Isolation, Identification and Quantification of Essential Oils in Cyprus and Anatolia Thyme

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ABSTRACT

In this study our aim was to extract, identify and quantify the essential oils in locally obtained thyme samples grown in Cyprus and Anatolia. The samples comprised three Cypriot specimens, one of which grew wild and two of which were grown commercially; and two commercially grown Anatolian samples. All the specimens were obtained as the dry herb. The essential oils from these were isolated by hydrodistillation using a Clevenger type Apparatus. Analyses were carried out by Gas Chromatography-Mass Spectrometry. In the extracts, up to 45 different constituents could be identified which accounted for about 95% of the total essential oils extracted. The major constituents in all the samples were found to be carvacrol (about 90 % of the total) and *p*-cymene (around 3 %). We can therefore predict the chemotype of our five samples as carvacrol but exactly which species they are requires further botanical identification. The higher yields of essential oils were obtained from the Cyprus specimens, the highest being from the wild Cyprus thyme.

Keywords: Carvacrol, Clevenger-type Apparatus, essential oils, Gas Chromatography- Mass Spectrometry, Thyme.

ÖZ

Bu çalışmanın amacı Kıbrıs ve Anadolu'da yetişen ve yerel olarak temin edilen bazı kekik örneklerindeki uçucu yağların damıtma yoluyla elde edilmeleri ve içeriklerinin ne olduğunu ve her bir maddeden ne kadar olduğunu tespit etmektir. Çalışılan örneklerin üçü Kıbrıs'ta, ikisi ise Anadolu'da yetişmiştir. Temin edilen tüm örnekler kurutulmuş haldeydi. Uçucu yağlar Clevenger tipi bir sulu-damıtma düzeneği ile damıtılarak elde edildi. Analizler Gaz Kromatografi – Kütle Spektroskopisi ile gerçekleştirildi. Elde edilen uçucu yağlarda toplamın yüzde 95'ini oluşturan 45 farklı madde tanımlandı. Tüm örneklerde, uçucu yağın çoğunluğunu oluşturan madde karvakrol (takriben 90 %) olarak tespit edilirken ikinci sıradaki madde *p*-cymene (takriben 3 %) oldu. Bu sonuçlara göre beş örneğimizin kimotipinin karvakrol olarak tanımlanması gerekeceğini düşünüyoruz. Ancak nihai isimlendirme örneklerimizin öncelikle botanik olarak tür ve cinsinin tanımlanması ile mümkün olacaktır. En çok uçucu yağ miktarı Kıbrıs kekik örneklerinden elde edilmiştir.

Anahtar Kelimeler: Karvakrol, Clevenger-tipi cihaz, uçucu yağlar, Gaz Kromatografi-Kütle Spektroskopisi, Kekik.

To My dear sister SURA

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WIHAD

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LIST OF ABBREVIATIONS

bp	Boiling point
CAS	Chemical Abstracts Service
CT	Chemotype
D	Density
EOs	Essential Oils
FID	Flame Ionization Detector
GC	Gas Chromatography
GC-MS	Gas Chromatography-Mass Spectrometry
h	Hour
HD	Hydrodistillation
HPLC	High-Performance Liquid Chromatography
I	Kovats retention index
I.D	Internal Diameter
IUPAC	International Union of Pure And Applied Chemistry
LC	Liquid Chromatography
LLE	Liquid–liquid extraction
MAE	Microwave Extraction
MAHD	Microwave assisted Hydrodistillation
MDGC	Multidimensional GC
MGH	Microwave-generated hydrodistillation
min	Minutes
MS	Mass Spectrometry
MSPD	Matrix solid-phase dispersion

M.w.	Molecular Weight
OAHD	Ohmicassisted hydrodistillation
PHWE	Pressurized hot water extraction
PLE	Pressurized liquid extraction
Psi	pounds per square inch
R	Resolution
Ref.	Reference
R_t	Retention time
S-CO_2	Supercritical carbon dioxide
SE	Solvent Extraction
SFE	Supercritical fluid extraction
SPME	Solid phase micro-extraction
T	Thyme
TEO	Thyme Essential Oil
TIC	Total Ion chromatogram
UAE	Ultrasound Extraction

Chapter 1

INTRODUCTION

Over the years, scientists have developed an ever-increasing interest in natural products as alternatives for artificial chemicals for use in pharmacological and cosmetics industries. Among these natural products, essential oils (EOs) have gained the greatest popularity in the research and development activities of these industries. The reasons for this interest stems from the fact that they are natural renewable resource generally cheaper to produce, environmentally, biologically safer and less toxic than their manufactured counterparts. As a result, there has been much work and research done on improving the yield and variety of EOs that can be obtained from various plants, as well as methods for extraction and isolation, and rapid identification and quantification by instrumental methods [1].

One of these plants is thyme which is a valuable herb that has been used by humankind since ancient times for culinary and medicinal purposes. The botanical genus name for thyme is *Thymus.* There are around 350 species of thyme and all produce varying amounts of essential oils composed of terpenes. They can be easily obtained from particular species of plants that contain between 0.1 to 1 % by weight of the dried plant. The plants although similar in appearance differ widely in the amount and type of essential oils they produce. Even plants of the same species produce different mixtures of essential oils as a result of variations in genetic makeup as well as growing conditions such as the environment, climate, soil type and harvest

period. In these cases the plant is characterized by its chemotype, which indicates the main constituent of the essential oils it produces. Thyme, as the herb or as extracted essential oils, possesses numerous biological and pharmacological benefits such as antimicrobial, antiseptic and antioxidant activity [2].

In this present study the research objectives are:

- To extract by hydro-distillation the essential oils from the locally obtained Cyprus and Anatolia grown thyme samples.
- To determine and compare the yield of essential oils in each sample.
- To analyse by Gas Chromatography-Mass Spectrometry the extracts so as to identify and quantify the constituents in each thyme essential oils extract.

Chapter 2

LITERATURE REVIEW

2.1 Thyme

Thyme is a popular aromatic herb belonging to the Lamiaceae (Labiatae) or mint family. There are around 350 species of thyme. The name thyme, however, refers to all members of the plant genus *thymus*. Some vernacular names include: common thyme, english thyme, garden thyme, herba timi, herba thymi, mother of thyme, red thyme, rubbed thyme, thick leaf thyme, thumon, thymon, thymian, timi, tomillo, za'ater and kekik [3].

The ancient Egyptians used thyme in embalming. Ancient Greeks also used it as incense in their bathrooms, coffins and temples, believing that it was a source of bravery for the knights and warriors and helped them in the afterlife. Romans spread thyme throughout Europe during the middle ages. They used it to purify their rooms, to give aroma to cheese and alcoholic beverages and placed it under their pillows to prevent nightmares. Romans also believed that thyme ensured safe passage of the deceased to the afterlife [4].

Thyme is a bushy, woody, evergreen herb. The thin and wiry stems are full of small (few mm in length) grey green leaves. Flowers consist of clusters of pale purple, pink or white blossoms arranged in opposite pairs, along the tips of the stems. The seeds are round and remain fertile even upon prolonged storage. The plant grows to a

height of about 40 cm but it also has a tendency to spread horizontally. As the plant ages, its stems thicken and become woody. A hand drawing of thyme plant and its foliage and two photographs of thyme are shown in Figures 2.1, 2.2 and 2.3 respectively. Thyme grows in most temperate regions. This includes the Mediterranean basin countries as well those countries that have similar climatic conditions elsewhere around the world. The best time to harvest thyme to maximize essential oil yield is during spring, on hot days, preferably from sunny locations. The plant needs good sun light to grow to its best potential in soil with a pH of 5.0 to 8.0. Thyme is either planted from seeds or propagated by taking cuttings from stems [3],[5].

Figure 2.1: Hand-drawing of Thyme [6]

Figure 2.2: Thyme [7]

Figure 2.3: Common thyme [8]

2.2 Thyme Species

There are about 350 species of thyme around the world. The main and most widely cultivated and used species of thyme are:

1-*Thymus vulgaris*: is known as common thyme, English thyme, summer thyme, French thyme, or garden thyme. It is commonly used in culinary practice.

2-*Thymus citriodorus* shown in Figure 2.4 is known as lemon thyme or orange thyme.

Figure 2.4: Thymus citriodorus [9]

3-*Thymus serpyllum* shown in Figure 2.5 is known as wild thyme or creeping thyme which is an important plant for honey bees.

Figure 2.5: Thymus serpyllum [10]

4-*Thymus praecox* is called the mother of thyme.

5-*Thymus pseudolanuginosus* is woolly thyme. It is grown as a ground cover.

6-*Thymus herba-barona* is known as caraway thyme [4], [11].

There are also some other species such as mint thyme, lavender thyme, Italian oregano thyme, and coconut thyme [12], [13].

2.3 Essential Oils (EOs) or Volatile Oils

EOs are very complex mixtures of plant products and among other qualities they possess various biological properties. They are a massive category of extremely volatile oils that offer plants their characteristic odour which are used particularly in perfumes; flavourings; aromatherapy and medicine. They are isolated from the leaves, stems, flowers or twigs of the plants. Chemically, they contain about 20-60 compounds at different concentrations; but usually 2 or 3 specific compounds will constitute majority of the total EOs yield. Most of the other components will exist in minor or trace amounts. Terpenes are the main constituents of EOs which include alcohols, aldehydes, esters, ketones, oxides, phenols and other types of organic compounds such as resins which are called oleoresins or balsams. There are various methods available for extracting and isolating EOs. These general methods are: distillation, expressing consumption and extraction [14], [15].

2.4 Terpenes

Terpenes are a large and diverse mixture of isomeric unsaturated hydrocarbons occurring in most EOs and oleoresins of plants. Terpenes form different classes by the combinations of 5 carbon units which are called isoprene C_5H_8 (2-methyl-1,3-butadiene) as shown in Figure 2.6 from which they are biogenetically derived. Terpenes or terpenoids as some authors prefer to name them are made of two or more isoprene units joined together in a head to tail manner, as exemplified in Figure 2.7.

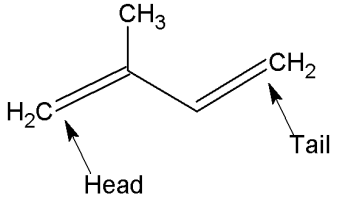


Figure 2.6: Isoprene (head-tail)

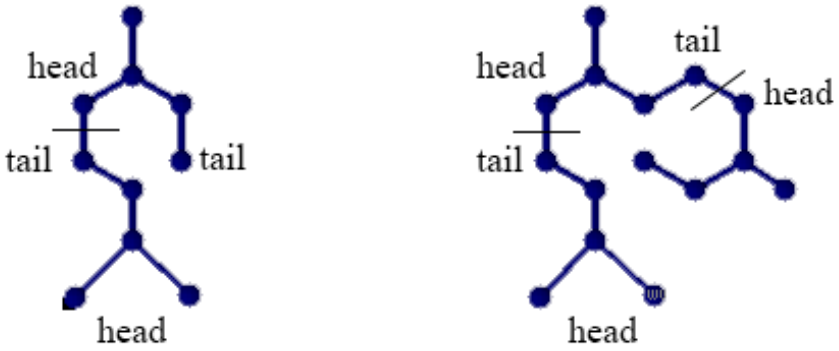


Figure 2.7: Joining types of Isoprene units [16]

2.4.1 Classification of Terpenes

There are two types of classification of terpenes. One type is based on the number of isoprene units making up the terpene and the other is based on whether it is acyclic or cyclic.

2.4.1.1 The Number of Isoprene Units

In this classification of terpenes, nomenclature is based on the number (n) of isoprene units $(C_5H_8)_n$ present in the structure as shown in Table 2.1.

Table 2.1: Classification of terpenes by number of isoprene units [16]

No.	Classification	No. of Isoprene Unit	Formula	Example
1	Hemiterpenes	1	C_5H_8	Prenyl and isovaleric acid.
2	Monoterpenes	2	$C_{10}H_{16}$	Open chain: Citral, Geraniol, linalool. Monocyclic: Limonene, Menthol, Thymol, Menthone, Carvone, Cineole. Bicyclic: Camphor, Pinene.
3	Sesquiterpenes	3	$C_{15}H_{24}$	Open chain: Farnesol. Cyclic: Cadinene.
4	Diterpenes	4	$C_{20}H_{32}$	Open chain: Phytol, Retinol. Cyclic: Gibberellins, resin acids.
5	Sesterterpenes	5	$C_{25}H_{40}$	Rare
6	Triterpenes	6	$C_{30}H_{48}$	Open chain: Squalene Cyclic: Triterpene alcohols and acids, Steroids,Gossypol, Cucurbitacine.
7	Tetraterpenes	8	$C_{40}H_{64}$	Carotenes, Xanthophylls and Lycopene.
8	Polyterpenes	>8	$(C_5H_8)_n$	Rubber, Gutta-percha, Balata.

2.4.1.2 By Acyclic or Cyclic Structure

(A) Acyclic Terpenes: They contain open structure such as myrecene and geraniol.

(B) Cyclic Terpenes are named according to the number of rings in the structure:

- Monocyclic Terpenes: contain one ring in the structure such as thymol and α-terpineol.
- Bicyclic Terpenes: contain two rings.
- Tricyclic Terpenes: contain three rings.
- Tetracyclic Terpenes: contain four rings.

Some terpene compounds, mostly cyclic, are shown in Figure 2.8.

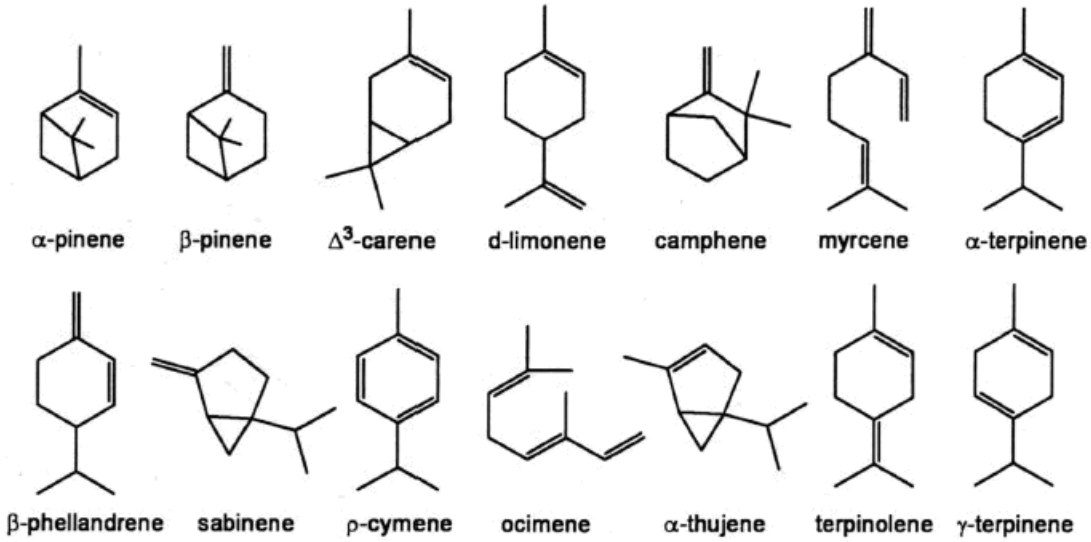


Figure 2.8: Some terpene compounds

Monoterpenes and sesquiterpenes make up almost 90% of EOs. Structurally, they belong to several different functional groups as shown Table 2.2.

