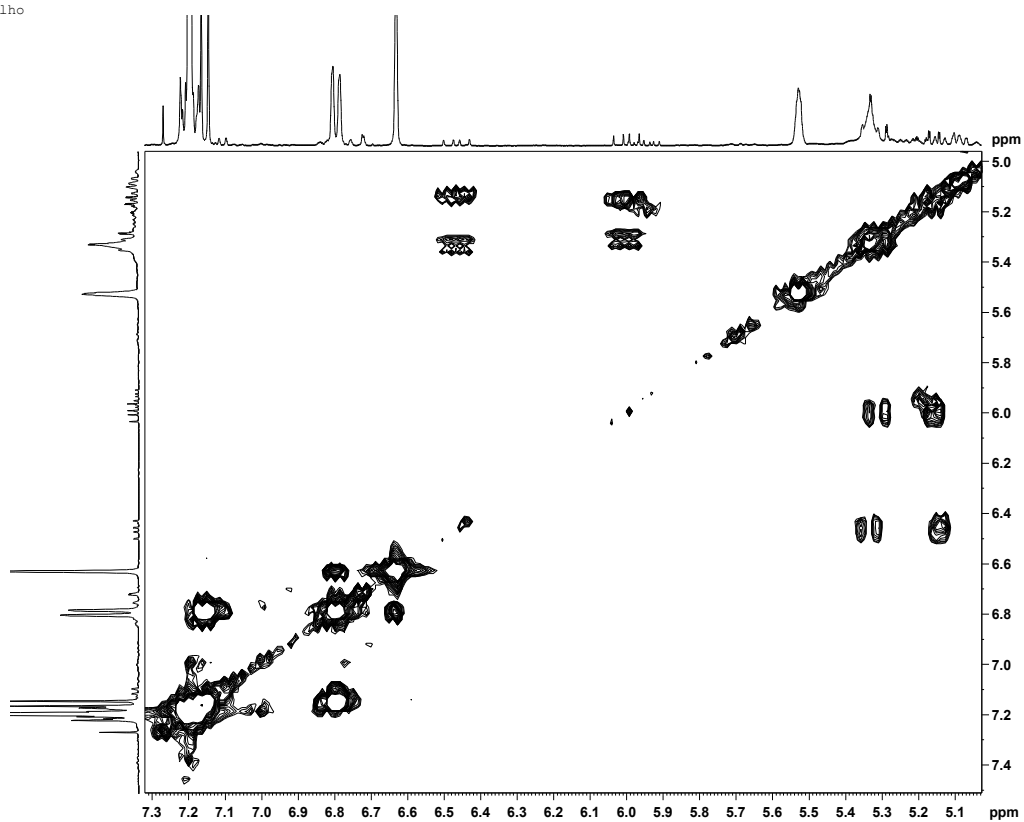


Figure A.6 (expansion 1) – COSY spectrum of thyme essential oil. 400 MHz, CDCl₃.

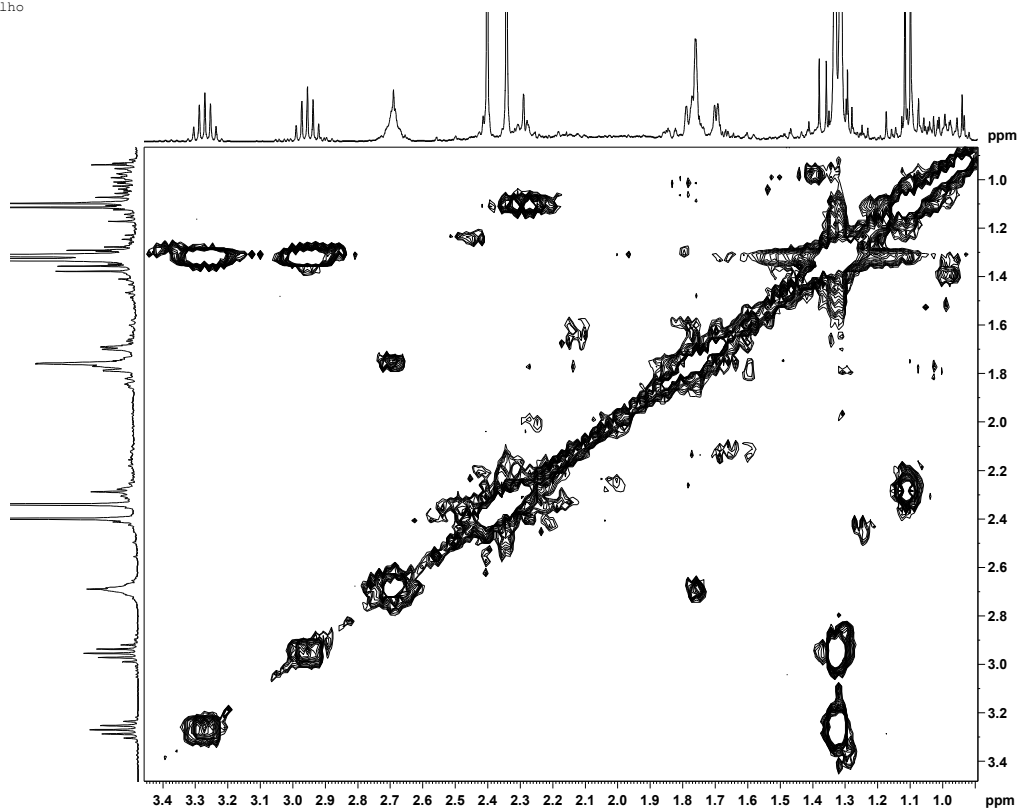


Figure A.6 (expansion 2) – COSY spectrum of thyme essential oil. 400 MHz, CDCl₃.

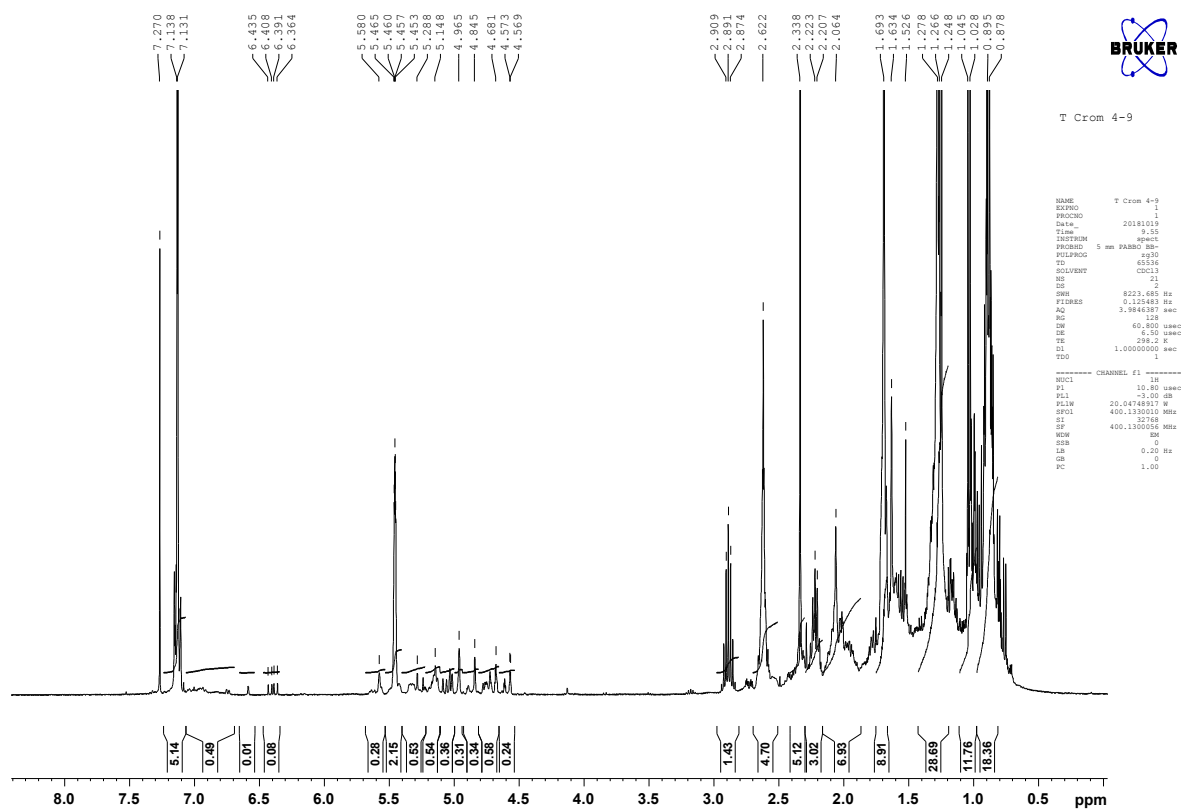


Figure A.7 – ¹H NMR spectrum of a fraction of thyme essential oil extracted with 90% petroleum ether - 10% ethyl acetate. 400 MHz, CDCl₃.

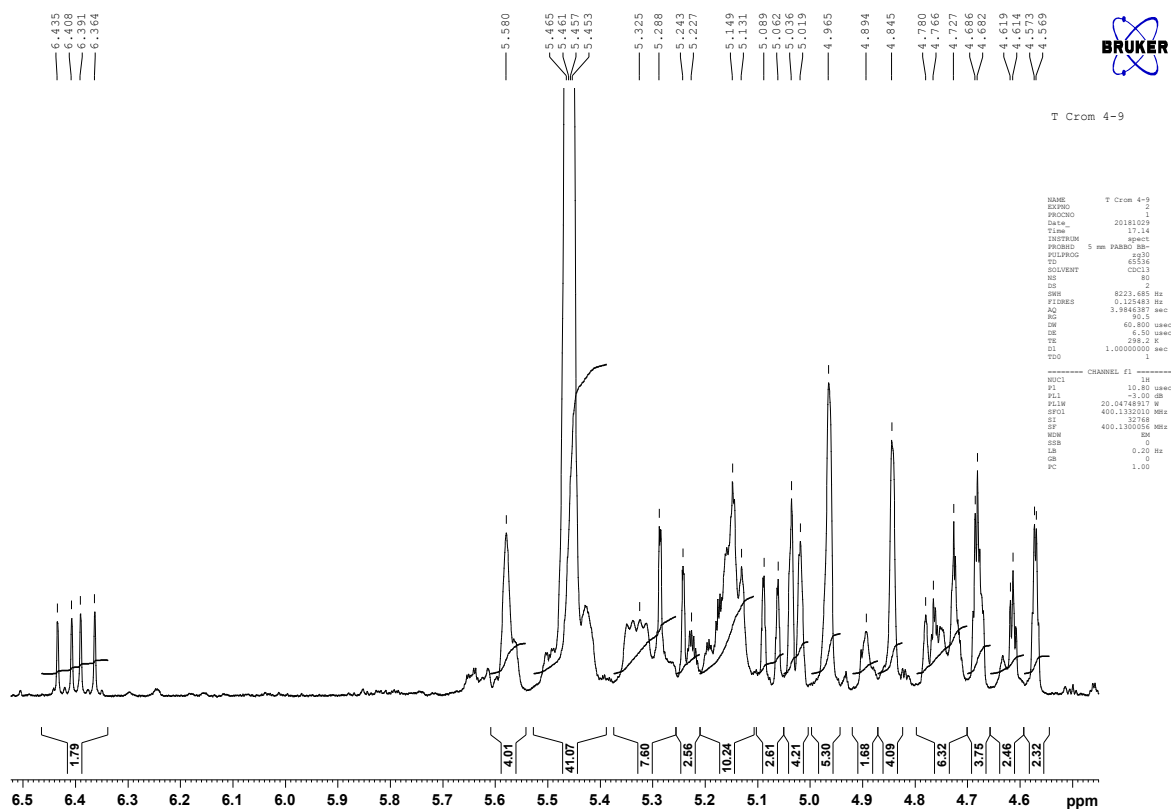


Figure A.7 (expansion 1) – ^1H NMR spectrum of a fraction of thyme essential oil extracted with 90% petroleum ether - 10% ethyl acetate. 400 MHz, CDCl_3 .

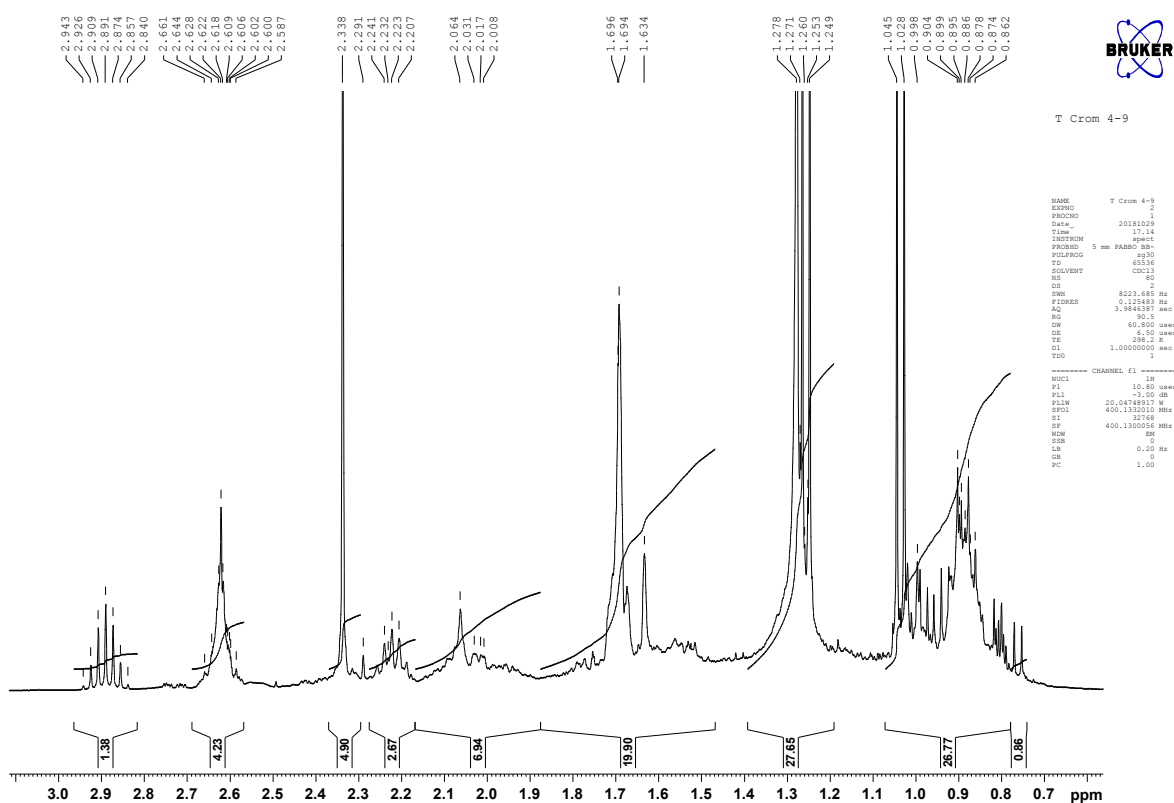


Figure A.7 (expansion 2) – ^1H NMR spectrum of a fraction of thyme essential oil extracted with 90% petroleum ether - 10% ethyl acetate. 400 MHz, CDCl_3 .

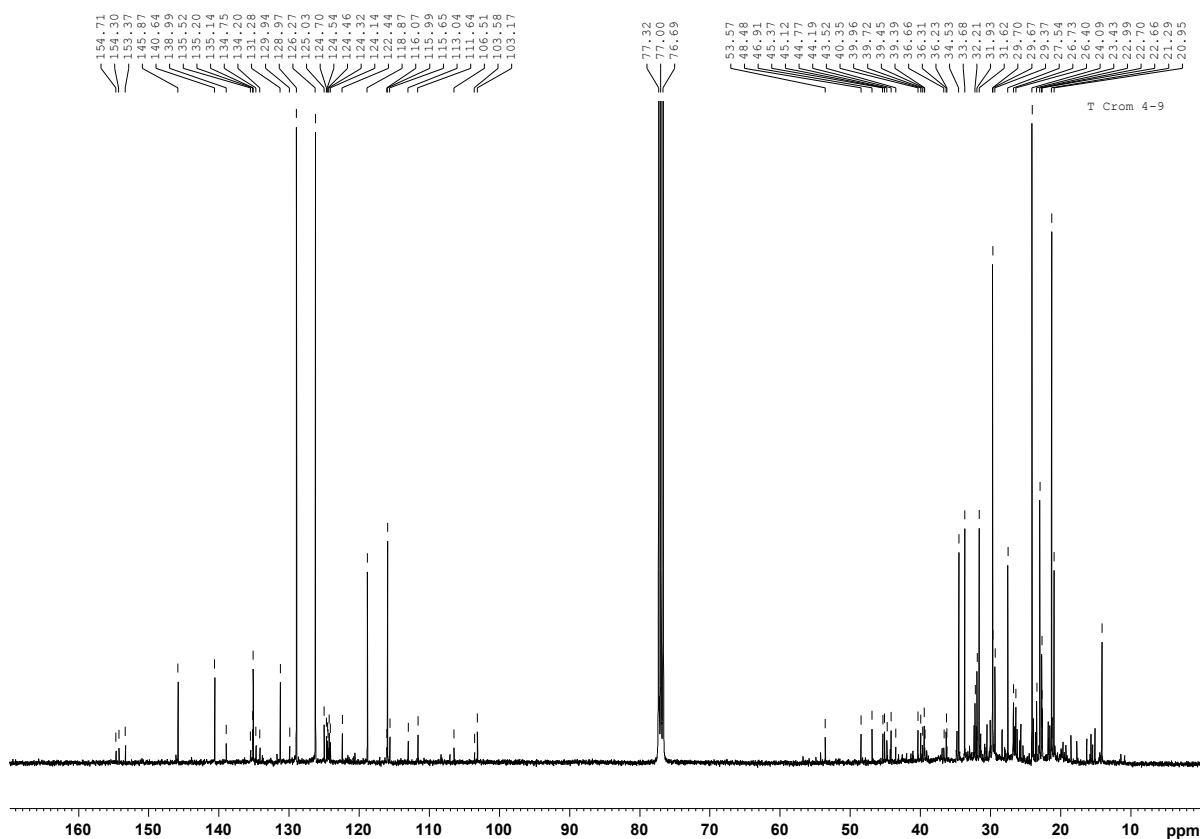


Figure A.8 – ^{13}C NMR spectrum of a fraction of thyme essential oil extracted with 90% petroleum ether - 10% ethyl acetate. 100 MHz, CDCl_3 .

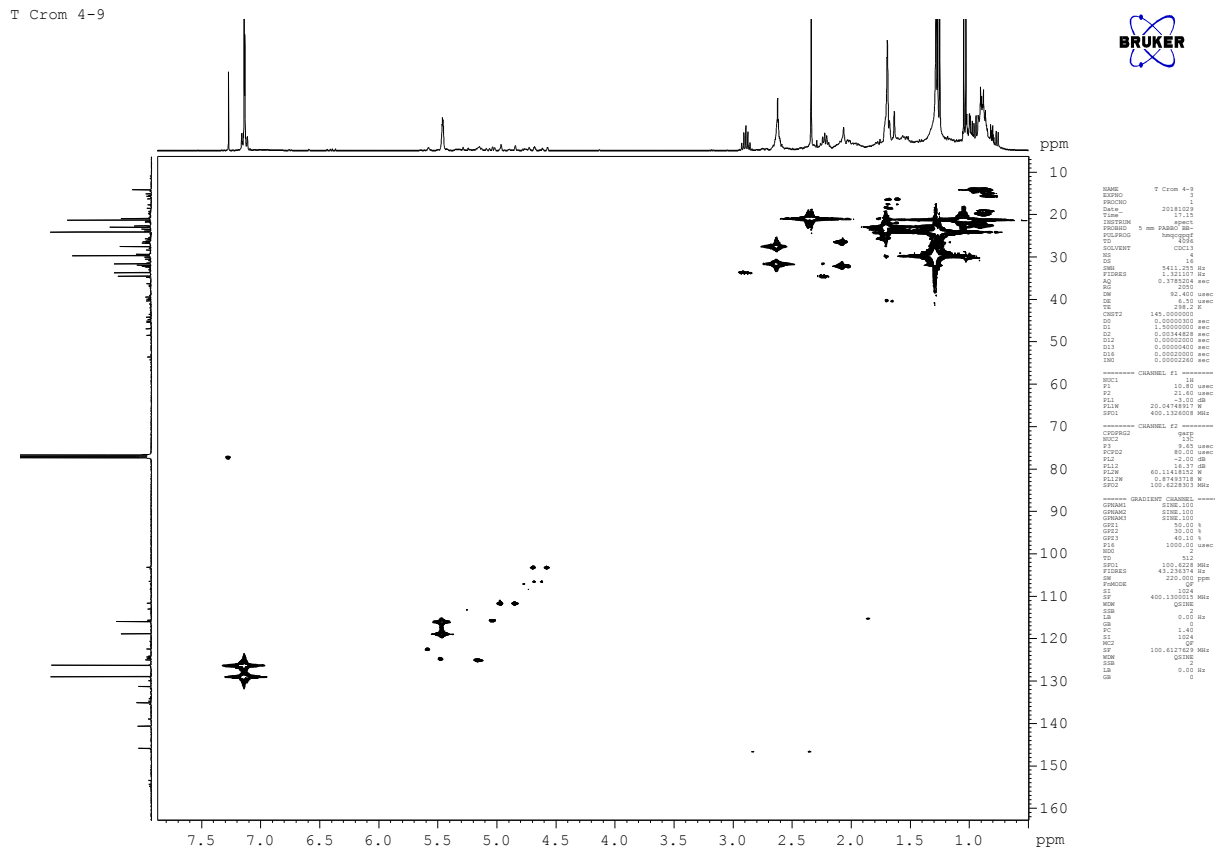


Figure A.9 – HMQC spectrum of a fraction of thyme essential oil extracted with 90% petroleum ether - 10% ethyl acetate. ^1H - 400 MHz, ^{13}C - 100 MHz, CDCl_3 .

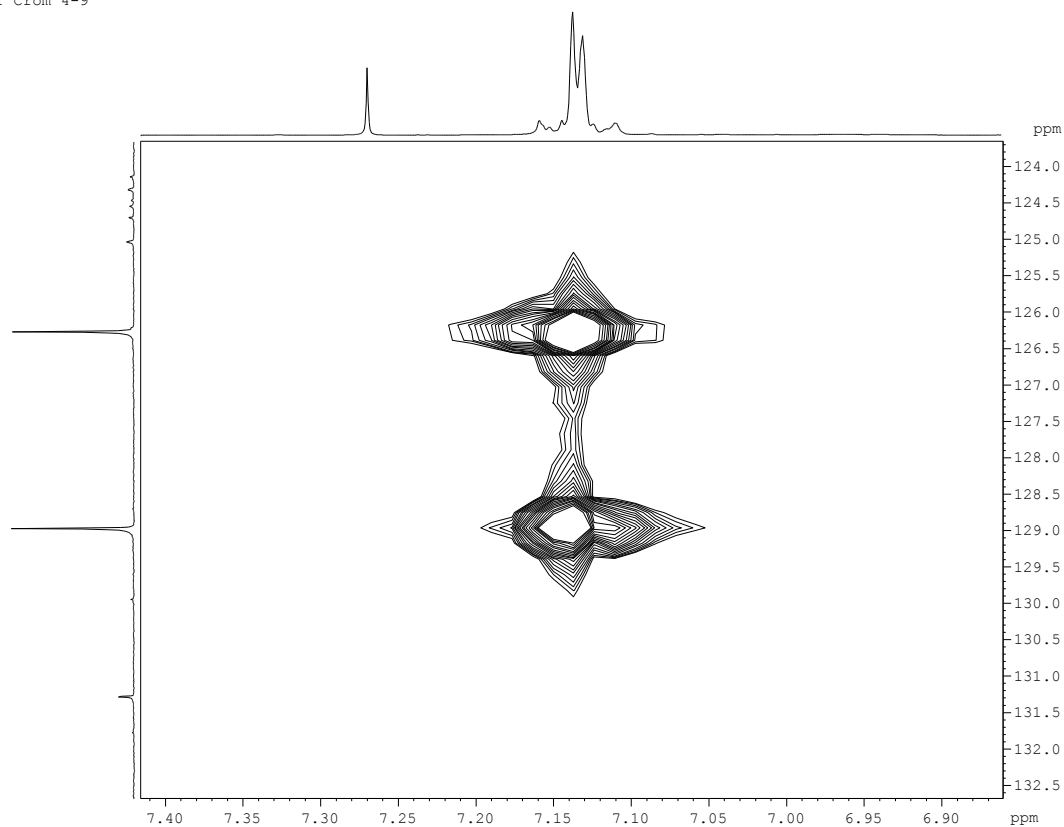


Figure A.9 (expansion 1) – HMQC spectrum of a fraction of thyme essential oil extracted with 90% petroleum ether - 10% ethyl acetate. ^1H – 400 MHz, ^{13}C – 100 MHz, CDCl_3 .

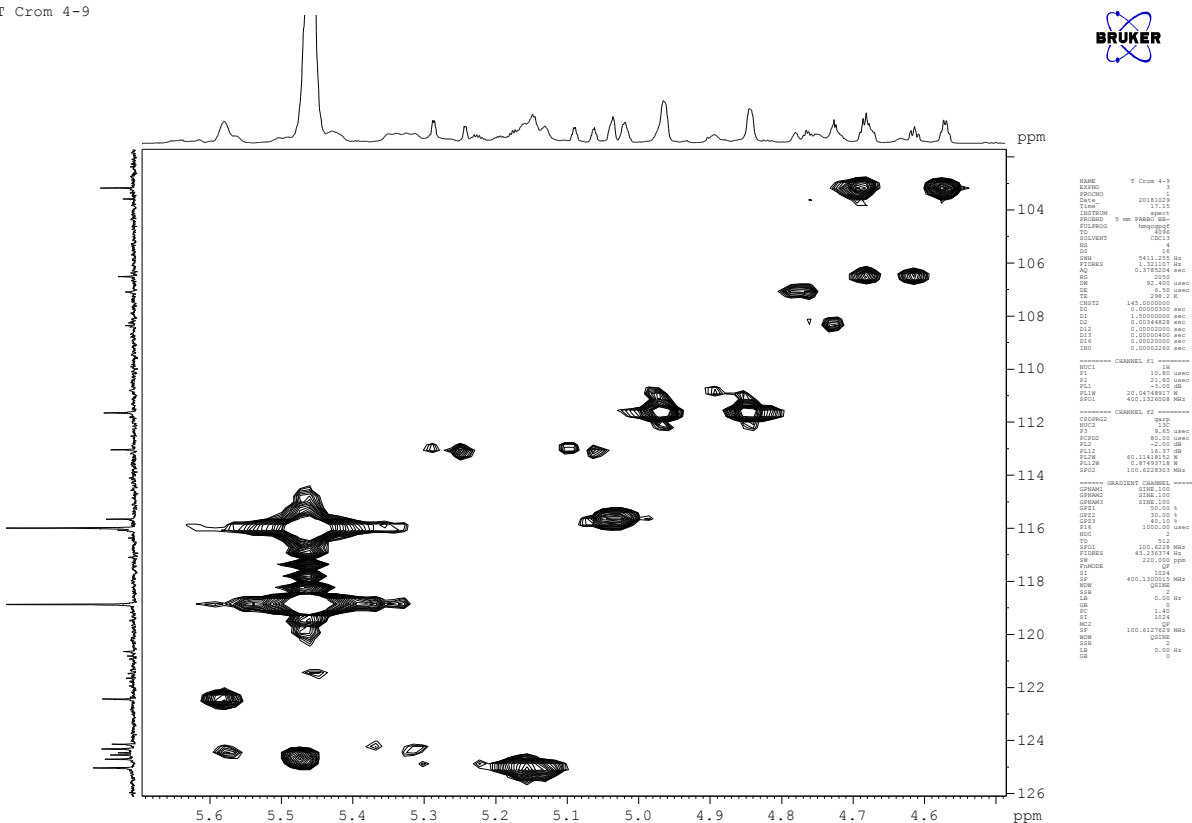


Figure A.9 (expansion 2) – HMQC spectrum of a fraction of thyme essential oil extracted with 90% petroleum ether - 10% ethyl acetate. ^1H – 400 MHz, ^{13}C – 100 MHz, CDCl_3 .

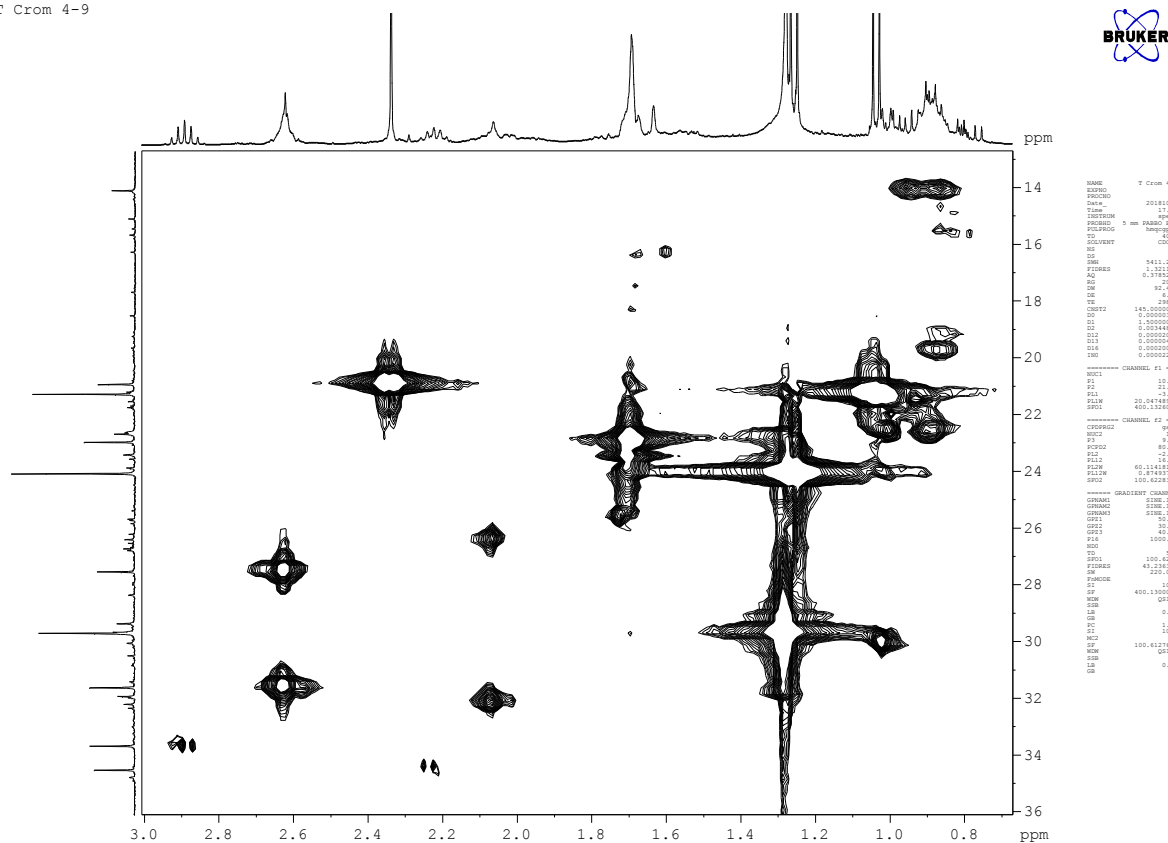


Figure A.9 (expansion 3) – HMQC spectrum of a fraction of thyme essential oil extracted with 90% petroleum ether - 10% ethyl acetate. ^1H – 400 MHz, ^{13}C – 100 MHz, CDCl_3 .

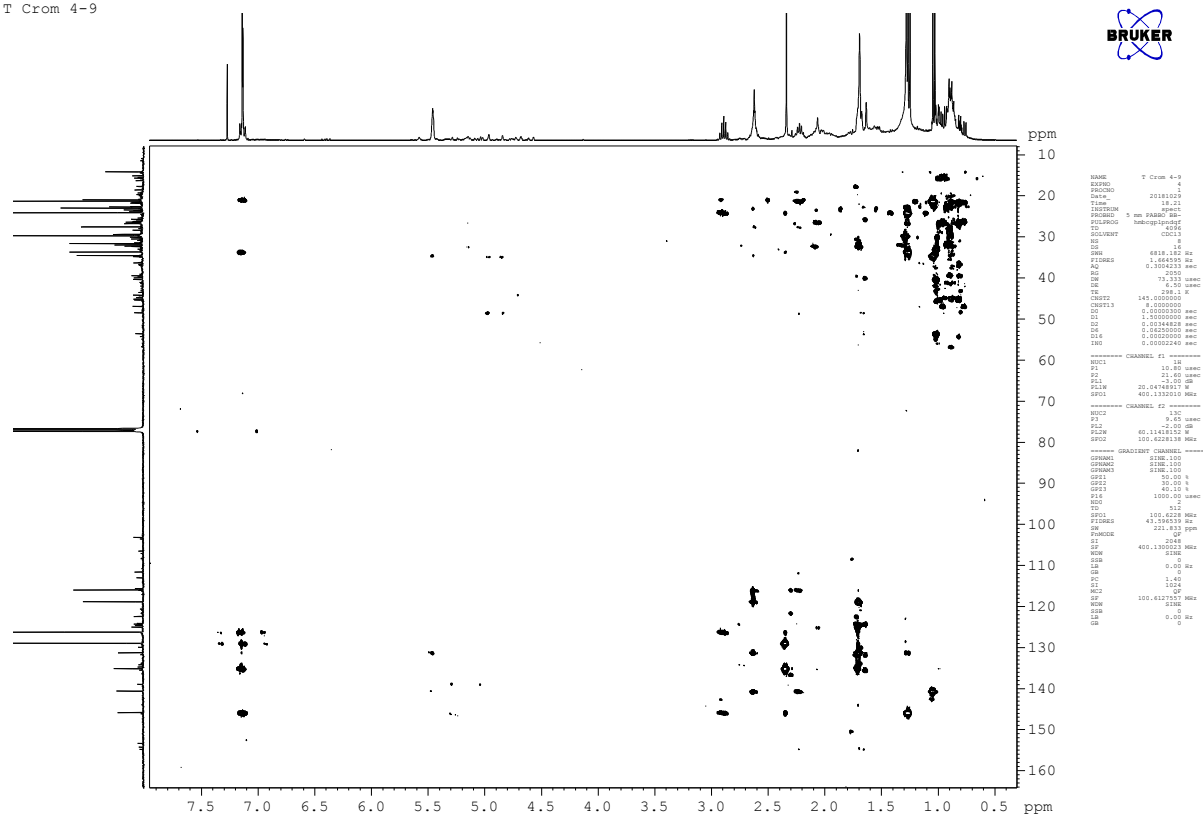


Figure A.10 – HMBC spectrum of a fraction of thyme essential oil extracted with 90% petroleum ether - 10% ethyl acetate. ^1H – 400 MHz, ^{13}C – 100 MHz, CDCl_3 .

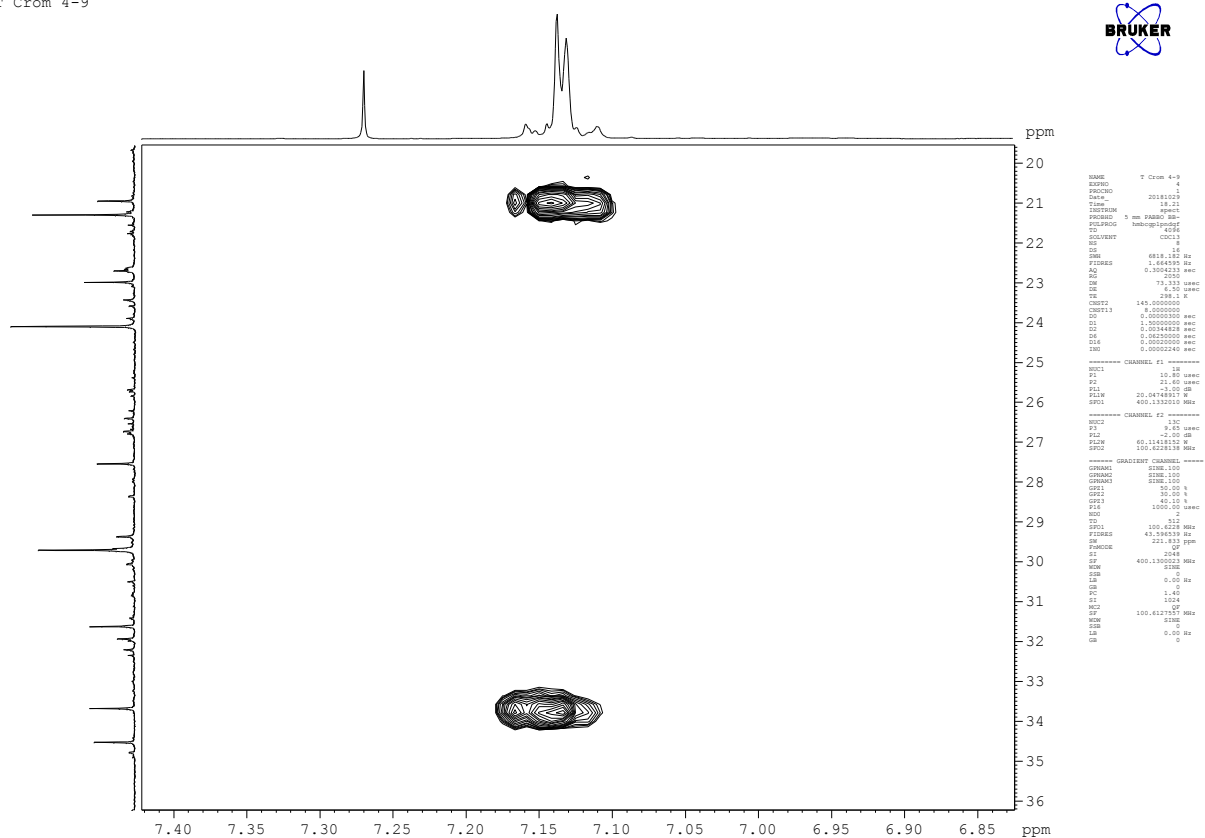


Figure A.10 (expansion 1) – HMBC spectrum of a fraction of thyme essential oil extracted with 90% petroleum ether - 10% ethyl acetate. ^1H – 400 MHz, ^{13}C - 100 MHz, CDCl_3 .

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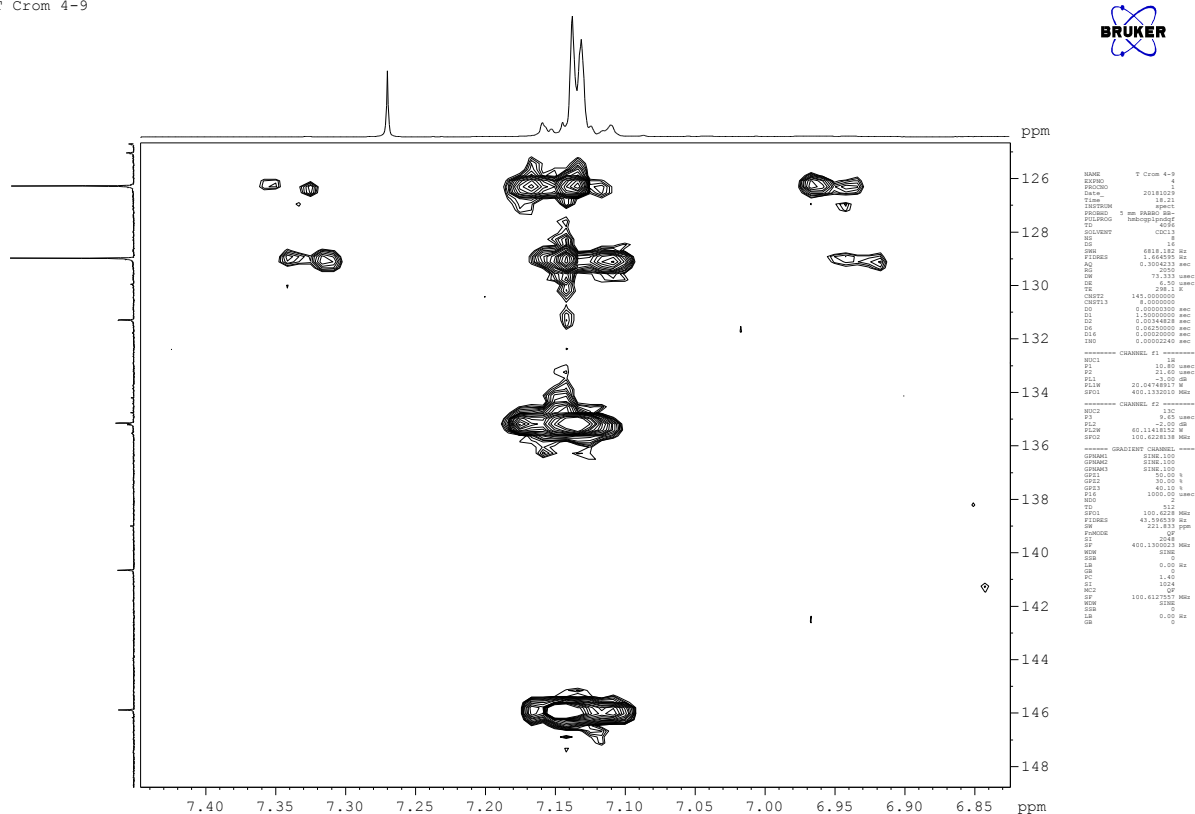


Figure A.10 (expansion 2) – HMBC spectrum of a fraction of thyme essential oil extracted with 90% petroleum ether - 10% ethyl acetate. ^1H – 400 MHz, ^{13}C - 100 MHz, CDCl_3 .

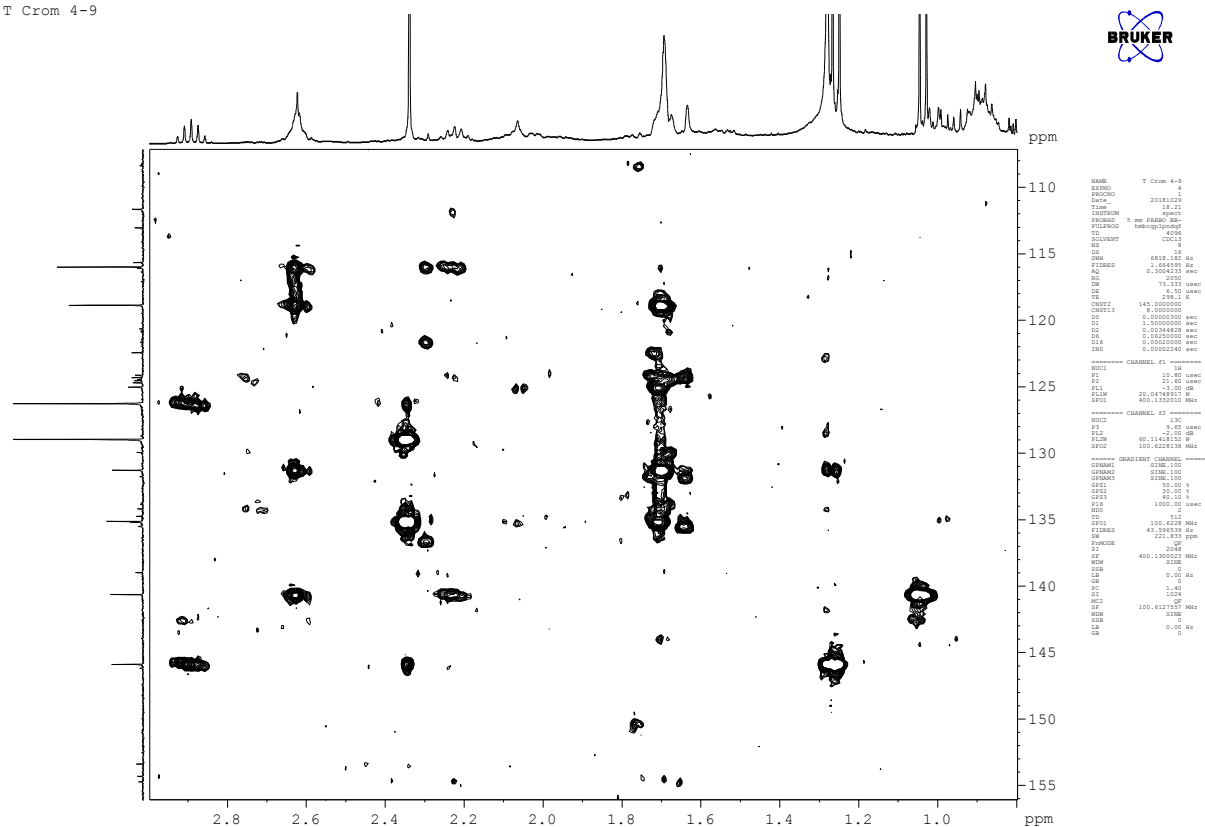


Figure A.10 (expansion 3) – HMBC spectrum of a fraction of thyme essential oil extracted with 90% petroleum ether - 10% ethyl acetate. ^1H – 400 MHz, ^{13}C - 100 MHz, CDCl_3

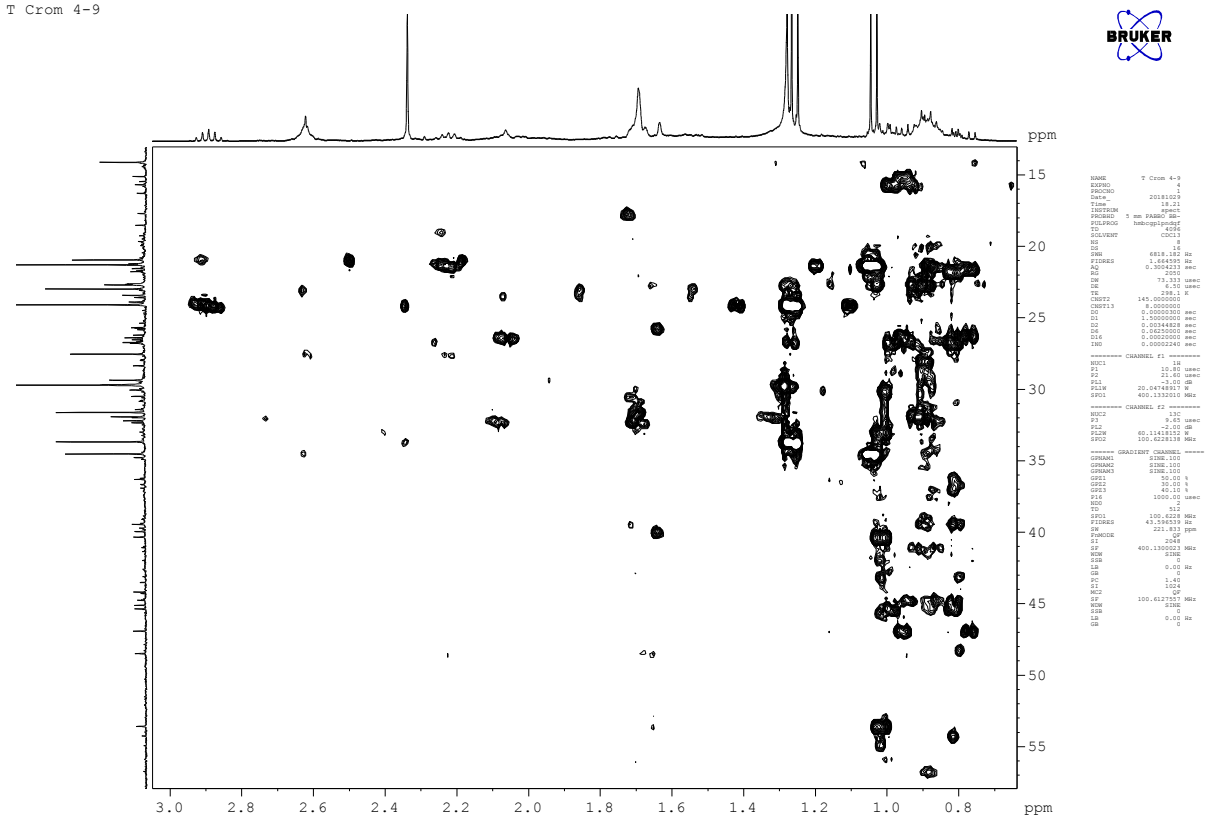


Figure A.10 (expansion 4) – HMBC spectrum of a fraction of thyme essential oil extracted with 90% petroleum ether - 10% ethyl acetate. ^1H – 400 MHz, ^{13}C - 100 MHz, CDCl_3 .

