Sudan University of Science and Technology

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Investigation of Phytochemicals From Parsley Fixed Oil by GC-MS and Antimicrobial Potency of the Oil

دراسة مكونات الزيت الثابت لنبات البقدونس بالكروموتغرافيا الغازية – طيف الكتلة ومقدرة الزيت التضادية للمايكروبات

A Thesis Submitted in Partial Fulfillment of the Requirements of the M.Sc. Degree in Chemistry

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استهلال

بسم الله الرحمن الرحيم

قال تعالى :

﴿قَالُوا سُبْحَانَكَ لَا عِلْمَ لَنَا إِلَّا مَا عَلَّمْتَنَا إِنَّكَ أَنْتَ الْعَلِيمُ الْحَكِيمُ﴾

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Dedication

To .....

Soul of my father

My precious mother

My brothers and sister

Acknowledgement

First I would like to thank **Almighty Allah** for giving me the will and strength to finish this study.

I would like to express my deepest indebtedness and gratitude to my supervisor; Prof. Mohammed Abdel Karim Mohammed for his helpful suggestions, guidance, encouragement and useful criticism throughout the period of the study.

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Special thanks due to Dr: Mazin Yousif, for his infinite support. I would like to extend my thanks to my colleagues for their valuable assistance.

My deepest thanks are due to my family and friends who encouraged me to complete this work.

Abstract

In the present study, the fixed oil of *Petroselinum crispum (Parsely)* was extracted from seeds.The oil was analyzed by gas chromatography – mass spectrometry (GC-MS) and 62 components were identified .Major constituents were: Apiol (36.44%), 9-octadecenoic acid (Z)-, methyl ester (27.17%), 1, 3-benzodioxole, 4-methoxy-6-(2-propenyl) - (12.12%), 9, 12-octadecadienoic acid (Z, Z)- methyl ester (8.32%), hexadecanoic acid methyl ester (4.08%) and benzene, 1, 2, 3-trimethoxy-5-(2-propenyl) (2.09%).

The oil was screened for antibacterial and antifungal activities using five standard human pathogens. Agar well diffusion technique was used to assess the antimicrobial activity of the oil against two Gram positive *(Bacillus subtili and Staphylococcus aureus)*, two Gram negative *(Escherichia coli and Pseudomonas aeroginosa)* bacteria and one fungal species *(Candida albicans).*The results indicated that *Petroselinum crispum* oil possess bioactive compounds having significant antimicrobial properties.

المستخلص

استخلص الزيت الثابت لبذور نبات البقدونس و درس بواسطة تقنية الكروماتوغرافيا الغازية-طيف الكتلة حيث اتضح ان الزيت يحتوي على 62 مكونا ، المكونات الرئيسية هي :

Apiol (36.44%), 9-octadecenoic acid (Z)-, methyl ester (27.17%), 1, 3-benzodioxole, 4-methoxy-6-(2-propenyl) - (12.12%), 9, 12-octadecadienoic acid (Z, Z)-, methyl ester (8.32%), hexadecanoic acid methyl ester (4.08%) and benzene, 1, 2, 3-trimethoxy-5-(2-propenyl) (2.09%).

ثم اخضع زيت البقدونس لاختبارات بيولوجية كمضاد للميكروبات حيث استخدمت اربعة أنواع من البكتريا القياسية وهي:

Gram positive *(Bacillus subtili and Staphylococcus aureus)*, Gram negative *(Escherichia coli and Pseudomonas aeroginosa)*.

ونوع واحد من الفطريات وهو:*(Candida albicans)*

وقد اوضحت نتائج الاختبارات البيولوجية ان الزيت الثابت لبذور نبات البقدونس يحتوي على مواد مثبطة لعمل الميكروبات.

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Chapter one

Introduction

1. Introduction

1.1. Essential Oils

Since ancient times, essential oils are recognized for their medicinal value and they are very interesting and powerful natural plant products. They continue to be of paramount importance until the present day. Essential oils have been used as perfumes, flavors for foods and beverages, or to heal both body and mind for thousands of years[1-4].

Record findings in Mesopotamia, China, India, Persia and ancient Egypt show their uses for many treatments in various forms. For example, in ancient Egypt, the population extracted oils by infusion. Later; Greeks and Romans used distillation and thus gave aromatic plants an additional value.

With the advent of Islamic civilization, extraction techniques have been further refined. In the era of the Renaissance, Europeans have taken over the task and with the development of science the composition and the nature of essential oils have been well established and studied[5-8].

Essential oils (also called volatile or ethereal oils, because they evaporate when exposed to heat in contrast to fixed oils) are odorous and volatile compounds found only in 10% of the plant

kingdom and are stored in plants in special brittle secretory structures, such as glands, secretory hairs, secretory ducts, secretory cavities or resin ducts[9-16].

The total essential oil content of plants is generally very low and rarely exceeds 1% [17], but in some cases, for example clove (*Syzygium aromaticum*) and nutmeg (*Myristica* fragrans), it reaches more than 10%. Essential oils are hydrophobic, are soluble in alcohol, non-polar or weakly polar solvents, waxes and oils, but only slightly soluble in water and most are colourless or pale yellow, with exception of the blue essential oil of chamomile (*Matricaria chamomilla*) and most are liquid and of lower density than water (sassafras, vetiver, cinnamon and clove essential oils being exceptions)[18,19].

1.1.1.Extraction of essential oils

Oils contained within plant cells are liberated through heat and pressure from various parts of the plant matter; for example, the leaves, flowers, fruit, grass, roots, wood, bark, gums and blossom.

The extraction of essential oils from plant material can be achieved by various methods, of which hydro-distillation, steam and steam/water distillation are the most common method of extraction[2,17,20].

Other methods include solvent extraction, aqueous infusion, cold or hot pressing, effleurage, supercritical fluid extraction and phytonic process[21-25].

This later process has been newly developed; it uses refrigerant hydrofluorocarbons solvents at low temperatures (below room temperature), resulting in good quality of the extracted oils.

Thus, the chemical composition of the oil, both quantitative and qualitative, differs according to the extraction technique. For example, hydro-distillation and steam-distillation methods yield oils rich in terpene hydrocarbons. In contrast, the super-critical extracted oils contained a higher percentage of oxygenated compounds[26-29].

Some of the extraction methods are given below:

i)Hydrodistillation

The technique involves distillation of water that is in direct contact with fresh or sometimes dried macerated plant materials. Plant material is grinded and weighed, then transferred into the Cleset up. Plant material is heated in two to three times its weight of water with direct steam.

The distillation vessel is heated over heating mantle and the water vapour and oil are removed through a water cool condenser.

ii)Hydrodiffusion

Hydrodiffusion is a method of extracting essential oils in which steam at atmospheric pressure (low-pressure steam <0-1 bar) is passed through the plant material from the top of the extraction chamber, thus resulting in the oils that retain the original aroma of the plants[30] .

iii)Cold pressing

Another method of extracting essential oil that has not found high application in scientific research is cold pressing. It is used to obtain citrus fruits oils such as bergamot, grape fruit, lemon, lime, etc.

The fruits to be extracted are rolled over a trough with sharp projections that penetrate the peels, this pierce the tiny pouches containing the essential oil. The whole fruit is pressed to squeeze the juice and is separated from the juice by centrifugation.

iv)Steam distillation

This is the most common method of extracting oils and is the oldest form of essential oils extraction. In this technique, the desired plant (fresh or sometimes dried) is first placed into the vessel. Next steam is added and passed through the plant that contains the plants aromatic molecules or oils.

Once upon, the plant releases these aromatic molecules, the fragrant molecules travel within a closed system towards the

cooling device. Cold water is used to cool vapours. As they cool, they condense and transform into a liquid state.

v)Microwave assisted process (MAP)

The MAP process uses microwave to excite water molecules in plant tissue causing the cells to rupture and release the essential oil trapped in the extra cellular tissue of the plants[31].

This technique has been developed and reported by many authors as a technique for extraction of essential oils in order to obtain a good yield of the essence and to reduce the time of extraction [32-35].

1.1.2. Chemical constituents of essential oils

Essential oils are highly complex mixtures of volatile compounds, and many contain about 20 to 60 individual compounds, but some may contain more than 100 different components[36-39], such as jasmine, lemon and cinnamon essential oils.

The major volatile constituents are hydrocarbons (e.g. pinene, limonene, bisabolene), alcohols (e.g. linalol, santalol), acids (e.g. benzoic acid, geranic acid), aldehydes (e.g. citral), cyclic aldehydes (e.g. cuminal), ketones (e.g. camphor), lactones (e.g. bergaptene), phenols (e.g. eugenol), phenolic ethers (e.g. anethole), oxides (e.g. 1,8 cineole) and esters (e.g. geranyl acetate)[40].

All these compounds may be classified into two main categories: hydrocarbons and oxygenated compounds[22,25,41-43].

Or also into terpenoids and phenylpropanoids[44-47] .

Terpenes usually refer to hydrocarbon molecules consisting of isoprene (2-methylbuta-1, 3-diene). The isoprene unit, which can build upon in various ways, is a five-carbon molecule. Two of the molecules of isoprene give monoterpenes, sesquiterpenes contain three molecule of isoprene, four isoprene gives diterpene. Isoprene units are obtained biosynthetically via mevalonate pathway[48].

1.1.3.Trade of essential oils

The knowledge of composition of essential oils and their therapeutic properties have contributed to the development of their cultivation and markets. Although only 100 species are well known for their essential oils, there are over 2000 plant species distributed over 60 families such as Lamiaceae, Umbelliferae and Compositae which can biosynthesize essential oils. There are about 3,000 essential oils, out of which approximately 300 are commercially important and are traded in the world market[5,49-51].

Essential oils constitute a major group of agro-based industrial products and they find applications in various types of industries, such as food products, drinks, perfumes, pharmaceuticals and cosmetics[52-57].

The world production and consumption of essential oils is increasing very fast [58]. Despite their high costs (due to the large quantity of plant material required), essential oil production has been increasing. The estimates of world production of essential oils

vary from 40,000 to 60,000 tonnes per annum and represent a market of approximately 700 million US $[59].

The predominately produced essential oils for industrial purposes are from orange, cornmint, eucalyptus, citronella, peppermint, and lemon[22], but the more commonly domestically used ones include lavender, chamomile, peppermint, tea tree oil, eucalyptus, geranium, jasmine, rose, lemon, orange, rosemary, frankincense, and sandalwood.

The countries that dominate the essential oils market worldwide are Brazil, China, USA, Indonesia, India and Mexico. The major consumers are the USA, EU (especially Germany, United Kingdom and France) and Japan.