Table 2.2: Monoterpenes and sesquiterpenes [14]

Classification		Acyclic	Monocyclic	Bicyclic
Monoterpenes	Carbure	Myrcene, ocimene	terpinenes, *p*-cymene, phellandrenes	pinenes, 3-carene, camphene, sabinene
	Alcohol	geraniol, linalol, citronellol, lavandulol, nerol	menthol, *α*-terpineol, carveol	borneol, fenchol, chrysanthenol, thuyan-3-ol
	Aldehyde	geranial, neral, citronellal		
	Ketone	tegetone	Ketone	tegetone
	Ester	linalyl acetate or propionate, citronellyl acetate	menthyl or *α*-terpinyl acetate	isobornyl acetate
	Ether	1,8-cineole, menthofurane		
	Peroxydes	Ascaridole		
	Phenols	thymol and carvacrol		
Sesquiterpenes	Carbure	azulene, *β*-bisabolene, cadinenes, *β*-caryophyllene, logifolene, curcumenes, elemenes, farnesenes, and zingiberene		
	Alcohol	bisabol, cedrol, *β*-nerolidol, farnesol, carotol, *β*-santalol, patchoulol and viridiflorol.		
	Ketone	germacrone, nootkatone, cis-longipinan-2,7-dione, *β*-vetinone and turmerones.		
	Epoxide	caryophyllene oxide and humulene epoxides		

2.4.2 General Properties of Terpenes

1-Most terpenes are usually colourless liquids when freshly extracted but upon prolonged storage and oxidation they tend to become darker. Few of them are solids like camphor.

2-They are volatile at normal temperature and pressure.

3-Their density are lower than water.

4-Terpenes are soluble in organic solvents and insoluble in water.

5-Terpenes have optical activity because they possess chiral centres, and occur in nature in enantiomeric form.

6. They are open chain or cyclic unsaturated compounds with one or more double bonds that enable them to undergo addition, polymerization and dehydrogenation reactions.

7. Most terpenes are strongly antiseptic and antibacterial.

8. They are easily oxidized by all oxidizing agents [14], [15], [16].

2.5 Chemical Composition of Thyme Essential Oil

The 350 species of thyme are similar in appearance and they all produce terpene based EOs such as thymol, α-thujone, α-pinene, camphene, β-pinene, *p*-cymene, α-terpinene, linalool, borneol, β-caryophyllene and carvacrol. However, different species produce different mixtures and amounts of EOs. Even plants of the same species manufacture different mixtures of EOs as a result of slight genetic variations and because of environmental, temporal and geographical factors. In such cases the plants belonging to a particular species are sub classified – called chemotype- according to the main terpenes they produce. Thus the chemotype is indicative of the main EO a particular plant will produce [17].

Because each chemotype produces a different mixture of EOs, correspondingly the pharmacological benefits afforded by each chemotype will be different. The complete identification of a chemotype requires the specification of the following criteria:

- Genus name of the plant.
- Country or region from where the plant was obtained.
- Whether the plant grows in the wild or it was cultivated.
- The growth stage of the plant at the time of harvest.
- The part of the plant from which the EOs were extracted and the method by which the EOs were obtained.
- Qualitative and quantitative analysis of the EO constituents, usually by GC-MS [17].

2.6 Thyme Chemotypes

The more commonly known and important chemotype and the major EOs they produce together with some data and explanations are listed in Table 2.3 below.

Table 2.3: Thyme Chemotypes [11], [18], [19], [20], [21],

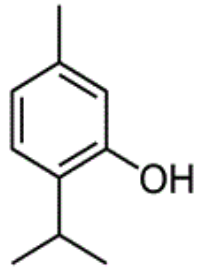
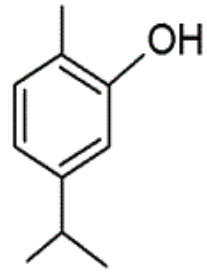
Chemotype	Major EOs	IUPAC name	Molecular Formula	M.w. g/mol.	Structure	Properties for major compound
Thymus vulgaris* CT-thymol Botanical name: *Thymus zygis	Thymol (60-70% of total EOs). It goes by the name of thyme or "red thyme".	2-isopropyl-5-methylphenol **CAS Reg. No.** 89-83-8	$C_{10}H_{14}O$	150.22		• White crystals or powder with a pungent aromatic smell, sweet-medicinal, herbaceous, warm. • D 0.979, mp 48–51°C, bp 233°C. • Soluble in organic solvents; slightly soluble in water and glycerol. • Combustible. • **Derivation**: From thyme oil or other oils, synthetically from *m*-cresol and isopropyl chloride. • **Use**: In perfume, musty preventive, antioxidant, flavoring, laboratory reagent, menthol synthetic.
Thymus vulgaris* CT-carvacrol Botanical name: *Thymus serpyllum	Carvacrol harvest in full (60 -80% of total EOs).But if harvest in spring it will be 30%. It is known as wild thyme.	5-Isopropyl-2-methylphenol **CAS Reg. No.** 499-75-2	$C_{10}H_{14}O$	150.22		• Thick, colorless oil to light yellow liquid, pungent, spicy odour. • D 0.976 ; bp 237°C • Insoluble in water; soluble in alcohol, ether, and alkalies. • Combustible. • **Derivation:** From *p*-cymene by sulfonation followed by alkali fusion. • **Use:** Perfumes, fungicides, disinfectant, flavoring, organic synthesis.

Table 2.3: Continued

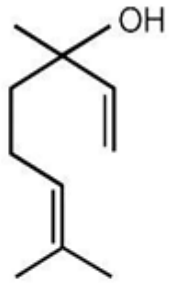
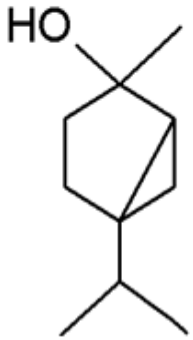
Chemotype	Major EOs	IUPAC name	Molecular Formula	M.w. g/mol.	Structure	Properties for major compound
***Thymus vulgaris* CT-linalool** **Botanical name:** **Thymus zygis**	linalool (80 to 95 % of EOs). It is known as garden thyme	3,7-Dimethyl-1,6-octadien-3-ol **CAS Reg. No.** 78-70-6	$C_{10}H_{18}O$	154.25		• Colorless liquid; with a pleasant, floral aroma comparable to bergamot oil and French lavender. • D 0.858–0.868 (25°C), bp 195–199°C. • Soluble in alcohol, ether, fixed oils. • Combustible. • **Derivation:** Citrus peel oils, especially from oranges. Made synthetically from geraniol. • **Use:** perfumery, flavoring agent.
***Thymus vulgaris* CT-4-thujanol**	4-thujanol (50% of total EOs). It is often called sweet thyme. 4-thujanol known as Sabinene Hydrate	1-Isopropyl-4-methylbicyclo [3.1.0]hexan-2-ol **CAS Reg. No.** 546-79-2	$C_{10}H_{18}O$	154.25		• Transparent liquid to pale yellow with a powerful and typical aroma. • D 1.0 ; bp 209.1°C • **Use:** Anti-bacterial, powerful antiviral, immune stimulant, hepatic cell, neurotoxin and nerve equilibrating and fungicide.

Table 2.3: Continued

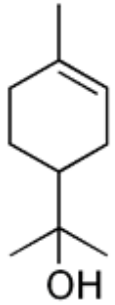
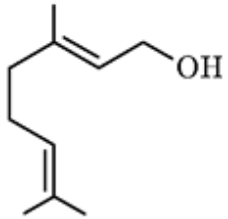
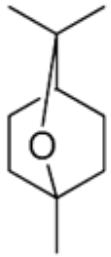
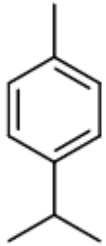
Chemotype	Major EOs	IUPAC name	Molecular Formula	M.w. g/mol.	Structure	Properties for major compound
***Thymus vulgaris* CT-*α*-terpineol**	*α*-terpineol (40 -90% of EOs) It has pepper smell.	1-Isopropyl-4-methyl-3-cyclohexen-1-ol **CAS Reg. No.** 98-55-5	$C_{10}H_{18}O$	154.25		• Mixture of the three isomers of colorless liquid or pale yellow, transparent crystals. • D 0.930–0.936 (25°C), bp 214 to 224°C. • Soluble in two volumes of 70% alcohol; slightly soluble in water, glycerol. • Combustible. • **Derivation:** terpene hydrate heated with phosphoric acid, distilled with dilute sulfuric acid using an azeotropic separation. • **Use:** Solvent for hydrocarbon materials, mutual solvent for resins and cellulose esters and ethers, perfumes, soaps, disinfectant, antioxidant, flavoring agent.
***Thymus vulgaris* CT-geraniol**	geraniol (75 to-85% of EOs)	Trans-3,7-Dimethyl-2,6-octadien-1-ol **CAS Reg. No.** 106-24-1	$C_{10}H_{18}O$	154.25		• Colorless to light yellow liquid with geranium scent. • D 0.870–0.890 (15°C), bp 230°C. • Soluble in alcohol, ether, mineral oil, fixed oils; insoluble in water and glycerol. • Combustible. • **Derivation:** From citronella oil (Java), citronellol-free grades from palmerosa oil, and (synthetically) from pinene. These are of higher quality. • **Use:** Perfumery, constituent of synthetic fragrances and synthetic linalool.

Table 2.3: Continued

Chemotype	Major EOs	IUPAC name	Molecular Formula	M.w. g/mol.	Structure	Properties for major compound
***Thymus vulgaris* CT-1,8 cineole**	1,8 cineole (80 -90 % of EOs). 1,8 cineole is called Eucalyptol.	1,3,3-Trimethyl-2-oxabicyclo [2.2.2]octan **CAS Reg. No.** 470-82-6	$C_{10}H_{18}O$	154.25		• Colorless with camphor fragrance. • D 0.921–0.923 (25°C); bp 174–177°C. • Slightly soluble in water; miscible with alcohol, chloroform, ether, glacial acetic acid, and fixed or volatile oils. • Combustible. • **Derivation:** By fractionally distilling eucalyptus oil followed by freezing. • **Use:** Pharmaceuticals (cough syrups, expectorants), flavoring, perfume.
***Thymus vulgaris* CT-*p*-cymene**	*p*-cymene It is mixture of isomers; ortho, meta and para cymene. It is known as winter thyme.	1-Isopropyl-4-methylbenzene **CAS Reg. No.** 99-87-6	$C_{10}H_{14}$	134.22		• Colorless to light yellow liquid. • D: 0.8551, bp 176.5°C. • Soluble in alcohol, ether, and chloroform; insoluble in water. • Combustible. • **Derivation:** Mixed cymenes are produced from toluene by alkylation. • **Use:** Solvents, synthetic-resin manufacture, metal polishes, organic synthesis (oxidation to hydroperoxides used as catalysts for synthetic-rubber manufacture; cymene alcohols are made by hydrogenating the hydroperoxides.

2.7 Utilization of Thyme and TEO

Thyme is one of those ancient herbs which is rich in chemical composition and beneficial properties, and finds widespread use in different areas of our lives. Throughout history thyme has been widely used for culinary, medicinal as well as ceremonial purposes. Ancient documents and abstracts of medicine include many references to the uses and benefits of thyme. Today, thyme still finds use in the kitchen as well as in food preparations and pharmaceutical industries. It is used both in alternative and conventional medicine with ever increasing popularity in use as well as applications[2], [22].

2.7.1 Culinary Use of Thyme

Thyme, both dried and fresh, is widely used in the kitchen for cooking as well as in preparing drinks all around the world. It is often used to flavour meats, soups, stews, cheese, tomatoes and eggs. But it is also used to flavour alcoholic and non-alcoholic drinks [4], [23].

2.7.2 Therapeutic Use of TEO

The synergy of the major compounds with the other minor compounds in TEO imparts useful and beneficial therapeutic and biological effects to these TEO and makes them useful as drugs or medicines [14].

The cytotoxic ability of TEO works as an excellent antiseptic, antimicrobial and antioxidant agent. TEO have the capacity to penetrate through bacterial membranes to the cell interior and inhibit activity on the functional, hydrophilic and lipophilic properties of the cell. Many researchers report that TEO is very effective reducers against the propagation of tumour cells. It has also been shown that TEO has an anticancer influence on the lung and colon cancer cells. As research continuous more

discoveries will be added regarding the pharmacological benefits of TEO against many other diseases [3], [14], [24].

2.7.3 Commercial Use of Thyme Oil

TEOs are widely used commercially, at an industrial level, in cosmetic and personal care products such as perfumes, soaps, shampoo, toothpaste, mouth wash and skin products. They are also used as flavoring agents, in veterinary, agricultural, food and other industries [4], [15].

2.8 Factors That Affect Composition of TEO

The factors that influence the composition (identity and quantity of individual components) of EOs are: growing environment, weather conditions, soil type, harvest period of the plant, drying conditions, and the method of extraction and isolation. Some studies about these factors are given below.

2.8.1 Seasonal Variation

A Spanish study of *Thymus hyemalis* L. showed that there are seasonal variations at every phenological stage in the quality and quantity of TEO; the highest yield of phenolic compounds was found for full flowering stage [25].

2.8.2 Vegetative Cycle Variations

The young *Thymus vulgaris* have the maximum yield of phenols (thymol and carvacrol) in June and July, and less in November and December with the same amount of monoterpene in both periods, whereas the yields of phenols and monoterpenes varied in the same periods of the vegetative cycle in old *Thymus vulgaris.* This study showed the importance of harvesting period to gain the best quality and quantity of each TEO components [26].

2.8.3 Time of Harvesting

In a study on the effects of harvesting time on EOs content and composition of Turkish *Thymus vulgaris* in cultivated plants for culinary purposes and small-scale EOs production showed that there were diurnal differences in EOs content. Fresh and dry leaves of *T. vulgaris* had greater oil content in early morning than in the hot noon hours of the day. The main compounds present in the EOs found in fresh and dry leaves were thymol, *γ*-terpinene, *p*-cymene and carvacrol. This study concluded that the best harvesting hours of *T.vulgaris* for higher thymol content were between early morning to before noon [27].

2.8.4 Drying Conditions

Drying is a preservation process of the plant to ensure the microbial safety of biological products. Studies have shown that changes in the concentrations of the volatile compounds in EOs during drying depend on the drying method (convective drying, vacuum–microwave drying, freeze drying and combination of convective pre-drying and VM finish drying). This study indicated the variation of time, temperature, and wattage on the aroma quality and composition of TEO [28].

2.9 Isolation and Separation Techniques

There are many techniques that have been used for EOs extraction, such as:

- Hydrodistillation (HD) by Clevenger type Apparatus which is the most widely used method in conventional processes. (Figure 2.9).
- Solvent Extraction (SE).
- Liquid–liquid Extraction (LLE).
- Headspace trapping techniques; Static headspace technique, Vacuum headspace technique, Dynamic headspace technique.
- Supercritical fluid Extraction (SFE).

- Solid phase micro-extraction (SPME).
- Microwave Extraction (MAE).
- Ultrasound Extraction (UAE).
- Multidimensional GC (MDGC).
- Microwave-generated hydrodistillation (MGH).
- Matrix solid-phase dispersion (MSPD).
- Ohmic-assisted hydrodistillation (OAHD).
- Pressurized hot water Extraction (PHWE).
- Pressurized liquid Extraction (PLE).

The main factors that influence the choice of extraction method include the chemical characteristics of EOs, time and cost of extraction, yields, efficiency of extraction, quality of the EOs extracted [29], [30].

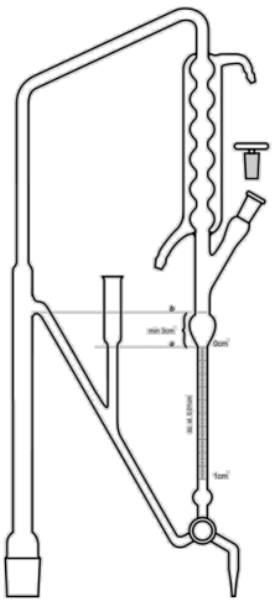


Figure 2.9: Clevenger type Apparatus

Table 2.4: Advantages and disadvantages of various extraction methods [reproduced from Ref. 29]

Extraction techniques	Advantages	Disadvantages
HD	Inexpensive, simplicity, easy to couple with other techniques	Time consuming, thermal degradation
SFE	High-quality extract, selective, eliminates solvent residues, minimized sample degradation	Costly, technical skills required, lipophilic nature of supercritical CO_2 makes it difficult for some active compounds extraction
UAE	Less temperature required, minor solvent quantity, time saving, improved yields	Particle size is a critical parameter for quantitative analysis
MAE	Efficient, less time of extraction, less sensitive to moisture content, less solvent usage	Sensitive temperature and pressure control for volatile compounds, limited sample analysis
MGH	Low cost, high-quality yield, reduced time of extraction	Nonefficient for volatiles. Solid residue removal is necessary, more suitable for fresh material or rehydrated
SPME	Simple, high screening, time saving, less solvent usage, easy to couple with other techniques	High selectivity of SPME fiber toward reagents, expensive fibers, limited lifetime of polymer coating, less efficient for quantitative analysis
PLE	Less consumption of solvent, short time of extraction	Limited amount of targeted samples, costly equipment
PHWE	Efficient, less cost, less consumption of organic solvents	High flow rates of water, less-concentrated extract
LLE	Efficient, fast, low operating and maintenance cost	Flow rate turndown, complex operability

Some studies showing the effects of the extraction methods on TEO composition are presented below.