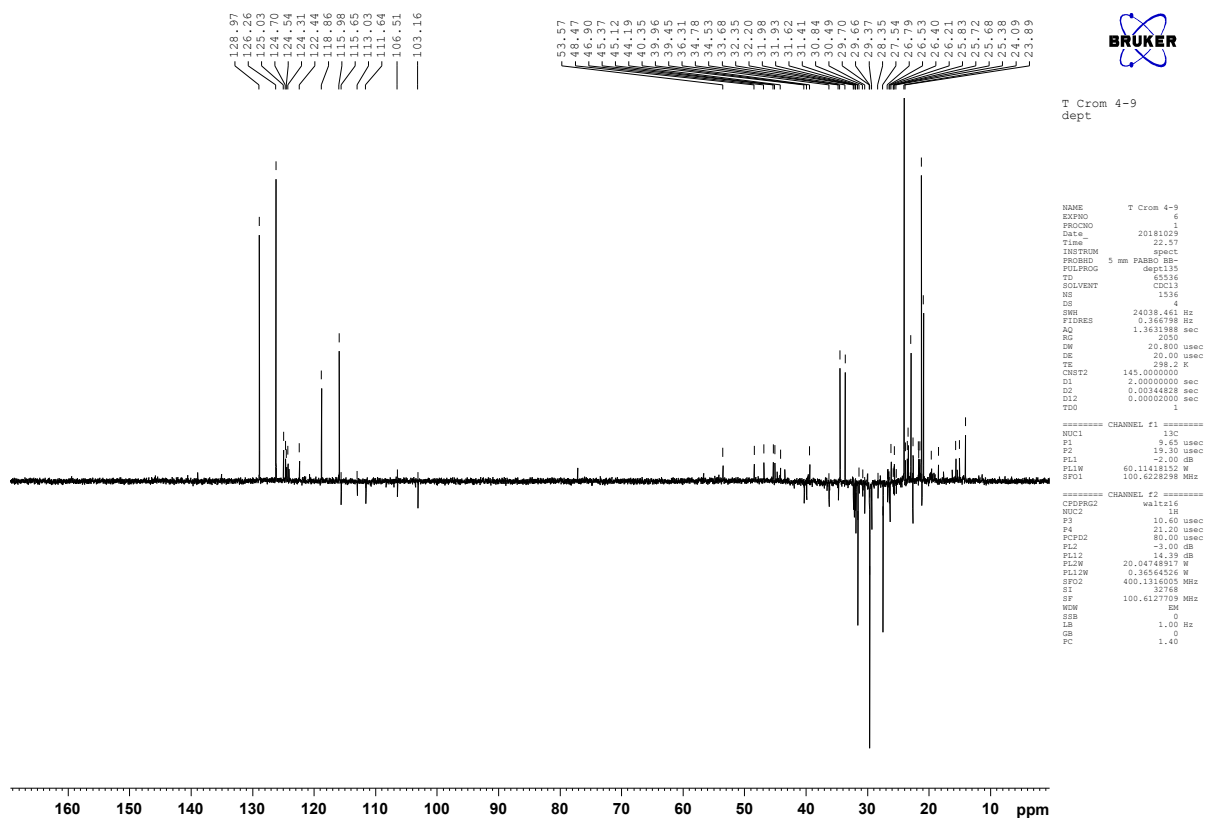


Figure A.11 – DEPT spectrum of a fraction of thyme essential oil extracted with 90% petroleum ether - 10% ethyl acetate. 100 MHz, CDCl₃.

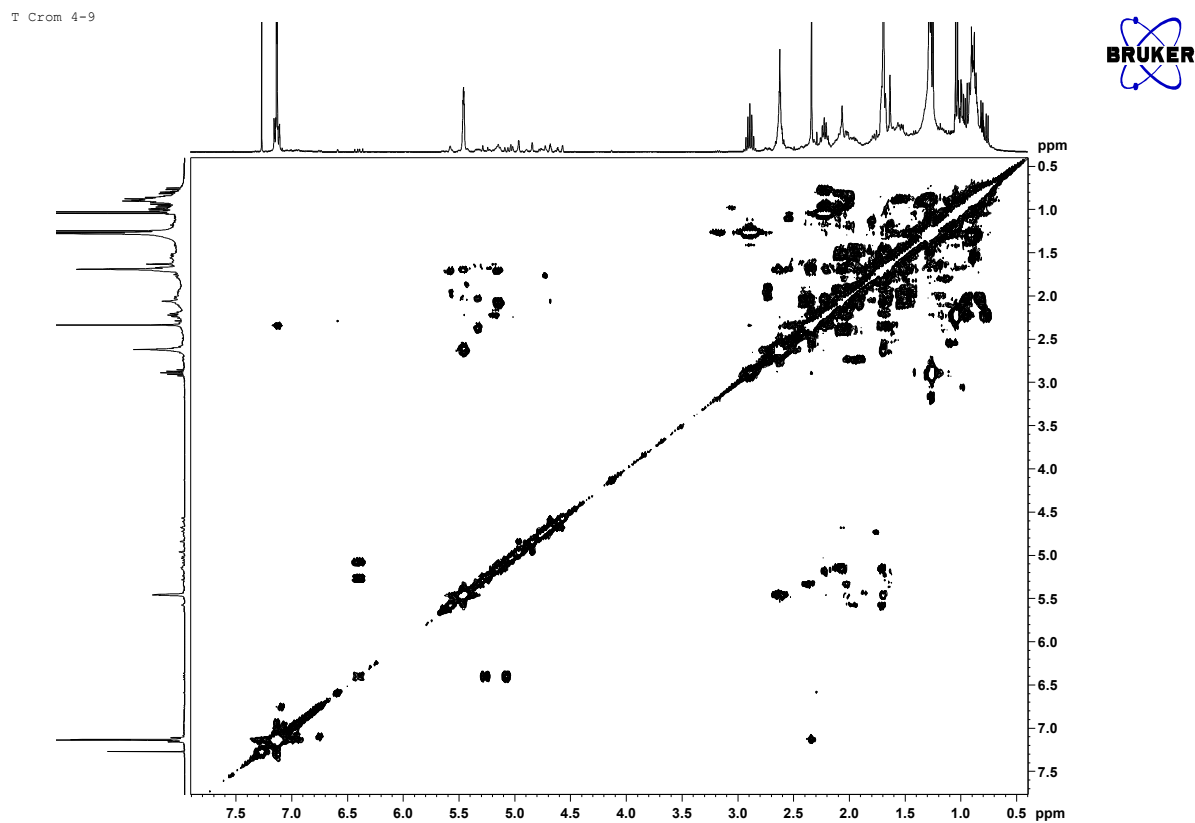


Figure A. 12 – COSY spectrum of a fraction of thyme essential oil extracted with 90% petroleum ether - 10% ethyl acetate. 400 MHz, CDCl₃.

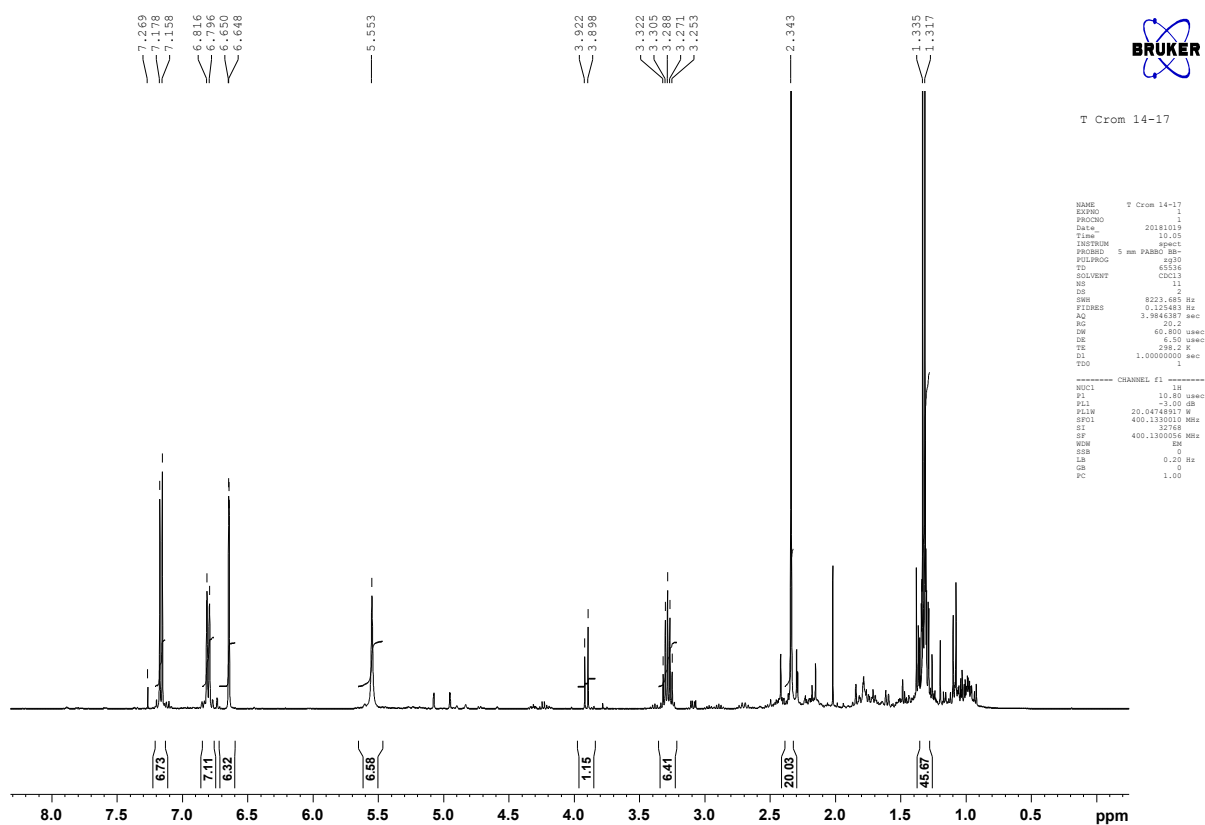


Figure A.13 – ^1H NMR spectrum of a fraction of thyme essential oil extracted with 75% petroleum ether - 25% ethyl acetate. 400 MHz, CDCl_3 .

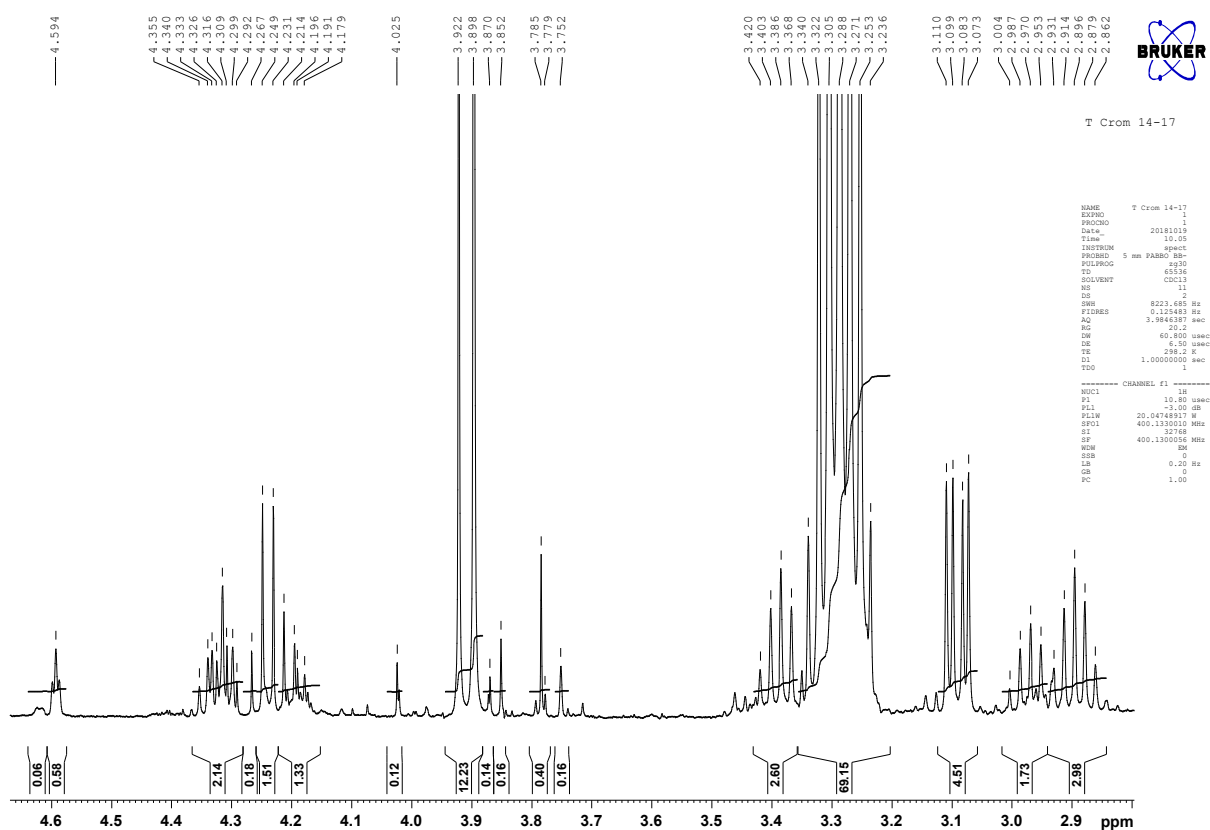
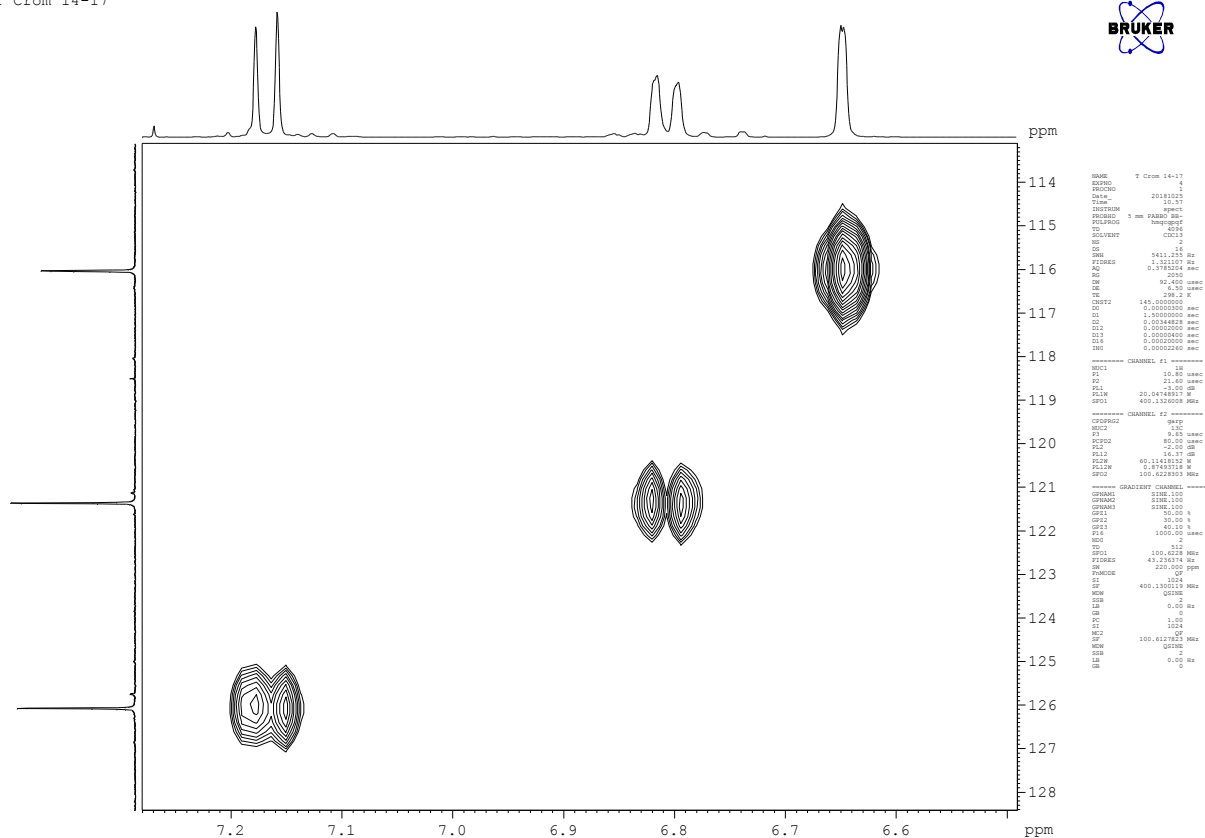
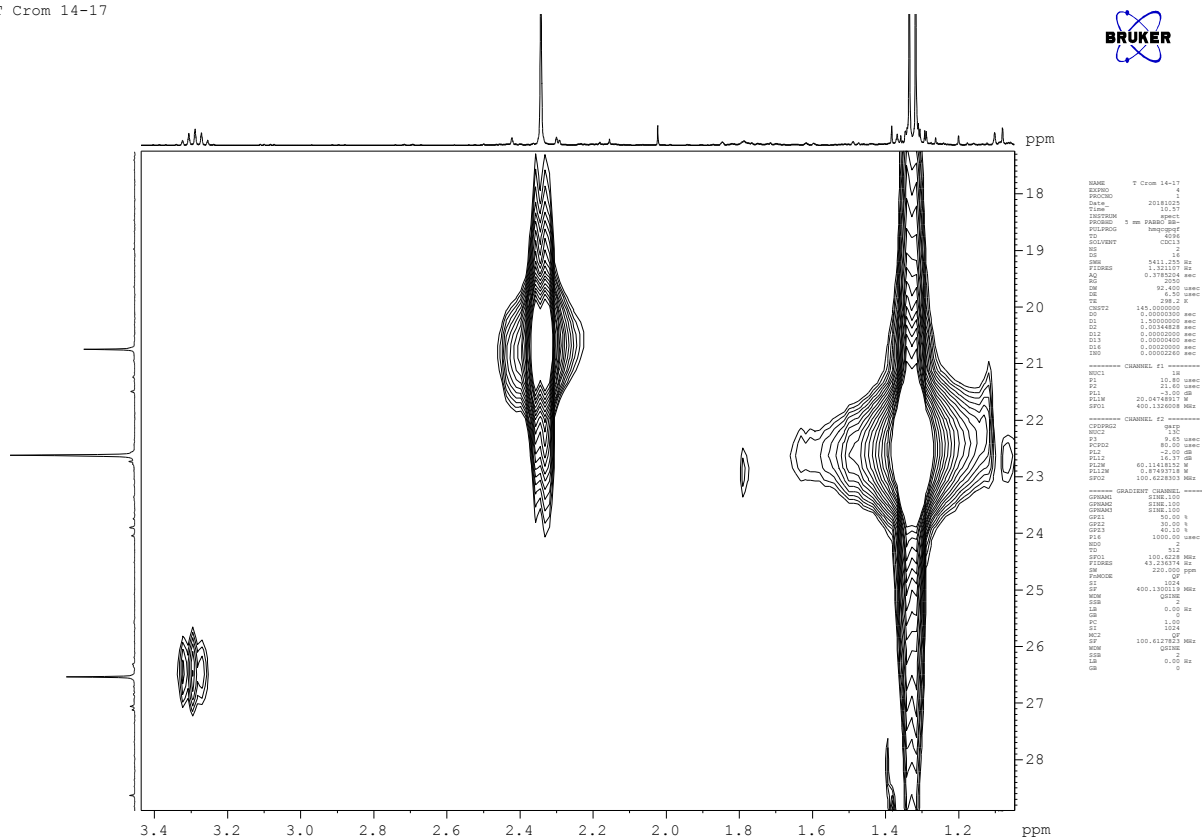


Figure A.13 (expansion) – ^1H NMR spectrum of a fraction of thyme essential oil extracted with 75% petroleum ether - 25% ethyl acetate. 400 MHz, CDCl_3 .

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T Crom 14-17



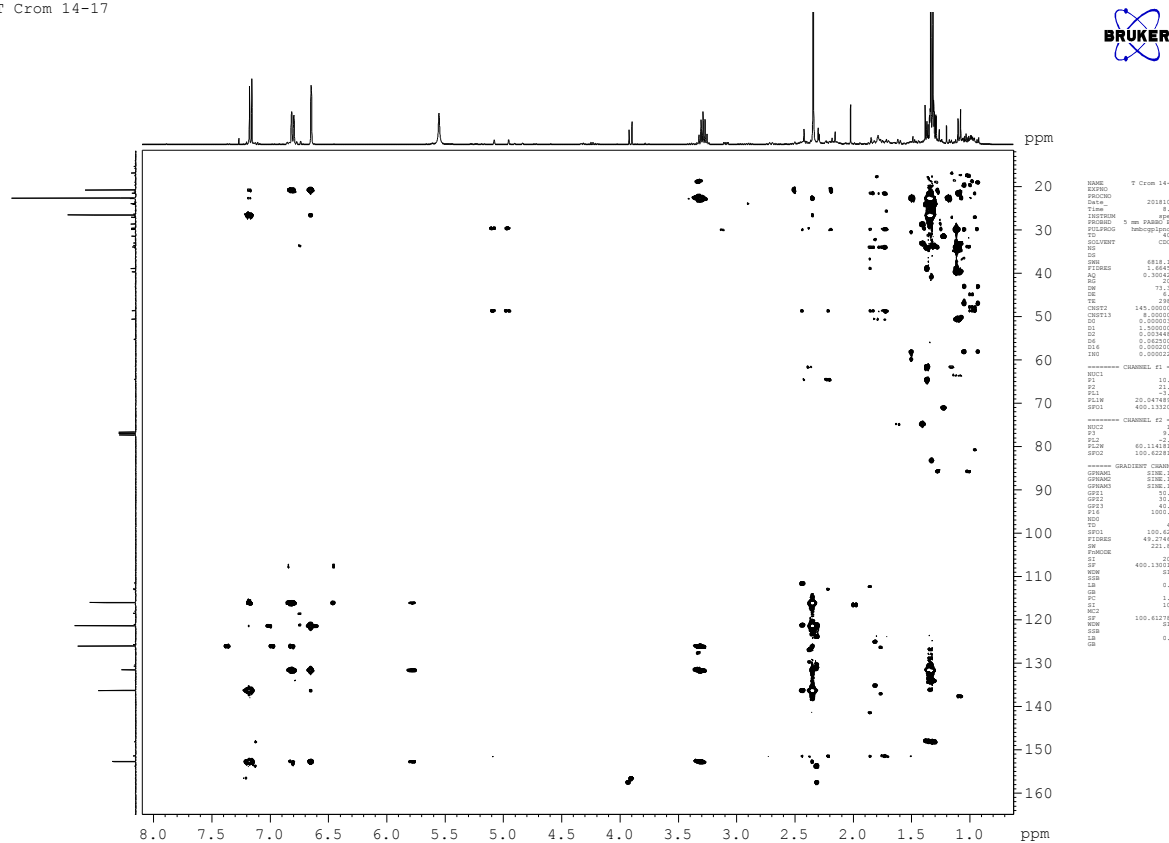


Figure A.16 – HMBC spectrum of a fraction of thyme essential oil extracted with 75% petroleum ether - 25% ethyl acetate. ^1H – 400 MHz, ^{13}C - 100 MHz, CDCl_3 .

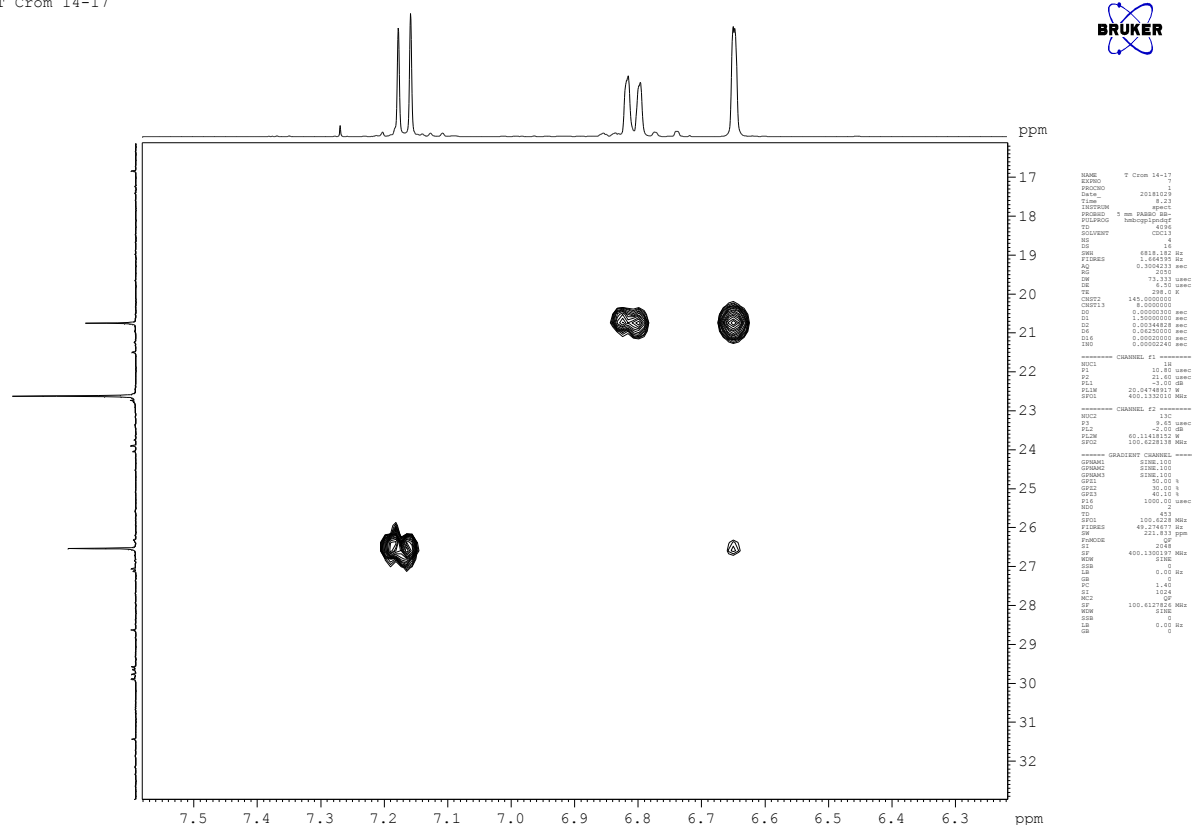


Figure A.16 (expansion 1) – HMBC spectrum of a fraction of thyme essential oil extracted with 75% petroleum ether - 25% ethyl acetate. ^1H – 400 MHz, ^{13}C - 100 MHz, CDCl_3

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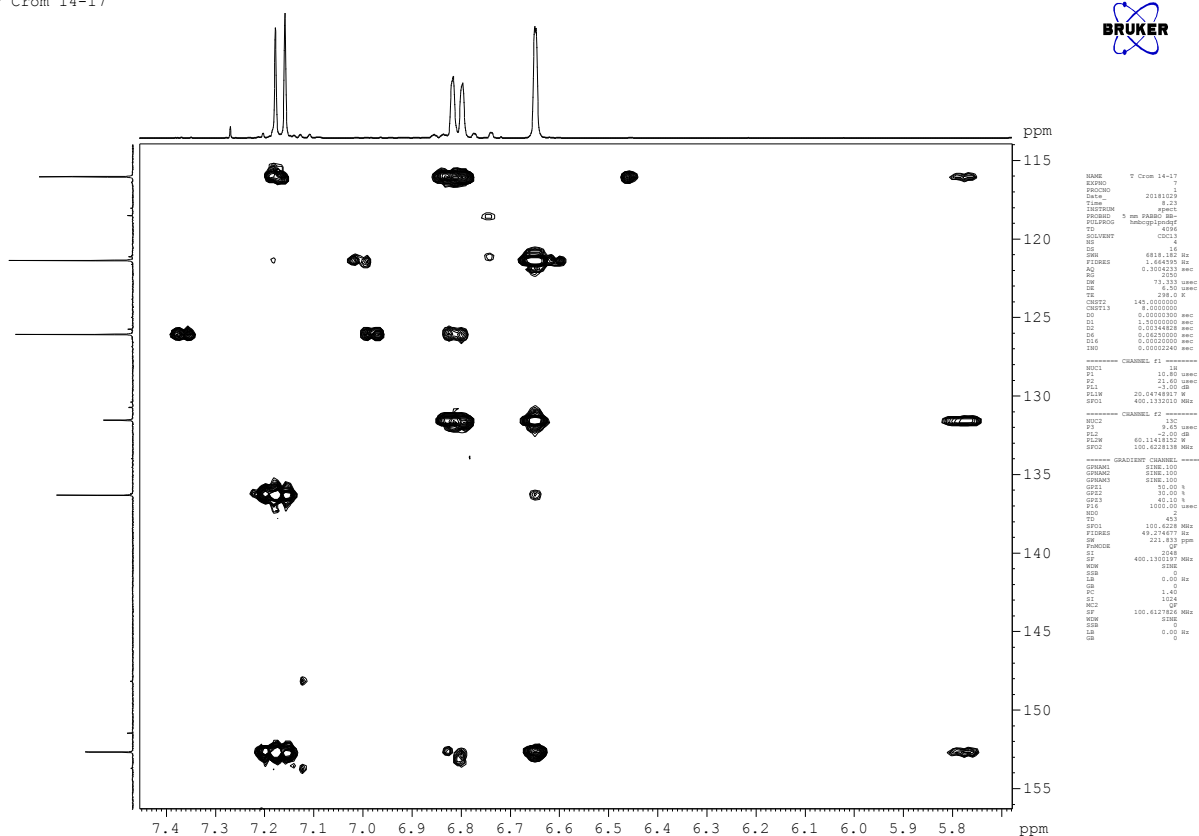


Figure A.16 (expansion 2) – HMBC spectrum of a fraction of thyme essential oil extracted with 75% petroleum ether - 25% ethyl acetate. ^1H – 400 MHz, ^{13}C - 100 MHz, CDCl_3 .

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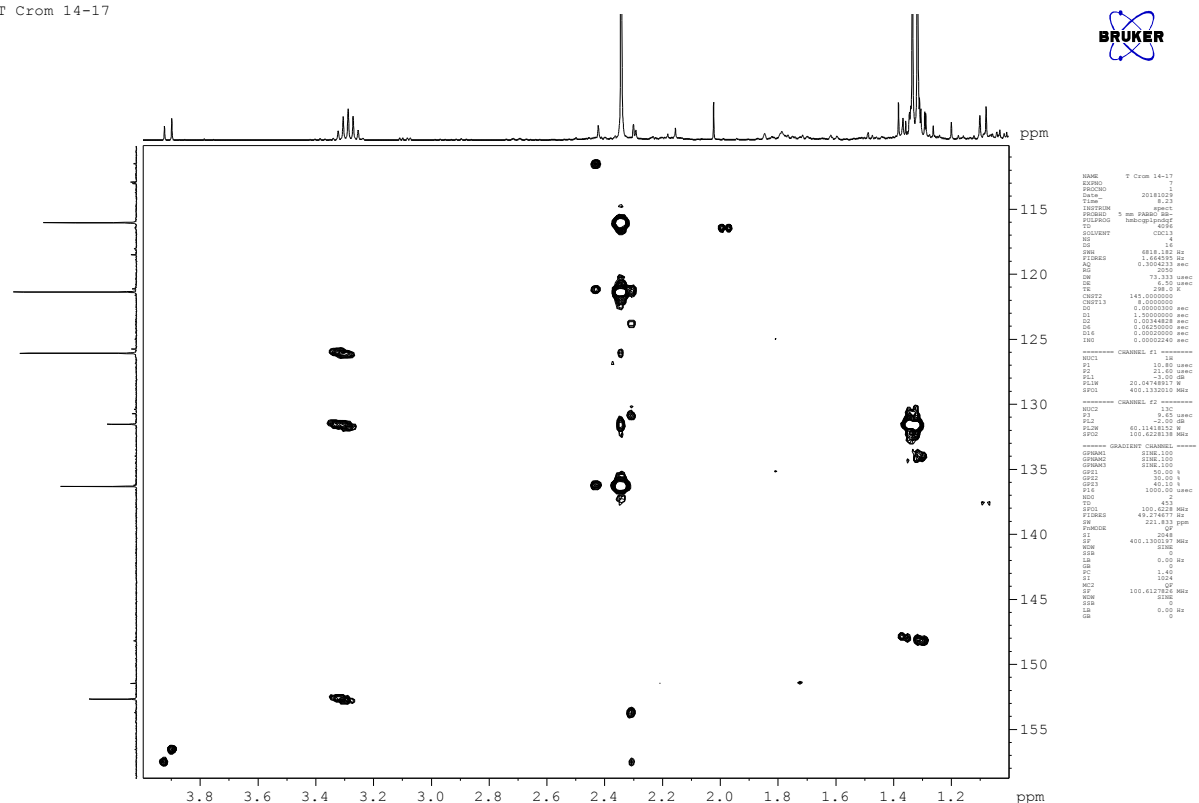


Figure A.16 (expansion 3) – HMBC spectrum of a fraction of thyme essential oil extracted with 75% petroleum ether - 25% ethyl acetate. ^1H – 400 MHz, ^{13}C - 100 MHz, CDCl_3 .

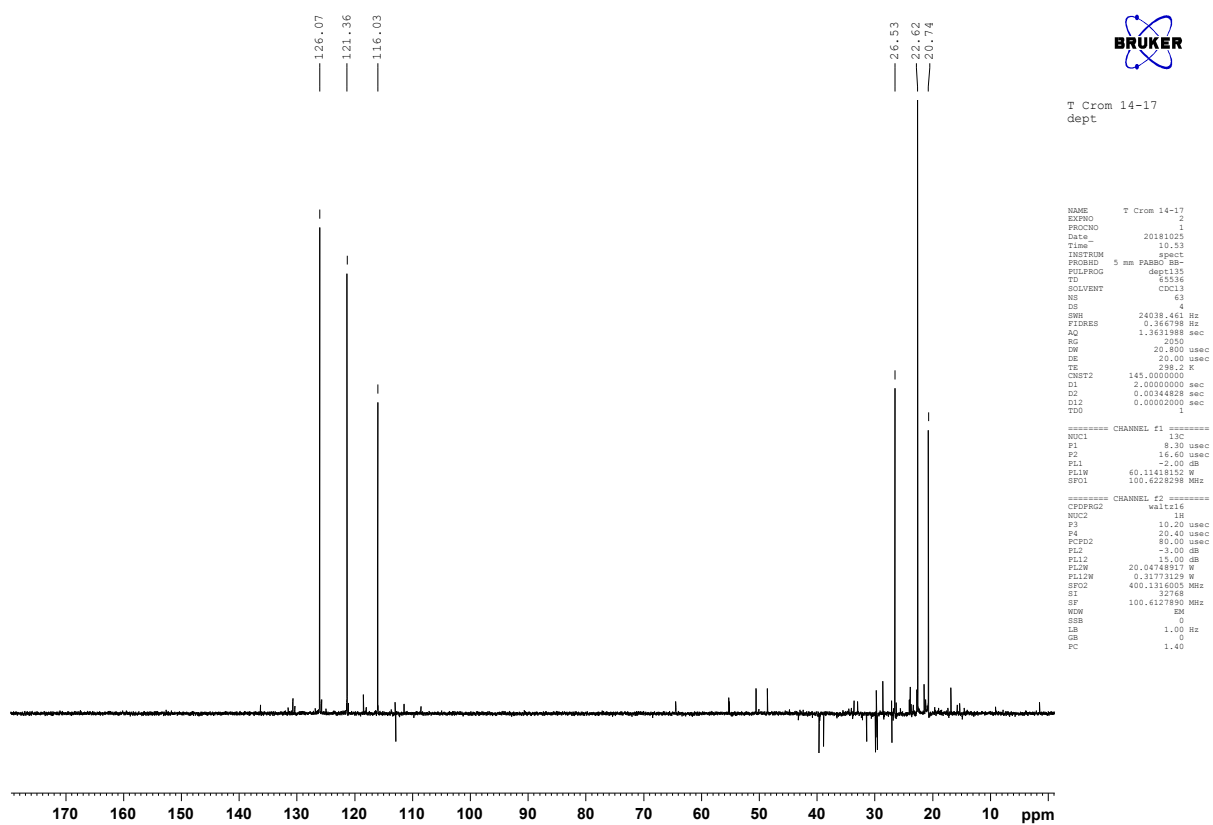


Figure A.17 – DEPT spectrum of a fraction of thyme essential oil extracted with 75% petroleum ether - 25% ethyl acetate. 100 MHz, CDCl₃.

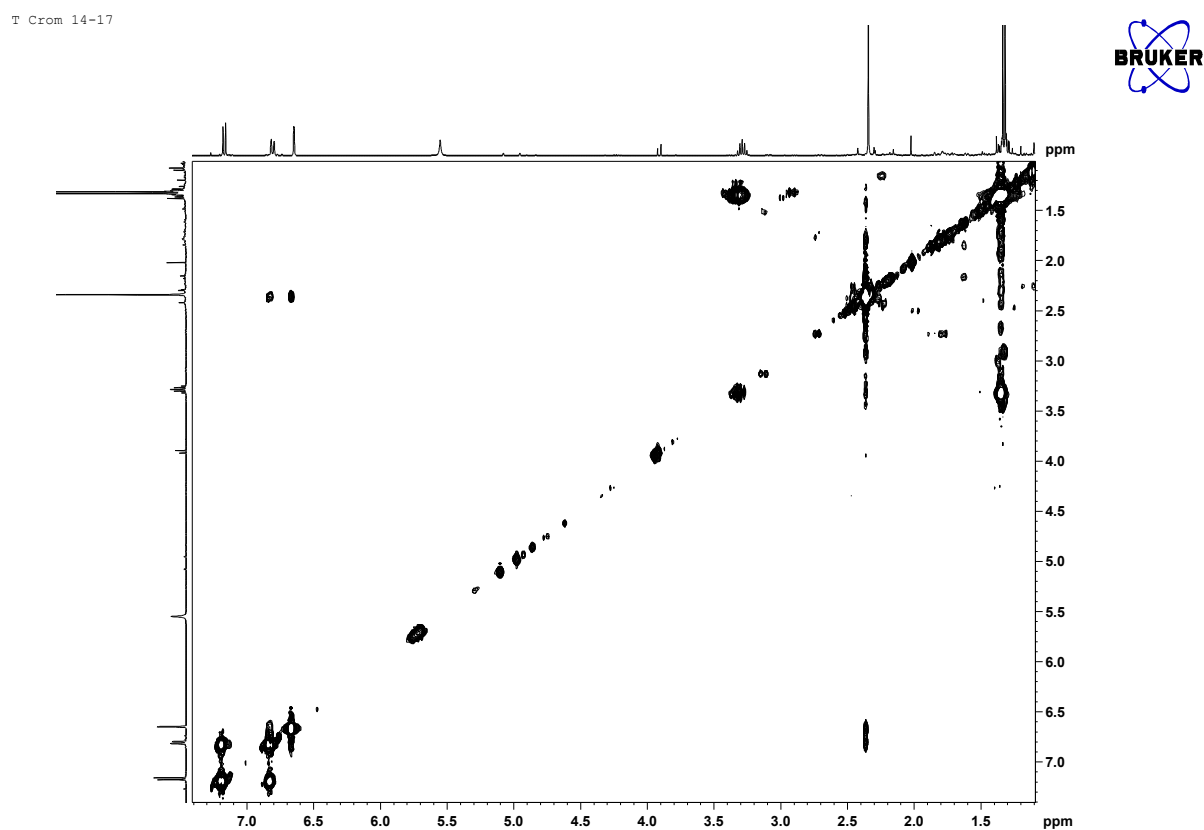


Figure A.18 – COSY spectrum of a fraction of thyme essential oil extracted with 75% petroleum ether - 25% ethyl acetate. 400 MHz, CDCl₃.

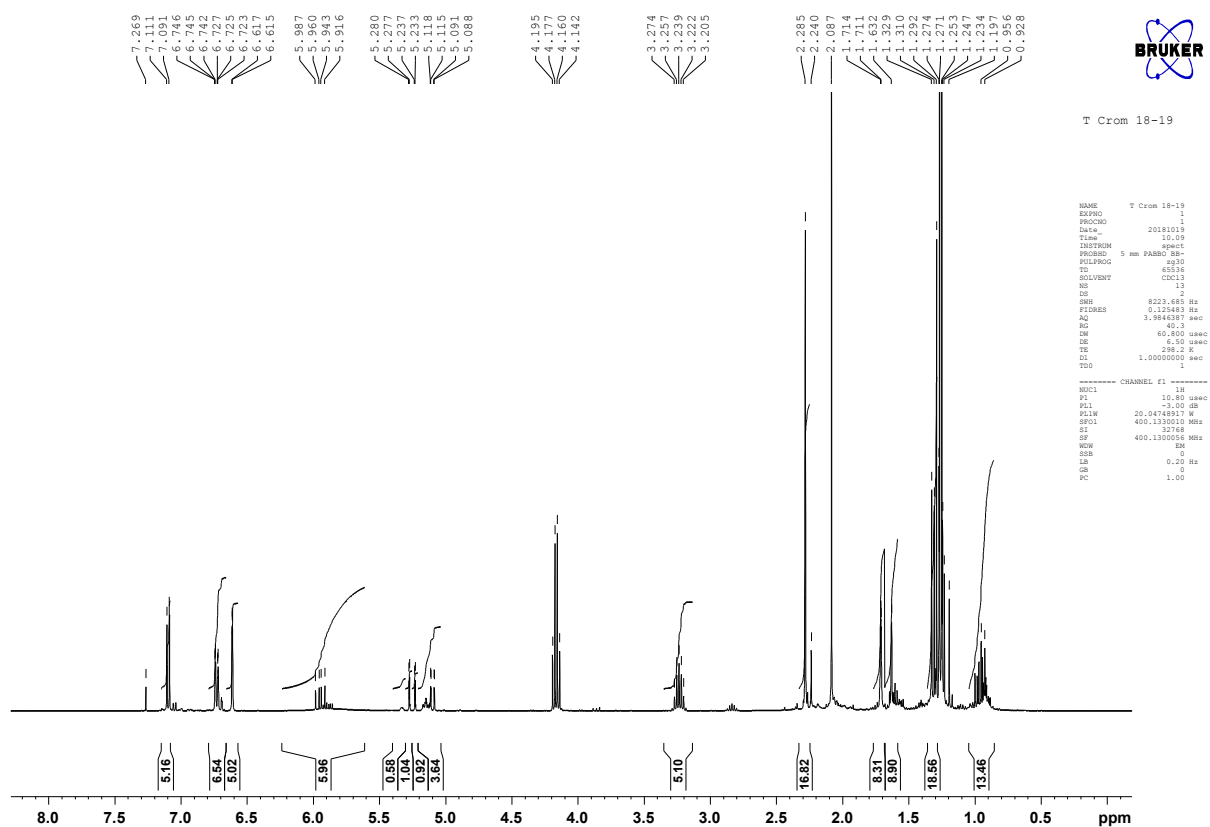


Figure A.19 – ^1H NMR spectrum of a fraction of thyme essential oil extracted with 50% petroleum ether - 50% ethyl acetate. 400 MHz, CDCl_3 .