1.1.4.Analysis of essential oils

The two main purposes of analysing essential oils are:

(i) To identify and quantify as many constituents as possible.

(ii) To evaluate the quality of the oils and detect any possible adulteration that may affect their usage. Analysis of essential oils is generally performed using Gas chromatography-mass spectroscopy [60].

1.1.5.Mechanism of the biological activities of essential oils

So far, there is no study that can give us a clear idea and be accurate on the mode of action of the essential oils. Given the complexity of their chemical composition, everything suggests that

this mode of action is complex, and it is difficult to identify the molecular pathway of action. It is very likely that each of the constituents of the essential oils has its own mechanism of action.

1.1.5.1. Antibacterial and antifungal action

Because of the variability of amounts and profiles of the components of essential oils, it is likely that their antimicrobial activity is not due to a single mechanism, but to several sites of action at the cellular level. Then, different modes of action are involved in the antimicrobial activity of essential oils. One of the possibilities for action is the generation of irreversible damage to the membrane of bacterial cells, that induce material losses (cytoplasmic), leakage of ions, loss of energy substrate (glucose, ATP), leading directly to the lysis of bacteria (cytolysis) and therefore to its death.

Another possibility of action is inhibition of production of amylase and protease which stop the toxin production, electron flow and result in coagulation of the cell content[61,62].

Antifungal actions are quite similar to those described for bacteria. However, two additional phenomena inhibiting the action of yeast are worth mentioning: the establishment of a pH gradient across the cytoplasmic membrane and the blocking of energy production of yeasts which involve the disruption of the bacterial membrane.

1.1.5.2. Antiviral activity

The complex mixture of essential oils usually shows a higher antiviral activity than individual compounds (due probably to synergism phenomena); with exception of β-caryophyllene which is the most famous antiviral compounds found in many different essential oils from different plant families. Different mechanisms of antiviral activity of different essential oils and their constituents seem to be present.

The antiviral activity of the essential oil is principally due to direct virucidal effects (by denaturing viral structural proteins or glycoproteins).

Proposed mechanisms suggest that essential oils interfere with the virus envelope by inhibiting specific processes in the viral replication cycle or by masking viral components, which are necessary for adsorption or entry into host cells, thus, they prevent the cell-to-cell virus diffusion [63].

1.1.6. Medicinal and Pharmacological uses of essential oils

Essential oils are valuable natural products used as raw materials in many fields, including perfumes, cosmetics, aromatherapy, phytotherapy, spices and nutrition, insecticides[30].

Aromatherapy is the therapeutic use of fragrances or at least mere volatiles to cure or mitigate or prevent diseases, infection and indisposition by means of inhalation[64].

Inhalation of essential oils or their individual volatile terpenes has a significant role in controlling the central nervous system. For instance, aroma inhibitition of storax pill essential oil and pre-inhalation of *Aconus gramineus* rhizome essential oils are used in Chinese folk medicine in the treatment of epilepsy[65,66].

The fragrance compounds, cis-jasmonate, which characterized the aroma of *Jasminum grandiflorum* have a tranquilizing effect on the brain upon inhalation[67].

They significantly increased the sleeping time of mice induced by pentobarbital. Cendrol, which is a major component of cardwood essential oil, shows a sedative effect and prolonged pentobarbital induced sleeping time on rats upon inhalation[68] .

The vapour of lavender essential oil or one of its main component linalool may also be applicable to the treatment of menopausal disorder through inhalation[69].

Lavender essential oil demonstrated an analgesic activity, mainly relevant after inhalation at the doses devoid of sedative side effects[70].

Strong in vitro evidence indicates that essential oils can act as antibacterial agent against a wide spectrum of pathogenic bacteria strains including:

Listeria monocytogenes, Linnocua, Salmonella typhimurium, Shigella dysentria and *Bacilluscerus*[71-73].

Thyme and oregano essential oils can inhibit some pathogenic bacteria strains such as *E.coli, Salmonella typhimurium*, *Salmonella entritidis* and *Salmonella choleraesuis*[74], with the inhibition directly correlated to the phenolic components carvacrol and thymol. Eugenol and carvocrol showed an inhibitory effect against the growth of four strains of *Escherichia* and *Listeria monocytogens*[75]

Also, the presence of phenolic hydroxyl group in carvacrol particularly is credited with its activity against pathogens such as *Bacillus cereus* [76,77]. Essential oil with high concentration of thymol and carvacrol e.g. oregano, savory and thyme, usually inhibit Gram positive more than Gram-negative pathogenic bacteria[78] .

Essential oils show bactericidal activity against oral and dental pathogenic microorganisms and can be incorporated into rinses or mouth washes for pre-procedural mouth control[79], general improvement of oral health, interdental hygiene and to control oral malodour [80].

Mouth rinses containing essential oils with chlorhexine gluconate are commonly used as preprocedural preparations to prevent possible disease transmission, decreases chances of postoperative infections, decreases oral bacterial load and decrease aerolization of bacteria[81] .

Besides their antibacterial and antifungal activities, essential oils have also been reported to possess interesting antiviral activities alternative to synthetic antiviral drugs. They have demonstrated virucidal properties with the advantages of low toxicity[82]; *Herpes simplex* virus (type III) causes some of the most common viral infections in human and can be fatal. Synthetic antiviral drugs have been used to treat Herpes infection[83].

Essential oils and their individual aroma components showed cancer suppressive activity when tested on a number of human cancer cells lines including glioma, tumours, breast cancer, leukaemia and others. Glioma is one of the most malignant human tumours[84].

A significant effect on the treatment of glioma using the sesquiterpene hydrocarbon element which is found in small amounts in many essential oils, it prolonged quality survival time of patients with glioma[85].

Antiangiogenic therapy is one of the most promising approaches to control cancer. Perillyl alcohol (POH) which is the hydroxlated analogue of d-limonene has the ability to interfere with angiogenesis[86].

POH either alone or with PA (perillic acid, the major metabolite of POH in the body), has the potential use as an anticancer drug that stimulates different types of tumour to apoptosis and inhibit their

proliferation of overcomes their resistance to chemo/radiotherapy[87].

Treatment of human leukaemia cells with eucalyptus oil showed morphological changes (fragmentation of DNA) indicating an induction of apoptosis[88]. The essential oil of lemon balm (*Mellisa oficinalis* L) was found to be effective against a series of human (A549, MCF-7, Caco-2, HL-60, K562) and a mouse cell line (B16F10)[89] and that of *Artemisia annua* L. Induced apoptosis of cultured SMMC-7721 hepatocarcinoma cells[90].

Essential oils are reported to have insecticidal properties essentially as ovicidal, larvicidal, growth inhibitor, repellence and antifeedant[91-93] .The influence of certain oils and their constituents on the reproduction of some insect species and on morphological changes in other has also been discussed[94].

1.1.7. Therapeutic benefits of essential oils

The feeding with aromatic herbs, spices and some dietary supplements can supply the body with essential oils. There are a lot of specific dietary sources of essential oils, such as example orange and citrus peel, caraway, dill; cherry, spearmint, caraway, spearmint, black pepper and lemon grass. Thus, human exposure to essential oils through the diet or environment is widespread. However, only little information is available on the estimation of essential oil intake. In most cases, essential oils can be absorbed

from the food matrix or as pure products and cross the blood brain barrier easily.

This later property is due to the lipophilic character of volatile compounds and their small size.

The action of essential oils begins by entering the human body via three possible different ways including direct absorption through inhalation, ingestion or diffusion through the skin tissue.

Essential oil compounds are fat soluble, and thus they have the ability to permeate the membranes of the skin before being captured by the micro-circulation and drained into the systemic circulation, which reaches all targets organs[95,96].

Another way by which essential oils enter the body is inhalation. Due to their volatility, they can be inhaled easily through the respiratory tract and lungs, which can distribute them into the bloodstream[2,97]. In general, the respiratory tract offers the most rapid way of entry followed by the dermal pathway

Oral ingestion of essential oils needs attention due to the potential toxicity of some oils. Ingested essential oil compounds and/or their metabolites may then be absorbed and delivered to the rest of the body by the bloodstream and then distributed to parts of the body.

Once essential oil molecules are in body, they interrelate with physiological functions by three distinct modes of action:

-Biochemical (pharmacological): Interacting in the bloodstream and interacting chemically with hormones and enzymes such as farnesene.

-Physiological: By acting (for example phytohormones) on specific physiological function. For example, the essential oil of fennel contains a form of estrogen-like compounds that may be effective for female problems such as lactation and menstruation.

-Psychological: by inhalation, the olfactory area of the brain (limbic system) undergoes an action triggered by the essential oil molecules and then, chemical and neurotransmitter messengers provide changes in the mental and emotional behavior of the person[43,98].

1.2. Gas chromatography (GC)

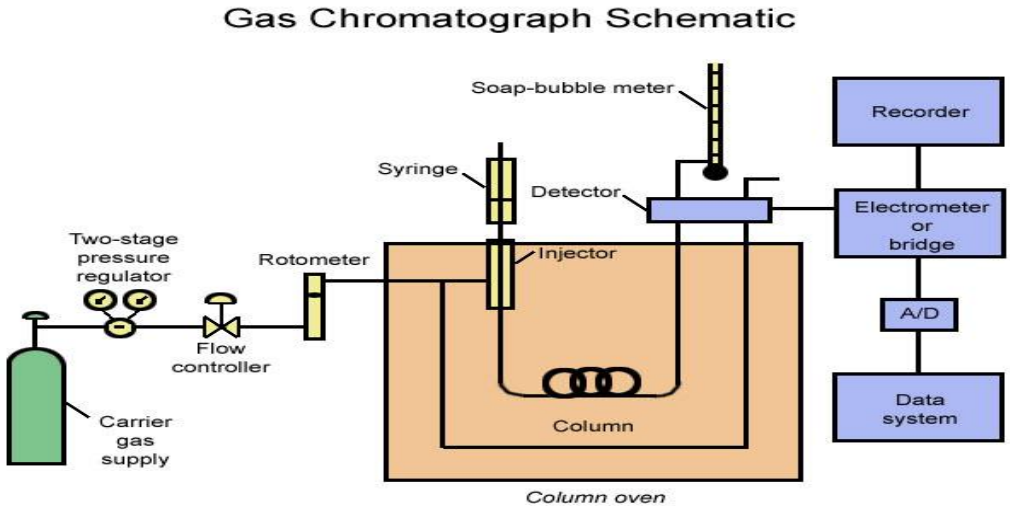
Gas chromatography is a powerful separation technique used in various fields of science such as forensic, environmental, food, agriculture and petrochemical industries[99,100].Gas chromatography is that form of chromatography in which a gas is the moving phase. The important seminal work was first published in 1952[101] when Martin and his co-worker James acted on a suggestion made 11years earlier by Martin himself in a Nobel-prize winning paper on partition chromatography[102].