2.9.1 Microwave-assisted Hydrodistillation

Microwave-assisted hydrodistillation (MAHD), shown in Figure 2.10, is an advanced technique for TEO extraction. A microwave oven is used for heating the thyme with water. This method gave better results compared with normal HD. The time of extraction is reduced by a factor of 3 (75 minutes with MHD in contrast to 4 h with HD), thereby reducing the operation costs (energy saving). Additionally, increase in the yield and the quality of EOs have been found. GC-MS analysis has shown that microwave radiation on TEO constituents is safe and does not degrade the components during extraction [31].

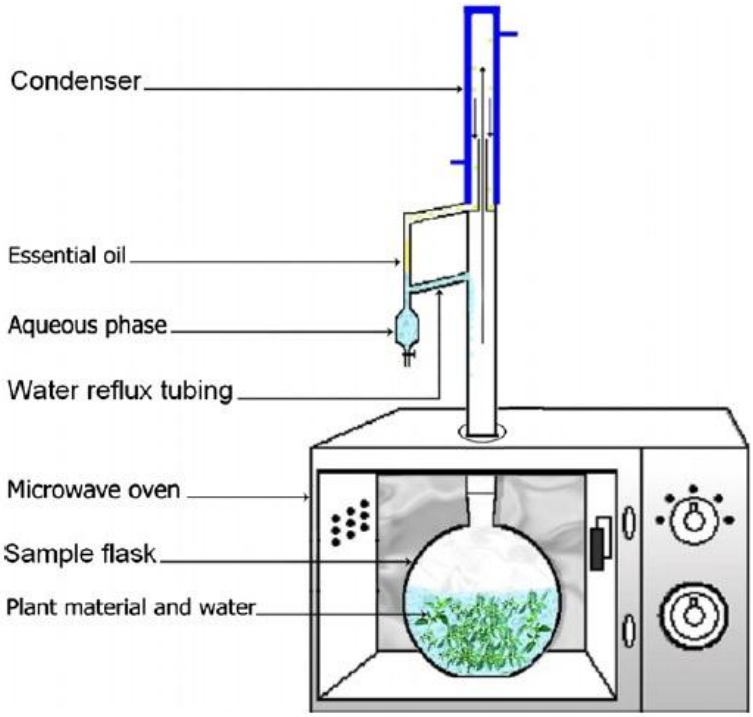


Figure 2.10: MAHD [31]

2.9.2 Ohmic-assisted Hydrodistillation

Ohmic-assisted hydrodistillation (OAHD) is another advanced technique for TEO extraction. A detailed diagram of OHD is shown in Figure 2.11. This method also gave better results than with normal HD. While the whole extraction process was complete in only 24.75 minutes with OAHD, it took 27.79 minutes just to begin to get the first drop of TEO by traditional HD. Thus, OAHD is a faster, low cost and environment-friendly method [32].

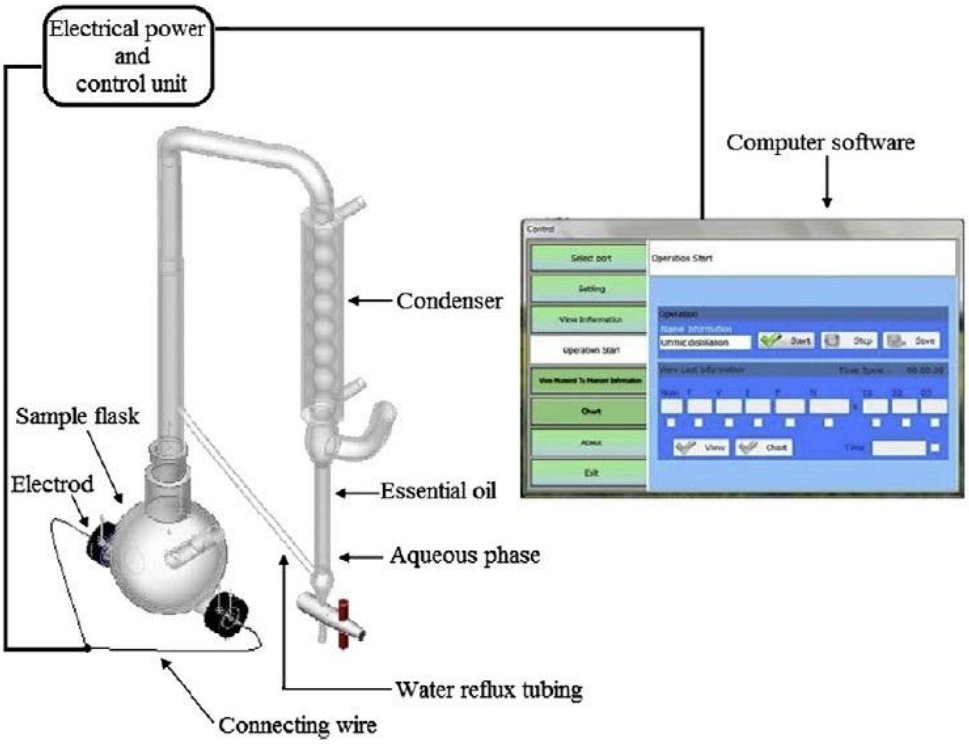


Figure 2.11: OAHD [32]

2.9.3 Supercritical Fluid Extraction

Supercritical fluid Extraction (SFE) technique is based on the critical point or the critical temperature at which point the barrier between the liquid and gaseous phase disappears and the solvent becomes a supercritical fluid. Carbon dioxide is one of materials that can be converted to a supercritical fluid relatively easily and therefore has found wide application in research and industry. Supercritical carbon dioxide ($S\text{-}CO_2$) extraction has also been used for the extraction of EOs. In a number of studies, the TEO of *Thymus lotocephalus* was isolated by HD and SFE. Researchers reported higher yields when using SFE compared to HD [29], [33], [34].

2.9.4 Pressurized Liquid Extraction

Pressurized liquid extraction (PLE) is based on the Soxhlet extraction method using the solvent in its supercritical state, as shown in Figure 2.12. In this way the solvating and penetrating power of the solvent is enhanced. PLE system has been

used with three different “green” solvents, namely ethanol, limonene and ethyl lactate to extract TEO of *Thymus vulgaris*, *Thymus zygis* and *Thymus citriodorus* at different extraction temperatures (60°C, 130°C, 200°C). PLE has been found to be a suitable technology to obtain higher yields of TEO in shorter extraction times with lower consumption of extraction solvents [35].

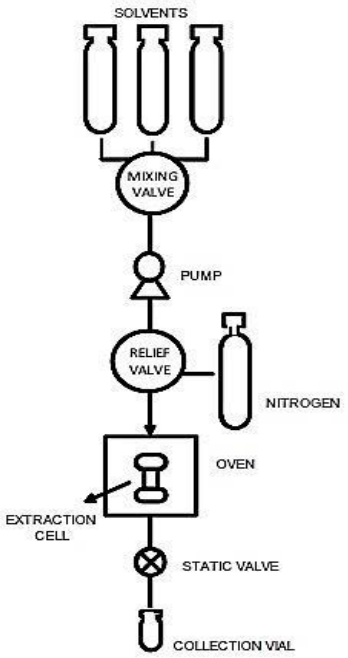


Figure 2.12: PLE [35]

2.10 Gas Chromatography-Mass Spectrometry Analysis Conditions

Chromatography is now about 150 years old as a separation technique both traditional and more recently instrumental which remains one of the most important tools for analysts in a variety of disciplines. In various configurations such as GC, GC-FID, GC-MS, GC-FID/MS, LC-MS and HPLC, chromatography is used for EOs analysis. Among all the chromatographic methods, GC-MS is the most frequently used method which is the combination of GC with an MS as the detector. These instrument are capable of separating complex mixtures into their individual components, and identify and quantify them [36], [37].

A schematic illustration of a typical GC-MS is shown in Figure 2.13 Samples of around a few μL volume are injected either manually or automatically by an autosampler through the septum of the hot inlet where the sample immediately vaporizes and is introduced into the moving stream of inert gas (mobile phase) which carries the sample vapour through the capillary column coated with a stationary phase. The components are separated from each other due to differences in their speed through the column, which depends on factors such as the molecular weight and chemical properties of the individual components. They reach the ionization chamber of the quadrupole rods of MS. In the MS the constituents are ionized and sent forward in a magnetic field. Each constituent is then identified on the basis of its mass spectrum (fragmentation pattern) and quantified on the basis of its total ion chromatogram (TIC) produced at the detector. Typical detection limits in GC-MS ranges between 0.25 to 100 pg [38].

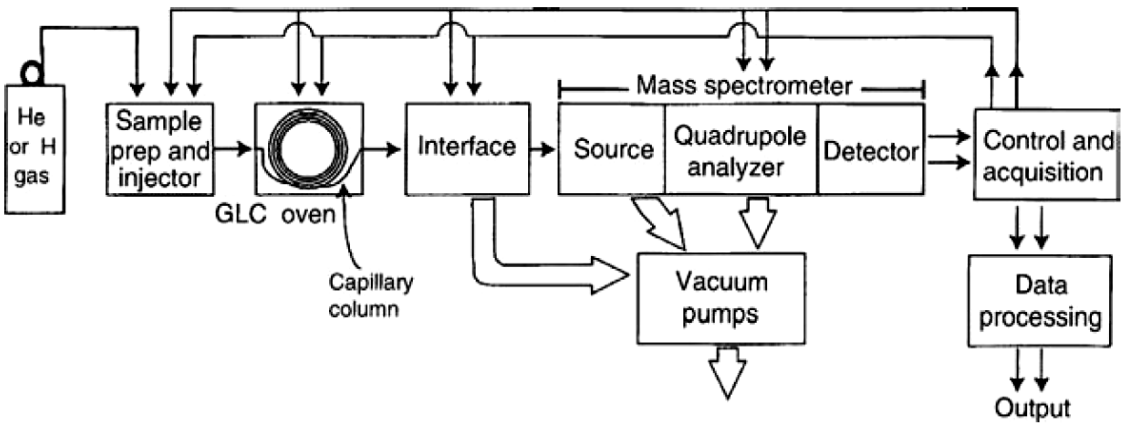


Figure 2.13: A typical GC-MS system diagram [36]

In GC-MS there are many factors that affect the separation. The more significant ones are discussed below.

2.10.1 Carrier Gas

The carrier gas is the mobile phase in GC. It must be chemically inert; gases that qualify and that have been used are He, H_2, Ar and N_2. They need to be safe, nontoxic, inexpensive and available in highly pure form. The most commonly used carrier gas is Helium. The choice of the gas is often dependent upon the type of detector. The flow rate in gas chromatography is regulated simply by controlling the gas inlet pressure. Inlet pressures usually range from 10 to 50 psi (lb/in^2) above atmospheric pressure, yielding flow rates of 1 to 25 mL/min for capillary columns. With pressure-controlled devices, it is assumed that flow rate remains constant as the inlet pressure remains constant. If the flow rate of the carrier gas through the column is too fast, little or no separation of the components take place because there isn't enough time for. On the other hand if the rate is too slow, the separation will take a long time (Retention time R_t) making the R_t excessively large and some of the less volatile components may be held up in the column. Also the peaks will be very broad [38], [39].

2.10.2 Inlets and Sample Injection

A micro-syringe is the device handling the injection of the liquid sample. The typical sample volume ranges from 0.1–10 µL, 1 µL being the most common volume.

An autoinjector or autosampler - called ALS - is capable of injecting very reproducibly, with injection-to-injection precision of typically 0.1%. However, the volume reproducibility from syringe-to-syringe may be ± several percent. With manual injections, precision of ± 1–5% in the resulting peak areas may be expected. There are two modes of injection: split and splitless. In the splitless mode, all the injected volume is put through the column whereas with the split mode only a

predetermined fraction of the injected amount is guided to the column**,** the advantages/disadvantages of the two modes are listed in Table 2.5 below [37].

Table 2.5: Advantages and disadvantages of common inlet modes [37]

Inlet	Advantages	Disadvantages
Split	• Simple • Starting point for method development • Use with isothermal and temperature programmed GC • Fast, very sharp peaks	• Choice of glass sleeve not trivial • Limits detection concentration to ppm • Most sample wasted through spilt vent • Loss of low volatility labile analytes
Splitless	• Trace analysis (ppb) possible • Cold trapping and solvent effects provide sharp peaks	• More complex than split • Limited to temperature programming. • Several parameters to optimize. • Loss of low volatility labile analytes

2.10.3 Column and Oven Parameters

Many publications describe the column as the ‘‘heart’’ of chromatography, as this is where separation occurs. The parameters that influence separation include

- Column temperature.
- Column physical dimensions such as length and internal diameter.
- The type of GC columns - whether packed or capillary.
- The nature and thickness of the stationary phase in the column.

2.10.3.1 Column Temperature

Temperature is the most important user-controllable variable in GC. The column is operated inside a temperature-controlled oven with a low thermal mass so that controlled rapid heating and cooling is possible. During a chromatographic analysis, the oven may be operated in two modes: isothermal (constant temperature) and

temperature programmed (linear temperature increase). Typically, ovens can be heated at rates of up to 40°/min. Some specialty ovens can reach temperature rates of up to 120°/min. The operating column temperature required for elution and good separation of components needs to be near the boiling point of the components or slightly above it. If the column temperature is too high, all the volatile components in the sample will pass through the column at the same rate as the carrier gas and no separation will occur because there will be no equilibration of the components with the stationary phase. In other words, when the oven/column temperature is well above the boiling point of the components their retention time, R_t, will be zero! If on the other hand, the oven/column temperature is too low, then the components will remain adsorbed inside the column or in the stationary phase and will not elute from the column even after a long time. In this case the retention time, R_t, of the components will be prohibitively high [37],[39].

2.10.3.2 Column Length

The column length has a direct influence on retention time (R_t); the quality of resolution (R); and on the carrier gas pressure. The influence of column length on resolution of two components with similar R_t is illustrated in Figure 2.14 the long columns (50, 60 and 105 m) give high R_t by requiring a long time for analysis but the peaks are well separated from each other. Such long columns are suitable for complex samples. However, the commonly used column length is 25 to 30 m which provides a compromise between analysis time and resolution. Shorter length columns of around 10 to 15 m are also sometimes used for easily separated few component samples, because the time of analysis is much shorter [37], [40].

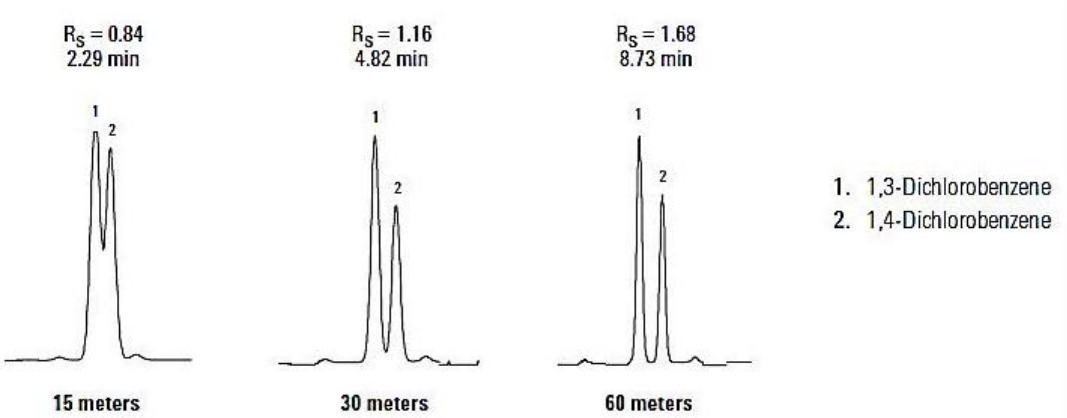


Figure 2.14: Effect of column length [40]

2.10.3.3 Column Internal Diameter

The small diameter columns (I.D) 0.15, 0.18 and 0.25 mm increase retention time, R_t, and therefore provide good peak resolution, R, but they lack the capacity for sample load, which means only a very small sample can be injected. On the other hand columns with larger diameters such as 0.32 mm I.D have larger sample capacity. Samples of 2 or more µL may be injected in the splitless mode whereas smaller diameter columns are injected less and are operated in the split mode. The effect of column internal diameter on peak resolution is illustrated in Figure 2.15 [40].