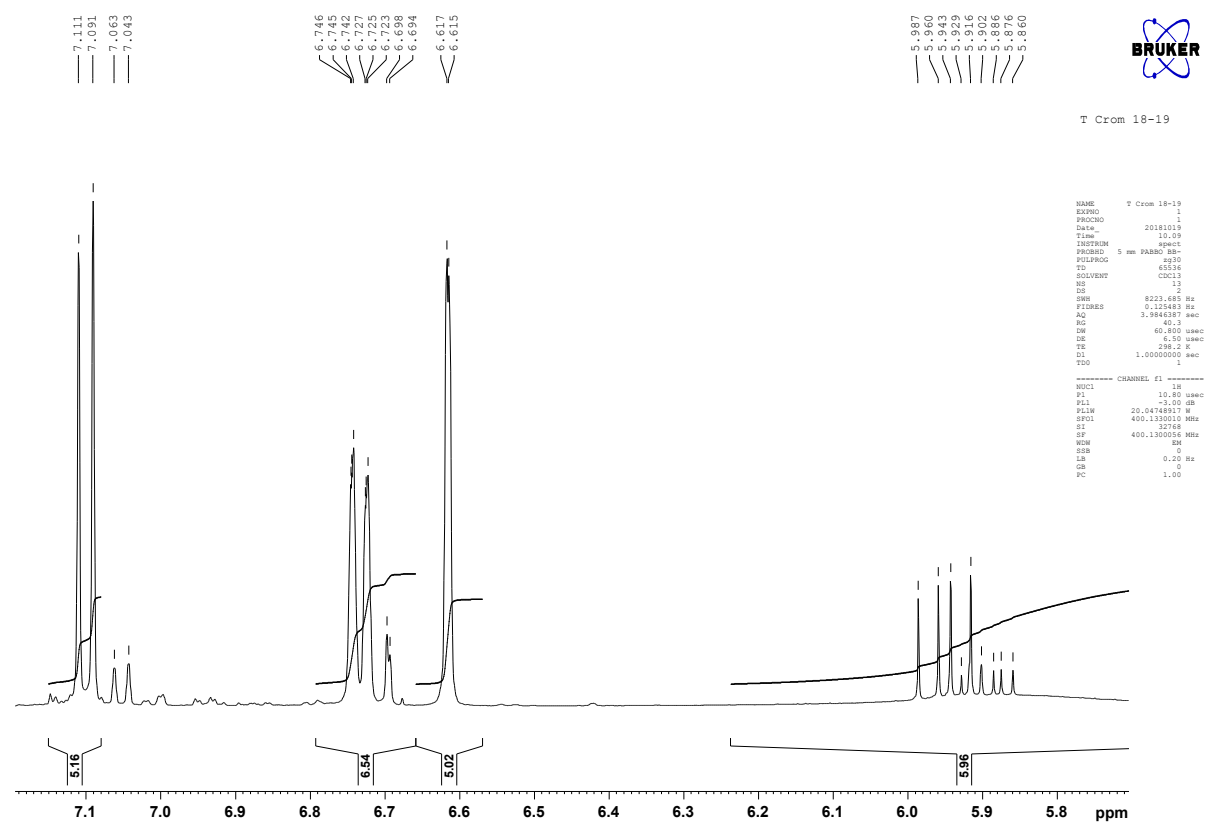


Figure A.19 (expansion 1) – ^1H NMR spectrum of a fraction of thyme essential oil extracted with 50% petroleum ether - 50% ethyl acetate. 400 MHz, CDCl_3 .

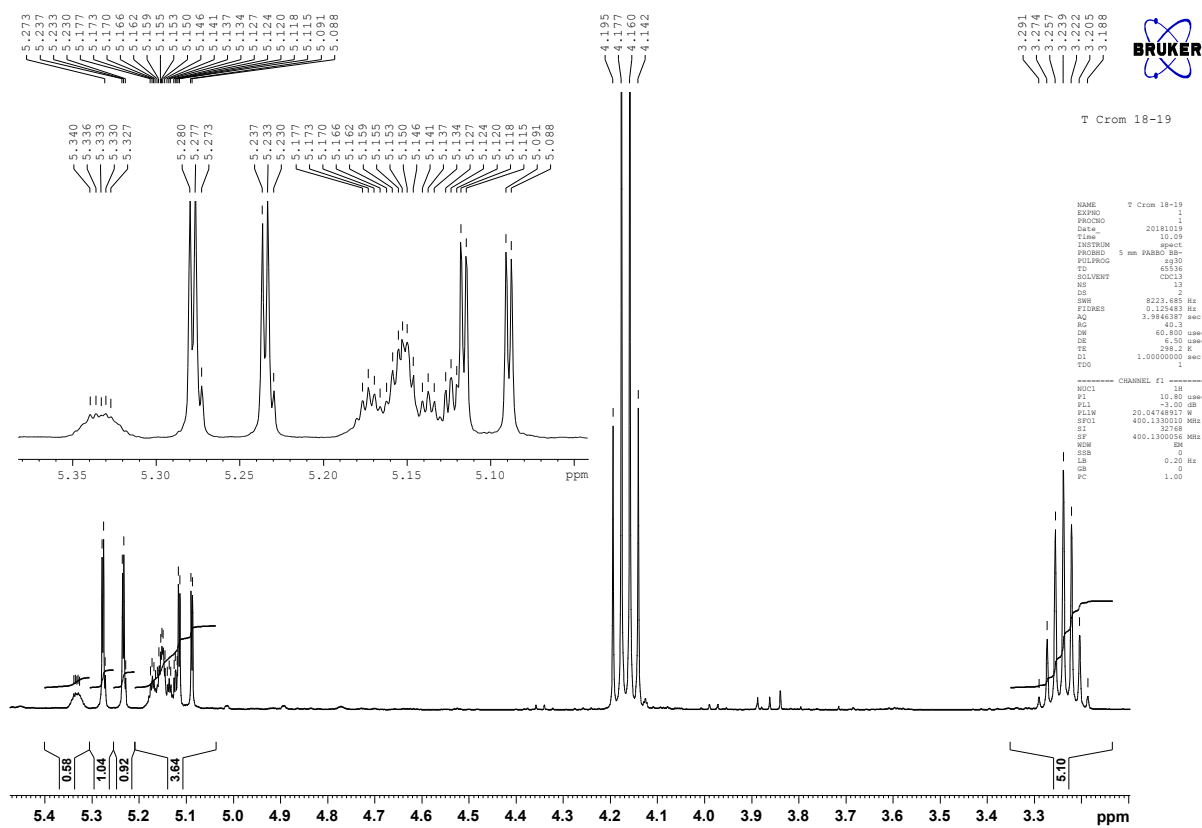


Figure A.19 (expansion 2) – ^1H NMR spectrum of a fraction of thyme essential oil extracted with 50% petroleum ether - 50% ethyl acetate. 400 MHz, CDCl_3 .

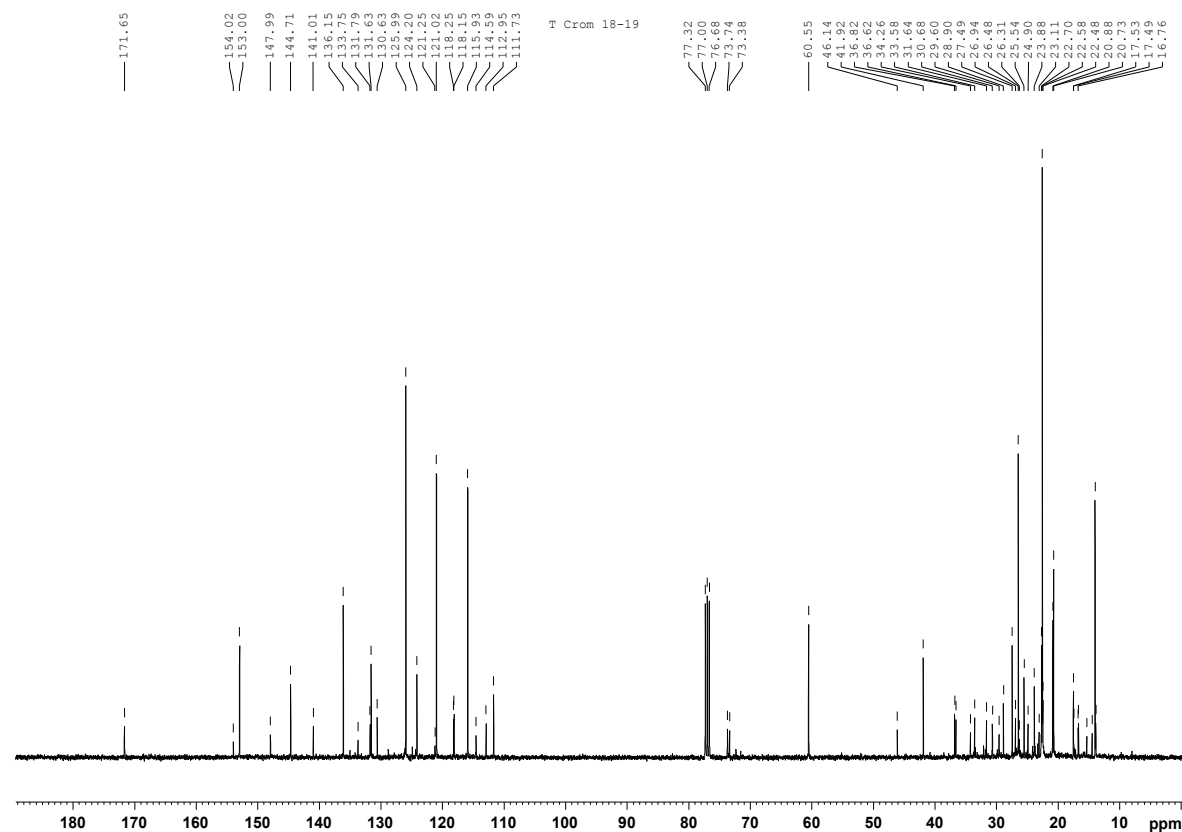


Figure A.20 – ^{13}C NMR spectrum of a fraction of thyme essential oil extracted with 50% petroleum ether - 50% ethyl acetate. 100 MHz, CDCl_3 .

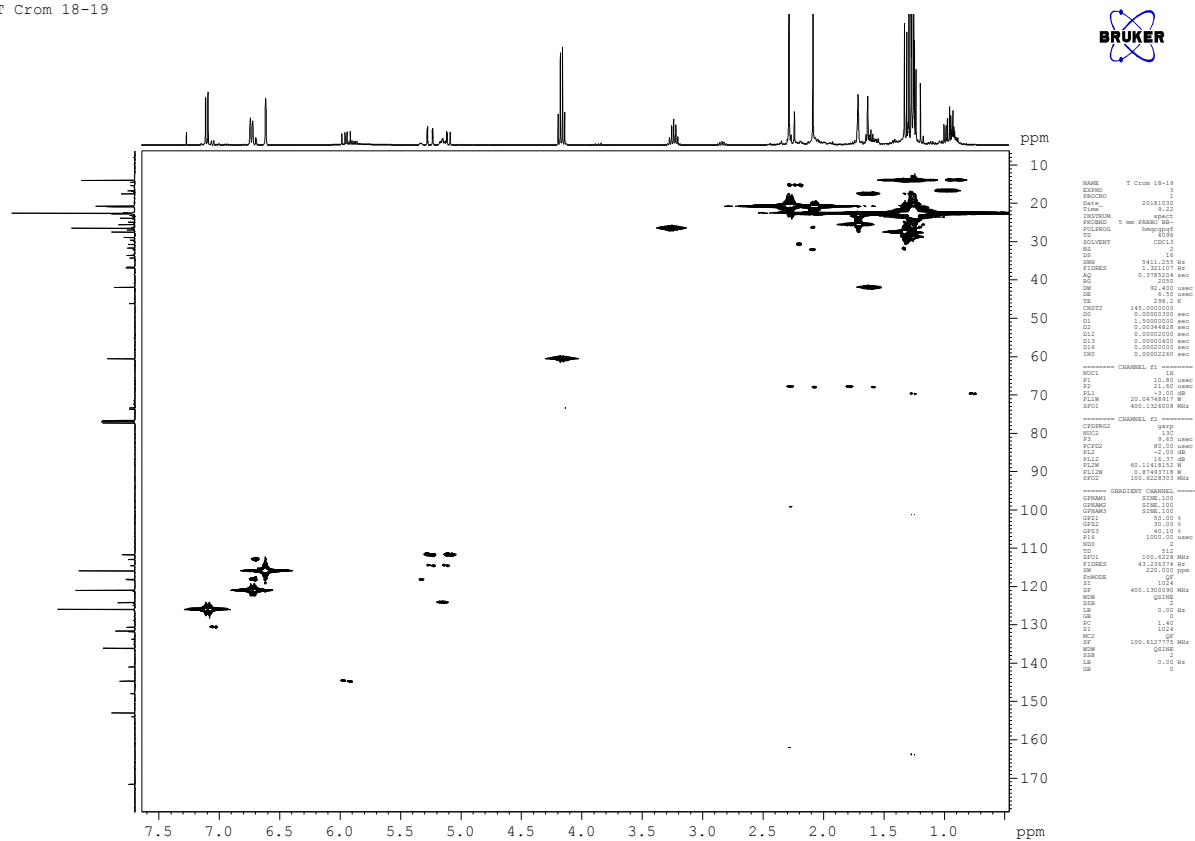


Figure A.21 – HMQC spectrum of a fraction of thyme essential oil extracted with 50% petroleum ether - 50% ethyl acetate. ^1H – 400 MHz, ^{13}C – 100 MHz, CDCl_3 .

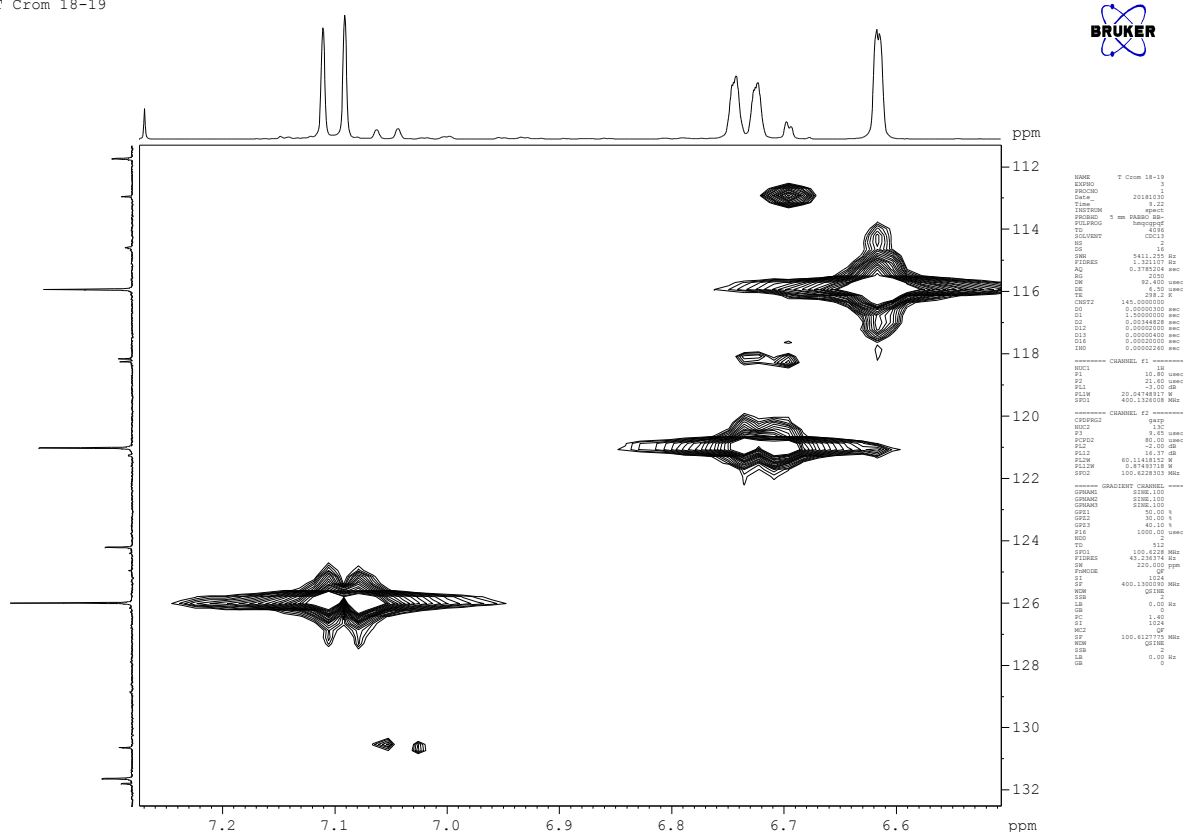


Figure A.21 (expansion 1) – HMQC spectrum of a fraction of thyme essential oil extracted with 50% petroleum ether - 50% ethyl acetate. ^1H – 400 MHz, ^{13}C – 100 MHz, CDCl_3 .

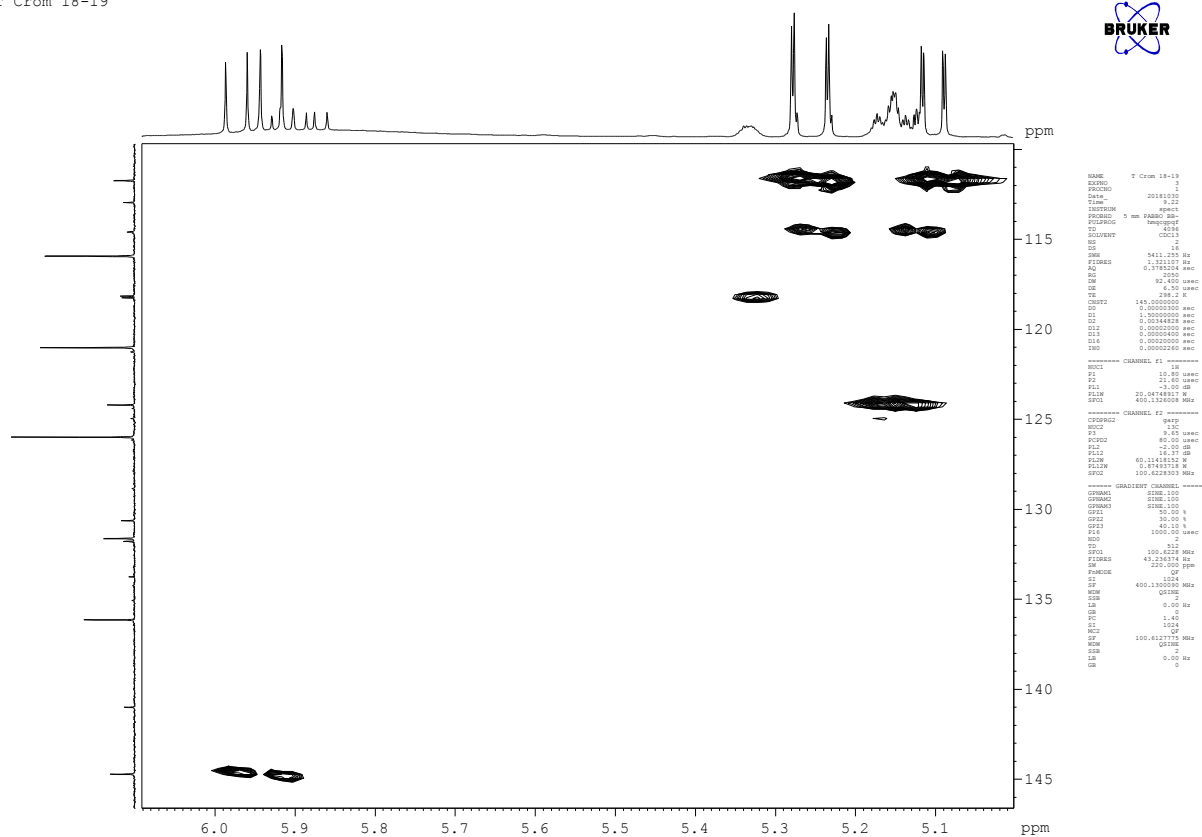


Figure A.21 (expansion 2) – HMQC spectrum of a fraction of thyme essential oil extracted with 50% petroleum ether - 50% ethyl acetate. ¹H – 400 MHz, ¹³C - 100 MHz, CDCl₃.

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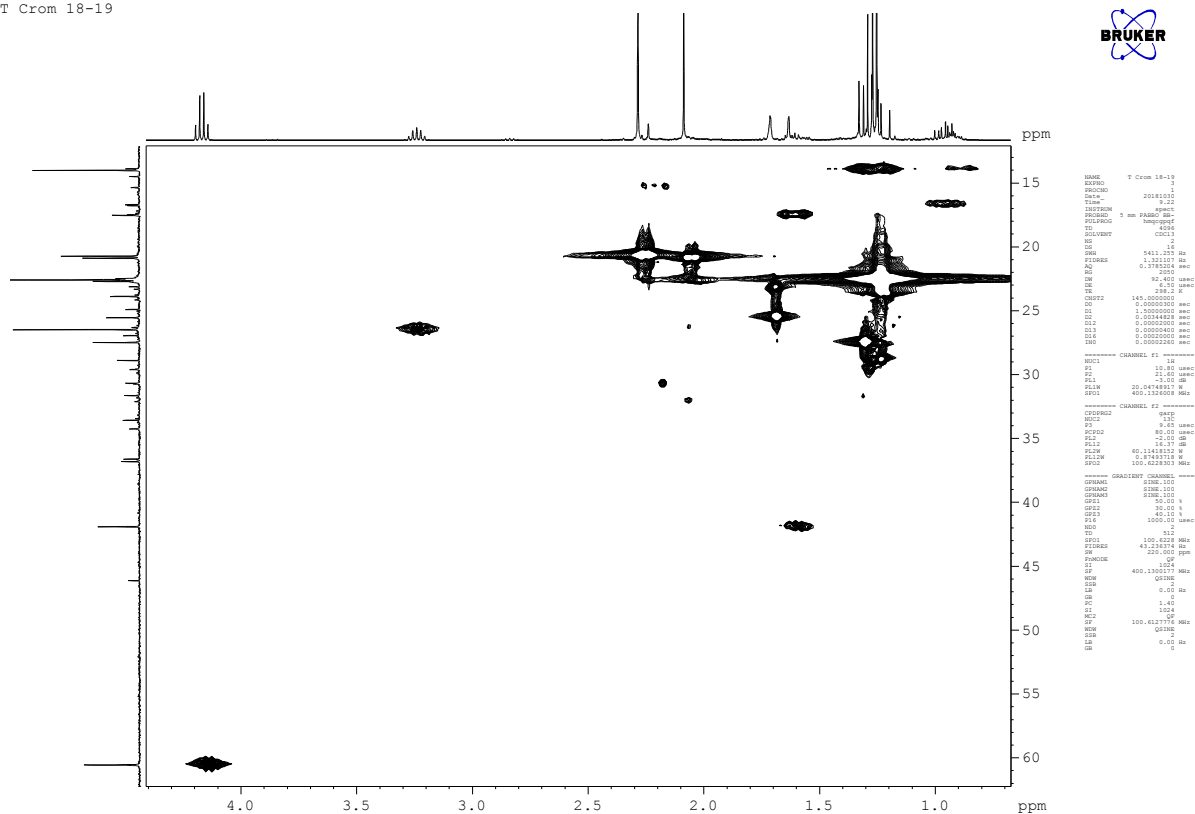


Figure A.21 (expansion 3) – HMQC spectrum of a fraction of thyme essential oil extracted with 50% petroleum ether - 50% ethyl acetate. ¹H – 400 MHz, ¹³C - 100 MHz, CDCl₃.

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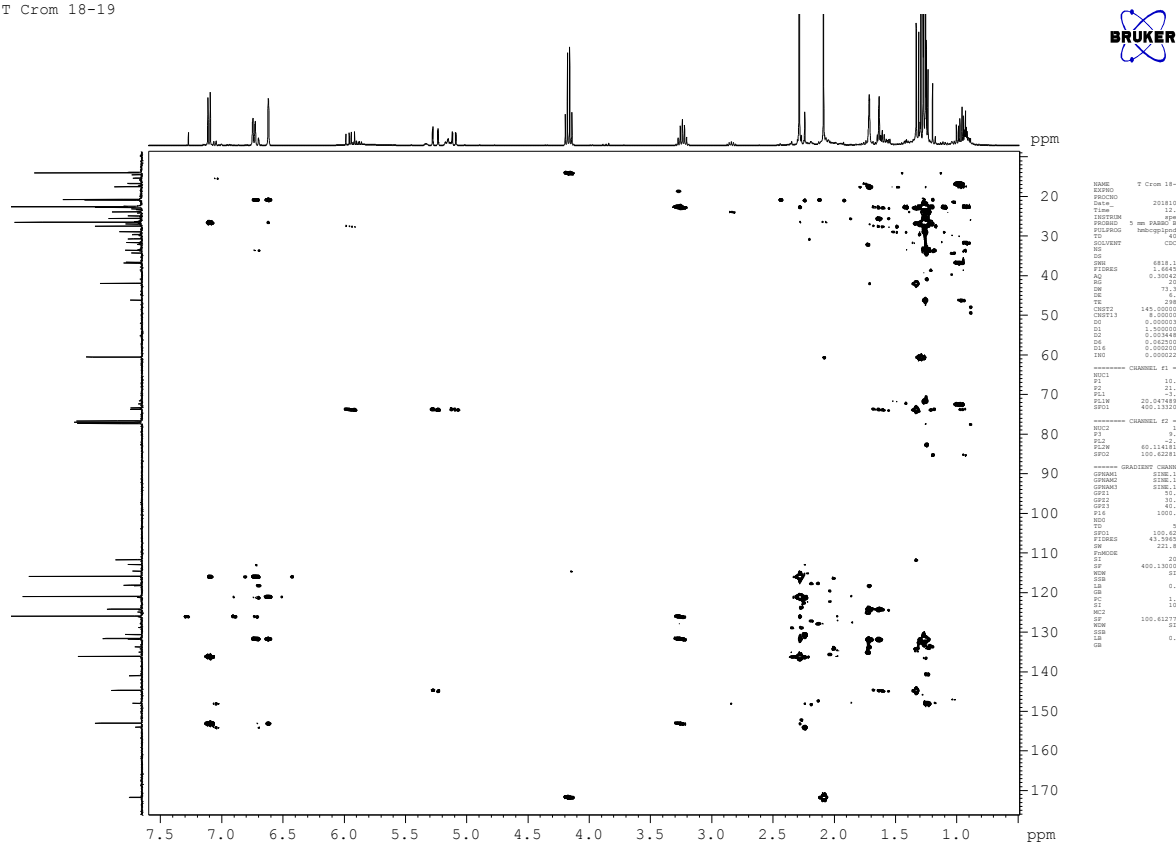


Figure A.22 – HMBC spectrum of a fraction of thyme essential oil extracted with 50% petroleum ether - 50% ethyl acetate. ^1H – 400 MHz, ^{13}C – 100 MHz, CDCl_3 .

T Crom 18-19

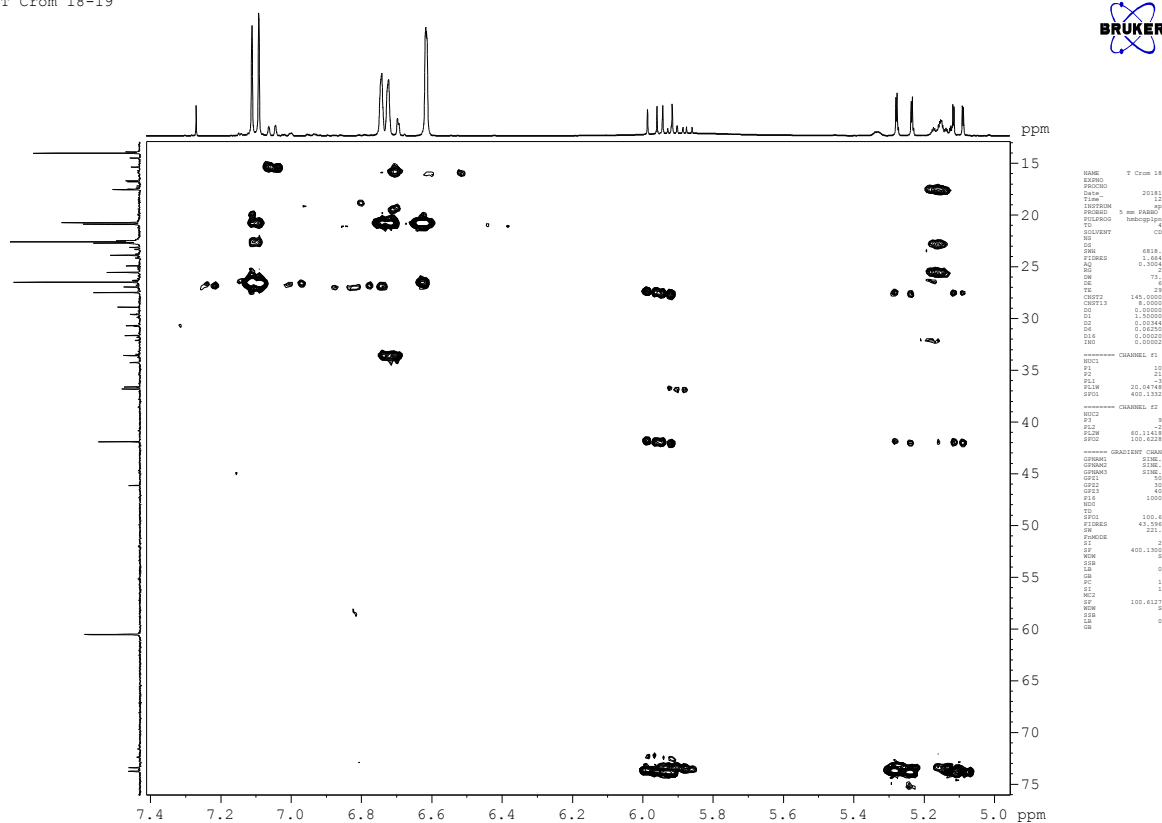


Figure A.22 (expansion 1) – HMBC spectrum of a fraction of thyme essential oil extracted with 50% petroleum ether - 50% ethyl acetate. ^1H – 400 MHz, ^{13}C – 100 MHz, CDCl_3 .

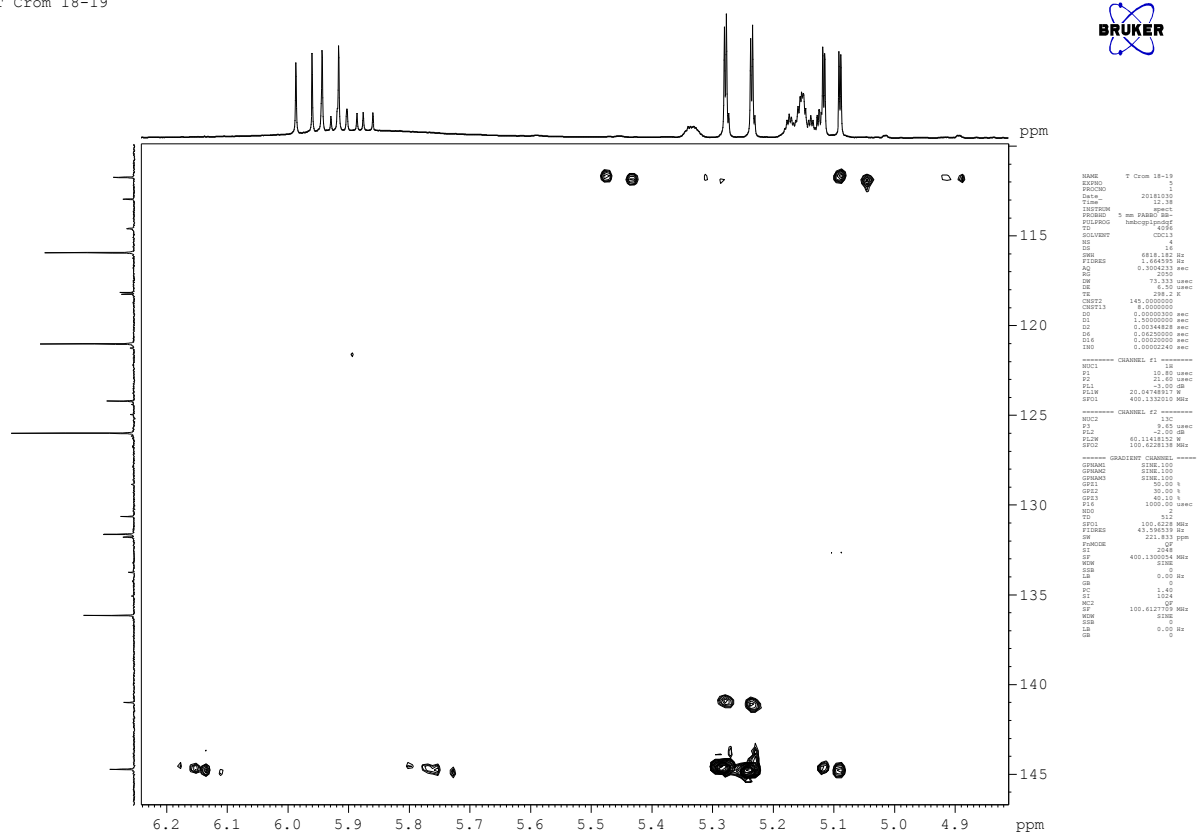


Figure A.22 (expansion 2) – HMBC spectrum of a fraction of thyme essential oil extracted with 50% petroleum ether - 50% ethyl acetate. ^1H – 400 MHz, ^{13}C – 100 MHz, CDCl_3 .

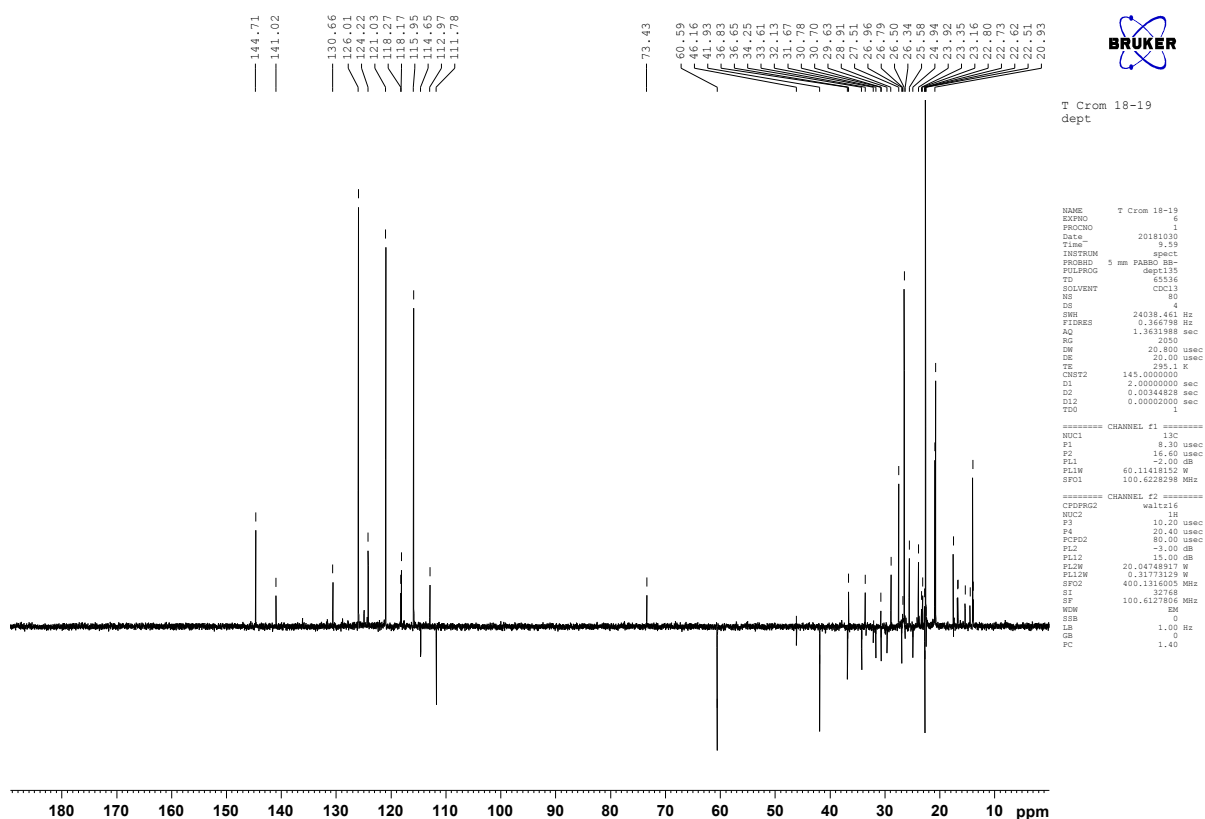


Figure A.23 – DEPT spectrum of a fraction of thyme essential oil extracted with 50% petroleum ether - 50% ethyl acetate. 100 MHz, CDCl_3 .

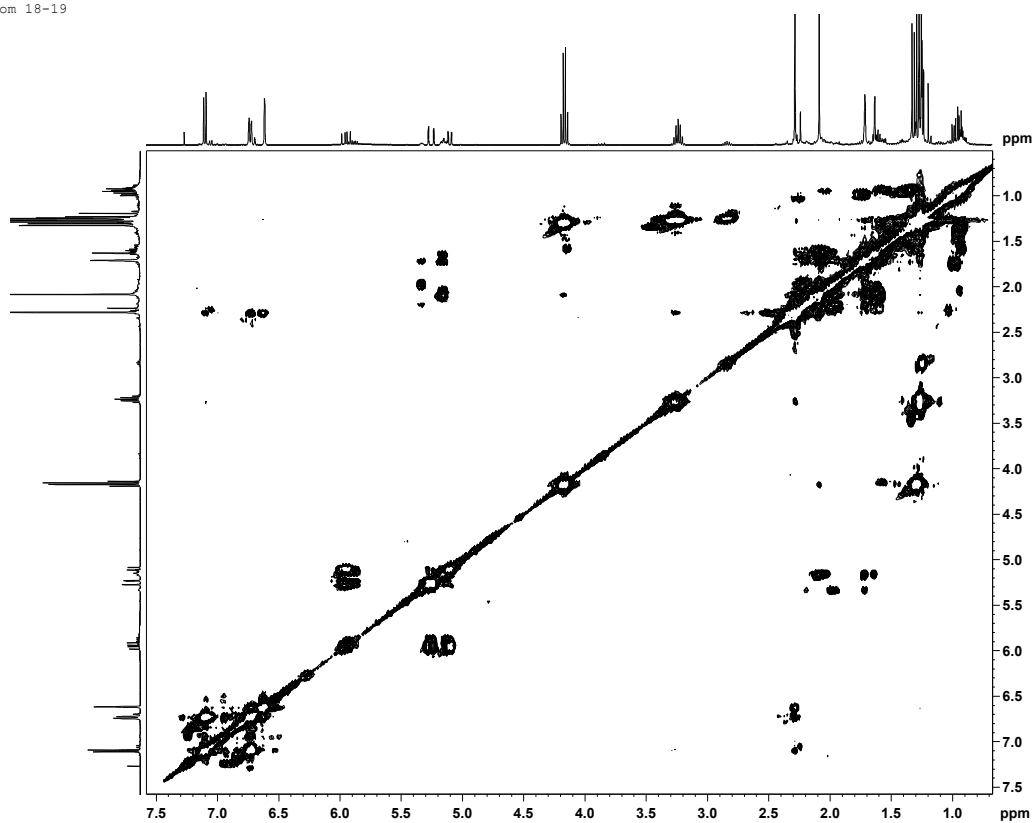


Figure A.24 – COSY spectrum of a fraction of thyme essential oil extracted with 50% petroleum ether - 50% ethyl acetate. 400 MHz, CDCl₃.

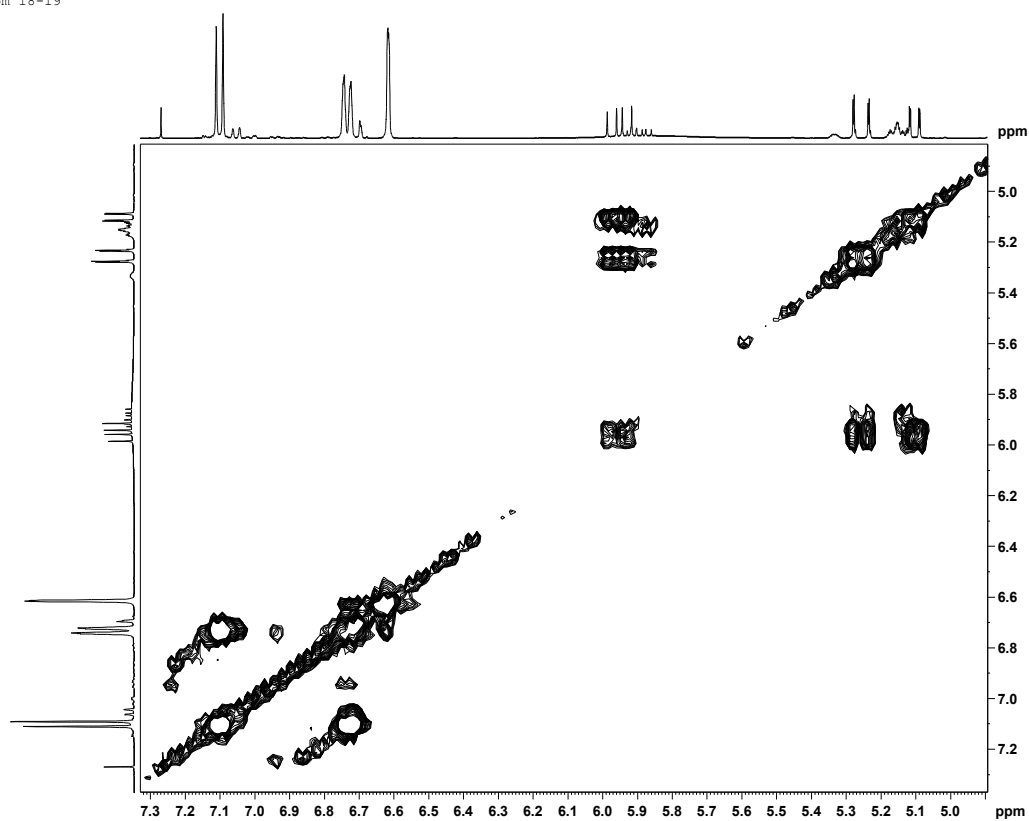


Figure A.24 (expansion) – COSY spectrum of a fraction of thyme essential oil extracted with 50% petroleum ether - 50% ethyl acetate. 400 MHz, CDCl₃.

Lemon balm essential oil

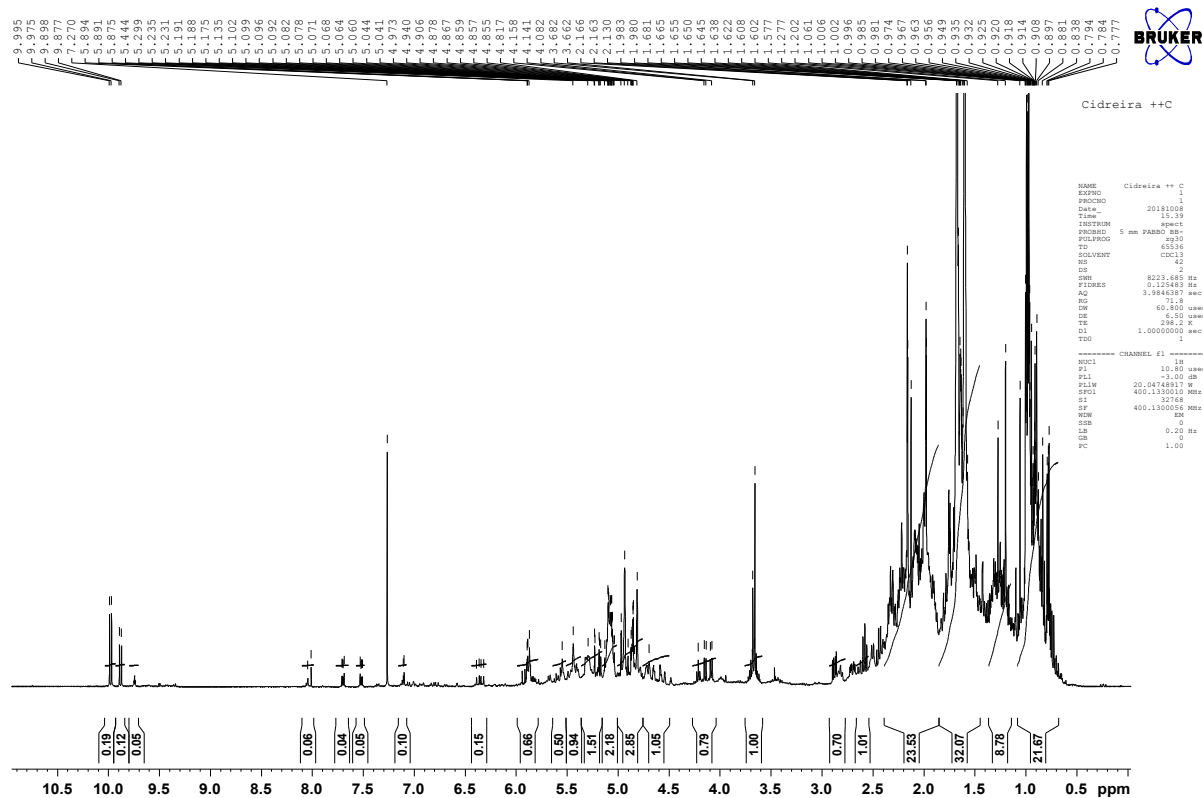


Figure A.25 – ^1H NMR spectrum of lemon balm essential oil. 400 MHz, CDCl_3 .