It was quickly discovered that GC was simple, fast and applicable to the separation of many volatile materials-especially

petrochemicals, for which distillation was the preferred method of separation at that time. Theories describing the process were readily tested and led to still more advanced theories.

Simultaneously the demand instruments gave rise to a new industry that responded quickly by developing new gas chromatographs with improved capabilities. Three of the most relevant articles are: one focused on the work of Tswett, Martin, Synge and James[103], one emphasizing the development of GC instruments[104]; And the third, which contained over 200 references on the overall development of chromatography[105].

Today GC is a mature technique and a very important one. The world- wide market for GC instruments is estimated to be about $1 billion or over 30,000 instruments annually.



1.2.1. Gas chromatography instrument

In gas chromatography, the separation is mainly achieved as a result of partitioning of analytes between the gaseous mobile phase and a static phase (stationary phase) while transporting the volatile and semi-volatile analytes through an open-tubular capillary column. A capillary GC column is coated with a thin film of liquid-like stationary phase which serves to retain the gaseous analytes transported by the mobile phase. The most commonly used mobile phases, referred to as carrier gases, include helium or hydrogen. Stationary phases are differentiated based on their polarity. Differential partitioning of analytes occurs as a function of properties such as polarity and boiling points[106].

Compounds which have greater affinity for the stationary phase spend more time in the column, whereas those with lower affinity spend less time in the stationary phase and thus elute earlier[107].

A GC instrument consists of a carrier gas supply, sample introduction unit (injector), capillary column, oven and a detector; the operation of the most important instrumental parts will be discussed briefly below.

1.2.1.1. Injection in GC

The injection port allows the volatile sample to be introduced in vapour form via the carrier gas stream into the capillary column. The most common injector used in contemporary GC is the

vaporising split/splitless injector. This injector was invented to prevent overloading of the capillary column due to its low volume and capacity, which may affect resolution. The sample is introduced into a heated chamber, where vaporisation occurs.

Two modes of injection, split and splitless, can be used depending on the concentration of the target analyte. Split mode is mostly used when the analyte is present at high concentrations, while splitless is used when the concentration of the analyte is low[108].

Splitless injection requires effective utilisation of focusing mechanisms such as the solvent effect, cold trapping and stationary phase focussing to avoid injection band broadening.

1.2.1.2. The capillary column

The capillary column is coated with a stationary phase that permits separation of compounds to take place. Stationary phases in a capillary GC are differentiated according to their polarity. Non-polar stationary phases such as PDMS, sometimes with 5 to 50% phenyl PDMS groups added are commonly used for the separation of compounds ranging from non-polar to medium polar. In these phases, separation is governed primarily by differences in vapour pressure, since non-specific dispersion interactions occur between the analytes and the stationary phase.

A polar phase has been used for the analysis of petrochemical samples and also for TDOs, since they primarily contain

hydrocarbons. On semi-polar (14% cyanopropyl-phenyl 86% PDMS) or polar phases, mostly PEG, selective interactions such as hydrogen bonding and dipole interactions occur, and compounds are separated according to their polarity.

The column is housed in an oven for accurate temperature control. Since the separation of compounds in GC is primarily based on differences in the vapour pressures of compounds, temperature plays a crucial role.

Temperature programming, where an initial low oven temperature is increased as a function of time, is used to provide optimal resolution for a range of weakly and strongly retained analytes within an acceptable analysis time.

Column dimensions such as length, internal diameter, film thickness and stationary phase are selected based on the analysis goals. Short (10-20 m) columns are used for fast separation of relatively simple mixtures. For complex samples, longer columns (50-60 m) provide improved separation efficiencies at the cost of longer analyses.

Furthermore, reduction in the internal diameter (e.g. from standard 0.25 mm i.d. to 0.1-0.18 mm) increases the efficiency per unit length and also provides higher optimal mobile phase flow rates, thereby allowing speeding up of the analysis. This is evident from

the relationship between the column length, efficiency and optimal flow rate and the internal diameter:

$$N = \frac{L}{H} = \frac{L}{d_c}$$

$$u_{opt} = \frac{2D_M}{r_c}$$

Where N is the plate number, L is the length of the column, H is the height equivalent of a theoretical plate, u_{opt} is the optimal mobile phase linear velocity, D_M is the diffusion coefficient of the analyte in the mobile phase and dc and r_c are the column internal diameter and radius, respectively[106].

1.2.1.3. Detection in GC

Detectors in chromatography should ideally obey certain characteristics such as adequate sensitivity, stability and reproducibility, linear response range to solute concentration over a wide dynamic range, as well as being reliable and easy to use.

A wide range of detectors are compatible with GC, such as the nitrogen phosphorus detector (NPD), atomic emission detector (AED), thermal conductivity detector (TCD), sulphur chemiluminescence detector (SCD), electron capture detector (ECD), FID and MS, amongst others. Detectors are normally

selected depending on the analyte of interest and the analysis goals (i.e. selective detection of the target analytes or screening of unknowns, trace level analysis, etc).

Some detectors are universal, meaning that they respond to any or most sample constituents, for example FID, MS and AED. In contrast, selective detectors respond to certain group of compounds, for example the NPD (for nitrogen and phosphorus containing compounds) and the ECD (for halogenated compounds).

Among all these detectors, MS and FID are the most commonly used detectors for analysis of a wide range of samples containing organic compounds. These detectors are also most commonly used in petrochemical analysis. MS is the most powerful and universal detector that provides detailed information about the identity of the chemical constituents, while FID only gives information about the quantitative chemical composition of the sample.

The Flame ionization detector (FID) is one of the most widely used detectors in gas chromatography. In this detector, the effluent from the column is directed into a small air and hydrogen flame; ions formed in the combustion of organic compounds in the flame are detected.

Detection involves monitoring the current produced by collection of these ions by the collector electrode. The response of the FID is

related to the number of carbon atoms entering the detector, thus it is a mass sensitive detector.

This detector is not sensitive towards non-combustible permanent gases such as CO2, SO2, NO2, etc.[109].The FID is extensively used in a variety of fields for qualitative and quantitative analyses[110-112]. Since FID is a mass sensitive universal detector for hydrocarbons, it can be used to estimate the mass % composition of hydrocarbon mixtures[113], and indeed has been used for this purpose in TDO analysis[114].

1.2.2.Advantages and Disadvantages

GC has several important advantages as summarized in the list below:

• Fast analysis, typically minutes

• Efficient, providing high resolution

• Sensitive, easily detecting ppm and often ppb

• Nondestructive, making possible on-line coupling; e.g., to mass spectrometer

• Highly accurate quantitative analysis, typical RSDs of 1-5%

• Requires small samples, typically IJ-L

• Reliable and relatively simple

• Inexpensive

Recently, a more efficient column was made by connecting nine 50-m columns into a single one of 450 m total length[115]. While

much shorter than the Chrompack column, its efficiency was nearly 100% of theoretical, and it was calculated to have a plate number of 1.3 million and found capable of separating 970 components in a gasoline sample.

Because GC is excellent for quantitative analysis, it has found wide use for many different applications. Sensitive, quantitative detectors provide fast, accurate analyses, and at a relatively low cost.

Disadvantages of Gas Chromatography

• Limited to volatile samples

• Not suitable for thermally labile samples

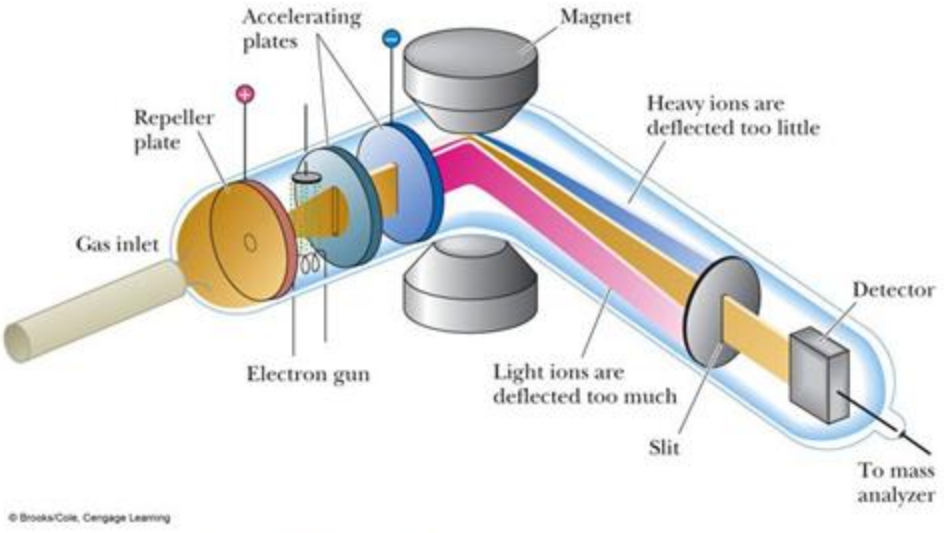
• Fairly difficult for large, preparative samples

• Requires spectroscopy, usually mass spectroscopy, for confirmation of peak identity.

1.3. Mass Spectrometry

1.3.1. Background

Mass spectrometry has progressed extremely rapidly during the last decade, between 1995 and 2005. This progress has led to the advent of entirely new instruments. New atmospheric pressure sources were developed[116]



Schematic of a Mass spectrometer

Mass spectrometry measures the mass to charge ratio (m/z) of ions produced from the analytes. MS detection essentially involves 3 steps: ionization, separation and detection. Each of these is briefly addressed below. The analyte enters the mass spectrometer via the ionization source.

Two types of ionization sources are used in GC, namely electron impact (EI) and chemical ionization (CI), with the former being more common. In EI, the molecules are bombarded with a high energy (70 eV) beam of electrons that ionise the molecules entering the ion source in the gas phase by removing an electron.

Because the formed molecular ions are unstable under such low pressure conditions, they fragment easily, and may be identified according to the characteristic fragmentation patterns formed[107]. In

the second step, ions are separated according to their mass to charge ratio (m/z) in vacuum in the mass analyser.

Two of the most common mass analysers were used: quadrupole (q) and time-of-flight (TOF) systems. In quadrupole MS (qMS), separation according to mass to charge ratio is performed by changing the rf and dc voltages applied across the four rods comprising the quadrupole. This changes the field in the quadrupole and allows only ions of a particular m/z ratio through to the detector for a given rf/dc ratio.