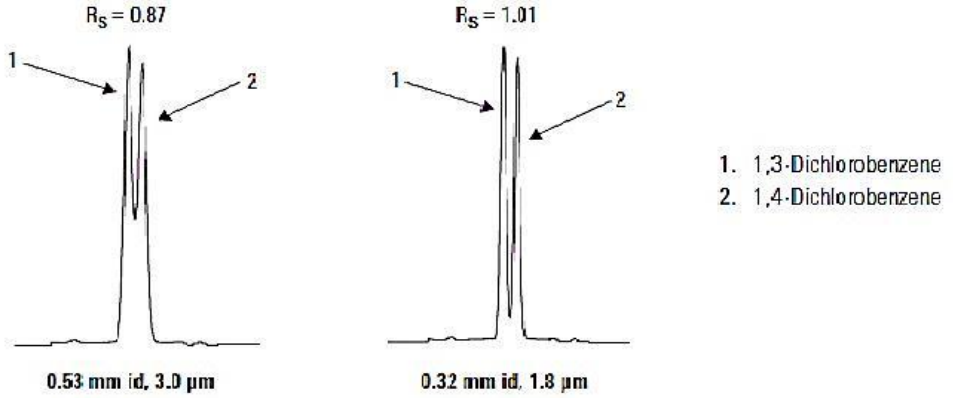


Figure 2.15: Effect of column internal diameter [40]

2.10.3.4 Column Film Thickness

The film thickness of the stationary phase in the column vary between 0.1 to 5 µm. It has a direct influences on R_t, and on the quality of resolution (R) as shown in Figure 2.16.

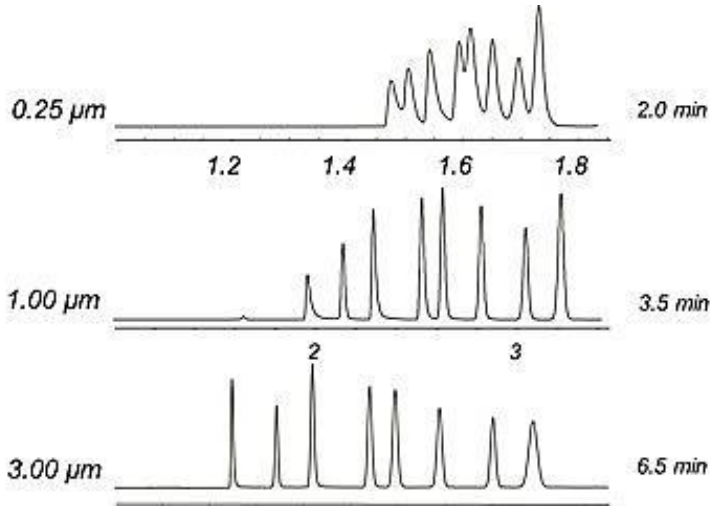


Figure 2.16: Effect of column film thickness [40]

Thicker films give higher R_t and better R, which is perfect for volatile and complex substances. Also the loading capacity increases with increasing film thickness. Typical commercially available film thickness are 0.18, 0.25, 0.32, 0.45 and 0.50 µm but most commonly used or recommended ones are 0.25 and 0.32 µm [40]. Table 2.6 Summarizes the relationship between column characteristics and column dimensions below.

Table 2.6: Summary of the relationship between column characteristics and column dimensions [37]

Inlet	Values	Characteristics and Applications
Length	10 m	• Fast separations • Use with thin films, small inside diameter • Pneumatics and flows most critical
	15-30 m	• Most common • Use with medium film thickness and inside diameter
	100 m	• Slowest • Very high peak capacity, complex samples.
Inside diameter	0.1 mm	• High pressure drop required • Lowest sample capacity • Fast separations • Most efficient but usually short column
	0.2, 0.25 and 0.32 mm	• Most common • Combination of good resolution and ease of use.
	0.53 mm	• Alternative to packed column • Used with thick film
Film thickness	0.1µm	• Thinnest • High boiling analytes • Fastest separations • Most efficient
	0.1 - 1µm	• Most common • Compromise between ease of use and efficiency.
	> 1 µm	• Thickest • Low boiling analytes (gases solvent)

2.10.3.5 Stationary Phase

The stationary phase is the film coating material in the inner wall of a capillary column. It can be quiet difficult to choose which stationary phase to use for a particular analysis because there is a very large selection available. In order to resolve this issue one must consider the polarity, volatility, thermal stability, inertness and solvating properties of the stationary phase as well as the nature of the intermolecular interaction between the solutes and the stationary phase [38], [41].

Some commonly used commercial stationary phases are listed in Table 2.7.

Table 2.7: Some Common Stationary Phase for GC [38]

Stationary Phase	Common Trade Name	Maximum Temperature °C	Common Applications
Polydimethyl siloxane	OV-1, SE-30	350	General purpose nonpolar phase, hydrocarbons, polynuclear aromatics, steroids, PCBs
5% Phenyl-polydimethyl siloxane	OV-3, SE-52	350	Fatty acid methyl esters, alkaloids, drugs, halogenated compounds
50% Phenyl-polydimethyl siloxane	OV-17	250	Drugs, steroids, pesticides, glycols
50%Trifluoropropyl-polydimethyl siloxane	OV-20	200	Chlorinated aromatics, nitroaromatics, alkyl-substituted benzenes
Polyethylene glycol	Carbowax 20M	250	Free acids, alcohols, ethers, essential oils, glycols
50% Cyanopropyl-polydimethyl siloxane	OV-275	240	Polyunsaturated fatty acids, rosin acids, free acids, alcohols

The column frequently used in GC for EOs is HP-5ms, which is reported by the manufacturer to have the following characteristic properties:

- (5%-Phenyl)-methylpolysiloxane.
- Maximum temperature limit 325°C to 350°C.
- Identical selectivity to HP-5.
- Non-polar.
- Very low bleed characteristics, ideal for GC-MS.
- Excellent inertness for active compounds including acidic and basic compounds.
- Suitable for Semivolatiles, halogenated compounds, pesticides, drugs and amines.
- Improved signal-to-noise ratio for better sensitivity and mass spectral integrity.
- Bonded and cross-linked.
- Solvent rinsable.

- Equivalent to USP Phase G27.
- Similar phases: DB-5, HP-5, DB-5ms, Rtx-5, Rtx-5ms, Rxi-5ms, Rxi-5Sil MS, Equity-5, PTE-5, BPX-5, AT-5ms, ZB-5ms, SLB-5ms [40],[41].

The molecular structure of the stationary phase is shown in Figure 2.17.

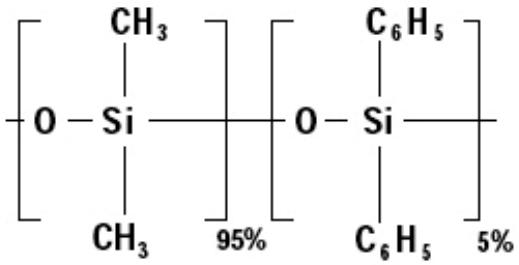


Figure 2.17: HP-5ms Stationary Phase

2.11 Qualitative Analysis

GC retention times R_t is the simplest qualitative tool that can be used to identify components in mixtures by comparison of the retention times, R_t, of the components with known standards. If R_t of a peak in the unknown sample matches a known standard then a positive identification may be made. However, when complex mixtures are analysed, some constituents elute together (co-elution) which can lead to false identifications and misinterpretations. In order to overcome this problem, Kovats developed a parameter called the Kovats' Index, which is based on the observation that if the symmetric series of logarithm of the adjusted, R_t are plotted against the carbon number, a linear relationship is obtained. From this he developed an equation to calculate a relative retention index that is almost constant and independent of GC operational parameters for isothermal chromatography. A similar

equation to calculate relative retention indices for temperature programmed chromatography was also developed [41], [43], [44].

2.11.1 Kovats Index

Kovats' Index is used to convert retention times, R_t, or adjusted retention times, $R_t^{'}$ into "constant" relative retention values that are more or less independent of operating and column conditions. This is achieved by relating the R_t of the species (unknown) to the R_t of two closely eluting n-alkanes one immediately before (*n*) and the other immediately after (*N*) the species in question. Two equations, one for isothermal and another for temperature programmed chromatography have been developed to calculate the relative retention time [41], [44], [45].

These two equations are reproduced below:

For isothermal chromatography,

$$I_{isothermal} = 100 \times \left[n + \frac{log\left(R'_{t(unknown)}\right) - log\left(R'_{t(n)}\right)}{log\left(R'_{t(N)}\right) - log\left(R'_{t(n)}\right)} \right]$$

Where:

$I_{\text{isothermal}}$ = Retention index,

n = the number of carbon atoms in the smaller n-alkane,

N = the number of carbon atoms in the larger n-alkane,

R'_t = the adjusted retention time.

For temperature programmed chromatography,

$$I_{temp\ prog} = 100 \times \left[n + \frac{R_{t(unknown)} - R_{t(n)}}{R_{t(N)} - R_{t(n)}} \right]$$

Where:

$I_{\text{temp prog}}$ = Retention index,

n = the number of carbon atoms in the smaller n-alkane,

N = the number of carbon atoms in the larger n-alkane,

R_t = the retention time.

2.12 Quantitative Analysis

The revolution in information technology in the past two decades has also transformed analytical chemistry, including GC-MS. Today, almost every stage of the analyses is automated and controlled by software and all data processing and calculations such as integration, quantitation as well as identification are carried out automatically by computers. Specifically in GC-MS, the whole instrument is automated and software controlled. Injection is done by an automatic sampler; the column oven temperature is programmed and controlled by the software. All operating parameters are continuously monitored and displayed on the computer screen. Data is collected and processed by the computer, and the chromatogram and detector data are electronically stored. After the analysis the stored data is further analysed either automatically or manually to provide qualitative and quantitative information about the constituents; namely what the individual components are and how much of each is present in the mixture. Finally, reports comprising the data in table form and plots of graphs selected by the operator are prepared almost instantaneously and made available as printable files [36].

The GC chromatogram and the MS data are used in two ways. First, the total ion chromatogram obtained for a particular peak is used to provide quantitative information about the amount of the component. Second the retention time together with MS spectrum of a component is used to identify its molecular structure. The latter is done by comparing the fragmentation pattern of the components mass-

spectrum against a large database of MS spectrums of known compounds whose spectrums were obtained under similar operating conditions [37].

For better quantification, however, there are two methods that are utilized in GC-MS to ensure a greater degree of accuracy and precision of the quantitative data. The first of these is the Calibration with Standards and the second is the Internal Standard Method. In the first method, a set of standards whose composition is similar to the "unknown" is prepared and its chromatogram obtained. Then, either the heights or areas of the peaks for the standards are plotted against their known concentration and a working calibration curve is obtained. This curve is then used to estimate the concentration of the "unknown". This method however requires frequent standardization in order to have high accuracy because of variations in the operational parameters of the GC [38].

The second method of using internal standards is somewhat better and eliminates some of the problems associated with variations in operational parameters of the GC. An accurately known amount of an internal standard is added to the samples as well as the external standards. The calibration plot for the standards is next plotted not for the peak height/area of the standard but for the ratio of standard analyte peak (either height or area) divided by the internal standard peak. This ratio plot provides the calibration curve. So when determining the unknown, data used is the ratio of the peak area of the unknown to the peak area of the internal standard. In this way much higher accuracy is obtained. This method requires that the peak for the internal standard is close to but still well separated from all the other constituents in the sample. Furthermore the sample to be analysed must not contain the compound to be used as the internal standard [38].

2.13 Thyme Essential Oils Around the World

In the Table 2.8 below, a summary of recent work on isolation and analyses of thyme EOs from around the world are provided. Also the table includes the method of extraction as well as the column types used in the GC-MS analyses.

Table 2.8: TEO around the world

T Species	T Country	Year	Isolation Method	GC-MS Column	Major components of TEO %	Ref.
Thymus dreatensis Batt.	Algeria	2014	HD Clevenger	HP-5ms capillary column (30 m x 0.25 mm x 0.25 µm)	thymol (28.1), γ -terpinene (18.7), thymyl methyl ether (10.9), *p*-cymene (8.8), and linalool (5.0)	46
Thymus vulgaris L.	Brazil	2012	HD Clevenger for 2h	DB-5 capillary column (30 m x 0.32 mm x 0.50 mm)	carvacrol (45.5), α-terpineol (22.9) *endo*-borneol (14.3) and caryophyllene (3.2)	47
Thymus vulgaris L.	Czech Republic	2013	Supercritical fluid extraction and HD	DB-5ms column (30 m x 0.25 mm x 0.25 µm)	thymol (15.3, 40.6), *p*-cymene (2.56, 9.2), γ –terpinene (1.78, 11.6)	48
Thymus capitatus and *Thymus vulgaris*	Egypt	2011	HD Clevenger for 3h	Fused silica capillary column HP (50 m x 0.32 mm) coated with Carbowax 20 M	carvacrol (53.16 & 48.23), thymol (15.17 & 12.74), *p*-cymene (4.19 & 3.63), α-pinene (4.30 & 3.35) and sabiene (4.30 & 2.10)	49
Thymus vulgaris L.	France	2013	HD Clevenger for 3h	HP-5ms capillary column (30 m x 0.25 mm x 0.25 µm	thymol (41.33), *p*-cymene (18.08), and γ-terpinene(13.12).	50
Thymus daenensis Celak.)	Iran	2014	HD	HP-5ms (60 m x 0.25 mm x 0.25 µm) capillary column	thymol (20 - 60.5) and carvacrol (20.1 - 63.4)	51
Shirazi thyme	Iran	2011	Ohmic-assisted hydrodistillation for 2h	HP-5ms capillary column (phenyl methyl siloxane,30 mm, 0.25 mm; 0.25 µm)	linalool (71.8) , α-pinene (4.3) carvacrol (4.1), linalyl acetate (1.7) , *p*-cymene (1.4), 1,8 cineol (1.4) and thymol (1)	52
Thymus serpyllum L.	Italy	2012	HD Clevenger for 3h.	Fused silica column; Equity-5 column (Supelco, Bellafonte, PA) (30 m x 0.25 mm x 0.25 µm)	carvacrol (35.8), carvacrol methyl ether (9.1), *p*-cymene (10.6), γ-terpinene (8.6) and thymol (6.0)	53
Thymus vulgaris L.	Jordan	2014	HD Clevenger for 4h	HP-5ms capillary column (30 m x 0.25 mm x 0.25 µm)	thymol (37.05), cis-dihydrocarvone (9.34), carvacrol (8.45), hydroxy-3-(3-methyl-2-butenyl)-3-cyclopenten-1-one (8.41), *p*-cymene (5.73), cis-sabinene (4.42), z-isoeugenol (3.342), and aromadendrene (3.42)	54

Table 2.8: Continued

T Species	T Country	Year	Isolation Method	GC-MS Column	Major components of TEO %	Ref.
Thymus vulgaris L.	Morocco	2009	HD Clevenger for 3h	HP-5 (cross-linked Phynel-Methyl Siloxane) column (30 m x 0.25 mm x 0.25 µm)	camphor (38.54), camphene (17.19), *α*-pinene (9.35), 1, 8-cineneole (5.44), borneol (4.91) and *β*-pinene (3.90)	55
Thymus serpyllum L.	Poland	2014	HD Deryng apparatus 3h and HD Clevenger for 2h	HP-5ms capillary column (30 m x 0.25 mm x 0.25 µm)	carvacrol (42.81–45.24), *γ*-terpinene (7.68–9.04), *β*-caryophyllene (5.28-9.10), *β*-bisabolene (5.76–6.91) and carvacrol methyl ether (4.92–6.09)	56
Thymus pulegioides L.	Romania	2009	Solid-phase extraction of infusion and hydrodistillate	Fused silica column DB-5MS (30 m x 0.32 mm x 0.25 µm)	In infusion : carvacrol (38.96), thymol (25.17), linalool (9.75) and thymoquinone (7.27), and in hydrodistillate: carvacrol (63.20) ,linalool (16.95) and thymol (15.55)	57
Thymus glabrescens	Serbia	2014	HD Clevenger for 4h	HP-5ms capillary column (30 m x 0.25 mm x 0.25 *µ*m)	geraniol (22.33), geranyl acetate (19.38) thymol (13.79), *α*-cubebene (5.51), linalool (5.49), o-cymene (4.73), *β*-bisabolene (4.08), isobornyl formate (2.87), *γ* –terpinene (2.75), neral (2.25)	58
Thymus capitatus	Sicily	2010	HD for 3h.	Fused silica capillary column Supelco, SPB-5 (15 m x 0.1 mm x 0.1 µm)	carvacrol ranging between 49 to 83 in 30 samples	59
Thymus sipyleus subsp. *sipyleus* var. *rosulans*	Turkey	2011	HD Clevenger for 4h.	SGE-BPX5ms capillary column (30 m x 0.25 mm x 0.25 µm)	carvacrol (30.0), thymol (14.5), *p*-cymene (10.2), *α*-terpinyl acetate (10.4), linalool (6.8) and *γ*-terpinene (3.4)	60

Chapter 3

MATERIALS AND METHODS

3.1 Chemicals

The details of the chemicals used in this work are listed below. They were used without further purification or treatment.