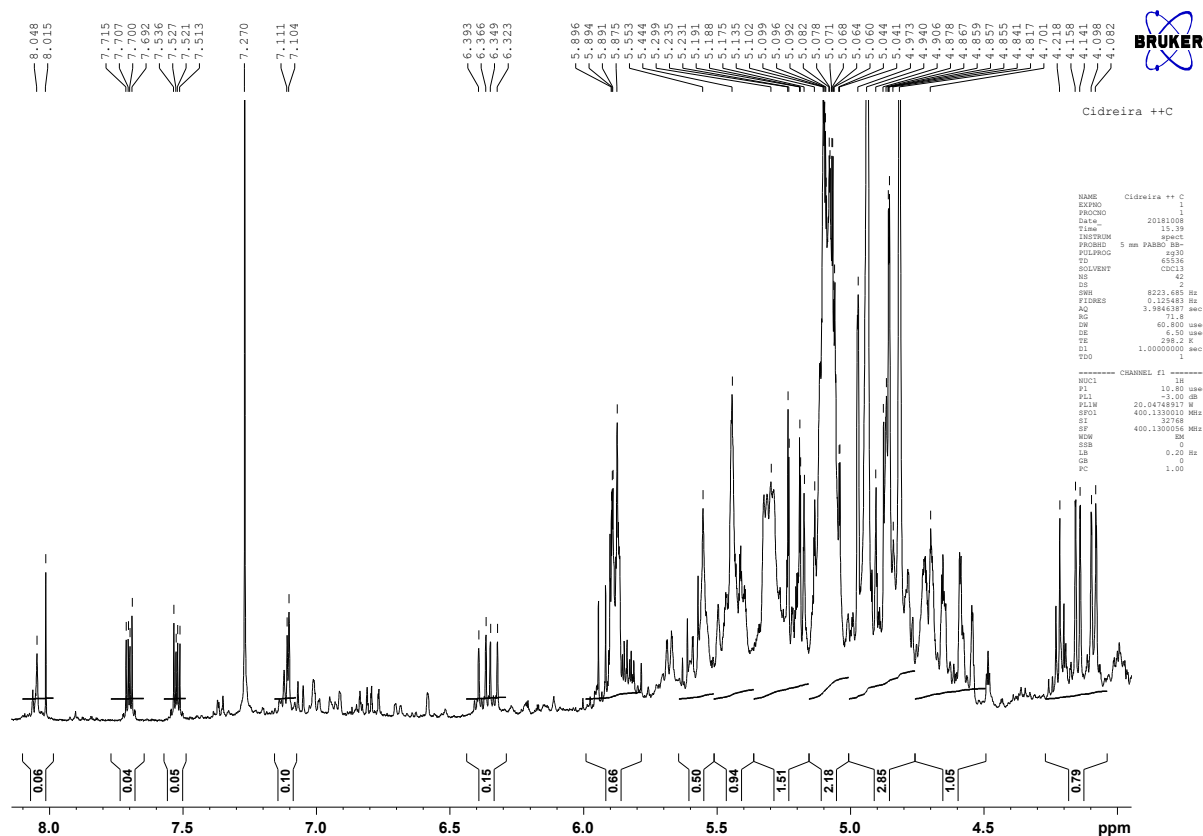


Figure A.25 (expansion) – ^1H NMR spectrum of lemon balm essential oil. 400 MHz, CDCl_3 .

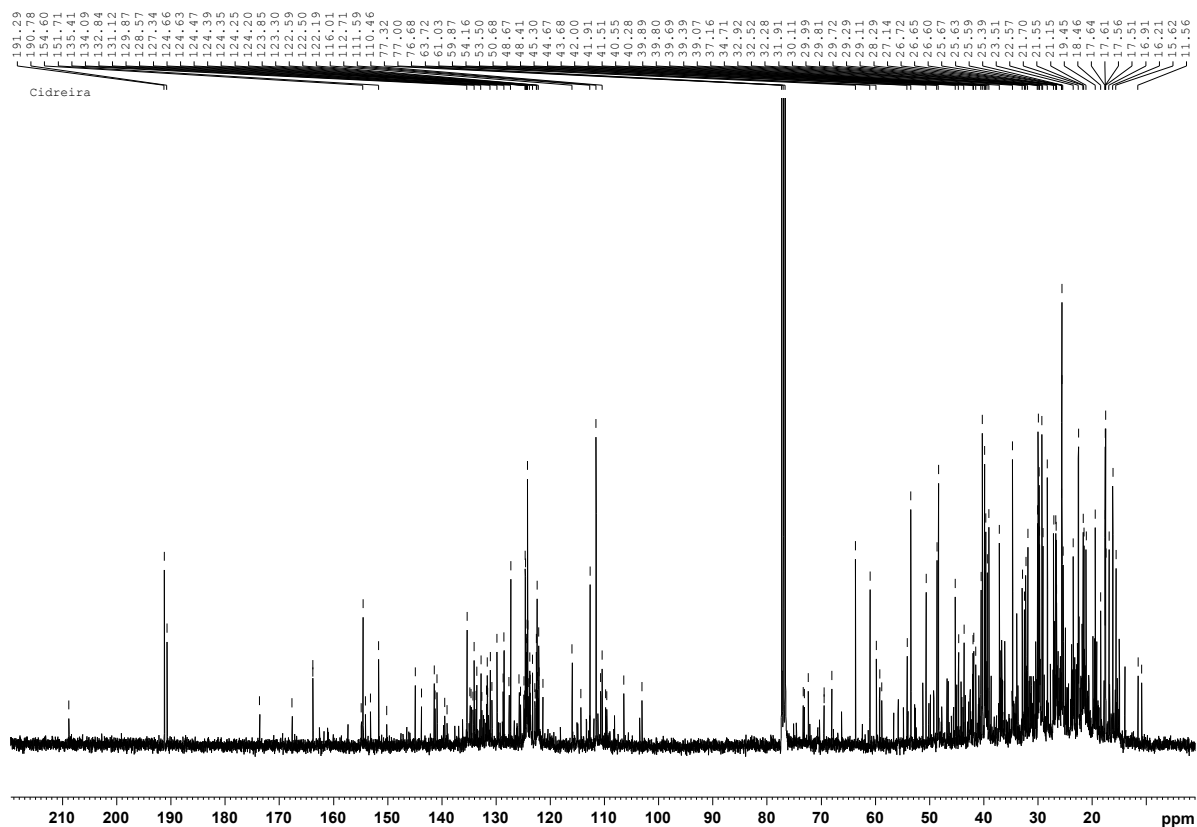


Figure A.26 – ^{13}C NMR spectrum of lemon balm essential oil. 100 MHz, CDCl_3 .

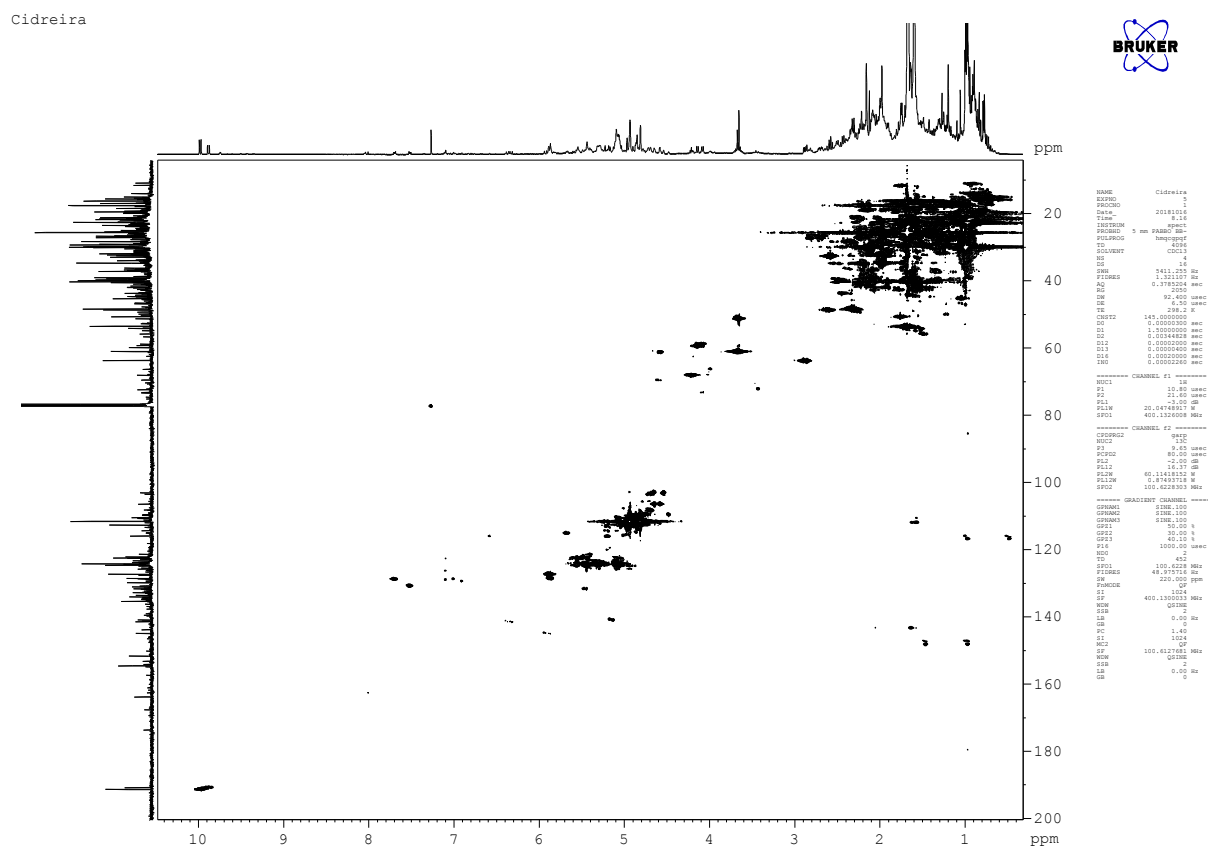
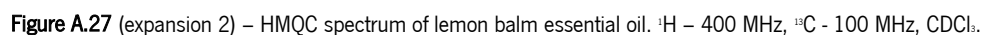
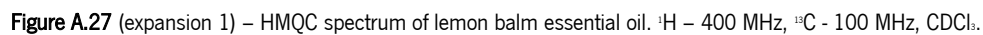
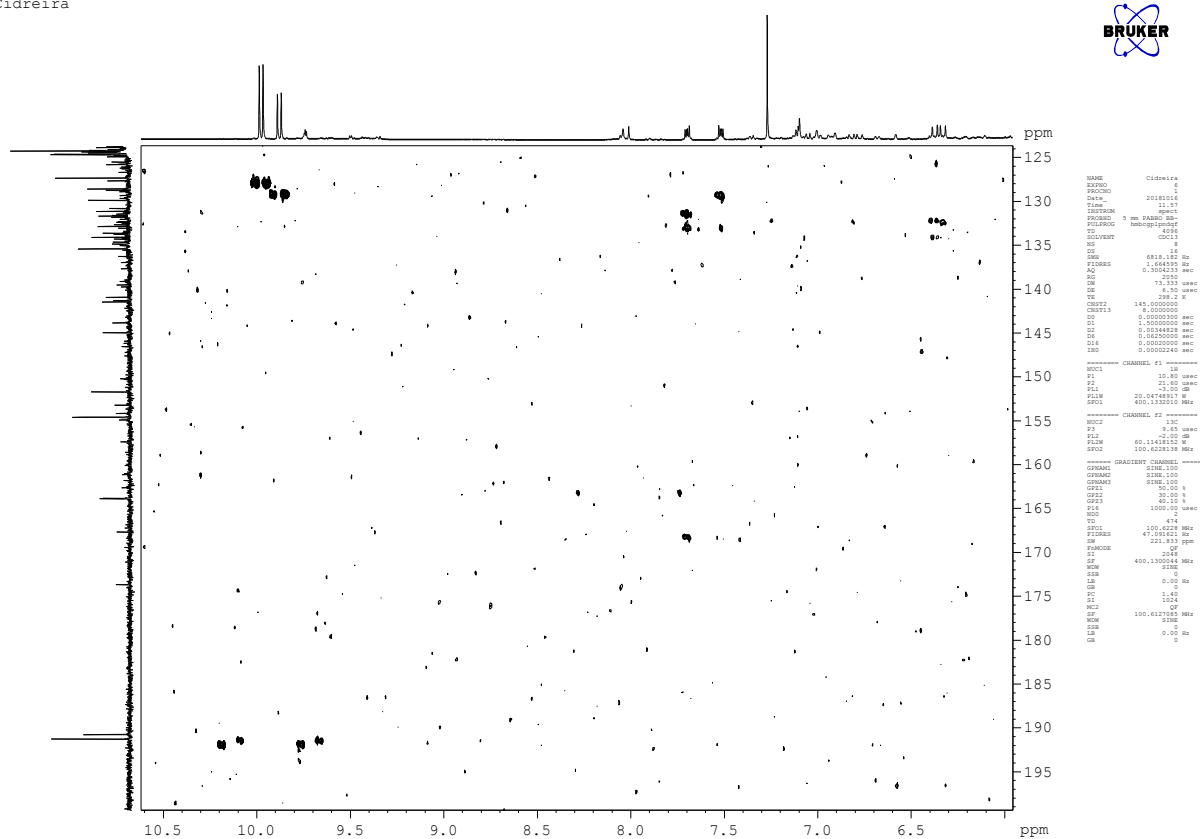
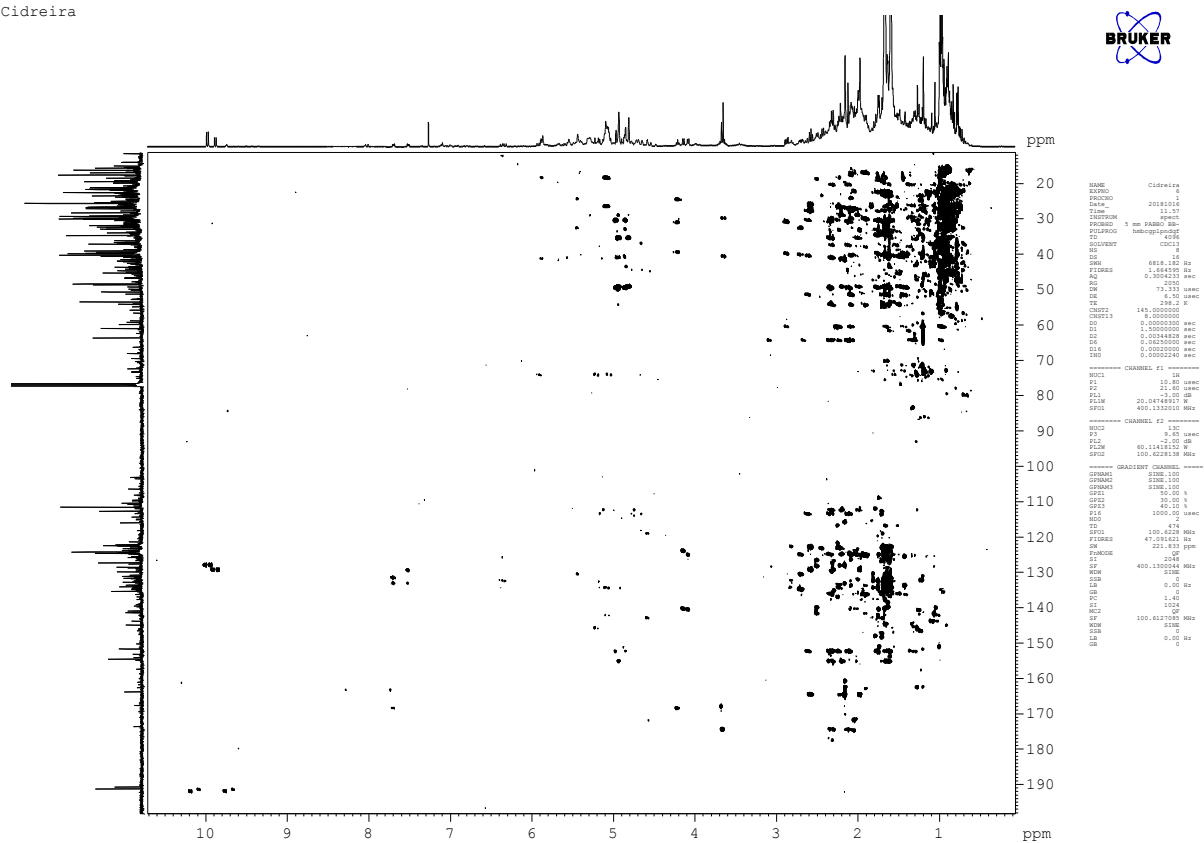
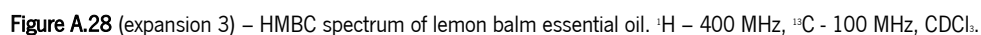
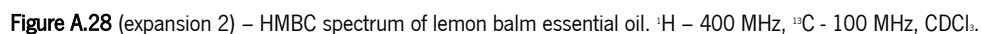


Figure A.27 – HMQC spectrum of lemon balm essential oil. ^1H – 400 MHz, ^{13}C – 100 MHz, CDCl_3 .







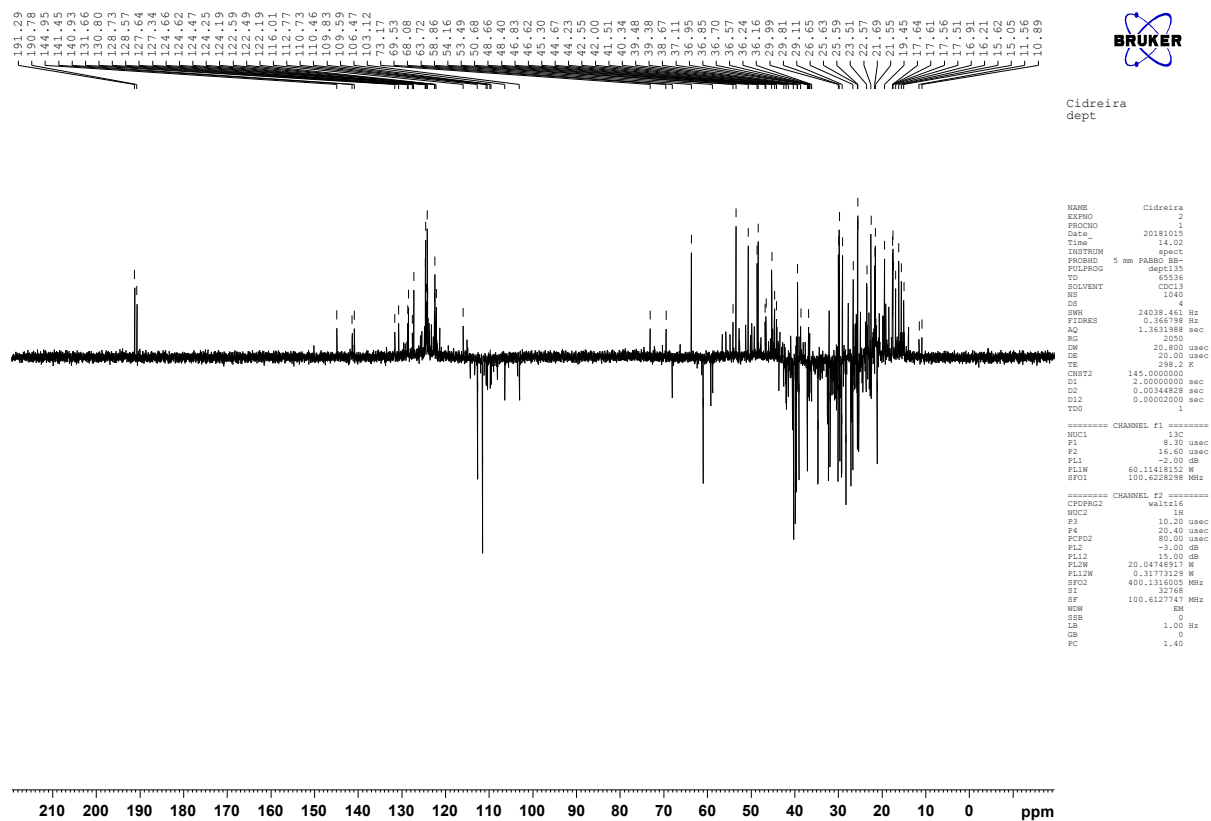


Figure A.29 – DEPT spectrum of lemon balm essential oil. 100 MHz, CDCl₃.

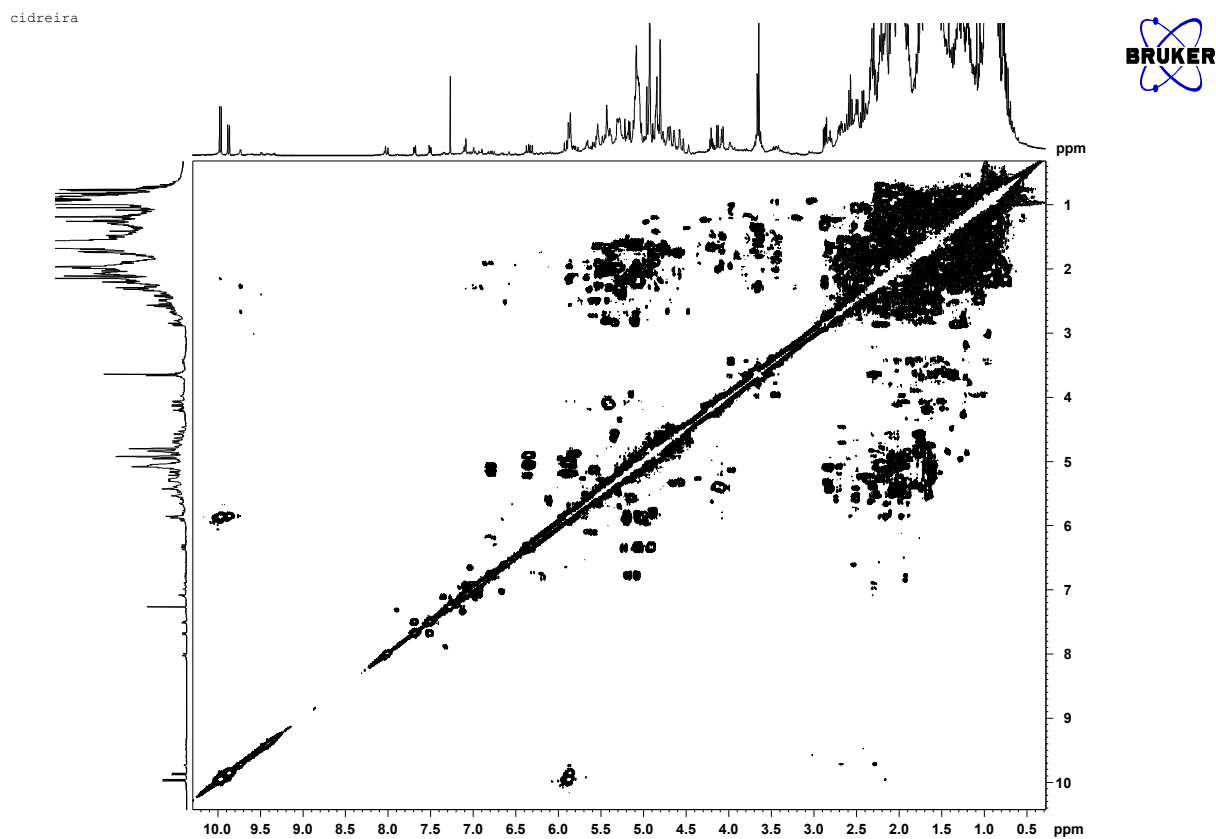


Figure A.30 – COSY spectrum of lemon balm essential oil. 400 MHz, CDCl₃.

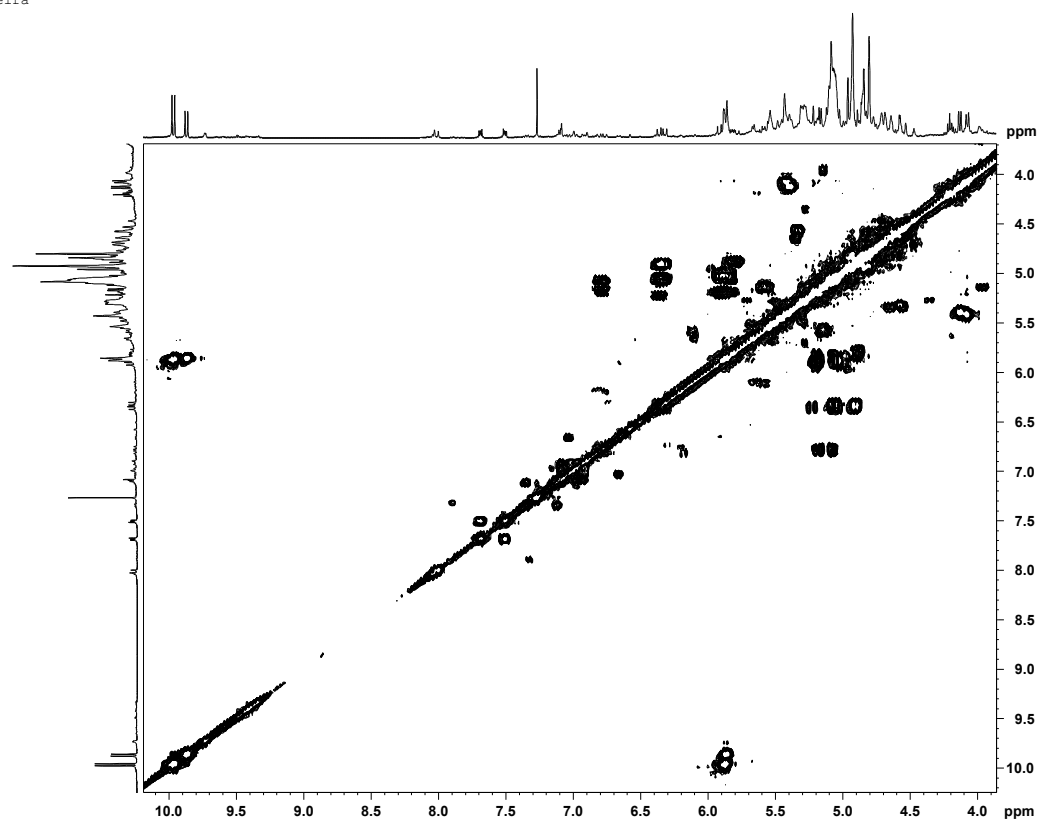


Figure A.30 (expansion) – COSY spectrum of lemon balm essential oil. 400 MHz, CDCl₃.

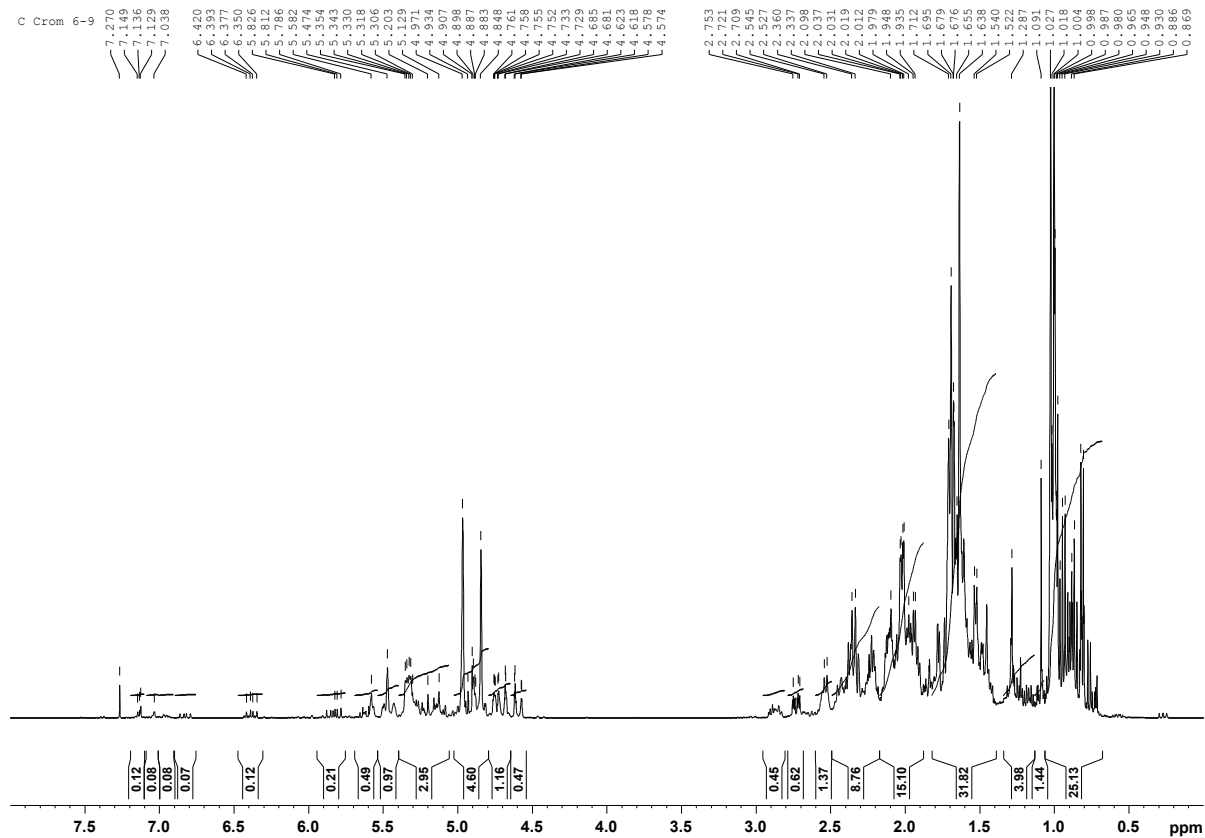


Figure A.31 – ¹H NMR spectrum of a fraction of lemon balm essential oil extracted in petroleum ether. 400 MHz, CDCl₃.

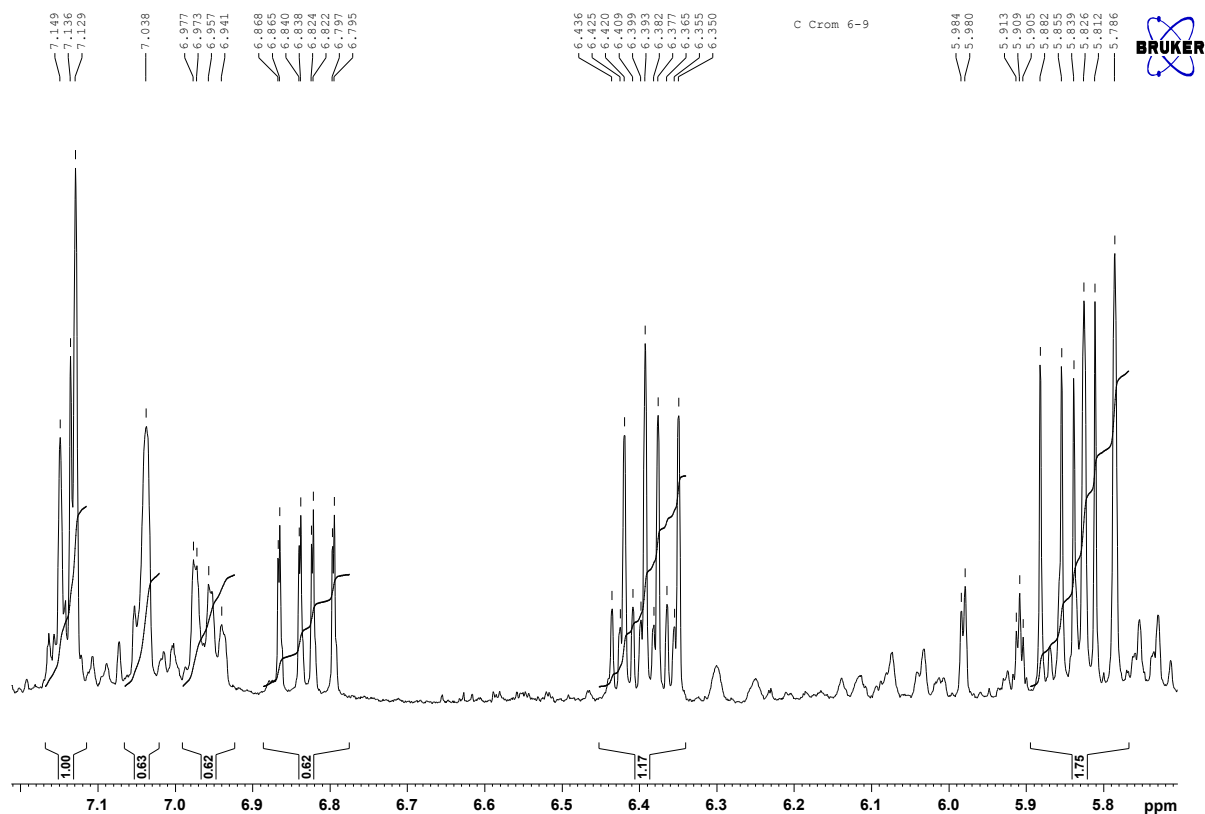


Figure A.31 (expansion 1) – ^1H NMR spectrum of a fraction of lemon balm essential oil extracted in petroleum ether. 400 MHz, CDCl_3 .

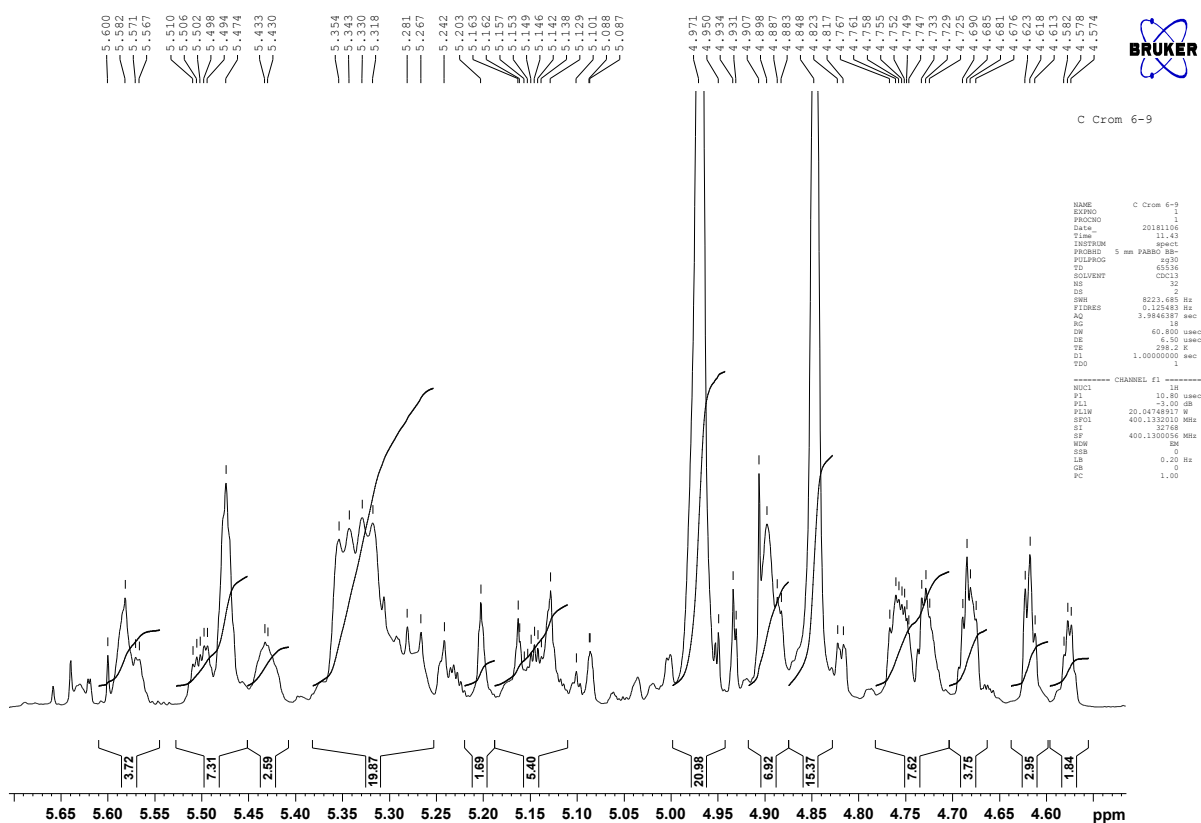


Figure A.31 (expansion 2) – ^1H NMR spectrum of a fraction of lemon balm essential oil extracted in petroleum ether. 400 MHz, CDCl_3 .

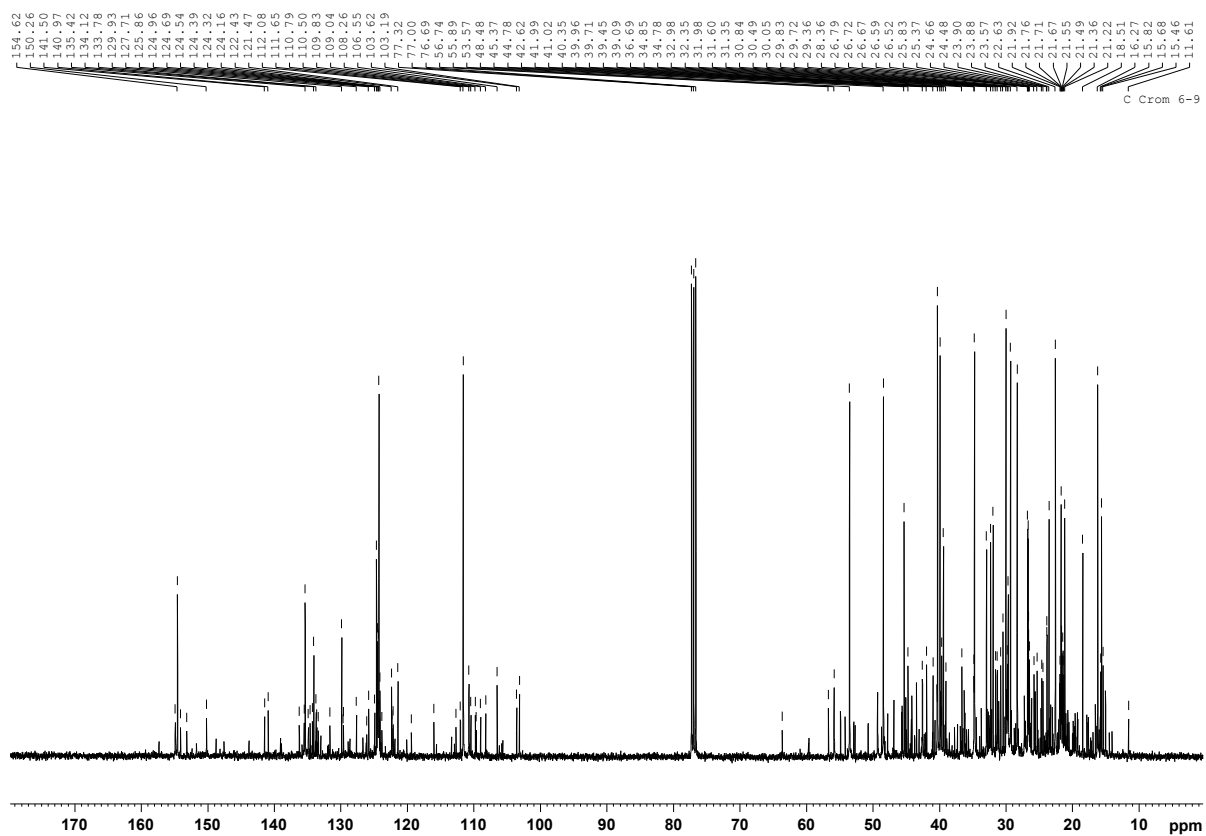


Figure A.32 – ^{13}C NMR spectrum of a fraction of lemon balm essential oil extracted in petroleum ether. 100 MHz, CDCl_3 .

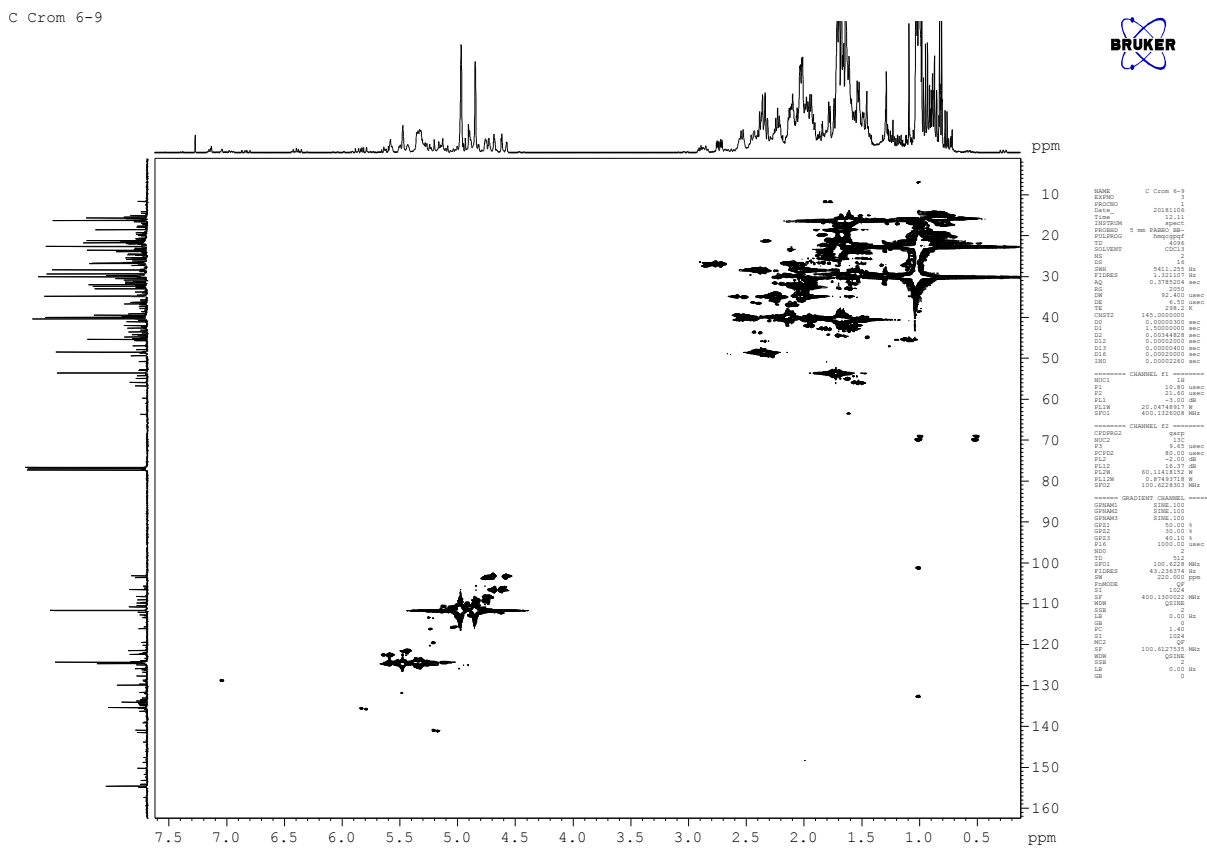


Figure A.33 – HMQC spectrum of a fraction of lemon balm essential oil extracted in petroleum ether. ^1H – 400 MHz, ^{13}C – 100 MHz, CDCl_3 .

