EXTRACTION AND CHARACTERISATION OF THE ESSENTIAL OIL FROM CENTELLA ASIATICA (PENNYWORT) GROWING IN SOUTH AFRICA

By

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A dissertation submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the Degree of Master of Science.

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Declaration

I declare that this Theses is my own, unaided work. It is being submitted for the Degree of Master of Science at the University of the Witwatersrand, Johannesburg. It has not been submitted before before for any degree or examination at any other University.

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(Signature of candidate)

on this _____day of _____ 2015.

Supervisor:

Prof. Ewa Cukrowska (University of the Witwatersrand, School of Chemistry)

Abstract

Aromatic plants and oils have been used for thousands of years in perfumes, and cosmetics and for their culinary and medicinal purposes. The essential oil from *Centella Asiatica* growing specifically in South Africa has many therapeutic uses and is used to treat various diseases. Different extraction methods were used on the leaves of *Centella Asiatica* and the composition of each sample of oil obtained was analysed to see how the composition of each sample is affected. The methods of extracting were optimised specifically for Centella Asiatica. The different extraction methods used were steam distillation, water distillation, solvent extraction and soxhlet extraction. Steam and water distillation were preformed with three different apparatus to compare the efficiency of the extraction and the affect on composition of the oil. It was found that steam distillation using the apparatus described by the British Pharmacopedia Volume IV was the most sufficient apparatus to use to obtain the greatest yield of oil. Soxhlet extraction was found to be the worst extraction method for volatile compounds

The optimised parameters for extraction of essential oils from *Centella Asiatica* using this apparatus were 100 g of leaves at a distillation rate of 2/3 ml min^{-1} for 75 minutes using 0.4 ml of xylene initially. It was also necessary to perform a 30 minute initial distillation with no plant matter. Steam distillation with this apparatus was found to yield the best quality oil.

The major constituents that were found in all the methods were α-carophyllene, carophyllene and germacrene D. There were some similarities found in the compositions of the essential oil between extraction methods in terms of the constituents found. However the abundance of those constituents varied between extractions. Each constituent has a different therapeutic effect. Therefore if an extraction method were to be chosen to extract a some specific constituent like germacrene D and α-carophyllene, steam distillation with the apparatus described by the British Pharmacopedia should be used. However if an extraction method were to be used to extract carophyllene, water distillation should be used.

The essential oil extracted using steam distillation yielded a greater amount sesquiterpenoid hydrocarbons. However monoterpenoid hydrocarbons were present in greater amount in the essential oil extracted using water distillation.

In the essential oil extracted from *Centella Asiatica*, 43 constituents were identified from steam distillation extraction representing 98.60% of the composition of oil and 54 constituents representing 98.29% from water distillation extraction.

It was found that from steam distillation using fresh leaves compared to dry leaves extraction a greater number of constituents were identified. Salting out was also used for extraction and compared to water distillation and it was found that the salting-out extractions yielded a greater amount of oxygenated polar compounds.

A commercial oil of Centella was also analysed and compared to the natural oil. It was found that the commercial oil was a synthetic oil and its composition was completely different from the natural oil and therefore synthetic oils cannot be used therapeutically for substitutes for natural essential oils.

Centella Asiatica prefers to grow in damp environments, therefore they are extremely prone to pollution. This was found to also affect the chemical composition of the oil obtained since the soil quality of where the plants were growing was important. This was investigated by spiking the soil of some *Centella asiatica* plants with chromium(VI), mercury(II) and lead(II). It was found that *Centella Asiatica* can store heavy metals in the leaves. Since it is a medical plant with many therapeutic uses, this is causes great concern about heavy metal contamination of herbal raw materials of *Centella Asiatica*. This also highlights the importance of good quality control on *Centella Asiatica*, so that heavy metals are not ingested. The people in the rural areas who use it as a raw plant for herbal preparations could be at risk of ingesting heavy metals if grown in a polluted area.

Acknowledgements

The completion of my dissertation and subsequent MSc has been a long journey. It's true that 'Life is what happens' when you completing your dissertation. Life does not stand still, nor wait until you are finished and have time to manage it. A lot has happened and changed in the time I have been involved in this project. My dissertation has always been a priority but as you know there are several priorities in a person life at one time. Unfortunately due to life's challenges and the changes that followed, my dissertation could not always be the number one priority. The business in which I am involved and my family, especially my little daughter, always had to be priority but at last it is complete, I have finished and could not have finished without the support of several important people.

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List of symbols and abbreviations

The following are the symbols and acronyms used in the dissertation

ADP	Adenosine Diphosphate
ATP	Adenosine Triphosphate
SFME	Solvent free microwave extraction
GC	Gas chromatograph
GC-MS	Gas chromatography mass spectrometry
ICP-OES	Inductively coupled plasma optical emission spectroscopy
FID	Flame ionization detector
MS	Mass spectrometer
SEM	Scanning electron microscope
TIC	Total ion current
BP apparatus	the apparatus described by the British Pharmacopedia IV
Std. dev	Standard deviation
nd	not detected
ROS	Reactive oxygen species

1 Introduction

For thousands of years aromatic plants and oils have been used as perfumes, incense and in cosmetics, as well as for culinary and medicinal purposes. Their traditional uses in early cultures are where their religious and therapeutic functions became combined. (Lawless, 1992)

In recent decades aromatherapy has become more popular, which has renewed interest in essential oils. Lawless (1992) described "Aromatherapy as a branch of alternative medicine which claimed that the specific aromas carried by essential oils have healing effects". The term aromatherapy was first introduced in 1928 by a French chemist named Gattefosse. He worked for his families perfume business and became interested with the therapeutic possibilities of the oils. He also found that "many of the essential oils were more effective in their entirety than their synthetic substituent's or their isolated active ingredients". (Lawless, 1992)

New forms of disease are increasingly affecting people that live in today's highly pressurized society and these diseases are less responsive to treatment with conventional medicines. Many people who have been disappointed with mainstream medicine have turned to complementary therapies which do not rely on scientific evidence but rather on historical and observational evidence. (Bowles, 2003) Modern research has largely confirmed the traditional uses regarding the therapeutic applications of certain plants. (Lawless, 1992)

The therapeutic potential of plant derived treatments, including essential oils, still need to be fully discovered. Many medicinal plants have been examined to provide biologically active compounds, on which most of our contemporary drugs are based. However, there is still much more to discover about their exact pharmacology. This is especially relevant in terms of essential oils which have such a concentrated yet complicated composition. But as written by Lawless (1992) in the Encyclopedia of essential oils that "only a small proportion of the

world floral has been examined for pharmologically active compounds, but with the ever increasing danger of plants becoming extinct, there is a real risk that many important plant sources may be lost ". Therefore, it is important to study and conserve the environment as they may provide important leads for the cure of diseases such as cancer, AIDS and others that society faces these days.

A specific plant with many therapeutic uses is *Centella Asiatica*, a perennial herbaceous creeper commonly found in moist places. (Gohil, 2010) It is indigenous to Africa, Asia, and South America. In South Africa, it is found along the moist eastern parts, widely distributed from the Cape Peninsula northwards. (Van Wyk et al, 2002)

Commonly, the plant is known as Pennywort and in Chinese as Gotu Kola. It is an important traditional medicinal herb used by communities of Asia and southern and central Africa since ancient times. *Centella Asiatica* has become very popular due to its effectiveness and versatility. It has, inparticular, a reputation of being a brain stimulant and as a wound healing agent. (Zheng, 2007)

Extensive experimental and clinical investigations have been conducted by scientists from around world which have been focused on searching for some potential compounds that have low toxicity and a high efficacy which could benefit human health. (Zheng, 2007)

There have been many studies on the therapeutic uses of the entire plant. However, the species varies considerably in different parts of the world and is sometimes treated as several distinct species. (Van Wyk et al, 2002) Therefore, the composition of *Centella Asiatica* growing in South Africa can vary greatly from *Centella Asiatica* growing in India and other countries due to the different factors such as geo-climatic location, soil type, life stage of plant, pollution and time and day harvesting done. (Bowles, 2003) Even though *Centella Asiatica* has been used

extensively in traditional medicine in South Africa, many studies have not been carried out in the country. (Oyedeji and Afolayan, 2004) There have been more studies carried out worldwide on the tincture of C*entella Asiatica* compared to the essential oil.

Heavy metals found in the environment can easily contaminate many medicinal herbs like *Centella Asiatica* during their manufacturing and growth processes when their ready made products are produced. Heavy metals can be found in the air, soil and water and examples of such sources are atmospheric dust, rainfall, fertilisers and plant protective agents. Since *Centella* has many therapeutic uses, good quality control is imperative to protect consumers from contamination. (Abu- Darwish, 2009)

1.1 Essential oils

Elsharkawy (2014) describes an "essential oil as a concentrated, hydrophobic liquid containing volatile compounds from plants". These oils are manufactured in the green (chlorophyll bearing) parts of the plant and are transported to other plant tissues as the plant matures. (Galadima et al, 2012) It is then stored in small sacs in the roots, shoots, leaves, flowers and seeds of fragrant plants. (Whitton, 1995) Whitton (1995) explains that this oil gives the plant its distinctive smell which "help attract insects for pollination and allow the plant to protect itself from invading bacteria and fungi. We extend this protection to our benefit when we extract and use the oils because they can be antiseptic, bactericidal, viricidal and fungicidal as well as anti-inflammatory, anti-spasmodic, digestive or sedative depending on their individual plant chemistry." Every oil extracted from various plants has a concentrated, distinctive combination of natural chemicals. (Whitton,1995)

In general, carbon, hydrogen and oxygen make up the composition of essential oils which can be subdived into two groups, oxygenated compounds and hydrocarbons. Examples of oxygenated compounds would be esters, alcohols,

aldehydes, phenols, oxides and ketones although sometimes lactones, nitrogen and sulphur compounds can be present as well. Hydrocarbons consist mainly of terpenes (monoterpenes, diterpenes and sesquiterpenes). (Lawless, 1992)

1.1.1 Essential oil extraction methods

Essential oils can be extracted by different methods such as distillation, solvent extraction, expression, maceration and enfleurage. (Whitton,1995)

Distillation

Distillation is the best and most commonly used method for obtaining the purest essential oils. The plant material is put into a flask and heated with water, steam or both. The heat causes the little sacs of oil to burst, releasing the volatile contents into the resulting vapour. The vapour is then cooled through a condenser and becomes a liquid once more. The liquid is then separated into the essential oil and fragrant water. (Whitton, 1995) This recondensed water is referred to as a hydrosol. The disadvantage of steam distillation is that some delicate chemical components may denature easily by the extreme heat. (Whitton, 1995)

Solvent extraction

The plant material is placed in a suitable flask with a solvent (e.g. hexane or supercritical carbon dioxide) and allowed to sit for a period of time (hours to days). (Bowles, 2003) Whitton (1995) states the method further that " The resulting mixture is then filtered and becomes what is known as a ‘concrete’. The concrete is then mixed with alcohol, chilled, filtered and the alcohol evaporated off, leaving behind a highly perfumed oil which is called an ‘absolute’."

The disadvantage of this method is that some of the solvent is present in the collected essential oil product that is consumed. This could be a problem as a reaction may occur if applied on the skin by a consumer who is allergic to the

solvent. Therefore, solvent extraction is usually used for oils that are put into perfumes and not used on the skin. (Kububa, 2009)

Expression

Expression is only used on citrus oils. The rinds are separated from the fruit either by hand or machine and squeezed to release their oils. (Whitton, 1995)

Maceration

The plant material is placed in hot vegetable oil. The heat causes the cell membranes to burst and the vegetable oil absorbs the plant oil creating an "infused" oil. (Whitton, 1995)

Enfluerage

This specialised method is not very popular and mainly used to extract oils from flowers. Whitton (1995) explains this method of extraction which consists of placing fresh flower petals on glass frames which have been layered in fat. The fat absorbs the oils from the flowers and the petals are replaced after 24 hours until the fat is saturated with oil. The final compound, is now called a “pommade”. It is then washed with alcohol. The alcohol is then evaporated off which leaves a scented oil. (Whitton,1995)

The following study will only focus on steam and water distillation and solvent extraction on *Centella Asiatica* leaves.

1.1.2 Factors that affect the composition (quality) of essential oils

The composition of essential oils between different species of the same genus are evident, however there are many other factors that influence the composition of oils from different specimens of the same species. As explained well by Hussain (2009) "Factors that determine the composition and yield of the essential oil obtained are numerous. In some instances it is difficult to segregate these factors from each other, since many are interdependent and influence one another." Geo-climatic location is one of the most significant factors that can cause different chemotypes of essential oils. Some factors that can affect the essential oil molecules ratio produced by the plant include genetic variations, seasonal and maturity variation, the presence of fungal diseases and insects (Hussain, 2009). Other factors also include soil type, life stage of plant and even the time of day when harvesting is done, since the volatile content of the leaf increases with time and also with the size of the leaf. (Bowles, 2003) The part of the plant used, the post-harvest drying and storage are also important factors that can affect the composition of the oil. (Hussain, 2009)

The extraction methods used also has an effect on the essential oil quality. Steam distillation for example can affect the chemical composition of the essential oils, as heat and water vapour can cause molecular rearrangement, hydrolysis of double bonds, and in general will produce substances not originally found in plant. (Bowles, 2003) In solvent extraction, it is impossible to remove every single molecule from the concrete or absolute which will also affect its composition.

Another issue is the degradation of essential oils; some of their constituents, such as monoterpenes and monoterpenoid aldehydes, readily combine with oxygen from the air, especially if there is free energy in the form of heat or light. Some will form resins and others will be oxidized. The position of the double bonds can change, open chains can close to from rings and the nature of functional groups can change. These processes will alter the therapeutic effects of the essential oil

constituents so it is important to store the essential oils in such a way as to minimize contact with air and minimize contact with free energy (i.e. store in amber bottles and refrigerate). However, no definitive studies have been done on the length of time for significant degradation of constituents, or the effects of such degradation on the therapeutic qualities of oils. For example, oxidation of some oils may enhance their therapeutic properties. (Bowles, 2003)

Pollution is another factor that influences the quality of the essential oil. For example *Centella Asiatica* in India and South-East Asia, suffers from high levels contamination because it is collected from sewage ditches. Because *Centella Asiatica* is an aquatic plant, it can easily take up pollulants from water into the plant. Devkota (2013) reported that essential oil production in *Centella Asiatica* plants are also affected by the variation of sunlight the plant receives. Accumulation of essential oil in herbs directly or indirectly depends upon light. *Centella Asiatica* produces high amounts of α-carophyllene and carophyllene oxide in open areas compared to shaded areas.

Another factor that is explained by Lawless (1992) is synthetic essential oils called ''natural identical'' oils versus natural oils. Currently many oils and perfumes that use to be extracted from different plants are being produced almost entirely synthetically. These synthetic oils are preferred by the perfumery and flavouring industries as they need consistency in their products and any seasonal changes will cause the natural oils to change. When the naturally occurring essential oils are compared to the so-called ''natural identical'' products, they have an entirely different character. For that reason the synthetic oils are much cheaper to produce than the genuine oils. Many aromatic oils contain a relatively small number of major constituents, several minor constituents and also a very large number of trace elements. To reconstruct such a complex combination of components including all trace elements would be virtually impossible. (Lawless, 1992)

Most ‘'natural identical’' oils are only 96% pure or accurate, but it is the other 4% which are usually the trace elements that often truly characterise a particular fragrance. A real essential oils has a specific combination of constituents, however its' therapeutic value is often a result of the trace elements. Lawless (1992) explains the reason for this might be that " these minute amounts of trace elements have synergistic or controlling effect on the main ones. Natural identical oils cannot be used therapeutically as substitutes for the naturally occurring aromatic materials, not only because the subtle balance of constituents is lost but also because they lack the vital ‘life force’ of oils of natural origin."

1.1.3 Uses of essential oils

The main uses of essential oils are in the fragrance industry as perfumes and aftershaves, as flavourings in the food industry and for their functional properties in the pharmaceutical industry. (Burt, 2004) Essential oils are very important in medicine. Their therapeutic uses are usually based on historical purposes rather than scientific evidence. Their uses range from skin treatments to remedies for cancer (Elsharkawy, 2014) as well as for their antiviral and antibacterial properties. (Burt, 2004)

1.1.4 Dangers of essential oils

Most essential oils are not used in their concentrated form but are diluted in a vegetable-based carrier oil such as olive oil or sweet almond oil usually at a concentration in the range 0.5 - 3 %. If applied directly to the skin in its concentrated form it could cause contact dermatitis usually caused by an allergic reaction. Some essential oils can increase the light sensitivity of the skin making it more likely to burn. (Tisserand and Young, 2014)

If a pure essential oil is ingested at amounts greater than its therapeutic use, it can be poisonous. Medical practitioners prefer organically produced oils as there is

some concern of contamination of essential oils with pesticides. (Tisserand and Young, 2014)

When essential oils are burned they release volatile organic compounds, which can have health effects. They may also release polycyclic aromatic hydrocarbons which are known carcinogens. The LD_{50} of most essential oils or their main components are 0.5-10 g kg^{-1} (orally or skin test). (Tisserand and Young, 2014)

1.2 Centella Asiatica

Centella Asiatica is a perennial herbaceous creeper usually found in moist places. It is a found in Africa, Asia, Australia and South America. (Van Wyk et al, 2002) It has kidney shaped leaves on long slender stalks with pink or red flowers in small bunches. (Gohil et al. , 2010) The species varies considerably in different parts of the world and is sometimes treated as several distinct species. (Van Wyk et al, 2002)

Centella has many therapeutic properties and has been used both externally and internally for thousands of years. It is used medicinally throughout the world such as in Ayurvedic medicine in India and in traditional Chinese medicine. (Gohil, 2010) The health potential of this common plant is only just beginning to be recognized. (Van Wyk and Gericke, 2000)

Centella is also known to help wound healing and for the treatment of various skin conditions. (Gohil, 2010) Extracts and tinctures of the plant material may also be used, or the active components may be isolated for use in standardised products. The whole plant may be used but mainly the leaves are used. (Van Wyk et al, 2002) However as written by Zheng (2007) "In view of its versatile medicinal properties, the requirement of *Centella Asiatica* in pharmaceutical industries has been sharply increasing, thus leading to the over exploitation of this

herb, it has already been listed as a threatened species in the International Union for Conservation of nature and national resources."

1.2.1 The chemical composition of Centella Asiatica and the predominant constituents

The extract of *Centella Asiatica* contains the following active components known as triterpenes: asiaticoside, asiatic acid and madecassic acid. It has also recently been found to contain other constituents which can also contribute to the healing property of *Centella* namely madecassoside, terminolic acid and asiaticoside-B. (Oyedeji and Afolayan, 2004)

Oyedeji and Afolayan (2004) found that the essential oil from *Centella Asiatica* grown in South Africa contained 14 sesquiterpenoid hydrocarbons (68.80%), 9 oxygenated monoterpenoids (5.46%), 5 oxygenated sesquiterpenoids (3.90%), 11 monoterpenoid hydrocarbons (20.2%), and 1 sulfide sesquiterpenoid (0.76%).

The predominant constituents that Oyedeji and Afolayan (2004) identified were α-carophyllene (21.06%), caryophyllene (19.08%), germacrene B (6.29%), bicyclogermacrene (11.22%) and myrcene (6.55%).

α-Carophyllene

α-Caryophyllene is a monocyclic sesquiterpine. It is also known as Humulene or α-humulene. (Fig 1.1) It is an isomer of caryophyllene, and in nature they are usually found together. (Elsharkawy et al, 2013) It was reported that this is one of the constituents that are responsible for the anti-inflammatory effects of the oil. (Passos et al, 2007)

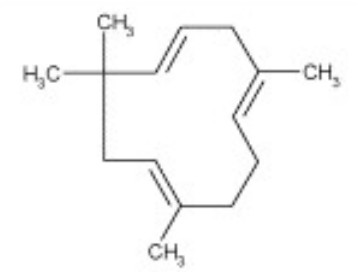


Figure 1.1 Structure of α-Carophyllene (Passos, 2007)

Caryophyllene

Carophyllene is a bicyclic sesquiterpine also known as β-Carophyllene. It is usually found together with its isomers isocaryophyllene and α-humulene. (Elsharkawy et al, 2013) It contains a cyclobutane ring, which is rare in natural structures. It is also known to have anti-inflammatory and antiviral effects as well as antibacterial properties. (Rajeswari and Muthuchelian, 2011)

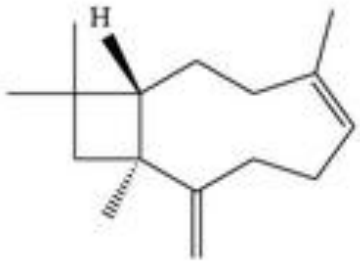


Figure 1.2 Structure of Carophyllene (Soares et al, 2013)

Bicylcogermacrene

Bicyclogermacrene has been reported by Santos et al. (2013) to have good antibacterial properties, especially against Gram-positive bacteria.

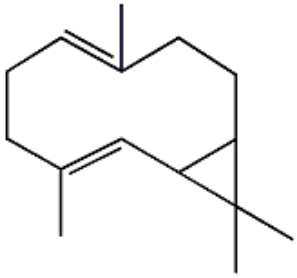


Figure 1.3 Structure of Bicyclogermacrene (Nishimura, 1969)

Germacrene B

Germacrenes are naturally occurring sesquiterpenes. They have been reported to have a number of purposes from being important intermediates in the biosynthesis of other sesquiterpenes to having antimicrobial, anti-inflammatory and antifungal properties. Two other important molecules are germacrene A and germacrene D. (Adio, 2009)

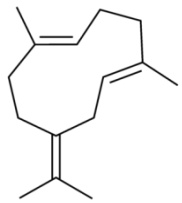


Figure 1.4 Structure of Germacrene B (Nishimura, 1969)

Myrcene

Myrcene is a natural acyclic monoterpene. It is often used in cosmetics, detergents and as a flavouring agent. It is the major constituent in hop, which is used to manufacture alcoholic beverages. It is an important intermediate in the production of terpene alcohols (e.g.: linalool, geraniol). Terpene alcohols are then used as

intermediates in the production of flavouring agents. (Chan et al, 2010)

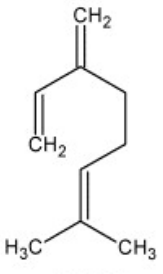


Figure 1.5 Structure of Myrcene (Kolicheski et al, 2007)

1.2.2 Properties and uses of Centella Asiatica

Centella Asiatica has been used for thousands of years in Ayurvedic medicine in India. It was often used for revitalising brain cells and nerves,i.e. used as a memory enhancer and for treating emotional disorders such as depression. In recent times there has been an increased interest in *Centella Asiatica*. The constituents responsible for the versatility of therapeutic actions of the plant are thought to be triterpenoids. (Gohil et al, 2010)

The whole plant of Centella Asiatica can be used, it can be eaten fresh or as an infusion or tincture. (Van Wyk and Gericke, 2000) Its has been reported by Gohil (2010) that *Centella* can be used internally as a sedative, antidepressant as well as for increasing concentration. It also has antioxidant properties and can be used to lower blood pressure.

Studies on *Centella* were reported by Van Wyk and Gericke (2000) demonstrating that *Centella* has anti-cancer activity, by preventing cell division possibly due to the triterpenoids that act as spindle poisons.

Externally *Centella* can be used for wound healing and for the treatment of various skin conditions such as eczema, leprosy and psoriasis. It has also been found to strengthen the arteries and veins and improve circulation. It also has excellent anti-inflammatory properties. (Gohil, 2010)

The essential oil of C*entella* also has antibacterial activity against Gram-positive and Gram-negative organisms as established by Oyediji and Afolayan (2004). It was found that the activity against Gram-positive bacteria was greater than against the Gram-negative bacteria. This is thought to be due to the high content of germacrene compounds which are known to be strong antimicrobial agents. (Oyediji and Afolayan, 2004)

1.2.3 Toxicity and safety

The safety profile of *Centella Asiatica* is good. There is no known toxicity if it is taken in recommended doses. Infrequent adverse reactions have been reported usually with high doses taken internally such as nausea, dizziness and headaches. External side effects such as allergic contact dermatitis and a burning sensation have also been reported. (Gohil et al, 2010)

1.3 *Centella Asiatica* and heavy metal contamination

Medicinal plants have been used for their therapeutic properties for thousands of years and with the popularity of complementary medicines their use has increased in recent times. Their safety has however been questioned due to illnesses and fatalities. Some medicinal plants in Europe, Asia and the United States were reported to contain toxic metals which were associated with poisonings. (Caldas and Machado, 2003)

The essential oil and heavy metal contents in *Centella Asiatica* can be affected by environmental conditions. It is an aquatic plant and pollutants in the water can be easily taken up into the plant. Heavy metals found in the soil of the plant can therefore also affect it. Rainfall, atmospheric dust as well as fertilisers are examples of other sources of heavy metal contamination. (Abu-Darwish, 2009) Elevated levels of heavy metal contamination were found in areas where agricultural mediums were used such as organic mercury or lead based pesticides as well as cadmium containing fertilisers which can also cause the irrigation water to become contaminated. (Caldas and Machado, 2003)

Plants require macronutrients (Ca, K, Mg, P, N) and much smaller amounts of micronutrients (Mo, Cu, Se, Co, Mn). It was reported by Abu-Darwish et al. (2009) that medicinal plants are natural sources for trace elements which are necessary for maintaining life processes in plants. A high intake of these trace elements can also cause toxicity. (Abu-Darwish et al, 2009)

The level of trace elements in plants can be affected by a variety of conditions such as the chemical characteristics of the soil as well as the ability of the plant to accumulate certain elements. Aromatic medicinal herbs have a complex composition and natural biological differences. It is therefore important to assess their quality, safety and effectiveness. Since *Centella Asiatica* has many therapeutic uses, it is necessary to assess the quality of any extracts or oils to protect the end user from contamination. (Abu-Darwish et al, 2009)

Heavy metals such as copper and zinc are essential elements to plants in small concentrations, however at high concentrations they can be toxic. The amount of these essential elements present in the plant are usually regulated homeostatically, and the amount taken up from the environment is controlled according to the plants nutritional demand. When there is an excess of these metals or if there are inadequate amounts, these regulating mechanisms are negatively affected which in turn affect the plants' health. (Pawlowski, 2011)

There are a number of factors that determine the toxicity of metals and their compounds. Factors such as the mechanisms by which the plant uptakes the metals through it cell membranes as well as the bioavailability such as intercellular distribution and the metals ability to bind to cellular macromolecules. The relative toxicity of different metals to plants can be affected by the experimental conditions as well as the genotype of the plant. (Azevedo and Rodriguez, 2012) Azevedo and Rodriguez, (2012) explained that "the mechanism evolving the toxicity of heavy metals is thought to originate by a complex pattern of interactions between cellular macromolecules and the metal ions". When a metal enters a cell, it triggers a number of genetic, metabolic and signal transduction processes to neutralise the source of toxicity. "Most heavy metals act through one of the following mechanisms: reactions of sulphydryl (–SH) groups with cations, changes in the permeability of the cell membrane, affinity for reacting with phosphate groups and active groups of ADP or ATP, replacement of essential ions and oxidative stress." (Azevedo and Rodriguez, 2012)

Lead, mercury and chromium are the best-known heavy metals and have been used by mankind for thousands of year. Whereas organic pollutants over time degrade to water and carbon dioxide, however heavy metals do not degrade since they are elements and accumulate in the environment. This is a serious risk to the environment as they tend to accumulate in marine sediments, lakes and estuaries and then transported from one "environmental compartment" to another. (Pawlowski, 2011) Mercury, lead and chromium can have adverse effects even at low concentrations exposure. (Pawlowski, 2011) It was reported by Caldas and Machado (2003) that lead and mercury can have potential toxic effects on the fetus, as it can cross the placental barrier as well as adverse affects on the nervous and renal systems.

1.3.1 The fate of lead in the environment and its effects on plants

Lead is a natural element found in the earth's crust mainly in the mineral form of galena (PbS) with smaller concentrations of cerussite ($PbSO_4$). It is not often found in its elemental form. Lead minerals are found usually with silver, gold, iron sulphides, copper and zinc. (Krishnanandan and Srikantaswawy, 2013)

Lead is dispersed throughout the environment mainly due to anthropogenic activities such as the mining and smelting of ore, manufacturing of lead-containing products and the combustion of coal and oil. Natural sources of lead include volcanic eruptions, soil erosions as well as bushfires. The concentration of lead from natural sources is much lower than anthropogenic sources (Abadin et al., 2007) As the toxicity of lead has become known, more stringent controls for some anthropogenic sources of lead have been implemented such as forbidding the use of lead based paint. Since lead does not degrade, these previous uses of lead have left great amounts in the environment. (Abadin et al. , 2007) Once lead enters the environment (air, water or soil) animals and plants can bioconcentrate it and it enters into the food chain, but no biomagnification has been found in the aquatic or terrestrial food chains. (Abadin et al. , 2007)

Humans can be exposed to lead by inhaling contaminated ambient air or smoking (increase lead levels in the blood) or by ingesting contaminated food and water. It is known that some fish have high levels of lead contamination. Exposure to lead at small concentrations does not cause any symptoms, but high concentrations of lead can have severe health effects. (Abadin et al., 2007) They can affect the processes of the central nervous system with symptoms such as headaches, poor attention span (Abadin et al., 2007), loss of memory, and irritability (Papanikolaou et al, 2005). They have been reported to cause renal dysfunction (Papanikolaou et al., 2005) as well as cardiovascular problems such as high blood pressure. (Schwartz and Howard, 2006) They have also been reported to cross the placental barrier and hence can affect the fetus. Other symptoms of lead exposure

include gastric problems, anaemia and pain in the joints. (Papanikolaou et al., 2005)

Lead particles in the air can be a result of natural sources or they are also emitted during some manufacturing processes (e.g. coal combustion). These lead particles can then settle onto the ground by gravitational settling and then can be deposited into the waters and onto soils. They can also suspend themselves again back into the air when disturbed. The natural concentration of lead in the air is less than 0.1 ug m^{-3}. (Abadin et al, 2007)

As reported by Abadin et al. (2007) "the solubility of lead compounds in water is a function of pH, salinity, hardness, and the presence of humic matter. Solubility is highest in soft, acidic water. The sink for lead is the soil and sediment". Lead does not usually leach into ground water and subsoil because it is adsorbed strongly to the upper layers of soil and usually retained there. In the environment lead compounds may undergo reactions to produce other lead compounds but since it is an element it does not decay. Therefore when lead is deposited in the soil, the plants do not take it up rapidly so it remains in the soil at high concentrations. (Abadin et al, 2007)

Abadin et al. (2007) reported that "The fate of lead in soil is affected by the adsorption at mineral interfaces, the precipitation of sparingly soluble solid forms of the compound, and the formation of relatively stable organic-metal complexes or chelates with soil organic matter." These processes were found to be dependent on certain factors such as the soil type, the pH of the soil, the particle size, cation exchange capacity (CEC), the amount of humic matter, inorganic colloids and iron oxides present in the soil. The solubility of lead in the soil naturally is very slow but it increases with increased acidity and can then leach into groundwater or subsoil. Leaching into the subsoil can also be induced if the concentration of lead in the soil comes near to or above the CEC limit of the soil and if the soil contains materials that can form soluble chelates with it. (Abadin et al. , 2007)

Plants and lead

Abadin et al. reported that the content of lead present in plants is mainly due to atmospheric deposition where these particles adsorb strongly onto the surface of the plant. Lead was found in internal plant tissues which showed that certain plants can take up lead from the soil by translocation or from the leaf surfaces (adsorption of dust). This absorption was not found to be significant, as lead prefers to adsorb strongly to organic matter in soil. However if the acidity of the soil increases the lead compounds bioavailablity also increases. Besides pH there were other factors that could affect the bioavailablity of lead to the plant such as soil moisture content, CEC, temperature and the amount of humic matter available. Abadin et al. found that the greatest concentration of lead was in the roots and smaller amounts were found in the leaves and stems. If the plants were harvested they could possibly enter the food chain or if the plants decay this lead will eventually return to the soil unless the plants are removed for other reasons. (Abadin et al., 2007)

Lead is an intermediate acid and can act as a soft-metal cation. Toxic metals can affect the natural processes of a plant by forming strong complexes with soft functional groups on biomolecules. These soft-metal cations were reported by Abadin et al. (2007) to cause phytotoxicity by a number of processes. An essential metal (e.g. Ca) can be bound to a bio-ligand and be displaced by a soft-metal (e.g. Cd). This complexation between a bio-ligand and the soft-metal cation can change it structurally and also prevent it from reacting normally and therefore interfering in its intended purpose. These soft-metal cations can also effect normal enzyme function by blocking the enzymes active or catalytic sites which they need to bind to biological substrates. For example the active sites of some enzymes contain amino acids such as cysteine and methionine that contain the soft ligands –SH and SCH which form strong covalent bonds with soft-metal cations such as Pb, Cd, Hg, Ag and Cu. The formation of these complexes can affect the normal functions of the enzymes which can be toxic to the affected organism. (Abadin et al., 2007)

Therefore, when high concentrations of lead are present in plants it can cause an inhibition of enzyme activities as well as affecting the minerals the plant takes up by modifying the membrane permeability. Lead contamination can have negative effects on photosynthesis which in turn will have negative effects on the growth of the plant as well as the morphology. It was also reported by Abadin et al. (2007) that high amounts of lead in plants can increase the production of reactive oxygen species (ROS) and cause oxidative stress.

1.3.2 The fate of mercury in the environment and its effect on plants

Mercury can be found in the environment in air, water and soil. It exists in several forms: elemental mercury (Hg), organic mercury (e.g., CH_3-Hg) and inorganic mercury (Hg^{2+}). It can also be found associated with ions (HgS) or as mercurous chloride (Hg_2Cl_2). (Azevedo and Rodriguez, 2012) HgS is the form most natural mercury occurs, however weathering processes can release mercury into the environment. (Rytuba and James, 2002)

Examples of natural sources of mercury are forest fires and volcanic emissions (Rytuba and James, 2002) Anthropogenic sources of mercury are from solid waste incineration (Wang et al. , 2004), coal combustion (Pacyna et al. , 2006), the manufacturing of gold, metal smelting, cement production and chlorine production. (Pacyna et al., 2006)

In the atmosphere, mercury exists as vapour, particles or in aqueous form when attached to water droplets. (Wang et al. , 2004) The ability of mercury to shift into a vapour form and its high solubility in water, makes it a huge environmental hazard as it is able to move in a number of ecosystems and remain in the atmosphere for long periods. (Azevedo and Rodriguez. 2012) These particles then settle into water or are deposited onto soil. (Wang et al. , 2004) Some organisms

(e.g. fungi or bacteria) then convert this mercury into methylmercury; this is the first introduction of mercury into the trophic chain. It is a highly toxic form of mercury that can bioaccumlate and biomagnify in aquatic organisms (shellfish, fish) and then be passed onto animals that consume fish including humans. This is usually the main source of methymercury exposure to humans. Certain fish can bioaccumalte it more than others but it usually depends on the organisms diet, how high they are in the food chain and their life span. (Acha et al., 2012)

The various forms of mercury can cause a extensive range of toxic effects in humans depending on the amount of exposure, age and the duration of exposure. (Homes et al., 2009) Homes et al. (2009) reported that at high concentrations, mercury targets particularly the central nervous system, the kidneys, thyroid and immune system can be negatively affected. Azevedo and Rodriguez (2012) reported that "the intact organomercurial cation is believed to be the toxic agent responsible for the damage provoked in cells; for instance, in humans, mercury can be modified to methyl–Hg that is capable of causing damage to the nervous system, liver, and ultimately cause death by multiorgan failure."