1- Diethyl ether $(C_2H_5)_2O$ (AR grade), MedikoKimya – Turkey.

2- Anhydrous sodium sulphate Na_2SO_4 (AR grade), MedikoKimya – Turkey.

3- Methanol CH_3OH 99.7% (GC grade) PhEur, Sigma Aldrich – Germany.

4- Commercial Thyme Oil, MedikoKimya – Turkey.

3.2 Plant Materials

Five separate thyme samples (as dry herb) were purchased from a local market in January 2015, in Famagusta – North Cyprus. Two of the samples were packaged dried herbs (Kekik) exported from Anatolia. The remaining three were the same species but two samples were specifically cultivated for commercial purposes while one had been collected from plants growing wild in the mountains in Morphou region. These samples were coded and labelled according to their country of origin, appearance (colour) and whether grown in the wild or cultivated, as shown in Table 3.1. They were also air dried before hydrodistillation.

Table 3.1: Thyme Samples

No.	Country of origin	Appearance	Code
1	Anatolia	Dark green	TRGD
2	Anatolia	Light green	TRGL
3	Cyprus	Light yellow	CYLY
4	Cyprus	Light green	CYLG
5	Cyprus (Wild)	Light yellow	CYWLY

3.3 Plant Drying Conditions

The five samples were air dried for 12 days at room temperature. The dried samples were then stored in tightly sealed dry glass jars until the extraction of the essential oils.

The photographs of each of the thyme samples after air-drying are shown in Figures 3.1 to 3.5 (taken by Wihad)

Figure 3.1: Sample TRGD

Figure 3.2: Sample TRGL

Figure 3.3: Sample CYLY

Figure 3.4: Sample CYLG

Figure 3.5: Sample CYWLY

3.4 Instruments

1- Mantle heater, Elektro.mag

2-Clevenger type Apparatus.

3- Agilent Technologies Model 7890A GC System coupled with 5975C VL MSD with Triple-Axis Detector with ALS.

3.5 The Isolation of the Essential Oils and Yields

The isolation of thyme EO was carried out by Hydrodistillation (HD) using a Clevenger type Apparatus according to the method recommended in the current European Pharmacopoeia [61]. Each sample extraction was carried out in duplicate.

1- Clevenger type Apparatus was setup (washed with distilled water (DW)).

2- The dried raw material sample was transferred into 1000 mL round bottomed flask and covered with DW, heated by heating mantle with regulator at 190°C with the apparatus as shown in Figure 3.6.

Figure 3.6: HD for Thyme with Clevenger-type Apparatus (taken by Wihad)

3- After the operating temperature reached the boiling point of water in the flask HD was continued for 4 hours.

4- Extracted TEOs were collected individually in weighed glass test tubes from the graduated part of the apparatus.

5- To each test-tube, 1.00 mL of diethylether was added to dissolve the TEO.

6- The test-tubes containing the TEOs were left open so as to ensure the evaporation of the added solvent diethylether.

7- Sufficient anhydrous sodium sulphate was put in plastic conical bottom screw-cap sample vial to eliminate any remaining water in the extracts and the mass of the vials recorded. Each vial was then labelled with the code of a TEO extract.

8- A micropipettor was used to quantitatively transfer each TEO extract from the glass tube to the weighed, appropriately labelled plastic vial. Each glass tube was then washed with a small portion of diethylether to dissolve any adhering EO and the washings were transferred to their corresponding plastic vial.

9- The vials were kept open for three days to allow for the diethylether to evaporate. No diethylether could be detected by smell in any of the vials after the first day.

10- The weight of each vial was measured twice; once, as M2 after one day of drying and again as M3, on the third day.

11- The mass of the extracts after one day (M2-M1) and after 3 days of drying (M3-M1) were calculated as the yield of TEO for each sample.

Thereafter all the TEO extracts in the vials were tightly sealed and kept in a dark and cool cupboard until further analysis. They varied in appearance from very light yellow to orange as shown in Figure 3.7. All the samples were treated in exactly the same way.

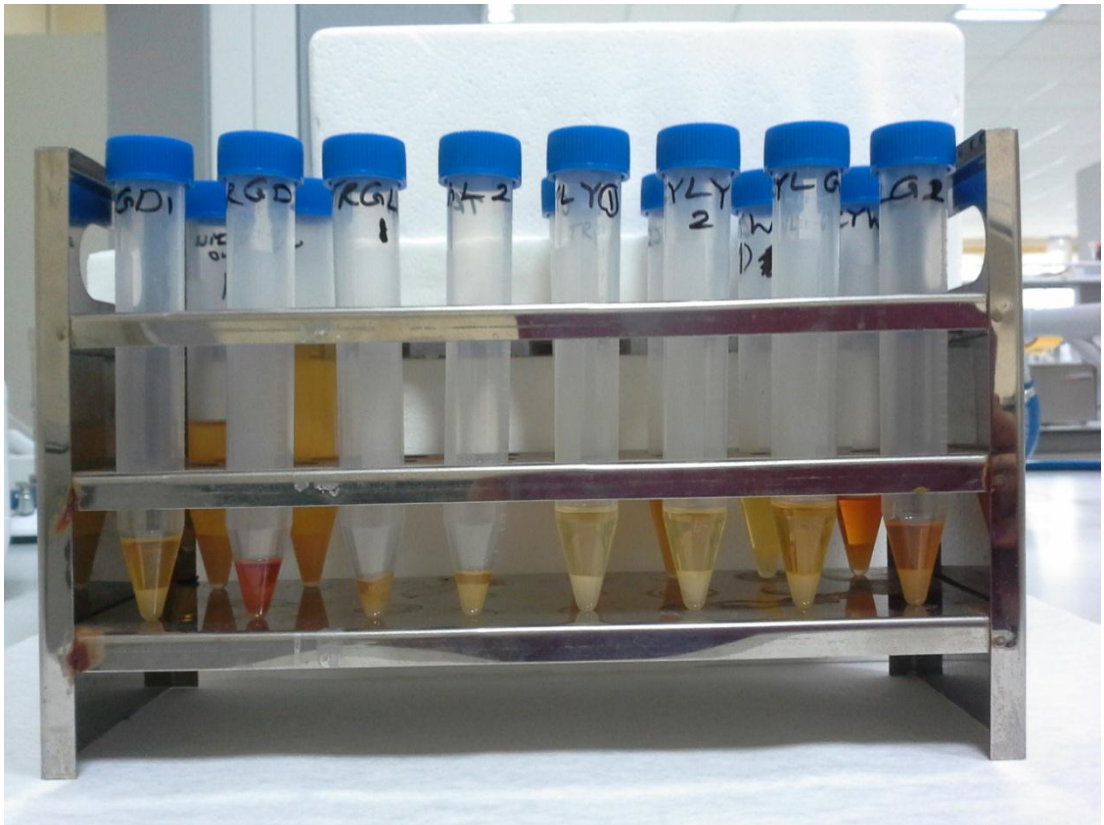


Figure 3.7: TEO after HD (taken by Wihad)

3.6 Sample Preparations for GC-MS Analysis

For analysis of the extracts by GC-MS, each extract was diluted with methanol and injected (by the automatic sampler) in to GC-MS without any further treatment.

For each extract, 20.0 μL of the extract was removed and placed in a vial using an automatic micro-pipette. To this 2.00 mL of GC grade Methanol (99.7% pure) was added. The vial was stoppered and shaken well to ensure through mixing. Subsequently these solutions were transferred into clean labelled GC-MS vials.

$$Concentration\ of\ extract = \frac{20\text{x}10^{-6}\ \text{L Extract}}{2\text{x}10^{-3}\ \text{L Methanol}} = \frac{20\mu\text{L}}{2\text{x}10^{-3}\ \text{L}}$$
$$= 10000\ \text{ppm}\ (by\ volume)$$

Following the analyses of these prepared solutions by GC-MS, one of the original extracts, namely sample CYLY1, was chosen for further treatment. For this extract, four separate concentrations were prepared and analysed by GC-MS. These four solutions were prepared by pipetting 5.0, 10.0, 15.0 and 20.0 μL of the extract CYLY1 into separate vials and to each 2.00 mL of GC grade methanol was added. To ensure the samples were dry, a small amount of anhydrous sodium sulfate on the tip of a microspatula was added. The solutions were then stoppered, shaken well and allowed to stand for 20 min, after which 1 mL was pipetted in to the GC-MS vials. In this way a range of concentrations that were 2500, 5000, 7500 and 10000 ppm by volume were obtained. These samples were labelled as EO20, EO21, EO22, EO15B respectively. The aim here was to attempt to see if we could obtain a meaningful relationship between EO concentration and total ion chromatogram. This, we thought, would help ensure better quantification of the unknowns.

3.7 Analyses of the TEO Extracts by GC-MS

The analyses of TEO samples were performed on an Agilent instrument GC System coupled with MSD with Triple - Axis Detector with ALS using the operating

conditions given in Table 3.2 [56]. Because of technical problems with the GC-MS, the samples prepared were analysed after a delay of 2 months.

Figure 3.8: GC-MS for TEO Analysis (taken by Wihad)

Table 3.2: GC-MS Method Parameter

GC Column	HP-5ms capillary column (5% Phenyl 95% dimethylpolysiloxane, non-polar) 30 m, 0.25 mm, 0.25 μm film thickness
Carrier Gas	Helium
Flow	1 mL / min
Injection mode	Split
Split ratio	30 : 1
Sample Injection Volume	1 μL
Inlets Heater	250°C
Pressure	24.768 psi
Total flow	65 mL / min
Septum Purge Flow	3 mL / min
Temperature Program	40°C hold for 5 min Then increase to 60°C at rate of 30°C / min Next to 230°C at rate of 6°C / min kept constant for 10 min then to 280°C at rate of 30°C / min
Final Temperature	280°C
Total run time	60.667 min
FID Heater H_2 flow Air flow	 280°C 40 mL / min 450 mL/min
MS Volt Solvent Delay Gain Factor	 70 eV 7.00 min 7.00 = 1224 V

For each TEO sample injected in to the GC-MS, a total ion chromatogram (TIC) was obtained by the MSD ChemStation (G1701EA E.02.02.1431 Copyright 1989 – 2011 Agilent Technologies, Inc.) software covering the molecular mass region from 50 to 500 Daltons. Each TIC was later used to identify and quantify the individual components in the TEOs.

Chapter 4

RESULTS AND DISCUSSION

4.1 Percent Yield of Essential Oils

The yield of essential oils extracted from the thyme samples by hydrodistillation are given in Table 4.1 below. It was observed that percent yield of EO varied from 0.3 % (for one of the Anatolian samples, TRGD1) to 6.8 % (for the wild Cyprus thyme CYLWY2). Generally the Cyprus thymes yielded greater percentage of EOs than the Anatolian specimens. Whether this is indicative of Anatolian thyme in general or is due to the low quality of the particular (imported) samples we obtained cannot be decided at this stage. More data about the origin, date of harvest and drying conditions of the samples need to be known before a conclusion can be made. On the other hand, however, the Cyprus thymes provided good yields. In fact, the intensity of the aroma from the Cyprus thymes at the time of purchase was much richer and stronger than the Anatolian samples, indicating a greater EO content.

4.2 Identification and Quantification of TEO Constituents

All TEO components were identified and quantified using the MSD ChemStation software. Identification of the components (peaks) were achieved by electronic comparison of their mass spectra against the mass spectrum library of Wiley8N05ST.L and NIST05a.L databases. Further confirmation of the identified compounds were also done by matching the order of their retention times against the corresponding retention time data provided in the book “Identification of Essential Oils” by Adams for the DB-5 column [62]. To illustrate the nature and format of the

Table 4.1: Hydrodistillation results and yield for thyme samples

No	Sample Name		HD Date	DW Qt. ml	Sample Qt. g	M1 g on 18/3	M2 g on 20/3	EO1 g on 20/3	M3 g on 23/3	EO2 g on 23/3	% Yield	EO Colour
1	TRGL1	Replicates	11/3	450	50	7.0750	8.0896	1.0146	8.0587	0.9837	1.97	Yellow
2	TRGL2		13/3	450	50	7.0826	7.5827	0.5001	7.5600	0.4774	0.95	Yellow
3	TRGD1	Replicates	10/3	450	50	7.1047	7.2828	0.1781	7.2774	0.1727	0.34	Yellow
4	TRGD2		13/3	225	25	7.0808	7.2455	0.1647	7.2402	0.1594	0.64	Yellow
5	CYLY1	Replicates	11/3	450	50	7.1090	8.9389	1.8299	8.8134	1.7044	3.41	Light yellow
6	CYLY2		12/3	225	25	7.1032	8.7081	1.6049	8.6014	1.4982	5.99	Light yellow
7	CYLG1	Replicates	12/3	450	50	7.1034	8.7977	1.6943	8.7079	1.6045	3.21	Yellow
8	CYLG2		13/3	300	29	7.0772	8.0640	0.9868	8.0132	0.9360	3.23	Orange
9	CYWLY1	Replicates	12/3	450	50	7.0595	10.3606	3.3011	10.2419	3.1824	6.36	Dark yellow
10	CYWLY2		13/3	200	20	7.0980	8.5719	1.4739	8.4579	1.3599	6.80	Orange
11	TRC1	Replicates	This is the commercial thyme oil, filtered and dried before use									Dark Yellow
12	TRC2											

data provided by the system and the software for a typical chromatogram, we reproduce in Figure 4.1 the screen image for the sample CYLG2, showing its TIC and FID based chromatograms, and the MS of one of its peaks eluting at 20.877 min.