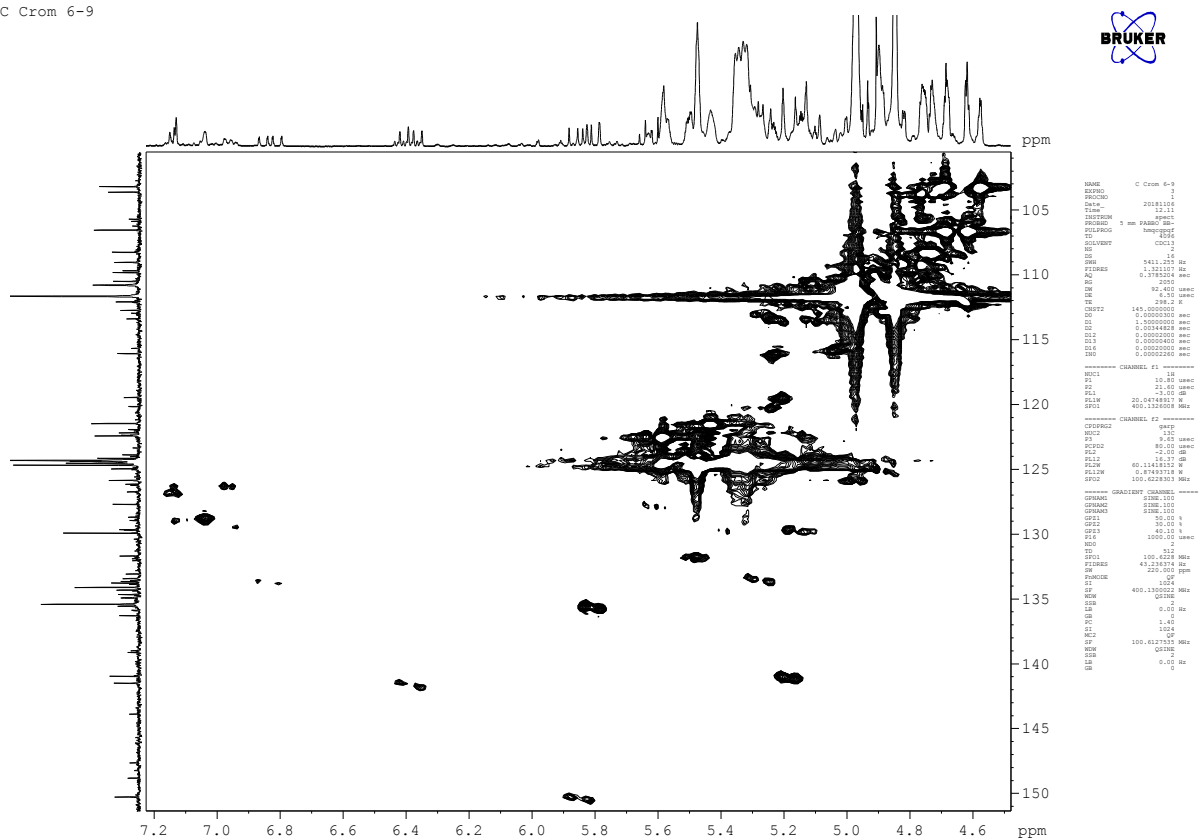


Figure A.33 (expansion) – HMQC spectrum of a fraction of lemon balm essential oil extracted in petroleum ether. ^1H – 400 MHz, ^{13}C – 100 MHz, CDCl_3 .

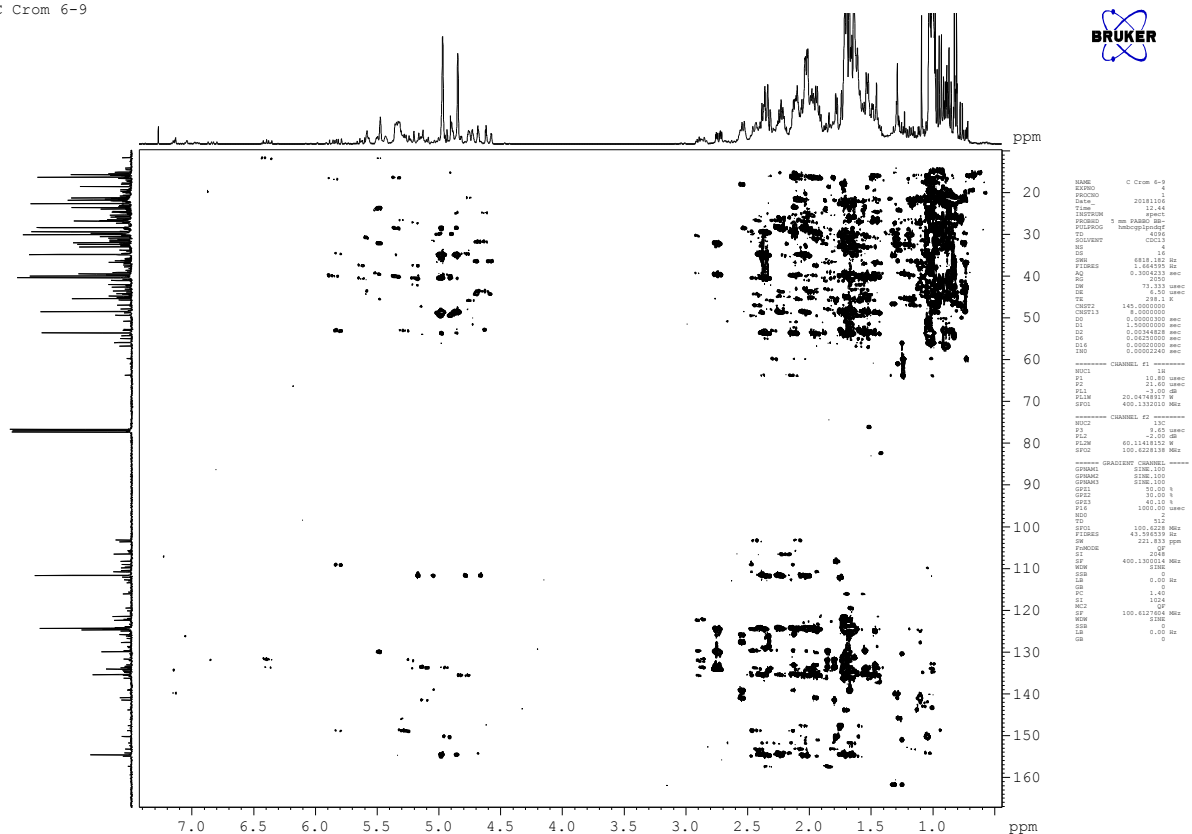
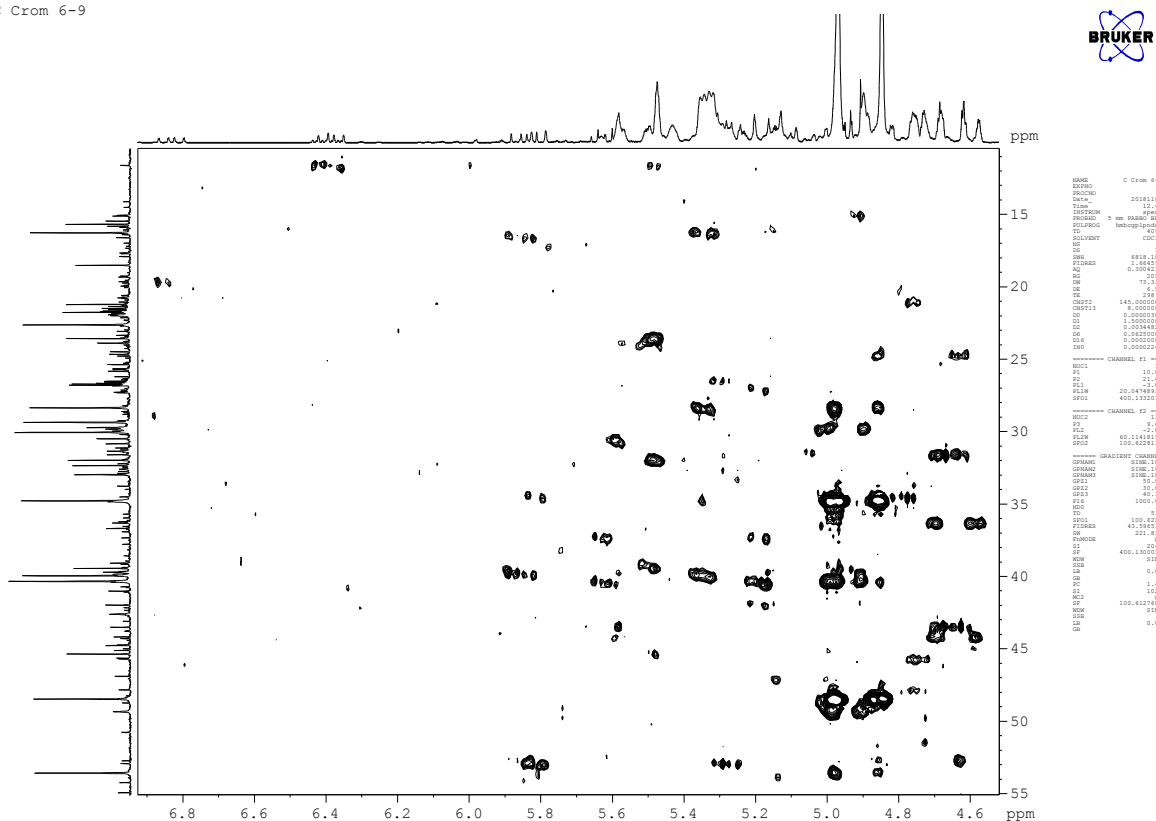
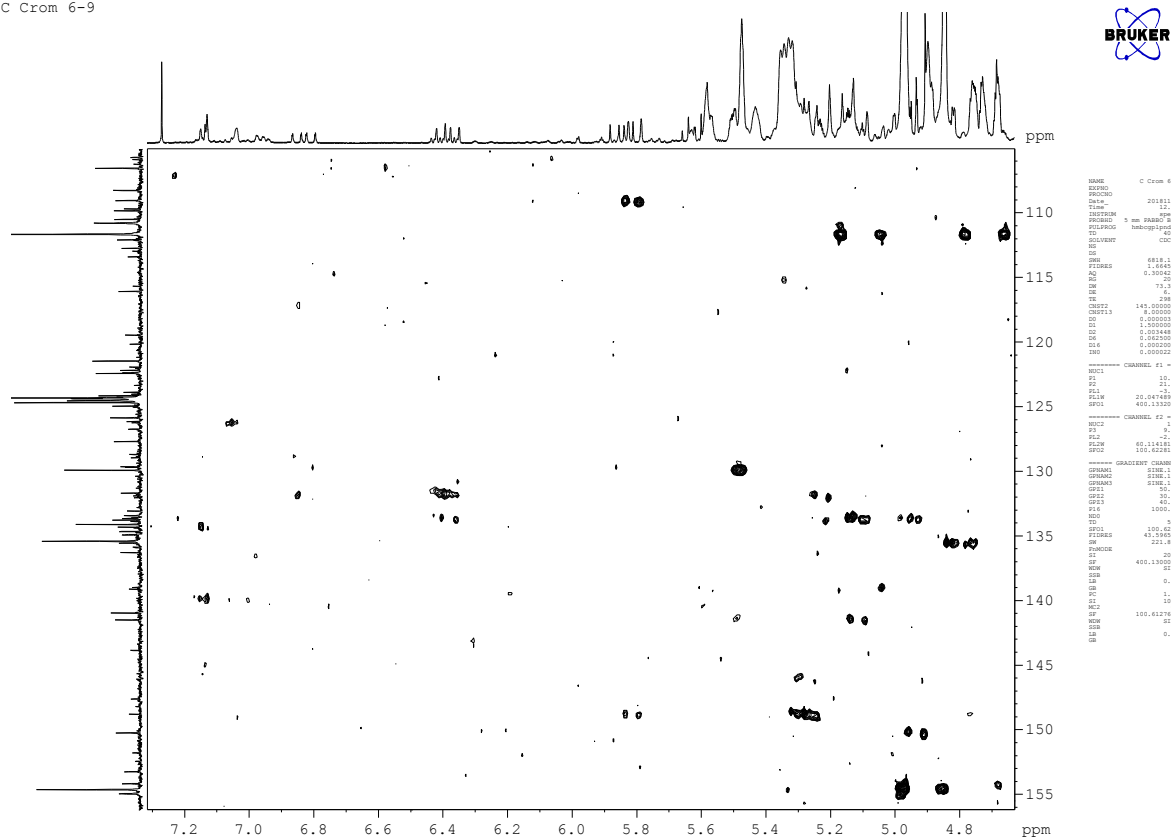


Figure A.34 – HMBC spectrum of a fraction of lemon balm essential oil extracted in petroleum ether. ^1H – 400 MHz, ^{13}C – 100 MHz, CDCl_3 .



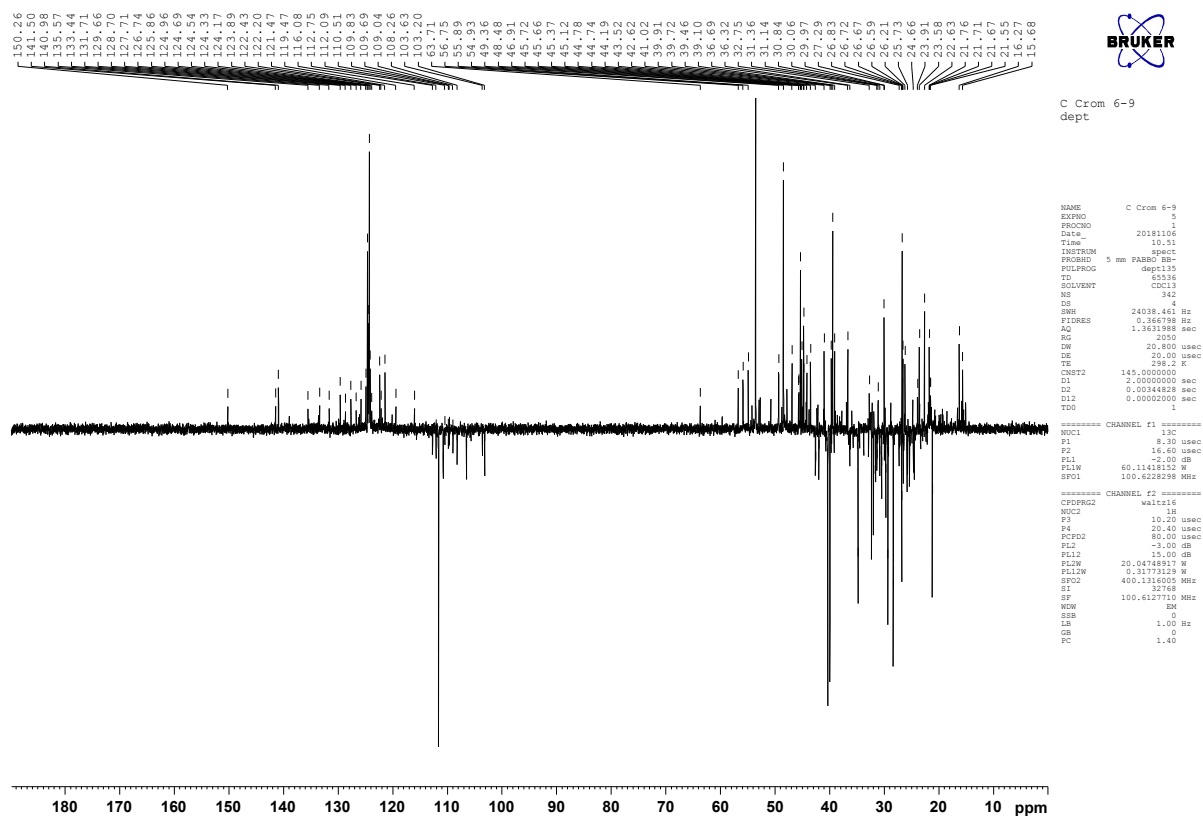


Figure A.35 – DEPT spectrum of a fraction of lemon balm essential oil extracted in petroleum ether. 100 MHz, CDCl₃.

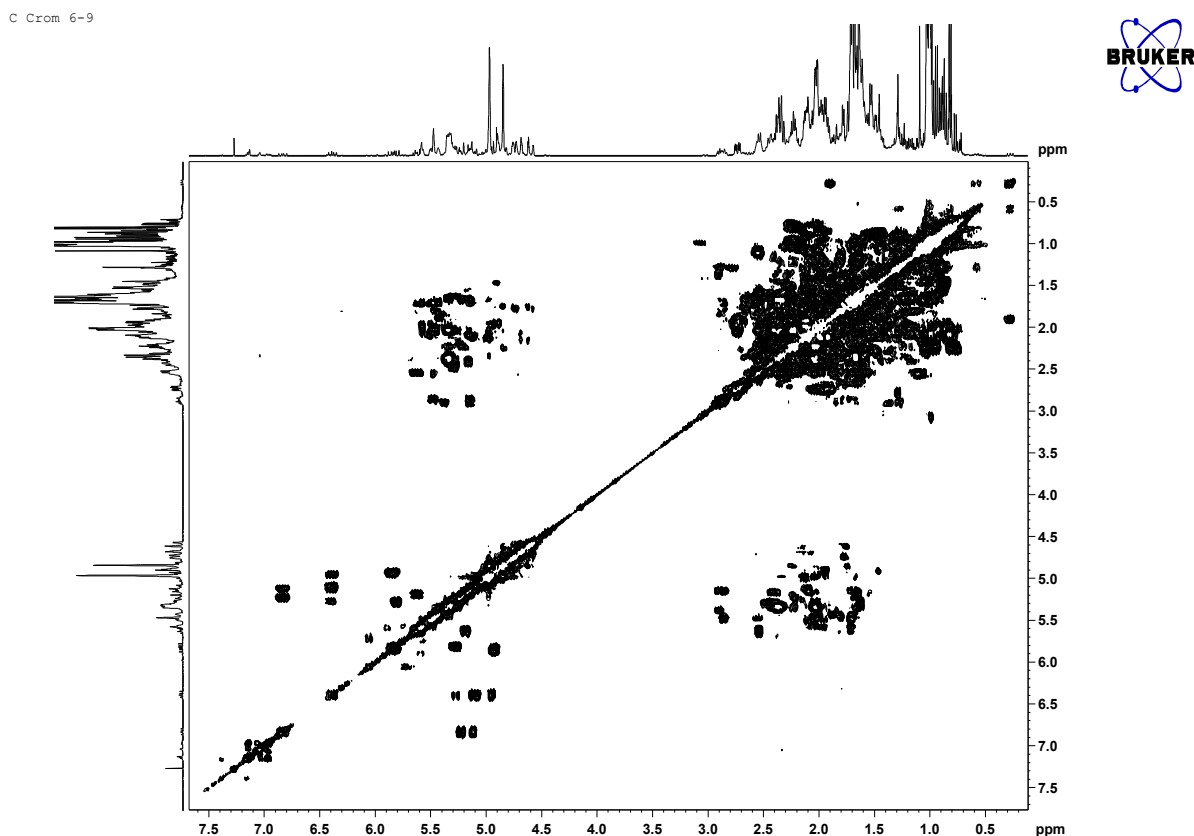


Figure A.36 – COSY spectrum of a fraction of lemon balm essential oil extracted in petroleum ether. 400 MHz, CDCl₃.

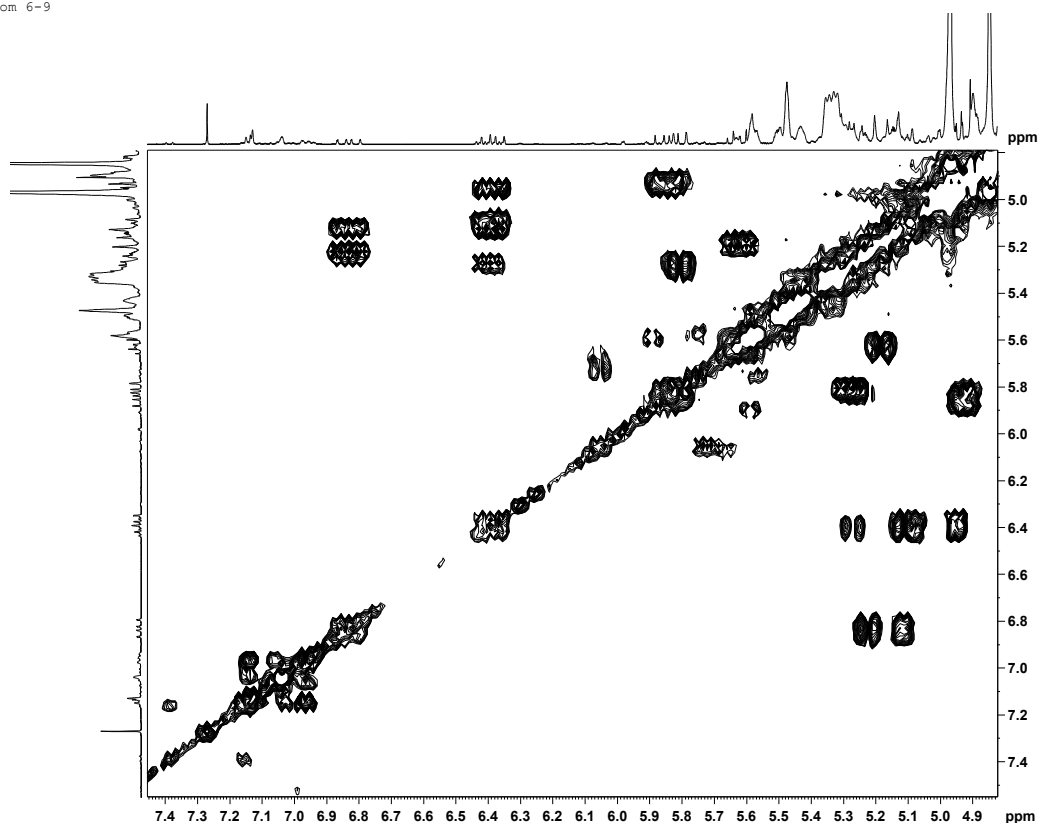


Figure A.36 (expansion) – COSY spectrum of a fraction of lemon balm essential oil extracted in petroleum ether. 400 MHz, CDCl₃.

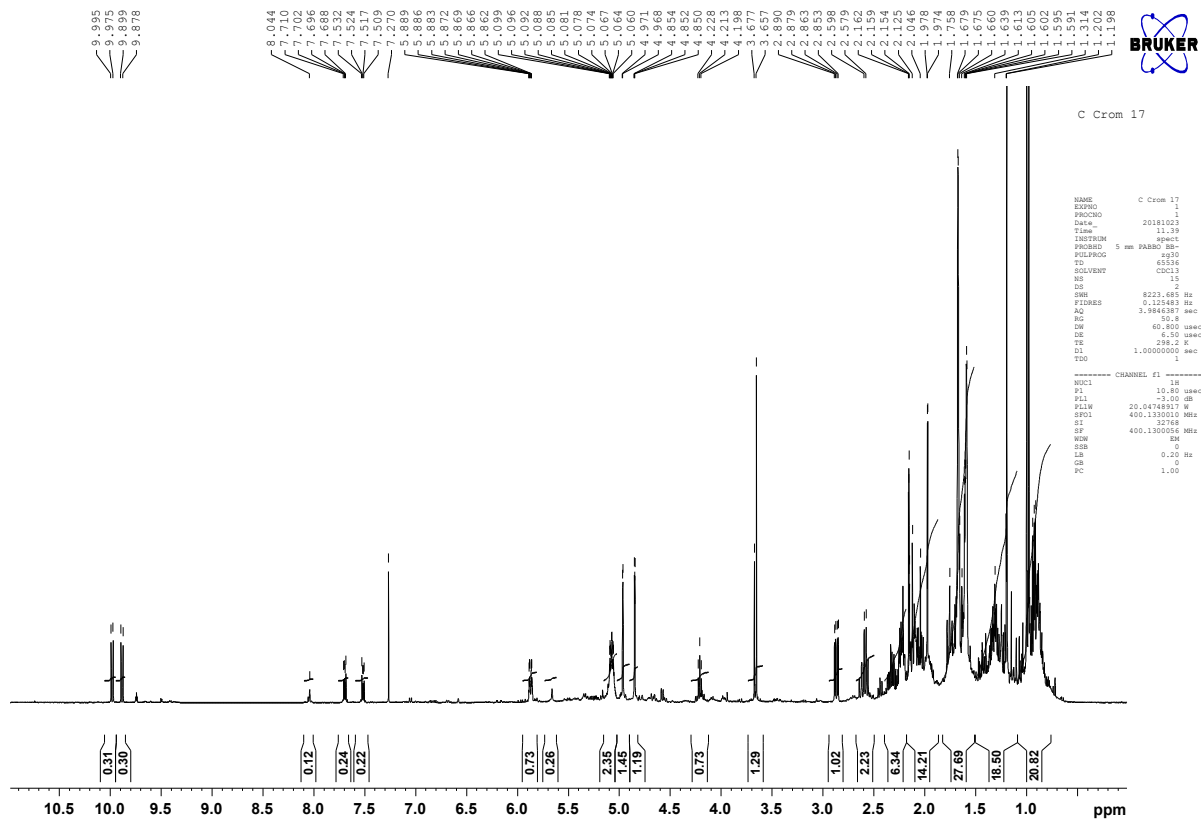


Figure A.37 – ¹H NMR spectrum of the first fraction of lemon balm essential oil extracted in 50% petroleum ether - 50% ethyl acetate. 400 MHz, CDCl₃.

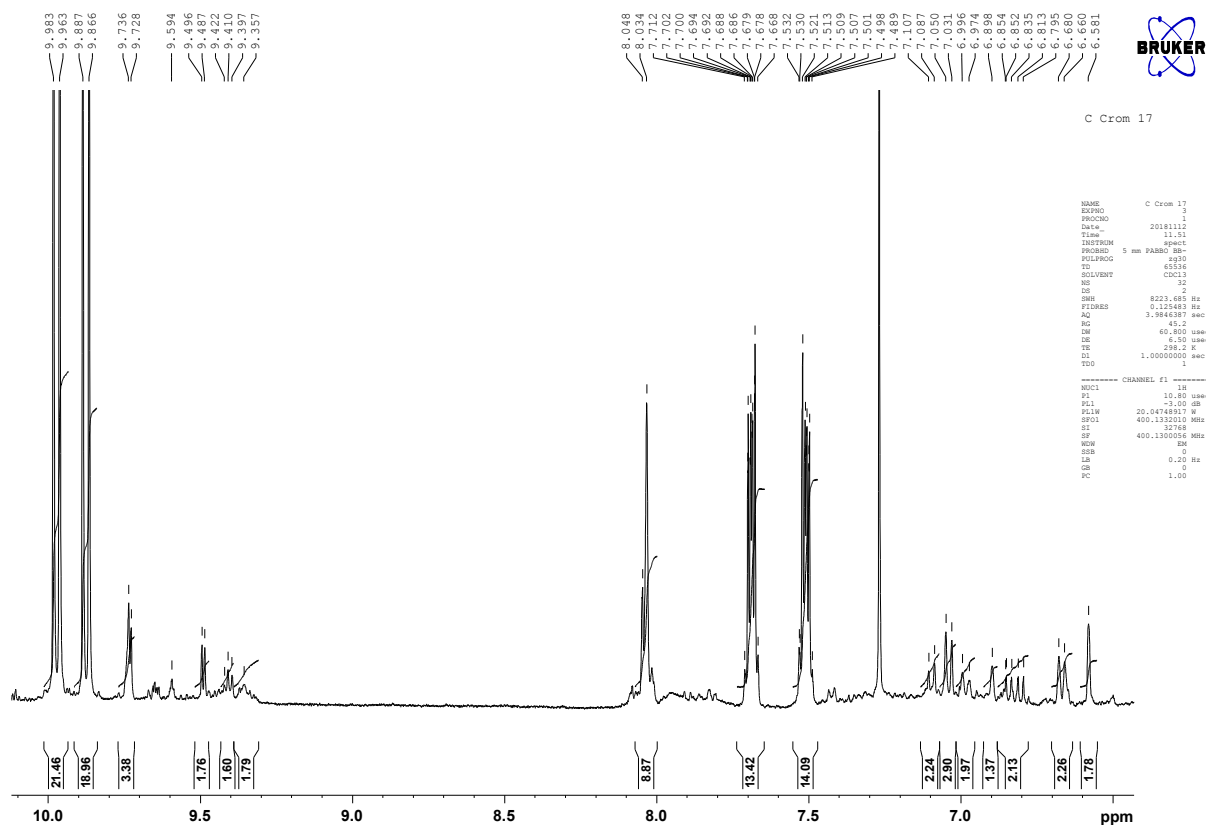


Figure A.37 (expansion 1) – ^1H NMR spectrum of the first fraction of lemon balm essential oil extracted in 50% petroleum ether - 50% ethyl acetate. 400 MHz, CDCl_3 .

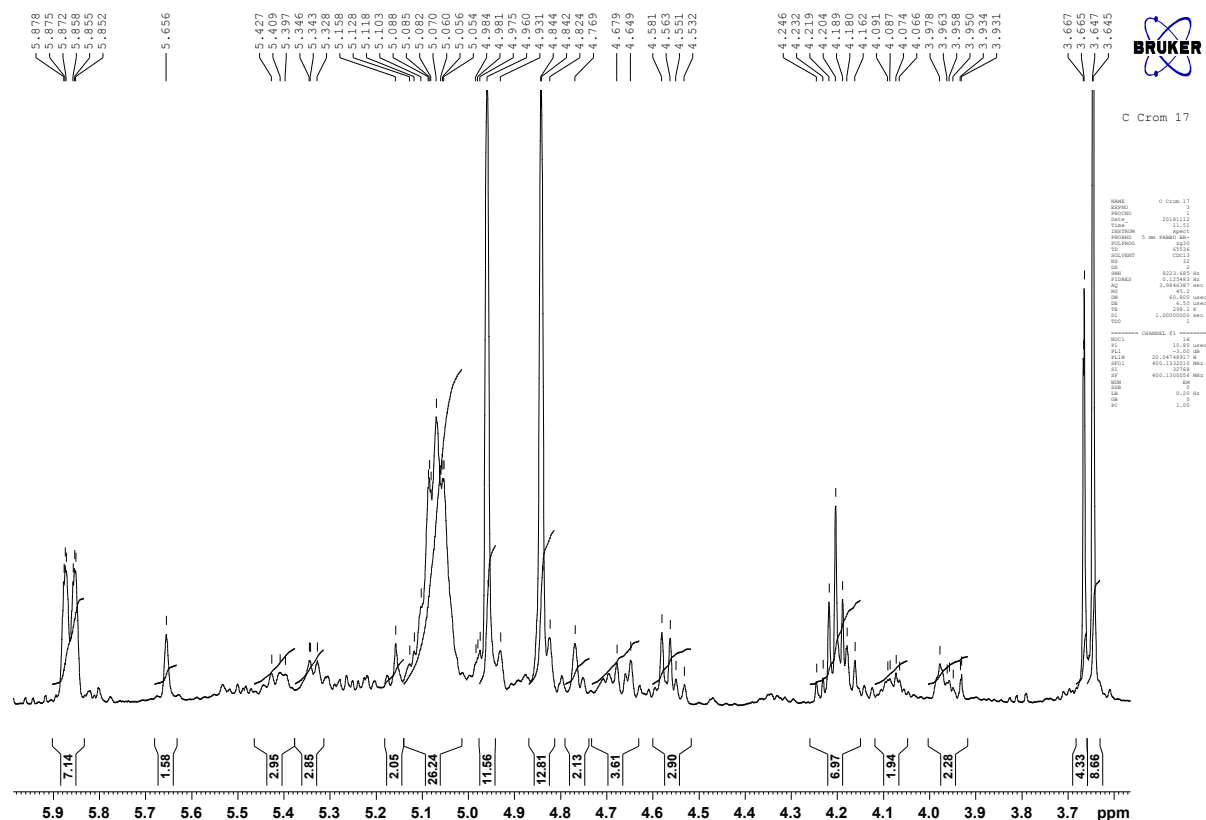


Figure A.37 (expansion 2) – ^1H NMR spectrum of the first fraction of lemon balm essential oil extracted in 50% petroleum ether - 50% ethyl acetate. 400 MHz, CDCl_3 .

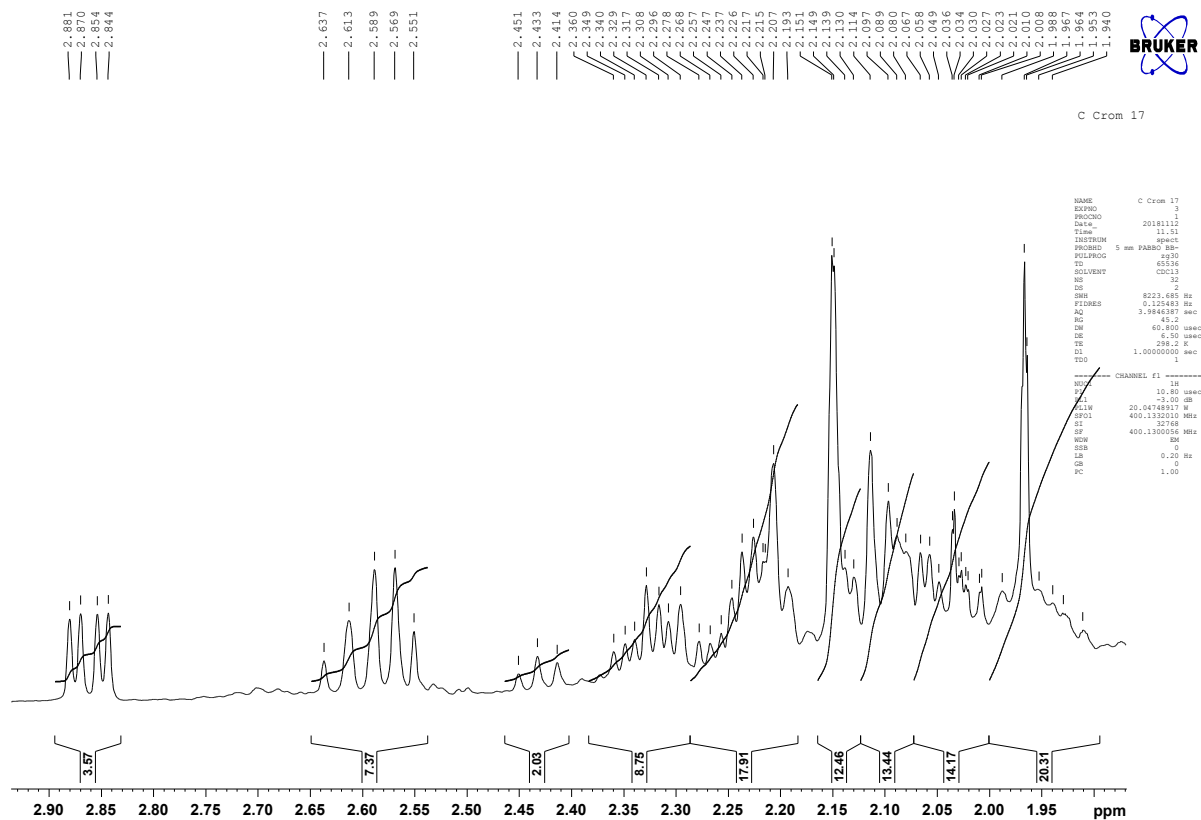


Figure A.37 (expansion 3) – ^1H NMR spectrum of the first fraction of lemon balm essential oil extracted in 50% petroleum ether - 50% ethyl acetate. 400 MHz, CDCl_3 .

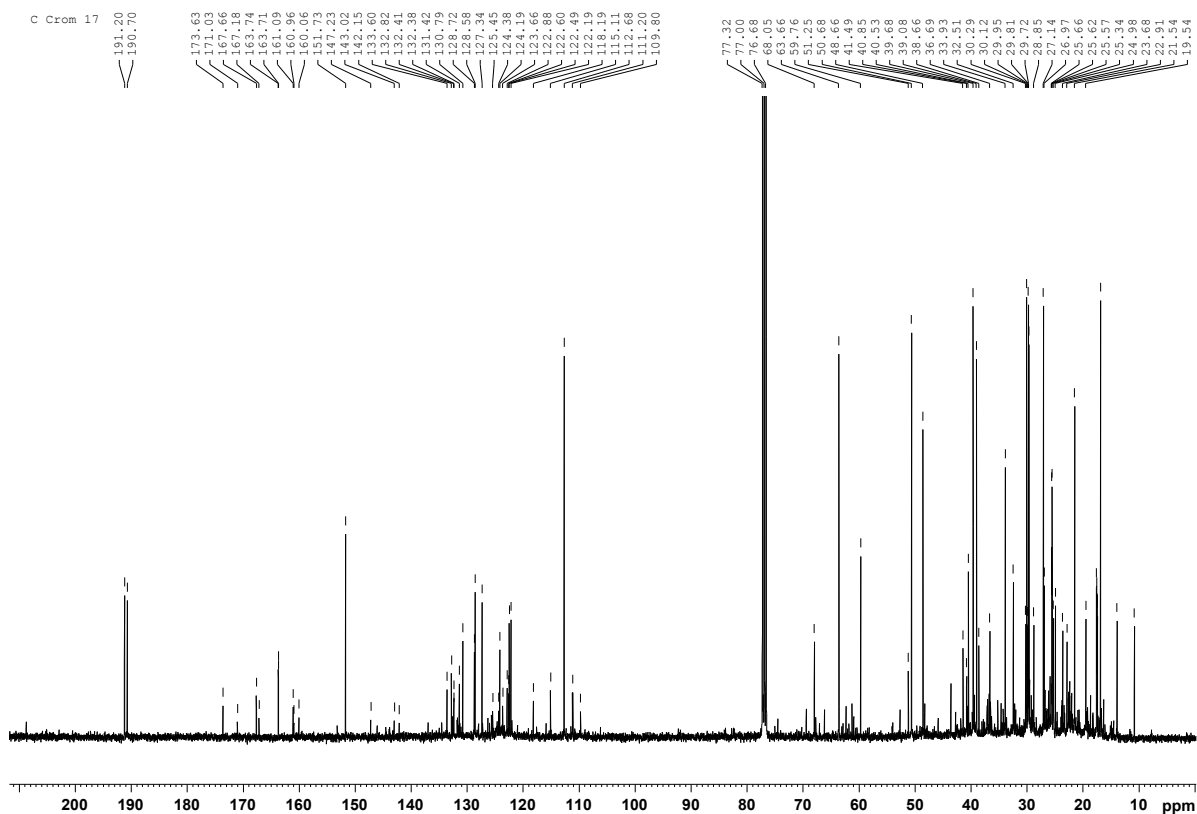


Figure A.38 – ^{13}C NMR spectrum of the first fraction of lemon balm essential oil extracted in 50% petroleum ether - 50% ethyl acetate. 100 MHz, CDCl_3 .

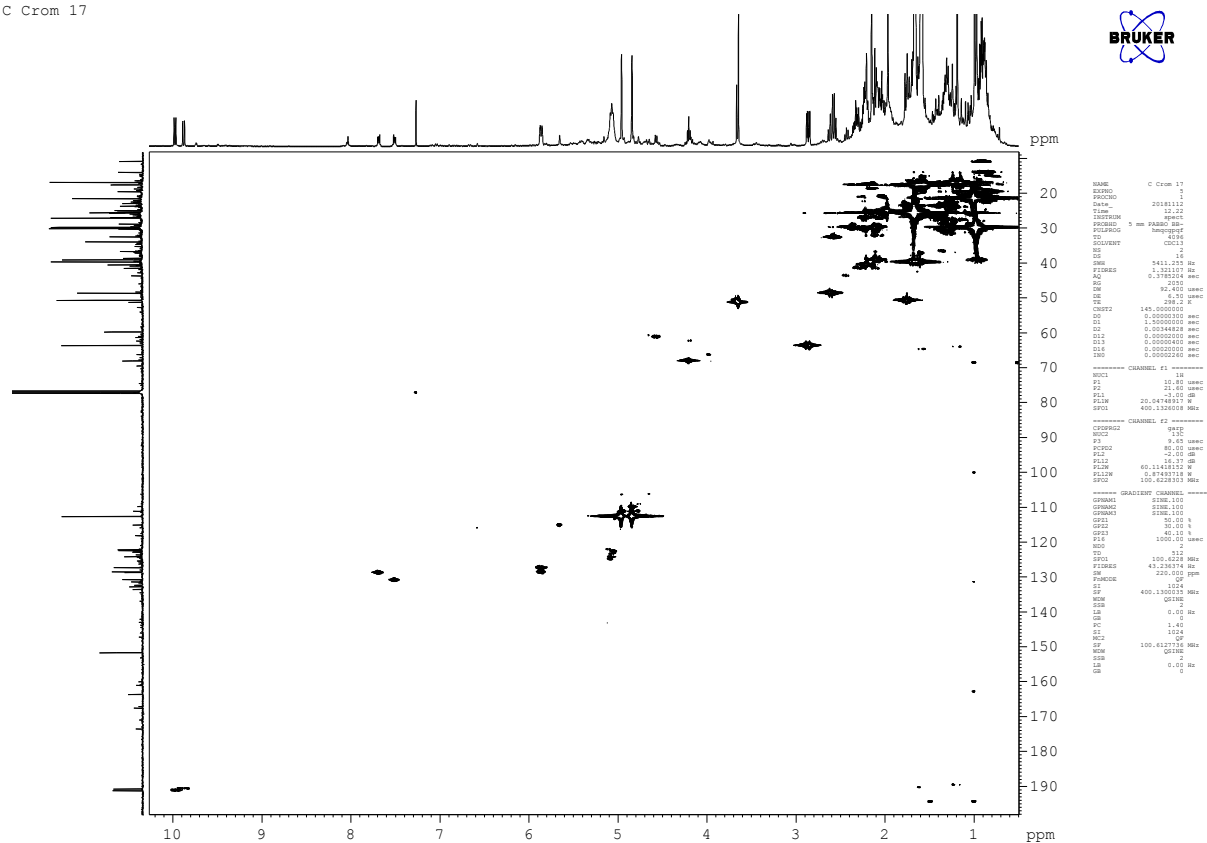


Figure A.39 – HMQC spectrum of the first fraction of lemon balm essential oil extracted in 50% petroleum ether - 50% ethyl acetate. ^1H - 400 MHz, ^{13}C - 100 MHz, CDCl_3 .

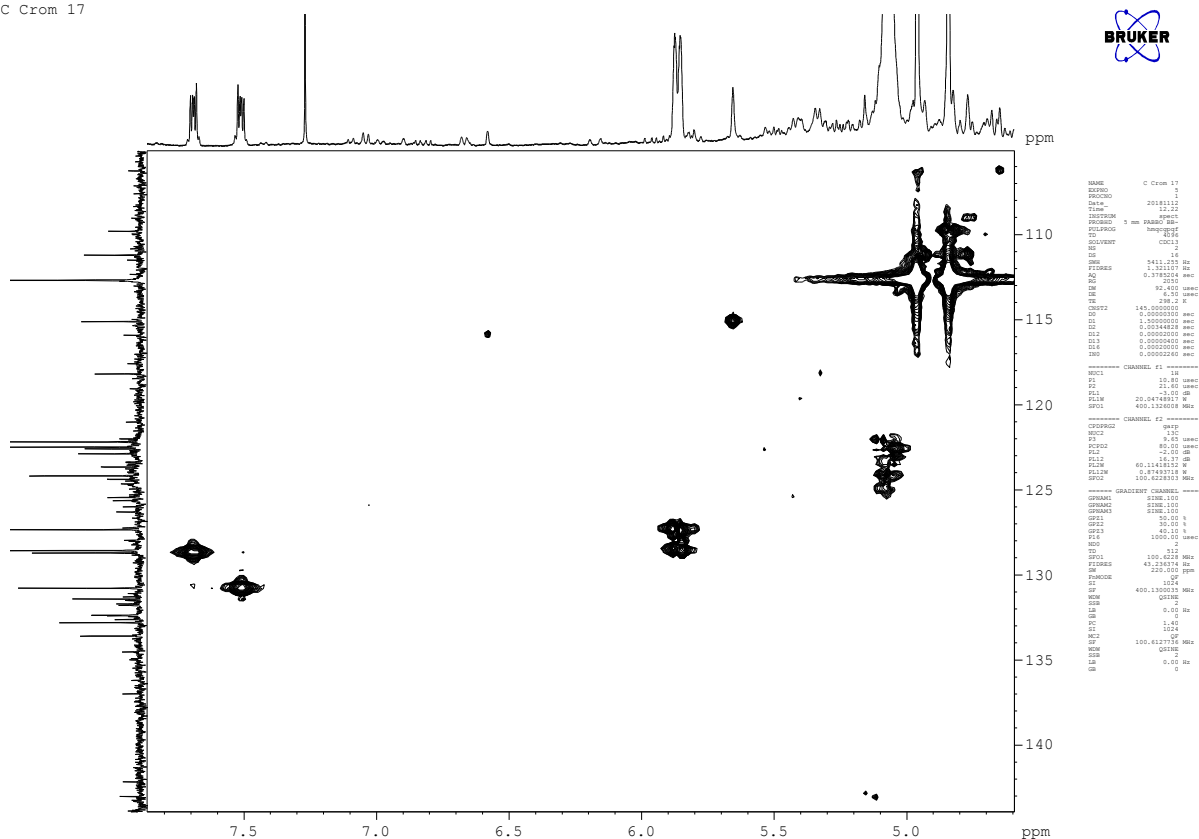


Figure A.39 (expansion 1) – HMQC spectrum of the first fraction of lemon balm essential oil extracted in 50% petroleum ether - 50% ethyl acetate. ^1H - 400 MHz, ^{13}C - 100 MHz, CDCl_3 .