The fate of mercury in the environment is dependent on the reactions mercury (Hg) undergoes with the aquatic environment and these are dependent on a number of factors such as the concentration of metallic mercury, the oxidation rate of the metal, the pH, the amount of other ions available, and the concentration of particulated matter. (Canela and Jardim, 1997) Mercury species reaching the aquatic system are, in a broad sense, classified into inorganic (Hg^0, Hg^{2+} and Hg_2^{2+}) and organic (CH_3Hg^+, $(CH_3)_2Hg$, etc.) forms. The lipid solubility of organic mercurial compounds are the reason that they are considered to be the most toxic form of the metal, these species can quickly enter the blood stream and damage the central nervous system. (Canela and Jardim, 1997)

Inorganic forms of mercury have much lower toxicity than the organic ones, with a decrease in toxicity from to Hg^{2+} > Hg_2^{2+} > Hg . (Canela and Jardim, 1997) Mercury vapour can exist in an oxidation state of 2+ (mercuric) or 1+ (mercurous). It forms a key role in the mercury cycle. Elemental mercury (Hg^0) can be oxidised and can form complexes with major inorganic ligands such as OH^-, Cl^- and S^{2-} and dissolved organic carbon which increases its solubility. (Pacyna et al., 2006) It can also form amalgams with most metals (except platinum and iron) Mercuric ions have a greater affinity for solids than the elemental form and they are responsible for majority of the organic and inorganic forms found in the environment. (Azevedo and Rodriguez , 2012)

Plants and mercury

The source of mercury contamination in soil is usually due to the addition of lime, fertilisers and manure. The concentration of mercury in the soil and the amount taken up by the plant are dependent on a number of factors such as plant type, the soils cation exchange capacity, aeration and pH. (Azevedo and Rodriguez, 2012)

It was reported by Azevedo and Rodriguez (2012) that plants which take up mercury by translocation or absorption of the vapour form have a tendency to accumulate it in the roots and in some plants it was found in the shoots as well.

It is thought that toxic metal ions enter the plant cells through the ionic channels (usually bound to nitrogen or sulphur) competing with other essential metals like copper, zinc and iron. These are the same processes which plants use to absorb their micronutrients.

This heavy metal absorption into the plant can cause damage in the plant cells by affecting the permeability of cell membranes, disrupting the essential ion transport throughout the plant, preventing the normal functioning of enzymes by blocking the active sites, substituting or displacing metal ions (e.g. Mg) from molecules (e.g. chlorophyll) and by denaturing proteins. Mercury can specifically cause phyotoxicity in plants by a number of mechanisms such as its ability to favour reactions with phosphate and sulphydryl (SH) groups, by substituting the plants

essential ions with mercury and its ability to disrupt functions involving important proteins. (Azevedo and Rodriguez, 2012)

When a plant is contaminated by mercury it can affect its normal functions by reducing its chlorophyll synthesis, photosynthesis and water uptake. It was found that organic and inorganic mercury can cause a loss of the plants' essential ions such as magnesium, manganese, potassium and an increase in iron. The toxicity observed in the aerial part of plants could be explained by the ability of the mercuric ion (Hg^{2+}) to affect the plasma membrane; however it is believed that the damage in the roots can explain the toxicity observed in the shoots. (Azevedo and Rodriguez, 2012)

1.3.3 The fate of chromium in the environment and its effects on plants

Natural sources of chromium in the environment are from the erosion of soil and rocks and from volcanic ash. Chromium and its compounds have various uses in the industrial industry and are released into the environment (in solid, liquid and gas phases) from sources such as metallurgical, chemical (pigments, electroplating, hide tanning etc.), combustion of fuels and used in the refractory brick production process. (Kotas and Stasicka, 2000) These anthropogenic sources have caused an increase of chromium compounds biomobility and bioavailability in the environment. (Shanker et al., 2005)

In the environment chromium is found in two oxidation states, Cr(III) and Cr(VI), and its toxicity in plants depends on its oxidation state. Cr(III) is a trace element vital for the proper functioning of living organisms. (Kotas and Stasicka, 2000) Cr(VI) species are generally very soluble and therefore more mobile and bioavailable, hence more toxic than less soluble Cr(III) species. (Shanker et al., 2005) Cr(VI) is a strong oxidant with a high redox potential responsible for a producing a large amount of reactive oxygen species (ROS) quite quickly which

accounts for its toxicity. (Shanker et al., 2005) The other properties of chromates also add to its toxicity such as their ability to form free radicals inside the cell when Cr(VI) is reduced to Cr(III) and its ability to diffuse freely across cell membranes. To evaluate the toxic effects of chromium in the environment, it is therefore necessary to understand the chemistry of each of its oxidation states in air, water and soil rather than the total chromium concentration as well as its transport and distribution throughout the environment. (Kotas and Stasicka, 2000)

A number of health problems can be caused by exposure to Cr(VI) compounds. If Cr(VI) is inhaled it can cause asthma, bronchitis, damage to the nasal septum and pneumonitis. If Cr(VI) compounds come into contact with the skin, they can cause dermatitis or skin allergies. The Cr(III) compounds are able to coordinate certain organic compounds which can result in the disruption of certain metallo-enzyme processes when present in considerably large amounts. (Shanker et al., 2005)

In the environment chromium can be found in air, water and soil. Shanker et al. (2005) reported that chromium occurs naturally in soil from 10 to 50 mg kg^{-1}. In fresh water and sea water it was found to range from 0.1 to 117 µg L^{-1} and from 0.2 to 50 µg L^{-1} respectively. Shanker et al. (2005) found that in the atmosphere the chromium concentration vary greatly from air samples taken from remote areas (Greenland and Antarctica) from $5.0 \times 10^{-6} – 1.2 \times 10^{-3}$ µg m^{-3} compared to samples taken from urban areas of 0.015 – 0.03 µg/ m^3. (Shanker et al., 2005)

Chromium enters the terrestrial environment by settling out of the air (e.g. chromium containing particles) or being washed out. It also enters through the disposal of chrome containing substances from industrial activities. The weathering of their parent materials is the main source of chromium in natural soil. It was found that on average the concentration of chromium in a number of soils varied from 0.02 to 58 mmol g^{-1}. (Kotas and Stasicka, 2000) Chromium is found in soil as Cr (III) adsorbed to soil particles or as insoluble $Cr(OH)_3$ which prevents it from leaching into the ground water or being absorbed by plants.

Cr(III) can also bind to humic acids which also make it insoluble. In soils with a neutral or alkaline pH, the Cr(VI) is present mainly as soluble chromates. When the soil becomes more acidic (pH less than 6), $HCrO_4^-$ becomes the main form of chromium present. $HCrO_4^-$ and CrO_4^{2-} are the most mobile forms in soil and can be easily leached into water or taken up by plants. (Kotas and Stasicka, 2000)

The redox reactions can convert Cr(III) to Cr(VI) and visa versa. However these reactions are dependent on a number of factors such as the amount of oxygen present, the pH, and the right reduceing agents which act as catalysts or ligands. An important mechanism in the environment is called dechromifiction which reduces Cr(VI) to Cr(III) with the help of catalysts such as Fe(II). Under the right conditions when Cr(III) is converted to Cr(VI) it increases the solubility of chromium and it can then move from the soil into the water system or enter a water body through surface run-off. Cr(III) can also enter the water system by forming soluble complexes with organic ligands. Once chromium contamination occurs in water three sub-systems need to be considered namely lake, river and ocean. Specific conditions control the transport routes in these subsystems such as depth, amount of humic matter present, oxidation conditions and temperature. (Kotas and Stasicka, 2000)

Chromium in plants

Chromium contamination in plants is dependent on the speciation of chromium which is responsible for the mobility of the ion and consequent uptake into the plant which causes the toxic effects. (Shanker et al., 2005)

As mentioned before, Cr (III) is less toxic and Cr(VI) being more mobile and more toxic. Plants have no specific mechanism by which they take up chromium. It can be taken up by the plant by carriers of essential ions such as iron or sulphate. Chromium toxicity can have adverse affects on the plant by affecting the growth of its roots, leaves and stems it can also alter its germination mechanisms and also cause detrimental effects on photosynthesis, mineral nutrition and water

absorption. Chromium can further cause alterations in metabolic activities in the plant such as effecting the function of enzymes and its capability to cause oxidative stress by producing reactive oxygen species. (Shanker et al., 2005) Shanker et al. (2005) reported that at low chromium concentrations (3.8×10^{-4} µM) some plants are not affected but it is toxic to most plants at 100 µM kg^{-1} dry weight. A common symptom of chromium toxicity is the leaves turning brown which is usually due to the high amount of ROS present in the cells, which cause oxidative stress.(Shanker et al., 2005)

In summary, a surplus of chromium can cause an imbalance in the nutrients, root injury, plant growth and chlorophyll synthesis inhibition, young leaves chlorosis, and the wilting of the plant. Chromium exposure can also cause alterations to the plants metabolism by effecting enzymes and by generating ROS. (Yadav, 2010)

1.4 Similar studies

There are not many literature reports on *Centella Asiatica* growing in SA despite its wide spread use in traditional medicines.

A study was conducted in South Africa on *Centella Asiatica* by Oyedeji and Afolayan (2004) to determine the composition and antibacterial activity of the essential oil. They found that *Centella* consists of 40 constituents. The predominant constituents were found to be β-caryophyllene (19.08%), α-humulene (21.06%), germacrene B (6.29%), bicyclogermacrene (11.22%) and myrcene (6.55%). It was also found that the essential oil extracted showed evidence of broad spectrum antibacterial activities against gram-negative and gram-positive organisms. (Oyedeji and Afolayan, 2004)

Other similar studies have been conducted on different essential oils but they were not conducted in South Africa. For example, a study was conducted by Babu and

Kaul (2003) to compare the different composition of essential oil from rose-scented geranium extracted by different distillation techniques. The samples were analyzed by Gas Chromatography (GC) and Gas Chromatography Mass Spectrometry (GC-MS) to determine the essential oil compositions. It was found that the greatest oil yields were obtained by water distillation (0.16%-0.22%) followed by water-steam distillation (0.09-0.12%) and steam distillation (0.06-0.18%). (Babu and Kaul, 2003)

Another study conducted by Lucchesi et al. (2004) compared solvent-free microwave extraction (SFME) of essential oils from aromatic herbs to hydro-distillation. The three herbs investigated were basil, thyme and garden mint. SFME is a combination of microwave heating and dry distillation without any solvent or water added run at atmospheric pressure. Lucchesi et al. (2004) found that the essential oil extracted by SFME for 30 minutes were qualitatively and quantitatively similar to those extracted by hydro-distillation for 4.5 hrs. However, the oils extracted using SFME yielded a greater amount of valuable oxygenated compounds and had more advantages over hydro distillation by being more cost efficient in terms of energy, time and plant material needed. It appears to be a good alternative for extraction of essential oils from plants as well as being green technology. (Lucchesi et al., 2004)

Scaneberg and Khan (2002) investigated a number of extraction methods for the essential oil of lemon grass by comparing the main compounds identified. They developed a gas chromatography flame ionization detection method to quantify the main compounds in the essential oil of lemon grass. They compared four different extraction methods: steam distillation, super critical fluid extraction and solvent extraction and accelerated solvent extraction. It was found that the results from solvent extraction by sonication with non-polar solvents were comparable to the ones obtained from the steam distillation method. Different solvents were also used for solvent extraction and it was found that hexanes extracted the greatest

percentage of the main compounds compared to acetone, dichloromethane and methanol. (Scaneberg and Khan, 2002)

The other investigations that are most commonly carried out are on the composition of various essential oils from different plants. Studies have been conducted on the composition of the essential oil of *Juniperus Pseudosabina* and it was found that it contains 43 constituents. (Dembitskii et al. , 1969) Another study was done by Casterjon et al. (2003) to identify the chemical composition and investigate the antimicrobial activity of the essential oil from *Annona Cherimola* which was extracted by steam distillation of the fresh leaves, flowers and fruits from the plant and the oil was analyzed by GC-MS. Sixty constituents were identified from the oils. (Casterjon et al., 2003)

A study was conducted on essential oil yields and heavy metal contents of some aromatic plants which were grown in the south of Jordan. The herbs analysed were *Thymus serpyllum L*., *Thymus Vulgaris L*. and *Salvia officinallis L*. The essential oil was extracted from dried aerial parts of the plant using hydro-distillation with a Clevenger type apparatus. The heavy metal contents in the herbal samples were analysed using atomic absorption flame emission spectrometry. The mean values of the essential oil yields of *T.serpyllum, T.Vulgaris* and *S.officinalis* were 2.5, 4.0 and 1.9% respectively. The plants were tested for the contents of Ni, Pb, Cu, Zn, Mn and Fe. Cobalt was only found in *T. serpyllum*. The highest content of Ni, Pb and Cu content was found in *T.vulgaris*. (Abu- Darwish, 2009) It was therefore concluded that environmental conditions can affect the heavy metal contents and the essential oil in the plants. It was also reported by Abu-Darwish (2009) that the plants investigated and grown in the South of Jordan could be used safely for pharmaceutical and other purposes without any harmful effects.

A investigative report on medicinal plants and heavy metal contamination was carried out in Brazil. The extensive use of medicinal plants in therapeutics have

questioned the safety of their use due to reports of illness. Samples of medicinal herbs were analysed including *Centella Asiatica*. Atomic absorption spectrophotometry was used to test for the amounts of lead, cadmium and mercury in the herbs. Caldas and Machadob (2004) found that guarana, egg plant and artichoke had no lead, cadmium or mercury detected. In the other herb samples mercury was found up to 0.087 ug g^{-1} and cadmium at concentrations up to 0.74 ug g^{-1}. The greatest amount of lead in the herb samples was found to be 22 ug g^{-1}, however three samples of horsechest were found to have the greatest concentrations of 153, 156 and 1480 ug g^{-1}. Caldas and Macadob hence reported that "The estimated lead intake through the consumption of horse chestnut reached 440 % of the provisional tolerable weekly intake (PTWI) and might be of concern to consumers if the medicine is taken on a long term basis". They also reported that exposure to cadmium and mercury by herbal medicines did not appear to be a health concern. The results of this study concluded that it is necessary to control the amount of toxic heavy metals present in plants used as medicines.

A study on heavy metal contamination and *Centella Asiatica* was conducted by Yup et al. (2010) on plants grown in metal-contaminated soils under laboratory conditions to examine the uptake of these heavy metals as well as the effect on growth. The uptake of heavy metals by different parts of the plants (roots, stems and leaves) were investigated. It was found that metals were taken up by the plant mainly in the roots, followed by the stems and then the leaves. They also found that there was a close positive relationship between the concentration of metal in the soil and the amount of metal accumulated in different parts of the plant. Therefore, *Centella Asiatica* was found to have the "potential of being used as a biomonitoring plant for heavy metal pollution in polluted soils". (Yup et al., 2010)

1.5 Aim of the study

There have been many studies conducted to investigate the therapeutic uses of the entire plant of *Centella Asiatica*. The species varies considerably in different parts of the world and is sometimes treated as several distinct species. There have been more studies conducted on the extract of C*entella Asiatica* rather than the essential oil.

Even though *Centella Asiatica* is used extensively in traditional medicine in South Africa there have not been many studies conducted to investigate the particular effect of the active ingredients from the oil and different extraction methods that could be used to optimize the therapeutic activity of the essential oil.

This study was therefore focused on *Centella Asiatica* growing in South Africa and because the different parts of the plant (e.g. stem, root, leaves) have different amounts of essential oil and different compositions, it was decided to focus on the essential oil extracted from the leaves only. The different extraction methods have been compared by the composition of the essential oil from the leaves and the yield obtained.

The aim of the following study was therefore to:

Compare the different extraction methods such as different distillation techniques (water distillation, steam distillation) and solvent extraction and optimise the conditions of extraction to obtain highest possible yields of the essential oil from C*entella Asiatica* leaves growing in South Africa. The different extraction techniques were also evaluated on how they modify and affect the active ingredients in the essential oil. The extraction was also done with dry leaves to see how it would affect the composition of the essential oil and a comparison of a natural-identical oil of *Centella* with the natural oil was also conducted.

The essential oil and heavy metal contents in *Centella Asiatica* can also be affected by environmental conditions. As mentioned *Centella Asiatica* is an aquatic plant and particularly sensitive to pollutants in the water, which can be easily taken up by the plant. Heavy metals found in the soil of the plant can therefore affect it. Other sources of heavy metal contamination are atmospheric dust, fertilisers and rainfall. (Rytuba and James, 2002) Since *Centella Asiatica* has many therapeutic uses it is imperative to have high quality control to protect people who use it from contamination. Therefore, in this study heavy metal contamination effects on the plant, yield and composition of the essential oil in *Centella Asiatica* leaves were investigated.

1.6 Key questions

The following fundamental questions will be addressed in the proposed research

- What is the most suitable method of extracting the essential oil from *Centella Asiatica* leaves?

- How do the different methods of extraction affect and modify the quality and quantity of the essential oils?

- How does changing the solvent for extraction effect the yield of oil obtained and the composition?

- What method can be used to quantify the small amount of essential oil obtained?

- Will larger or smaller younger leaves contain more essential oil?

- How do dry leaves used in the extraction process affect the composition of the oil and the yield?

- How does the composition of the natural oil vary from a commercial (synthetic) oil of *Centella Asiatica* ?

- How does changing the soil quality by spiking it with heavy metals affect the plant and the oil composition and yield?

- Does the essential oil contain any heavy metals?

2 Experimental

The research was conducted in two parts:

- Part 1: Investigating the different extraction processes on dry and fresh *Centella Asiatica* leaves
- Part 2: Investigating the effect of heavy metals in the soil on the *Centella* plant and how it affects the composition and yield of the essential oil.

2.1 Part 1: Investigating the different extraction methods

2.1.1 Sample collection

Ten *Centella Asiatica* plants were bought from Margret Roberts farm in Magaliesberg South Africa and planted in the same moist area. After 4 - 8 weeks the leaves of 2.5 - 9 cm length were harvested and used for the experiments.

2.1.2 Isolation of the oil

Four different extraction methods were investigated: steam distillation, water distillation, solvent extraction and soxhlet extraction using various solvents. The steam distillation has 2 variations in apparatus which was investigated: using a Clevenger apparatus and using a variation of the Clevenger apparatus.

Once the initial results were obtained from the basic Clevenger apparatus, a further investigation was done in detail using a more complex apparatus for essential oil extraction as described by the British pharmacopeia Volume IV, using both steam and water distillation. The apparatus is used by many companies

professionally and it was therefore chosen for use in the investigation on *Centella Asiatica* leaves to determine if the amount of oil collected could be quantified and how the extraction method could affect the composition of the oil. All the extraction methods on this apparatus were carried out with the same amount of leaves within a 2.5 – 9 cm leaf size range. The best extraction method was then identified and tested on dry leaves to determine how this affects the yield and essential oil composition. Salting out was also investigated by adding salt into the water distillation to determine how it would affect the *Centella Asiatica* essential oil yield and essential oil composition. All distillation procedures and solvent extractions were carried out in a fume cabinet.

The oil was then analyzed by gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS). In addition a scanning electron microscope was used for surface morphology of the leaf in order to compare the essential oil sacs before extraction and after extraction.

2.1.3 The Different extraction methods investigated

A. STEAM DISTILLATION

Clevenger apparatus (Setup 1)

For the steam distillation two different setups were used. The first setup used for steam distillation is a Clevenger apparatus to which a false bottom extraction container is connected at the bottom of the apparatus and filled with leaves. The steam formed by heating the distilled water in the round bottom flask has to move through the extraction container where it causes the volatile components from the leaves to evaporate and travel with the steam through the connecting tube on the Clevenger apparatus to the condenser and condenses into the receiver tube. The

function of the recycle tube is to recycle the water back into the round bottom flask when the receiver tube is too full. (Fig. 2.1)

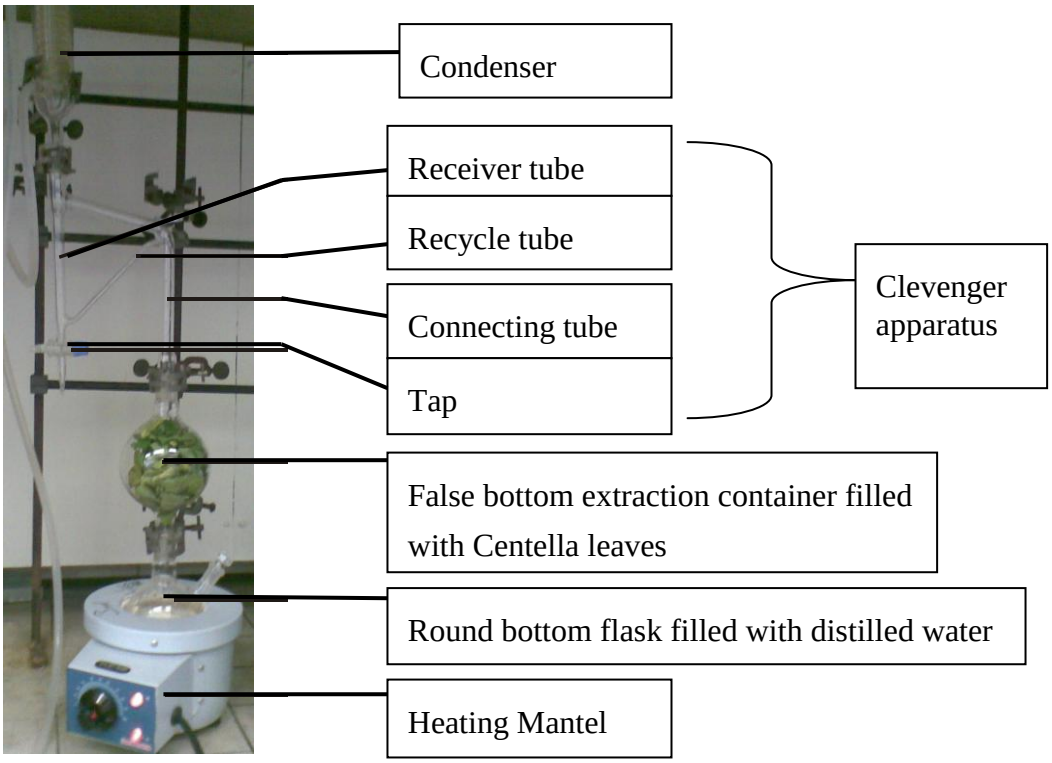


Figure 2.1 Steam distillation setup 1 using the Clevenger apparatus

Variation of the Clevenger apparatus (Setup 2)

The second setup used for steam distillation is a variation of the Clevenger apparatus. The main difference between this variation of the Clevenger apparatus and the Clevenger apparatus is that in the variation of the Clevenger apparatus the extraction container is part of the apparatus.(Fig. 2.2)

Experiment 1A: Setup 1 - Steam distillation with distilled water on fresh leaves (42 g)

- 42 g of fresh *Centella Asiatica* leaves were washed with distilled water and placed in a still.

- 250 ml of distilled water was placed in the round bottom flask at the bottom of the Clevenger apparatus and heated to produce steam. The steam is channelled into the flask with the leaves and then into a condenser

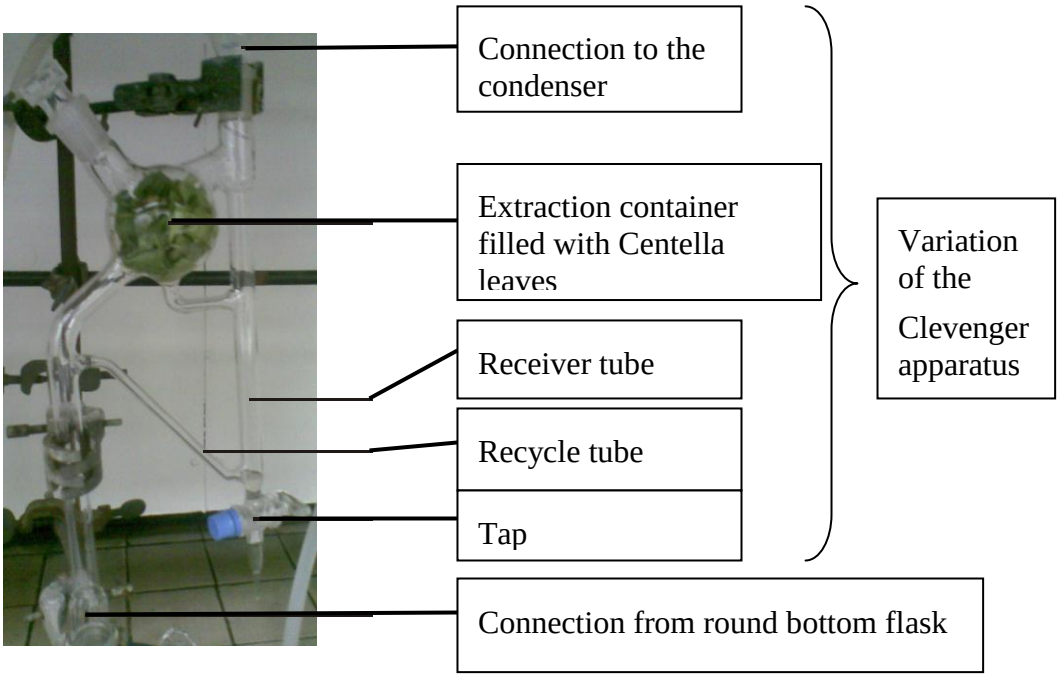


Figure 2.2 The variation of the Clevenger apparatus (Setup 2)

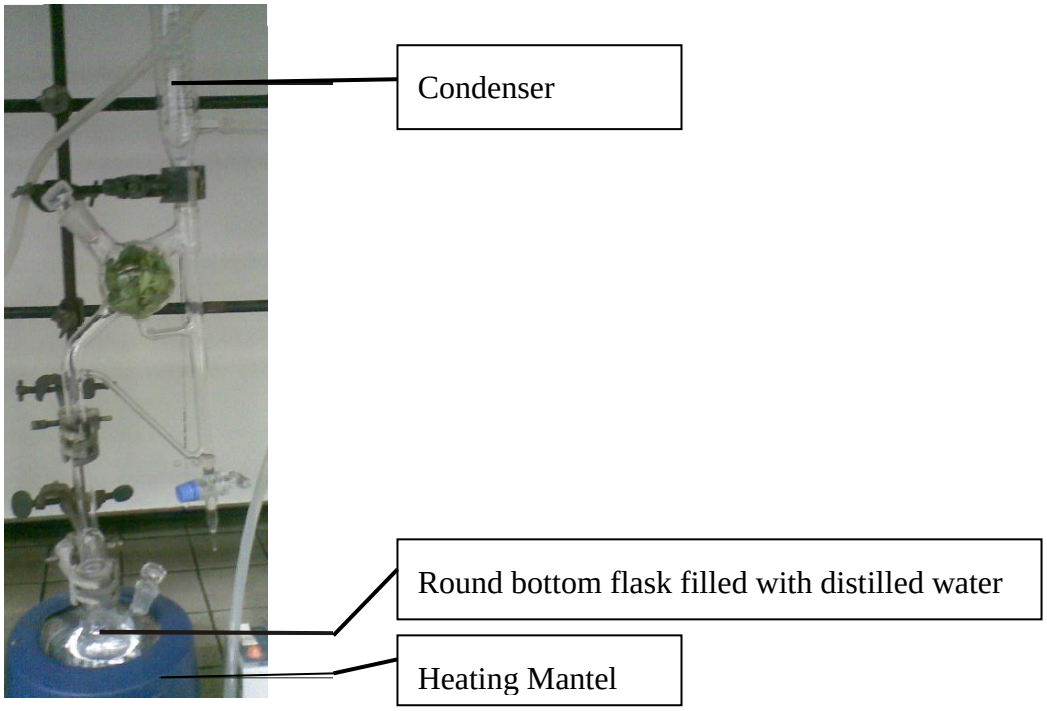


Figure 2.3 Steam distillation setup 2 using the variation Clevenger apparatus

and cooled, causing it to become a liquid once more, which is then collected in the receiver tube.

- The distillation is continued for 3 hours.
- The parts of the apparatus just below the condenser (the connecting tube) where the steam moves through are covered in cotton wool to prevent the steam and oil from condensing on the cooler walls of the connecting tube before it reaches the condenser. This minimizes any loss of oil before it reaches the receiver tube.

Figure 2.4 Picture to illustrate the connecting tube of the Clevenger apparatus covered in cotton wool

- After 3 hours, the liquid that now consists of oil and water in the receiver tube, was separated by using hexane.
- 1 ml of hexane was added to the receiver tube and mixed.
- Then the water and hexane (which contains the oil) is separated into two beakers. The hexane (and oil) mixture is then dried over anhydrous sodium sulphate and used for analysis.
- Sample named SDP-1

Experiment 1B: Setup 1 - Steam distillation with distilled water on fresh leaves (10 g)

- The same procedure above was repeated 3 times with 10 g of fresh *Centella* leaves instead of 42 g.
- In this case, 0.7 ml of hexane was used for separating the oil and water and 75 ml of distilled water were added to the round bottom flask to produce steam.
- The experiment was repeated 3 times.
- Samples were named SD-1, SD-2 and SD-3.

Experiment 2A: Setup 2 - Steam distillation with distilled water on fresh leaves (10 g)

- 10 g of *Centella* leaves were washed with distilled water and placed in the varied Clevenger apparatus into the extraction container.
- 75 ml of distilled water were placed in the round bottom flask and heated to produce steam.
- The distillation was carried out for 3 hours.
- Then 0.7 ml of hexane was used to separate the oil from the water and dried over anhydrous sodium sulphate and analysed.
- The experiment was repeated twice.
- Samples were named SDS2W1, SDS2W2.

Experiment 2B: Setup 2 - Steam distillation with vinegar on fresh leaves (25 g)

- The same procedure as for experiment 2A was used but with vinegar in the round bottom flask instead of distilled water .
- 25 g of fresh leaves were used in this case and 0.7 ml of hexane.
- The experiment was repeated twice.
- Samples were named SD-VIN and SD-VIN-2.

Experiment 2C: Setup 2 - Steam Distillation with distilled water on dry leaves (5 g)

- The same procedure as experiment 2A was repeated but with 5 g of dry leaves instead of 10g of fresh leaves.
- Fresh leaves were placed on a tray in a cupboard for 3 weeks to dry.
- 0.7 ml of hexane was used in this case.
- This was repeated twice.
- Samples were named SD-DRY1, SD-DRY2.

B. WATER DISTILLATION

Experiment 3A: Water distillation on fresh leaves (40 g)

- 40 g of leaves were washed with distilled water and placed in the round bottom flask in the Clevenger apparatus below:

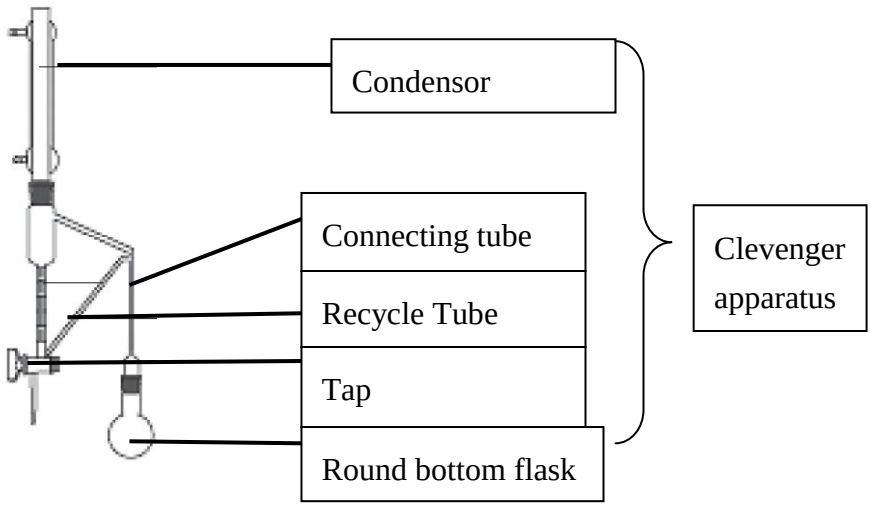


Figure 2.5 Clevenger apparatus used for water distillation of leaves

- 200 ml of distilled water were added to the round bottom flask to make sure the leaves are completely covered.
- The round bottom flask was then heated for 3 hours.

- 1 ml of hexane was used to separate the oil and water and the hexane/oil mixture was dried over anhydrous sodium sulphate and analysed.
- The experiment was repeated twice. Samples were named WD1 and WD2.

Experiment 3B : Water distillation on dry leaves (4 g)

- The same procedure as for experiment 3A was used for 4 g of dry leaves.
- 75 ml of distilled water was used and 0.7 ml of hexane.
- The experiment was repeated twice.
- Samples were named $WD_{DRY}1$ and $WD_{DRY}2$.

C. SOLVENT EXTRACTION

Experiment 4:

- In solvent extraction, the plant material was placed in a 100 ml round bottom flask with the different solvent used.
- It was then placed in a sonicator for 15 minutes and then clamped in a fume cabinet and allowed to sit for 65 hours.
- The resulting mixture was then filtered.
- The oil was then separated from the solvent by 0.7 ml of hexane in a separating funnel.
- The oil was then dried over anhydrous sodium sulphate and then analysed.