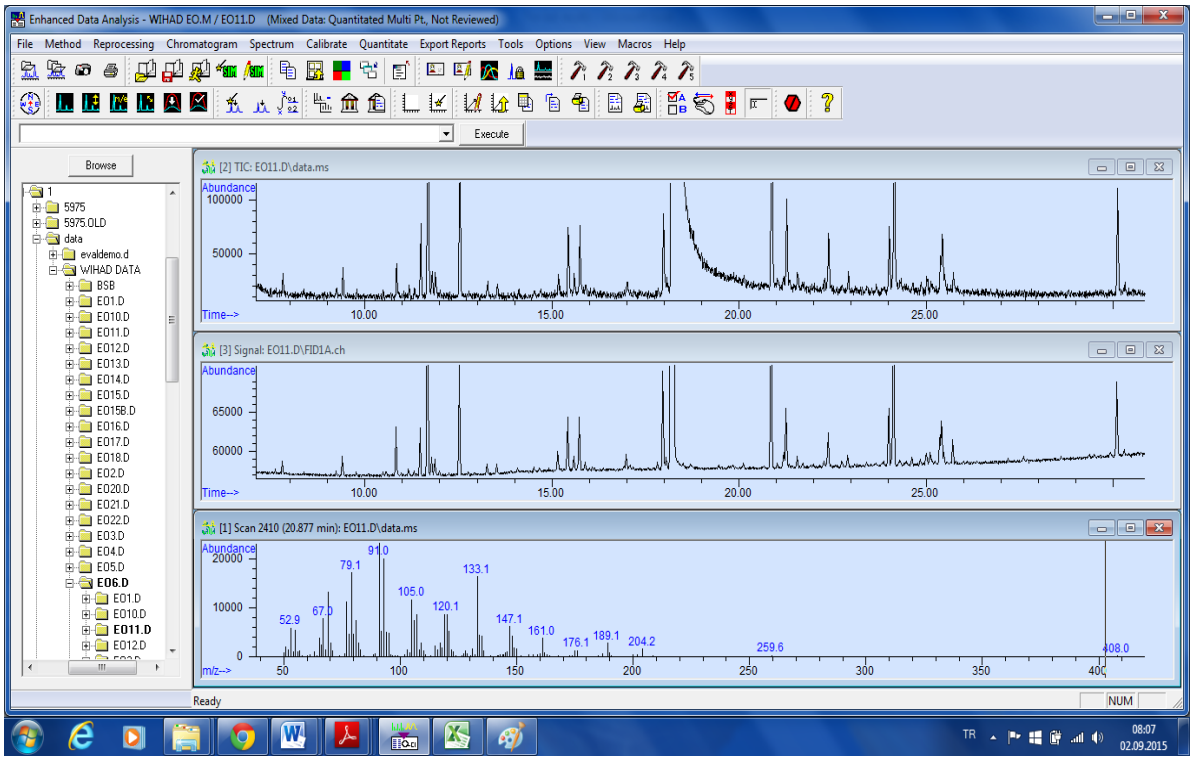


Figure 4.1: Screen image of TIC and FID of the sample CYLG2, and the MS of the peak at 20.877 min

In Figure 4.2 the screen image for the sample CYLG2, showing the MS spectrum of a peak eluting at 20.877 min compared to MS library indicate the identical matches of Caryophyllene.

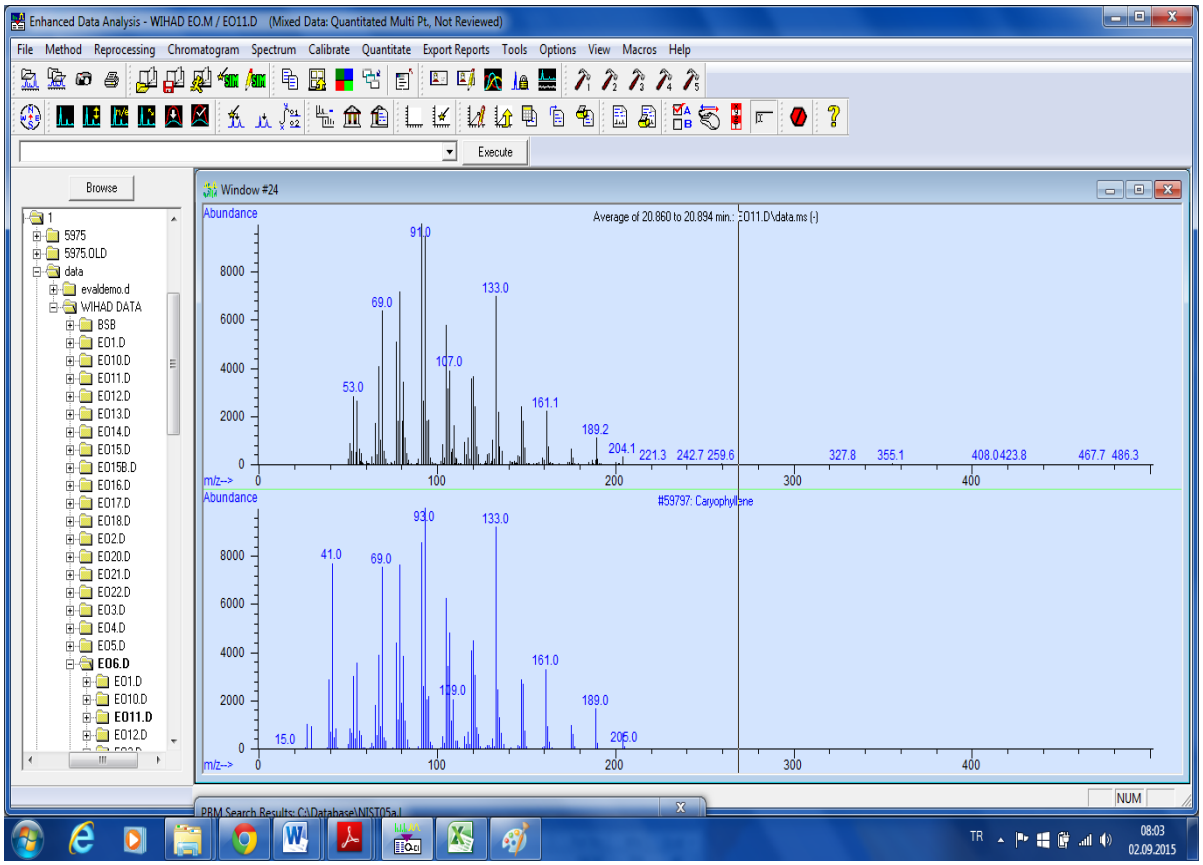


Figure 4.2: Screen image of MS spectrum for a peak at 20.877 min compared to MS library

The MSD ChemsStation software enables either automatic or manual peak identification or integration. Figures 4.3 and 4.4 shows the automatically identified TIC chromatograms of the samples CYWLY2 and TRGD2. In the first, many of the smaller peaks remain “undetected” whereas in the second chromatogram many of the smaller peaks are detected and identified. The difference is because of the “peak detection” settings. If set too high the software misses many peaks in the chromatogram and does not identify them. If set to very low, then even noise is detected as peaks! So it is either necessary to set the sensitivity at optimal level, or do the integration and identification manually.

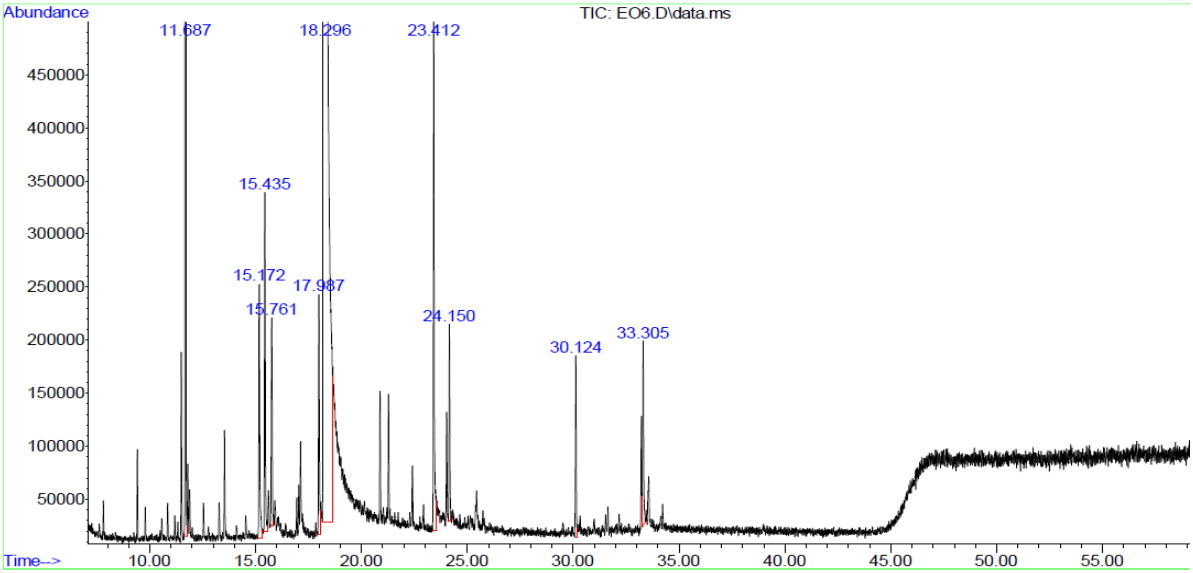


Figure 4.3: TIC Chromatogram of sample CYWLY2

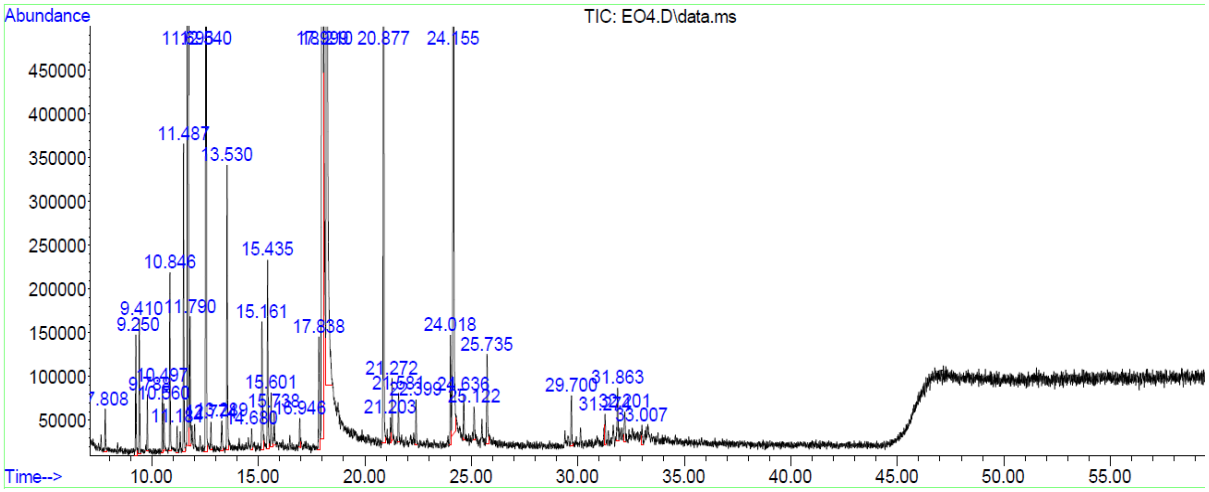


Figure 4.4: TIC Chromatogram of sample TRGD2

In this work, peak and component identifications were done manually by selecting each peak; integrating it with background correction and then identifying its structure/name by searching the peaks MS spectrum from the MS database. The Figures 4.5 and 4.6 given below illustrate the identification process of a peak at the retention time, R_t, of 18.239 minute. This peak was manually selected, the nearby background was subtracted, and the MS spectrum was searched from the database. The system returned a set of possible matches, specifying the compound to be 2-

methyl-5-(1-methyethyl) phenol (the IUPAC name of carvacrol). The percent quality of each match is also indicated adjacent to the name. For this peak, the % quality of the matches for the first three matches were 87, 91 and 72. More "match quality" parameters, and confidence level data are also calculated and provided by the software.

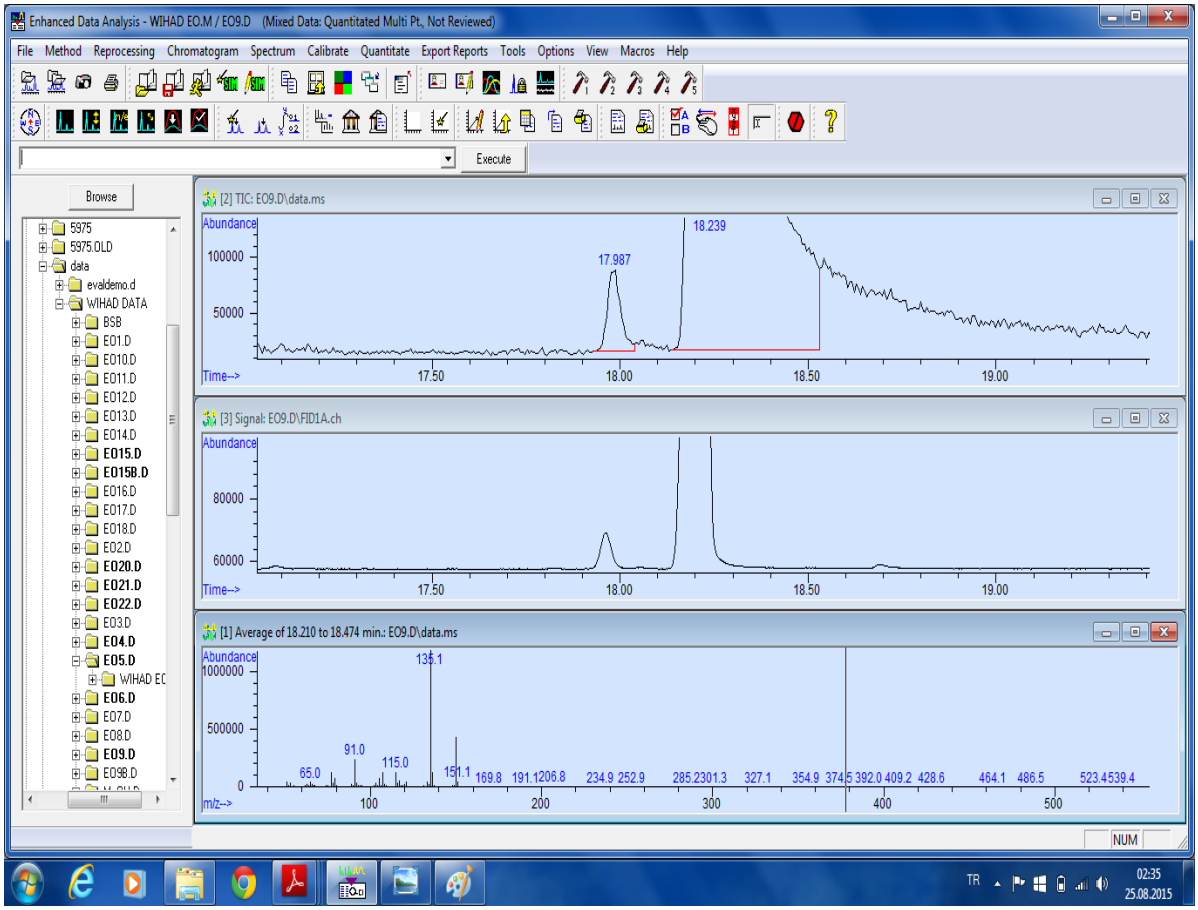


Figure 4.5: Screen image of identification process (1) of a peak at R_t 18.239 min

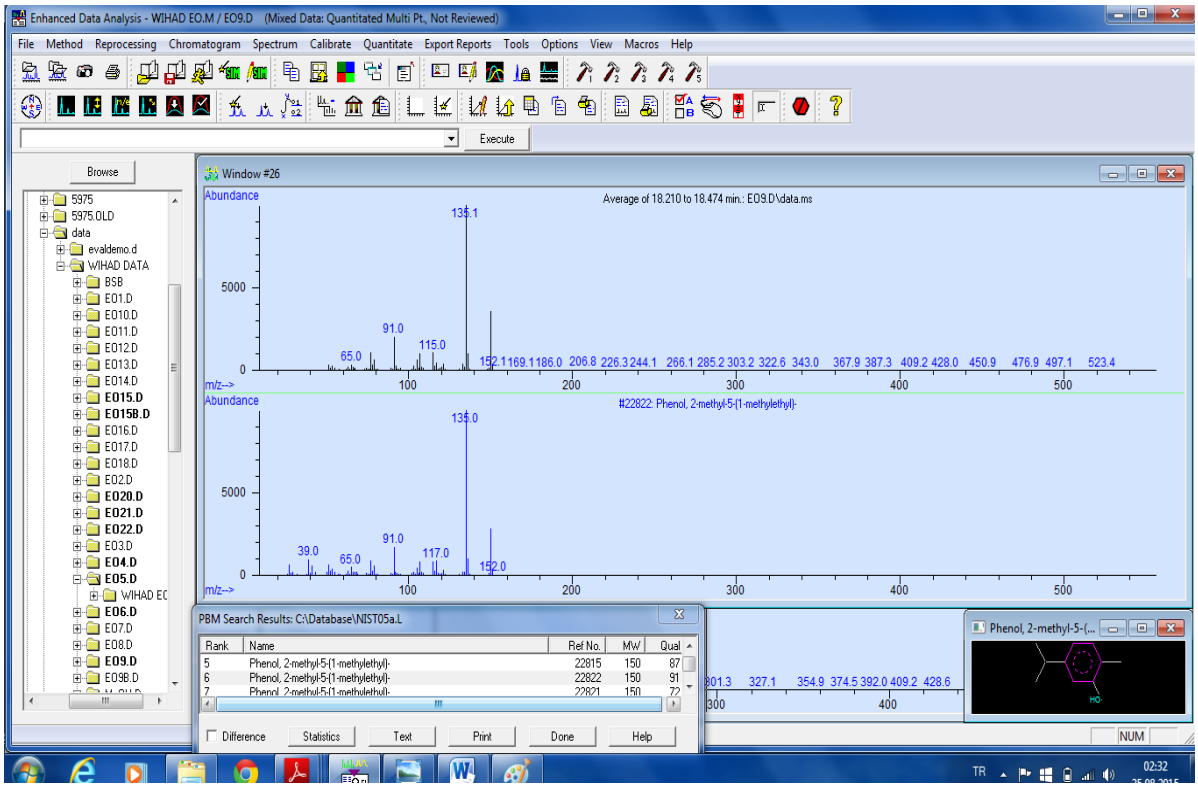


Figure 4.6: Screen image of identification process (2) of a peak at R_t 18.239 min

The same identification procedure was done for the peak at $R_{t\ of}$ 11.687 minute, which are shown in Figures 4.7 and 4.8. In this instance, the peak was identified as 1-methyl-4-(1-methylethyl) benzene (the IUPAC name of *p*-cymene) with a match quality of 91, 90 and 97% for the first three matches.