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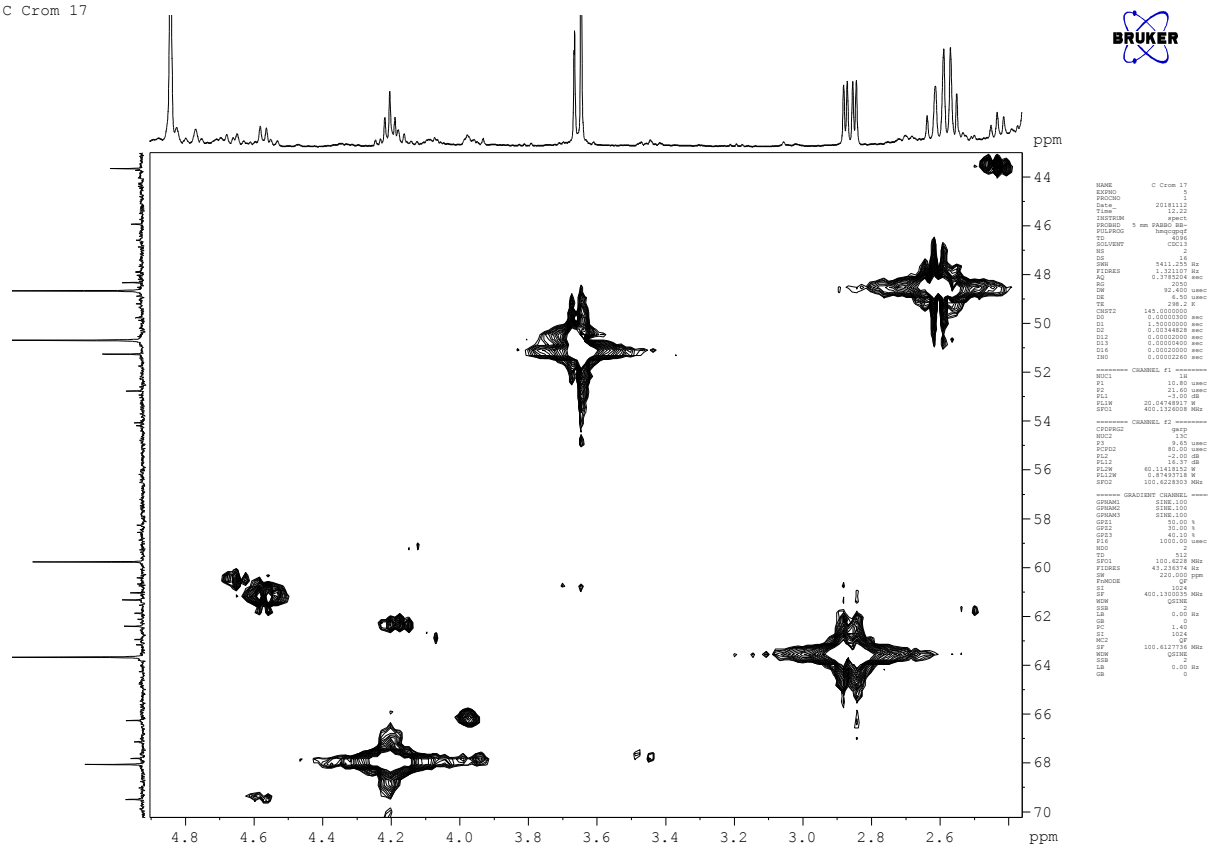


Figure A.39 (expansion 2) – HMQC spectrum of the first fraction of lemon balm essential oil extracted in 50% petroleum ether - 50% ethyl acetate. ^1H - 400 MHz, ^{13}C - 100 MHz, CDCl_3 .

C Crom 17

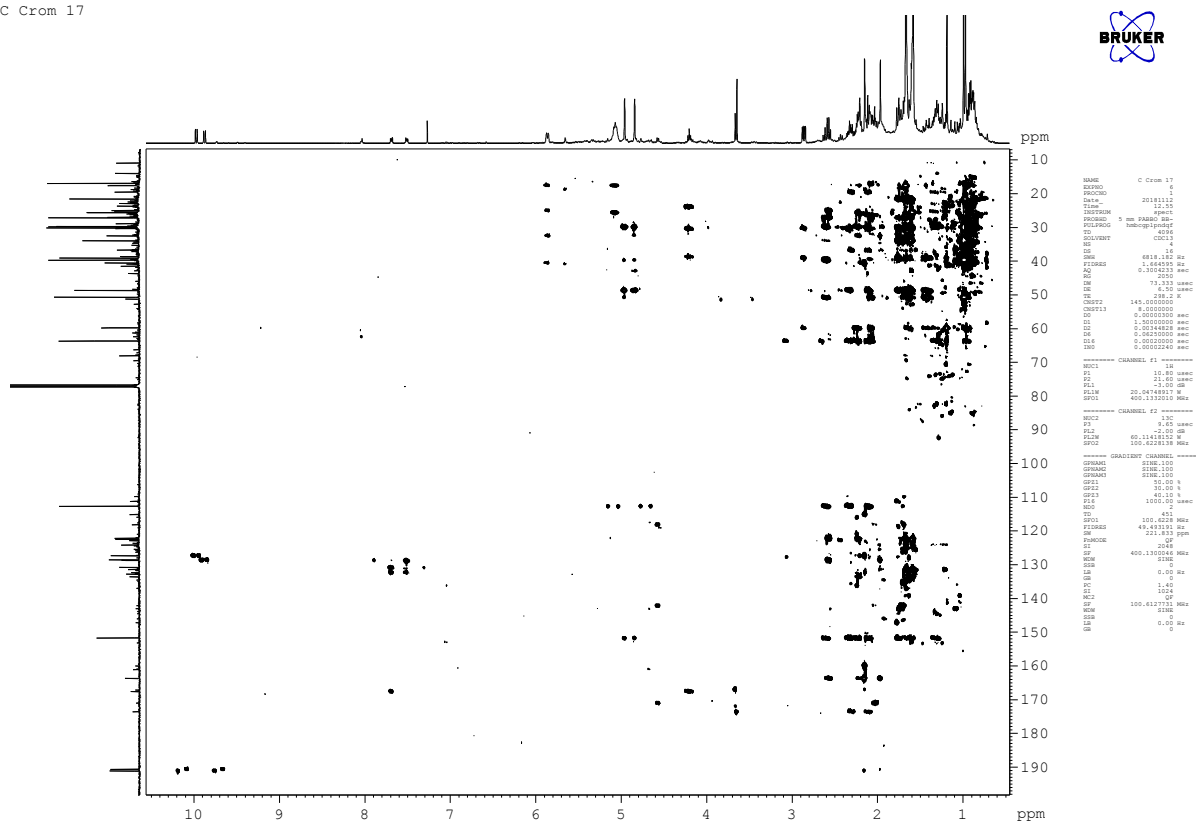
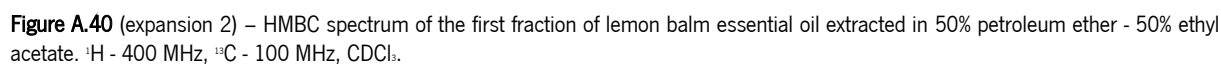
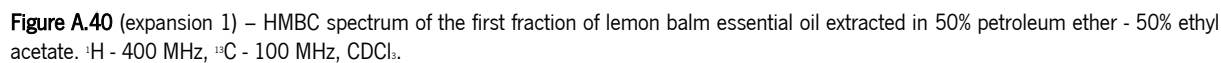


Figure A.40 – HMBC spectrum of the first fraction of lemon balm essential oil extracted in 50% petroleum ether - 50% ethyl acetate. ^1H - 400 MHz, ^{13}C - 100 MHz, CDCl_3 .



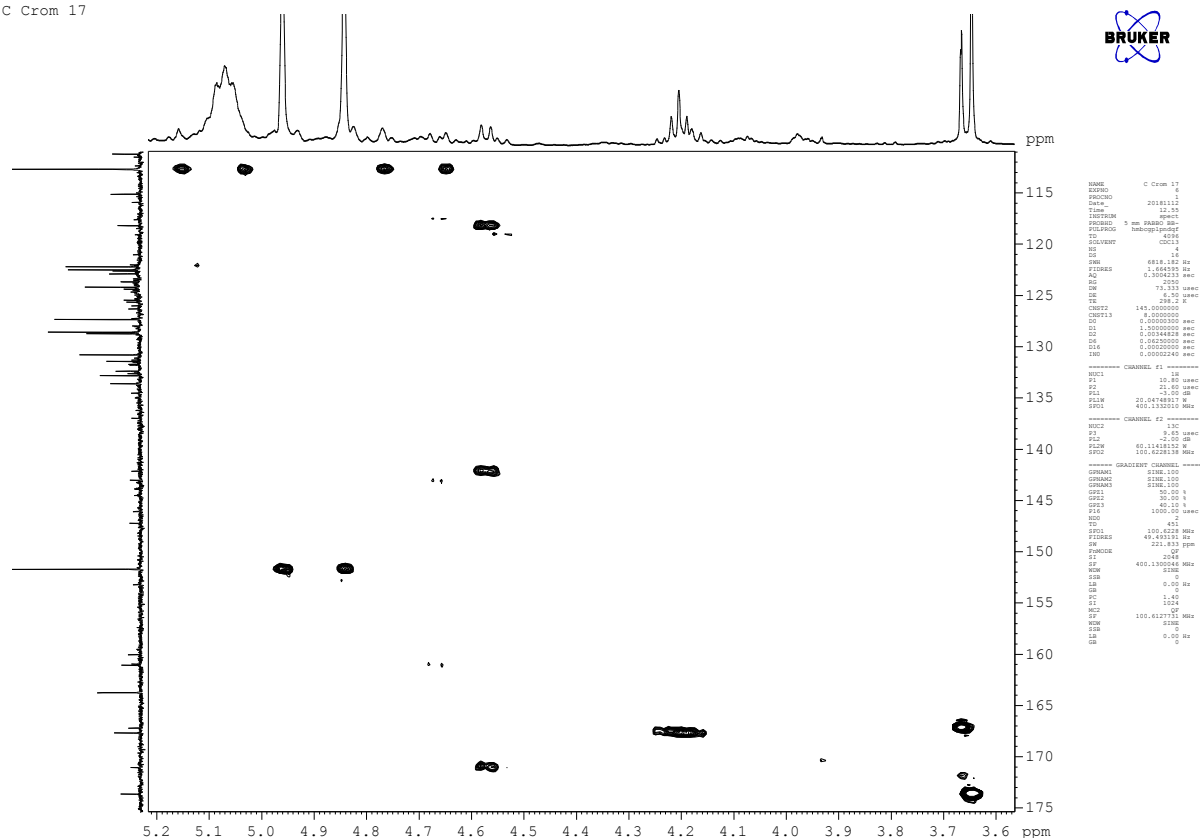


Figure A.40 (expansion 3) – HMBC spectrum of the first fraction of lemon balm essential oil extracted in 50% petroleum ether - 50% ethyl acetate. ^1H - 400 MHz, ^{13}C - 100 MHz, CDCl_3 .

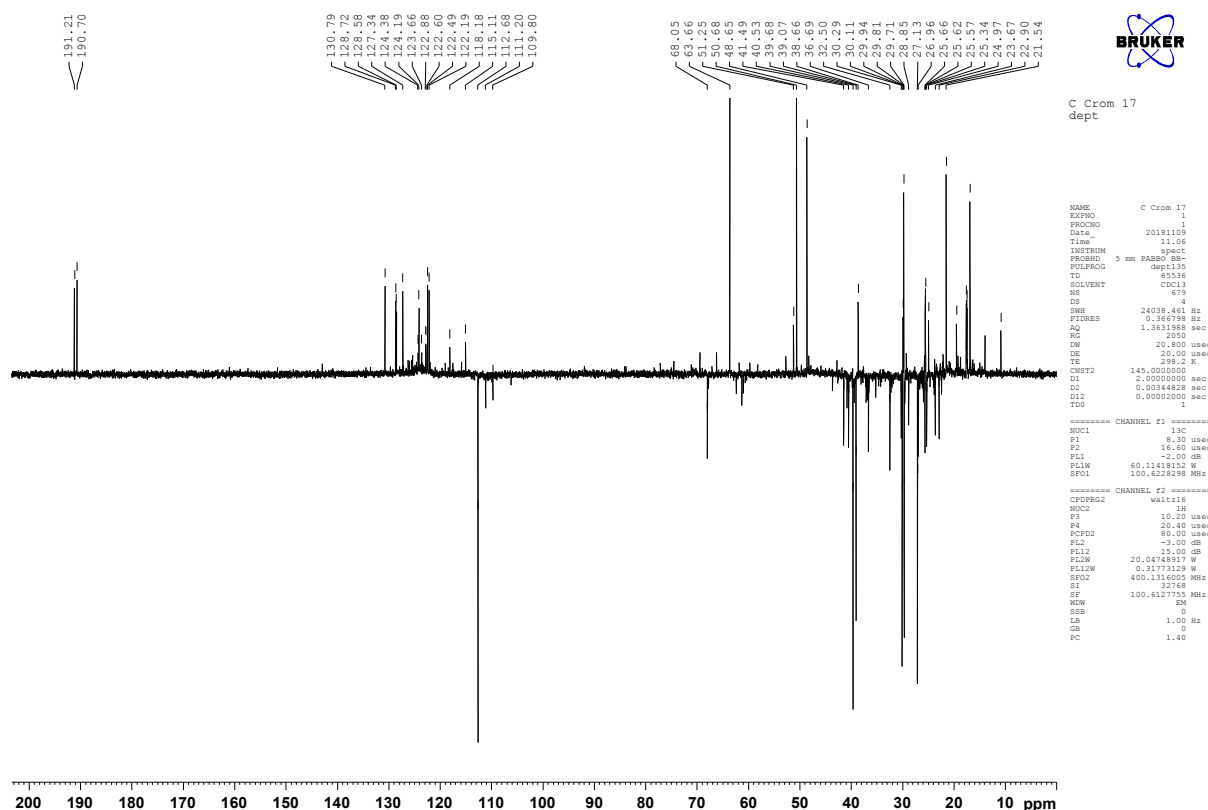


Figure A.41 – DEPT spectrum of the first fraction of lemon balm essential oil extracted in 50% petroleum ether - 50% ethyl acetate. 100 MHz, CDCl_3 .

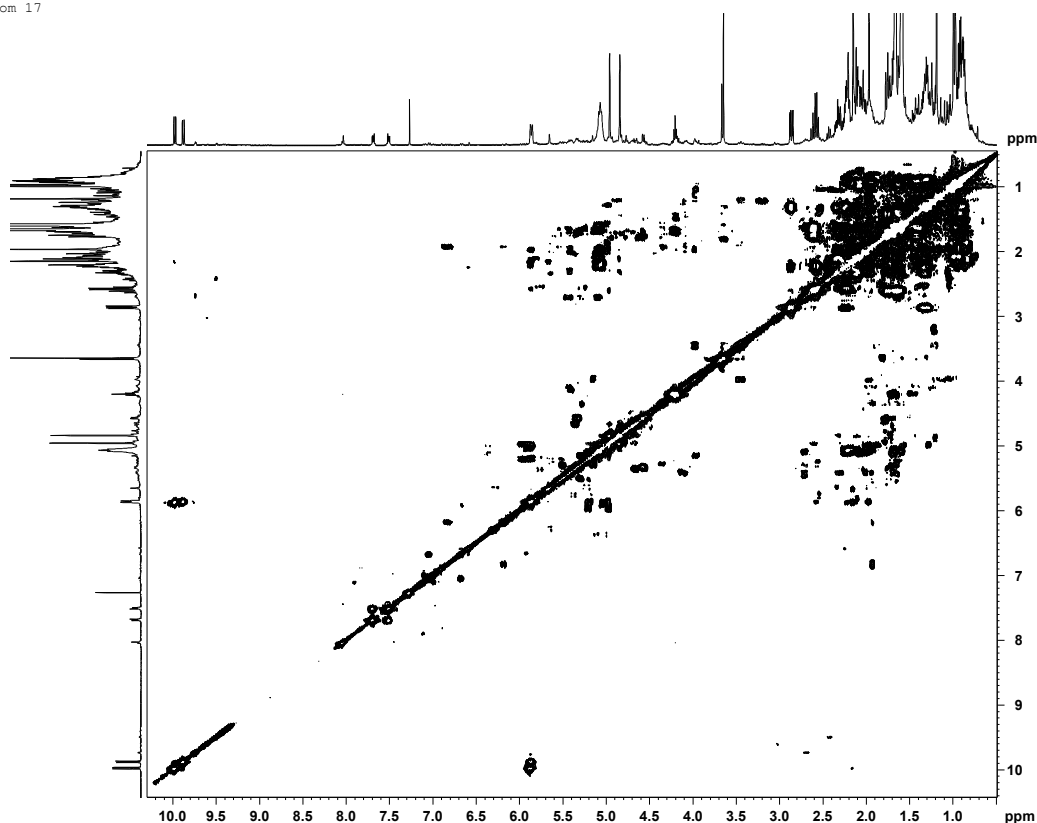


Figure A.42 – COSY spectrum of the first fraction of lemon balm essential oil extracted in 50% petroleum ether - 50% ethyl acetate. 400 MHz, CDCl₃.

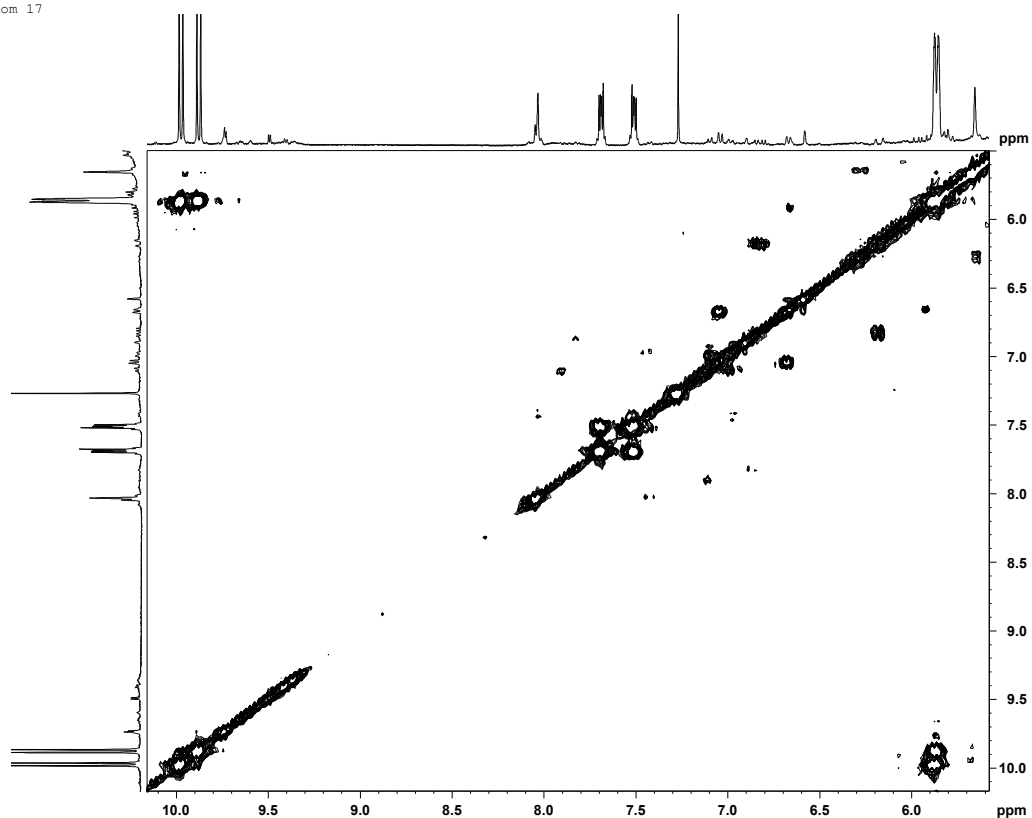


Figure A.42 (expansion 1) – COSY spectrum of the first fraction of lemon balm essential oil extracted in 50% petroleum ether - 50% ethyl acetate. 400 MHz, CDCl₃.

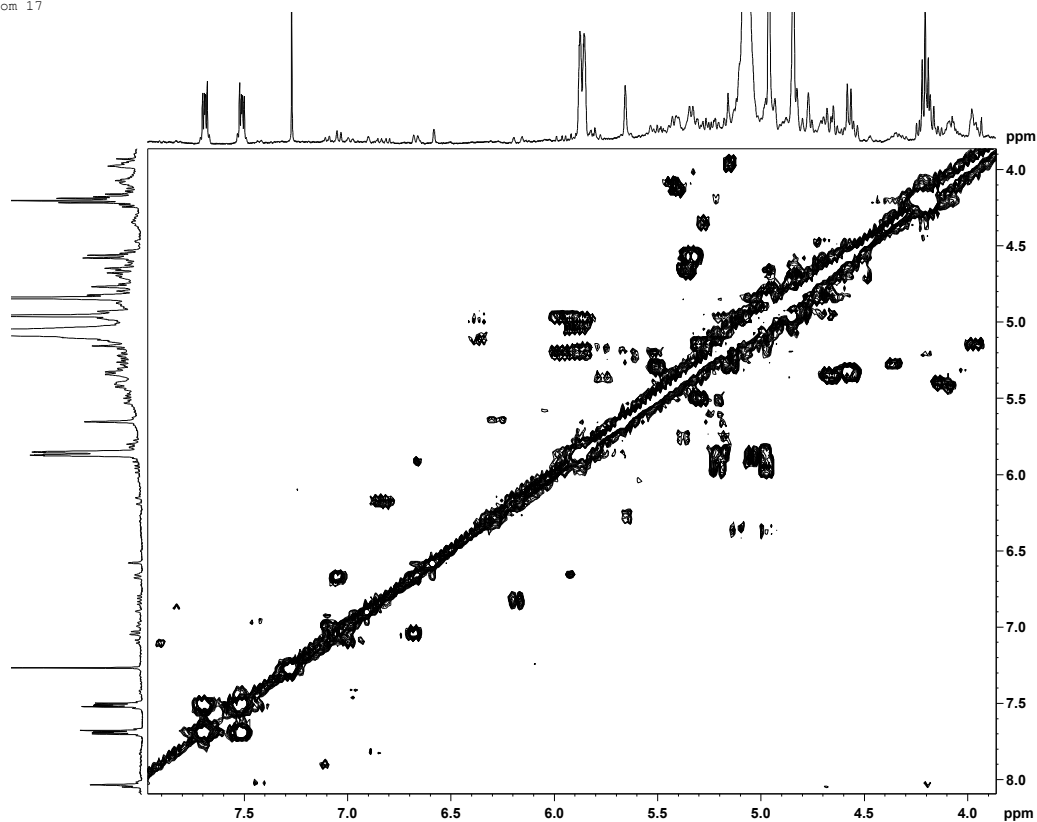


Figure A.42 (expansion 2) – COSY spectrum of the first fraction of lemon balm essential oil extracted in 50% petroleum ether - 50% ethyl acetate. 400 MHz, CDCl₃.

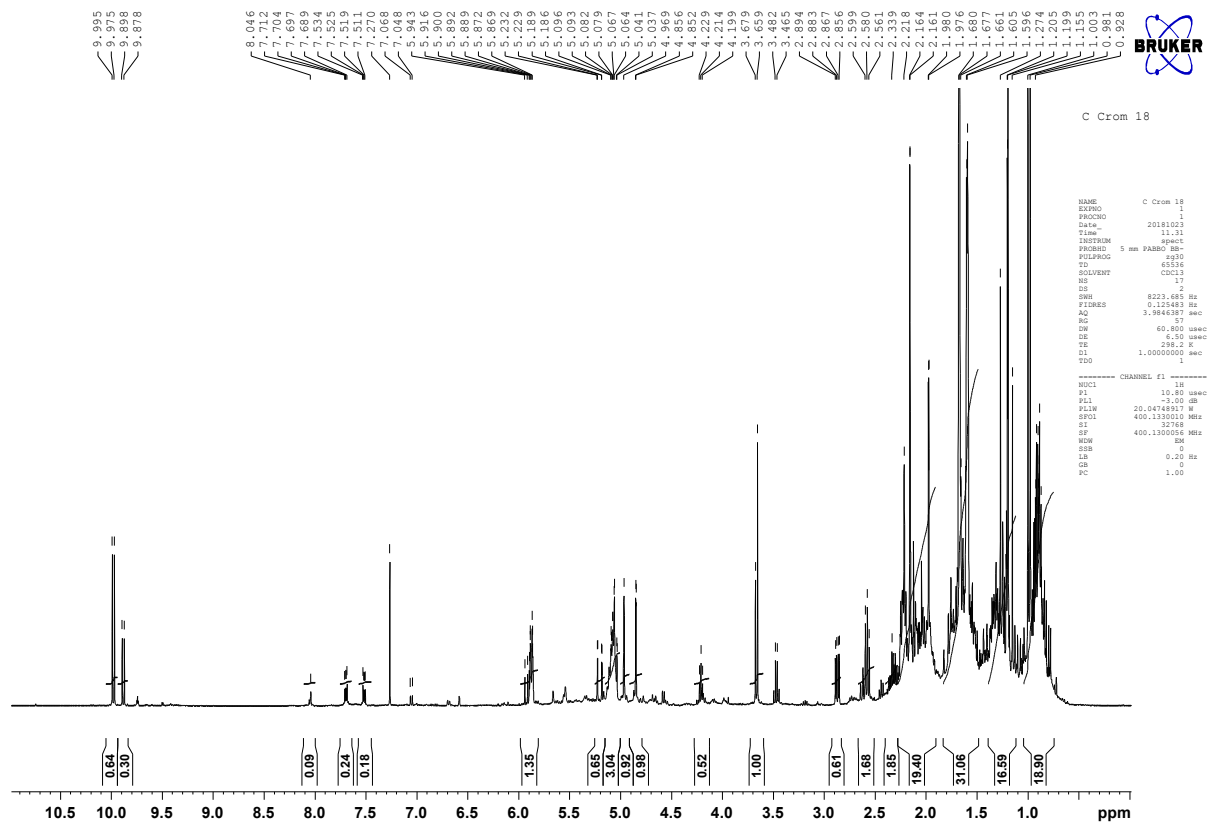


Figure A.43 – ¹H NMR spectrum of the second fraction of lemon balm essential oil extracted in 50% petroleum ether - 50% ethyl acetate. 400 MHz, CDCl₃.

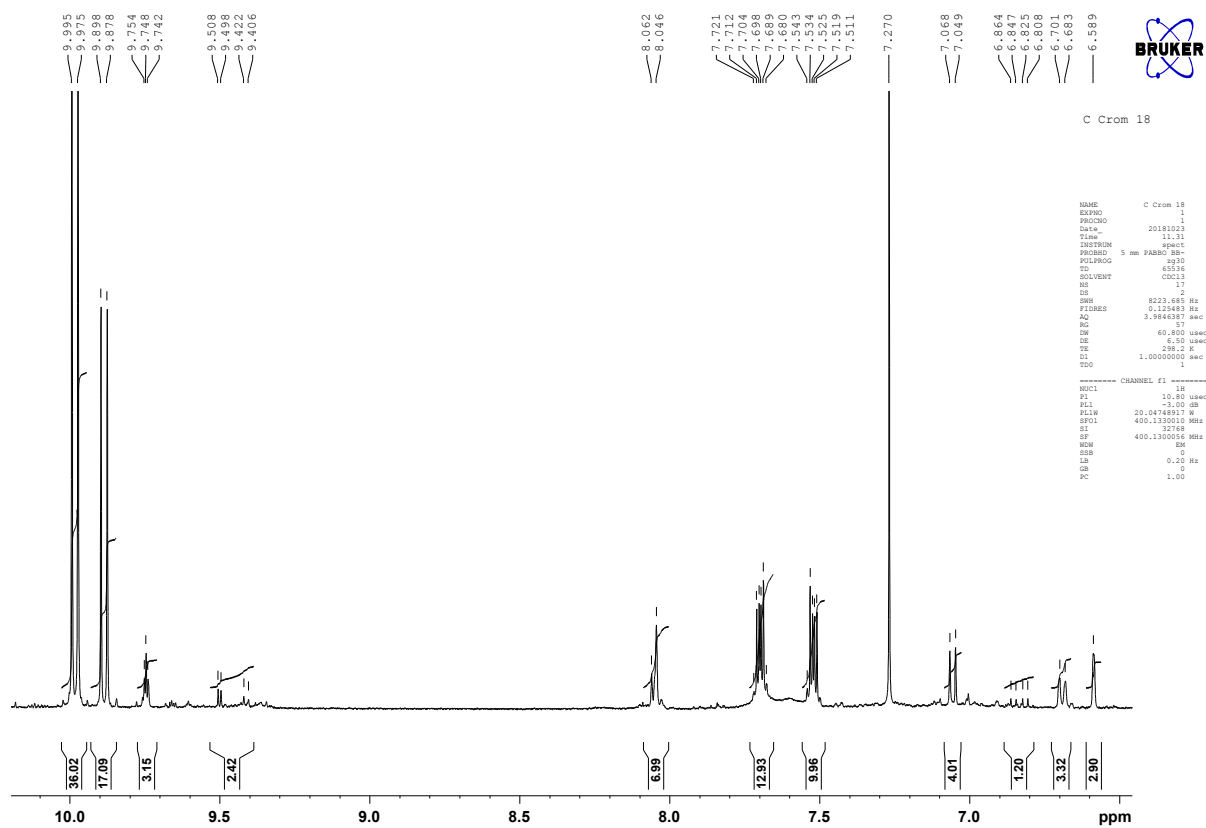


Figure A.43 (expansion 1) – ^1H NMR spectrum of the second fraction of lemon balm essential oil extracted in 50% petroleum ether - 50% ethyl acetate. 400 MHz, CDCl_3 .

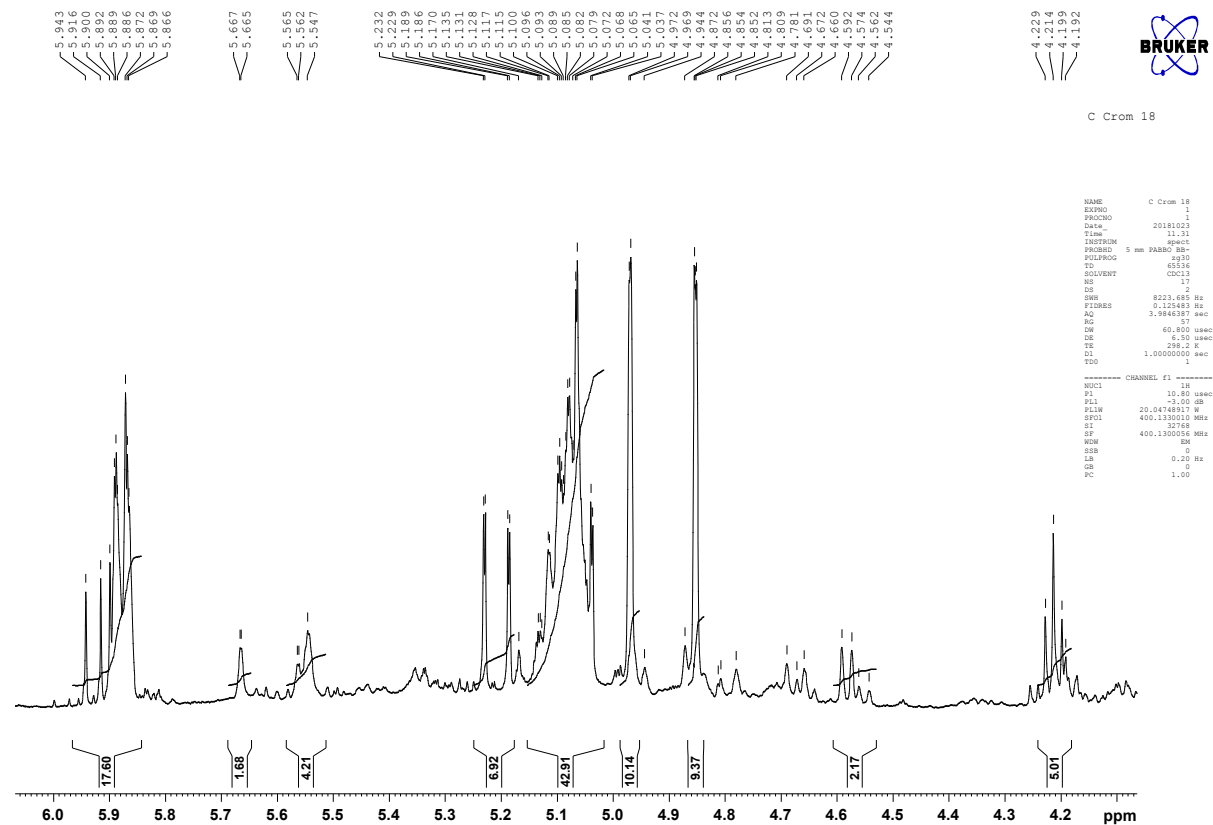


Figure A.43 (expansion 2) – ^1H NMR spectrum of the second fraction of lemon balm essential oil extracted in 50% petroleum ether - 50% ethyl acetate. 400 MHz, CDCl_3 .

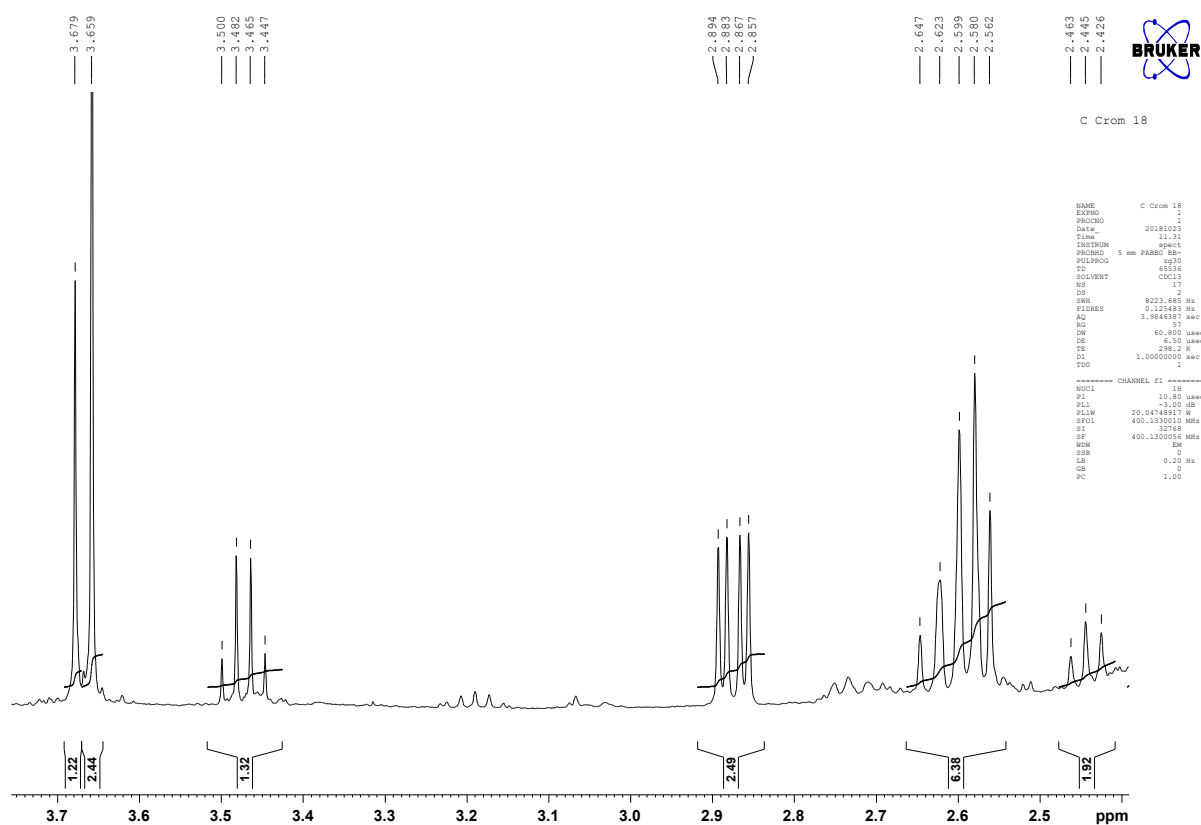


Figure A.43 (expansion 3) – ^1H NMR spectrum of the second fraction of lemon balm essential oil extracted in 50% petroleum ether - 50% ethyl acetate. 400 MHz, CDCl_3 .

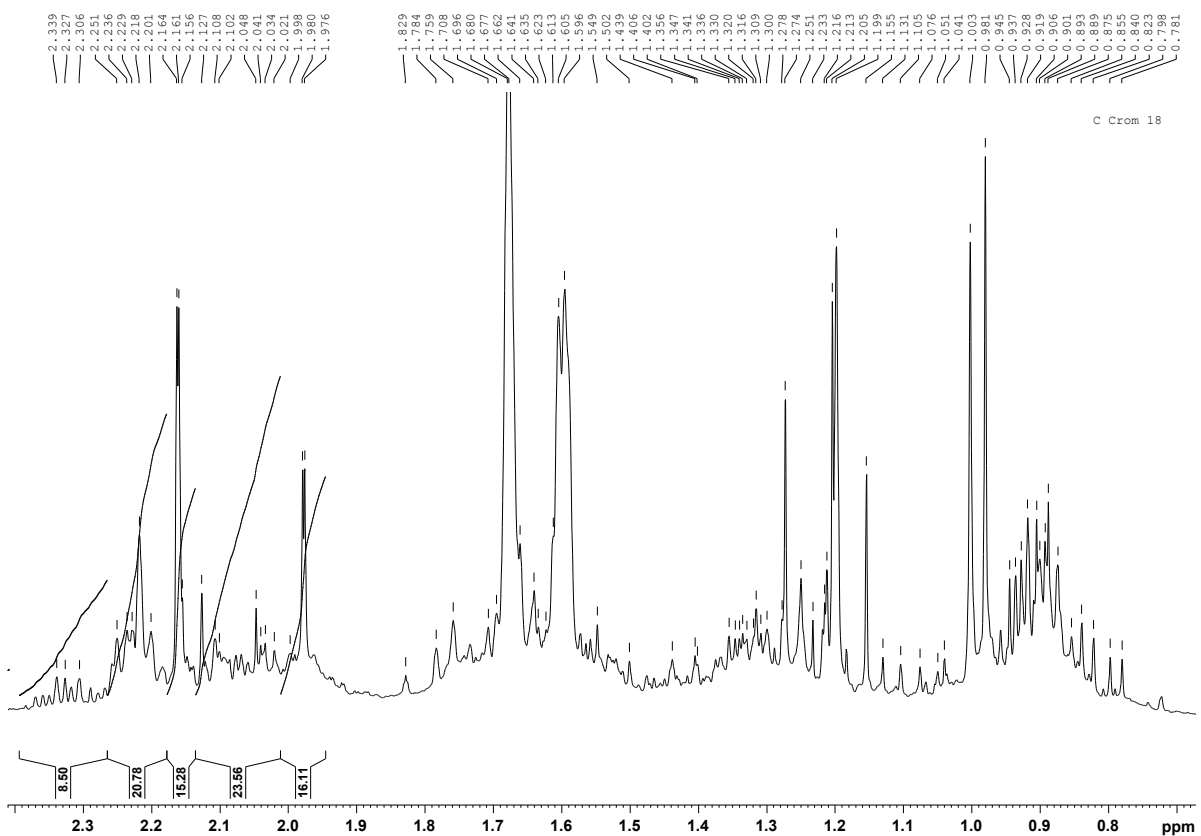


Figure A.43 (expansion 4) – ^1H NMR spectrum of the second fraction of lemon balm essential oil extracted in 50% petroleum ether - 50% ethyl acetate. 400 MHz, CDCl_3 .

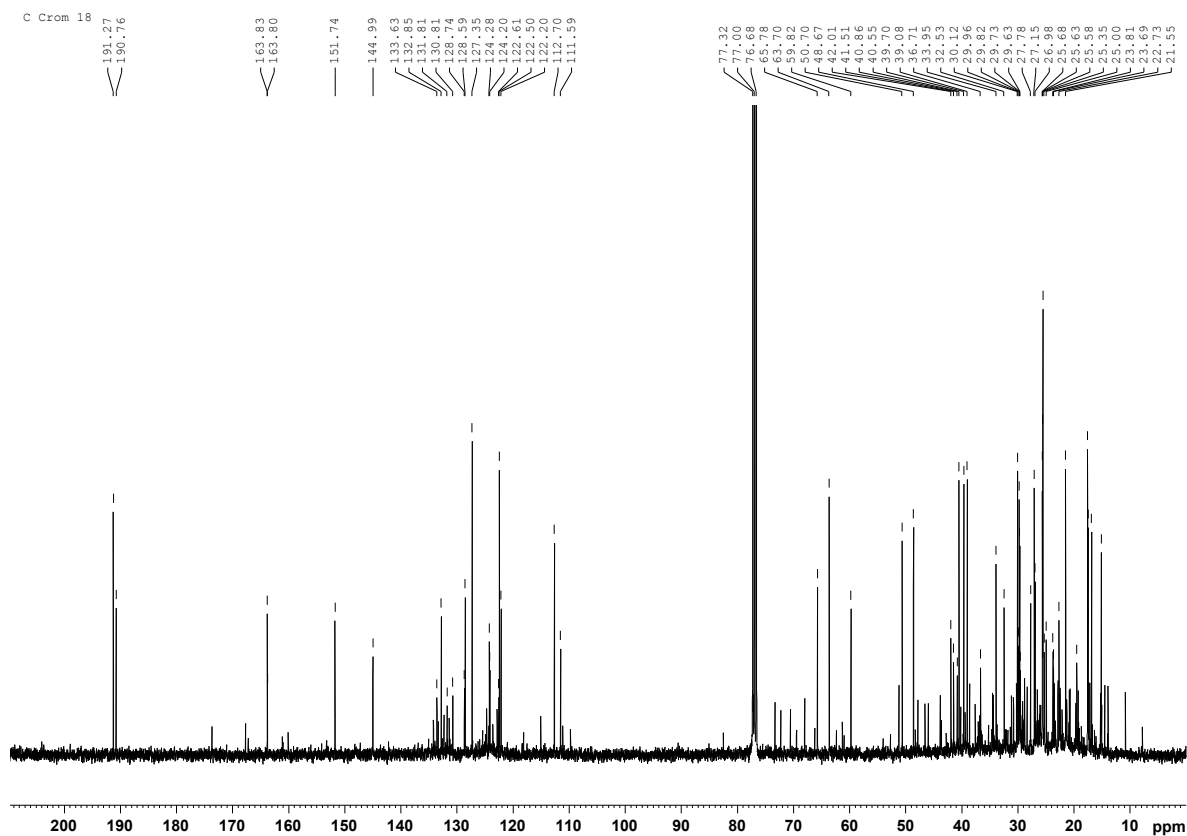


Figure A.44 – ^{13}C NMR spectrum of the second fraction of lemon balm essential oil extracted in 50% petroleum ether - 50% ethyl acetate. 100 MHz, CDCl_3 .