The solvents used were:

Experiment 4A: Glacial acetic acid using 20 g of fresh *Centella* leaves. (Sample SEGA)

Experiment 4B: Distilled water using 13 g of fresh *Centella* leaves. (Sample SEW)

Experiment 4C: Vinegar using 10 g of fresh *Centella* leaves. (Sample SEV)

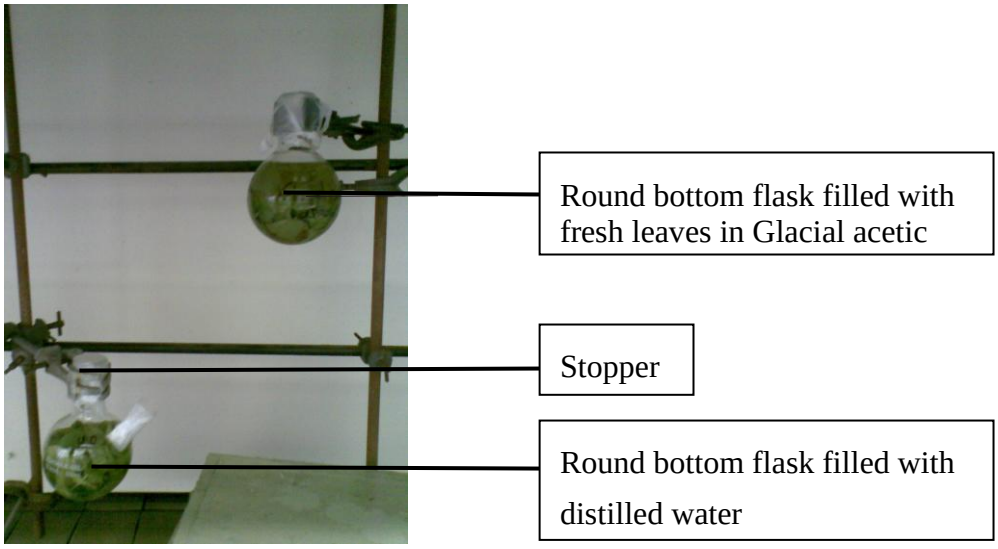


Figure 2.6 Solvent extraction

D. SOXHLET EXTRACTION

Soxhlet extraction is a procedure for extracting non-volatile and semi-volatile organic compounds from solids. The sample was placed in cellulose thimbles covered in cotton wool as shown in figure 2.7.

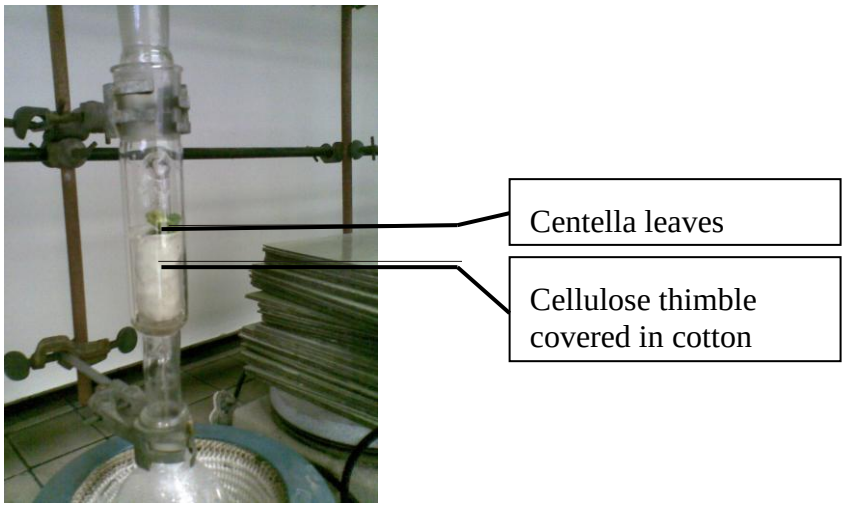


Figure 2.7 Soxhlet extraction apparatus

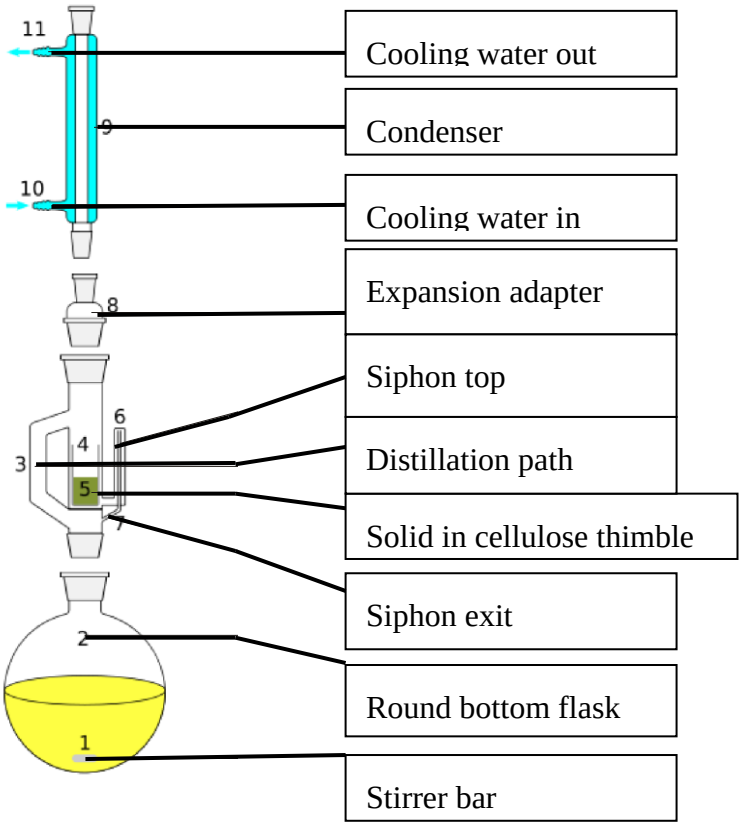


Figure 2.8 A more detailed diagram of the soxhlet extraction apparatus (Soxhlet extractor, 2009)

A certain amount of solvent was added to the system into the round bottom flask and heated to an suitable temperature, which caused the solvent to boil. Then the solvent vapour condensed and drops of solvent fell down onto the solid sample particles. Once the side vessel was full, the solvent ran back to the round bottom flask. This process was then repeated for several hours until a sufficient amount of analytes was obtained. During each cycle, a portion of the non-volatile compound dissolved in the solvent. (Nollet and Grob, 2006)

The water and oil mixture was separated with 0.7 ml of hexane and dried over anhydrous sodium sulphate.

Experiment 5A :

- 6 g of leaves were used and placed in a thimble and soxhlet extraction was carried out with 100 ml of distilled water (the solvent).

- The water was heated for 3 hours and then 1 ml of hexane was used to separate oil and water and the oil was then analysed.
- The experiment was repeated twice.
- Samples were named SOX1 and SOX2.

E. Investigating the apparatus for the determination of essential oils in plant materials described by the British Pharmacopedia Volume IV (Setup 6)

This apparatus is described by the British Pharmacopedia Volume IV. It was specifically chosen for use in this research project because many professional companies use the apparatus to extract essential oils from plants. Therefore, it was chosen in this investigation on *Centella Asiatica* leaves and also to investigate if it can be used to quantify the oil collected. The companies specifically use steam distillation with the apparatus, but both water and steam distillation were used with the apparatus.

All leaves in the experiments using the apparatus were measured from the widest part of the leaf, just where the stem attaches to the leaf (Fig. 2.9) and then an average of the size of the leaves used per experiment was calculated.

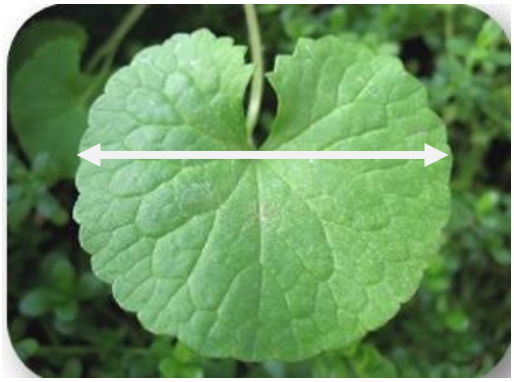


Figure 2.9 A Centella leaf showing where the leaves were measured at the widest part.

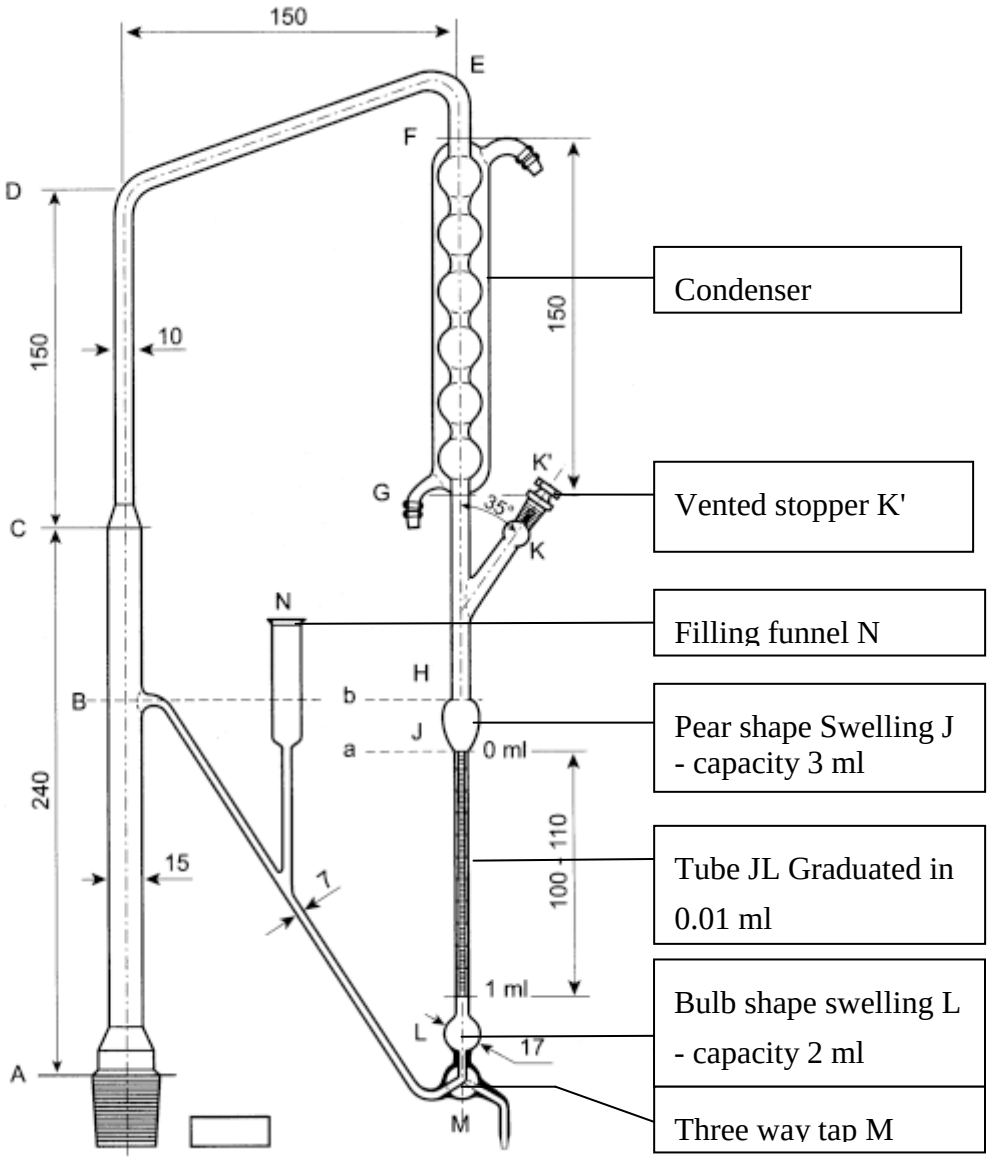


Figure 2.10 Apparatus for the determination of essential oils in plant material (British Pharmacopeia volume VI)

The determination of essential oils was carried out by steam distillation in the special apparatus described above (Fig. 2.10). A Round bottom flask of 500 ml is connected to the end of the apparatus (at point A - Fig. 2.10) and a false bottom extraction container filled with leaves is also added in the case of steam distillation. In the case of water distillation no false bottom extraction container was added and a larger 1000 ml round bottom flask is used. There is a heating mantel below the round bottom flasks. Once the water is heated, the distillation rate is adusted to 2/3 ml per minute. The distillate is collected in the graduated

tube (JL) using xylene to take up the essential oil. The aqueous phase is automatically returned to the distillation flask.

Method used for apparatus above: (Fig. 2.10)

1. Add 400 ml of distilled water into a 500 ml round bottom flask for steam distillation

 * Add 700 ml of distilled water into a 1000 ml round bottom flask for water distillation

2. Position a heating mantel under the round bottom flasks.

3. To the round bottom flask connect the false bottom extraction container. (Steam distillation only)

4. Attach the BP apparatus (Fig. 2.10) to the round bottom flask. Then attach the condenser pipe to the tap.

5. Introduce distilled water through funnel N until it is at level B.

6. Remove the vented stopper K' and introduce the prescribed quantity of xylene using a pipette with its tip at the bottom of the tube K. This is the initial xylene used (Xi).

7. Replace the stopper K'.

8. Switch on heating mantel and heat the water in the round bottom flask to boiling and adjust the distillation rate to 2/3 ml per minute.

(To determine the rate of distillation, during distillation the water level was dropped using the three-way tap until the meniscus is at the lower mark (a) (Fig. 2.10). Close the tap and measure the time taken for the liquid to reach the upper mark (b). The pear shape swelling J has a capacity of 3 ml. Open tap and continue the distillation, modifying the heat to regulate the distillation rate)

9. Distil for 30 minutes, stop the heating and after 10 minutes read off the volume of xylene in the graduated tube (X_{30}). (This step is necessary to determine a more or less constant amount of xylene in the apparatus because xylene is volatile)

10. Introduce into the false bottom extraction container the prescribed quantity of washed Centella leaves and continue the distillation as described above for the time and rate (2/3 ml min^{-1}) prescribed. (Steam distillation)

*Introduce into the round bottom flask the prescribed quantity of leaves and continue the distillation at a time and rate (2/3 ml per minute) prescribed. (Water distillation)

11. Stop the heating after the time prescribed and let the apparatus cool for 10 minutes.

12. Remove the stopper K' and introduce 0.1 ml of a 1 g L^{-1} solution of sodium fluoresceinate and 0.5 ml of distilled water. (It colours the water florescent yellow to make it easier to separate the non-polar xylene/oil mixture from the distilled water)

13. Read off the volume of liquid collected (X_{after}) in the graduated tube (JL). (Then subtract the amount of xylene previously noted (X_{30}). The difference represents the quantity of essential oil in the quantity of leaves used.

The water-free mixture of xylene and essential oil is collected as follows for analysis:

14. Lower the mixture of xylene and essential oil into the bulb shaped swelling L by means of the three-way tap, allow to stand for 5 minutes and then lower the mixture slowly until it reaches the level of tap M.

15. Open the tap anti-clockwise so that the water flows out of the connecting tube BM.

16.Wash the tube with acetone and with a little toluene introduced through the filling funnel N.

17. Turn the tap anti-clockwise in order to recover the mixture of xylene and essential oil in a appropriate flask.

18. Then analyse the mixture using a GC-MS.

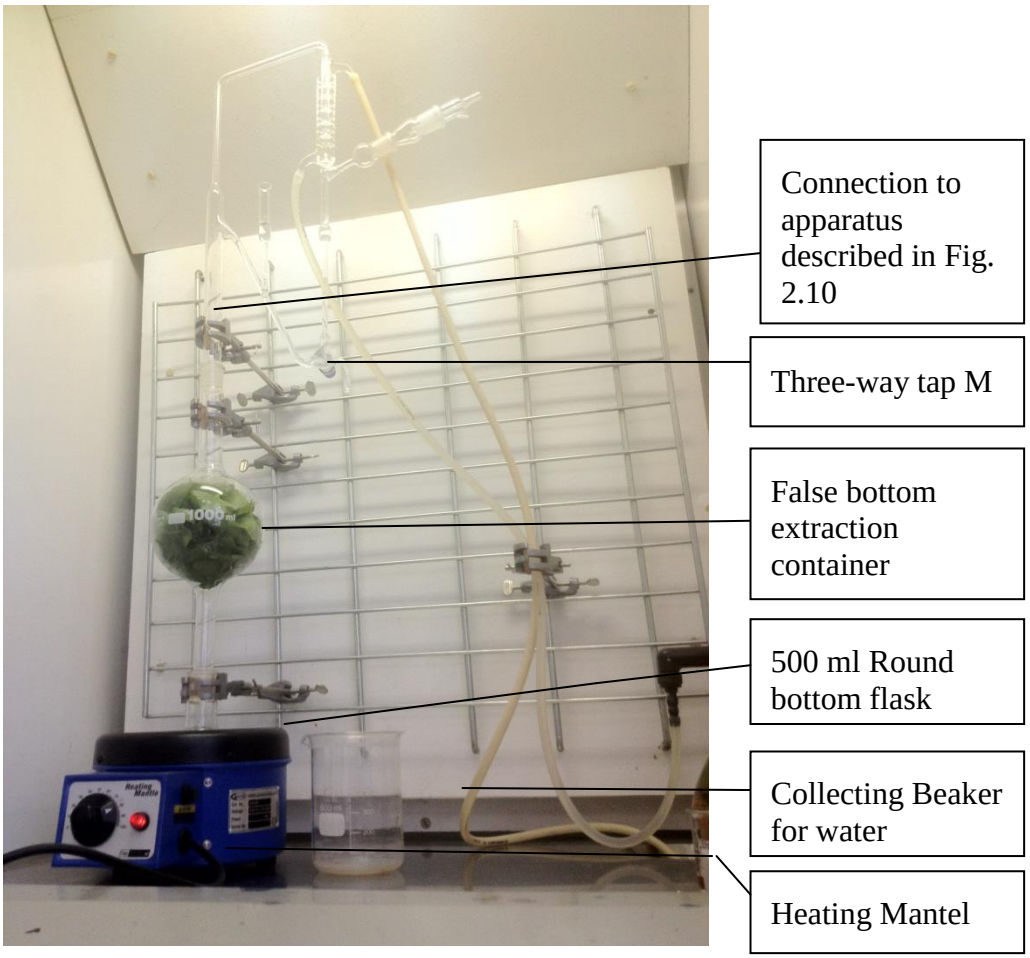


Figure 2.11 Actual steam distillation setup used (Setup 6)

THE FOLLOWING EXPERIMENTS WERE RUN USING STEAM DISTILLATION (SETUP 6):

Experiment 6: Initial steam distillation experiments

Initial steam distillation experiments on fresh leaves were run to familiarise ourselves with the apparatus and how to use it effectively.

The experiment names were ST2A (using 0.2 ml xylene and no water distillation initially), ST3A (1st did water distillation for 30 minutes), ST4AHEX (using hexane) and STEAM T1 (no xylene initially to see what happens).

Experiment 7: Optimisation of the extraction time for Centella Asiatica

Steam distillation was chosen for optimisation because it is mainly used with this apparatus to extract essential oils. The experiments were run using setup 6 with the same amount of leaves (50 g) at times of 35 min, 60 min, 75 min and 90 min. These experiments were named TM1, TM2, TM3 and TM4 respectively.

Experiment 8: Optimisation of the amount of Centella leaves used for experiments

Steam distillation using setup 6 was run with 30 g, 50 g and 100 g of leaves. The experiments were named LEAF 1, LEAF 2 and LEAF3.

Experiment 9: Steam distillation experiments

Steam distillation experiments using setup 6 at the optimised extraction time (determined from experiment 7) and with the optimised amount of leaves (determined from experiment 8) were then run. The experiment names were SQ1, SQ2 and SQ3.

THE FOLLOWING EXPERIMENTS WERE RUN USING WATER DISTILLATION (SETUP 6B):

Experiment 10: Initial water distillation experiments

Initial water distillation experiments were run for familiarisation with the process of water distillation. Experiments were named WD1A and WD2A.

Experiment 11: Water quantitative experiments

Water distillation experiments were run using setup 6B and using the optimised amount of leaves and extraction time determined from Experiments 7 and 8. The experiment names were WDQ1, WDQ2 and WDQ3.

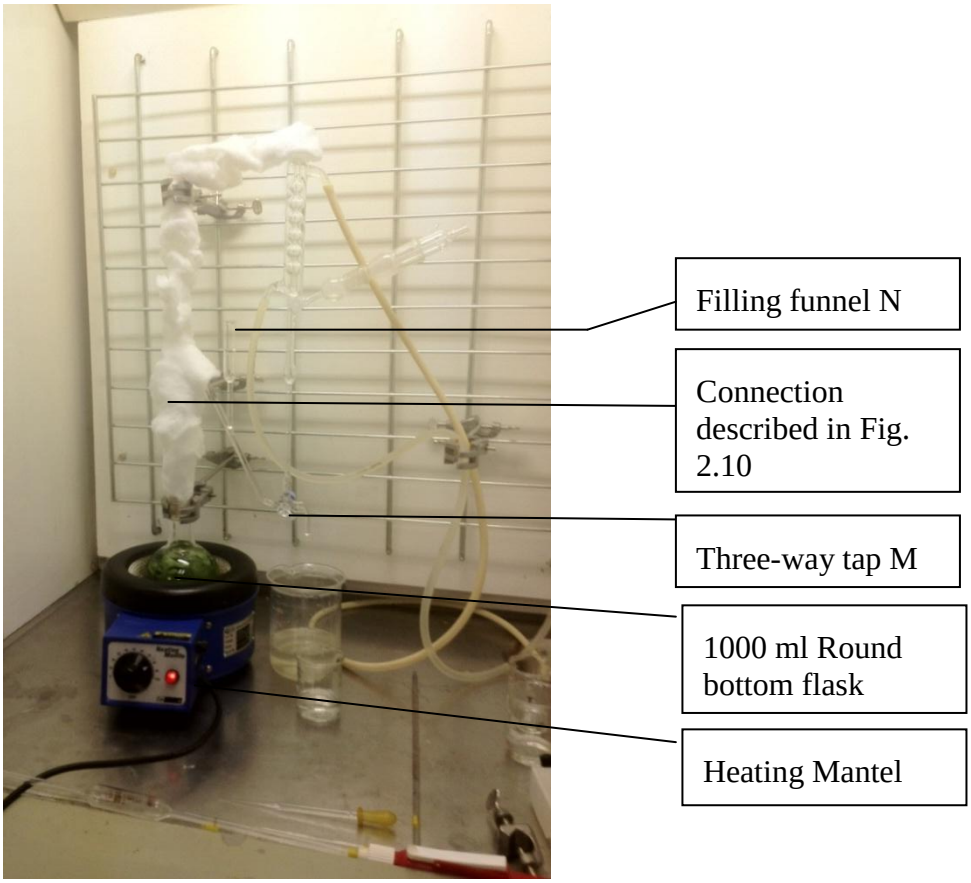


Figure 2.12: Actual water distillation setup used (Setup 6B)

Experiment 11: Water distillation experiments with a 20% salt solution

Water distillation experiments were then run with a 20% salt solution in the round bottom flask instead of just distilled water. A 20 % w/w solution was prepared and the experiments were run at the optimised extraction time and with the optimised amount of leaves. The experiments were named WDSO1, WDSO2, WDSO3.

Experiment 12: Dry leaves essential oil extraction

After experiment 9 (steam distillation) was run and experiment 11 (water distillation) the results were compared to determine which is the best distillation method for extracting essential oils from *Centella Asiatica* leaves using the apparatus. Then this distillation method will be used to run a further three

experiments on dry leaves. The experiments were named DRY1, DRY2 and DRY3.

2.2 Part 2: Investigating the effect of heavy metals in the soil on the *Centella* plant and the composition and yield of the essential oil

2.2.1 Sample collection

A further twelve *Centella Asiatica* plants were obtained from Margret Roberts farm and each of them were grown in the same size pots for 6 - 8 weeks. The plants were watered once every two days.

2.2.2 Spiking of the soil with heavy metal contaminants

- The soil of three pots were spiked with 10 ppm of lead (Pb^{2+}). This was done using a lead nitrate ($Pb(NO_3)_2$) solution (Appendix A). Sample names were Pb1, Pb2 and Pb3
- The soil of another three pots were spiked with a 10 ppm of Mercury (Hg^{2+}), this was done using a solution of mercurous nitrate ($HgNO_3 \cdot H_20$) (Appendix A). Sample names were Hg1, Hg2 and Hg3.
- Three pots were also spiked with chromium (Cr(VI)). This was done using a sodium dichromate solution ($Na_2Cr_2O_7 \cdot 2H_20$) (Appendix A). Sample names were Cr1, Cr2 and Cr3.
- The remaining three pots were the control pots. Sample names were control 1, control 2 and control 3.

The pots were watered once every two days. Care was taken to avoid leaching of water from the pots by keeping a plastic dish underneath each pot to collect the leachates. The collected leachates were again returned to the experimental pots.

The soil and leaf samples of the plants were then harvested after 1 week for metal analysis.

2.2.3 Determination of heavy metal contents in *Centella Asiatica* leaf, soil and essential oil samples

The harvested leaves were used to run triplicate experiments using setup 6 to see if the metal contamination in the soil affected the yield of the essential oil obtained or if any of the metal contamination was seen in the essential oil collected. (Sample names Cr1, Cr2, Cr3, Pb1, Pb2, Pb3, Hg1 , Hg2, Hg3)

The *Centella Asiatica* leaf samples from each pot were oven dried at 70°C for at least 24 hours until the dry weight was constant. The leaf samples were then digested. Approximately 0.1 g of the leaf samples were weighed and 8 ml of nitric acid and 2 ml of hydrogen peroxide were added and the mixture was put in the microwave preparation system for 30 minutes. The solution was then run on the inductively coupled plasma optical emission spectrophotometer (ICP-OES). The measurements were done in triplicate and statistically analysed.

The soil from which the plants were grown were also analysed. 0.25 g of the soil sample was weighed and then 3 ml of nitric acid and 9 ml of HCl were added and put in the microwave preparation system for 30 minutes. The solution was analysed using the ICP-OES to determine the metal ion concentration in the soil.

2.3 Analysis

2.3.1 Gas Chromatography

Gas chromatography (GC), is a commonly used for analysing and separating organic compounds that can be vaporised without decomposition. Common uses of GC include separating the different components of a mixture or testing the purity of a particular substance. (Donald et al., 2006)

The mobile phase in gas chromatography is usually a inert carrier gas, such as helium or an unreactive gas such as nitrogen. The stationary phase is a inert solid support with a layer of microscopic liquid or polymer, inside a column which is usually a metal tube or a piece of glass. It is essential to control the temperature of the column and depends on the boiling point of the sample. (Donald et al., 2006)

As the gaseous compounds which are being analysed move through the column they react with the walls of the stationary phase and elute at different times which is known as a retention time of the compound. These retention times is what gives GC its analytical value as they can be compared. There are a number of factors that can be used to vary the retention time such as the temperature and the flow rate of the carrier gas. (Bowles, 2003)

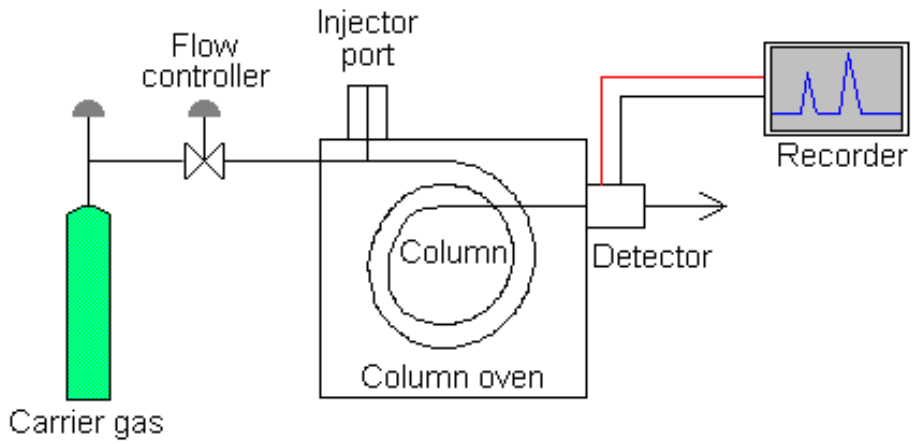


Figure 2.13 A schematic diagram of a gas chromatograph (Sheffield Hallam University, 2008)

Gas chromatography in essential oil analysis is a useful technique since it separates out essential oil constituents by their volatilities and polarities. A small sample of the oil is injected into a thin coated tube (the column) via the injector port (Fig. 2.13) using a microsyringe. The column is found inside an oven that is slowly heated to about 250^{o}C (the boiling point of essential oils). The vaporized oil constituents are carried along the column in a flow of inert gas, usually helium. As the carrier gas moves the analyte molecules through the column, their movement is disrupted by the adsorption of the analyte molecules either onto the packing materials in the column or onto the column walls. The speed at which the molecules move along the column depend on the strength of adsorption, which in turn depends on the type of molecule and on the substance used in the stationary phase. (Bowles, 2003) This causes the constituents to separate and elute at different times called retention times. As they reach the end of the tube, they are identified by a special detector. A graph is printed out showing the different constituents as peaks separated by time, each peak representing the percentage of the total amount of oil tested. (Bowles, 2003) The detector that can be used is a flame ionization detector (FID) shown in Fig 2.14.

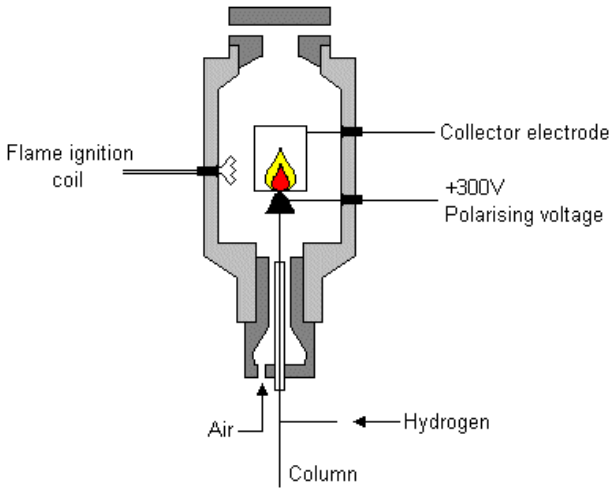


Figure 2.14 Flame ionization detector (Sheffield Hallam University, 2008)

The effluent from the column is mixed with hydrogen and air (Fig. 2.14), and ignited. Ions and electrons are produced when organic compounds are burnt in the flame which can conduct electricity through the flame. This causes a large electrical potential at the burner tip and above the flame a collector electrode is situated. (Raaman, 2006) The ions are then attracted to the collector electrode and when they hit the electrode a current is induced which is measured using a high-impedance picoammeter and fed into an integrator. Depending on the computer and software will depend on the way the final data is displayed. However generally a graph is displayed that has total ion on the y-axis and time on the x-axis. (Raaman, 2006)

FIDs are sensitive to mass rather than concentration. this is one of the advantages of FID's because if there are any changes in the flow rate of the mobile phase it will not affect the response of the detector. The other advantages is that it has low noise and is highly sensitive which is specially useful for analysing organic compounds, but has the disadvantage that it destroys the sample. (Raaman, 2006)

Gas spectrometry is usually combined with another detector, a mass spectrometer (MS). Mass spectrometry is the measurement of molecular weights of a compound by first ionizing them. The compounds, which can either gain or lose electrical charges are then separated according to their mass to charge ratio and detected. As each individual substance emerges from the GC, it is fed into the MS, ionized and separated. The end spectrum is a series of peaks (a spectrum) where individual substances are separated in time and according to their mass-to-charge-ratio. (Bowles, 2003) A photo of the inside of a GC-MS is shown is Figure 2.15 below.

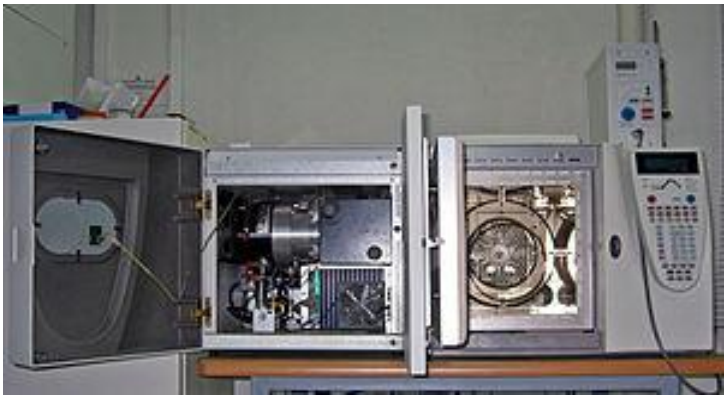


Figure 2.15 The inside of the GC-MS, with the column of the gas chromatograph in the oven on the right and the MS on the left

Types of ionisation

When the molecules travel through the column they pass through into the mass spectrometer where they are ionised by different methods, usually only one method is used at a time. Once the sample is fragmented, it will then be detected, usually by an electron multiplier diode, which 'converts' the ionised mass fragment into an electrical signal that is then detected. (Lakshimi Himabindu et al., 2013)

In this experiment the electron ionisation (EI) method was used. This is the most common form of ionisation. The molecules enter into the MS where they are subjected to a continuous flow of free electrons emitted from a filament. When the electrons bombard the molecule it causes a them to fragment in a distinctive and reproducible way. This is called "hard ionisation". (Lakshimi Himabindu et al., 2013)

GC with FID detector is less effective than with MS because to identify the peaks one has to first discover the retention times of each constituent in the oil. A way around this is to run a GC analysis of a standard sample and then compare each subsequent batch to the standard. However, a standard may not be 100% pure. Therefore it is not as accurate as a GC-MS which usually has an internal library

and the components can be identified more accurately. (Lakshimi Himabindu et al., 2013)

The GC and GC-MS method used for the analysis of the essential oil from Centella Asiatica

GC analysis of the essential oil samples was performed on a Agilent Technologies Gas Chromatograph GC-682 fitted with a TRB1 capillary column (30 m x 0.32 mm i.d., film thickness 0.25 μm), using hydrogen as the carrier gas at a flow rate of 1 ml min^{-1} equipped with FID detector. The data was processed using Agilent Cerity QA-QC processing system. Temperature programme was 70 - 240^{o}C at a rate of 5^{o}C min^{-1}, with a 0 min hold at 70^{o}C and 2 min at 240^{o}C. The hexane peak was eliminated by only starting the analysis after 4 minutes. The samples were injected manually.

GC-MS analysis was conducted on Trace GC 2000 gas chromatograph equipped with a DB5-MS column interfaced with a DFS magnetic sector mass spectrometer. Electron ionisation was at 70 eV with an ion source temperature of 250^{o}C. The temperature programme was 70 - 240^{o}C at a rate of 5^{o}C min^{-1} , with a 0.5 min hold at 70^{o}C, a 0.5 min hold at 100^{o}C and a 1 min hold at 240^{o}C. Helium was used as the carrier gas, with a flow rate of 1.5 ml min^{-1}. The samples were injected manually.

GC-MS Analysis for Setup 6 and Setup 6B samples had to be run on a different GC-MS using a different column but with the same method above because the initial GC-MS used was not available. The separation of the volatile compounds was performed on a gas chromatograph, Agilent 6890 N (Agilent, Palo Alto, CA), coupled with an Agilent mass spectrometer detector Agilent 5975 MS (Agilent, Palo Alto, CA). The GC-MS system was equipped with an Zebron-ZB 274305 Semi Volatiles with 5m integrated guard (30 m, 0.25 mm ID, 0.25 μm film thickness) GC column from Phenomenex. Analyses were carried out using helium as carrier gas with a flow rate of 1.5 ml min^{-1}. The injector temperature was maintained at 280ºC with a split ratio of 20:1.