By varying this ratio, ions of different m/z ratios can be detected. The quadrupole mass analyser consists of four parallel rods around the flight path of the ions. On two opposite rods a radio frequency (rf) is applied, whilst on the remaining two a direct current (dc) voltage is applied.

This results in a magnetic field through which the ions travel which is changed continuously so that at any given setting of the rf and DC voltages only one ion will be resonant and arrive at the detector, while other ions are non-resonant and collide with the rods[107]. MS instruments can be operated in one of two modes: full scan mode, which is used for identification of unknown compounds, and selected ion monitoring (SIM), which is used for analysis of target compounds and is more sensitive than scan mode.

In time of-flight mass analysers, ions formed in the source are accelerated into a flight tube by application of an extraction field on a back-plate or repeller. Ions attain the same kinetic energy in this process, and are forced through the acceleration region into the field-free drift region[117].

Because all ions have the same kinetic energy, but different masses, the time taken by the ions to travel through the flight tube depends on their mass to charge ratios[118]. Lighter ions reach the detector earlier, while the heavier ones reach the detector last.

1.3.2.Ionization techniques

Depending on the purpose of analysis, ionization potentially resulting in extensive fragmentation of particular molecule yielding characteristic ions pattern or soft ionization providing information on molecular ion are the basic options.

1.3.2.1. Electron ionization (EI)

In GC, the most widely used ionization technique is electron ionization (formerly called electron impact ionization), in which sample molecules are in the first phase bombarded by high-energy electrons (70 eV), creating high-energy, single charged molecular ions followed by the loss of excess energy via fragmentation, producing a collection of fragment ions characteristic of the compound[119].

1.3.2.2. Chemical ionization (CI)

In some cases, however, EI fragmentation is too extensive, leaving little or no trace of a molecular ion, which makes the determination of the molecular weight (and, consequently identification of particular compound) somewhat difficult. This problem can be solved by applying of low energy or "soft" techniques such as positive chemical ionization(PCI)[120].

In PCI, the ion source is charged with a reagent gas (e.g.methane), at a relatively high pressure (0.1–100 Pa), which undergoes EI ionization, producing an excess of reagent ions.

Sample molecules are subsequently ionised by the reagent gas ions via proton transfer, producing pseudomolecular ions and, depending on the choice of a reagent gas, adduct ions may be formed.

The pseudomolecular ions generally have low internal energy and, consequently, they are less prone to the fragmentation than the molecular ions generated under EI conditions, providing unambiguous molecular-weight information. Due to little or no fragmentation, PCI is less suitable for confirmation, what can be, however, highly appreciate in some analysis, since the pseudomolecular ion is more intensive and specific than any lower-mass fragment ions[121] . In other words, PCI can offer both increased sensitivity and improved detectability (reduced chemical

ionization from background or co-eluting analytes results in increased S/N ratio).

The production of a large population of low-energy electrons during CI operation provides an opportunity for another ionization technique:

Negative chemical ionization (NCI), alternatively called electron capture negative ionization (ECNI) or negative ion chemical ionization (NICI). The basic mechanism of this technique is the same as that of an ECD: a low-energy electron is captured by an electronegative sample molecule, forming the molecular anion, which may undergo fragmentation, depending on its structure[119]. ENCI has two important advantages over EI and CI:

(i) allowing a 100-fold or greater improvement in sensitivity, and (ii) it is highly selective, since only a limited number of analytes are prone to efficient electron capture (e.g.analytes containing a halogen atom, a nitro group, or an extended aromatic ring system).

1.4. Gas chromatography–mass spectrometry (GC-MS)

GC/MS-a combination of two different analytical techniques, Gas Chromatography (GC) and Mass Spectrometry (MS), is used to analyze complex organic and biochemical mixtures[122]

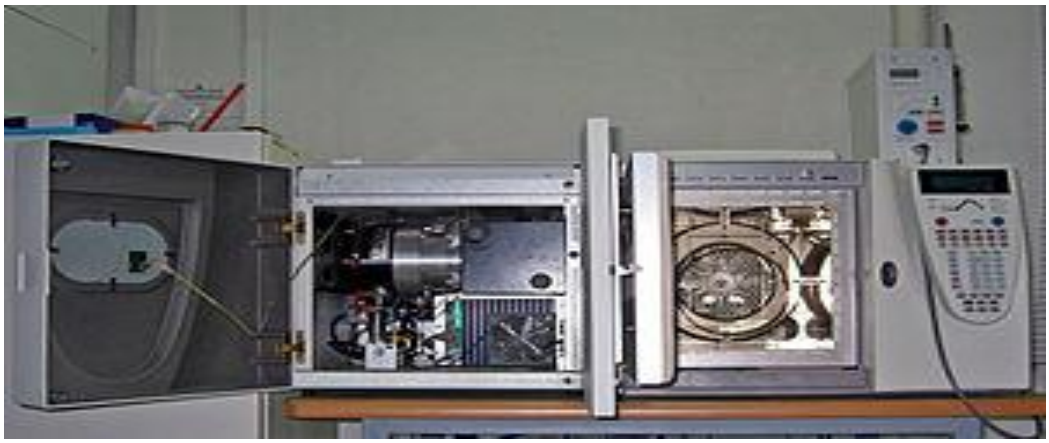


Diagram of Gas chromatography–mass spectrometry (GC-MS)

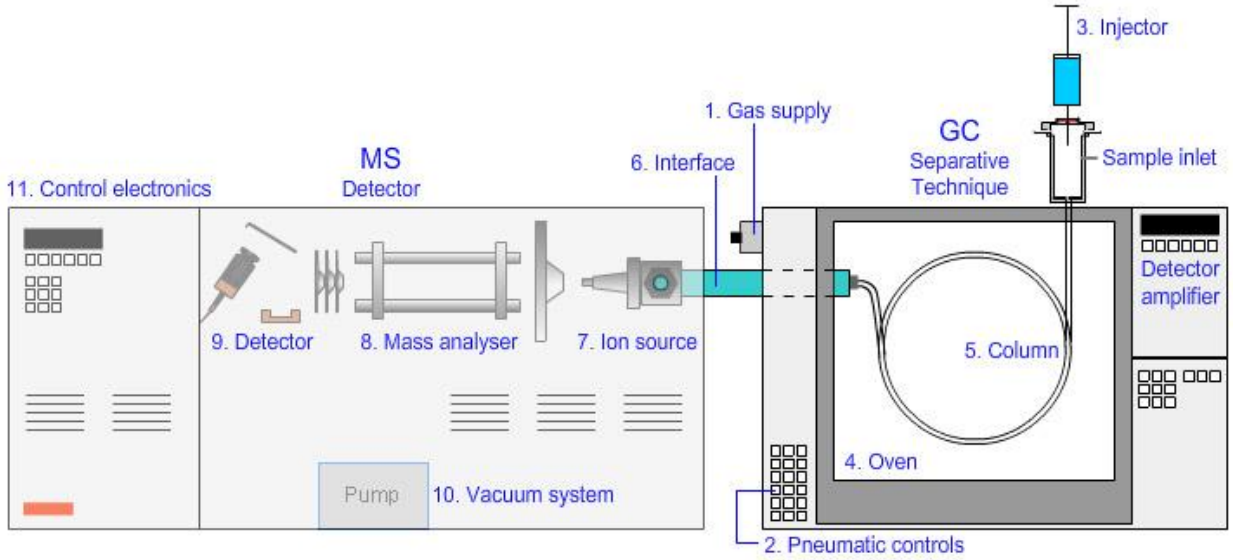
The use of a mass spectrometer as the detector in gas chromatography was developed during the 1950s by Roland Gohlke and Fred McLafferty . These sensitive devices were bulky, fragile, and originally limited to laboratory settings.

The development of affordable and miniaturized computers had helped in the simplification of the use of this instrument, as well as allowed great improvements in the amount of time it takes to analyze a sample.

In 1996 the top-of-the-line high-speed GC-MS units completed analysis of fire accelerants in less than 90 seconds, whereas first-generation GC-MS would have required at least 16 minutes. This has led to their widespread adoption in a number of fields[123]

1.4.1.Instrumentation and Working of GC-MS

The different parts of GC-MS and its functions are discussed below:



Different parts of GC-MS

i)Gas supply

Carrier gas is fed from the cylinders through the regulators and tubing to the instrument. It is usual to purify the gases to ensure high gas purity and gas supply pressure.

ii)Injector

Here the sample is volatilized and the resulting gas entrained into the carrier stream entering the GC column .

iii)Column

Gas Chromatography uses a gaseous mobile phase to transport sample components through columns either packed with coated silica particles or hollow capillary columns containing, the stationary phase coated onto the inner wall.

Capillary GC columns are usually several meters long (10-120 m is typical) with an internal diameter of 0.10-0.50mm, whilst packed GC columns tend be 1-5 meters in length with either 2 or 4mm internal diameter.

iv)Oven

Gas chromatography have ovens that are temperature programmable, the temperature of the gas chromatographic ovens typically ranges from 5 ^{o}C to 400 ^{o}C but can go as low as -25 ^{o}C with cryogenic cooling.

v)Mass Spectrometer

The separation of the phase ions is achieved within the mass spectrometer using electrical and/or magnetic fields to differentiate ions.

vi)Ion source

In the ion source, the products are ionized prior to analysis in the mass spectrometer.

vii)Mass analyzer

There are several very popular types of mass analyzer associated with routine GC-MS analysis and all differ in the fundamental way in which they separate species on a mass-to-charge basis.

viii)Vacuum system

Mass analyzers require high levels of vacuum in order to operate in a predictable and efficient way.

ix)Detector

The ion beam that emerges from the mass analyzer, have to be detected and transformed into a usable signal. The detector is an important element of the mass spectrometer that generates a signal from incident ions by either generating secondary electrons, which are further amplified, or by inducing a current (generated by moving charges).

x)Control electronics

The MS parameters can be selected and controlled from this panel. Modern instruments will also allow to control MS parameters from a computer by using specially designed software.

The mobile-phase called as carrier gas, must be chemically inert. The helium gas is most commonly used, however, argon, nitrogen, and hydrogen are also used.

These gases are held in pressurized tanks and use pressure regulators, gauges, and flow meters to control the flow rate of the gas. Flow rates usually range from 25-150 mL/min with packed columns and 1-25 mL/min for open tubular capillary columns, and are assumed to be constant if inlet pressure is constant. This is often accompanied by a molecular sieve to purify the gas before it is used. Samples are introduced as a plug of vapor.