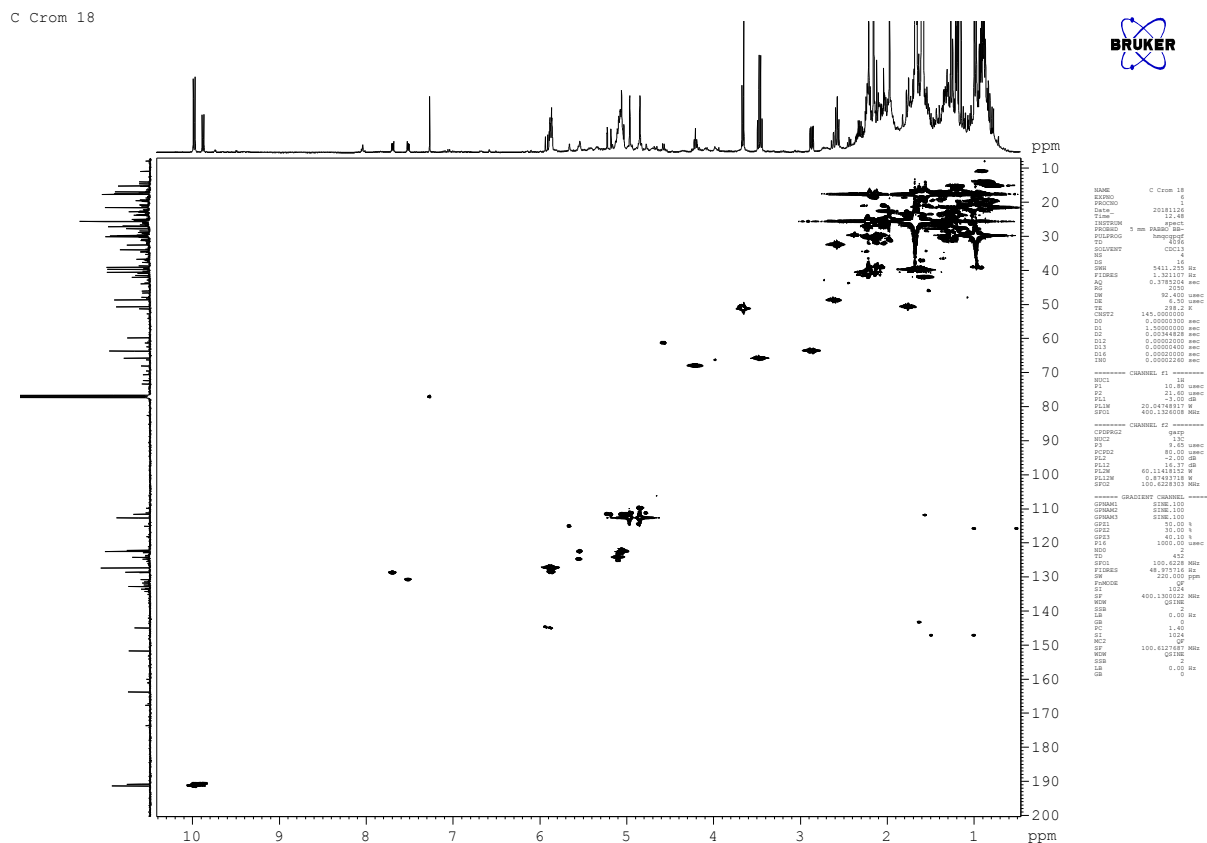
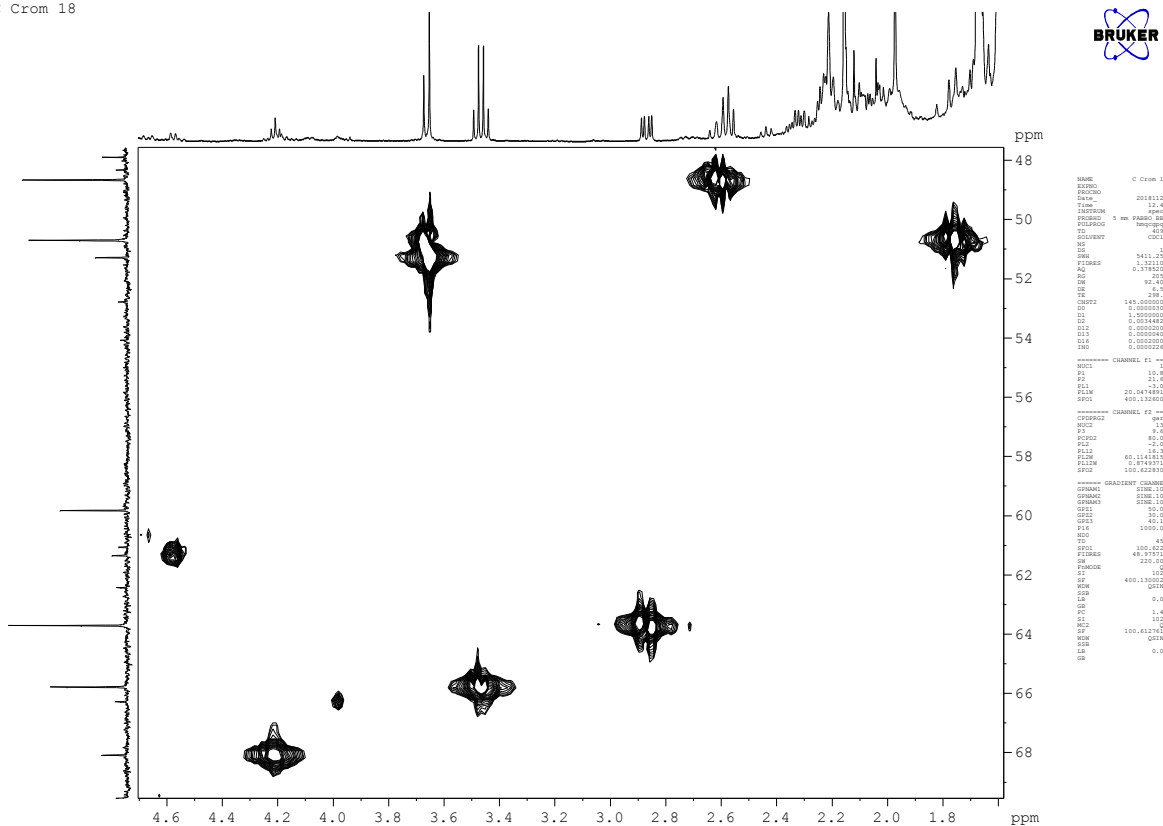
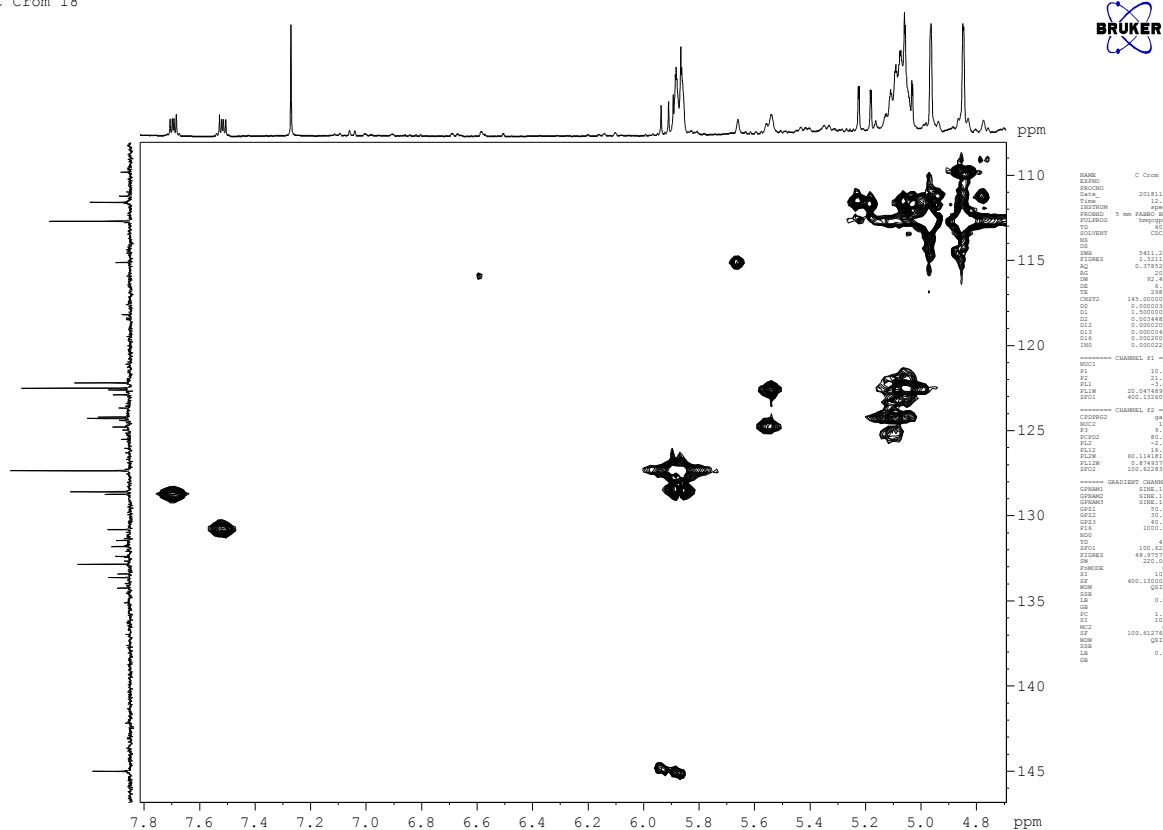


Figure A.45 – HMQC spectrum of the second fraction of lemon balm essential oil extracted in 50% petroleum ether - 50% ethyl acetate. ^1H - 400 MHz, ^{13}C - 100 MHz, CDCl_3 .



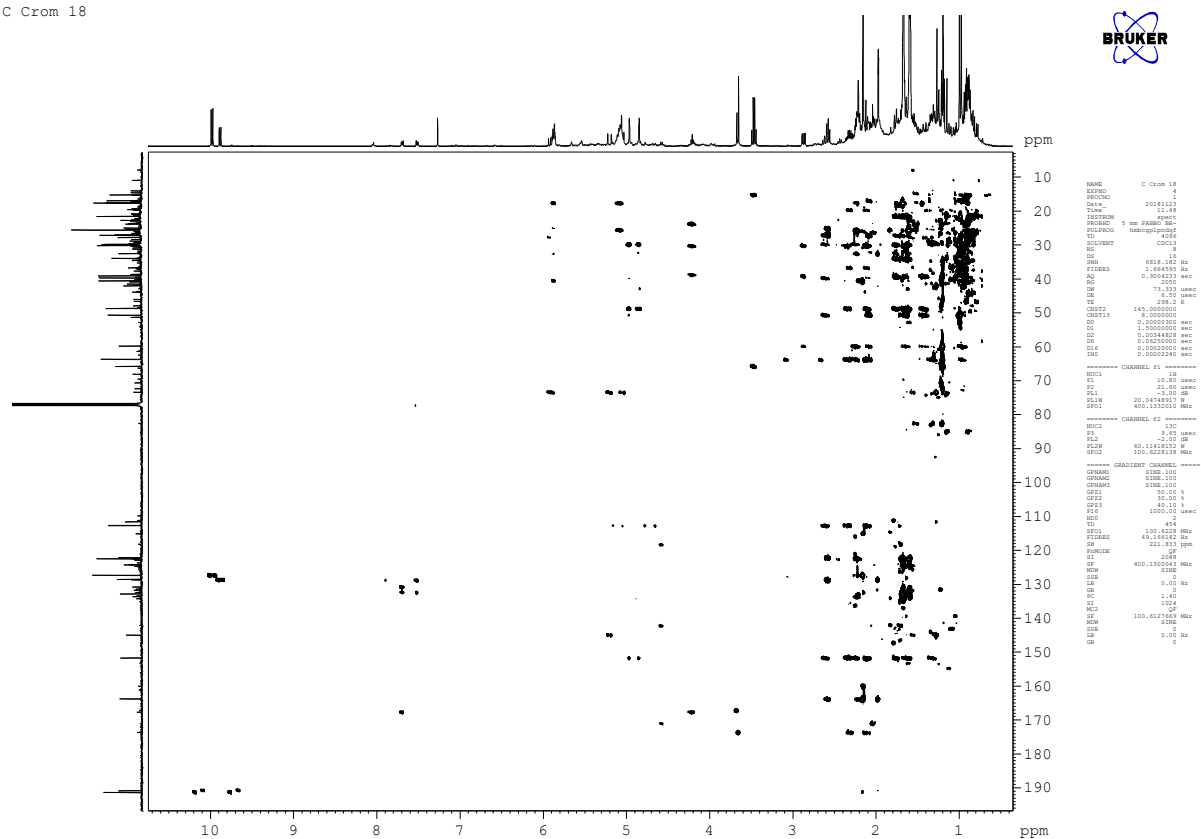


Figure A.46 – HMBC spectrum of the second fraction of lemon balm essential oil extracted in 50% petroleum ether - 50% ethyl acetate. ^1H - 400 MHz, ^{13}C - 100 MHz, CDCl_3 .

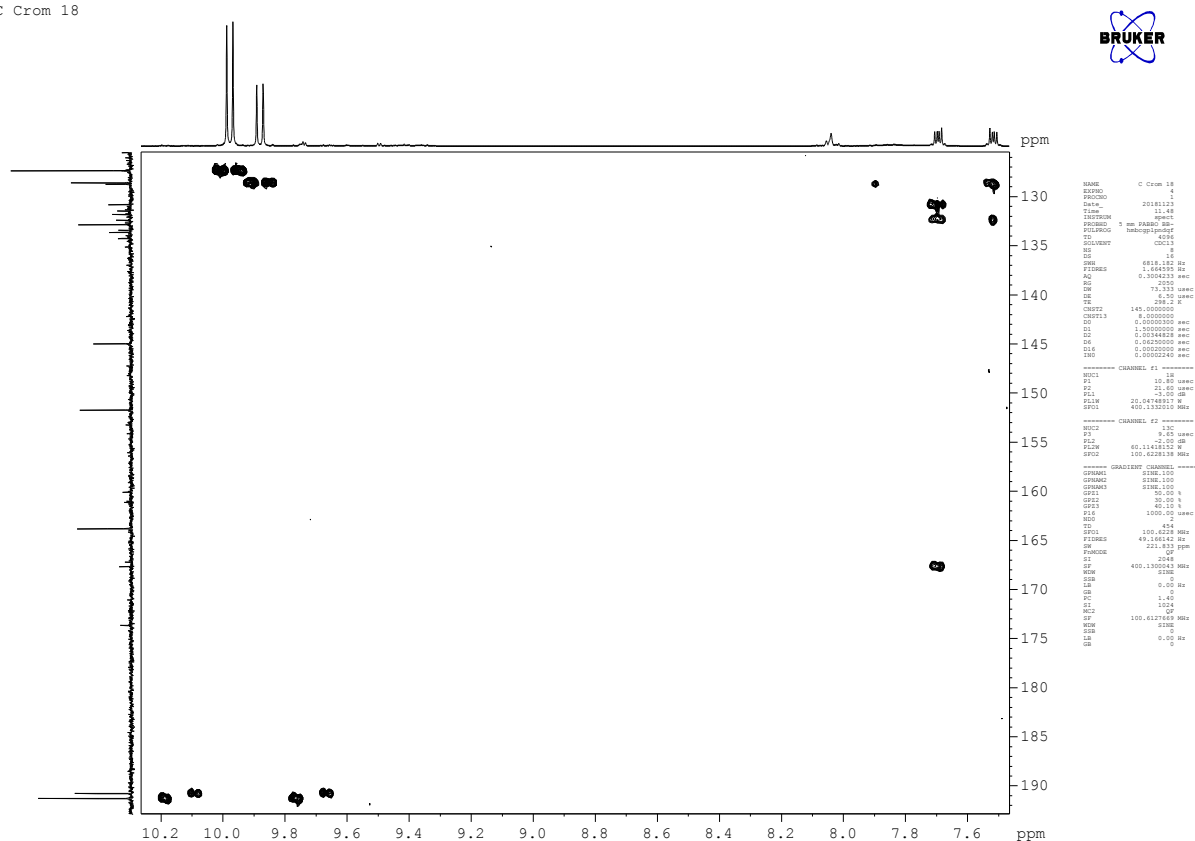
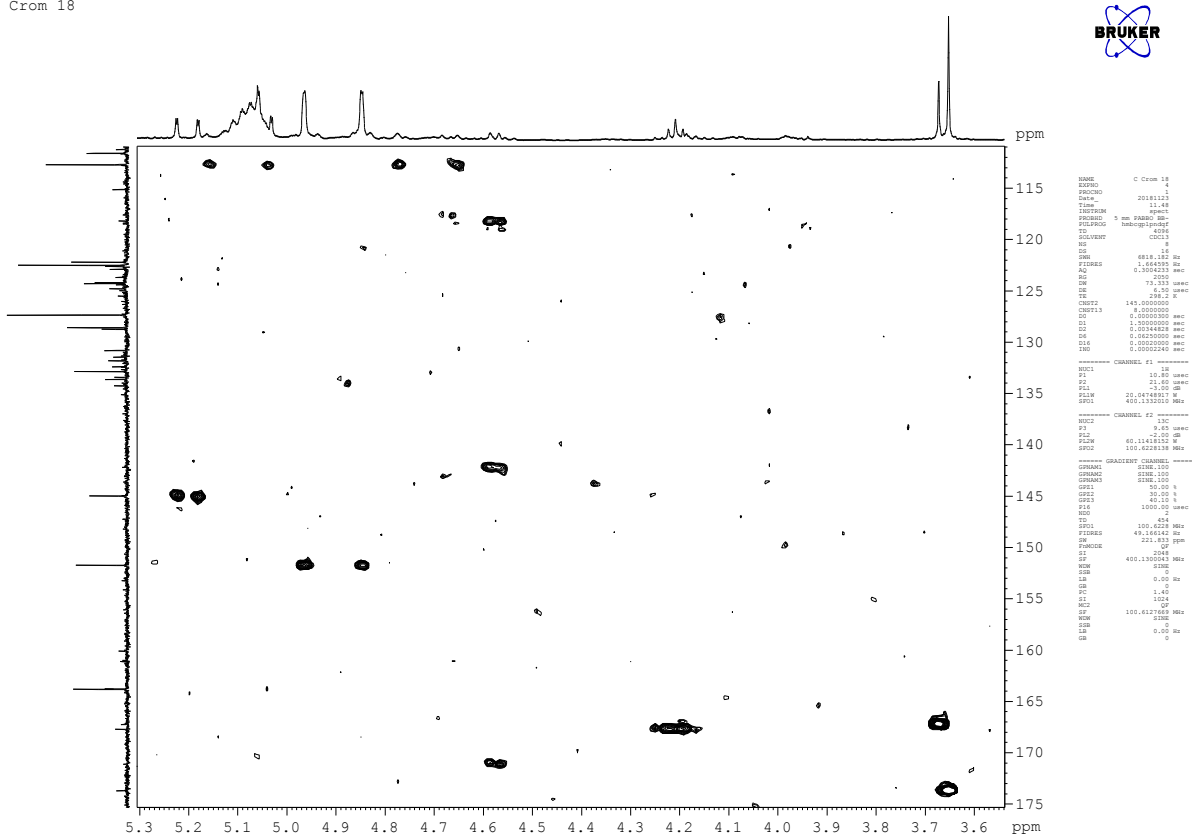
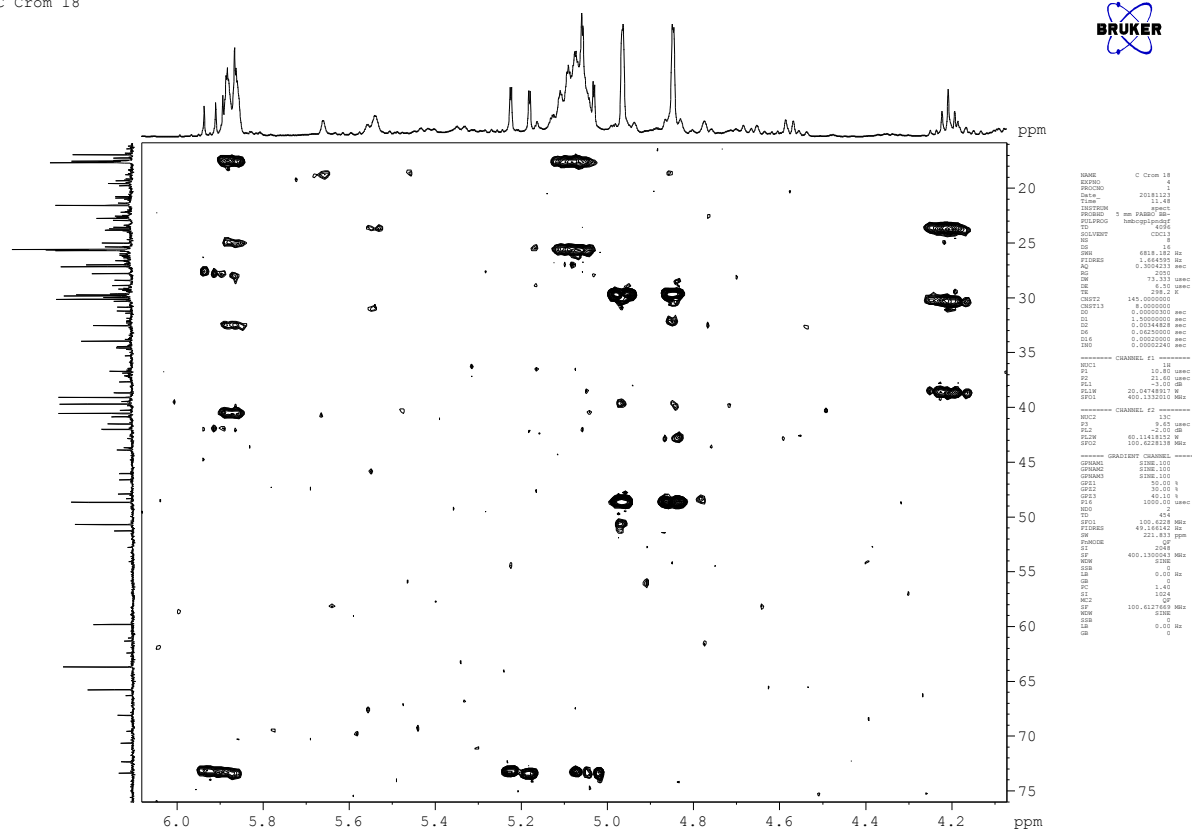
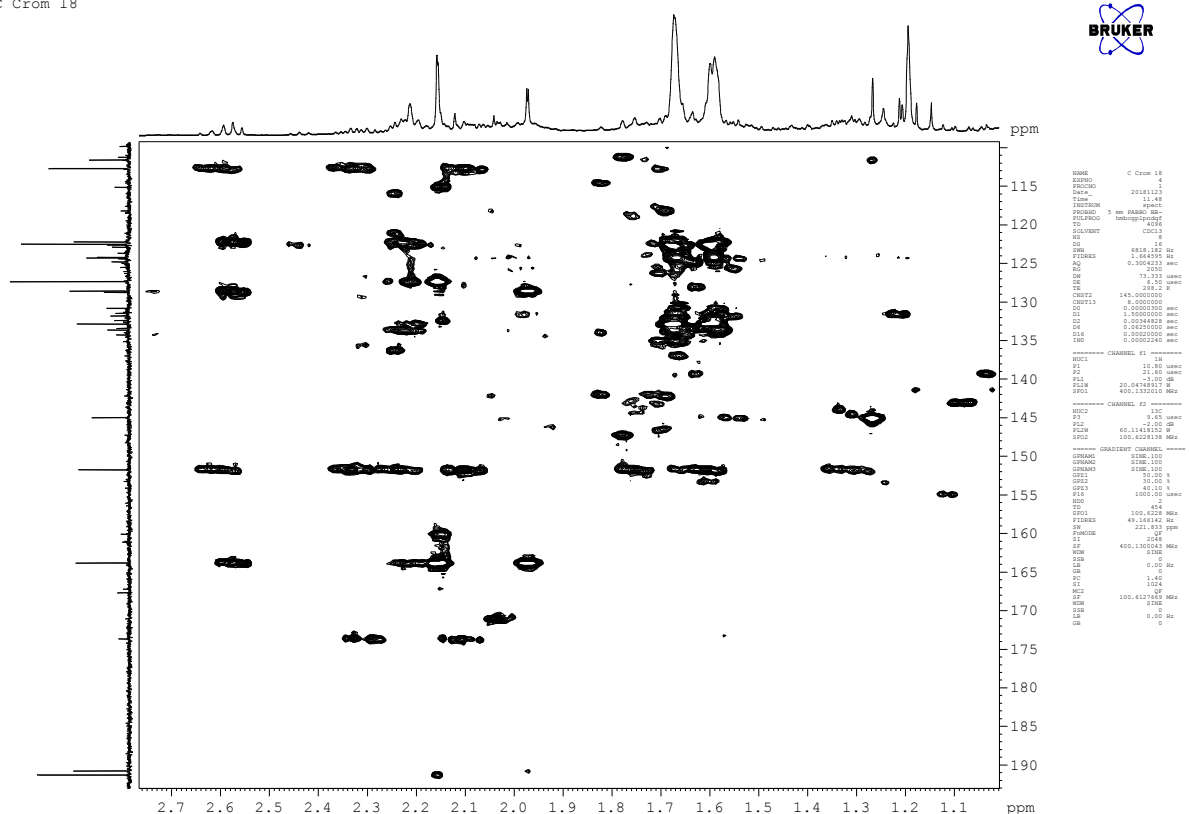
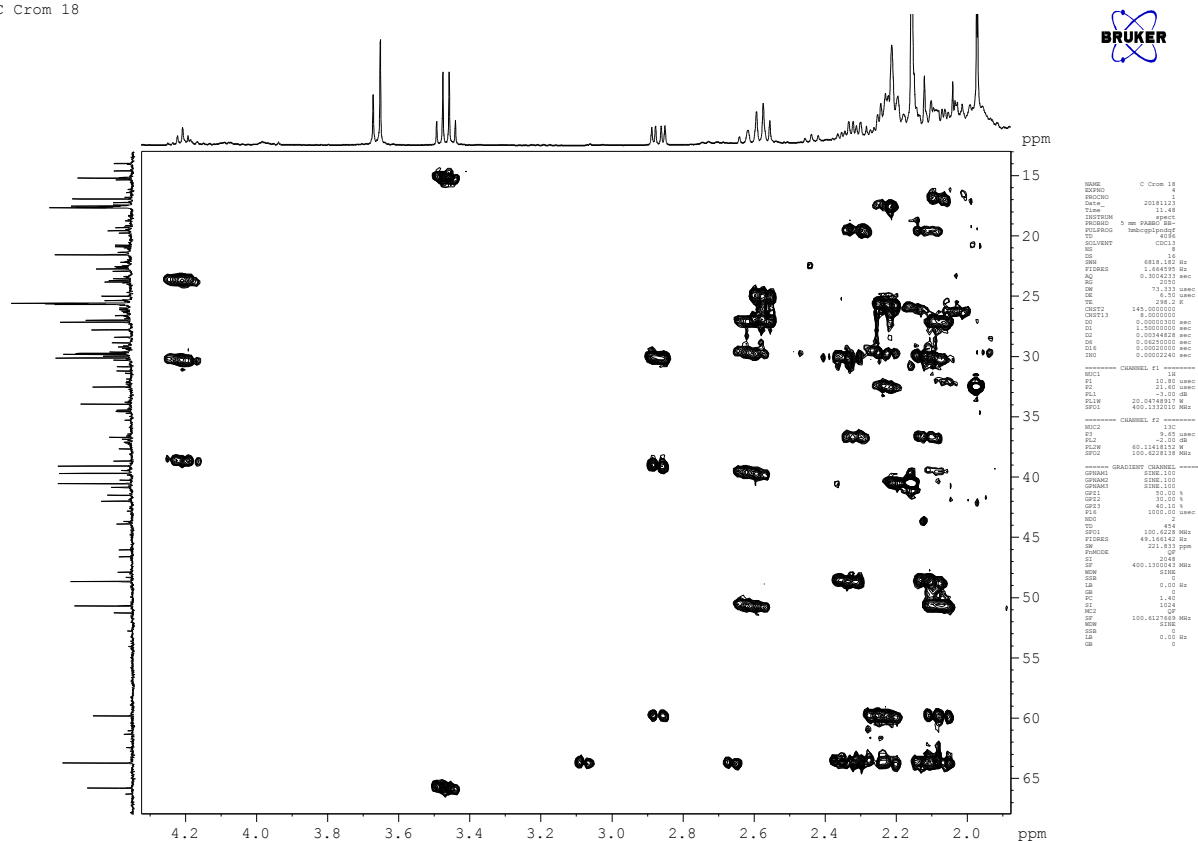


Figure A.46 (expansion 1) – HMBC spectrum of the second fraction of lemon balm essential oil extracted in 50% petroleum ether - 50% ethyl acetate. ^1H - 400 MHz, ^{13}C - 100 MHz, CDCl_3 .





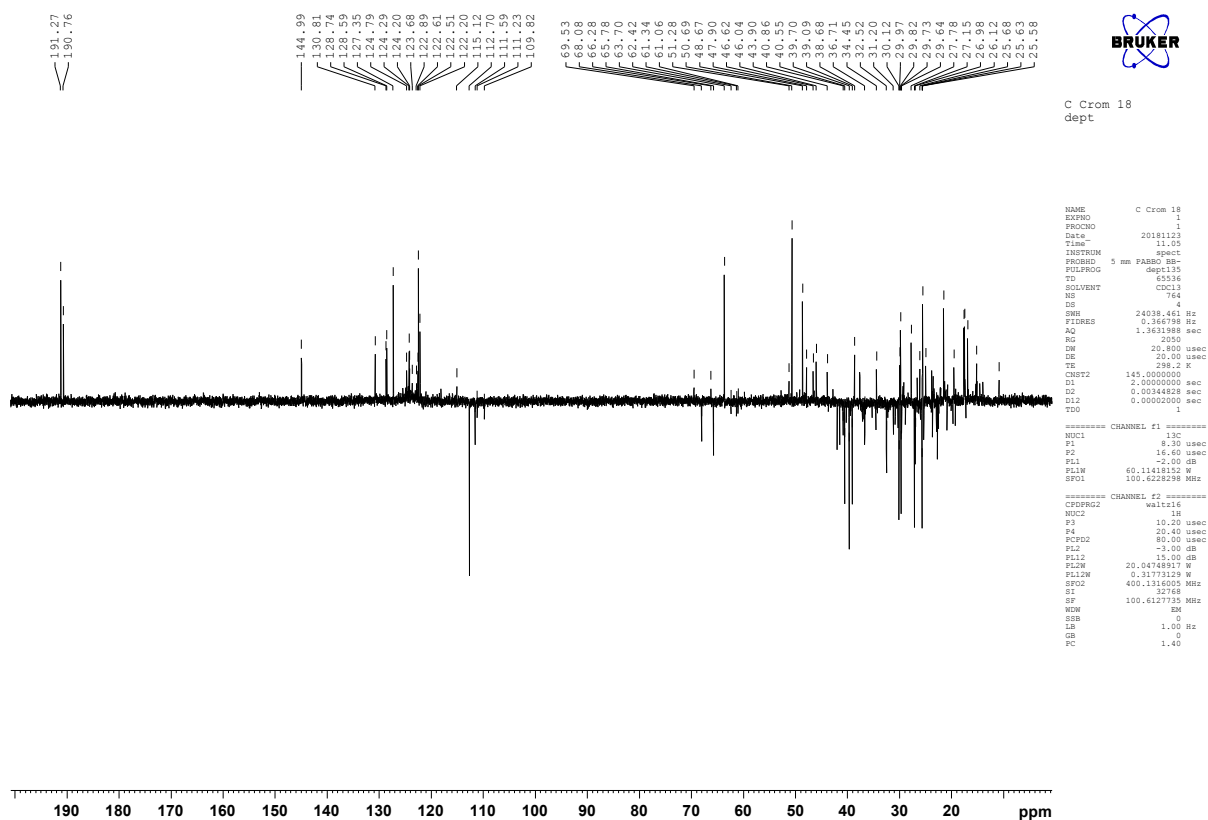


Figure A.47 – DEPT spectrum of the second fraction of lemon balm essential oil extracted in 50% petroleum ether - 50% ethyl acetate. 100 MHz, CDCl₃.

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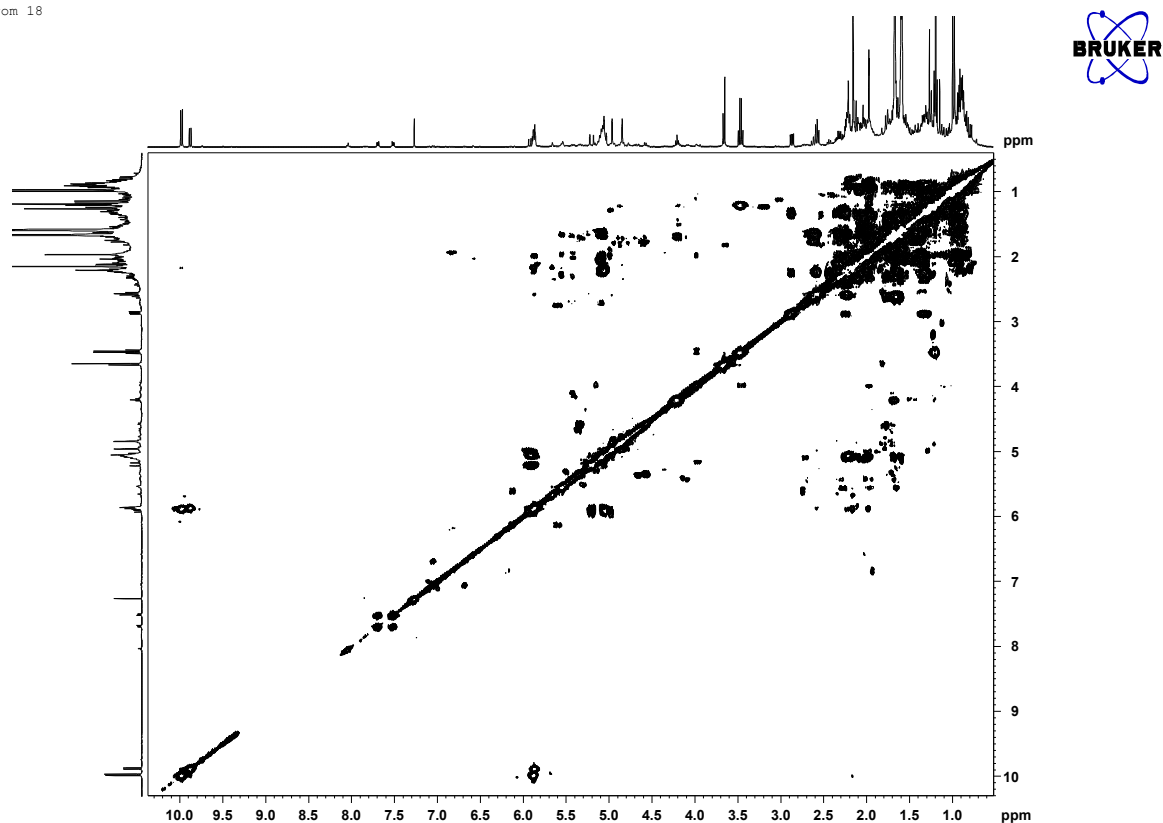


Figure A.48 – COZY spectrum of the second fraction of lemon balm essential oil extracted in 50% petroleum ether - 50% ethyl acetate. 400 MHz, CDCl₃.

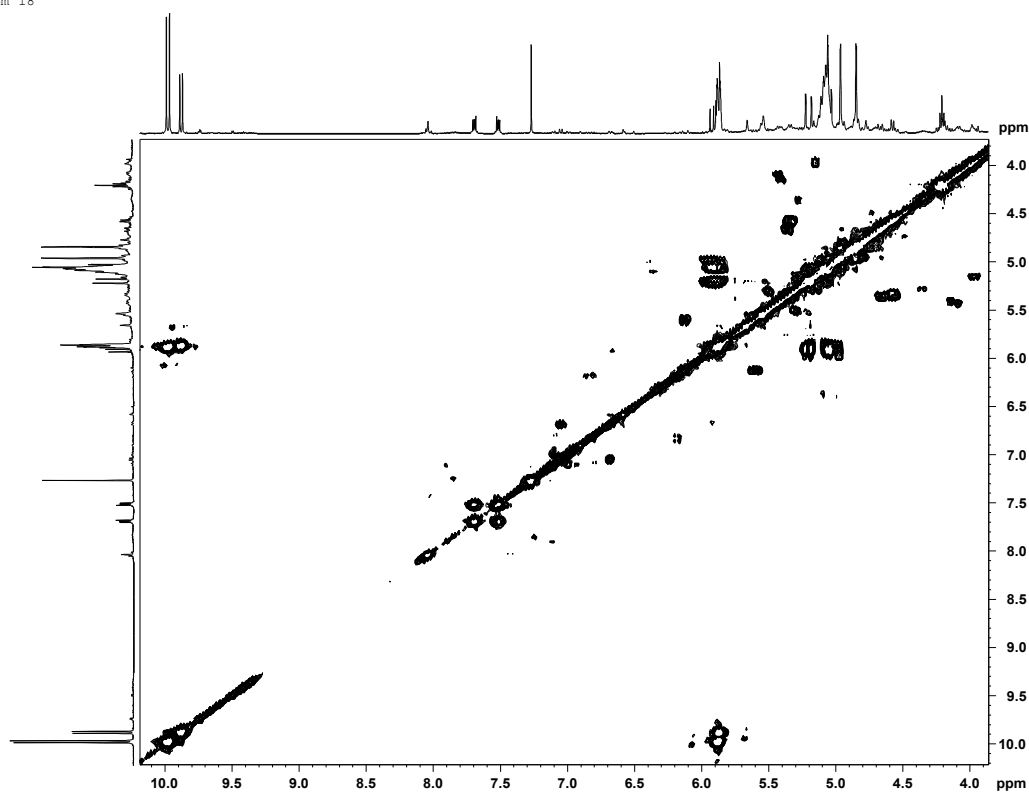


Figure A.48 (expansion) – COZY spectrum of the second fraction of lemon balm essential oil extracted in 50% petroleum ether - 50% ethyl acetate. 400 MHz, CDCl_3 .

Group B – NMR spectra used in the quantitative characterization of essential oils

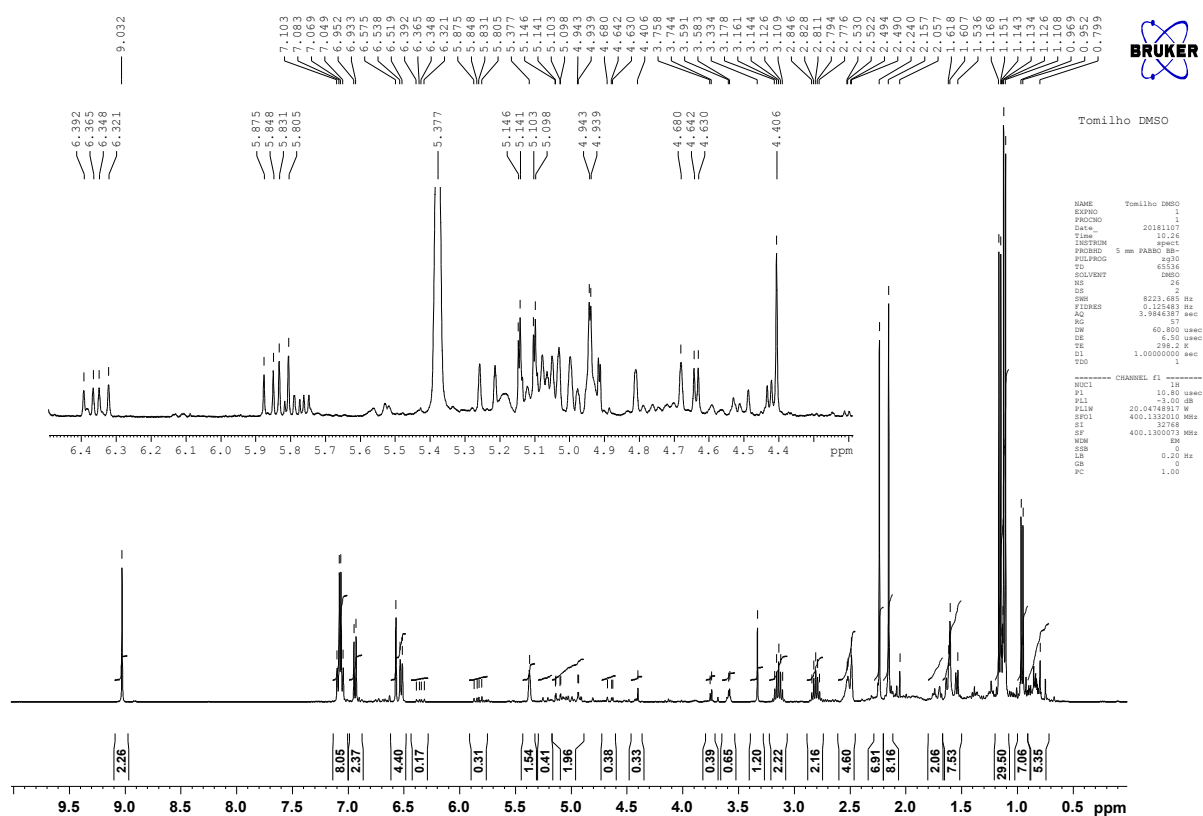


Figure B.1 – ^1H NMR spectrum of thyme essential oil. 400 MHz, $\text{DMSO}-d_6$.

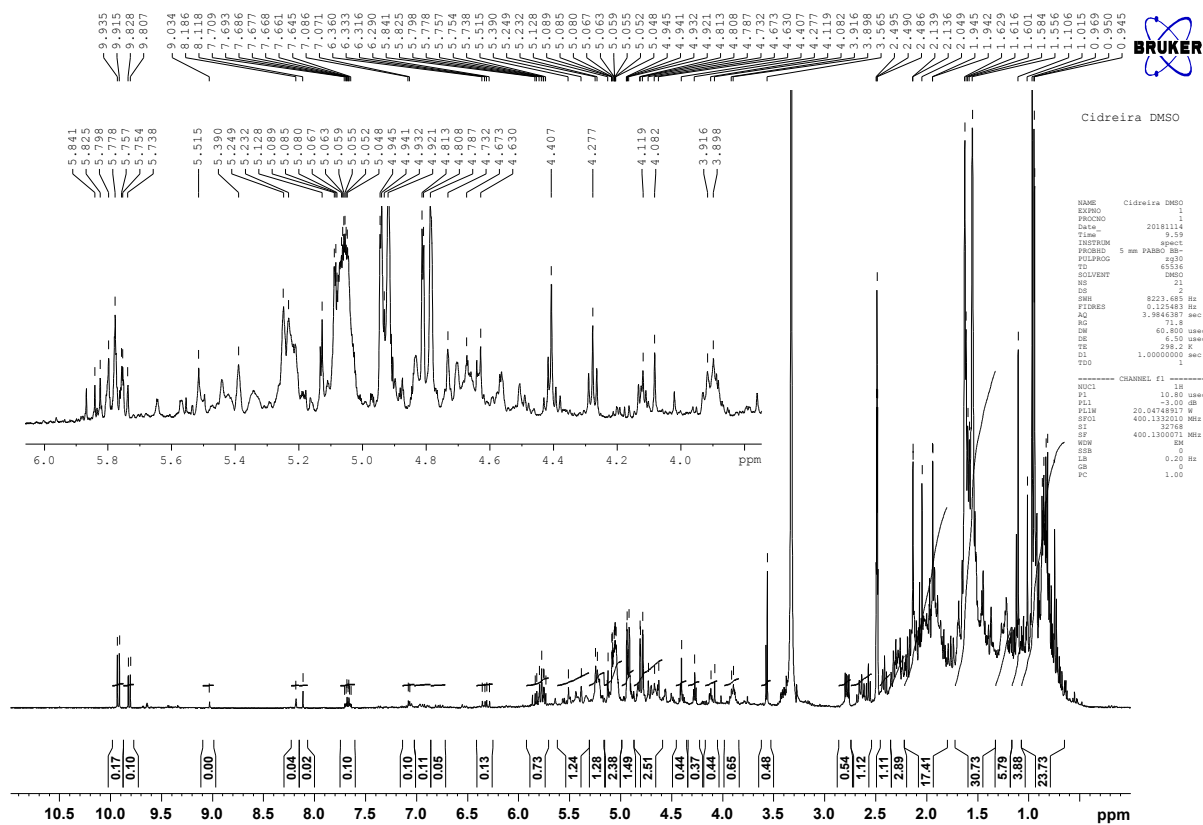


Figure B.2 – ^1H NMR spectrum of lemon balm essential oil. 400 MHz, $\text{DMSO}-d_6$.

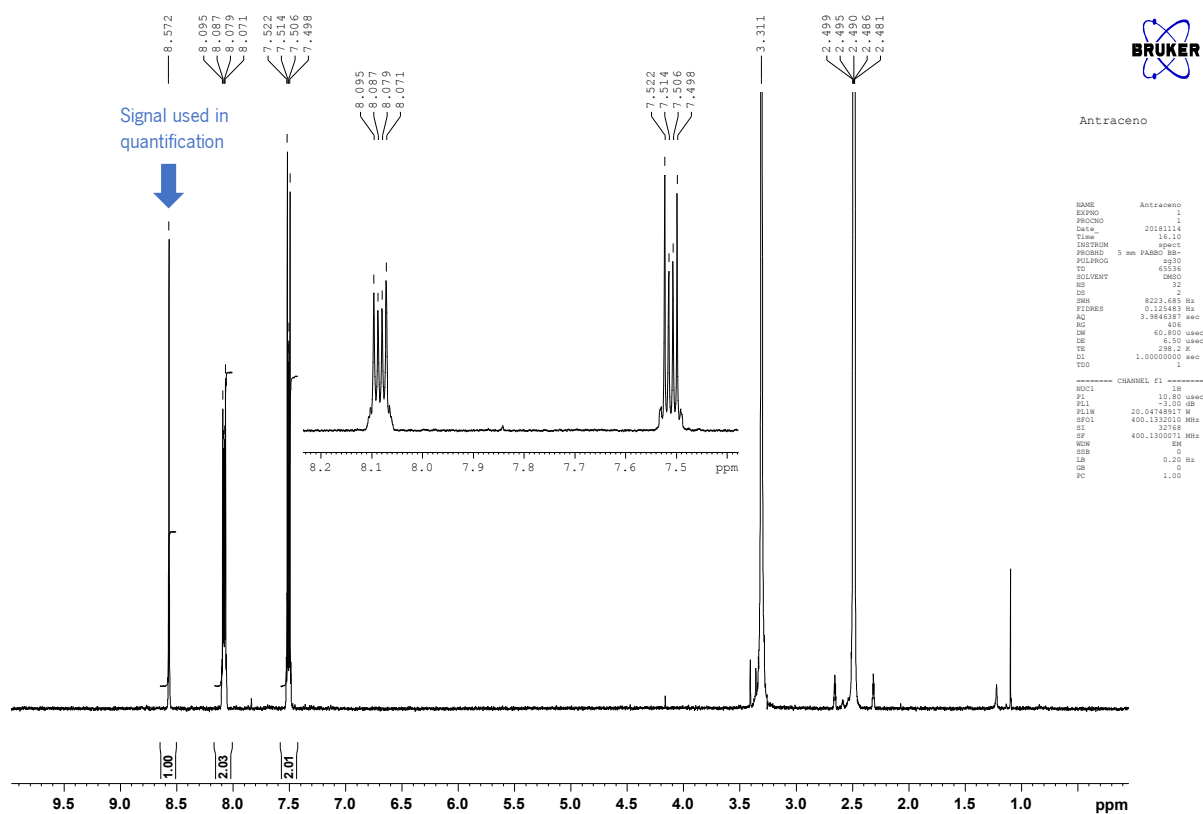


Figure B.3 – ^1H NMR spectrum of anthracene. 400 MHz, $\text{DMSO}-d_6$.