The temperature programme was 70 - 240^{o}C at a rate of 5^{o}C min^{-1}, with a 0.5 min hold at 70^{o}C, a 0.5 min hold at 100^{o}C and a 1 min hold at 240^{o}C. The MSD was operated in full scan mode and the source and quad were maintained at 240ºC and 150ºC, respectively. The transfer line temperature was maintained at 280ºC. The solvent delay was at 5.23 minutes. The GC-MS used an automated sampler.

Identification of constituents

Identification of the constituents was carried out by comparison of their mass spectral pattern and retention indices with those of standard sample and with the data from the NIST-MS library version 2.0.

The compounds in the samples from Setup 6 and Setup 6 B were tentatively identified by mass spectra and retention indices which were compared to authentic chemicals and the NIST05 spectral library collection.

2.3.2 Inductively coupled plasma optical emission spectroscopy (ICP-OES)

Inductively coupled plasma optical emission spectrometry (ICP-OES) is an analytical technique used for the detection of trace metals. As described by Stefansson et al. (2007) "it is a type of emission spectroscopy that uses the inductively coupled plasma to produce excited atoms and ions that emit electromagnetic radiation at wavelengths characteristic of a particular element." The strength of this emission is an indication of an elements concentration within the sample. (Mermet, 2005) It was employed in this research to determine the concentration of metal ions in the soil and leaf samples.

In this analytical method a liquid or gas sample is injected into the ICP-OES. If analysis of a solid sample is required it usually needs to undergo acid digestion so that the analytes are found in solution. This sample of liquid or gas is converted to an aerosol and directed into the central channel of the plasma. The plasma core has a temperature of approximately 10 000 K, so the aerosol is rapidly vaporized

by a process called nebulisation. These analyte elements are now present as free atoms in the gaseous state (Fig. 2.16). Additional energy is passed on to the atoms when they collide, promoting them to excited states. Adequate amounts of energy are often available to convert the atoms to ions and consequently promoting the ions to their excited states. The atomic and ionic excited state molecules prefer to be in the ground state and they have the ability to emit energy by emitting a photon. These photons have distinctive energies that are determined by the quantized energy level structure for the atoms or ions. Therefore, to identify the elements, the wavelength of the photons are used. The relationship between the total number of photons and the concentration of the element in the original sample are directly proportional. (Hou, 2000)

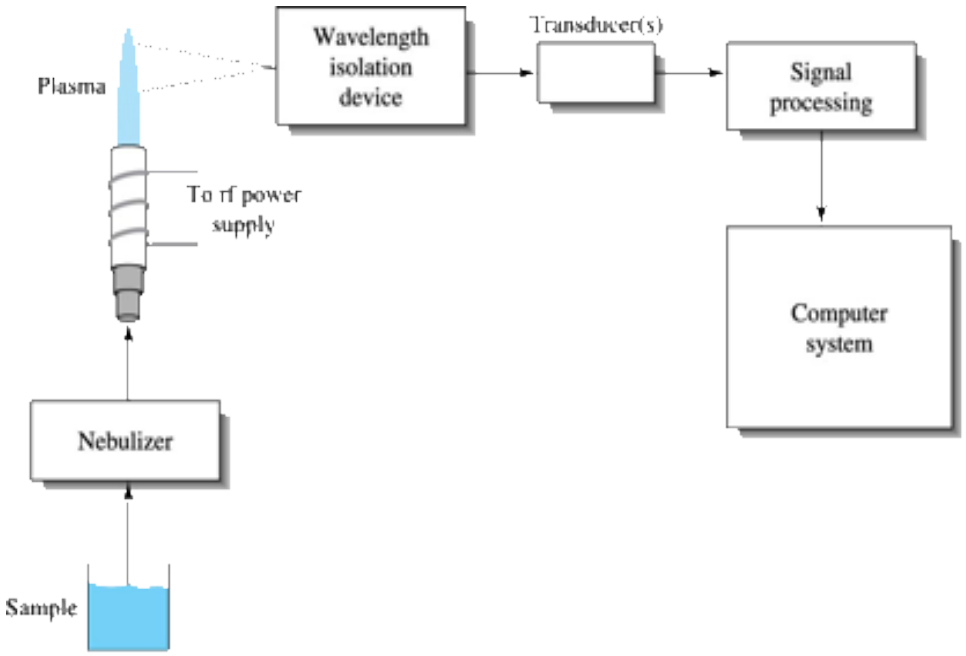


Figure 2.16 A simplified diagram to explain the components of the ICP-OES (Mermet, 2005)

The ICP-OES is composed of two parts: An ICP torch and an optical spectrometer. The ICP torch consists of 3 concentric quartz glass tubes. The output of the radio frequency (RF) generator surrounds part of this quartz torch. The plasma is usually generated using argon gas. An intense electromagnetic field

is created within the coil by the high power RF signal flowing in the coil, after the torch is switched on. This RF signal is created by the RF generator (Fig. 2.17). (Hill, 2007)

The argon gas flowing through the torch is ignited with a Tesla unit that creates a brief discharge arc through the argon flow to initiate the ionization process. Once the plasma is "ignited", the Tesla unit is turned off.

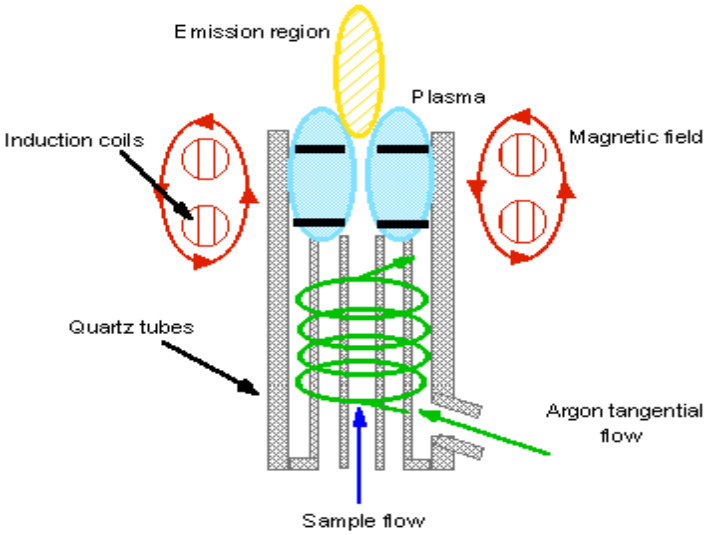


Figure 2.17 Diagram of an ICP-OES Torch (Mermet, 2005)

According to Hill (2007), "The argon gas is ionized in the intense electromagnetic field and flows in a particular rotationally symmetrical pattern towards the magnetic field of the RF. A stable, high temperature plasma of about 10000 K is then generated as a result of the inelastic collisions created between the neutral argon atoms and the charged particles."

An aqueous sample is then delivered into a nebuliser where it is atomised and directed into the plasma. In the plasma, electrons and other charged ions collide with the sample and the latter is broken down into charged ions. The various molecules fragment into their individual atoms which then lose electrons and recombine continually in the plasma, which results in radiation being emitted at the characteristic wavelengths of the specific elements. (Hill, 2007)

The emitted light is then focused on a diffraction grating using one or two lenses where it is separated into its component wavelengths in the optical spectrometer. The light intensity (colour) of each different wavelength is then measured with a photomultiplier tube. The intensity of each line is compared to formerly measured intensities of known concentrations of the elements. (Hill, 2007)

2.3.3 Scanning electron microscope (SEM)

The scanning electron microscope (SEM) uses a beam of high energy electrons in a raster scan pattern for imaging the surface of a sample. Interactions of the samples atoms with electrons produce signals that enclose information about the sample's electrical conductivity, surface topography and composition. (Sachdeva et al., 2014)

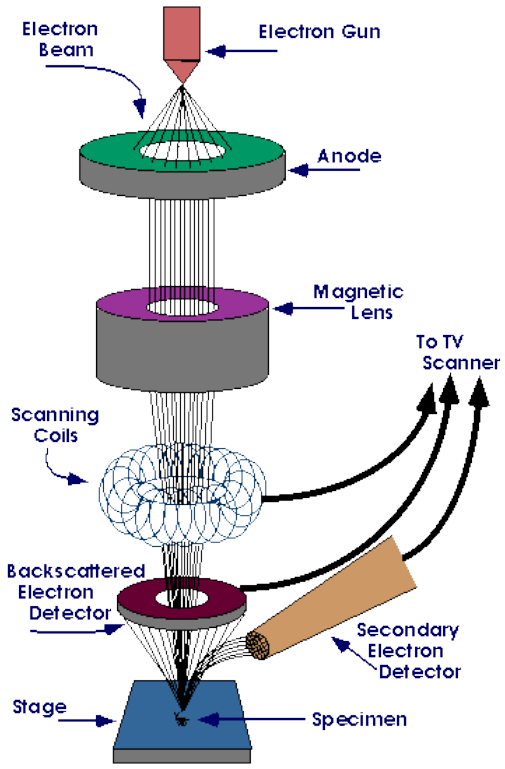


Figure 2.18 A diagram of a Scanning electron microscope (Sachdeva et al., 2014)

At the top of the microscope is an electron gun that generates a beam of electrons which travel vertically in a vacuum through the microscope. The beam moves

through lenses and an electromagnetic field which focuses the beam on the sample. When it hits the sample, electrons and X-rays are ejected from the sample. (Sachdeva et al. , 2014)

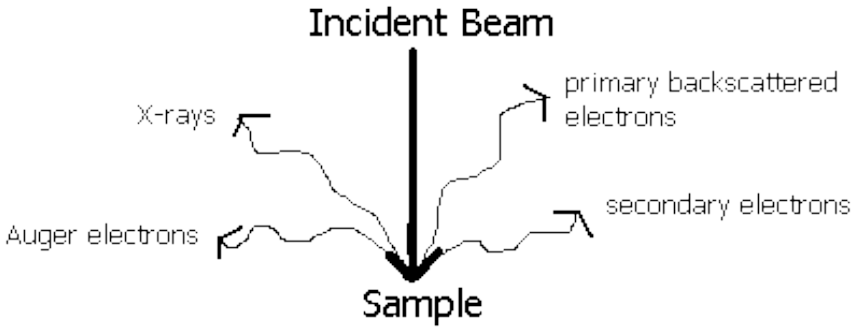


Figure 2.19 Diagram showing what particles are emitted once an incident beam hits a sample in the SEM (Sachdeva et al. , 2014)

A detector collects these backscattered electrons, secondary electrons and X-rays and converts them into a signal that is sent to a screen. This produces the final image (Sachdeva et al., 2014).

Sample preparation for SEM

The SEM operates under a vacuum and electrons are used to form an image. All samples to be examined under a SEM must be completely dry as any water would vaporise in the vacuum. No sample preparation is necessary for metals because they are conductive, however non-metals must be coated in a thin layer of conductive metal which is usually done be a device called a "sputter coater" (Sachdeva et al., 2014).

For biological samples (such as leaves) in the SEM, as mentioned earlier the sample must be dry because of the vacuum. Dry, hard materials such as bones, or wood can be examined with little additional preparation, however living tissues and cells generally need to undergo chemical fixation to stabilise and preserve their structure. Fixation of a sample is typically done by placing the sample into a

buffered chemical fixative such glutaraldehyde. The fixed tissue is then dehydrated. This is carried out usually by critical point drying because air-drying can cause shrinkage and collapse and hence change the samples structure. Critical point drying uses organic solvents (e.g. ethanol, acetone) to replace water in the cells and then these solvents are replaced with a transitional fluid such as liquid carbon dioxide at high pressure. The carbon dioxide is eventually removed while in a supercritical state, so that no gas-liquid interface is present within the sample during drying. An epoxy resin or a conductive double sided tape is used to mount the dry sample on a specimen stub and then it is sputter coated with gold or gold/palladium alloy before examination in the microscope (Sachdeva et al. , 2014). All samples must also be of an appropriate size to fit in the specimen chamber .

The SEM used was the Jeol JSM 840. The easy usable magnification range is from 20x up to 10 000x, which is used in most cases. A must fit criteria for a sample to be used in a SEM is:

a) The sample must be solid

b) Samples must be dry, since water degrades in the vacuum system. Critical point drying is therefore done.

c) The sample must be conductive. By applying a conductive coating, this can be overcome if your sample is non-conductive.

d) Preferable size is anything less than 30 mm in diameter and less than 20 mm high to be able to go through the airlock and easy manipulation. However up to 100 mm diameter and 30 mm high can be used.

The following steps were taken in the preparation of material for SEM

1. Fixation
2. Dehydration
3. Examination in the electron microscope

Fixation

The aim is to maintain material in as 'life-like' condition as possible. This is achieved by exposing the material to a chemical agent which binds the protein of the cells and renders them susceptible to damage during the procedures necessary to visualize the ultrastructural detail. To achieve this result, it is important to take some important precautions. These are:

Size: For SEM – up to 1 - 2 cm^2.

Time: Material should be transferred into the fixative solution as soon as possible to avoid any autolytic changes. Another danger is traumatic damage. This can be caused by the crushing of material during dissection. Therefore, great care should be exercised at this stage.

Choice of fixative: This is dependent on the type of material, method of fixation or type of procedure to be used. The most common fixative used is Glutaraldehyde,which is obtained as a 25% solution.

Dehydration

This is achieved by putting the material through a graded series of absolute ethyl alcohol in distilled water (20%, 40%, 50%, 60%, 70%, 80%, 90%) with 5 - 15 minutes in each dilution and then into 100% ethanol which has been dried over a water-absorbing chemical e.g. silica gel or sodium sulphite.

2.3.4 Sample preparation method used for the SEM on *Centella* leaves

Preparation of Phosphate buffer (0.2 M)

Take 6.41 g of Sodium dihydrogen phosphate and 41.3 g of Disodium hydrogen phosphate ($Na_2HPO_4 \cdot 7H_2O$) and place in a 1 L volumetric flask. Fill the volumetric flask with distilled water up to the 1 L mark.

Fixation

The leaves were fixed in 2 - 4% Glutaraldehyde in a phosphate buffer for a period of 48 hours:

The fresh leaf of *Centella* was cut up with a scalpel to small pieces (about 0.3 mm x 0.3 mm -0.4 mm x 0.4 mm) and placed in a vial. The same procedure was carried out on a leaf of *Centella* after steam distillation was completed for 3 hours.

The small pieces of leaf in the vial were then washed in buffer (3 x 5 minutes each wash)

4ml of Glutaraldehyde was placed in a 100 ml volumetric flask and then the volumetric flask was filled with phosphate buffer up to the 100 ml mark.

The 4% Glutaraldehyde solution prepared was poured in each vial just covering the pieces of the leaf for 48 hours.

Dehydration

The samples were dehydrated through 20% ethyl alcohol in distilled water for 5 - 15 minutes.

Then through each 40, 50, 60,70 ,80 and 90% ethyl alcohol in distilled water for 5 - 15 minutes and lastly through 100% ethyl alcohol twice for 5 -15 minutes and left in the 100% alcohol.

The samples then went for super critical drying and were mounted on a specimen stub using an electrically-conductive double-sided adhesive tape before viewing under the SEM.

In this investigation, a Scanning electron micrograph was taken of the surface of the leaf of *Centella Asiatica* before extraction and after extraction and compared.

3 Results and discussion

3.1 Investigations using the Clevenger and a variation of the Clevenger apparatus

One of the aims of the experiment was to determine which extraction method give the highest yield. However, this proved to be very difficult because *Centella Asiatica* leaves do not contain a high concentration of essential oil. Therefore, to produce a sufficient yield, one that would be possible to quantify, about 500 g of leaves would be needed for every extraction and a much bigger extraction container. To obtain this amount of leaves for every extraction was not possible due to the difficulty in obtaining such a large amount of leaves for each experiment, a field of *Centella Asiatica* would be necessary. So the initial experiments (1 to 5) were conducted using approximately 10 - 42 g of leaves and the extraction processes yielded very little oil. A receiver tube was calibrated and the oil was collected to see if it could be quantified. It was found that this was not possible because only a few drops floating on the surface of the water were seen and this was not a sufficient amount to even form a meniscus. An empty sample vial was weighed and then the sample vial with the hexane and oil after extraction and subtracting the weight of hexane added. This did not work either since the hexane evaporates during the separation process and some is lost on the walls of the receiver tube. 0.7 ml of hexane (since that is the amount added to the receiver flask) is weighed initially, after the separation the oil and water is weighed and a much smaller weight than 0.7 ml of hexane is observed and this is the hexane and oil combined. Therefore the method failed as well.

Therefore with such a small amount of leaves it is very difficult to quantify the yield. To find an explanation as to why the yield obtained was in such a small amount, the surface of the fresh *Centella* leaf was examined under a SEM to see what the essential oil sacs look like before and after extraction.

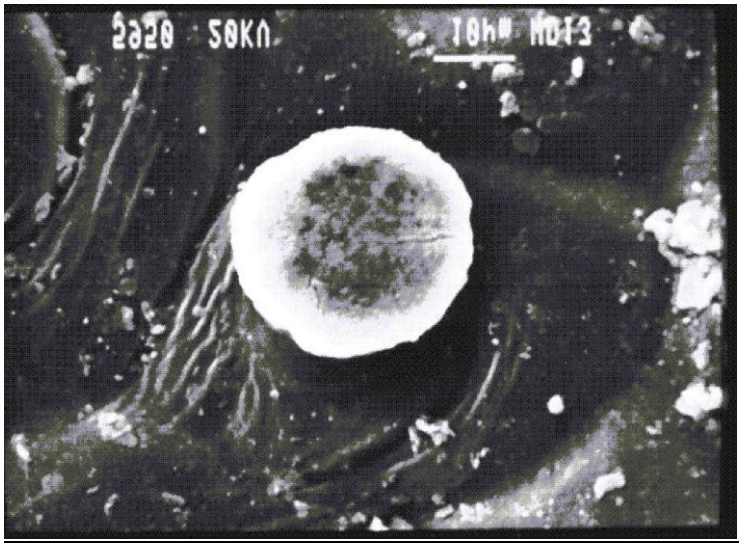


Figure 3.1 Scanning electron micrograph of a fresh leaf before extraction (1600x , 20keV)

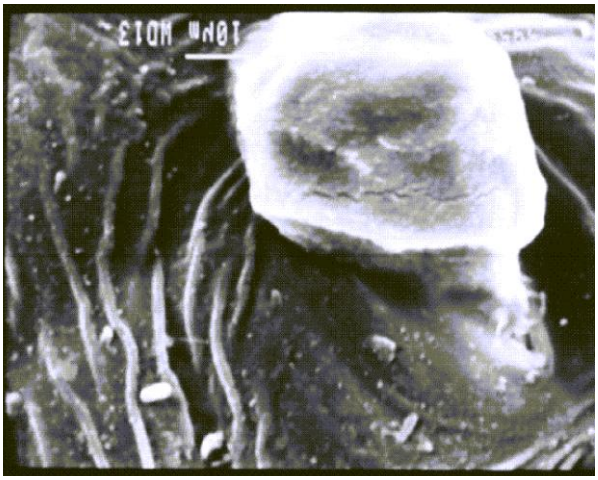


Figure 3.2 Scanning electron micrograph of a fresh leaf before extraction (3500x, 15keV)

From figure 3.1 the essential oil sac of *Centella* can be seen on the fresh leaf. It is formed on a sort of stalk projecting from the leaf (as seen more clearly in Fig. 3.2). The essential oil sacs are located near the stomata. This can be seen in the figure 3.3.

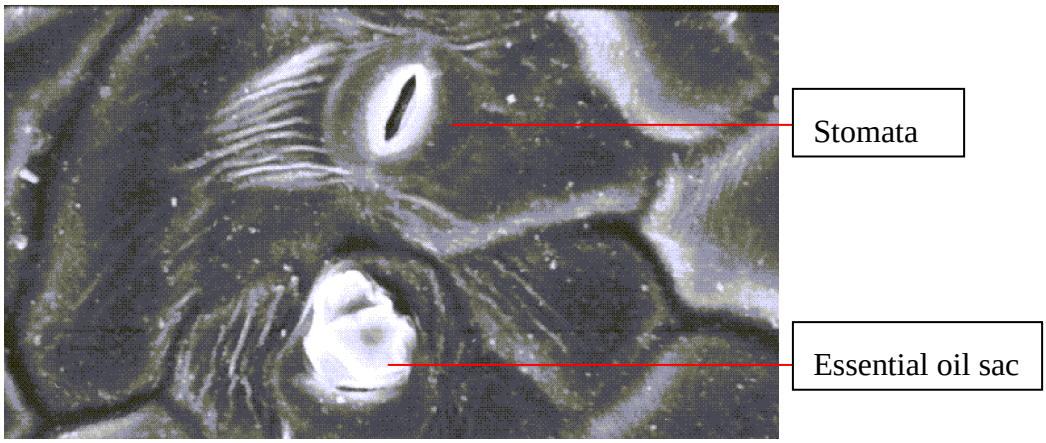


Figure 3.3 Scanning electron micrograph of a fresh leaf before extraction (1100x, 20keV)

In figure 3.3 the essential oil sac looks slightly collapsed. This could probably due to natural causes like the sun and insects or the preparation of the sample could have caused the oil sac to collapse.

After a steam distillation extraction was carried out, a leaf was prepared for viewing under the SEM.

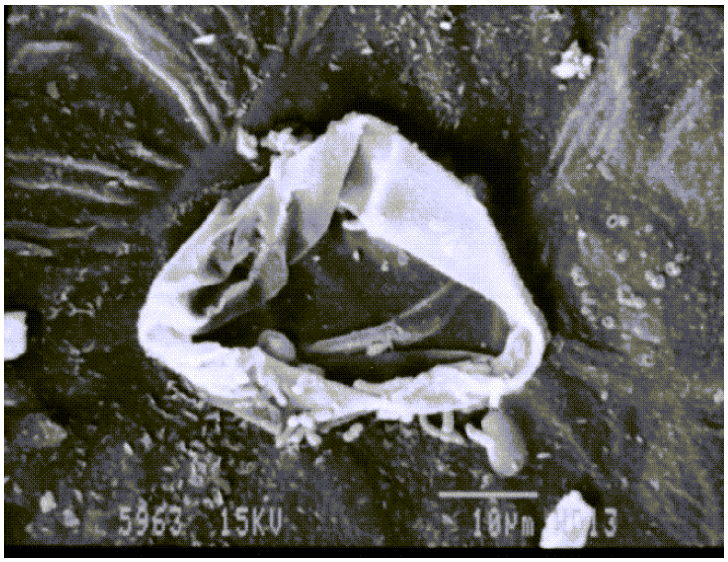


Figure 3.4 Scanning electron micrograph of a leaf after extraction (1900x, 15keV)

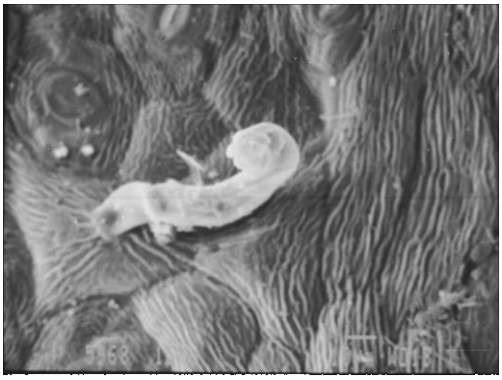


Figure 3.5 Scanning electron micrograph of a distilled leaf after extraction (850x ,20keV)

Figure 3.4 shows that after extraction the essential oil sac bursts to release the oil. In figure 3.5 the stalk over which the oil sac forms can be seen, but there is no oil sac present after extraction. Figure 3.6 below shows the burst oil sac and the stalk it was attached to.

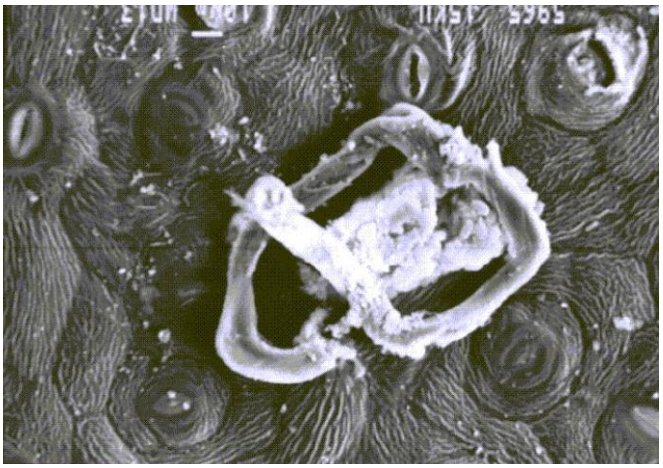


Figure 3.6 Scanning electron micrograph of a distilled leaf after extraction (650x, 15keV)

When looking at the overall surface of the *Centella* leaf (Fig. 3.7) before extraction and comparing it to a SEM of a lavender leaf (Fig. 3.8), it is observed that the lavender leaf contains a large number of oil sacs as expected because it is a very aromatic plant and there are not many essential oil sacs present in the *Centella* leaf. This could be a reason so little oil is obtained in the *Centella* extraction processes and why a very large amount of leaves is needed to obtain a

sufficient yield that can be quantified (Fig. 3.7). (More scanning electron micrographs taken of the centella leaves can be seen in Appendix B)

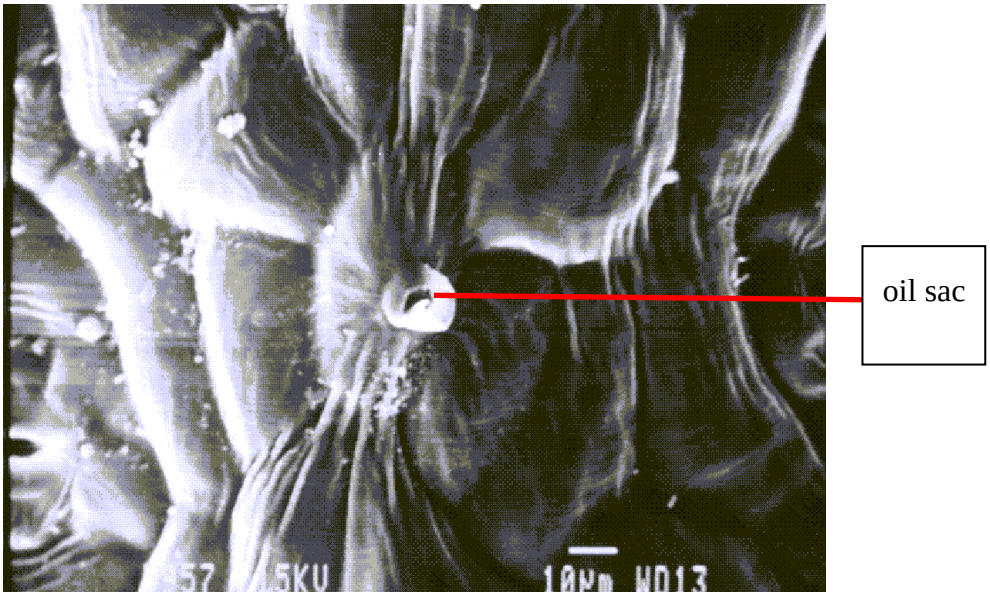


Figure 3.7 Scanning electron micrograph of a fresh leaf before extraction (800x, 15keV) where an essential oil sac can be seen and it looks like it is slightly collapsed. There are not many essential oil sacs visible on the surface of the leaf.

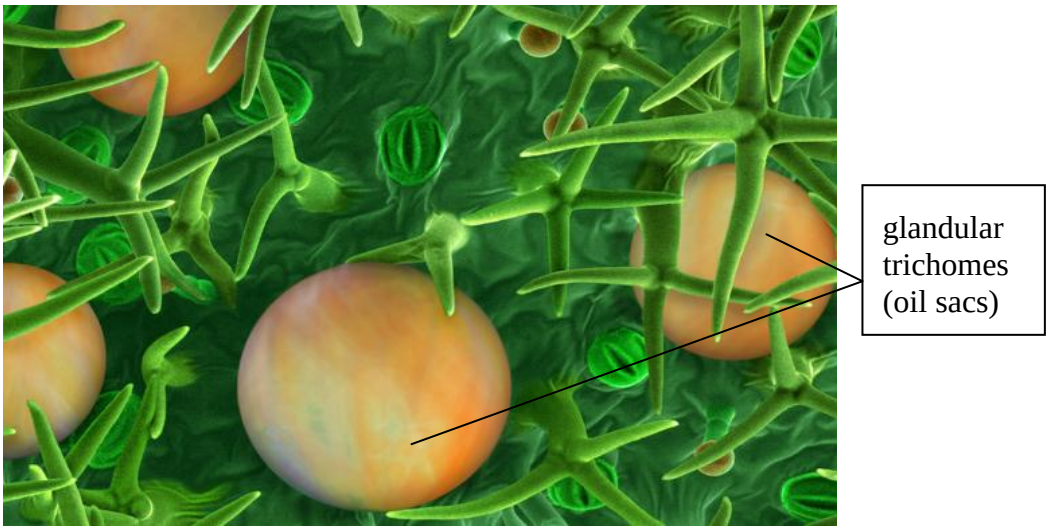


Figure 3.8 A false-coloured scanning electron micrograph of a lavender leaf imaged at 200 microns. The surface of the leaf is covered in fine hair-like outgrowths made from specialised epidermal cells called non-glandular trichomes, which protect the plants against pests and reduce

evaporation from the leaf. Glandular trichomes are also present, containing the oil produced by the plant. (http://www.labnews.co.uk/features/speaking-a-thousand-words/)

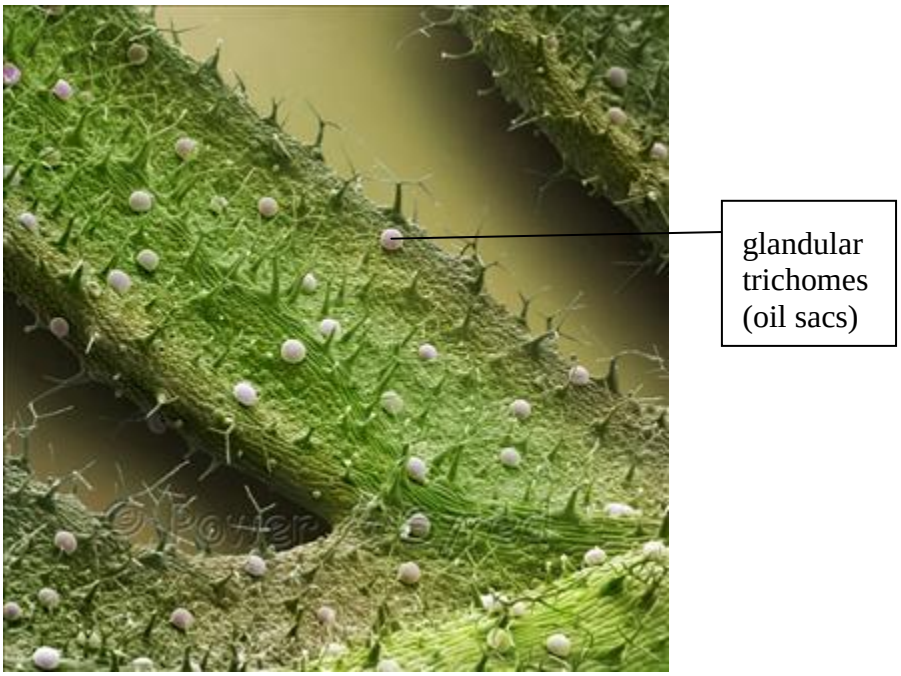


Figure 3.9 A Coloured scanning electron micrograph (SEM) of a section of French lavender (Lavandula dentata) leaf, showing the tooth-like structures (trichomes, spikes). A number of oil glands (spheres) can also be seen. When the leaf is touched or damaged, the glands rupture and release the oil that gives lavender its distinctive fragrance. Magnification: x526 when printed 10 centimetres wide. (http://www.psmicrographs.co.uk/lavender-leaf-showing-oil-glands-lavandula-dentata-/science-image/80200166)

In figure 3.9, one can see that the surface of the lavender leaf has a great amount of essential oil sacs present. (Another SEM of lavender can be found in Appendix B)

Based on the chemical composition of *Centella Asiatica* growing in South Africa from a previous study by Oyediji and Afolayan (2004) some of the pure constituents (for standards) were ordered to help identify the constituents in the samples of *Centella Asiatica* from the different extractions done. The following standards were used: α-Pinene, camphene, β-pinene, myrcene, α-phellandrene, α-terpinene, γ-terpinene, terpinolene, menthone and terpinen-4-ol. Unfortunately

only one of the predominant constituents was obtained (i.e. myrcene) since the rest were not available.

The standards were then run on the GC and GC-MS. The GC did not help much in identifying the retention times of the standard samples since the gas chromatogram was expected to have one main peak for a certain standard but actually more than one peak was formed and it was uncertain to which peak (and retention time) the certain constituent actually correlates. (Graphs of the standards analysed by the GC are given in Appendix C)

The GC-MS was then used to analyse the standards to obtain their mass spectra so that they can be used as a comparison to identify the components of the actual *Centella* essential oil. The retention times however could not be compared to the gas chromatograms obtained from the actual *Centella* samples from each extraction because the standards were run on a different temperature gradient. They were run on a normal gradient to see at what temperature the peaks would arise and to optimize the GC-MS for running the mixture of standards to obtain good separation between peaks.

The chromatograms and mass spectra obtained from each of the standards can be found in appendix D.

A mixture of standards were analysed as well to see if they co-elute.The spectrum obtained is given in Figure 3.10.

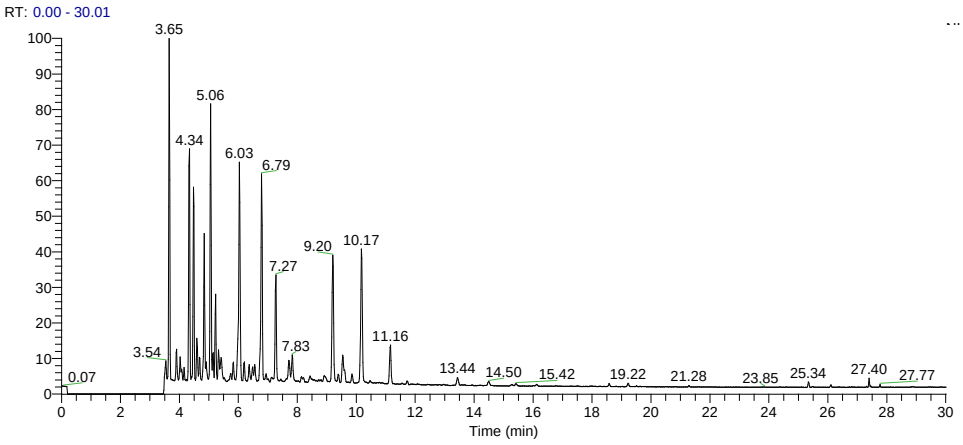


Figure 3.10 Gas chromatogram of the mixture of the standards

The graph shows good separation and the retention times correlate to the following constituents gviven in Table 3.1.