Liquid samples are introduced using calibrated micro-syringes to inject sample through a septum and into a heated sample port

which should be about 50°C above the boiling point of the least volatile constituent of the sample. After the sample is introduced, it is carried to the column by the mobile phase. The temperature of the column is an important variable, so the oven is equipped with a thermostat that controls the temperature to a few tenths of a degree. Boiling point of the sample and the amount of separation required determines the temperature the sample should be run with. As the mobile phase carrying the sample is passed through the stationary phase in the column, the different components of the sample are separated. After being separated, the sample is run through a detector[122], which ionizes the sample and then separates the ions based on their mass-to-charge ratio.

This data is then sent to a computer to be displayed and analyzed. The computer linked to the GC-MS has a library of samples to help in analyzing this data[124]. Data for the GC-MS is displayed in several ways. One is a total-ion chromatogram, which sums the total ion abundances in each spectrum and plots them as a function of time. Another is the mass spectrum at a particular time in the chromatogram to identify the particular component that was eluted at that time. A mass spectra of selected ions with a specific mass to charge ratio, called a mass chromatogram, can also be used.

1.4.2. Principle

The mass spectrometer is a universal detector for gas chromatographs since any compound that can pass through a gas chromatograph is converted into ions in mass spectrometer. At the same time, the highly specific nature of mass spectrum makes the mass spectrometer a very specific gas chromatographic detector[124].

Gas chromatography is an ideal separator, whereas mass spectrometry is excellent for identification. The aim of an interfacing arrangement is to operate both a gas chromatograph and a mass spectrometer without degrading the performance of either instrument. The problem is compatibility. One incompatibility problem is the difference in pressure required for the operation of a gas chromatograph and the mass spectrometer. Whereas the former operates at high pressures, the latter is designed to run under high vacuum. An associated problem is the presence of much carrier gas and little sample in the effluent from the gas chromatograph[125]

If the gas chromatograph is using packed column the flow of carrier gas may be in excess of 30ml/min, which would collapse the vacuum of the mass spectrometer. Therefore carrier gas must be substantially removed and various designs have to be developed[126]

1.4.3. Gas Chromatograph – mass spectrometer interface

The interface must provide the link between the two instruments. Almost all GC-MS interface systems contain an enrichment device. However, the high pumping speeds used in mass spectrometers may permit the total effluent from capillary GC columns to be transported to the ion source of the mass spectrometer. When the chemical ionization reagent gas is used as carrier gas, the effluent can be introduced directly into the mass spectrometer.

1.4.3.1. Effusion separator

Since the carrier gas molecules are usually much lighter than those of samples they can be removed preferentially by an effusion chamber. Effluent from the gas chromatograph passes through a tube constructed of ultrafine porosity sintered glass with average pore size of about 10^{-4} Cm.

1.4.3.2. Jet/Orifice Separator

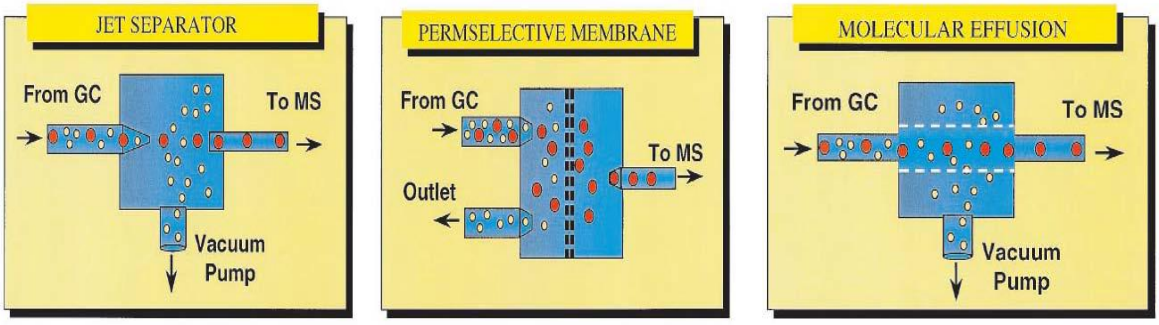
A precisely aligned supersonic jet/orifice system is effective in removing the carrier gas by effusion. Effluent from the carrier gas chromatograph is throttled through a fine orifice, where it rapidly expands into a vacuum chamber. During this expansion, the faster diffusion rate of helium results in a higher sample concentration in the core of the gas stream, which is directed towards a second jet

or orifice aligned with first jet. The distances between jets must be changed for a change in flow rates. Yields are about 25%.

1.4.3.3. Membrane separator

The membrane separator takes advantage of large differences in permeability between most organic molecules and the carrier gas when both are confronted by a membrane. Effluent from a gas chromatograph enters a cavity that is separated from the mass spectrometer vacuum system by a dimethyl silicone rubber membrane usually about 0.025-0.040 mm thick.

Helium has a low permeability, whereas the organic molecules pass through the membrane and directly into the high vacuum of mass spectrometer system. Enrichment values are 10-20 fold the yield may be 30-90% [125]



Interfaces for GC-MS coupling(The analyte and carrier gas molecules are represented by the red and yellow spheres, respectively)

1.4.4.Major applications of the supersonic GC-MS

The Supersonic GC-MS can be applied with few major advantages to practically all GC-MS analysis types. The main features of enhanced molecular ion, improved confidence in sample identification, significantly increased range of thermally labile and low volatility samples amenable for analysis, much faster analysis, improved sensitivity particularly for compounds that are hard to analyze and the many other features and options provide compelling reasons to use the Supersonic GC-MS in broad range of areas including:

i)Petrochemical and hydrocarbons analysis

Significantly enhanced molecular ions that are always observed, isomer and structurally significant mass spectral peaks and extended range of low volatility hydrocarbons that are amenable for analysis including waxes up to $C_{74}H_{150}$ makes the Supersonic GC-MS a most valuable GC-MS, particularly in comparison with standard GC-MS that often fails to enable hydrocarbon identification due to lack of molecular ions and similarity of low mass fragments. Broad range of petrochemicals could be analyzed by this technique including: fuels and hydrocarbon mixtures, including gasoline, kerosene, naphthenic acids, diesel fuel, various oil types, transformer oil, biodiesel, wax and broad range of

geochemical samples. A special isomer abundance analysis method was developed for fuel characterization was analyzed.

ii)Geochemical research

Significantly enhanced molecular ions that are always observed , major isomer and structurally significant mass spectral peaks, extended range of low volatility hydrocarbons that are amenable for analysis and unique isotope ratio information (without combustion) and the ability to perform parent scans MS-MS of biomarkers make the Supersonic GC-MS a most valuable GC-MS for organic geochemical applications.

iii)Forensic (arson, explosives, drugs, unknowns)

Enhanced and trustworthy molecular ions, extended range of thermally labile compounds amenable for analysis including all the major labile explosives, isotope abundance analysis software for improved sample identification, ChromatoProbe solid sample introduction device, Open Probe and ultra-fast GC-MS analysis make the Supersonic GC-MS the ideal Forensic GC-MS system. With the Supersonic GC-MS we could get a step closer to make the CSI (Crime Scene Investigation) TV scenes with GC-MS a reality.

Touch the sample, push it inside the GC-MS and get in a few seconds an accurate answer on the type of mixture or compound analyzed.

iv)Environmental analysis

Enhanced molecular ions, extended range of compounds amenable for analysis, superior sensitivity particularly for compounds that are the bottleneck of the whole analysis and faster analysis are the main attractive features of the supersonic GC-MS.

v)Pesticide analysis and food safety

Enhanced molecular ions, extended range of compounds amenable for analysis, superior sensitivity for those pesticides that are the bottleneck of the whole analysis and faster analysis are the main attractive features of the Supersonic GC-MS. The enhanced molecular ion and lack of ion source peak tailing and degradation can further serve for improved sensitivity in MS-MS. Isotope abundance analysis provides greater confidence level in the pesticide identification.

Pulsed flow modulation GCxGC-MS can further improve pesticide analysis via the reduction of matrix interferences. The ability to analyze the full range of carbamate pesticides and many other pesticides makes the Supersonic GC-MS the ideal system to provide confirmatory pesticide identification to supplement and complement LC-MS and further analyze those few pesticides that are difficult by both LC-MS and GC-MS.

vi)Pharmaceutical and drug analysis

The supersonic GC-MS is the only GC-MS that can analyze medium size drugs that are not amenable for standard GC-MS analysis including Reserpine Itraconazole, underivatized steroids etc.

In addition, unlike LC-MS the Supersonic GC-MS provides quantitative information on drug synthesis yield and on the abundance of drug contamination, it has no ion suppression effects and the analysis are fast, under 8 min analysis cycle time.

vii)Clinical toxicology

Enhanced molecular ions, extended range of compounds amenable for analysis, superior sensitivity for compounds that are the bottleneck of the whole analysis and faster analysis are the main attractive features of the Supersonic GC-MS for clinical toxicology.

In addition, the Supersonic GC-MS can serve to confirm (or reject) LC-MS identifications.

viii)Food and fragrance

Enhanced molecular ions, isotope abundance analysis software for improved unknown sample identification, unique isomer mass spectral effects and compatibility with pulsed flow modulation GCxGC-MS make the Supersonic GC-MS the ideal GC-MS for

the food and fragrance industry as an information generation machine[127].

*1.5. Petroselinum crispum (*parsley*)*

1.5.1.Taxonomy

Family: Umbelliferae

Names: Common parsley, garden parsley, rock parsley, Italian parsley; Flat-leaf parsley; Blattpetersilie, Petersilie (German); persil (French); prezzemolo (Italian); Perejil (Spanish); Persilja (Swedish); Baqdunis (Arabic); Peterselie (Dutch); Salsa (Portuguese); Pyetrushka (Russian); Paseri(Japanese); Yang-Hu-Sul (Chinese); Pietruszka (Zwyczajna) (Polish); Maidanos, Petroselino (Greek)

1.5.2.Description

Soft, rounded, leafy mound. Height 2-3 feet; width 10 inches. Flowers are green-yellow in umbels. Leaves are divided, feathery with strong aroma. Fruit is oval and compressed. The long taproot is spindly, thick and resembles small parsnips. Blooms in the second summer.[127]

Flat-leaved parsley flower

parsley leaves

Parsley roots

parsley seeds

The main parts of Parsley (*Petroselinum crispum*)

Held in high esteem by the Greeks, parsley was used to crown victors at the Isthmian Games and to decorate tombs, being linked with Archemorus, the herald of death.