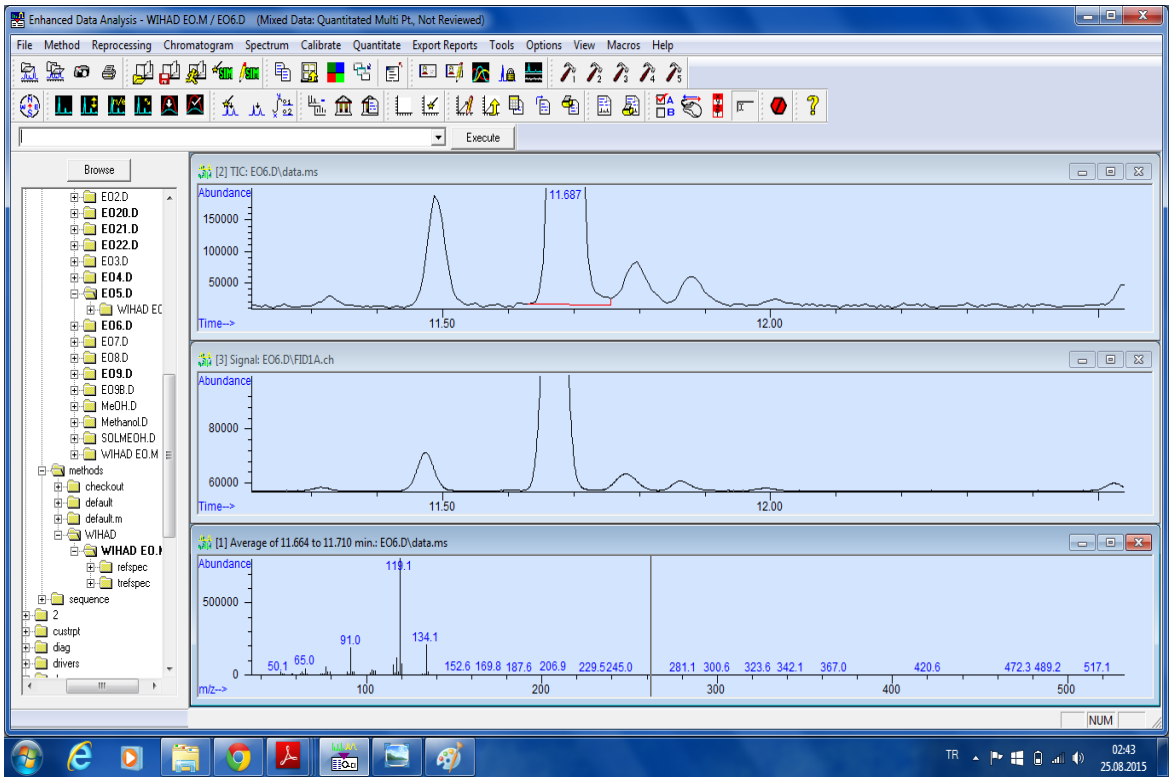


Figure 4.7: Screen image of identification process (1) of a peak at R_t 11.687 min

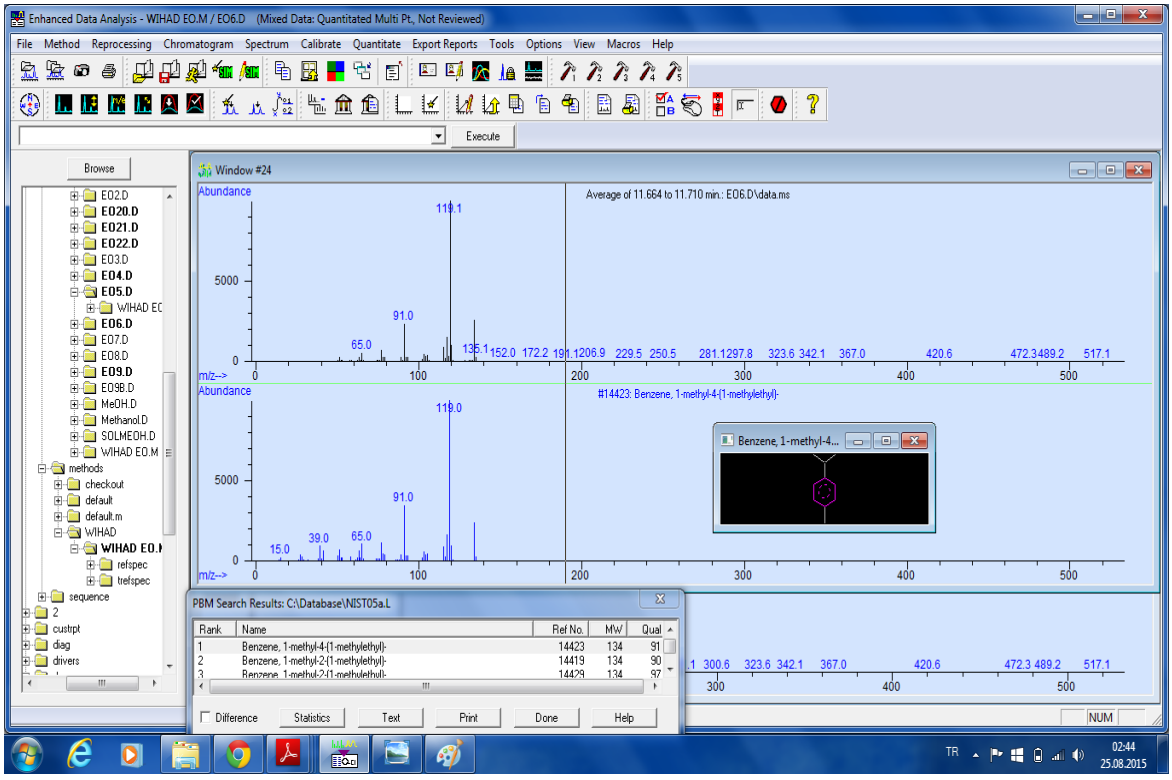


Figure 4.8: Screen image of identification process (2) of a peak at R_t 11.687 min

The software allows users to print predesigned reports or prepare their own custom made reports that include various parameters about the peaks that have been identified. These parameters include retention time, compound identity (common or IUPAC name) compound CAS number, peak height and/or peak area, and percentage of the component to the total.

In the present work, all the chromatograms were processed both automatically by autointegration and manually. More peaks and components were identified when manual integration was used.

4.3 Autointegration

The autointegration was done by RTE Integrator with minimum peak area 0.2% of largest peak. It gave two separate reports. The first report tabulated the peak heights and peak area percentages of the identified peaks in the chromatogram. The second report tabulated the first three matches for each identified peak, together with match quality percentage, IUPAC and common names, CAS number and the reference from which the MS library data was taken for the particular peak.

For the extracts analysed in this work, a total of 31 different constituents were detected but only 30 compounds were identified. Not all the identified compounds were present in every sample. The peaks were also quantified and were found to account (on average) for about 97 % of the total TEO extract. In all the samples, the major compound was carvacrol with an average presence of 93 %, followed by *p*-cymene at 3 % as shown in Table 4.2.

4.4 Manual Integration

As explained before (and illustrated in Figures 4.3, 4.4) a number of peaks were not identified during autointegration. Therefore all the chromatograms were manually

integrated to ensure identification and inclusion of all the peaks. Manual integration identified and quantified 45 constituents representing on average 95 % of the total extracted TEO. The major compounds were again found to be 91.7 % carvacrol and 2.8 % *p*-cymene as shown in Table 4.3. The additional peaks detected by manual integration, as well as some other differences between the two integration method results are summarised below:

- The autointegration had missed some minor compounds such as; 3-carene (11.327 min), trans-β-ocimene (12.008 min), *p*-cymen-8-ol (15.595 min), trans-dihydro carvone (15.910 min), D-darvone (17.003 min), duroquinone (17.106 min), 7-epi-α-cadinene (22.927 min) , *α*-selinene (25.437 min).
- Although manual integration detected three peaks at about 0.01 % level, no chemical identification could be made from the MS library.
- In general, the percentage of the major and minor TEO constituents are very similar whether integrated automatically or manually. As for the very small minor or trace components, they appear only with the manual integration.
- Replicate extract results (similarly named samples ending with 1 and 2) show that the level of agreement amongst some of the components is only about an order of magnitude. This means the percent value of a particular (especially minor or trace) component can differ by a factor of ten. The difference or variation reduces to about 10% for the major components. However, this variation does not occur with every component, some pairs agreeing very well with each other.

Table 4.2: Compounds identified and their percentage in TEO samples, determined by AutoIntegration

Compound	R_t Adams[1] min.	KI[2]	R_t Exp.[3] min.	TRC1	TRC2	TRGD1	TRGD2	TRGL1	TRGL2	CYLY1	CYLY2	CYLG1	CYLG2	CYWLY1	CYWLY2
m-Xylene			7.808				0.135								
α-Thujene	5.117	925	9.250				0.323								
α-Pinene	5.317	939	9.416	0.255	0.299	0.106	0.367			0.287				0.277	
Camphene	5.667	951	9.788				0.133								
1-Octen-3-ol	6.383	978	10.497				0.167								
β-Pinene	6.433	986	10.566		0.219	0.136									
β-Myrcene	6.800	992	10.846	0.184	0.201	0.309	0.466			0.560		0.189		0.191	
α-Phellandrene	7.250	1007	11.184				0.076								
α-Terpinene	7.617	1017	11.487	0.487	0.416	0.588	0.794			0.754	0.226	0.429		0.479	
p-Cymene	7.850	1026	11.687	7.316	7.281	18.861	27.531	0.414		6.139	2.057	4.903	1.017	6.972	2.905
β-Phellandrene	8.033	1045	11.796		0.163		0.406			0.303				0.228	
1,8-Cineole	8.083	1046	11.870		0.173					0.384					
γ-Terpinene	9.083	1060	12.540	1.403	1.423	6.955	6.314			1.728	0.498	1.117	0.440	0.582	
cis-*p*-Menth-2-en-1-ol	9.317	1123	12.759			0.127	0.082								
Terpinolene	10.133	1093	13.272			0.128	0.138			0.192					
1,3,6-Octatriene, 3,7-dimethyl-, (Z)-		1051	13.536	0.904	0.910	0.973	0.852	1.030	0.620	0.236					
Isoborneol	12.733	1146	15.166	0.353	0.329	0.529	0.532	0.606	0.495	0.291	0.211			0.324	0.329
Terpinen-4-ol	13.667	1177	15.435	0.544	0.602	0.560	0.609	0.481	0.296	0.629	0.396	0.453		0.398	0.366
α-Terpineol	14.200	1190	15.744	0.190	0.213	0.095	0.103			0.354		0.325		0.305	0.287
Thymol methyl ether	16.133	1235	16.940			0.121	0.087								
Thymol	18.550	1297	17.987	11.791	11.758	0.459	38.057	1.503	0.890	0.263	0.308	0.253	0.203	0.239	0.273
Carvacrol	18.950	1317	18.239	75.745	74.677	60.880	12.689	93.551	95.932	86.962	95.162	91.408	96.918	89.789	94.599
Caryophyllene	24.033	1417	20.877	0.426	0.410	3.949	3.333	1.681		0.918	0.664	0.703	0.689	0.217	
Aromadendrene	24.850	1436	21.272			0.219	0.198								
α- Humulene	25.450	1452	21.575			0.179	0.163								
(+)-Ledene		1482	22.404			0.139	0.141								
Ethanone,1-(2-hydroxy-4-methoxyphenyl)-			23.406												0.579
Isolongifolene, 9,10-dehydro-			24.018		0.156	0.360	0.379		0.213						
Caryophyllene oxide	30.617	1578	24.150	0.194	0.262	3.386	3.710	0.575	0.504		0.200	0.219	0.458		0.228
Not identified			25.730			0.254	0.361								

Table 4.3: Compounds identified and their percentage in TEO samples, determined by Manual Integration