Table 3.1 Retention times of the standard samples run on the GC-MS

Retention time [min]	Standard
3.65	Alpha -pinene
3.91	Camphene
4.34	B-Pinene
4.48	Myrcene
4.84	Phellandrene
5.06	Terpinolene
6.03	y-terpinene
6.79	α-terpinene
9.20	Menthone
10.17	Terpinen-4-ol

Then the samples from each experiment were run on the GC and on the GC-MS.

If no peaks were visible on the GC chromatogram, a GC-MS was not run since there was no constituents to identify.

Since the standards obtained were not 100% pure, the GC results ran were not helpful in identifying the constituents. The GC was also run with a different column then the GC-MS (for experiments 1 to 5) and therefore the components had different retention times and the results could not be compared to the GC-MS results. Therefore, from these results all that can be said is that from each different

extraction method, a different chromatogram was obtained and each extraction method resulted in a different composition of the oil.

When the samples were run on a GC-MS the results obtained from the different extraction methods were are shown below:

The setup 1 steam distillation on fresh leaves (42 g) was run first and the peaks were identified using the NIST –MS library and the standards.

The chromatogram in Fig. 3.11 was obtained for steam distillation setup 1 on fresh leaves.

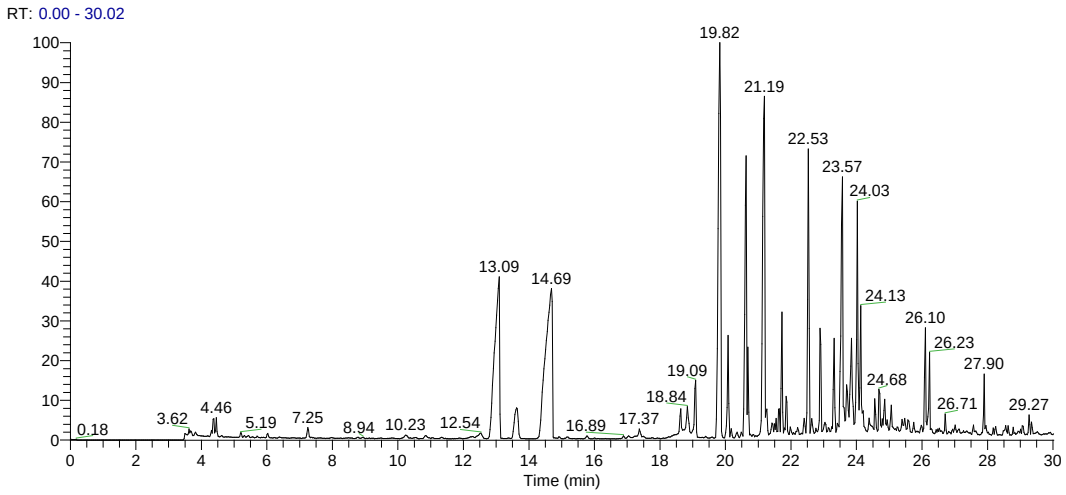


Figure 3.11 Gas chromatogram from the GC-MS of steam distillation setup 1 with distilled water on fresh leaves of *Centella* (42 g) (SDP1)

Each prominent peak of the chromatogram was examined and compared to the NIST-MS library and the standards to identify the constituents. The first part of the chromatogram was examined between the retention times 3.5 - 7.6 minutes shown in Fig.3.12.

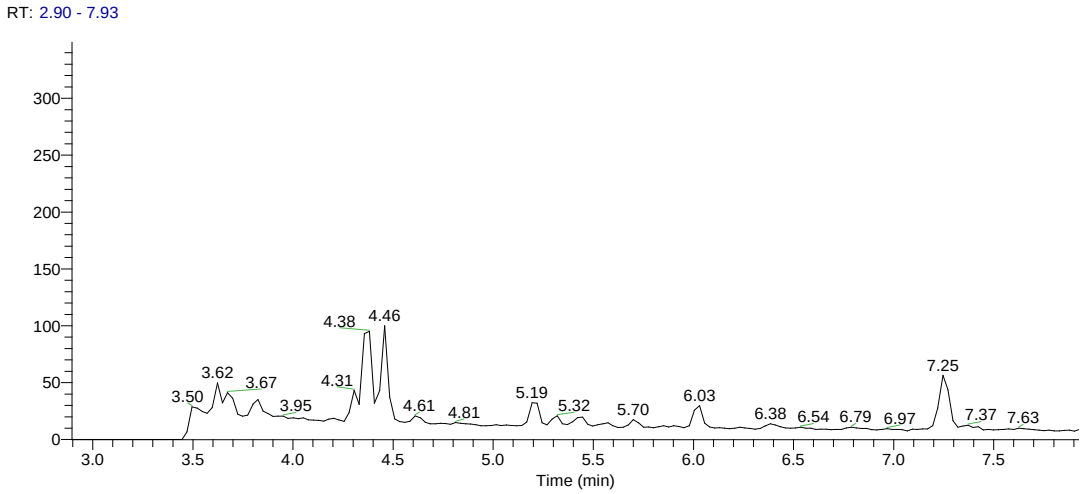


Figure 3.12 Gas chromatogram from the GC-MS of SDP1 between the retention times from 3.5–7.6 minutes

The peak at 4.46 min had the following mass spectrum:

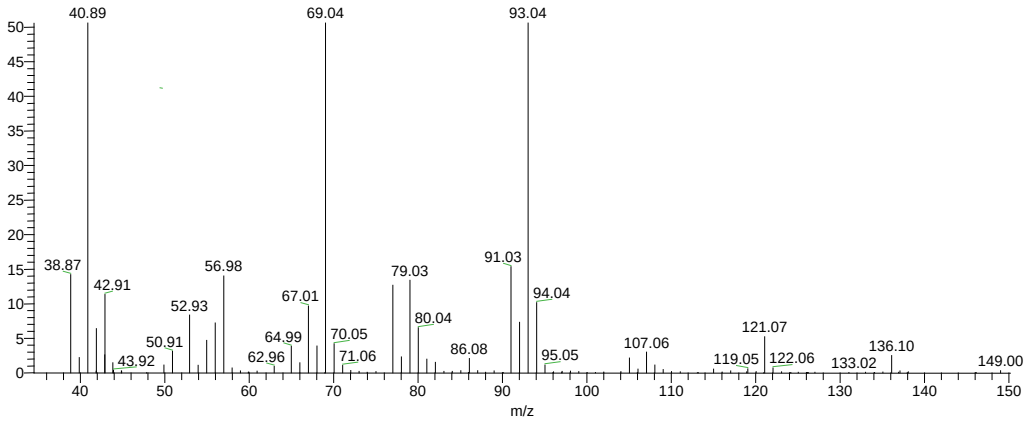


Figure 3.13 Mass spectrum of peak at 4.46 min in the gas chromatogram of SDP1

This mass spectrum (Fig. 3.13) was then compared to the mass spectrums from the standards and it was found to correlate to the mass spectrum of myrcene (Fig.3.14) since the peak ratios were the closest to this standard.

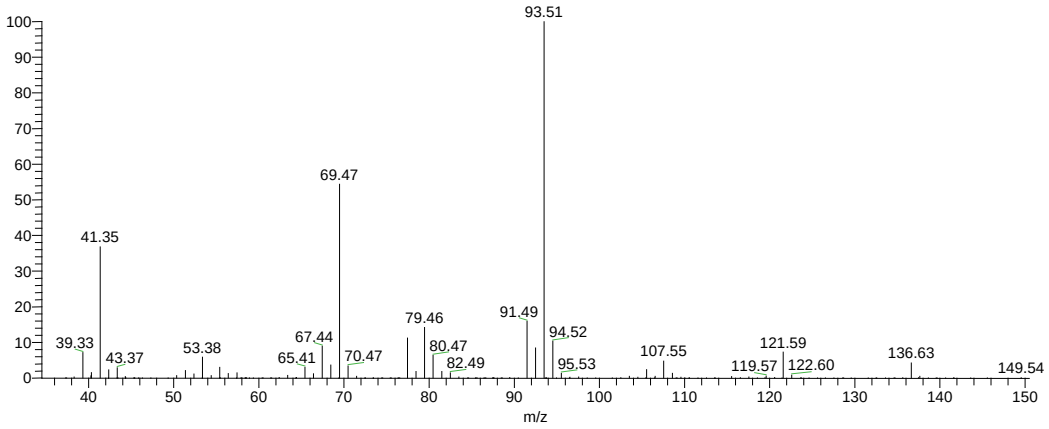


Figure 3.14 Mass spectrum of myrcene

The next peak 6.03 min was examined and the following mass spectrum was obtained (Fig.3.15):

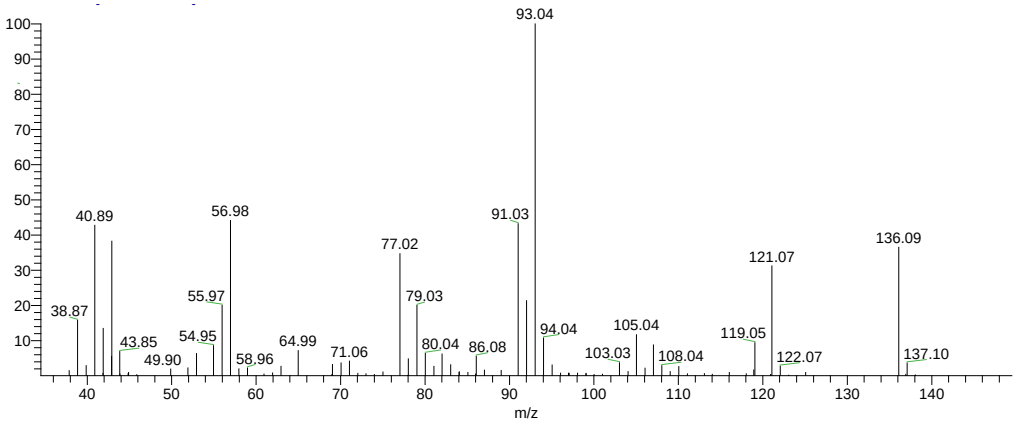


Figure 3.15 Mass spectrum of peak at 6.03 min in the gas chromatogram of SDP1

The mass spectrum (Fig. 3.15) when compared the standards and the NIST-MS library was found to correlate to y-terpinene. The ratios of the peaks in figure 3.15 (the 77.02, 91.03 and 93.04) are similar to the ratios in figure 3.16 (below) (the 77.45, 91.49 and 93.51). The peak at 6.03 minutes therefore correlates to y-terpinene.

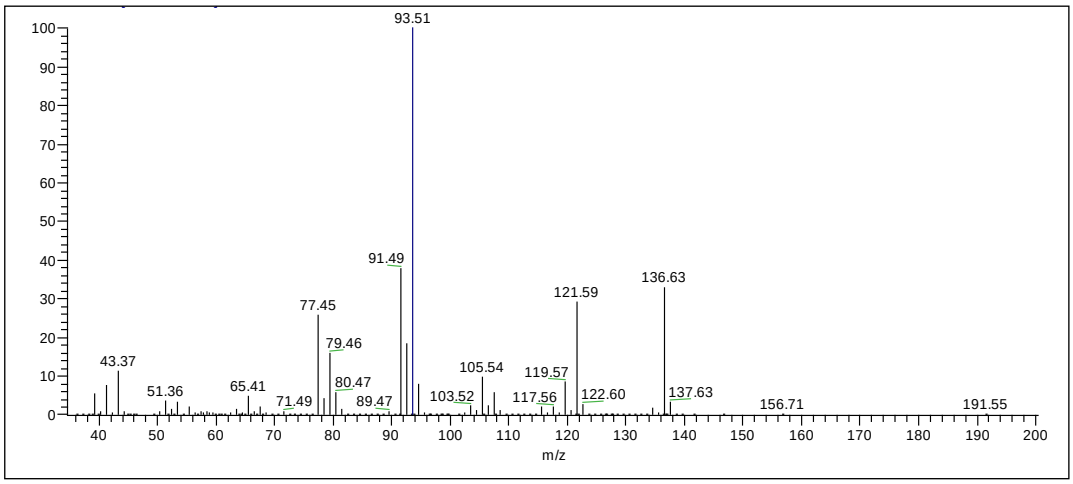


Figure 3.16 Mass spectrum of y-terpinene

This process was repeated to identify all the other peaks in the chromatogram the identifying process and results are attached in Appendix E.

After all the dominant peaks were examined, the constituents identified in the sample of SDP1 (the steam distillation with distilled water (45 g) of leaves) are shown in table 3.2

Table 3.2 Peaks (retention times) and the components identified at those specific retention times in the sample of SDP1.

Retention time [min]	Compound
4.46	Myrcene
6.03	Terpinene
7.25	Linalol
13.09	Citral
13.62	Neral
14.69	α-Citral
17.37	δ- Elemene
19.82	B-Carophyllene
20.08	y-Elemene
20.63	α-Humulene
20.96	allo-Aromadendrene
21.19	B-Cuvebene
21.72	B-Bisobolene
22.53	λ-Gurjunene
22.91	Carophyllene oxide
23.32	2 – (4a8-Dimethyl-1,2,3,4,4a,5,6,8a-octahydro-2-napphthalenyl-2-propanol
23.55	Juniper Campher
23.85	tau-Muurolol
24.03	α-Cadinol
26.23	phytol

The samples for all the extraction methods were analysed and the peaks compared to the sample of SDP1 to identify the constituents in the other samples. The following graphs were obtained and compared to each other and SDP1 (Fig. 3.17). (Each separate graph can be seen in Appendix F)

The labels to the graphs shown in Figure 3.17 are:

GRAPH 1: Gas chromatogram of sample from steam distillation setup 1 on fresh leaves of Centella (42 g) using distilled water (SDP1).

GRAPH 2: Gas chromatogram of sample from steam distillation setup 1 on fresh leaves of Centella (10 g) using distilled water (SD-1).

GRAPH 3: Gas chromatogram of sample from steam distillation setup 1 on fresh leaves of Centella (10 g**)** using distilled water (SD-2).

GRAPH 4: Gas chromatogram of sample from steam distillation setup 2 on fresh leaves of Centella (10 g) using distilled water (SDS2W1).

GRAPH 5: Gas chromatogram of sample from steam distillation setup 2 on fresh leaves of Centella (10 g) using distilled water (SDS2W2).

GRAPH 6: Gas chromatogram of sample from steam distillation setup 2 on fresh leaves of Centella (25 g) using vinegar (SD-VIN).

GRAPH 7: Gas chromatogram of sample from steam distillation setup 2 on fresh leaves of Centella (25 g) using vinegar (SD-VIN-2).

GRAPH 8: Gas chromatogram of sample from steam distillation setup 2 on dry leaves of Centella (5 g) using distilled water (SD-DRY1).

GRAPH 9: Gas chromatogram of sample from solvent extraction on fresh leaves of Centella using water (40 g)(SEW).

GRAPH 10: Gas chromatogram of sample from water distillation on fresh leaves of Centella (40g) (WD1).

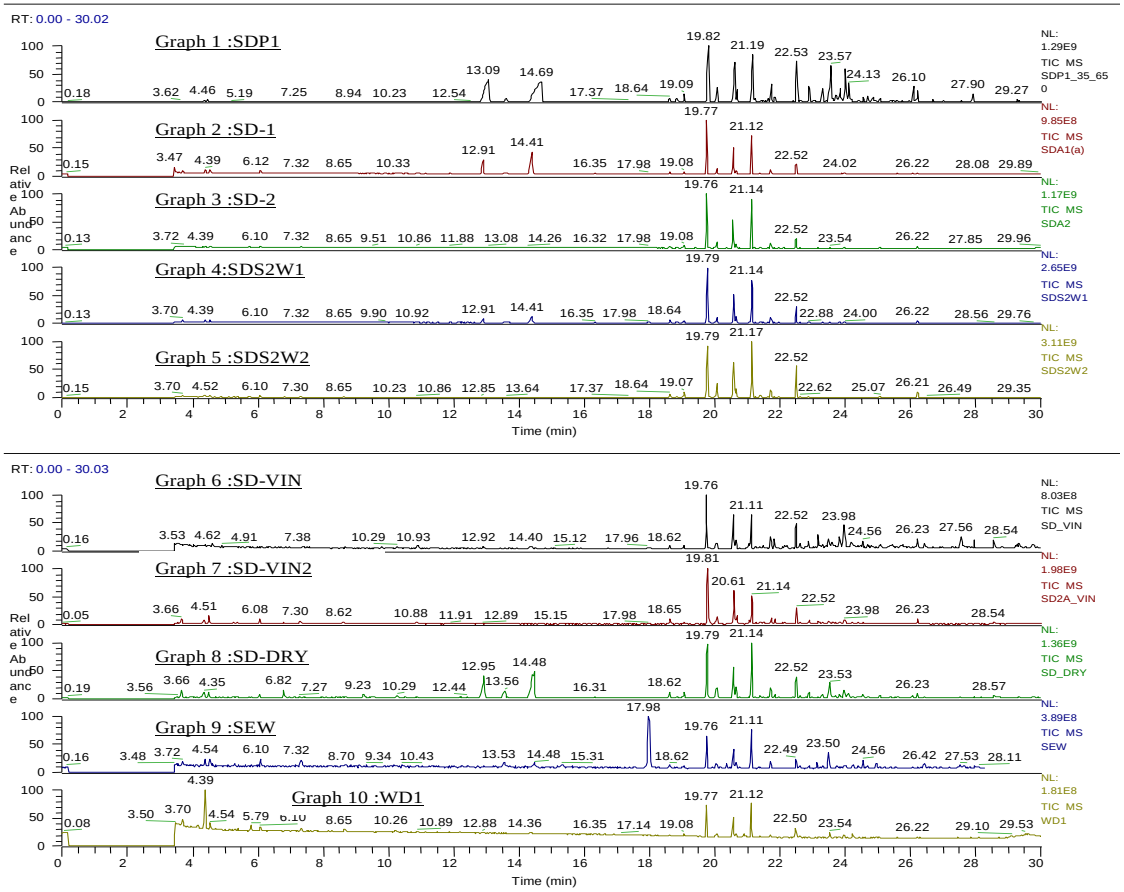


Figure 3.17 Comparison of the gas chromatograms of the *Centella* oil after each extraction process is conducted (A larger copy of the comparison graphs is available in appendix M)

Table 3.3 Comparison of the different constituents present in the samples obtained from the different extraction methods

	STEAM DISTILLATION [minutes]								SOLVENT EXTRACTION [min]	WATER DISTILLATION [min]	LITERATURE[7]
Constituents	SETUP 1			SETUP 2							Steam distillation
	SDP1	**SD-1**	**SD-2**	**SDS2W1**	**SDS2W2**	**SD-VIN**	**SD-VIN-2**	**SD-DRY**	**SEW**	**WD1**	
Myrcene	4.46	4.54	4.54	4.54	4.52	4.62	4.51	4.35	4.54	4.54(s)	p
Terpinene	6.03	6.12	6.10	6.10	6.10		6.08	6.05	6.10	6.10	p
Linalol	7.25	7.32	7.32	7.32	7.30	7.38	7.30	7.27	7.32	7.32	-
Citral	13.09	12.91	12.88(s)	12.91	12.85	12.92	12.89	12.95	trace	12.88	-
Neral	13.62	-	13.54	13.54(s)	13.64(s)	trace	trace	13.56	13.53	trace	-
α-Citral	14.69	14.41	14.26(s)	14.41(s)	trace	trace	14.43(s)	14.48	14.48(s)	trace	-
δ- Elemene	17.37	-	-	-	17.24	17.24	-	-	17.98	-	-
B-Carophyllene	19.82	19.77	19.76	19.79	19.79	19.76	19.81	19.79	19.76	19.77	p
y-Elemene	20.08	20.08	20.07	20.07	20.07	20.05	20.08	20.08	20.05	20.07	p
α-Carophyllene	20.63	20.58	20.58	20.61	20.60	20.58	20.61	20.58	20.58	20.58	p
allo-Aromadendrene	20.69	20.66	20.66	-	20.68	20.66	2.69	20.66	20.66	20.69	p

B-Cuvebene	21.19	21.12	21.14	21.14	21.17	21.11	21.14	21.14	21.11	21.12	-
B-Bisobolene	21.72	21.70	21.70	21.70	21.70	21.70	21.72	21.70	21.70	21.71	-
λ-Gurjunene	22.53	22.52	22.52	22.52	22.52	22.52	22.52	22.52	22.49	22.50	-
Carophyllene oxide	22.89	trace	trace	22.88	22.87	22.89	22.89	22.89	trace	trace	p
2 – (4a8-Dimethyl-1,2,3,4,4a,5,6,8a-octahydro-2-napphthalenyl-2-propanol	23.32	-	-	23.31	23.31	23.31	23.31	trace	trace	trace	-
Juniper Campher	23.57	trace	23.54	trace	trace	trace	trace	23.53	23.50	23.54	-
tau-Muurolol	23.85	-	-	-	-	23.82	23.82	-	-	-	-
α-Cadinol	24.03	trace	-	24(s)	-	23.98	23.98	23.98	24.00	23.98	-
phytol	26.23	26.22	26.22	26.22	26.21	26.23	26.23	26.23	trace	26.22	-

Note: The numbers in each cell are the retention time in minutes of that specific peak for that specific constituent in each sample (This information was obtained from Fig. 3.17)

KEY : (s) =small peak (ie: small amount of constituent present) trace = trace amount of the constituent present p = constituent present

From the graphs of figure 3.17, a table shown in Table 3.3 was constructed identifying the different constituents present in each sample of oil from each different extraction method.

In table 3.3 it can be observed that the retention times of the same constituent vary slightly between extraction methods. This is because manual injection was used to introduce the sample into the GC-MS and more consistent retention times would have been achieved if an automatic injector was used.

3.1.1 Steam distillation

Setup 1

From Graph 1 (Fig. 3.17) for experiment 1A: the steam distillation of fresh leaves (42 g) (sample SDP1) it was observed that there were more peaks i.e. more constituents present in graph 1 then graph 2 (sample SD-1) and graph 3 (sample SD-2) for experiment 1B: The steam distillation of fresh leaves (10 g). When the total ion current (TIC) was compared it was found that graph 1 has TIC of 1.29 x 10^9 ions, which was greater than graph 2 and 3 with TIC of 9.85 x 10^8 ions and 1.17 x 10^9 ions, respectively. It is observed that the concentration of ions extracted in SDP1 is higher than in sample SD-1 and SD-2. Hence using more leaves for extraction will give a more concentrated oil for analysis and more constituents will be present. This can be justified since the more leaves used in the extraction process, the more oil sacs are available that burst and release the oil which contains the different constituents that are collected and separated with hexane for analysis. The amount of hexane used also affects the concentration of the oil since the analysis was done with hexane. However 1 ml of hexane was used in the case of experiment 1A: SDP1 with 42 g of leaves and this was decreased to 0.7 ml for experiment 1B (SD-1, SD-2, SD-3) using 10 g of leaves and therefore the TIC in this case is affected more significantly by the amount of leaves used in the extraction and not by the amount of hexane used as sample

SDP1 1 still had a higher TIC than the other two samples. Therefore using more leaves will give a graph with more signals hence with more constituents.

When graph 2 (SD-1) and 3 (SD-2) (Fig. 3.17) from the steam distillation of fresh leaves setup 1 (10 g) are compared it can be seen that graph 2 has two more peaks visible at 12.91 and 14.41 minutes, even though the extraction was performed on the same amount of leaves. The peaks at 12.91 and 14.41 min were however present in graph 3 but in very small amounts (table 3.3). The difference between these two graphs can be explained by the amount of hexane used. It is hypothesised that all though 0.7 ml was used in each case, hexane is very volatile, some of it could have been lost by vaporising in the extraction of SD-1 and a more concentrated oil occurred with more constituents present compared to sample SD-2 where less could have vaporised and the oil collected was less concentrated. The more solvent mixed with the oil, the smaller the amount of each constituent (ions) present in solution and hence a smaller TIC is observed. Another reason could have been that in the sample SD-1 extraction process, the 10 g of leaves used consisted of larger leaves than the leaves used in the extraction of sample SD-2 . The bigger (and older) the leaves, the more oil they contain, and this could have affected the concentration of consituents extracted.

This experiment 1B was repeated 3 times, but only 2 samples were run on the GC-MS because the 3rd sample (SD-3) yielded so little oil and dried up by the time it could be analysed. The extraction methods yield very little oil and in this case even more oil was lost when drying with anhydrous sodium sulphate because some oil gets attached to the particles as well and is left behind. Therefore, only GC was used to analyse the sample. The GC obtained had a number of peaks present (Appendix G - figure G4) but it could not be compared to the GC-MS obtained by the other 2 samples because different columns were used. However the gas chromatograms of the other two samples (SD1 and SD2) can be compared. (Appendix G - Fig. G2 and G3) It was determined that the number of peaks were approximately the same with similar retention times and hence the composition was similar.

Setup 2

In experiment 2A: Analysis of the oil from steam distillation setup 2 using fresh leaves (10 g) generated graph 4 (sample SDS2W1) and graph 5 (SDS2W2) (Fig 3.17). It was observed that the number of constituents obtained was less than sample SDP1 (with 42 g of leaves). The reason for this could be because less leaves were used. The particular constituents that were not present in SDS2W1 and SDS2W2 are shown in table 3. When the graphs were compared to each other, it was noted that graph 4 (SDS2W1) had 2 additional peaks at 12.91 (citral) and 14.41 min (α-citral) compared to graph 5 (SDS2W2). Even though graph 5 had a peak at 12.85 min (table 3.3) which corresponded to the citral constituent that SDS2W1 had at 12.91 min, it was in a very low abundance and it had trace amounts of alpha-citral. The reason for this difference could also be due to the hexane evaporating and more concentrated oil was extracted in sample SDS2W1 than SDS2W2 and since the constituents were present in very low concentrations even a drop more of hexane could have diluted a certain constituent to an even lower concentration and thus it could not be picked up by the mass spectrometer.

When comparing distilled water steam distillation setup 1 and setup 2, the ion concentrations for setup 1 experiments were 1.29×10^9 ions (SDP1), 9.85×10^8 ions (SD-1), 1.17×10^9 ions (SD-2) and for setup 2 experiments were 2.65×10^9 ions (SDS2W1) and 3.11×10^9 ions (SDS2W2). It can clearly be seen that the ion concentration is higher for setup 2. Therefore, setup 2 was more efficient since a higher concentration of ions in each of the setup 2 steam distillations were observed. This makes sense because in setup 2, the variation of the Clevenger apparatus was used and the extraction vessel is connected directly to the receiver tube (Fig. 2.2) and any oil/water formed in the extraction vessel falls into the receiver tube yielding a higher concentration of oil. In setup 1, the Clevenger apparatus was used where there is a long path for the steam and extracted volatile oil to travel before it reaches the receiver tube, giving it more time to condense

and hence a greater chance of some oil can be left on the walls of the connector tube which could result in less oil and less constituents collected in the receiver tube (Fig. 2.1).

Since setup 2 gave a higher concentration of constituents of the oil it was used to investigate the effect a different solvent (vinegar in this case) on the composition and concentration of the essential oil. (Experiment 2B)

When the steam distillation using setup 2 was conducted with vinegar instead of distilled water, graphs 6 (sample SDVIN-1) and 7 (SD-VIN-2) (Fig. 3.17) were obtained.

Comparing the graphs to SDS2W1 (graph 4) and SDS2W2 (graph 5), it is observed that they have approximately the same peaks from 3.53 min to 22.52 min with the only difference being the peaks in SDS2W1 (graph 4) which has 2 extra peaks at 12.91 and 14.41 min compared to SDVIN (graph 4) and SDVIN-2 graphs (graph 5). This could again be due to the amount of hexane used as previously mentioned because the peaks (constituents) are present but at low concentrations. However, from 22.52 min to 30 min the graphs of SD-VIN (graph 4) and SDVIN-2 (graph 5) have a better abundance of constituents with retention times between 22.52 min and 30 min than the graphs of the setup 2 steam distillation with distilled water (graph 4 and 5). This was probably because more leaves were used in the vinegar extraction (25 g).

When the TIC of SD-VIN (8.03×10^8 ions) and SDVIN-2 (1.98×10^9 ions) are compared to the TIC of SDS2W1 (2.65×10^9 ions) and SDS2W2 (3.11×10^9 ions), it is observed that the SDS2W samples were more concentrated since their TIC values are higher than those for the SDVIN and SDVIN-2. The amount of hexane used in both experiments was the same. Therefore, steam distillation with water gives a higher concentration of the constituents of the oil even though the distillation with vinegar was preformed with 25 g of leaves instead of 10 g of leaves like in experiment 2A (SDS2W1 and SDS2W2). Vinegar was investigated as one of the solvents because *Centella* oil was extracted traditionally by tribes and medicine men instead of water. However, from the results it shows that

vinegar was not as effective in extracting more constituents as compared to distilled water.

When the SD-VIN and SD-VIN-2 graphs are compared there is not much difference between them. The only slight difference being that SDVIN-2 (graph 6) has a slightly higher concentration of constituents between 3.66 and 8.22 min and between 22.52 and 30 minutes. This was probably due to the amount of hexane used and because it tends to volatise quickly decreasing the concentration of oil for analysis in SD-VIN2.

The steam distillation setup 2 with distilled water was used to investigate the effect of dry leaves on the composition and concentration of the oil. (Experiment 2C) The gas chromatogram obtained is given as graph 8 (SD-DRY1). When this graph is compared to graph 4 and 5 (SDS2W1 and SDS2W2) it was noted that even though the concentration of the oil in graph 8 is lower (TIC = 1.36×10^9 ions) compared to graph 4 and 5 (TIC = 2.65×10^9 ions and 3.11×10^9 ions) the %abundance of constituents is greater. The amount of leaves used would not account for any difference in the graphs since 5 g of dry leaves is approximately 10 g of fresh leaves. Therefore dry leaves seem to be more effective than fresh leaves yielding more constituents with a greater abundance than fresh leaves. However fresh leaves were found to give a higher total ion concentration.

The experiment 2C was preformed twice, the other sample (SD-DRY2) when run on the GC (Appendix G: figure G10) gave a very noisy graph due the sample being too dilute. Even though 0.7 ml of hexane was used, the same reasons as explained before apply, even if 1 extra drop of hexane is causes the sample to be too dilute. Therefore a GC-MS was not run on the sample. Since this experiment was only repeated successfully once, a repeat of the experiment is needed to make a better conclusion on dry leaves.

3.1.2 Water distillation

Water distillation was conducted on fresh leaves to see how this affects the oil composition and concentration. (Experiment 3A) Graph 10 (WD1) (Fig. 3.17) was obtained. When this is compared to the SDP1 (graph 1), the number of peaks in the water distillation graph was less than in graph 1 even though approximately the same amounts of leaves were used (40 g in WD1 and 42 g in SDP1). However the peak at 4.39 min, which corresponds to the constituent myrcene (table 3.3) has the highest abundance compared to any other extraction method. There are no other prominent peaks present in the water distillation graph 10 that were not present in SDP1 (graph 1). This shows that the same composition of the oil was obtained with water distillation as with steam distillation but fewer peaks i.e. less constituents are present of some compounds.

Two samples were run for water distillation. However sample 2 (WD2) when run on the GC (Appendix G: Figure G12) gave a very noisy graph due the sample being too dilute. 1ml of hexane was used in this experiment and this was possibly too much since the concentration of oil was very low as the sample was too dilute. Therefore, the sample was not analysed by GC-MS.

Water distillation was also done on dry leaves to see the effect it has on the composition and concentration. Two samples were extracted ($WD_{DRY}1$ and $WD_{DRY}2$). The samples were run on the GC and the graphs obtained had no peaks present (Appendix G –Fig. G13 and G14). The one peak present in figure G13 could be an experimental artefact due to noise.

Since from the steam distillation of dry leaves (SDDRY-1) it is known that there should be some oil sacs present even after drying, there should have been a graph obtained with a number of peaks. However, the reason nothing was obtained could have been that the amount of leaves used was very small (4 g) in each case and very little oil was obtained which when extracted with 0.7 ml of hexane became too dilute and no peaks were obtained in the gas chromatogram.

3.1.3 Solvent extraction

Solvent extraction was performed using:

- Glacial acetic acid and 20 g of fresh *Centella* leaves. (Sample SEGA) Experiment 4A)
- Distilled water and 13 g of fresh *Centella* leaves. (Sample SEW) (Experiment 4B)
- Vinegar and 10 g of fresh *Centella* leaves. (Sample SEV) (Experiment 4C)

The oil obtained from the leaves left in glacial acetic acid was analysed using the GC and a graph was obtained with no peaks. (Appendix G – Fig. G15)
The small peak at 2.10 min is probably due to some solvent (hexane). The reason that no peaks (constituents) were obtained is probably because the glacial acetic acid was too concentrated and destroyed the leaf and the oil sacs and it was observed that no oil was obtained.

The oil obtained from the leaves left in distilled water was analysed using GC-MS and graph 9 (SEW) (Fig. 3.17) was obtained. When compared to SDP1 (graph 1), it was noted that SDP1 still has the greatest number of constituents in a greater abundance. However, in graph 9 (SEW) the peak at 17.98 min which correlates to delta-elemene, has the highest abundance than any other extraction method. The total ion concentration is low (3.89×10^8 ions) compared to SDP1 (1.29×10^9 ions). When compared to water distillation (graph 10), the peaks were similar apart from the most abundant peak of myrcene at 4.39 min in WD1 (graph 10) and the peak at 17.98 min (delta-elemene) in SEW (graph 9).

From the solvent extraction done with vinegar, the sample was analysed on the GC and the graph obtained had no peaks (Appendix G – Fig. G17). This could be because so little leaves (10 g) were used and the leaves were very young and they did not possess much oil.

3.1.4 Soxhlet extraction

Two soxhlet extractions were run (SOX1 and SOX 2) .The samples were analysed using the GC and the graphs obtained had no peaks (Appendix G - G18 and G19). The possible explanation is that soxhlet extraction is used for extracting non-volatile and semi-volatile organic compounds from solids, and essential oils are very volatile compounds. As a result no peaks were obtained when the sample was analysed.

In all the extraction processes, one of the problems seemed to be the amount of hexane used and this caused the samples to be too dilute for analysis. However, 0.7 ml of hexane was found to be the optimum amount to use.and was used in most extractions. If less hexane was used, then the amount of the hexane and oil mixture obtained tended to be very small after separating from the oil and water. This was because even though 0.7 ml is added to separate the oil and water, a lot of hexane is lost because it is volatile and by the time you dry the sample with anhydrous sulphate and transfer it to a sample bottle a even smaller amount is left. Some of the hexane can also gets adhered onto the glass and total sample yield obtained for analysis is very small. If more than 0.7 ml of hexane was used, the sample became too dilute.