The Greeks also planted parsley and rue along the edges of herb beds, thereby instigating the expression "being at the parsley and rue," meaning to be at the start of an enterprise. Although the

Greeks used parsley medicinally, and Homer recorded that warriors fed parsley to their horses, it appears that the Romans were the first to use it as a food.

They consumed parsley in quantity and made garlands for banquet guests to discourage intoxication and to counter strong odors. *Petros selinon* (rock celery) which could refer to parsley's ability to relieve kidney and bladder stones.

Parsley is one of the first herbs to appear in spring and has been used for centuries in the Seder. In European folklore, parsley's notoriously slow germination period gave rise to the superstition that its roots went down to the devil seven times before the plant would grow.[128]

1.5.3.Constituents

Parsley oil comprises about 0.1% of the root, about 0.3% of the leaf and 2%-7% of the fruit. Essential oil includes: apiol, apiolin, myristicin, pinene; flavonoids (apigenin); glycoside; vitamins A,C ; minerals (iron, manganese, calcium, phosphorus);protein. Parsley contains psoralen and related compounds that can induce photosensitivity; these include ficusin, bergapten, majudin and heraclin.[129]

1.5.4. Medicinal Use

Chew the leaf raw to freshen the breath and promote healthy skin. Infuse for a digestive tonic. Bruised leaves have been used to treat

tumors, insect bites, lice and skin parasites and contusions. Parsley tea at one time was used to treat dysentery and gallstones. Other traditional uses reported include the treatment of diseases of the prostate, liver and spleen, in the treatment of anemia, arthritis and cancers, and as an expectorant, antimicrobial, aphrodisiac, hypotensive, laxative and as a scalp lotion to stimulate hair growth. Use in a poultice as an antiseptic dressing for sprains, wounds and insect bites. Decoction of the root is used for kidney troubles and as a mild laxative.

Juice is applied to reduce swellings. It also stimulates appetite and increases blood flow to digestive organs, as well as reducing fever. Another constituent, the flavonoid apigenin, reduces inflammation by inhibiting histamine and is also a free-radical scavenger.

The seed, when decocted, has been used for intermittent fevers. It has also being traditionally used as a carminative to decrease flatulence and colic pain. The seeds have a much stronger diuretic action than the leaves and may be substituted for celery seeds in the treatment of gout, rheumatism and arthritis. It is often included in "slimming" teas because of its diuretic action.

Oil of the seed (5-15 drops) has been used to bring on menstruation.

1.5.5.Homeopathic Uses:

Used for very itchy hemorrhoids, as well as for urinary complaints such as a deep itch in the urinary tract, and gonorrhea with a sudden urge to urinate and a milky discharge.[130]

1.5.6.Aromatherapy Uses:

1.5.6.1. Extraction

Essential oil by steam distillation from the seed and the herb. An essential oil is occasionally extracted from the roots; an oleoresin is also produced by solvent extraction from the seeds.

1.5.6.2. Actions

Antimicrobial, antirheumatic, antiseptic, astringent, carminative, diuretic, drpurative, emmenagogoue, febrifuge, hypotensive, laxative, stimulant (mild), stomachic, tonic (uterine)

1.5.6.3. Constituents

Seed: mainly apiol, with myristicin, tetramethoxyally-benzene, pinene and volatile fatty acids.

Herb: Mainly myristicin with phellandrene, myrcene, apiol, terpenolene, menthatriene, pinene and carotel, among others.

1.5.6.4. Uses

Circulation, Muscles and Joints: accumulation of toxins, arthritis, broken blood vessels, cellulites, rheumatism, sciatica.

Digestive System: colic, flatulence, indigestion, hemorrhoids.

Genito-urinary system: amenorrhea, dysmenorrhea, to aid labor, cystitis, urinary infections.[131]

1.5.7.Cosmetic Use

Infusion of the leaf is used as a hair tonic and conditioner. The whole plant is added to facial steam and lotion for dry skin and to minimize freckles. Infusion is used as a soothing eyebath. The essence from the seeds is used in the manufacture of certain strong masculine scents[132].

Aim of this study

This study was designed to :

- Extraction of fixed oil from Parsley seeds .

-Analysis of extracted oil by GC-MS.

-Screening the oil for antimicrobial potency.

Chapter Two

Materials and Methods

2. Materials and Methods

2.1-Materials

2.1.1-Plant material

Petroselinum crispum seeds were purchased from the local market-Khartoum and authenticated by the Department of Phytochemistry and Taxonomy, National Research Center, Khartoum-Sudan.

2.1.2- Instruments

A Shimadzo GC-MS-QP2010 Ultra instrument with a RTX-5MS column (30m,length ; 0.25mm diameter ; 0.25 μm, thickness)was used**.**

2.1.3-Test organisms

Petroselinum crispum fixed oil was screened for antimicrobial activity using the standard pathogenic microorganisms shown below:

Table 2.1: Test organisms

Ser. No	Micro organism	Type
1	*Bacillus subtilis*	G+ve
2	*Staphylococcus aureus*	G+ve
3	*Pseudomonas aeroginosa*	G-ve
4	*Escherichia coli*	G-ve
6	*Candida albicans*	fungi

2.2- Methods

2.2.1- Preperations of reagents for phytochemical screening.

a)-Flavonoid test reagents

- Aluminium chloride solution

(1 g) of aluminum chloride was dissolved in 100 ml methanol

- Potassium hydroxide solution

(1 g) of potassium hydroxide was dissolved in 100 ml water.

-Ferric chloride solution

(1 g) of ferric chloride was dissolved in 100 ml methanol.

b)- Alkaloid test reagents

Mayer reagent

- **Mercuric chloride solution**: 1.36 g in 60 ml. water.

- **Potassium iodide solution** : 5 g in 10 ml. water

The two solutions were combined and then diluted with water up to 100 ml.

-Wagner reagent

(1.27 g) iodine and(2 g) of potassium iodide in (100 ml) water.

2.2.2- Phytochemical screening

Powdered air- dried seeds of *Petroselinum crispum* were extracted with 95% ethanol (soxhlet) until exhaustion. This prepared extract(PE) was used for phytochemical screening. *Petroselinum crispum* oil was screened for major secondary metabolites.

i)Test for unsaturated sterols and for triterpenes

(10 ml)of the (PE) was evaporated to dryness on a water bath, and the cooled residue was stirred with petroleum ether to remove most of the coloring materials. The residue was then extracted with 10 ml chloroform. The chlorform solution was dehydrated over anhydrous sodium sulphite . (5 ml) portion of the solution was mixed with(0.5 ml) of acetic anhydride, followed by two drops of concentrated sulphuric acid.

ii) Test for flavonoids

(20 ml) of the (PE) were evaporated to dryness on water bath. The cooled residue was defatted with petroleum ether and then dissolved in 30 ml of 30% aqueous methanol and filtered. The filtrate was used for the following tests:

- To 3 ml. of filtrate a fragment of magnesium ribbon was added, shaken and then few drops of concentrated hydrochloric acid were added.

- To 3 ml. of the filtrate few drops of aluminium chloride solution were added.
- To 3 ml. of the filtrate few drops of potassium hydroxide solution were added.

iii) Test for alkaloids

(10 ml) of the (PE) were evaporated to dryness on a water bath and 5 ml of 0.2N hydrochloric acid were added and the solution was heated with stirring for 10 minutes, then cooled and filtrated. Filtrate was divided into two portions:

To one portion a few drops of Maeyer reagent were added., to the other portion few drops of Wagner reagent were added.

iv)Test for tannins

(10 ml) of (PE) were evaporated to dryness and the residue was extracted with n-hexane and then filtrated. The insoluble residue was stirred with n-hexane and (10 ml) of hot saline (0.9% w/v of sodium chloride and freshly prepared distilled water) were added. The mixture was cooled , filtrated and the volume adjusted to 10 ml. with more saline solution. (5 ml) of this solution were treated with few drops of ferric chloride solution.

v)Test for saponins

(1g) of dried powdered plant material was placed in a clean test tube. (10 ml) of distilled water were added and the tube was

stoppered and vigorously shaken for about 30 seconds, and allowed to stand.

2.2.3-Extraction of oil from seeds of *Petroselinum crispum*

Petroselinum crispum seeds(300g) were exhaustively extracted with n-hexane (maceration).The solvent was removed under reduced pressure and the oil was kept at 4^{o}C for further manipulation.

The oil(2ml) was placed in a test tube and 7ml of alcoholic sodium hydroxide were added followed by 7ml of alcoholic sulphuric acid.The tube was stoppered and shaken vigorously for five minutes and then left overnight.(2ml) of supersaturated sodium chloride were added, then (2ml) of normal hexane were added and the tube was vigorously shaken for five minutes .The hexane layer was then separated.(5µl) of the hexane extract were mixed with 5ml diethyl ether . The solution was filtered and the filtrate(1µl) was injected in the GC-MS vial.

2.2.4- GC-MS analysis

Petroselinum crispum oil was analyzed by gas chromatography – mass spectrometry where a Shimadzo GC-MS-QP2010 Ultra instrument with a RTX-5MS column (30m,length ; 0.25mm diameter ; 0.25 µm, thickness) was used.Helium (99.99 %) was used as carrier gas. Oven temperature program and other chromatographic conditions are displayed below:

Table 2.2: Oven temperature program

Rate	Temperature(°C)	Hold Time (min.$^{-1}$)
-	150.0	1.00
4.00	300.0	0.00

Table2.3: Chromatographic conditions

Column oven temperature	150.0°C
Injection temperature	300.0°C
Injection mode	Split
Flow control mode	Linear velocity
Pressure	139.3KPa
Total flow	50.0ml/ min
Column flow	1.54ml/sec.
Linear velocity	47.2cm/sec.
Purge flow	3.0ml/min.
Spilt ratio	- 1.0

2.2.5-Antimicrobial test

a)-Preparation of bacterial suspensions

One ml aliquots of 24 hours broth culture of the test organisms were aseptically distributed onto nutrient agar slopes and incubated at 37°C for 24 hours.

The bacterial growth was harvested and washed off with sterile normal saline, and finally suspended in 100 ml of normal saline to produce a suspension containing about 10^8-10^9colony forming units per ml.The suspension was stored in the refrigerator at 4°C until used. The average number of viable organism per ml of the stock suspension was determined by means of the surface viable counting technique.