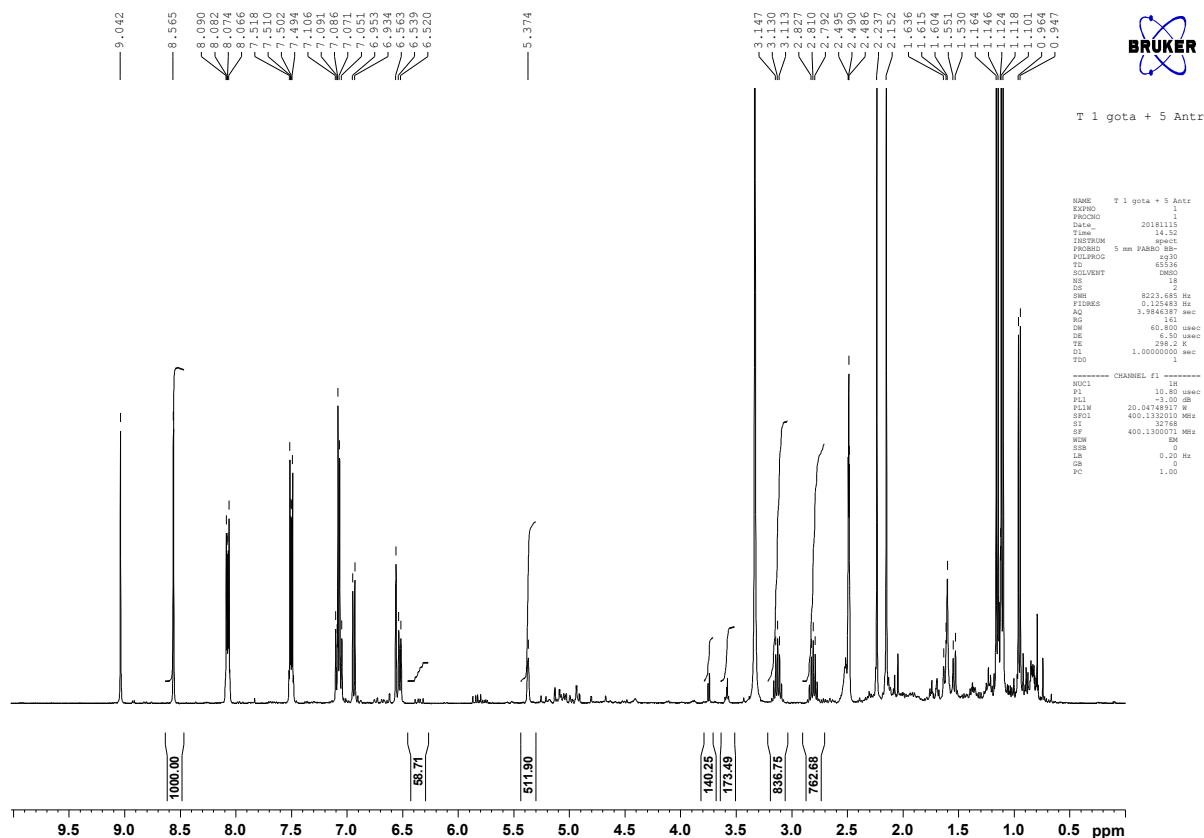


Figure B.4 – ^1H NMR spectrum of thyme essential oil and anthracene – example of 1 replicate. 400 MHz, $\text{DMSO}-d_6$.

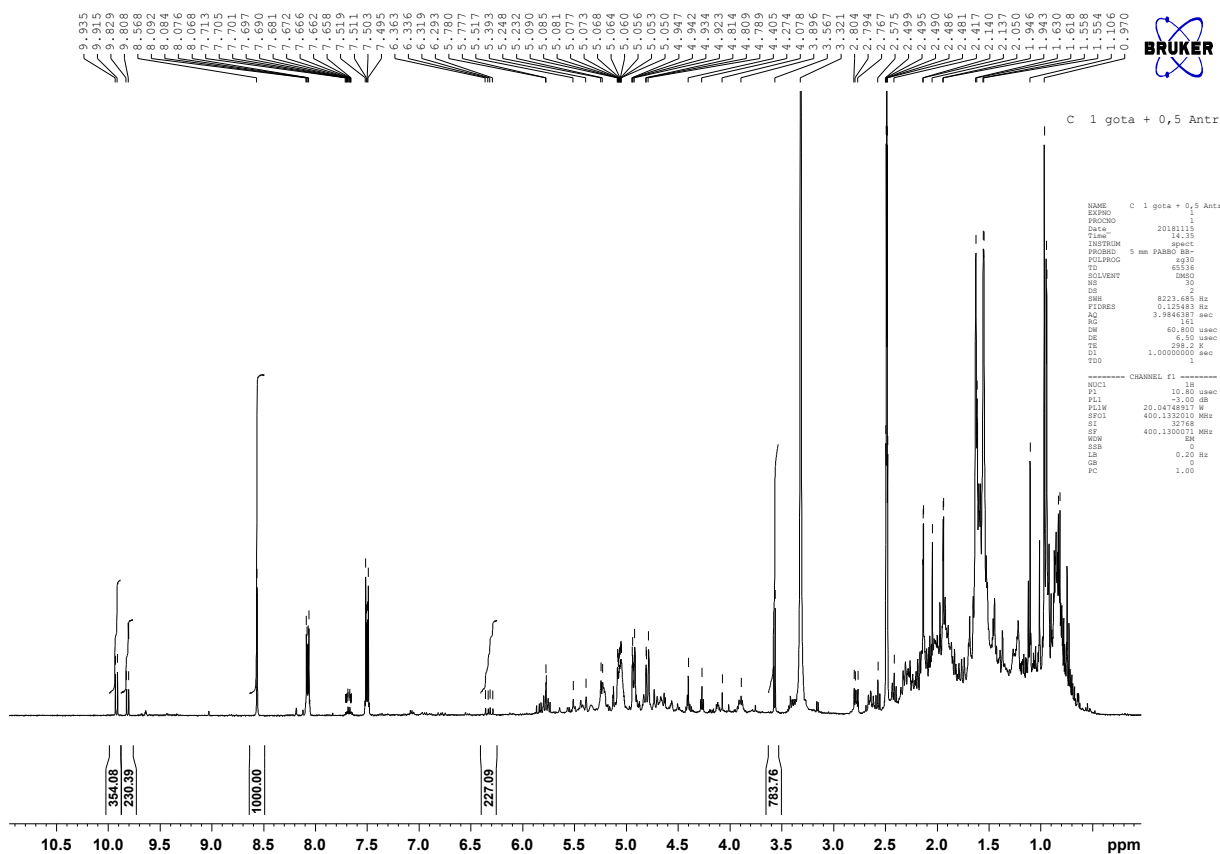


Figure B.5 – ^1H NMR spectrum of lemon balm essential oil and anthracene – example of 1 replicate. 400 MHz, $\text{DMSO}-d_6$.

Group C – GC-MS spectra of essential oils

Thyme essential oil

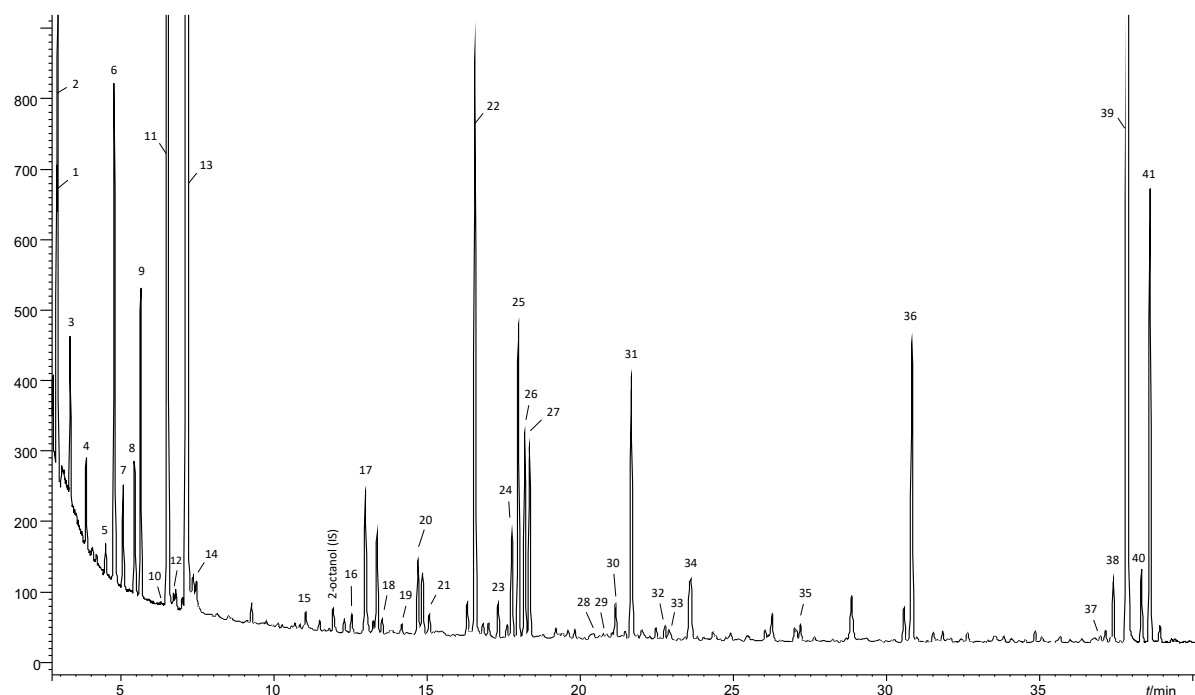


Figure C.1 – GC spectrum of thyme essential oil and 2-octanol as internal standard – example of 1 replicate. Sapiens-WaxMS column.

List of identified compounds in thyme essential oil: (1) α -pinene; (2) α -thujene; (3) camphene; (4) β -pinene; (5) δ -3-carene; (6) myrcene; (7) α -terpinene; (8) limonene; (9) 1,8-cineole; (10) cis-ocimene; (11) γ -terpinene; (12) trans-ocimene; (13) *p*-cymene; (14) terpinolene; (15) 3-octanol; (16) furan linalool oxide I; (17) 1-octen-3-ol; (18) furan linalool oxide II; (19) α -copaene; (20) camphor; (21) β -bourbonene; (22) linalool; (23) bornyl acetate; (24) β -caryophyllene; (25) thymol methyl ether; (26) terpinen-4-ol; (27) carvacrol methyl ether; (28) α -humulene; (29) neral; (30) γ -muurolene; (31) borneol; (32) β -bisabolene; (33) geranial; (34) δ -cadinene; (35) geraniol; (36) caryophyllene oxide; (37) eugenol; (38) thymol derivative; (39) thymol; (40) carvacrol derivative; (41) carvacrol.

Lemon balm essential oil

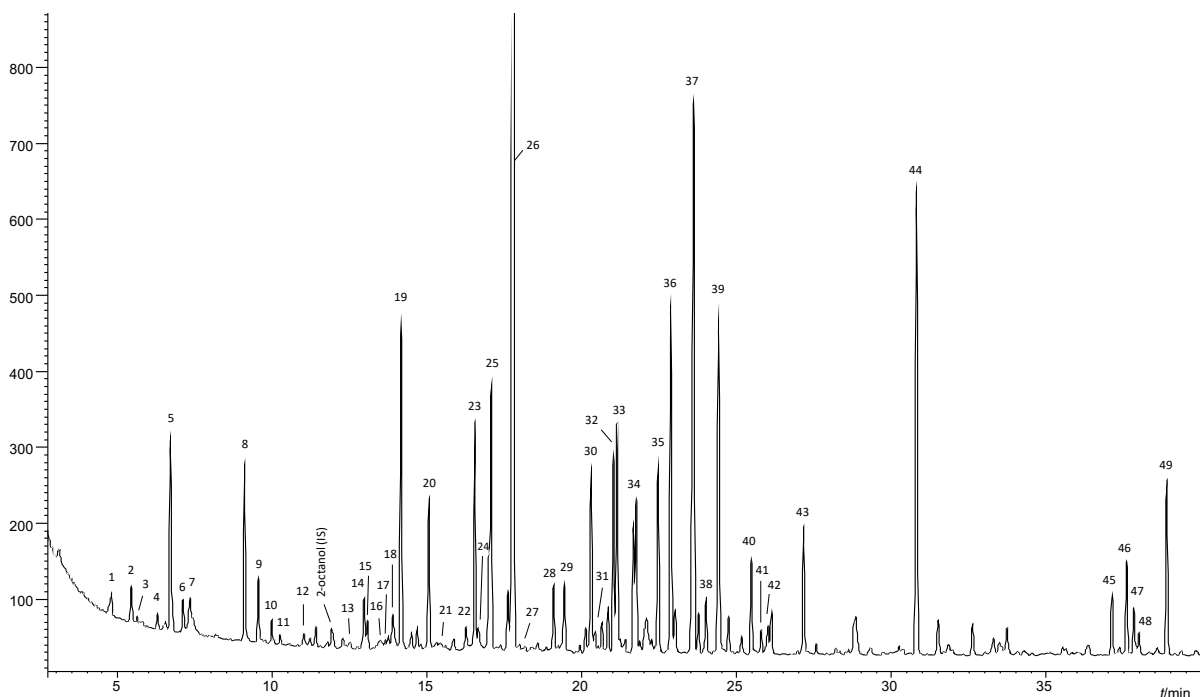


Figure C.2 – GC spectrum of lemon balm essential oil and 2-octanol as internal standard – example of 1 replicate. Sapiens-WaxMS column.

List of identified compounds in lemon balm essential oil: (1) myrcene; (2) limonene; (3) 1,8-cineole; (4) cis-ocimene; (5) trans-ocimene; (6) *p*-cymene; (7) terpinolene; (8) 6-methyl-5-hepten-2-one; (9) rose oxide I; (10) rose oxide II; (11) allocymene I; (12) 3-octanol; (13) furan linalool oxide I; (14) 1-octen-3-ol; (15) α -cubebene; (16) 6-methyl-5-hepten-2-ol; (17) furan linalool oxide II; (18) citronellal; (19) α -copaene; (20) β -bourbonene; (21) vitispirane; (22) α -ionene; (23) linalool; (24) isopulegol I; (25) methyl citronellate; (26) β -caryophyllene; (27) terpinen-4-ol; (28) citronellyl formate; (29) allo-aromadendrene; (30) α -humulene; (31) citronellyl acetate; (32) neral; (33) α -amorphene/ γ -muurolene; (34) germacrene D; (35) α -muurolene; (36) geranial; (37) δ -cadinene; (38) geranyl acetate; (39) citronellol; (40) nerol; (41) β -damascenone; (42) *cis*/*trans*-calamenene; (43) geraniol; (44) caryophyllene oxide; (45) τ -cadinol; (46) τ - α -muurolol; (47) thymol; (48) δ -cadinol; (49) α -cadinol.

Group D – Zeta Potential of nanoemulsions in stability tests

Nanoemulsions with sunflower oil

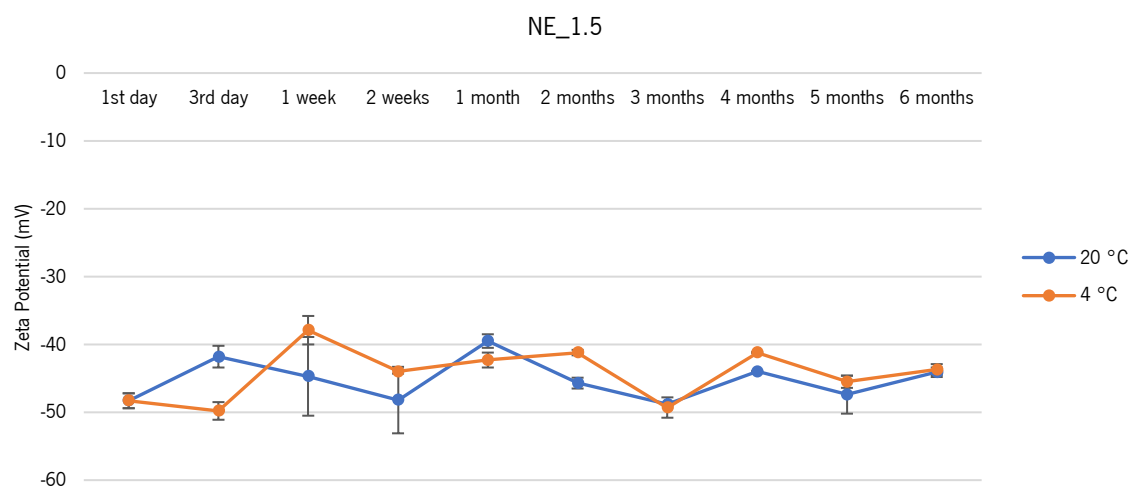


Figure D.1 – Variations of zeta potential of NE_1.5 (SOR 1.5, 10 wt% sunflower oil), for 6 months at two storage conditions, 20 °C and 4 °C.

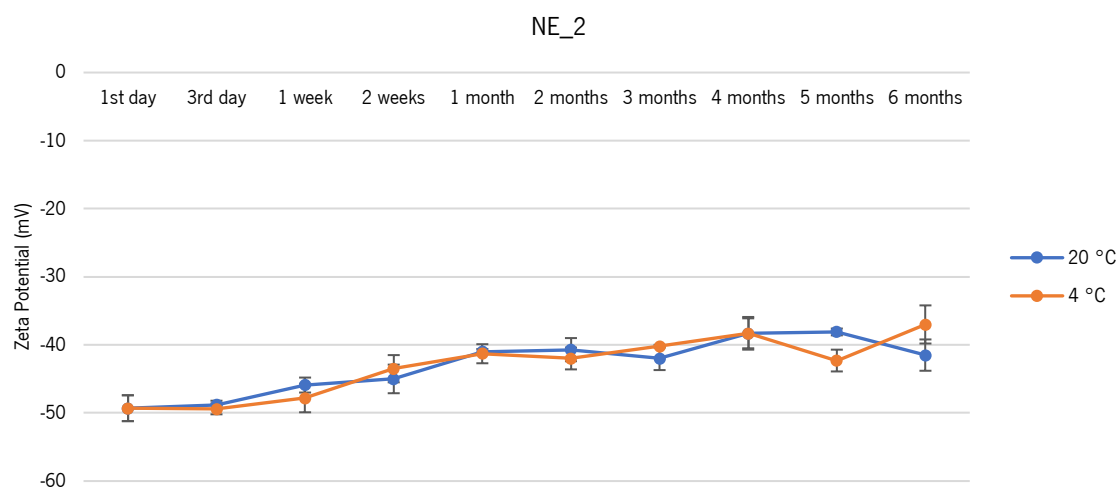


Figure D.2 – Variations of zeta potential of NE_2 (SOR 2, 10 wt% sunflower oil), for 6 months at two storage conditions, 20 °C and 4 °C.

Nanoemulsions with thyme essential oil

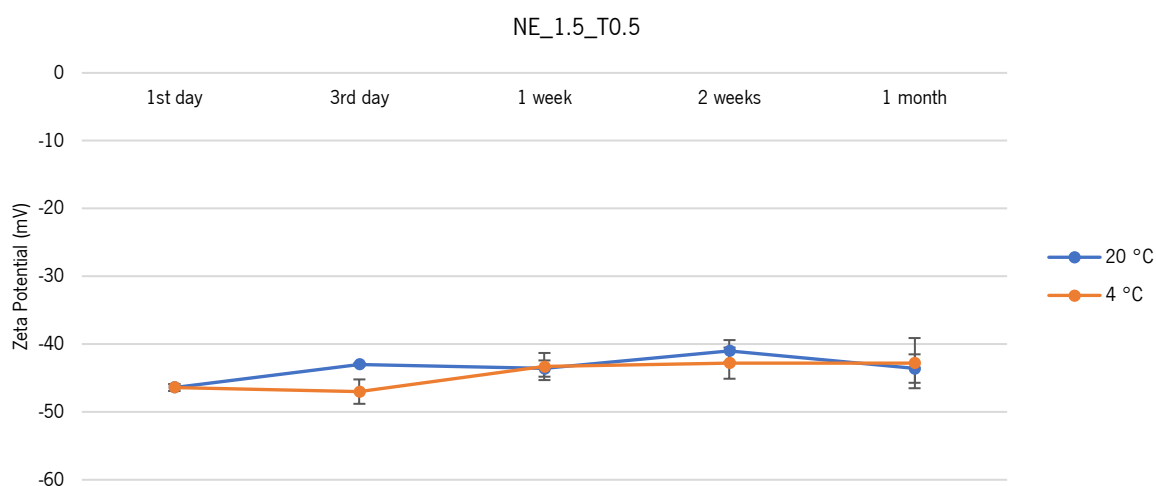


Figure D.3 – Variations of zeta potential of NE_1.5_T0.5 (SOR 1.5, 0.5 wt% of thyme essential oil and 9.5 wt% sunflower oil), for 1 month at two storage conditions, 20 °C and 4 °C.

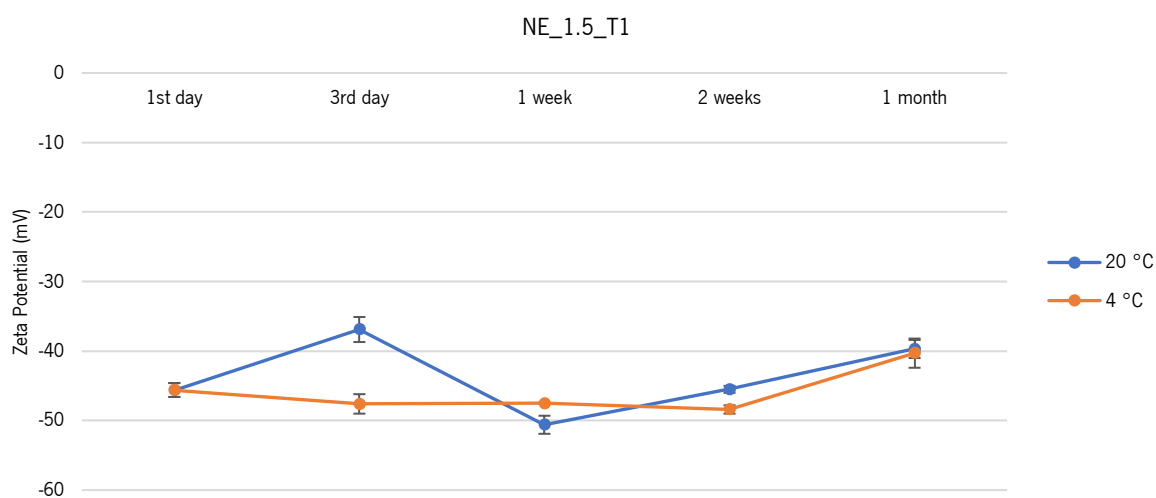


Figure D.4 – Variations of zeta potential of NE_1.5_T1 (SOR 1.5, 1 wt% of thyme essential oil and 9 wt% sunflower oil), for 1 month at two storage conditions, 20 °C and 4 °C.

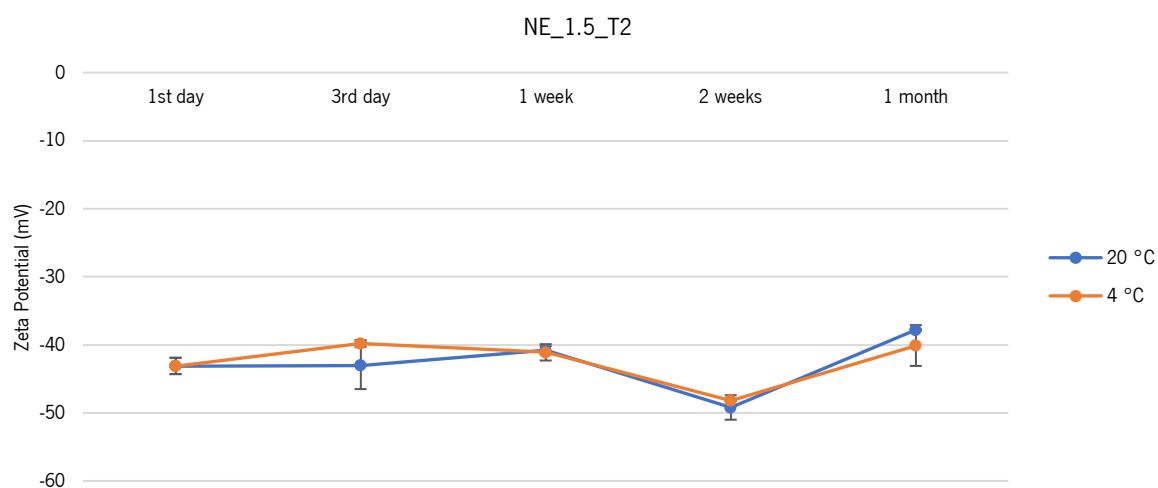


Figure D.5 – Variations of zeta potential of NE_1.5_T2 (SOR 1.5, 2 wt% of thyme essential oil and 8 wt% sunflower oil), for 1 month at two storage conditions, 20 °C and 4 °C.

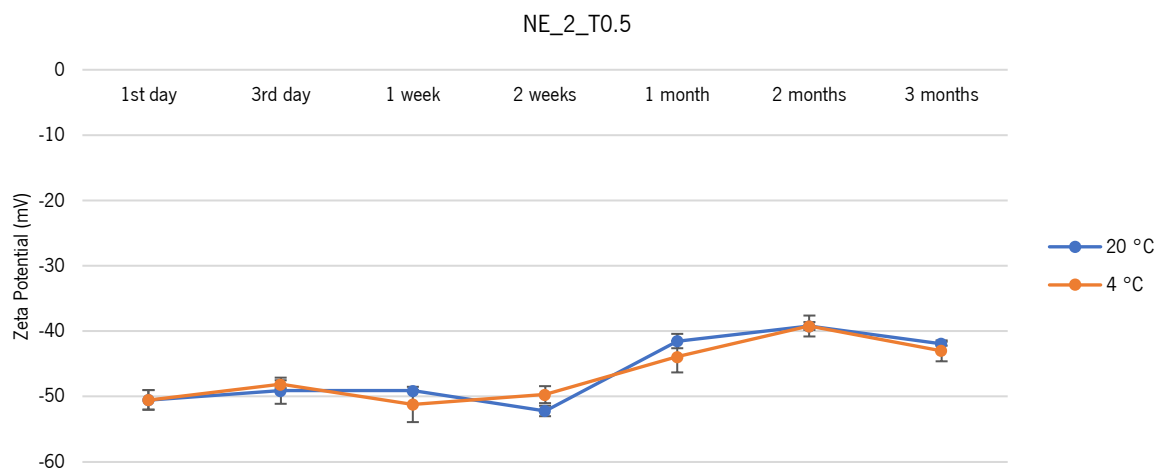


Figure D.6 – Variations of zeta potential of NE_2_T0.5 (SOR 2, 0.5 wt% of thyme essential oil and 9.5 wt% sunflower oil), for 3 months at two storage conditions, 20 °C and 4 °C.

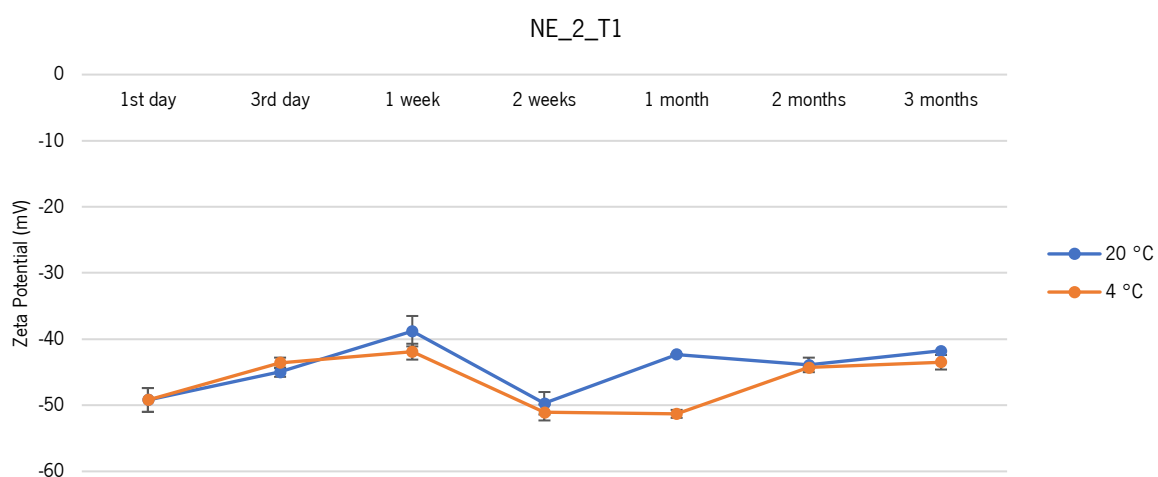


Figure D.7 – Variations of zeta potential of NE_2_T1 (SOR 2, 1 wt% of thyme essential oil and 9 wt% sunflower oil), for 3 months at two storage conditions, 20 °C and 4 °C.

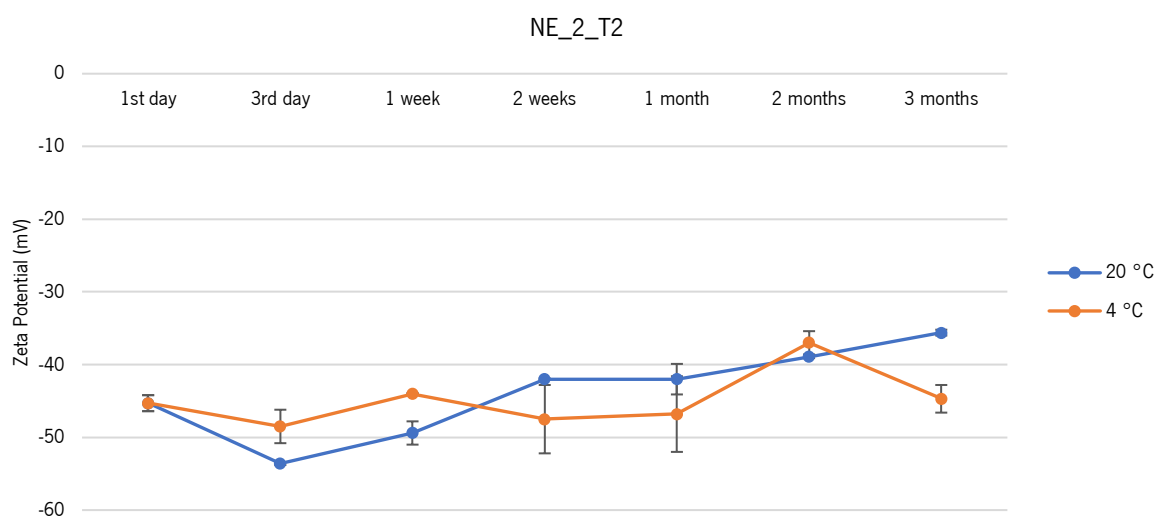


Figure D.8 – Variations of zeta potential of NE_2_T2 (SOR 2, 2 wt% of thyme essential oil and 8 wt% sunflower oil), for 3 months at two storage conditions, 20 °C and 4 °C.

Nanoemulsions with lemon balm essential oil

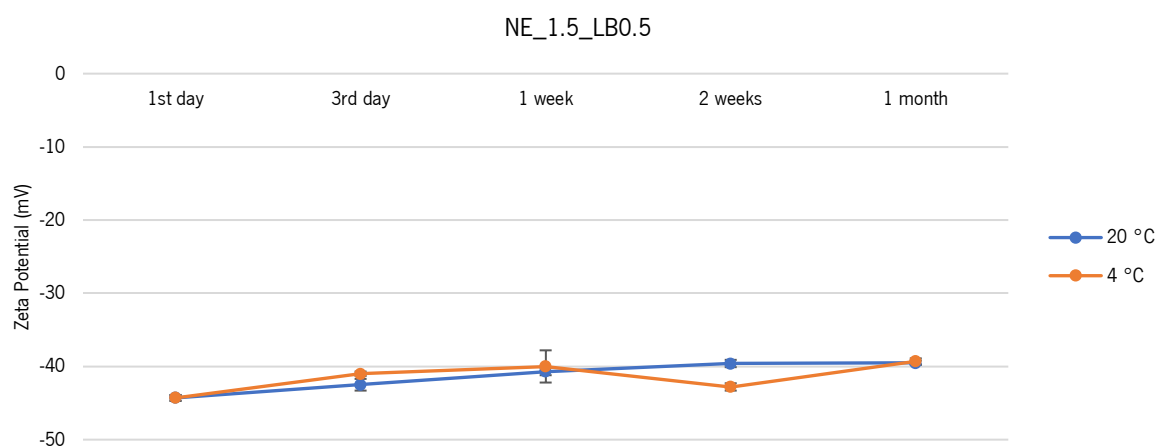


Figure D.9 – Variations of zeta potential of NE_1.5_LB0.5 (SOR 1.5, 0.5 wt% of lemon balm essential oil and 9.5 wt% sunflower oil), for 1 month at two storage conditions, 20 °C and 4 °C.

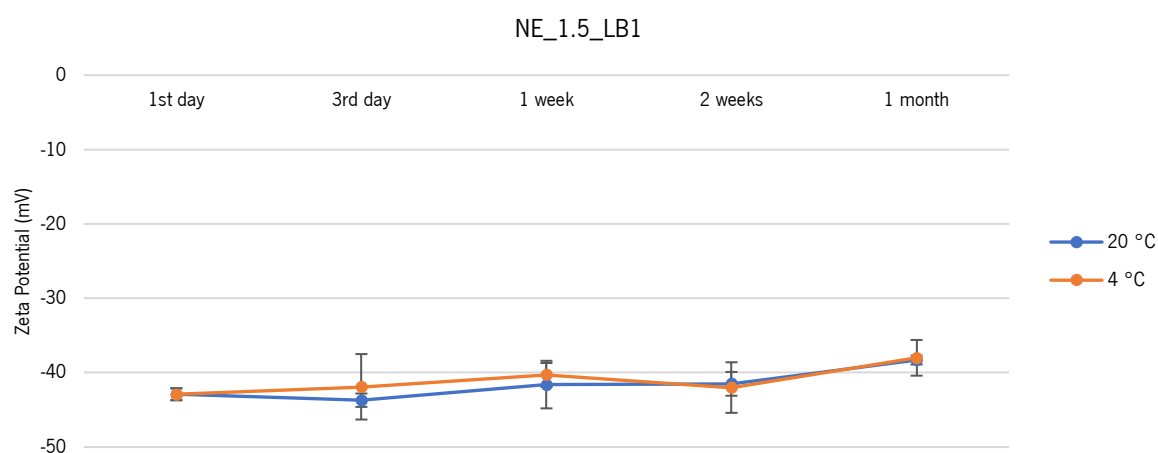


Figure D.10 – Variations of zeta potential of NE_1.5_LB1 (SOR 1.5, 1 wt% of lemon balm essential oil and 9 wt% sunflower oil), for 1 month at two storage conditions, 20 °C and 4 °C.

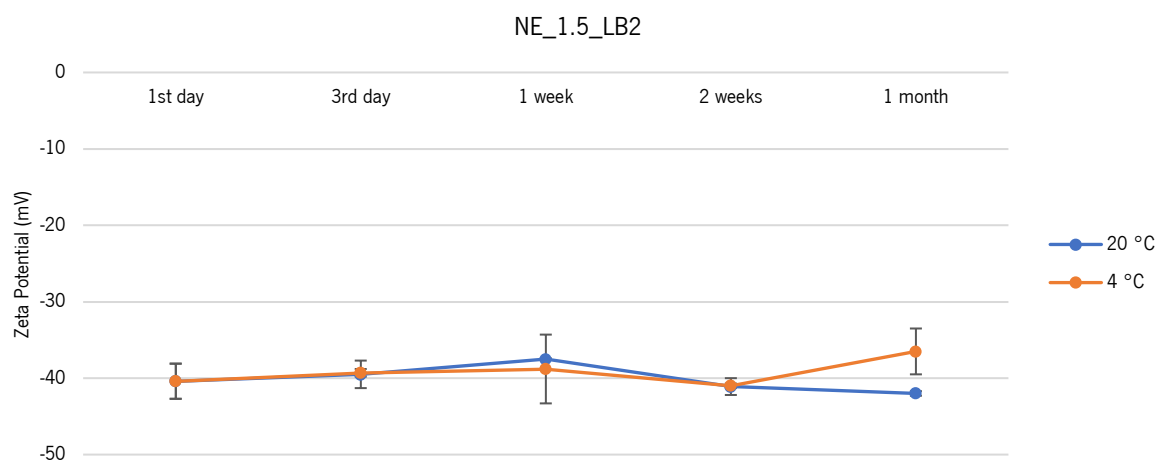


Figure D.11 – Variations of zeta potential of NE_1.5_LB2 (SOR 1.5, 2 wt% of lemon balm essential oil and 8 wt% sunflower oil), for 1 month at two storage conditions, 20 °C and 4 °C.

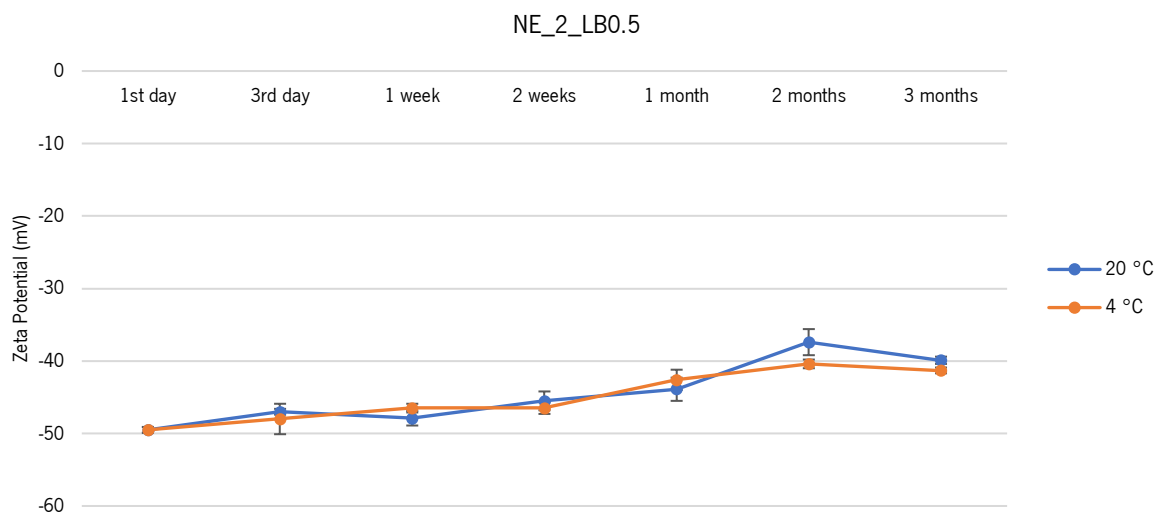


Figure D.12 – Variations of zeta potential of NE_2_LB0.5 (SOR 2, 0.5 wt% of lemon balm essential oil and 9.5 wt% sunflower oil), for 3 months at two storage conditions, 20 °C and 4 °C.

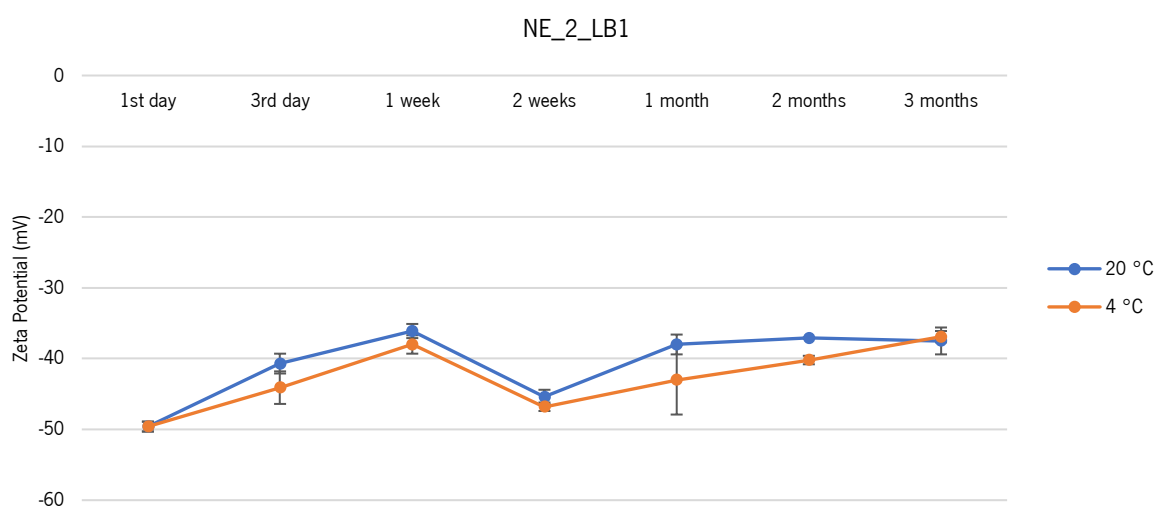


Figure D.13 – Variations of zeta potential of NE_2_LB1 (SOR 2, 1 wt% of lemon balm essential oil and 9 wt% sunflower oil), for 3 months at two storage conditions, 20 °C and 4 °C.

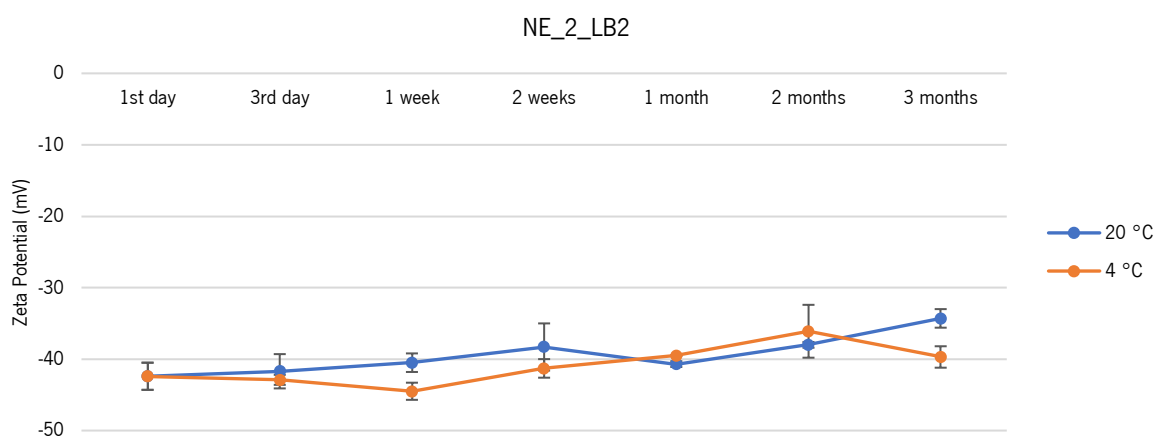


Figure D.14 – Variations of zeta potential of NE_2_LB2 (SOR 2, 2 wt% of lemon balm essential oil and 8 wt% sunflower oil), for 3 months at two storage conditions, 20 °C and 4 °C.

Group E – Calibration curves: antioxidant activity tests

DPPH assay

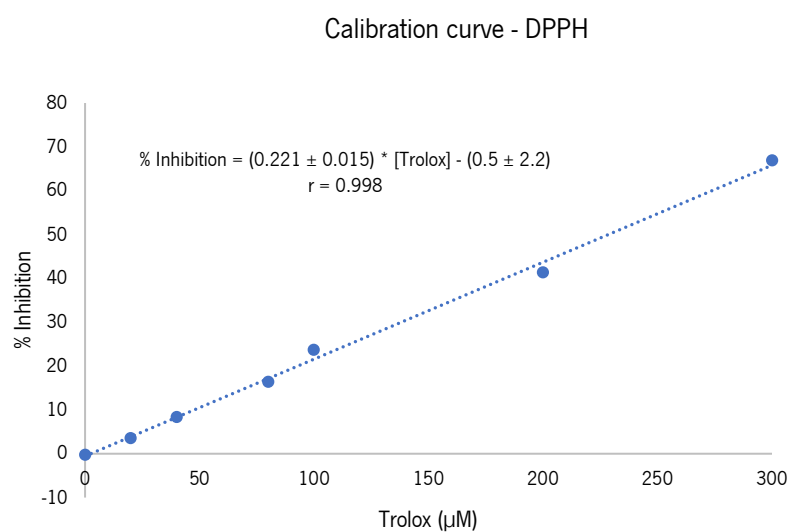


Figure E.1 – Linear part of the calibration curve obtained by the DPPH free scavenging test for different concentrations of Trolox.

ABTS assay

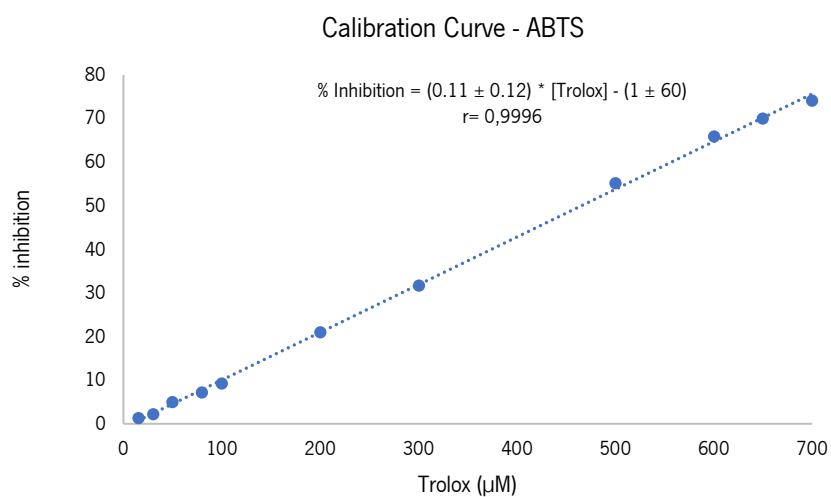


Figure E. 2 – Calibration curve obtained by the ABTS antioxidant test for different concentrations of Trolox.