3.1.5 Results compared to previous studies

The constituents obtained from the steam distillation of fresh leaves were compared to the previous study done in South Africa on the chemical composition of Centella Asiatica which was extracted by steam distillation. When compared to the previous study (Table 3.3–literature column), only 7 out of 20 constituents identified from the steam distillation experiments were the same. These were myrcene, terpinene, B-carophyllene, y-elemene, α-humulene, allo-aromadendrene and carophyllene oxide. The predominant constituents in the previous study were

α-humulene (21.06%), β-caryophyllene (19.08%), bicyclogermacrene (11.22%), germacrene B (6.29%) and myrcene (6.55%).[7] Therefore, only 3 out of the 5 predominant constituents were identified.

In the oil sample from the steam distillation of fresh *Centella* leaves, the predominant constituents were citral (13.09 min), α–citral (14.69 min), β-carophyllene (19.82 min), α-carophyllene (20.63 min), allo-aromadendrene (20.69 min), β-cuvebene (21.19 min), λ-gurjunene (22.53 min) and juniper campher (23.55 min). Myrcene at 4.46 min is actually in a very low concentration and is not a predominant constituent as in the similar study done.

The only similarity between the similar study results and the steam extraction done, therefore, is that B-carophyllene and α-carophyllene are predominant constituents in both studies and 7 constituents were identified in the steam distillation (SDP1) compared to the similar study. In this initial study, only the predominant constituents were identified. If some of the minor constituents would have been identified, there would most probably be more similarities to the literature study constituents. The total composition of the oil still varies though from the literature study since the predominant constituents are different besides B-carophyllene and α-carophyllene. Although *Centella* leaves used in this study and the literature study were both grown in South Africa, the essential oil composition differs greatly. This proves that the composition of the essential oil is affected by many factors such as geo-climatic location, soil type, life stage of plant and even the time of day when harvesting is done, since the volatile content of the leaf increases with time and also with the size of the leaf.

When comparing the variation of constituents between the different extraction methods it can be seen from table 3.3 that myrcene, linalool, β-carophyllene, γ-elemene, α-carophyllene, allo-aromadendrene, β-cuvebene, β-bisobolene and λ-gurjunene are present in the composition of all the essential oils from the different extraction processes (Table 3.3). The trace constituents found in the oil samples

analysed are observed in such small amounts compared to the predominant constituents, they were not included in the comparisons between extractions.

The experiments explained above were all used to determine which extraction method to focus on further. Unfortunately because of the size of the apparatus and the small amounts of leaves used, quantification was not possible as explained at the beginning of this chapter. Therefore this study focused more on the quality of the essential oil, since the more constituents present the more therapeutic and the better the quality of the oil.

3.2 Extraction of essential oils from *Centella Asiatica* using the apparatus described by the British Pharmacopedia

The experiments (no.6 to 12) were done on setup 6 and 6B, using the apparatus described by the British Pharmacopedia IV. In this study this apparatus will be referred to as the BP apparatus for ease of explanations. The apparatus was investigated in this study in the hope that the amount of oil collected from *Centella Asiatica* could be quantified which was not successfully determined in the initial distillation experiments using normal Clevenger apparatus (experiments no.1 to 5).

A few initial steam distillation extractions were preformed to get familiar with the apparatus and to determine what parameters were needed. The extractions were preformed as shown in Table 3.4.

Table 3.4 Initial steam distillation experiments on fresh leaves using the BP apparatus and a distillation rate of 2/3 ml min^{-1}.

Experiment Name	Weight of fresh leaves (g)	Average size of leaves (mm)	Distillation Time (min)	Xylene used initially (ml) (Xi)	Xylene after 30 minute distillation with no leaves (ml) (X_{30})	Xylene and oil after extraction (ml) (X_{after})
ST2A	36.2	64.05	60	0.2	n/a	0.20
ST3A	50.40	58.31	60	0.2	0.15	0.15
STEAM T1	53.86	56.44	60	0.2	n/a	0.15
ST4AHEX	44.88	56.09	60	0.4 (HEXANE)	0.1	0.1

Once the BP apparatus was set up, a known amount of xylene was added into bottom of tube K (Fig. 2.10) which was taken as the initial xylene (Xi). After the initial distillation was preformed for 30 minutes without leaves, the amount of xylene in the graduated tube (X_{30})(JL - Fig. 2.10) was read and the leaves were

added into the false bottom extraction container and the extraction was distilled for the prescribed amount of time. The meniscus in the graduated tube (X_{after})(JL - Fig. 2.10) was read and the amount of xylene read off earlier (X_{30}) subtracted from it. This calculation then gave the amount of essential oil collected.

In experiment ST2A, the steam distillation extraction was performed for 60 minutes with 36.2 g of leaves and 0.2 ml of xylene initially. The 30 minutes distillation was not preformed without leaves as described in the original method in order to see how it affects the results. After the extraction the amount of xylene and oil was 0.2 ml (Table 3.4), which indicates that there was no oil collected. Since xylene is volatile, it could have evaporated and some essential oil could have collected in the graduated tube which would cause the total amount of oil and xylene collected equal to the same amount of xylene added initially. Since a GC-MS graph was obtained for experiment ST2A, it is known that some oil had to be collected. So some xylene could have also been lost on the walls of the apparatus.

In experiment ST3A, the steam distillation extraction with 50.40 g of leaves was preformed for 60 minutes and 0.2 ml of xylene was used. The 30 minutes distillation without leaves was initially carried out and then the leaves were added to the apparatus after cooling. After the 30 minutes distillation without leaves, 0.15 ml of the xylene was measured. *Centella Asiatica* leaves were then added to the false bottom extraction container and the distillation was preformed for 60 minutes. After the distillation, the amount of xylene and oil collected was read off in the graduated tube JL. This value was 0.15 ml. Therefore some xylene has been lost after the 30 minute extraction where it was 0.2 ml initially. However, the amount of xylene after 30 minutes (0.15 ml) and after the extraction (0.15 ml) is the same, this is because some xylene could have evaporated and replaced by some essential oil collected to get the same amount of xylene present as before. Also some xylene could have been lost on the walls of the apparatus.

When comparing the experiment ST2A and ST3A, it is observed that running the distillation with no leaves for 30 minutes is a necessary step. This is because it allows the amount of xylene present in the apparatus to reach a point where it is in a more or less constant balance in the apparatus. Xylene is however volatile and some of it will vaporize during distillation.

In experiment STEAM T1 the steam distillation extraction was preformed for 60 minutes with 53.86 g of leaves and 0.2 ml of xylene was used initially. It was decided to not add the collecting medium (xylene) to the water in order to investigate if it would be possible to read the amount of essential oil collected in the graduated tube without the xylene. For this reason the initial 30 minute distillation without leaves was not necessary. From this experiment it was observed that the drops of the essential oil were collected in minute droplets that were too far apart from each other to combine together to form a meniscus and vaporized shortly after they were collected in the pear shaped swelling J (Fig. 2.10). Hence a collecting medium such as xylene is definitely necessary.

When experiments ST2A, ST3A and STEAM T1 were completed, it was realised that the amount of xylene and oil collected was very small and for analysis a slightly larger sample was necessary. This is because the GC-MS used had an automatic sampler and a larger amount of sample was needed in each sample vial for the sample to be taken up by the needle. As a result the amount of xylene used initially (Xi) was increased to 0.4 ml.

The steam distillation experiment was then preformed with hexane as the collecting medium instead of xylene. Steam distillation was preformed with 44.88 g of leaves and 0.4 ml of hexane was initially used. After the experiment was carried out for 30 minutes only about 0.1 ml of hexane was left. It was necessary to add an extra 1.1 ml to make sure there would be enough for the distillation with leaves. After the extraction, only 0.1 ml of hexane and oil was collected.

From the experiment it was realised that hexane is too volatile to use as a collecting medium in this case because the apparatus gets hot and it volatises too quickly, which would possibly also remove some of the essential oil collected.

This can be explained by comparing the volatility of xylene and hexane. Volatility is the tendency of a substance to vaporize. It is directly related to a substance's vapour pressure. At a given temperature, a substance with higher vapor pressure vaporizes more readily than a substance with a lower vapour pressure. The vapour pressure of xylene at 25°C is 9 mmHg (Daubert and Danner, 1989) and the vapour pressure of hexane at 25°C is 153 mmHg (Chao et al.,1983). If the two vapour pressures were compared it can be seen that hexane has a higher vapour pressure than xylene. and hence hexane vaporizes more readily. So xylene is therefore the better collecting medium because it does not vaporize as readily.

In principle, the method for the extraction using the BP apparatus should work to quantify the amount of essential oil collected. However, when the extractions were preformed on *Centella Asiatica* leaves it was not possible to quantify the amount of oil successfully. If the amount of essential oil collected in all the experiments (Table 3.5) are compared, it is observed that the amount of oil collected in majority of the experiments is zero or a negative value. It is not possible that no essential oil was collected because from the GC-MS results (which all had a number of peaks present), it is evident that the essential oil from *Centella asiatica* leaves was definitely collected. There are a number of reasons why there would be no increase in the volume of the xylene and oil mixture after the leaves distillation.

Firstly xylene is volatile, so trying to keep it as constant as possible it not completely possible. It can reach a more or less balance in the apparatus during the distillation process at a constant rate, nevertheless some of it can be lost on the walls of the apparatus. The slightest adjustment to the rate of distillation can affect the amount of xylene in the apparatus and the temperature of the apparatus as well. If the outside temperature is warm (e.g. in summer) as in the case when the

experiments were preformed, the xylene was likely to volatize quicker than had it been performed in winter.

Table 3.5 Comparison of the xylene quantities used and measured in the experiments

Experiment name	Xylene used initially (ml) (Xi)	Xylene after 30 minute distillation with no leaves (ml) (X_{30})	Xylene and oil after extraction (ml) (X_{after})	Essential oil collected (X_{after} - X_{30}) (ml)
SQ1	0.4	0.31	0.31	0
SQ2	0.4	0.28	0.30	0.02
SQ3	0.4	0.35	0.36	0.01
WD1A	0.4	0.36	0.36	0
WD2A	0.4	0.25	0.20	- 0.05
WDQ1	0.4	0.35	0.33	- 0.02
WDQ2	0.4	0.31	0.30	- 0.01
WDQ3	0.4	0.34	0.33	- 0.01
DRY 1	0.4	0.33	0.32	- 0.01
DRY 2	0.4	0.33	0.31	- 0.02
DRY 3	0.4	0.33	0.32	- 0.01
WDSO1	0.4	0.31	0.31	0
WDSO2	0.4	0.34	0.32	- 0.02
WDS03	0.4	0.32	0.30	- 0.02

The second reason why an increase in the xylene and oil mixture collected, was not observed could be because the amount of oil collected from *Centella Asiatica* leaves was so small. So when the essential oil is collected in such small amounts the only way to quantify it would be to have a smaller diameter graduated tube with graduations at every 0.001 point so that even the slightest change could be recorded. This was not possible because if the graduated tube was any smaller in diameter it would cause problems by causing air bubbles which will affect the

experiment and the measurements taken from the graduated tube. The only other way to increase the amount of oil collected would be to increase the amount of leaves used. It was ,however, challenging using 100 g for each experiment. So to collect 500 g of leaves would not be possible unless a field of *Centella asiatica* was available.

The main method which determines the amount of oil collected from an extraction with plant material, using the BP apparatus would probably work with a plant with a high essential oil content such as lavender. Since the amount of oil collected could not be measured, the results from each extraction could not be quantified and therefore qualitative data using total ion current (TIC) of the gas chromatograms was used to compare results.

A few experiments were first preformed to optimise the time of extraction and the amount of *Centella asiatica* leaves to use for each experiment using the BP apparatus. From the experiments (Table 3.4) it was determined that the optimum amount of xylene to use initially was 0.4 ml.

Optimisation of extraction time using BP apparatus (steam distillation)

To determine the optimum extraction time for *Centella Asiatica* leaves, 50 g samples of *Centella Asiatica* leaves were extracted for 35 min, 60 min, 75 min and 90 minutes and the TIC against the time of extraction was plotted. The experiments were performed at a rate of 2/3 ml min^{-1} with 0.4 ml of xylene used initially. (Table for plotting graph - Appendix H) Figure 3.18 shows the TIC versus time of the extraction using the BP apparatus for steam distillation.

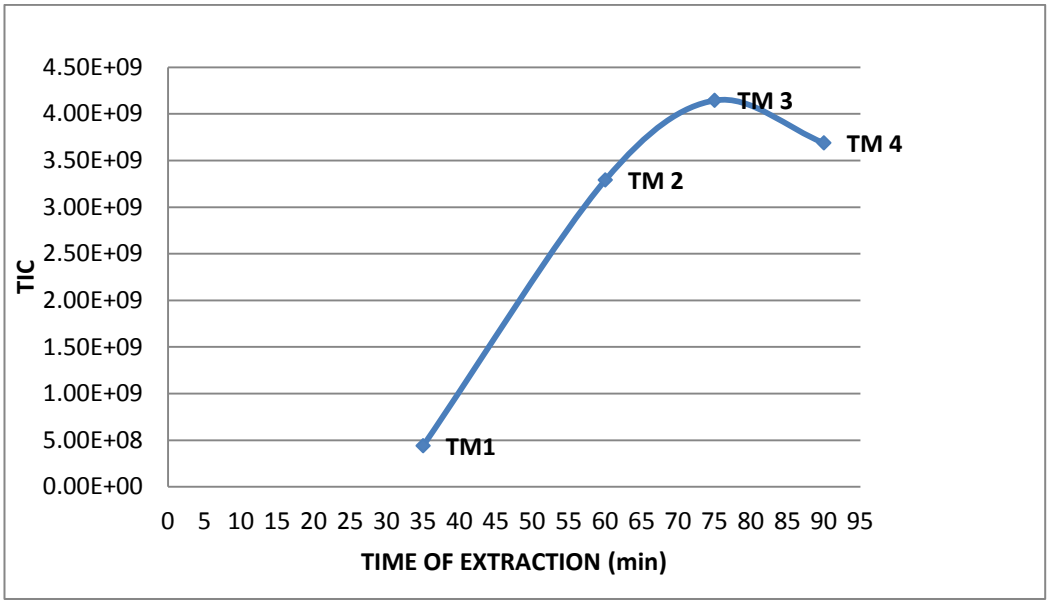


Figure 3.18 Total Ion Concentration vs Time of extraction using the BP apparatus (Steam distillation)

At 35 minutes (TM1), there is the smallest amount of ions present. This is probably because the distillation did not run for long enough for the all of volatile components to collect in the xylene. At 60 minutes (TM2), the amount of ions have increased with increasing extraction time. At 75 minutes (TM3) the amount of ions is at its highest. After 90 minutes (TM4), it can seen that the amount of ions has decreased, this is probably because the lighter volatile components could have been lost the longer the distillation time. The optimised distillation time for *Centella Asiatica* leaves using the BP apparatus was therefore 75 minutes which was used for all the further experiments.

Optimisation of the amount of leaves using BP apparatus (steam distillation)

Experiments for optimisation of the amount of leaves were preformed with 30 g, 50 g and 100 g of leaves at 60 minutes at a rate of 2/3 ml min^{-1} and using 0.4 ml of xylene. (Appendix I -table used for plotting graph)

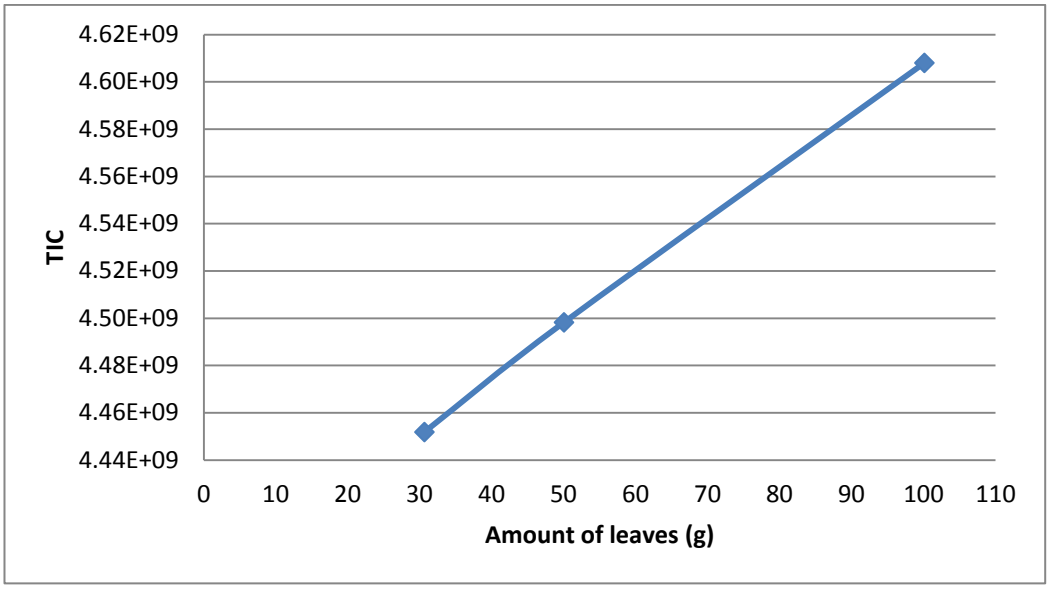


Figure 3.19 Total Ion Concentration vs amount of *Centella Asiatica* leaves used in the BP apparatus (Steam distillation)

From figure 3.19, it is observed that the greater number of leaves, the more essential oil sacs present, the more oil can be collected and therefore the more ions were collected. It can be concluded that using 100 g of leaves is the optimised amount to use for *Centella asiatica* leaf extraction. This amount of leaves was also the most practical as it was the maximum amount which is able to fit into a 1000 ml round bottom flask or false bottom extraction container, and it was also an amount that was the most possible to collect and sustain the plants than using for example 500 g of leaves for each experiment.

Therefore from the initial experiments and the optimisation experiments it was decided that the best parameters to use for the experiments on *Centella asiatica* using the BP apparatus was with 100 g of leaves at a distillation rate of 2/3 ml min^{-1} for 75 minutes using 0.4 ml of xylene initially. It was also necessary to preformed the 30 minute distillation with no plant material as well, for xyelen to reach a constant balance in the apparatus. These optimised conditions were then used to determine if water or steam distillation was more effective for the extraction of the essential oil using the BP apparatus.

3.2.1 Steam distillation - BP Apparatus (Setup 6)

Three steam distillation experiments were performed using the BP apparatus on fresh leaves at a distillation rate of 2/3 ml min^{-1} and 0.4 ml of xylene was used initially. Water distillation was preformed for 30 minutes before leaves were added to false bottom extraction container. The conditions and results are summarised in Table 3.6.

Table 3.6 Steam distillation experiments on fresh leaves using the BP apparatus

Experiment name	Weight of leaves (g)	Average size of leaves (mm)	Distillation time (min)	TIC
SQ1	101.5	56.43	75	6.09×10^9
SQ2	102.86	50.62	75	7.38×10^9
SQ3	101.96	47.76	75	3.51×10^8

The composition of the oil collected in these experiments was then determined. Since a different column for the GC-MS analysis was used with this BP apparatus, the peaks had to be identified in the BP apparatus gas chromatograms and then only be compared to the composition of the oil collected in the initial experiments which were performed using the clevenger apparatus (experiments 1 - 5).

The chromatogram in fig. 3.20 was obtained for steam distillation A on fresh leaves (SQ1).

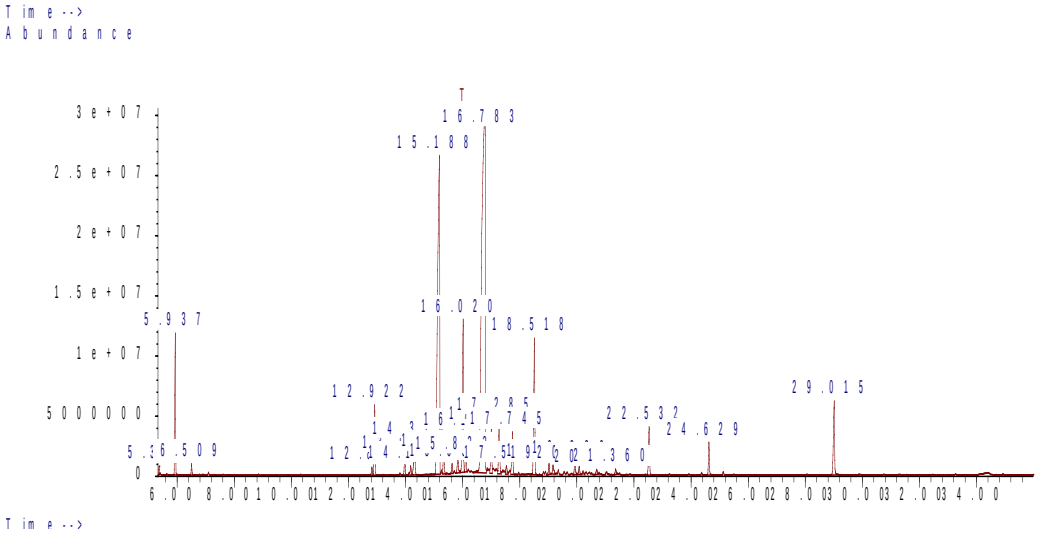


Figure 3.20 Gas chromatogram of experiment SQ1(steam distillation BP) using fresh leaves

Then each prominent peak of the chromatogram was examined and compared to the NIST-MS library and the standards to identify the constituents. The first part of the chromatogram was examined between the retention times of 5.4-8.4 min.

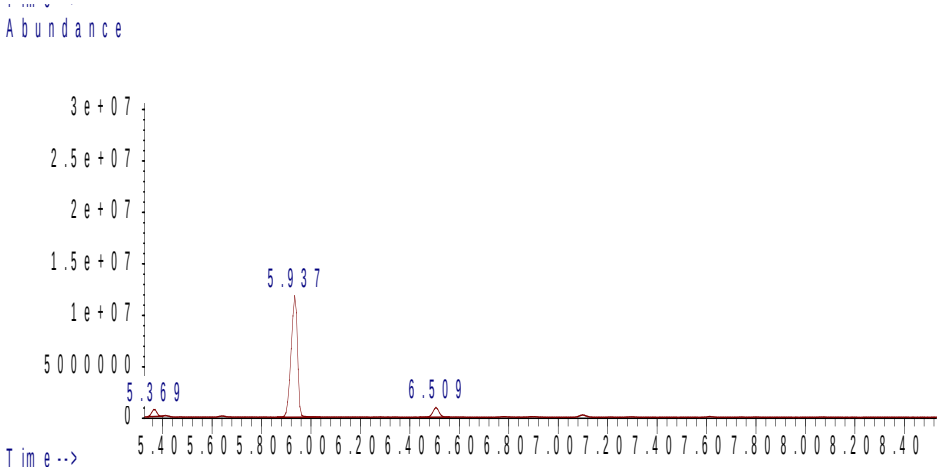


Figure 3.21 Gas chromatogram of experiment SQ1 between the retention times from 5.4 - 8.4 minutes

The peak at 5.937 min had the following mass spectrum (Fig. 3.22)

Figure 3.22 Mass spectrum of peak at 5.937 minutes in gas chromatogram SQ1

This mass spectrum (Fig. 3.22) was then compared to the standards and NIST-MS library and it was found to correlate to γ-terpinene (Fig. 3.23).

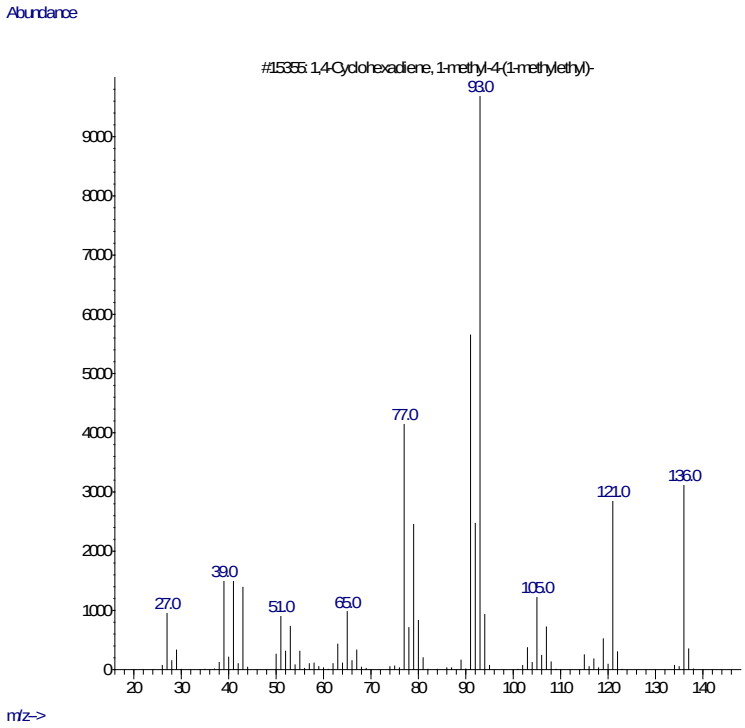


Figure 3.23 Mass spectrum of γ-terpinene

Table 3.7 Chemical composition of the essential oil from *Centella asiatica* identified from the sample of SQ1

Sample name	SQ1	
Constituents [a]	**Retention time** [b]	**% Composition** [c]
α-Terpinene	-	-
β-Cymene	-	-
D-Limonene	5.37	trace
γ-Terpinene	5.94	3.48
Terpinolene	6.51	0.26
Trans-3-Nonen-2-one	-	-
Isoterpinolene	12.83	0.26
δ-Elemene	12.92	2.26
Copaene	13.97	0.64
β-Bourbonene	14.18	0.30
β-Elemene	14.32	1.45
Caryophyllene	15.19	21.83
γ-Elemene	15.34	0.78
Isoledene	15.63	0.32
γ-Cadinene	-	-
α-Cubebene	-	-
B-Gurjunene	-	-
Bicyclo[4.4.0]dec-1-ene, 2-isopropyl-5-methyl-9-methylene-	-	-
Aromadendrene	15.83	0.65
α-Carophyllene	16.02	6.06

***Allo*-Aromadendrene**	16.11	1.24
γ-Muurolene	-	-
Naphthalene, 1,2,4a,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-	-	-
Germacrene D	16.78	41.52
Bicyclogermacrene	17.01	1.61
β-Cuvebene	-	-
α-Elemene	-	-
Isocarophyllene	-	-
Germacrene A	17.29	2.10
δ-Cadinene	17.54	0.35
(+)-4-Carene	17.75	1.64
Germacrene B	18.52	5.33
Caryophyllene oxide	19.03	0.37
1,7,7-Trimethyl-2-vinylbicyclo[2.2.1]hept-2-ene	19.17	0.38
Juniper camphor	19.93	0.51
Spathulenol	20.09	0.32
α-Cadinol	20.70	trace
Viridiflorol	21.36	0.22
β-Ionone epoxide	22.53	1.72
Phytol	24.63	1.12
n-Hexadecanoic acid	-	-
2-Naphthalenamine,N-methyl	29.02	2.92
Total		99.65

[a] Consitutents are listed in order of elution time on the Zebron-ZB 274305 column

[b] Retention times calculated on Zebron-ZB 274305capillary column

[c] Relative area percentage: peak area relative to total peak area percent

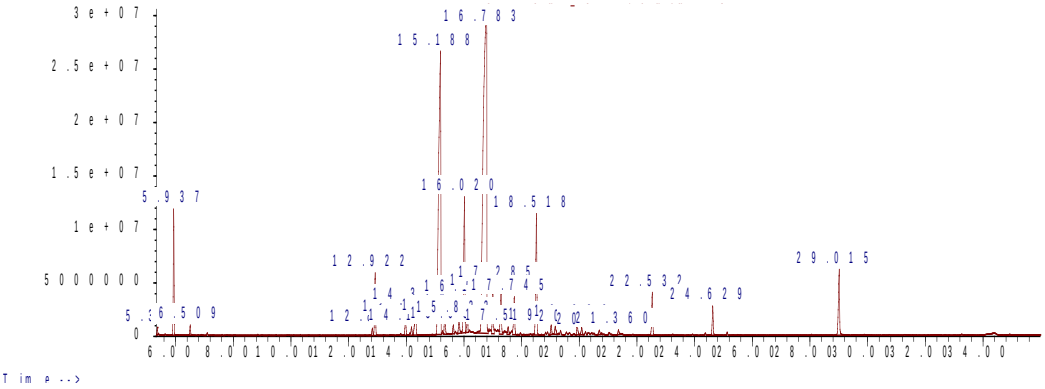
The peak at 5.936 minutes therefore correlates to γ-terpinene.
This process was then continued to identify all the peaks in the chromatogram.(Appendix J) After all the peaks were examined the constituents that were identified in the sample of SQ1 are shown in table 3.7.

The samples for all the extraction experiments were compared to the sample of SQ1 to help identify the constituents in the other samples. The first three steam distillation experiments (SQ1,SQ2 and SQ3) were compared to SQ1 retention times in order to help identify the peaks (Fig. 3.7).

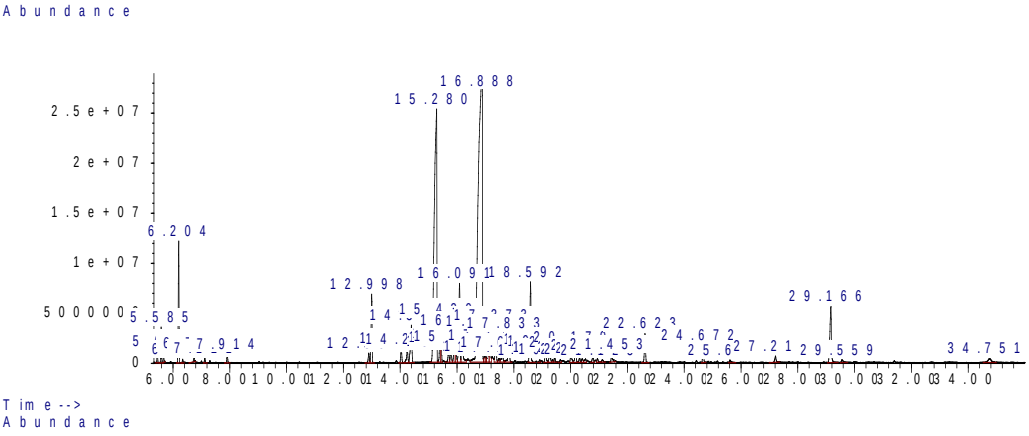
The graphs of the three steam distillation experiments (SQ1, SQ2 and SQ3) are compared in figure 3.24. It can be seen that graph B has the more peaks (i.e. more constituents) present than graph A (SQ1) and graph C (SQ3). Graph C has the least number of peaks. By comparing the total ion current (TIC) of the three experiments (Table 3.6), it can be seen that graph B has 7.38×10^9 ions which is greater than graph A and graph C with 6.09×10^9 ions and 3.51×10^8 ions, respectively. This correlates to the number of peaks seen. This shows that the concentration of ions in the oil collected in experiment SQ2 was higher than the concentration of ions collected in SQ1 and SQ3. Since the other parameters in the experiments were kept the same i.e. same distillation rate and same amount of leaves for the same amount of time, they will not contribute to the variations observed in the results.