Serial dilutions of the stock suspension were made in sterile normal saline in tubes and one drop volumes (0.02 ml) of the appropriate dilutions were transferred by adjustable volume micropipette onto the surface of dried nutrient agar plates. The plates were allowed to stand for two hours at room temperature for the drop to dry, and then incubated at 37°C for 24 hours.

b)-Preparation of fungal suspensions

Fungal cultures were maintained on sabouraud dextrose agar incubated at 25°C for four days. The fungal growth was harvested and washed with sterile normal saline, and the suspension was stored in the refrigerator until used.

c)-Testing for antimicrobial activity

By using the cup-plate agar diffusion bioassay , the antimicrobial potency of the oil was assessed. (2ml) of the standardized bacterial stock suspension were mixed with 200 ml of sterile molten nutrient agar which was maintained at 45°C in a water bath. (20 ml) Aliquots of the incubated nutrient agar were distributed into sterile Petri dishes, the agar was left to settle and in each of these plates which were divided into two halves, two cups in each half (10 mm in diameter) were cut using sterile cork borer (No 4), each one of the halves was designed for one of the compounds. Separate Petri dishes were designed for standard antibacterial chemotherapeutic, (ampicillin and gentamycin).

The agar discs were removed, alternate cup were filled with 0.1 ml samples of each compound using adjustable volume microtiter pipette and allowed to diffuse at room temperature for two hours. The plates were then incubated in the upright position at 37°C for 24 hours.

The above procedure was repeated for different concentrations of the test compounds and the standard antibacterial chemotherapeutics. After incubation, the diameters of the resultant growth inhibition zones were measured in triplicates and averaged.

Chapter Three

Results and Discussion

3. Results and Discussion

3.1-Phytochemical screening

Petroselinum crispum seeds gave positive reactions for: flavonoids, tannins, alkaloids, saponins and carbohydrates.

Table 3.1:Phytochemical screening of *Petroselinum crispum*

Species	Flavonoids	Tannins	Alkaloids	Saponins	Carbohydrates
Petroselinum crispum	+ve	+ve	+ve	+ve	+ve

3.2-GC-MS analysis

Petroselinum crispum oil was extracted by maceration from seeds and analyzed by GC-MS where 62 components were detected in total ions chromatograms (Figure 3.1).

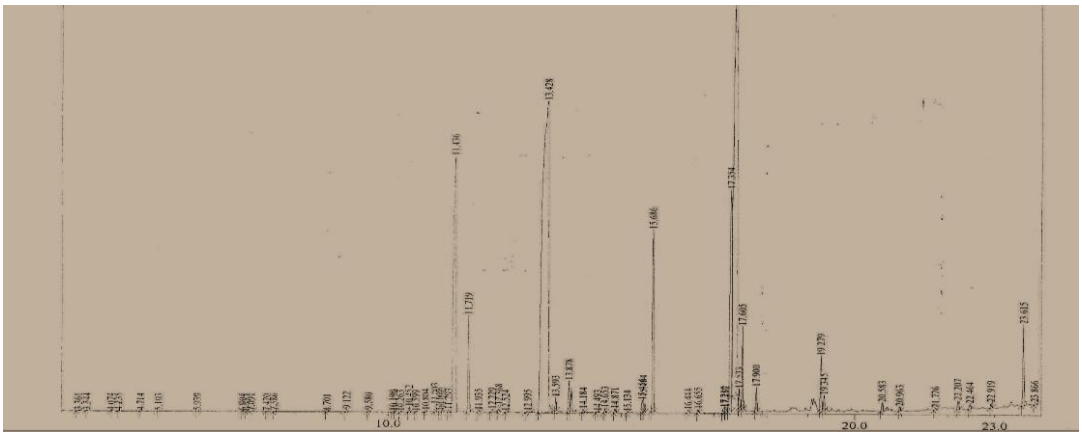


Figure 3.1: Total ions chromatograms of *Petroselinum crispum* oil

Different constituents of the oil were quantified and identified by retention time and their characteristic fragmentation pattern. A tabulation of these components is given in Table (3.2)

Table 3.2: Total ions chromatograms of *Petroselinum crispum* oil

Peak Report TIC

Peak#	R.Time	Area	Area%	Name
1	3.361	53417	0.00	Hexanoic acid, methyl ester
2	3.544	27962	0.00	.alpha.-Pinene
3	4.074	17027	0.00	.beta.-Pinene
4	4.235	33062	0.00	Pentanoic acid, 4-methyl-, methyl ester
5	4.714	32398	0.00	D-Limonene
6	5.103	32502	0.00	.gamma.-Terpinene
7	5.939	31056	0.00	Octanoic acid, methyl ester
8	6.904	58507	0.00	Thymol
9	6.984	85559	0.01	L-.alpha.-Terpineol
10	7.091	164344	0.01	Bicyclo[3.1.1]hept-2-ene-2-methanol, 6,6-di
11	7.420	38982	0.00	Citronellol
12	7.586	191489	0.01	3-(2-Hydroxy-cyclopentylidene)-2-methyl-p
13	8.701	54674	0.00	Decanoic acid, methyl ester
14	9.122	687866	0.05	3-Cyclohexene-1-methanol, .alpha.,.alpha.,
15	9.580	475090	0.04	Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-7
16	10.100	246718	0.02	1H-3a,7-Methanoazulene, octahydro-3,8,8-
17	10.150	257761	0.02	Caryophyllene
18	10.263	183234	0.01	Bicyclo[3.1.1]hept-2-ene, 2,6-dimethyl-6-(4
19	10.452	1348679	0.10	(E)-.beta.-Famesene
20	10.599	169551	0.01	1H-Benzocycloheptene, 2,4a,5,6,7,8,9,9a-oc
21	10.804	364047	0.03	.beta.-copaene
22	11.003	2135670	0.16	Naphthalene, decahydro-4a-methyl-1-meth
23	11.104	332939	0.02	Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-4
24	11.162	857025	0.06	.beta.-Bisabolene
25	11.293	701743	0.05	Dodecanoic acid, methyl ester
26	11.436	164112211	12.12	1,3-Benzodioxole, 4-methoxy-6-(2-propenyl
27	11.719	28343982	2.09	Benzene, 1,2,3-trimethoxy-5-(2-propenyl)-
28	11.935	551533	0.04	Asarone
29	12.229	1422825	0.11	1,3,5-Trimethoxy-2-propenylbenzene
30	12.368	1846488	0.14	Carotol
31	12.524	342394	0.03	1H-1,3-Benzimidazole-2-methanol, 5-meth
32	12.995	1088069	0.08	cis-3-Butyl-4-vinyl-cyclopentene
33	13.428	493477272	36.44	Apiol
34	13.593	3409538	0.25	Methyl tetradecanoate
35	13.878	8483012	0.63	1,3-Benzenediamine
36	14.184	1038200	0.08	2',4'-Dimethoxy-3'-methylpropiophenone
37	14.493	431625	0.03	6-Octadecenoic acid, methyl ester, (Z)-
38	14.653	1317809	0.10	Pentadecanoic acid, methyl ester
39	14.871	1037869	0.08	2-Pentadecanone, 6,10,14-trimethyl-
40	15.134	274448	0.02	.alpha.-Santalol
41	15.456	3622787	0.27	7,10,13-Hexadecatrienoic acid, methyl este
42	15.484	3712592	0.27	9-Hexadecenoic acid, methyl ester, (Z)-
43	15.686	55253847	4.08	Hexadecanoic acid, methyl ester
44	16.444	837198	0.06	7-Hexadecenoic acid, methyl ester, (Z)-
45	16.655	1030433	0.08	Heptadecanoic acid, methyl ester
46	17.210	2423051	0.18	6,9-Octadecadienoic acid, methyl ester
47	17.252	1556285	0.11	Cyclohexadecane
48	17.354	112655745	8.32	9,12-Octadecadienoic acid (Z,Z)-, methyl es
49	17.475	367962054	27.17	9-Octadecenoic acid (Z)-, methyl ester
50	17.533	4879794	0.36	Phytol
51	17.605	21959540	1.62	Methyl stearate
52	17.900	6525907	0.48	Tricyclo[5.1.0.0(3,5)]octane-2,6-dione, 1,3,4
53	19.279	15321503	1.13	2-Butenoic acid, 2-methyl-, 2-(acetyloxy)-1.
54	19.345	3892483	0.29	Eicosanoic acid, methyl ester

55	20.583	2845037	0.21	9-Octadecenoic acid, 1,2,3-propanetriyl est
56	20.963	1354208	0.10	Docosanoic acid, methyl ester
57	21.726	690902	0.05	Tricosanoic acid, methyl ester
58	22.207	2722116	0.20	Tetracontane
59	22.464	1603442	0.12	Tetracosanoic acid, methyl ester
60	22.919	1454924	0.11	Tetratriacontane
61	23.615	25362901	1.87	Hexatriacontane
62	23.866	634637	0.05	Hexacosanoic acid, methyl ester
		1354059963	100.00	

Major constituents are discussed below:

Apiol (36.44%)

Figure 3.2: Mass spectrum of apiol

The EI mass spectrum of apiol is shown in Fig. 3.2.The peak at m/z 222, which appeared at R.T. 13.428 in total ion chromatogram, corresponds to $M^+[C_{12}H_{14}O_4]^+$ .The peak at m/z 207 corresponds to loss of a methyl function and the peak at m/z 191 corresponds to loss of a methoxyl function.

9-Octadecenoic acid (Z)-, methyl ester (27.17%)

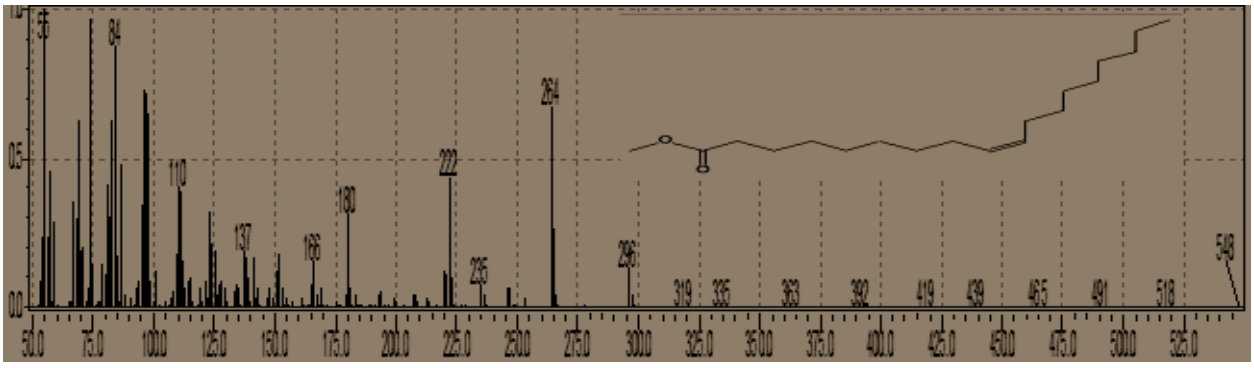


Figure 3.3: Mass spectrum of 9-octadecenoic acid (Z)-, methyl ester

The EI mass spectrum of 9-octadecenoic acid (Z)-, methyl ester is shown in Fig. 3.3.The peak at m/z 296, which appeared at R.T. 17.475 in total ion chromatogram, corresponds to $M^+[C_{19}H_{36}O_2]^+$. The peak at m/z 265 corresponds to loss of a methoxyl function.