Compound	R_t Adams[1] min.	KI[2]	R_t Exp.[3] min.	TRC1	TRC2	TRGD1	TRGD2	TRGL1	TRGL2	CYLY1	CYLY2	CYLG1	CYLG2	CYWLY1	CYWLY2
cis-Bicyclo[4,2,0]octa-3,7-diene			7.614				0.047			0.038				0.020	
m-Xylene			7.808			0.025	0.115		0.012	0.094	0.025	0.033	0.038	0.044	0.019
α-Thujene	5.117	925	9.250	0.123	0.110	0.080	0.280			0.036	0.017	0.035		0.018	
α-Pinene	5.317	939	9.416	0.258	0.224	0.097	0.355			0.267	0.104	0.103	0.055	0.263	0.063
Camphene	5.667	951	9.788	0.097		0.040	0.130			0.073	0.027	0.019		0.073	0.020
1-Octen-3-ol	6.383	978	10.497		0.066	0.061	0.161			0.059	0.014	0.020		0.032	
β-Pinene	6.433	986	10.566	0.136	0.162	0.120	0.176	0.02	0.008	0.044	0.023	0.025		0.023	0.013
β-Myrcene	6.800	992	10.846	0.203	0.205	0.284	0.463		0.015	0.529	0.150	0.177	0.056	0.179	0.026
α-Phellandrene	7.250	1007	11.184			0.042	0.060			0.121	0.043	0.045	0.020	0.059	0.012
3-Carene	7.400	1011	11.327			0.021	0.044			0.072	0.020	0.025	0.015	0.041	0.009
α-Terpinene	7.617	1017	11.487	0.368	0.420	0.565	0.773		0.009	0.735	0.219	0.407	0.124	0.460	0.146
p-Cymene	7.850	1026	11.687	7.149	7.330	17.977	26.723	0.105	0.405	6.017	2.018	4.767	1.000	6.772	2.905
β-Phellandrene	8.033	1045	11.796	0.130	0.178	0.267	0.379		0.013	0.294	0.093	0.147	0.043	0.200	0.0700
1,8-Cineole	8.083	1046	11.870	0.140	0.162	0.038	0.040	0.027	0.017	0.362	0.122	0.140	0.043	0.060	0.0400
trans-*β*-Ocimene	8.650	1032	12.008			0.049	0.072					0.019		0.030	
γ-Terpinene	9.083	1060	12.540	1.320	1.337	6.638	6.069			1.677	0.486	1.086	0.426	0.554	0.0200
cis-*p*-Menth-2-en-1-ol	9.317	1123	12.759			0	0.086		0.009		0.031				
Terpinolene	10.133	1093	13.272			0.116	0.114			0.175	0.054	0.061	0.033	0.110	0.027
1,3,6-Octatriene,3,7-dimethyl-,(Z)-		1051	13.536	0.844	0.840	0.926	0.797	1	0.589	0.206	0.129	0.137	0.018	0.105	0.082
Isoborneol	12.733	1146	15.166	0.354	0.278	0.495	0.524	0.568	0.476	0.213	0.175	0.134	0.046	0.271	0.271
Terpinen-4-ol	13.667	1177	15.435	0.487	0.514	0.546	0.594	0.472	0.304	0.592	0.373	0.43	0.172	0.379	0.344
p-Cymen-8-ol	13.950	1193	15.595	0.086		0.157	0.168	0.071	0.069		0.030	0.048	0.034	0.036	0.042
α-Terpineol	14.200	1190	15.744	0.181	0.209	0.096	0.065	0.106	0.083	0.342	0.266	0.327	0.143	0.281	0.274
trans-Dihydro carvone	14.750	1200	15.910								0.042	0.048		0.021	
Thymol methyl ether	16.133	1235	16.94	0.146		0.087	0.093	0.069	0.068	0.111	0.055	0.026		0.040	0.019
D-Carvone	16.400		17.003			0.044		0.138	0.146	0.065	0.049	0.036		0.053	0.043
Duroquinone			17.106									0.039		0.041	0.069
Thymol	18.550	1297	17.987	11.561	11.751	0.412	38.770	1.463	0.934	0.254	0.286	0.228	0.139	0.222	0.249
Carvacrol	18.950	1317	18.239	75.478	75.484	61.678	13.532	92.158	94.151	86.231	93.963	89.631	95.442	88.654	93.624
Caryophyllene	24.033	1417	20.877	0.438	0.378	3.766	3.235	1.639	0.042	0.892	0.651	0.665	0.689	0.193	0.111

Table 4.3: Continued

Compound	R_t Adams[1] min.	KI[2]	R_t Exp.[3] min.	TRC1	TRC2	TRGD1	TRGD2	TRGL1	TRGL2	CYLY1	CYLY2	CYLG1	CYLG2	CYWLY1	CYWLY2
Aromadendrene	24.850	1436	21.272			0.231	0.180	0.118	0.024	0.114	0.055	0.164	0.169	0.159	0.119
α- Humulene	25.450	1452	21.565			0.174	0.147	0.069		0.054	0.027	0.034	0.044		
(+)-Ledene		1482	22.404			0.142	0.109	0.072		0.052	0.039	0.102	0.118	0.069	0.055
7-epi-α-Cadinene	25.317	1522	22.927			0.032		0.068	0.039		0.014	0.025	0.043		
Ethanone, 1-(2-hydroxy-4-methoxyphenyl)-			23.406								0.019	0.092		0.111	0.540
Isolongifolene, 9,10-dehydro-			24.018	0.086		0.339	0.326	0.154	0.208			0.072	0.129	0.064	0.088
Caryophyllene oxide	30.617	1578	24.150	0.189	0.229	3.411	3.672	0.498	0.510	0.138	0.203	0.220	0.387	0.128	0.202
α-selinene	27.183	1494	25.437					0.053	0.130			0.062	0.100		
Not identified			25.730				0.324	0.068	0.055			0.028	0.050		
1,4-Cyclohexadiene, 1-methyl-4-(1-methylethyl)-			30.118					0.104	0.127	0.145	0.145	0.137	0.251	0.141	0.162
Not identified			33.210						0.017			0.031			
cis-β-Farnesene	25.617	1443	33.223										0.038	0.042	0.1080
Not identified			33.292						0.020			0.044			
(E)-β-Famesene		1439	33.304										0.039	0.038	0.204
Not identified			33.569												0.070

1. Adams R_t (min.).
2. Kovats Index.
3. Experimented Retention time (min).

4.5 Issues Adversely Affecting the Quality and Consistency of the Results

The main discrepancies and inconsistencies that have been observed in the results obtained during this work are:

Total yields and percentage composition of the replicate extracts show large variations even though extractions were carried out using homogenized samples and identical hydrodistillation conditions, and GC-MS analyses were carried out under constant conditions.

Possible causes for these may be:

1- The TEO extracts were left exposed to the atmosphere for two-three days in order to ensure evaporation of the solvent diethylether. This however may have resulted in the loss of volatile essential oil components, and also in the oxidative degradation of some of the components. Significant colour changes and darkening of the EOs of especially some samples were observed. Most noticeable were the samples TRC1, TRC2, TRGD1 and TRGD2. Table 4.4 shows the percentages of the main components in the extracts for these samples and the average values for the rest of the extracts.

Table 4.4: Potentially Degraded Samples

Compound	R_t min	TRC1	TRC2	TRGD1	TRGD2	The rest of samples
Carvacrol	18.239	75.5	75.5	61.7	13.5	86.2 to 95.4%
p-Cymene	11.687	7.1	7.3	17.9	26.7	0.1 to 6.8%
Thymol	17.987	11.6	11.7	0.41	38.8	0.14 to 1.5%

The large variations in carvacrol and thymol concentrations indicate the possible conversion of carvacrol to thymol.

2-Extracts were stored for 56 days at room temperature in plastic screw-capped vials until we were able to use the GC-MS. This may have also allowed time for degradation of the components.

3-Expert help was not available for operating the GC-MS instrument.

4-The "gas clean filter" which filters and dry the carrier gas for the GC-MS was saturated with water.

5-Structural rearrangements of terpenes may have taken place during the storage period which may have converted some compounds to their derivatives or isomers as proposed in Figure 4.9.

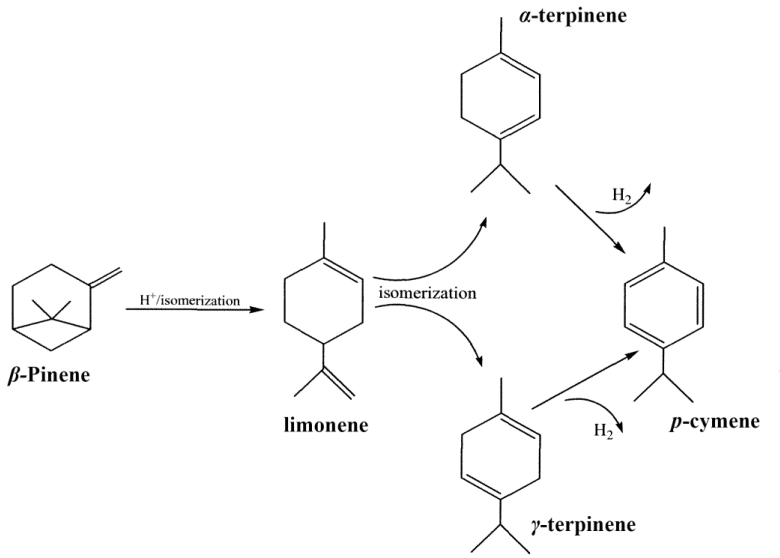


Figure 4.9: Potential mechanisms for the conversion of *β*-pinene to *p*-cymene [63]

4.6 Issues with Compound Identification Using the MS Library

The MS matching to identify compounds relies on the matching of the fragmentation patterns of the peak in question and the spectrum in the library database. When the peak eluting from the GC is too small or is contaminated with some impurity, the possibility of obtaining a good match is reduced and identification becomes difficult. However, beyond this problem, there are some compounds that are structurally so similar that even though they may have widely differing R_t values they may have

strongly similar MS spectrums. One such compound that we came across was *γ*-terpinene. In all the chromatograms in this work, the peaks with the retention time of 12.540, 12.759, 13.272, 15.435 and 15.744 minutes were identified as the first matched compound as *γ*-terpinene with high matching quality percentages. Why this is so is unclear. It may be that these compounds are closely related to *γ*-terpinene but have not been identified and included in the MS library yet. Based on data from Adams and Kovats, possible compounds that are causing this behaviour may be the derivatives shown below in Figure 4.10.

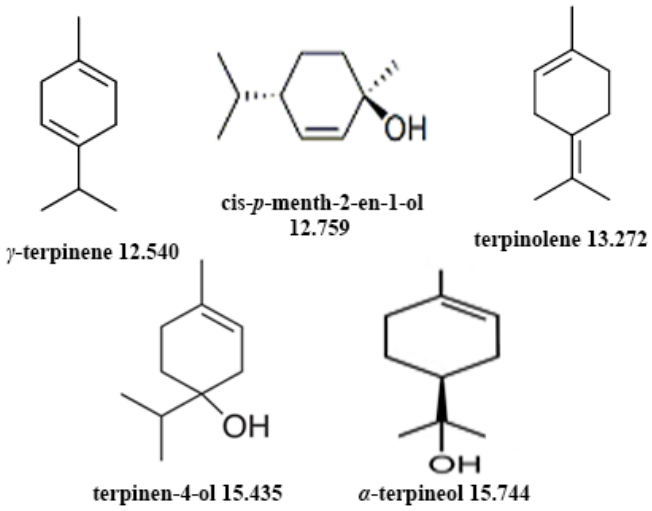


Figure 4.10: *γ*-terpinene derivatives and their R_t (min)

4.7 Yield and Composition Variations between Replicate TEO Extracts

The Cypriot samples CYLY, CYLG and CYWLY had been extracted in duplicate. The amount of starting material used in the extraction was not the same however as shown in the Table 4.5. The GC-MS results indicate that greater percent yields were obtained from smaller quantities extracted and similarly the percentage carvacrol in the extracts were greater for the smaller quantities. This may have to do with

improved efficiency of the hydrodistillation process when the flask is not too full or overcrowded. This is an issue that deserves further consideration.

Table 4.5: The variation of carvacrol percentages

Sample Code	Sample Qt. g	The Yield%	Carvacrol %
CYWLY1	50	6.36	88.6
CYWLY2	20	6.80	93.6
CYLY1	50	3.41	86.2
CYLY2	25	5.99	93.9
CYLG1	50	3.21	89.6
CYLG2	29	3.23	95.4

4.8 Composition Variations Within the same TEO Extract

Four different dilutions, namely 2500, 5000, 7500 and 10000 ppm, of the CYLY1 extract was prepared. These solutions were analysed with the GC-MS to see if the solutions would give the same composition and concentration. Concentration measurement and compound identification were done by manual integration and the results are listed in Table 4.6.

Table 4.6: Composition and Concentration of EOs in different CYLY1 solutions

Compound	**R_t Adams[1] min.**	**KI[2]**	**R_t Exp.[3] min.**	**CYLY1 2500ppm**	**CYLY1 5000ppm**	**CYLY1 7500ppm**	**CYLY1 10000ppm**
m-Xylene			7.808	0.049	0.030	0.061	0.036
α-Thujene	5.117	925	9.250		0.013	0.035	0.019
α-Pinene	5.317	939	9.416	0.174	0.092	0.211	0.105
Camphene	5.667	951	9.788	0.056	0.023	0.052	0.027
1-Octen-3-ol	6.383	978	10.497	0.030	0.011	0.037	0.016
β-Pinene	6.433	986	10.566	0.025	0.024	0.026	0.027
β-Myrcene	6.800	992	10.846	0.261	0.159	0.346	0.188
α-Phellandrene	7.250	1007	11.184	0.075	0.039	0.092	0.047
3-Carene	7.400	1011	11.327	0.032	0.023	0.042	0.026
α-Terpinene	7.617	1017	11.487	0.427	0.243	0.502	0.282
p-Cymene	7.850	1026	11.687	3.705	2.522	4.941	2.965
β-Phellandrene	8.033	1045	11.796	0.182	0.102	0.213	0.121
1,8-Cineole	8.083	1046	11.870	0.264	0.189	0.285	0.197
trans-β-Ocimene	8.650	1032	12.008			0.023	0.014
γ-Terpinene	9.083	1060	12.540	0.914	0.588	1.217	0.717
cis-*p*-Menth-2-en-1-ol	9.317	1123	12.759		0.018		0.014
Terpinolene	10.133	1093	13.272	0.118	0.061	0.114	0.071
1,3,6-Octatriene, 3,7-dimethyl-, (Z)-		1051	13.536	0.173	0.152	0.161	0.154
Isoborneol	12.733	1146	15.166	0.229	0.200	0.21	0.209
Terpinen-4-ol	13.667	1177	15.435	0.487	0.493	0.475	0.491
p-Cymen-8-ol	13.950	1193	15.595		0.040	0.045	
α-Terpineol	14.200	1190	15.744	0.260	0.315	0.299	0.294
trans-Dihydro carvone	14.750	1200	15.910		0.030	0.055	0.023
Thymol methyl ether	16.133	1235	16.94	0.095	0.069	0.072	0.058
D-Carvone	16.400		17.003	0.047	0.039	0.035	0.035
Duroquinone			17.106				0.008
Thymol	18.550	1297	17.987	0.255	0.264	0.230	0.260
Carvacrol	18.950	1317	18.239	91.157	93.004	88.994	92.360
Caryophyllene	24.033	1417	20.877	0.693	0.760	0.784	0.763
Aromadendrene	24.850	1436	21.272		0.077	0.081	0.077
α- Humulene	25.450	1452	21.565		0.030	0.049	0.036
(+)-Ledene		1482	22.404		0.042	0.039	0.050
7-epi-α-Cadinene	25.317	1522	22.927		0.025		0.020
Isolongifolene, 9,10-dehydro-			24.018		0.021		0.022
Caryophyllene oxide	30.617	1578	24.150	0.138	0.159	0.121	0.132
1.4-Cyclohexadiene. 1-methyl-4-(1-methylethyl)-			30.118	0.156	0.143	0.112	0.125

1. Adams R_t (min.).
2. Kovats Index.
3. Experimented Retention time (min).

Results show that:

- for some of the trace compounds, they are undetected in some of the solutions.
- Within an order of magnitude, there are no significant differences in the concentrations but for minor and trace constituents there are large variations in the different solutions.
- For the major constituent, carvacrol, there is about 5 % difference in the concentration for the different solutions.

Why there is such variation is difficult to explain at this moment. We suspect the differences do not arise from the actual composition of the solutions but that they arise from the variations in the GC-MS data. More work with high quality standards needs to be carried out to calibrate and tune the method.

Chapter 5

CONCLUSION

In this study locally obtained Cyprus and Anatolian dried thyme samples were subjected to hydrodistillation to extract their essential oils and then analysed by GC-MS in order to establish the composition and concentrations of the essential oils. Motivation for this undertaking was to try and assess the possible yields and quality of the essential oils that may be obtained from this locally available, renewable and pharmaco-chemically valuable source due to the many useful biological and pharmacological properties of these essential oils.

The results obtained showed that;

- Yields of EOs obtained were as high as 6.8% by dry plant weight, especially from Cyprus wild grown thyme sample. Anatolian thyme samples produced much lower yields.
- GC-MS results showed that at least 45 identifiable compounds were isolated from the samples, in varying concentrations; from a few tenth of a percent to around 90%.
- The major component in all the samples were found to be carvacrol at a concentration of around 90% of the total EO extracted, with lesser but significant quantities of *p*-cymene (average of 3 %).
- The variability and apparent inconsistencies in replicate or duplicate results in composition of EOs indicate that better extraction and storage regimes need

to be employed in the future so as to obtain more consistent and reproducible data.

- Thyme samples grown under different conditions and harvested at different times should be further investigated to increase the yield, and improve the quality of the EOs.

In conclusion, our results show that local production of EOs from locally available thyme is viable and that further research should continue in this field.

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