Sample SQ3 had the smallest TIC, this is probably because there could have been some xylene and oil left behind in the apparatus as it can easily get adhered to the walls of the apparatus and therefore less oil and less oil constituents were collected. Some oil could have also volatised while the sample was being collected from a beaker. If the size of the leaves used in experiments were compared, the leaves used in experiment SQ3 were slightly smaller compared to SQ1 and SQ2 and hence could have also had a smaller number of oil sacs present resulting in a smaller amount of constituents collected and a smaller TIC was

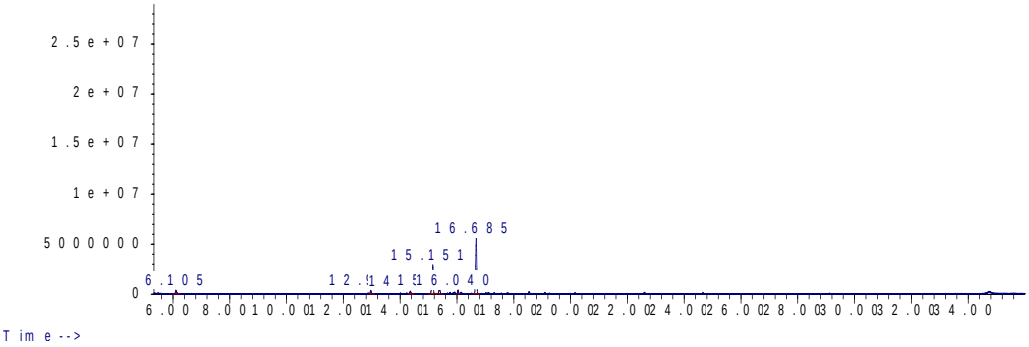
calculated. When the tube BM was rinsed with toluene and acetone before the sample was collected, some of the rinsing reagents could have



Graph A Gas chromatogram of sample SQ1



Graph B Gas chromatogram of sample SQ2



Graph C Gas chromatogram of sample SQ3

Figure 3.24 Comparison of the gas chromatograms of the Centella oil samples collected from steam distillation experiments SQ1, SQ2 and SQ3

Table 3.8 Comparison of the different constituents present in the samples obtained from steam distillation experiments SQ1,SQ2 and SQ3 ([**bold**] - constituent present in significant value , [blue] - predominant constituent

Sample name	SQ1		SQ2		SQ3		Averag
Constituents [a]	**Retention time** [b]	**% Composition** [c]	**Retention time** [b]	**% Composition** [c]	**Retention time** [b]	**% Composition** [c]	**% Composition**
α-Terpinene	-	-	5.45	0.28	-	-	0.09
β-Cymene	-	-	5.59	0.76	5.48	0.31	0.36
Limonene	5.37	trace	5.67	0.25	-	-	0.08
γ-Terpinene	5.94	3.48	6.20	3.10	6.10	2.98	3.19
Terpinolene	6.51	0.26	6.74	0.24	-	-	0.17
Trans-3-Nonen-2-one	-	-	7.92	0.24	-	-	0.08
Isoterpinolene	12.83	0.26	12.90	0.37	12.88	0.41	0.35
δ-Elemene	12.92	2.26	13.00	2.36	12.97	2.49	2.37
Copaene	13.97	0.64	14.04	0.54	14.02	0.42	0.53
β-Bourbonene	14.18	0.30	14.25	0.56	14.23	0.38	0.41
β-Elemene	14.32	1.45	14.39	1.72	14.37	1.98	1.71
Caryophyllene	15.19	21.83	15.28	21.05	15.15	23.54	22.14
γ-Elemene	15.34	0.78	15.41	1.50	-	-	0.76
Isoledene	15.63	0.32	-	-	-	-	0.11

γ-Cadinene	-	-	15.44	1.06	15.39	4.02	1.69
α-Cubebene	-	-	15.71	0.46	15.68	0.34	0.27
B-Gurjunene	-	-	15.79	0.58	15.75	0.91	0.50
Bicyclo[4.4.0]dec-1-ene, 2-isopropyl-5-methyl-	-	-	15.91	0.82	15.87	0.55	0.46
Aromadendrene	15.83	0.65	15.96	0.62	15.92	1.12	0.80
α-Carophyllene	16.02	6.06	16.09	3.21	16.04	2.62	3.96
***Allo*-Aromadendrene**	16.11	1.24	16.19	1.48	16.15	1.33	1.35
γ-Muurolene	-	-	16.39	0.26	-	-	0.09
Naphthalene, 1,2,4a,5,6,8a-hexahydro-4,7-	-	-	-	-	16.51	0.38	0.13
Germacrene D	16.78	41.52	16.89	38.24	16.69	42.00	40.59
Bicyclogermacrene	17.01	1.61	17.09	1.16	17.02	0.63	1.13
β-Cuvebene	-	-	17.17	0.69	17.11	0.98	0.56
α-Elemene	-	-	17.24	0.33	-	-	0.11
Isocarophyllene	-	-	17.37	1.38	17.31	0.68	0.69
Germacrene A	17.29	2.10	-	-	-	-	0.70
δ-Cadinene	17.54	0.35	17.60	0.54	17.56	0.36	0.42
(+)-4-Carene	17.75	1.64	17.83	1.24	17.78	0.66	1.18
Germacrene B	18.52	5.33	18.59	3.11	18.54	1.64	3.36
Caryophyllene oxide	19.03	0.37	19.12	0.55	19.10	1.02	0.65
1,7,7-Trimethyl-2-vinylbicyclo[2.2.1]hept-2-ene	19.17	0.38	19.26	0.49	19.25	0.36	0.41

Juniper camphor	19.93	0.51	20.02	0.46	20.00	0.29	0.42
Spathulenol	20.09	0.32	20.18	0.63	20.16	0.66	0.53
α-Cadinol	20.70	trace	20.79	0.31	-	-	0.10
Viridiflorol	21.36	0.22	21.46	0.41	-	-	0.21
β-Ionone epoxide	22.53	1.72	22.63	1.08	22.61	1.06	1.29
Phytol	24.63	1.12	24.67	0.61	24.67	0.64	0.79
n-Hexadecanoic acid	-	-	27.22	0.36	-	-	0.12
2-Naphthalenamine,N-methyl	29.02	2.92	29.17	2.65	29.11	0.24	1.94
Squalene	-	-	34.75	0.44	34.75	5.01	1.82
Total		99.65		96.14		100	98.60

[c]onstituents are listed in order of elution time on the Zebron-ZB 274305 column

[b] Retention times calculated on Zebron-ZB 274305capillary column

[c] Relative area percentage: peak area relative to total peak area percent

Table 3.9 Summary and comparison of the major constituents present in the samples obtained from the steam distillation experiments and a comparison to a similar study conducted in South Africa

Steam distillation experiments									
	No. identified	SQ1 (%)	No. identified	SQ2 (%)	No. identified	SQ3 (%)	No. identified	South African study* (%)	Average steam exp. %
Monterpenoid hydrocarbons	5	5.65	7	6.24	4	4.36	11	20.20	5.42
Oxygenated monoterpenoids	0	0.00	1	0.24	0	0.00	9	5.46	0.08
Sesquiterpenoid hydrocarbons	15	86.44	21	81.67	19	86.37	14	68.80	84.83
Oxygenated sesquiterpenoids	5	1.42	5	2.36	3	1.96	5	3.90	1.91
Sulfidesesquiterpenoid	0	0.00	0	0.00	0	0.00	1	0.76	0.00
Beta-Ionone epoxide	1	1.72	1	1.08	1	1.06	0	0.00	1.29
Phytol	1	1.12	1	0.61	1	0.64	0	0.00	0.79
2-Naphthalenamine,N-methyl	1	2.92	1	2.65	1	0.24	0	0.00	1.94
Squalene	0	0.00	1	0.44	1	5.01	0	0.00	1.82
Other	1	0.38	2	0.85	1	0.36	0	0	0.53
Total	28	99.65	38	96.14	30	100.00	40	99.12	98.60

* South African study data from Oyedeji (2004)

also collected in the sample vial with the oil and xylene and hence decreased the concentration of oil in the sample, which would cause the TIC of the sample to decrease. Even the smallest extra drop of xylene collected can cause a significant change to the concentration of the oil collected because of such minute amounts of the oil that are collected and hence such minute amounts of oil constituents collected.

The TIC of SQ1 (6.09×10^9 ions) to SQ2 (7.38×10^9 ions) were compared and it can be seen that less ions and were collected in experiment SQ1 than SQ2. Approximately the same amount of leaves were used for each experiment. The difference between the two TIC values can possibly be because of the varied amount of oil sacs in the leaves used between the experiments. The size of the oil sacs may vary from leaf to leaf because of environmental factors that affect the plant growth. For example if some leaves were grown in the sun and some in the shade, the ones in the sun will thrive more and have more oil sacs than the ones in the shade. (Devkota et al, 2013) Some oil sacs of the plant could also have been damaged before harvesting (e.g. mechanical damage by insects or weather such as hail). There could also been some oil lost on the walls of the apparatus and in the tube used to collect the oil which would cause a smaller constituents of oil to be collected and hence a lower TIC.

From Table 3.9 the constituents that make up the greatest composition of the oil from *Centella Asiatica* leaves in the steam distillation experiments were sesquiterpenoid hydrocarbons (81.67 - 86.44%) among which germacrene D at 38.24%, 41.52% and 42% and carophyllene was 21.05%, 21.83%, 23.54% in SQ2, SQ1 and SQ3 respectively. Monoterpenoid hydrocarbons (4.36 - 6.24%) were also identified in the essential oil, such as γ- terpenine (2.98-3.48%) as well as oxygenated sesquiterpenoids (1.42 - 2.36%) such as carophyllene oxide (0.37-1.02%). β-Ionone epoxide (1.06 - 1.72%), phytol (0.61 - 1.12%), 2-naphthalenamine, n-methyl (0.24 - 2.92%) and squalene (0.00 - 5.01%) were also identified.

The possible reason the greatest amount of monoterpenoid hydrocarbons and oxygenated sesquiterpenoids were observed in SQ2 is because it had the highest total ion concentration, hence it has a higher concentration of oil and hence a greater amount of each constituents present. The constituents which were already present in small amounts were therefore detected as well. Whereas in SQ1 and SQ3 much smaller amounts of monoterpenoid hydrocarbons and oxygenated sesquiterpenoids were present (because the samples were less concentrated), some of the constituents either were not detected when analysed or only detected in such small amounts. Sesquiterpenoid hydrocarbons were however present in the greatest amount in SQ1 (86.44%) ,then SQ3 (86.37%) and then SQ2 (81.67%) (Table 3.9). So SQ2 with the highest TIC has the lowest amount of sesquiterpenoid hydrocarbons, a possible explanation for this might be the higher the TIC i.e. more ions are present for analysis and therefore a lot of constituents present in smaller quantities were identified as well as the ones present in significant quantities, which all contribute to the total composition. Whereas in SQ1 and SQ3 where they have a smaller TIC, and hence a lower ion concentration, only the constituents that were present in significant amounts were detected when analysed and the ones in smaller concentrations were either present in too small quantities to analyse or were not even detected. SQ1 has a slightly higher amount of sesquiterpenoid hydrocarbons possibly due to the greater amount of leaves used for extraction and consequently more of the sesquiterpenoid hydrocarbons could have been available for extraction compared to sample SQ3.

From Table 3.8, the constituents that were identified in each experiment (SQ1, SQ2 ,SQ3) were compared. Forty two constituents were identified from the three different oil samples, accounting from 96.14% to 100% of the oil composition. Primarily the predominant constituents will be used for comparisons between the experiments. In all the steam distillation experiments (SQ1, SQ2, SQ3) the following constituents were the predominant ones: γ- terpenine, δ-elemene, carophyllene, γ-cadinene, α-carophyllene, germacrene D, geramacrene A, germacrene B, 2-naphthalenamine,n-methyl and squalene. (In blue in Table 3.8)

When the amounts of γ-terpenine, α-carophyllene, germacrene B and 2-Naphthalenamine,n-methyl were compared from the steam distillation experiments, the greatest amounts were detected in sample SQ1 (3.48%, 6.06%, 5.33% , 2.92%) followed by sample SQ2 (3.10%, 3.2%, 3.11%, 2.65%) and sample SQ3 (2.98%, 2.62%, 42%, 1.64%, 0.24%). The difference in sample SQ3 can be explained because of the much lower TIC, then in sample SQ1 or sample SQ2. Therefore the concentration of oil and hence the concentration of each constituent in the oil could be much less in sample SQ3 than sample SQ1 and SQ2. SQ1 had a smaller TIC than SQ2 but it has a greater amount of γ-terpenine, α-carophyllene, germacrene B and 2-naphthalenamine,n-methyl. One of the reasons could be that the slightly larger leaves were used for experiment SQ1 (average of 56.43 mm) than SQ2 (average of 50.62 mm). The larger the leaf, the more time it has had to mature and the greater volatile content it will have compared to smaller leaves and therefore a greater chance of having more of the predominant constituent present than in SQ2. Another reason could be that SQ1 was grown in an area that has more light than SQ2. Variations in light conditions can affect the composition of essential oils collected. α-Carophyllene is produced in higher amounts in *Centella asiatica* leaves when grown in an area with greater amounts of sunlight. (Devkota et al, 2013)

When the percentage composition of the predominant constituent of 2-naphthalamine, n-methyl were compared between the experiments, it can be seen that it is present in samples of SQ1, SQ2 and SQ3. However, in SQ1 and SQ2 it is present at 2.92% and 2.65% and in SQ3 at 0.24%. Hence it is a predominant constituent of sample SQ1 and SQ2 and not of sample SQ3. The reason for this could be that the TIC of SQ3 (3.51×10^8 ions) is much lower than SQ2 (7.38×10^9 ions). Therefore the concentration of oil and hence the concentration of each constituent in the oil is much less than in sample SQ3. Since the amount of oil collected is so small in all the experiments, if a drop more of xylene was collected in the sample vial, the concentration of the oil could also decrease by a significant amount. Slightly smaller leaves were also used in experiment SQ3 and therefore

less volatile content was available which could have affected the amount of 2-naphthalamine,n-methyl present.

The predominant constituent of germacrene A is compared in each experiment and it can be seen that it is a predominant constituent of sample SQ1 but not of samples SQ2 or SQ3. The reason for this can be that essential oils can also degrade, especially if the form of free energy such as light or heat is present which can cause the position of double bonds to change, open chains can close to form rings and the nature of the functional groups can change. These processes can all form different molecules that were extracted during the distillation process.

When the percentage composition of δ-elemene, γ-cadinene, and squalene were compared in each of the steam distillation experiments, it was noted that the greatest amounts were present in samples SQ3 (2.49% ,4.02%, 5.01%) followed by SQ2 (2.36%,1.06%,0.44%) and then SQ1 (2.26%, 0%, 0%). SQ3 has the lowest TIC but it has nearly all the predominant constituents present. The reason for this could be that the leaves harvested were from a much more favourable area than the other leaves. When the amount of γ-cadinene and squalene is compared in the samples, it is notable that γ-cadinene is a predominant constituent of SQ3 and SQ2 but not of SQ1 and that squalene is a predominant constituent of SQ3 only and not SQ1 and SQ2.

When the constituents of carophyllene and germacrene D were compared, it can be seen that they were present in the greatest amount in sample SQ3 (23.54%, 42.00%) followed by sample SQ1 (21.83%, 41.52%) and then sample SQ2 (21.05%, 38.24%). SQ3 leaves could have been harvested from a more favourable area than the other leaves. The difference between SQ1 and SQ2 can be explained by the size of the leaves used, SQ1 had a slightly larger leaf used in the experiments and therefore had more volatile content that SQ2 and a greater chance of having a larger amount of the predominant constituents.

The other significant peaks present were β-elemene, aromadendrene and carophyllene oxide which were present in the greatest amount in SQ3. γ-elemene, a*llo*-aromadendrene and isocarophyllene were present in the greatest amount in SQ2. Bicylcogermacrene, +(-)-4-Carene, β-ionone epoxide and phytol were present in the greatest amount in SQ1.

The reasons given for the difference in composition in the experiments above were all possibilities however there are a large number of other factors that could affect the ratios of essential oil molecules in the samples. Environmental factors play a huge role in the growth of the plant. If the plant is grown in a location where it gets enough sunshine, enough water, with good soil quality then the plant will undergo photosynthesis more and grow better and produce more oil than a plant which was grown in the shade, with little water and polluted soil. The composition can also be affected by the time and day the harvesting was done. The leaves for the experiments were not harvested all at the same time and therefore this could cause variations in the composition as well. If some leaves were harvested on a cloudy day and the other batch on a sunny day, the amount of certain volatile constituents could differ because of the amount of light the plant was getting in order to grow and produce more oil. If some leaves were harvested in a week with a lot of rain and the others in a week where there was little rain, the amount of each constituents will vary as well. *Centella Asiatica* prefers a damper environment, hence in a more rainy week, it will thrive better and a difference in oil composition would be seen. The location of the harvested *Centella Asiatica* leaves also can affect the composition. Even though the *Centella* plants were planted in the same location, because of the amount of leaves needed, some plants were planted closer to the shade of a tree than other plants. The ones under the tree would get less light and have a slower photosynthesis then the plants in the sunshine. Hence a difference in oil composition could also be seen. Another reason could be that some compounds could have vaporised during the extraction and therefore not collected.

3.2.2 Water distillation using the BP apparatus

Three water distillation experiments were performed using setup 6B on fresh leaves at a distillation rate of 2/3 ml min^{-1} and an initial volume of xylene of 0.4 ml. Water distillation was preformed for 30 minutes before leaves were added to the round bottom extraction container. Some initial experiments were preformed to get familiar with the apparatus. The conditions and results were summarised in table 3.10.

Table 3.10 Initial water distillation experiments on fresh leaves using setup 6B above and a distillation rate of 2 /3 ml min^{-1}. Water distillation extraction for 30 minutes before leaves were added to round bottom flask.

Experiment name	Weight of leaves (g)	Average size of leaves (mm)	Distillation time (min)	TIC
WD1A	45.59	60.71	60	6.26×10^9
WD2A	84.67	55.29	60	4.57×10^9

From the two initial water distillation experiments, it can be seen that WD1A has a greater TIC than WD2A. The experiments were performed for the same time at the same distillation rate. Therefore the reason WD1A has a higher TIC could be that more xylene vaporised during the experiment, consequently a higher concentration of ions was collected in the oil. The amount of leaves used in WD2A (84.67 g) compared to WD1A (45.59 g) was greater, so it would be expected that the amount of oil and hence the TIC in WD2A to be greater than WD1A. However this was not the case, the reason for this could be that WD1A has larger leaves, hence more oil sacs present on a larger surface area and more oil constituents can be collected, whereas WD2A has a smaller leaf size average and less oil sacs and less constituents were collected. Also sample WD2A could have lost some oil in the apparatus or in the beaker when collected.

After the initial experiments, three water distillations experiments were performed using setup 6B on fresh leaves at a distillation rate of 2/3 ml min^{-1} and 0.4 ml of

xylene was used initially. Water distillation was performed for 30 minutes before leaves were added to the round bottom extraction container.

Table 3.11 Water distillation experiments on fresh leaves using setup 6B above and a distillation rate of 2/3 ml min^{-1}. Water distillation performed for 30 minutes before leaves were added to round bottom flask.

Experiment name	Weight of leaves (g)	Average size of leaves (mm)	Distillation time (min)	TIC
WDQ1	101.11	63.08	75	2.02×10^8
WDQ2	100.06	50.93	75	6.60×10^9
WDQ3	100.91	49.60	75	5.76×10^9

The experiments shown in Table 3.11 were all performed with around 100 g of leaves, for 75 minutes using 0.4 ml of xylene to collected the oil.

When the experiments in table 3.11 were compared, it can be seen that WDQ1 had the smallest TIC (2.02×10^8 ions) whereas WDQ3 had more ions (TIC of 5.76×10^9 ions), and WDQ2 had the most ions with a TIC of 6.60×10^9 ions. Oil samples WDQ2 and WDQ3 have a similar average size of the leaves used and approximately the same amount of leaves were used, yet there is a difference in the TIC. The reason that WDQ3 has a lower TIC than WDQ2 is probably due to errors during the experiment, some oil staying in the apparatus when collected, or some molecules vaporised during extraction. Also any extra xylene could have decreased the amount of ions collected. The *Centella asiatica* leaves were also harvested at different times and the amount of oil and composition present in each leaf differs, so the even though a similar mass of leaves were used, many other factors can affect the oil composition which can contribute to the leaves having a smaller ion concentration.

The reason for sample WDQ1 contained the lowest amount of ions compared to WDQ2 and WDQ3 could be due to experimental errors. The leaves used in experiment WDQ1 were larger on average, it would be then expected for the amount of ions in the oil collected to have been larger than WDQ2 or WDQ3,

which was not the case. Some oil could have been lost when the water in the experimental tube was drained. Also if extra xylene was collected in sample vial, the concentration of the oil collected also decreases hence less ions were detected. The amount of oil collected is minute, so even a single drop extra of xylene or rinsing solution (toluene and acetone) could have decreased the concentration of ions collected. This coincides with the graphs (Fig. 3.25) where sample WDQ2 and sample WDQ3 (graph D and E respectively) have a similar number of peaks and WDQ1 has the least number of peaks.

The peaks in the water distillation experiments (WDQ1,WDQ2 and WDQ3) were then identified using the same method as explained in the steam distillation experiments (Table 3.12).

From Table 3.13, the constituents that make up the greatest composition of the oil from *Centella Asiatica* leaves in the water distillation experiments were sesquiterpenoid hydrocarbons (77.52 - 87.28%) amongst which germacrene D ranging from 29.27% (WDQ1), 35.38% (WDQ2), 39.81% (WDQ3) and carophyllene 20.46%, 20.88%, 32.24% in WDQ2, WDQ3 and WDQ1 respectively. Monoterpenoid hydrocarbons (8.04 - 9.00%) were also identified in the essential oil, such as γ- terpenine (3.36 - 5.64%) as well as oxygenated sesquiterpenoids (0.68 - 4.48%) such as carophyllene oxide (0.51 - 1.04%).β-ionone epoxide (0.81 - 1.47%), 2-naphthalenamine, n-methyl (4.05 - 4.24%) and squalene (0.11 - 0.94 %) were also identified.

The reason the greatest amount of monoterpenoid hydrocarbons and oxygenated sesquiterpenoids were observed in WDQ2 is possibly because it had the highest total ion concentration and thus it has a higher concentration of oil and a greater amount of each constituents is present, especially the constituents which were already present in small amounts can be detected. WDQ1 and WDQ3 had much smaller amounts of monoterpenoid hydrocarbons and oxygenated sesquiterpenoids and because the samples were less concentrated, some of the constituents were not detected when analysed or only detected in such small

amounts. However, the sesquiterpenoid hydrocarbons were present in the order : WDQ1 (87.28%) > WDQ3 (80.08%) > WDQ2 (77.52%) (Table 3.13). A possible reason could be again due to the TIC of each sample, since WDQ1 had the lowest TIC yet the highest sesquiterpenoid hydrocarbons. The smaller the TIC, the less ions there are to analyse and only the most significant constituents will be detected.

From Table 3.12 the constituents that were identified in each experiment (WDQ1, WDQ2 ,WDQ3) were compared. Fifty four constituents were identified from the three different oil samples, accounting from 97.18% to 99.80% of the oil contents. The predominant constituents were used mainly for comparisons between experiments. In all the water distillation experiments (WDQ1, WDQ2, WDQ3) the following constituents were the predominant ones: γ- terpenine, δ-elemene, β-elemene, carophyllene, γ-elemene, α-carophyllene, germacrene D, δ-cadinene, germacrene B and 2-naphthalenamine,n-methyl. (In blue in Table 3.12)

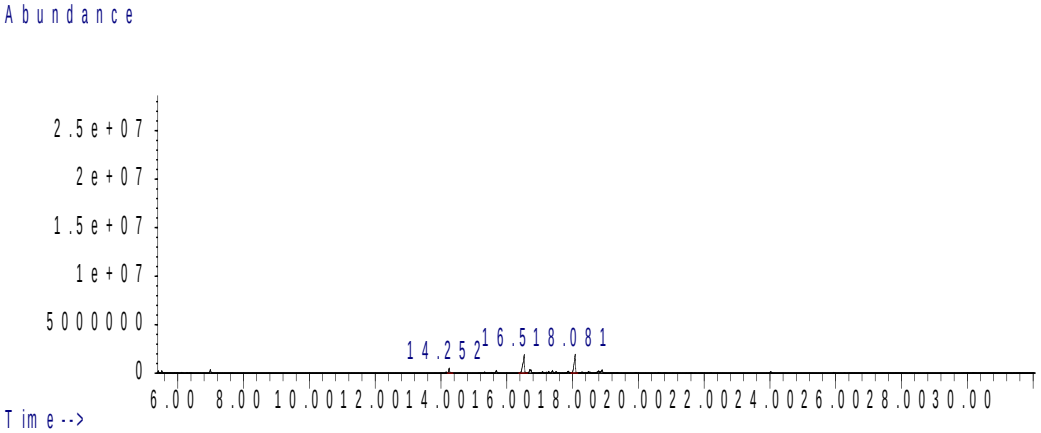
The gas chromatograms that were obtained for the water distillation experiments are shown in Fig. 3.25. Then peaks in the water distillation experiments (WDQ1,WDQ2 and WDQ3) were then identified using the method as before. (Table 3.12)

In the water distillation experiments the major constituent is seen to be germacrene D in WDQ2 (35.38%) and WDQ3 (39.80%) and then carophyllene in WDQ2 and WDQ3 at 20.46% and 20.88%, respectively. However in WDQ1 it was observed that carophyllene is the major constituent at 32.24% and then germacrene D at 29.27%. The reason for the difference could be that WDQ1 had a lower TIC, so not that many compounds of germacrene D were collected. It could be possible that the smaller leaf size used in experiment WDQ1 (49.60 mm) contained a greater amount of carophyllene than germacrene D. Also the life stage of plant and environmental factors could have played a role in the difference in constituents percentages present in each of the water distillation experiments .

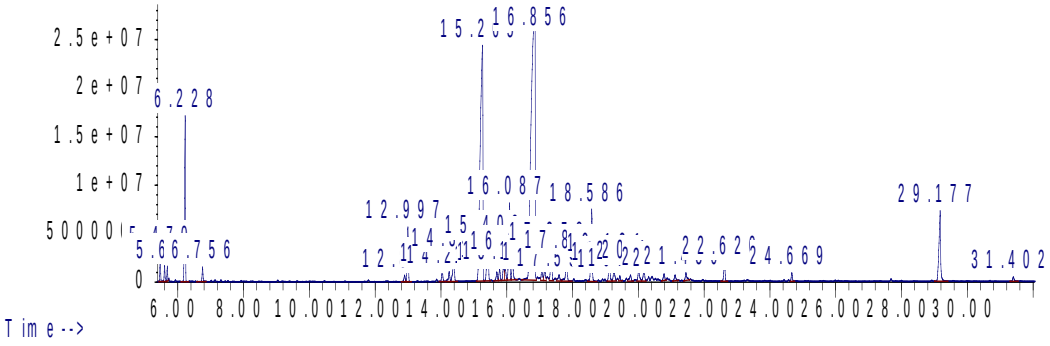
When the amount of γ- terpenine, gamma elemene, α-carophyllene, were compared in the water distillation experiments, the greatest amounts were detected in sample WDQ2 (5.64%, 2.47%, 3.52%) followed by sample WDQ3 (5.09%, 1.17%, 3.15%) and WDQ1 (3.36%, trace, 2.60%). The difference in the constituent percentages can be explained in terms of TIC of each sample. WDQ1 has the lowest TIC, followed by WDQ3 and the greatest TIC was detected in sample WDQ2. The concentration of oil and hence the concentration of each constituent in the oil would therefore be less in sample WDQ1 than sample WDQ3, with the highest concentration in sample WDQ2. It can also be noted that γ-elemene was a predominant constituent of WDQ2, a significant constituent in WDQ3 and it was only present in trace amounts in WDQ1.

When the percentage composition of δ-elemene, β-elemene were compared in each of the water distillation experiments, it can be seen that the greatest amounts were seen in samples WDQ1 (5.15% ,2.79%) followed by WDQ2 (2.09%,1.39%) and WDQ3 (1.70%, 1.12%). WDQ1 has the lowest TIC but it has nearly all the predominant constituents present. The reason for this could be that the leaves used for the WDQ1 experiment were harvested at a more favourable time, or because of different environmental conditions the leaves contained more of those constituents than the other experiments. Another reason could be that the slightly larger leaves were used for experiment WDQ1 (average of 63.08 mm) than WDQ2 (average of 50.93 mm) than WDQ3 (average of 49.60 mm). The larger the leaf the more time it has had to mature and the greater volatile content it will have compared to smaller leaves and therefore a greater chance of having more of the predominant constituent present than in WDQ2 and WDQ3.

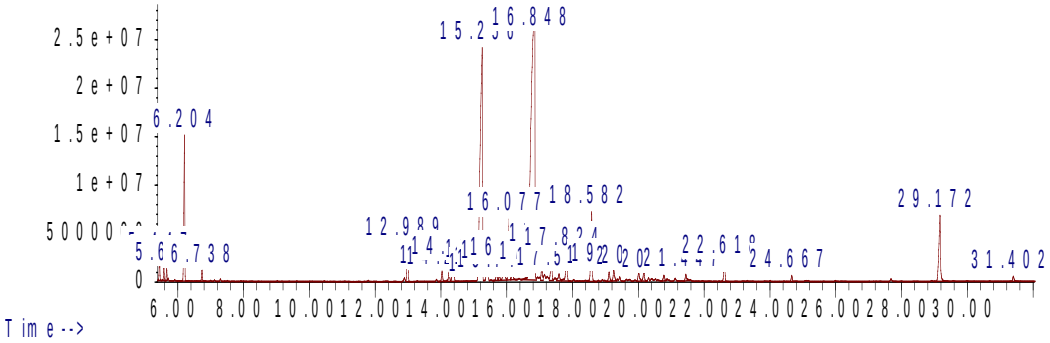
When the percentage composition of germacrene D, germacrene B and -naphthalenamine,n-methyl were compared in each of the water distillation experiments, it can be seen that the constituents were present in the greatest percentage in sample WDQ3 (39.81%, 3.50% , 4.24%) followed by WDQ2 (35.38%, 3.26 %, 4.05%) and then by WDQ1 (29.27%, 0%, 0%).



Graph D: Gas chromatogram of sample WDQ1



Graph E: Gas chromatogram of sample WDQ2



Graph F: Gas chromatogram of sample WDQ3

Figure 3.25 Comparison of the gas chromatograms of the Centella oil samples collected from water distillation experiments WDQ1, WDQ2 and WDQ3

Table 3.12 Comparison of the different constituents present in the samples obtained from water distillation experiments WDQ1,WDQ2 and WDQ3 ([**bold**] - constituent present in significant value , [blue] - predominant constituent

	WDQ1		WDQ2		WDQ3		Averag
Constituents [a]	**Retention time**[b]	**% Composition**[c]	**Retentio n time**[b]	**% Composition**[c]	**Retenti on time**[b]	**% Composition** [c]	**% Compo sition**[c]
β-Pinene	5.41	1.62	-	-	-	-	0.54
β-Myrcene	5.52	1.76	-	-	-	-	0.59
α-Phellandrene	5.91	trace	-	-	-	-	trace
α-Terpinene	6.12	0.35	5.47	0.69	5.45	0.62	0.55
β-Cymene	6.29	0.25	5.60	0.40	5.58	0.36	0.34
Limonene	6.38	-	5.69	0.38	5.67	0.34	0.24
γ-Terpinene	6.99	3.36	6.23	5.64	6.20	5.09	4.70
Terpinolene	7.66	0.25	6.75	0.42	6.74	0.36	0.34
Isoterpinolene	14.16	0.46	12.90	0.27	12.89	0.00	0.24
δ-Elemene	14.25	5.15	13.00	2.09	12.99	1.71	2.98
Copaene	14.56	0.27	14.04	0.48	14.04	0.46	0.40
α-Cubebene	15.33	0.82	-	-	-	-	0.27
β-Bourbonene	15.55	0.38	14.25	0.48	14.25	0.47	0.45
β-Elemene	15.68	2.79	14.39	1.39	14.39	1.12	1.77

Caryophyllene	16.53	32.24	15.27	20.46	15.25	20.88	24.53
γ-Elemene	16.71	-	15.41	2.47	15.42	1.17	1.21
Isoledene	17.00	0.38	15.70	0.47	15.70	0.49	0.45
γ-cadinene	17.10	1.29	15.78	0.54	15.78	0.29	0.71
Bicyclo[4.4.0]dec-1-ene, 2-isopropyl-5-methyl-9-	17.21	0.48	15.90	0.72	-	-	0.40
Aromadendrene	17.28	1.38	15.95	0.62	15.90	1.10	1.03
α-Caryophyllene	17.39	2.60	16.09	3.52	16.08	3.15	3.09
***Allo*-Aromadendrene**	17.50	1.61	16.18	1.18	16.17	1.04	1.28
Naphthalene, 1,2,4a,5,6,8a-hexahydro-4,7-	17.87	2.49	-	-	-	-	0.83
Ocimene	-	-	-	-	16.38	0.31	0.10
Germacrene D	18.08	29.27	16.86	35.38	16.85	39.81	34.82
Bicyclogermacrene	-	-	16.94	0.20	16.94	0.22	0.14
β-Gurjunene	18.29	0.66	-	0.00	16.99	0.21	0.29
α-Elemene	-	-	17.07	0.61	17.07	0.73	0.45
β-Cuvebene	18.43	0.30	17.16	0.62	17.15	0.46	0.46
Isocarophyllene	-	-	17.24	0.27	17.24	0.39	0.22
Germacrene A	-	-	17.36	1.56	17.36	1.62	1.06
γ-Muurolene	18.49	0.68	17.48	0.17	17.47	0.24	0.36
β-cadinene	18.80	0.85	-	-	-	-	0.28

δ-cadinene	18.90	3.28	17.60	0.44	17.60	0.58	1.44
(+)-4-Carene	-	-	17.82	1.20	17.82	1.73	0.98
Germacrene B	-	-	18.58	3.26	18.58	3.50	2.25
α-Muurolene	19.35	0.35	-	-	-	-	0.12
Nerolidal	19.94	0.53	-	-	-	-	0.18
Carophyllene oxide	-	-	19.12	1.04	19.11	0.52	0.52
1,7,7-Trimethyl-2-vinylbicyclo[2.2.1]hept-2-ene	20.61	0.23	19.26	0.67	19.25	0.65	0.52
y-Gurjunene	-	-	19.44	0.25	19.44	0.23	0.16
Humulene epoxide	-	-	19.76	0.41	-	-	0.14
Juniper camphor	-	-	20.02	0.72	20.01	0.56	0.43
Spathulenol	-	-	20.17	0.69	20.17	0.55	0.41
Allo-Aromadendrene oxide	-	-	20.32	0.25	-	-	0.08
Seychellene	-	-	20.42	0.33	20.42	0.20	0.18
α-Cadinol	22.21	0.15	20.79	0.41	20.78	0.36	0.31
Isoaromadendrene epoxide	-	-	21.12	0.37	-	-	0.12
Viridiflorol	-	-	21.45	0.58	21.45	0.32	0.30
3-Buten-2-one, 4-(2,2,6-trimethyl-7-	24.03	1.47	22.62	0.81	22.62	0.97	1.08
Bicyclo[3.1.1]heptane, 2,6,6-trimethyl-	25.94	0.52	24.67	0.33	24.67	0.26	0.37
2 -Naphthalenamine,N-methyl	-	-	29.17	4.05	29.17	4.24	2.76

Ethanone,1 -(2,5-dimethyl-4-phenylfuran-3-yl)-	32.86	0.64	31.40	0.22	31.40	0.28	0.38
Squalene	35.24	0.94	34.78	0.11	34.76	0.31	0.45
Total		99.80		97.18		97.90	98.29

[a] Constituents are listed in order of elution time on the Zebron-ZB 274305 column

[b] Retention times calculated on Zebron-ZB 274305capillary column

[c] Relative area percentage: peak area relative to total peak area percent

Table 3.13 Summary and comparison of the major constituents present in the samples obtained from the water distillation experiments and a comparison to a similar study conducted in South Africa

Water distillation experiments								
	No. identified	WDQ1 (%)	No. identified	WDQ2 (%)	No. identified	WDQ3 (%)	No. identified	South African study % [7]	Average water exp. % composition
Monterpenoid hydrocarbons	9	8.04	7	9.00	8	8.81	11	20.20	8.62
Oxygenated monoterpenoids	0	0.00	0	0.00	0	0.00	9	5.46	0.00
Sesquiterpenoid hydrocarbons	21	87.28	23	77.52	24	80.08	14	68.80	81.63
Oxygenated sesquiterpenoids	2	0.68	8	4.48	5	2.31	5	3.90	2.49
Sulfidesesquiterpenoid	0	0.00	0	0.00	0	0.00	1	0.76	0.00
β-Ionone epoxide	1	1.47	1	0.81	1	0.97	0	0.00	1.08
2-Naphthalenamine,N-methyl	0	0.00	1	4.05	1	4.24	0	0.00	2.76
Squalene	1	0.94	1	0.11	1	0.30	0	0.00	0.45
Other	3	1.39	3	1.21	3	1.19	3	0.00	1.26
Total	37	99.8	44	97.18	43	97.90	43	99.12	98.29

The reason the lowest amount is seen in WDQ1 could be because of the small TIC and therefore the constituents were not present in the sample or they were diluted too much when collected because they were present in minute amounts except in the case of germacrene D. Germacrene D is present in such high amounts in the oils collected from *Centella Asiatica* leaves that even if the TIC was lower, it is a major constituent and even though it is slightly lower than in WDQ2 and WDQ3 it is still present in a significant amount. However WDQ3 has a lower TIC than WDQ2 and it can been seen that these constituents were present in the greater amounts in WDQ3. This could be due to various factors that affect the quantity and quality of the oil collected such as enviromental factors. For instance, the conditions the plant grew in as well as the life stage of each plant that the leaves were harvested from.

Comparison of the percentage composition of carophyllene and δ-cadinene in the water experiments shows that it is present in the greatest amount in WDQ1 and then WDQ3 and WDQ2 in percentages of 32.24%, 20.88% and 20.46% and 3.2 %, 0.58% and 0.44%, respectively. Therefore δ-cadinene is a predominant constituent of WDQ1 and not WDQ2 and WDQ3 .