1, 3-Benzodioxole, 4-methoxy-6-(2-propenyl) - (12.12%)

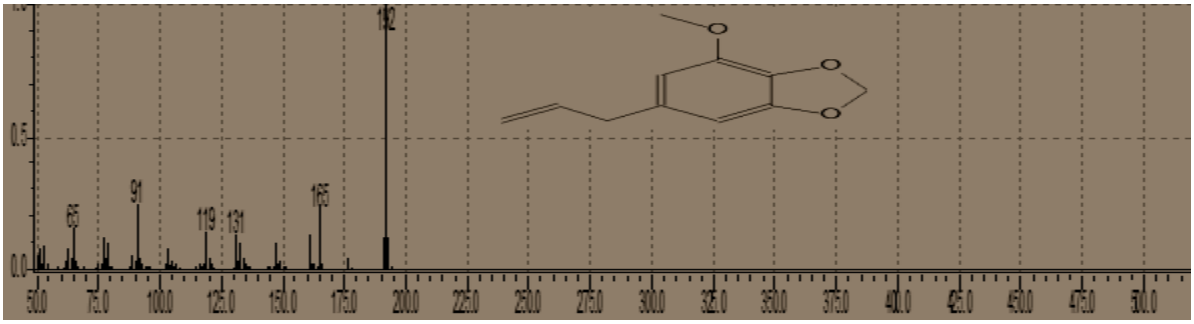


Figure 3.4: Mass spectrum of 1, 3-benzodioxole, 4-methoxy-6-(2-propenyl)

The EI mass spectrum of 1, 3-benzodioxole, 4-methoxy-6-(2-propenyl) is shown in Fig. 3.4.The peak at m/z 192, which appeared at R.T. 11.436 in total ion chromatogram, corresponds to $M^+[C_{11}H_{12}O_3]^+$ .The peak at m/z 177 corresponds to loss of a methyl function.

9, 12-Octadecadienoic acid (Z, Z)-, methyl ester (8.32%)

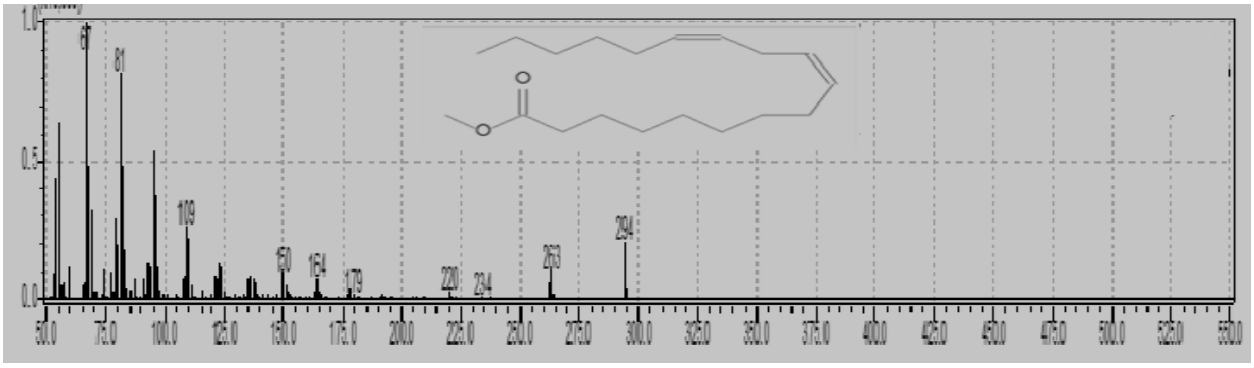


Figure 3.5: Mass spectrum of 9, 12-octadecadienoic acid (Z, Z)-, methyl ester

The EI mass spectrum of 9, 12-octadecadienoic acid (Z, Z)-, methyl ester is shown in Fig. 3.5.The peak at m/z 294, which appeared at R.T. 17.354 in total ion chromatogram, corresponds to $M^+[C_{19}H_{34}O_2]^+$ .The peak at m/z 263 corresponds to loss of a methoxyl function.

Hexadecanoic acid, methyl ester (4.08%)

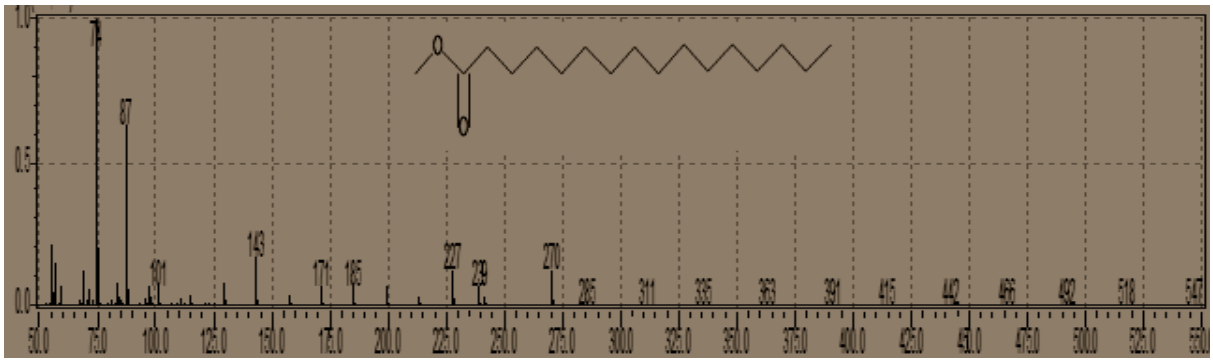


Figure 3.6: Mass spectrum of hexadecanoic acid, methyl ester

The EI mass spectrum of hexadecanoic acid, methyl ester is shown in Fig. 3.6.The peak at m/z 270, which appeared at R.T. 15.686 in total ion chromatogram, corresponds to $M^+[C_{17}H_{34}O_2]^+$ .The peak at m/z 239 corresponds to loss of a methoxyl function.

5-(2-propenyl) -1, 2, 3-trimethoxybenzene (2.09%)

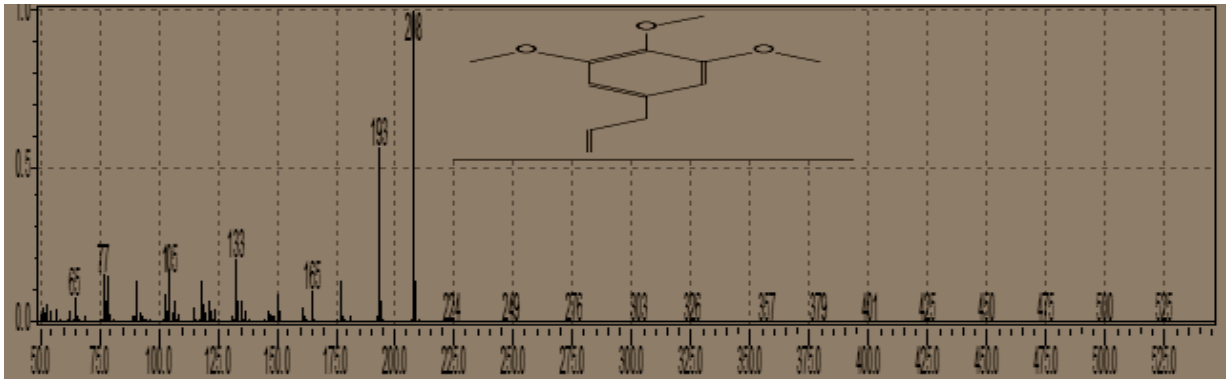


Figure3. 7: Mass spectrum of **5-**(2-propenyl) -1, 2, 3-trimethoxybenzene

The EI mass spectrum of **5-**(2-propenyl) -1,2, 3-trimethoxybenzene is shown in Fig. 3.7.The peak at m/z 208, which appeared at R.T. 11.719 in total ion chromatogram, corresponds to $M^{+}[C_{12}H_{16}O_3]^{+}$ .The peak at m/z 193 corresponds to loss of a methyl function.

3.3-Antimicrobial activity

The oil was screened for antimicrobial activity against standard organisms. The average of the diameters of the growth inhibition zones are shown in Table (3.3) .The results were interpreted in terms of the commonly used terms (<9mm: inative;9-12mm:partially active;13-18mm: active;>18mm:very active) .Tables (3.4) and (3.5) represent the antimicrobial activity of standard antibacterial and antifungal chemotherapeutic agents against standard bacteria and fungi respectively.

Table (3.3) : Antibacterial activity of *Petroselinum crispum* oil :M.D.I.Z (mm)

Drug	Conc.(mg/ml)	Ec	Ps	Sa	Bs	Ca
Petroselinum crispum	100	9	10	15	14	8

Table (3.4) : Antibacterial activity of standard chemotherapeutic agent :M.D.I.Z (mm)

Drug	Conc. mg/ml	Bs.	Sa.	Ec.	Ps.
Ampicillin	40 20	15 14	30 25	- -	- -

	10	11	15	-	-
Gentamycin	40 20 10	25 22 17	19 18 14	22 18 15	21 15 12

Table (3.5) : Antifungal activity of standard chemotherapeutic agents against standard fungi

Drug	Conc. mg/ml	An.	Ca.
Clotrimazole	30 15 7.5	22 17 16	38 31 29

- Sa.*: Staphylococcus aureus*
- Ec.*: Escherichia coli*
- Pa.: *Pseudomonas aeruginosa*
- An.: *Aspergillus niger*
- Ca.: *Candida albicans*
- *Bs.: Bacillus subtilis*
- M.D.I.Z: Mean diameter or growth inhibition zone (mm). Average or two replicates, inhibition zone >=15: sensitive, <15: resistant.

The oil showed very good activity against all test organisms : *Staphylococcus aureus* and *Bacillus subtilis* ,but it was partially active against : *Escherichia coli* and *Pseudomonas aeruginosa*. However, it was inactive against the yeast *Candida albicans*.

Recommendations

-Other phytochemicals of *Petroselinum crispum* can also be isolated, identified and their biological activity could be investigated.

-The extracted oil can also be evaluated for other biological activities (anti-inflammatory, antimalarial, antispasmodic…etc.).

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