The other significant peaks present were found to be β-pinene, β-myrcene, γ-cadinene, aromadendrene, allo-aromadendrene, napthalene 1,2,4a,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, β-ionone epoxide which were present in the greatest amount in WDQ1. Carophyllene oxide is present in the greatest amount in WDQ2 whereas germacrene A and +(-)-4-carene are present in the greatest amount in WDQ3.

The reasons given for the difference in ratios of essential oil molecules in the samples of the water distillation experiments are all possible reasons, however there are a large number of other factors that could affect the composition of the samples.

Environmental factors play a huge role in the growth of the plant. As discussed earlier, the more favourable the conditions for plant growth, such as plenty of water and sunshine to photosynthesis and good soil quality with no pollution, the better it will grow and mature and produce more oil sacs. The seasons can also affect the amount of oil sacs present in each leaf. For example, during winter the leaves are very small and because there is little or no rain, the plant grows much slower and develops slower than in summer. The composition can also be affected by the life stage of plant when it is harvested. The leaves for the experiments were not harvested all at the same time and therefore this could cause variations in the composition as well. The location of the harvested *Centella* leaves also can affect the composition i.e. soil planted in. Variation in the essential oil composition across the three samples could also be due to variation in light conditions. Accumulation of essential oils in herbs either directly or indirectly depend upon light. Although the light intensity received by the plants was not evaluated, higher levels of canopy cover above them generally result in higher levels of shading. The *Centella* plants were all planted in the same location next to each other, but because of the amount of leaves needed and the soil space necessary for all the plants, some plants were planted in an area where they get sun all day and others where they have minimal sunlight e.g. in the shade of a tree and consequently having a slower photosynthesis than the plants grown in the sunshine. Hence a difference in oil composition could also be seen. Another reason could be that some compounds might have vaporised during the extraction and hence not collected.

3.2.3 Comparison between steam and water distillation experiments

Once the steam and water distillations were complete, a comparison was made to determine which method yields the greatest amount of oil. Unfortunately as explained before at the beginning of section 3.2, since quantitative data could not be determined in the experiments, the amount of ions detected in the experiments were used for comparison. The greater the number of ions present, the greater the

oil concentration and the better the quality of the oil. As Lawless (1992) explained, trace elements in an essential oil are the ones responsible for the characteristic of the essential and the therapeutic value of the oil is often a result of these elements. This is because these trace elements are thought to have a controlling effect on the main constituents, hence if an oil has a greater TIC, it will have a greater amount of trace elements and therefore be of better quality than an oil with a lower TIC.

Comparison of the average TIC between the water and steam distillation experiments can be made because both sets of experiments were performed on 100 g of fresh leaves at a distillation rate of 2/3 ml min^{-1} with 0.4 ml of xylene used initially and the distillation performed for 75 minutes. Both sets of experiments were performed for 30 minutes before leaves were added to the extraction container.

Table 3.14 Comparison of the average TIC of steam and water distillation experiments

Experiment set	TIC	Std deviation
Steam distillation	4.61×10^9	3.74×10^9
Water distillation	4.18×10^9	3.47×10^9
Steam distillation (Dry leaves)	2.82×10^9	2.14×10^9
Water distillation (salt)	2.22×10^9	1.71×10^9

The average TIC of the water and steam distillation experiments are compared in Table 3.14. It can be seen that steam distillation yielded 4.61×10^9 ions which was greater than water distillation with 4.18×10^9 ions. Therefore it can be concluded that steam distillation yields a greater amount of ions and a better concentration of oil than water distillation. So if a method was to be used to collect the most amount of ions and hence a better quality oil, steam distillation is a more effective method in collecting the essential oil from *Centella Asiati*ca leaves than water distillation. One of the reasons could be that in water distillation the leaves were

in direct contact with the water and any constituent in the oil that is polar, could possibly stay behind dissolved in waterwhen distillation occurs and dissolve in the water, hence less ions were collected in the sample.

When the average of each constituent from Table 3.9 (steam distillation) and Table 3.13 (water distillation) were compared, it is observed that steam distillation (84.84%) yields a greater amount of sesquiterpenoid hydrocarbons present in the oil than water distillation (81.63%). β-Ionone epoxide and squalene is present in greater amounts in steam distillation (1.29%, 1.82%) than in water distillation (1.08%, 0.45%). Oxygenated monoterpenoids and phytol were only present in steam distillation not water distillation. However, monoterpenoid hydrocarbons and oxygenated sesquiterpenoids were present in greater amounts from oil samples from water distillation (8.62%, 2.49%) than steam distillation (5.42%, 1.91%). 2-naphthalenamine,n-methyl was present in greater amounts in water distillation (2.76%) than steam distillation (1.94%).
In steam distillation 43 compounds were identified, which on average represents 98.60% of the composition of the oil. In water distillation, 54 compounds were identified which on average represents 98.29% of the composition of the oil.

In all the steam distillation experiments (SQ1, SQ2, SQ3), the following constituents were the predominant ones: γ- terpenine, δ-elemene, β-carophyllene, γ-cadinene, α-carophyllene, germacrene D, germacrene A, germacrene B, 2-naphthalenamine,n-methyl and squalene. (In blue in Table 3.8) .

In all the water distillation experiments (WDQ1, WDQ2, WDQ3) the following constituents were the predominant ones: γ-terpenine, δ-elemene, β-elemene, carophyllene, γ-elemene, α-carophyllene, germacrene D, δ-cadinene, germacrene B and 2-naphthalenamine,n-methyl. (In blue in Table 3.12)

The constituents identified between the two different experimental procedures were compared and the common constituents were determined: γ-terpenine, δ-elemene, carophyllene, α-carophyllene, germacrene D, germacrene B and 2-

naphthalenamine,n-methyl. However, oil samples from water distillation experiments yielded on average 4.70%, 2.98%, 24.53%,3.38% and 4.14% of γ-terpenine, δ-elemene, carophyllene, germacrene B and 2-naphthalenamine,n-methyl, respectively, which was greater than steam distillation with percentage compositions of 3.19%, 2.37%, 22.14%, 3.36% and 1.19%, respectively.

Steam distillation yielded on average 3.96% and 40.59% of α-carophyllene and germacrene D, which was greater than water distillation yielding 3.09% and 34.82%.

The other major constituents identified were compared and it can be seen that γ-cadinene was found in both water and steam distillation samples. It was however present in much higher quantities in steam distillation samples (average of 1.69%) than water distillation experiments (0.71%). Germacrene A was identified in one steam distillation sample and in two of the water distillation samples, with an average of 0.7% and 1.06%, respectively, thus it is greater from water distillation than steam distillation samples. Also squalene is present in steam distillation (1.82%) at a greater value than water distillation (0.45%).

β-elemene, γ-elemene and δ-cadinene were present in percentage compositions of 1.77%, 1.21% and 1.44% in the water distillation samples, respectively, compared to steam distillation samples with percentage compositions of 1.71%,0.76%, 0.42%, respectively. The other significant constituents present in both water and steam distillation were aromadendrene, *allo*-aromadendrene, naphthalene, 1,2,4a,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-, bicyclogermacrene, isocarophyllene, (+)-4-carene and carophyllene oxide.

The following constituents on average were present in higher amounts in the steam distillation samples than in the water distillation samples:
allo-aromadendrene (1.35%, 1.28%), bicyclogermacrene (1.13%, 0.14%), isocarophyllene (0.69 %,0.22 %), (+)-4-carene (1.18 %,0.98 %) and carophyllene oxide (0.65 %,0.52 %).

However, aromadendrene and naphthalene, 1,2,4a,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)- were present in greater amounts in water distillation samples on average (1.03%, 0.83%) than steam distillation samples (0.8%, 0.13%). β-pinene and β-myrcene were both present in water distillation samples, but not present in steam distillation samples.

The water and steam distillation experiments were both performed for 75 minutes using approximately 100 g of leaves. The difference between the samples can be explained by the two different methods used for extraction. Each distillation technique can affect the composition of the oil, as heat and water can cause molecular rearrangement, hydrolysis of double bonds, and in general will produce substances not originally found in the plant. Another reason could be the degradation of the essential oil and post harvest storage. Even though care is taken to minimise time exposed to oxygen from the air and light, this form of free energy can cause the position of double bonds to change, open chains can form closed rings and the nature of functional group can change. In water distillation some polar compounds in the essential oil could be lost in the water (polar) and not vaporise because the leaves are in direct contact with the polar solvent.

There are many other factors that can contribute to the difference between the samples collected. "In some instances it is difficult to segregate these factors from each other, since many are interdependent and influence one another" (Hussain, 2009). These variables may include seasonal variations. For example leaves collected in winter when the leaves of *Centella Asiatica* were much smaller and because it is much dryer season, photosynthesis decreases and the growth of the plant slows down and hence it can affect the essential oil composition. Another variable is the maturity variation between plants and the growth stages. The more mature a plant, the larger its leaves and the more oil sacs it will contain and a greater amount of different constituents present. As mentioned before, light variation is another variable and since the variation of the composition of oil is directly or indirectly depended on light, the more light a plant gets the better it

will grow and produce different amounts of constituents than plants grown in shaded areas. Other variables include geographical origin and genetic variation.

Pollution can also affect the composition of the oil since the soil quality of where the *Centella Asiatica* plants are growing is important. The plant likes to grow in damp environments, therefore it is especially prone to pollution. If the *Centella Asiatica* plant is grown in a polluted area, the metabolism of the plant can be affected and variations in the oil collected can be observed.

3.2.4 Steam distillation on dry leaves using the BP apparatus

Since steam distillation was found to be more effective in extracting a better quality oil, this method was used to determine the constituents present in dry leaves. Three steam distillation experiments were performed using the BP apparatus on dry leaves at a distillation rate of 2/3 ml min^{-1} and 0.4 ml of xylene was used initially. Water distillation was performed for 30 minutes before leaves were added to false bottom extraction container. The conditions and results are summarised in Table 3.15.

Table 3.15 Steam distillation experiments on dry leaves using the BP apparatus

Experiment name	Weight of fresh leaves (g)	Weight of dry leaves (g)	Average size of leaves (mm)	Distillation time (min)	TIC
DRY1	101.05	19.34	36.23	75	2.87×10^9
DRY2	96.45	16.04	62.90	75	6.63×10^8
DRY3	100.1	15.8	52.52	75	4.94×10^9

The graphs of the three steam distillation experiments using dry leaves (DRY1, DRY2 and DRY3) are shown in figure 3.26, it can be seen that graph I has the most number of peaks i.e. more constituents present than graph G (DRY1) and graph H (DRY2) having the least number of peaks. By comparing the total ion

concentration (TIC) of the three experiments (Table 3.15), it can be seen that graph I has 4.94 x 10^9 ions which is greater than graph G and graph H with TIC of 2.87 x 10^9 ions and 6.63 x 10^8 ions, respectively. This correlates to the number of peaks seen. The concentration of ions in the oil collected in experiment DRY3 was therefore higher than the concentration of ions collected in DRY1 and DRY2. Since the other parameters in the experiments were kept the same i.e. same distillation rate, amount of leaves used and the experiments preformed for the same amount of time, these will not be contributing factors to the variations observed in the results. Sample DRY2 had the smallest TIC, probably due to some xylene and oil left behind in the apparatus and therefore less oil and hence less ions collected. Also some oil could have volatised while the sample was collected from a beaker. If even an extra drop of xylene was collected, this could have also decreased the concentration of oil collected. If the size of the leaves used in experiments were compared, the ones used in experiment DRY2 (62.90 mm) were the largest compared to DRY1 (36.23 mm) and DRY3 (52.52 mm) and therefore should have had a greater number of oil sacs present. However, a smaller TIC was calculated. A possible explanation is that even though more oil sacs can be present on a more mature leaf, the oil sacs are exposed to environmental factors (such as pollution, insects etc.) for a longer period of time than a younger leaf. Since the oil is volatile if something like an insect damaged an oil sac, the oil would have volatised hence less constituents were collected. Another reason could be if some of the rinsing reagents collected in the sample vial with the oil and xylene and therefore decreased the concentration of oil in the sample which would cause the TIC of the sample to decrease. The weight of the dry leaves used in experiment DRY2 was slightly less than in the other two experiments, hence this could also be a reason for a slightly lower TIC.

When a comparison between DRY1 and DRY3 is made, it can be seen that DRY 3 has a higher TIC of 4.94 x 10^9 ions than DRY1 with 2.87 x 10^9 ions. Approximately the same amount of fresh leaves were used, but after drying for 2 weeks the dried leaf weight of DRY1 leaves was higher (19.34 g) than DRY3 (15.8 g). This shows that DRY1 leaves had a greater plant matter content than

DRY3 possibly because smaller leaves were used in DRY1 (36.23 mm) and 100 g of smaller leaves means much more leaves would be needed than 100 g of the larger ones. Therefore each small leaf will lose a certain small amount of water proportional to its size ,compared to larger leaves which would store more water. So the larger the leaf the more water it stores, and hence the more water it will lose when drying. Since there were less larger leaves losing bigger amounts of water content, less plant matter will be collected.

Then peaks in the steam distillation experiments (DRY1, DRY2 and DRY3) were then identified using the same method explained in the steam distillation experiments. (Table 3.17)

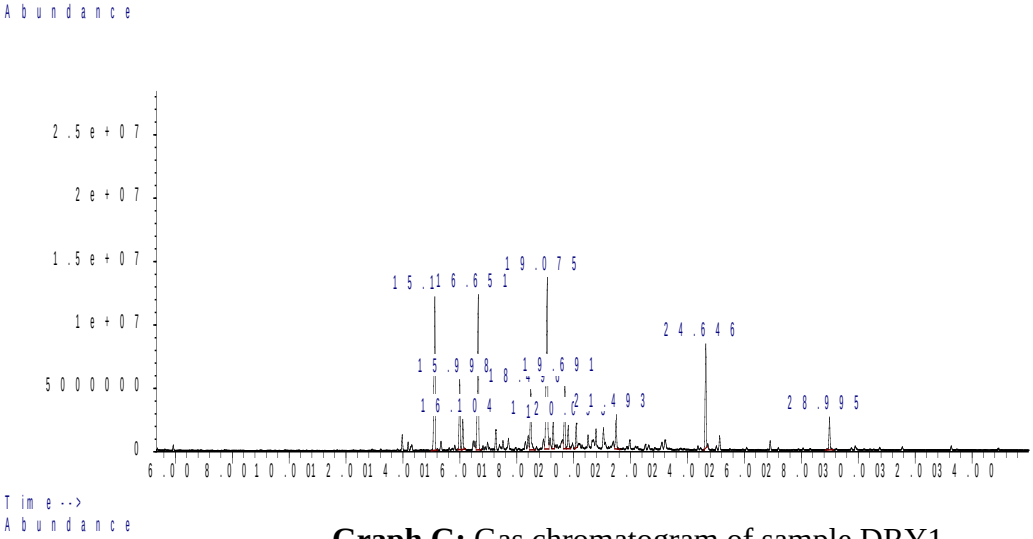
From Table 3.16 the constituents that make up the greatest composition of the oil from *Centella Asiatica* leaves in the steam distillation experiments using dry leaves were sesquiterpenoid hydrocarbons (44.74 - 87.52%) amongst which germacrene D ranging from 13.12% (DRY1), 30.00% (DRY2), 38.23% (DRY3) and carophyllene 12.28%, 16.13%, 23.46% in DRY1, DRY2 and DRY3 respectively. Oxygenated sesquiterpenoids (0.4 - 34.38%) were also identified in the essential oil, such as carophyllene oxide (0.4 - 17.17%). Monoterpenoid hydrocarbons were also identified (0.31 - 3.78%) such as γ- terpenine (0.31 - 2.29%) as well as oxygenated monoterpenoids (0.00 - 2.95%) . β-Ionone epoxide (0.00 - 1.49%), 2-naphthalenamine, n-methyl (2.33 - 5.66%) and phytol (1.14 - 7.47%) were also identified.

DRY3 sample has the greatest amount of monoterpenoid hydrocarbons (3.78%) and sesquiterpenoid hydrocarbons (87.52%). It had the highest total ion current, hence it has a higher concentration of oil and thus a greater amount of each constituents was present. On the other hand it contains very little oxygenated sesquiterpenoids at 0.4% compared to DRY1 (34.38%) and DRY2 (27.25%). DRY1 also has the greatest amount of oxygenated monoterpenoids (2.95%).

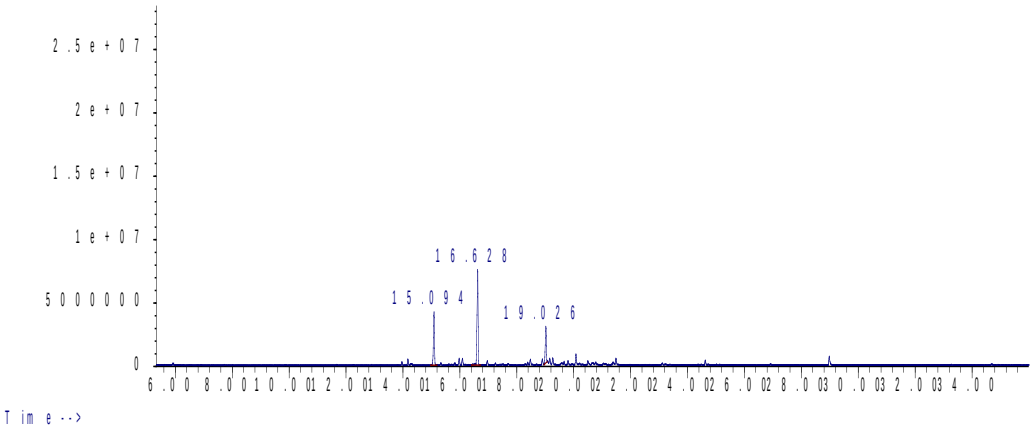
The reasons for the variations in composition of the oil could be due to the location where it was harvested from and the certain environmental factors that could have affected the oil production and composition.

In Table 3.17 the constituents that were identified in each experiment (DRY1, DRY2, DRY3) were compared. Forty five constituents were identified from the three different oil samples, accounting from 95.86% to 100% of the oil contents. However, the predominant constituents were used for comparisons between experiments. In all the steam distillation experiments with dry leaves (DRY1, DRY2, DRY3), the following constituents were the predominant ones: γ-terpenine; carophyllene; α-carophyllene; germacrene D; isocarophyllene; carophyllene oxide; 1,7,7-Trimethyl-2-vinylbicyclo[2.2.1]hept-2-ene; trans-chrysanthemal; humulene epoxide; ledene oxide; 7R,8R-8-Hydroxy-4-isopropylidene-7-methylbicyclo[5.3.1]undec-1-ene; phytol and 2-naphthalenamine, n-methyl. (In blue in Table 3.17) .

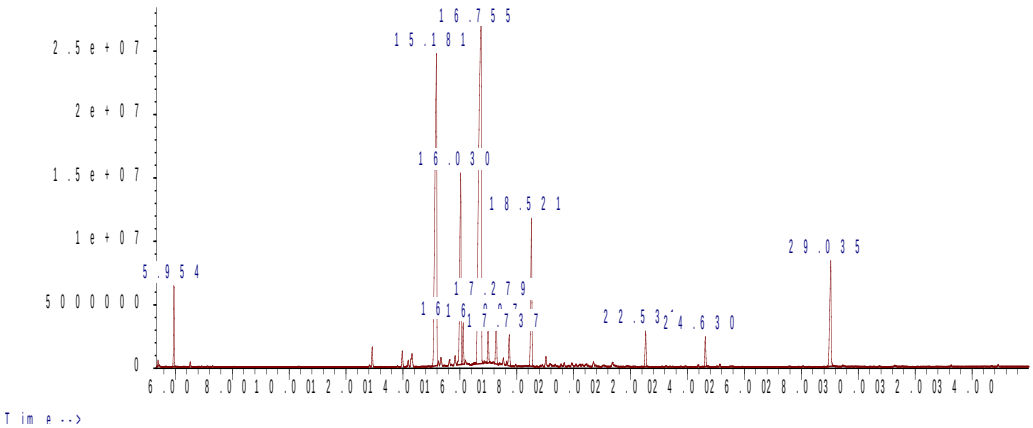
In the steam distillation experiments with dry leaves, the major constituent were determined to germacrene D in DRY3 (38.23%) and DRY2 (30.00%) and DRY1 (13.12%), carophyllene in DRY3 (23.46%), DRY2 (16.13%) and DRY1 (12.28 %). DRY3 sample also has the greatest amount of γ-terpenine (2.29%) and 2-naphthalenamine (5.66%) compared to DRY2 (0.52%,3.08%) and DRY1 (0.31%, 2.33%), respectively. The possible explanation is that DRY3 had the highest TIC and more ions were present for analysis, hence more constituents were identified. DRY2 had the smallest TIC but a greater amount of germacrene D, carophyllene, γ-terpenine, naphthalenamine, n-methyl than DRY1. The reason could be that DRY2 was grown in an area that has more favourable conditions for plant growth than DRY1 leaves. The life stage of plant could have also played a role in the difference in constituents percentages observed.



Graph G: Gas chromatogram of sample DRY1



Graph H: Gas chromatogram of sample DRY2



Graph I: Gas chromatogram of sample DRY3

Figure 3.26 Comparison of the gas chromatograms of the Centella oil samples collected from steam distillation experiments using dry leaves DRY1, DRY2 and DRY3

Table 3.16 Summary and comparison of the major constituents present in the samples obtained from the steam distillation experiments using dry leaves and a comparison to a similar study conducted in South Africa

Steam distillation experiments on dry leaves									
	No. identified	DRY1 (% composition)	No. identified	DRY2 (% composition)	No. identified	DRY3 (% composition)	No. identified	South African study* %	Average dry leaves % composition
Monterpenoid hydrocarbons	1	0.31	1	0.52	3	3.78	11	20.20	1.54
Oxygenated monoterpenoids	2	2.95	1	2.21	0	0.00	9	5.46	1.72
Sesquiterpenoid hydrocarbons	15	44.74	14	59.95	15	87.52	14	68.80	64.07
Oxygenated sesquiterpenoids	11	34.38	11	27.25	1	0.40	5	3.90	20.68
Sulfidesesquiterpenoid	0	0.00	0	0.00	0	0.00	1	0.76	0.00
β-Ionone epoxide	0	0.00	0	0.00	1	1.49	0	0.00	0.50
Phytol	1	7.47	1	1.25	1	1.14			3.29
2-Naphthalenamine,N-Methyl	1	2.33	1	3.08	1	5.66	0	0.00	3.69
Squalene	0	0.00	0	0.00	0	0.00	0	0.00	0.00
Other	4	3.68	4	4.51	0	0.00	3	0.00	2.73
Total	35	95.86	33	98.77	22	99.99	43	99.12	98.21

* South African study data from Oyedeji (2004)

Table 3.17 Comparison of the different constituents present in the samples obtained from steam distillation experiments DRY1,DRY2 and DRY3 ([**bold**] - constituent present in significant value , [blue] - predominant constituent

	DRY1		DRY2		DRY3		Avera
Constituents [a]	**Retention time**[b]	**% Composition**[c]	**Retenti on time**[b]	**% Composition**[c]	**Retenti on time**[b]	**% Composition**[c]	**% Comp osition**
Limonene	-	-	-	-	5.40	0.13	0.04
γ-Terpinene	5.93	0.31	5.92	0.52	5.95	2.29	1.04
δ-Elemene	-	-	-	-	12.92	0.74	0.25
Copaene	13.98	1.07	13.97	0.98	13.98	0.61	0.89
β-Bourbonene	14.19	0.57	14.18	1.70	14.19	0.26	0.84
β-Elemene	14.32	0.62	-	-	14.32	0.78	0.47
β-Cuvebene	-	-	14.27	0.73	-	-	0.24
Caryophyllene	15.12	12.28	15.09	16.13	15.18	23.46	17.29
γ-Elemene	-	-	-	-	15.34	0.40	0.13
Isoledene	-	-	-	-	15.64	0.24	0.08
γ-Cadinene	15.34	0.70	15.34	0.72	-	-	0.47
Aromadendrene	-	-	15.82	0.66	15.83	0.44	0.37
α-Caryophyllene	16.00	4.96	15.98	1.84	16.03	9.87	5.56

***Allo*-Aromadendrene**	16.10	1.96	16.09	1.81	16.12	1.51	1.76
Naphthalene, 1,2,4a,5,6,8a-hexahydro-4,7-dimethyl-	16.47	0.69	-	-	-	-	0.23
β-ionene	16.53	0.57	16.53	0.41	-	-	0.33
Germacrene D	16.65	13.12	16.63	30.00	16.76	38.23	27.12
Bicyclogermacrene	16.98	0.41	16.97	1.26	17.00	1.39	1.02
Isocarophyllene	17.26	1.32	17.25	0.54	17.28	2.41	1.42
δ-cadinene	17.52	0.66	-	-	17.53	0.30	0.32
1-Naphthalenol	17.71	0.96	-	-	-	-	0.32
(+)-4-Carene	-	-	-	-	17.74	1.36	0.45
2-(4a,8-Dimethyl-1,2,3,4,4a,5,6,7-octahydro-	18.40	1.02	18.39	0.64	-	-	0.56
Germacrene B	18.50	4.71	18.48	1.49	18.52	6.88	4.36
Isolongifolene,7,8-dehyro-8a-hydroxy-	18.94	0.87	18.90	1.94	-	-	0.94
Carophyllene oxide	19.08	17.17	19.03	12.81	19.03	0.40	10.13
Diethyl phthalate	-	-	19.09	1.23	-	-	0.41
1,7,7-Trimethyl-2-vinylbicyclo[2.2.1]hept-2-ene	19.17	0.96	19.16	2.26	-	-	1.07
Trans- chrysanthemal	19.28	1.99	19.27	2.21	-	-	1.40
y-Gurjunene	19.61	1.12	19.59	1.19	-	-	0.77
Humulene epoxide	19.69	5.17	19.67	1.00	-	-	2.05
Spathulenol	19.81	1.88	19.80	1.38	-	-	1.09

Ledene oxide	20.09	1.78	20.08	3.26	-	-	1.68
Allo-aromadendrene oxide	-	-	20.21	0.53	-	-	0.18
Aromadendrene oxide (2)	20.51	1.09	20.50	1.40	-	-	0.83
Seychellene	20.66	0.55	20.65	0.88	-	-	0.48
N-methyl-N-methoxy-5,6,7,8-tetrahydro-1-	20.79	1.48	20.78	0.61	-	-	0.70
Isoaromadendrene epoxide	21.05	1.53	21.05	0.52	-	-	0.68
Cycloisolongifolene,8-hydroxy-,endo-	21.40	0.74	21.39	1.31	-	-	0.68
7R,8R-8-Hydroxy-4-isopropylidene-7-	21.49	2.38	21.49	2.45	-	-	1.61
cis-alpha-Copaene-8-ol	21.97	0.75	-	-	-	-	0.25
β-Ionone epoxide	-	-	-	-	22.53	1.49	0.50
Phytol	24.65	7.47	24.63	1.25	24.63	1.14	3.29
Pthalic acid	26.91	0.66	-	-	-	-	0.22
2 -Naphthalenamine,N-methyl	29.00	2.33	28.99	3.08	29.04	5.66	3.69
Total		95.86		98.77		100.00	98.21

[a] Constituents are listed in order of elution time on the Zebron-ZB 274305 column

[b] Retention times calculated on Zebron-ZB 274305capillary column

[c] Relative area percentage: peak area relative to total peak area percent

Much smaller leaves were used for DRY2 (36.23 mm) than DRY3 (52.52 mm) and therefore a difference in composition occured.

When the amount of α-carophyllene, isocarophyllene and germacrene B were compared the greatest amounts were present in sample DRY3 (9.87%, 2.41%, 6.88%), followed by DRY1 (4.96%, 1.32%, 4.71%) and then DRY2 (1.84%, 0.54%, 1.49%). A possible explanation is the total ion concentration of DRY3 was greater than DRY1 which was greater than DRY2. Therefore, the greater number of ions present, the greater chance of certain constituents available for analysis. In experiment DRY1, larger leaves were also used than in DRY2, and therefore more oil sacs were present and thus a greater chance of certain constituents being present in the sample.

When the percentage composition of carophyllene oxide, humulene epoxide and phytol were compared in each of the steam distillation experiments on dry leaves the greatest amounts were seen in samples in the order : DRY1 (17.17%, 5.17%, 7.47%) > DRY2 (12.81%, 1.00%, 1.25%) > DRY3 (0.4%, 0.00%, 1.14%). DRY1 has a greater TIC than DRY2, and therefore can have a greater amount of constituents present. However, DRY3 has the smallest amount of these constituents present, but the highest TIC. The reason for this could be that the leaves for DRY1 and DRY2 harvested were from a much more favourable area with favourable conditions than the other leaves. When the percentage of carophyllene oxide and humulene epoxide present in the samples were compared, it can been seen that carophyllene oxide is a predominant constituent of DRY1 and DRY2 samples but not of DRY3 and that humulene epoxide is a predominant constituent of DRY1 only and not of DRY2 and DRY3.

When the amount of 1,7,7-Trimethyl-2-vinylbicyclo[2.2.1]hept-2-ene, trans-chrysanthemal, ledene oxide and 7R,8R-8-Hydroxy-4-isopropylidene-7-methylbicyclo[5.3.1]undec-1-ene were compared, the greatest amounts were present in sample DRY2 (2.26%, 2.21%, 3.26%, 2.45%), then DRY1 (0.96%, 1.99%, 1.78%, 2.38%) and they were not present at all in DRY3. The TIC of

DRY2 is lower than DRY1, but it has a higher amount of these constituents. A possible reason for this could be that younger leaves were used in DRY2 (36.23 mm) than DRY1 (62.90 mm). The younger leaves could possibly have more of these constituents than older leaves because the composition may vary of the oil as the leaf grows. However other environmental factors could have also affected the composition. If DRY2 was planted in a location that is more favourable for plant growth and development then it would have a greater number of oil sacs that contain certain constituents.

The reasons given for the difference in ratios of essential oil molecules in the samples of the steam distillation experiments with dry leaves were all possible reasons, however there were a large number of other factors that could affect the composition of the samples as explained in the other results.

The amount oil in a plant and the composition of the essential oil vary due to a number of factors. As discussed before environmental factors are probably the biggest reason and can affect the growth of the *Centella Asiatica* plant. The more favourable the conditions for plant growth, such as plenty of water and sunshine to photosynthesis and good soil quality with no pollution, it will grow and mature better and produce more oil sacs than a plant which was grown in the unfavourable conditions. The seasons can affect the plant growth and hence the amount of oil produced. For example in winter the plants growth slows down and this effects production of oil in the leaves. The life stage of the plant is also an important factor. Some smaller leaves may contain less oil sacs than larger more mature leaves. Since the leaves for the experiments were not harvested all at the same time and therefore this could cause variations in the composition as well. The location of the harvested *Centella* leaves also can affect the composition. For example the soil quality and the amount of light received would affect the composition.

Other reasons as discussed earlier could be that some compounds could have vaporized during the extraction and therefore not collected, some oil could have

been left behind in apparatus and some compounds may have been collected in minute amounts and not detected. If an extra drop of xylene was collected, it would decreased the concentration of some constituents present as well.

Steam distillation on fresh leaves compared to dry leaves

When the average TIC of the steam distillation experiments on fresh leaves were compared to dry leaves (Table 3.14), it can be seen that steam distillation on fresh leaves yields a higher TIC of 4.61×10^9 ions compared to dry leaves of 2.82×10^9 ions. Forty two constituents were identified in the steam distillation experiments on fresh leaves compared to forty five constituents identified using dry leaves. The reason fresh leaves yield a higher amount of ions is because some of the oil could have been lost during post harvest drying due to volatisation and mechanical damage to oil glands. Dry leaves yielded a greater amount of constituents present. The reason there is oil present in the leaves after drying could be due to postharvest metabolic activity within the plant. (Hussain, 2009)

The average percentage compositions of Tables 3.9 and Table 3.16 were compared, steam distillation on fresh leaves had a higher amount of sesquiterpenoid hydrocarbons (84.83%) and monoterpenoid hydrocarbons (5.42%) compared to the ones on dry leaves (64.07%, 1.54%). Steam distillation on dry leaves however yielded a higher amount of oxygenated sesquiterpenoids (20.68%) as well as oxygenated monoterpenoids (1.72%) compared to fresh leaves with 1.91% and 0.08% respectively. Dry leaves steam distillation also yielded more phytol (3.29%) and 2-naphthalenamine,n-methyl (3.69%) than fresh leaves steam distillation with 0.79% and 1.94%, respectively. However β-ionone epoxide was greater in fresh leaves (1.29%) than dry leaves (0.5%) and squalene was only detected in fresh leaves (1.82%) and not in dry leaves.

The major constituents obtained in steam distillation on fresh leaves and dry leaves were then compared (table 3.8 and table 3.17) and γ-terpenine, carophyllene, α-carophyllene, germacrene D, germacrene B and 2 -

naphthalenamine,n-methyl were the major constituents in both sets of experiments. γ-cadinene is a major constituent of steam distillation on fresh leaves and even though it is present in dry leaves, it is present in small amounts. δ-elemene, germacrene A and squalene were all present in the fresh leaves samples but they were not present in dry leaves samples.

Isocarophyllene, carophyllene oxide and phytol were present in both dry and fresh leaf distillations, however they were present in greater amounts in dry leaves (1.42%, 10.13%, 3.29%) than fresh leaves samples (0.69%, 0.65%,0.79%). Humulene epoxide, trans-chrysanthemal, ledene oxide and 7R,8R-8-Hydroxy-4-isopropylidene-7-methylbicyclo[5.3.1]undec-1-eneis present only as a major constituent in dry leaves steam distillation and is not detected in fresh leaf steam distillation.

One reason for the difference in composition can be explained by the drying process. When leaves dry, they can get damaged and oil sacs can burst and thus release their volatile oil. The drying process could also change bonds in some constituents and the oil can degrade with some constituents reacting with oxygen and free energy during the distillation process and therefore yielding slightly different constituents than in steam distillation on fresh leaves.

3.2.5 Water distillation on fresh leaves using salting out and the BP apparatus

After the water distillation experiments were performed, it was decided to investigate salting out i.e. adding salt to the water during water distillation. The purpose of this was to try and see if the amount of the oil constituents collected increased and if a better quality oil would be obtained than normal water distillation.

Table 3.18 Salting out water distillation experiments on around 100 g of fresh leaves using setup 6B above and a distillation rate of 2/3 ml min^{-1}. Water distillation performed for 30 minutes before leaves added to round bottom flask. A 10% w/w solution of salt was used.

Experiment name	Weight of leaves (g)	Average size of leaves (mm)	Distillation time (min)	TIC
WDSO1	102.2	55.95	75	4.16×10^9
WDSO2	100.1	50.15	75	1.56×10^9
WDSO3	98.24	39.08	75	9.48×10^8

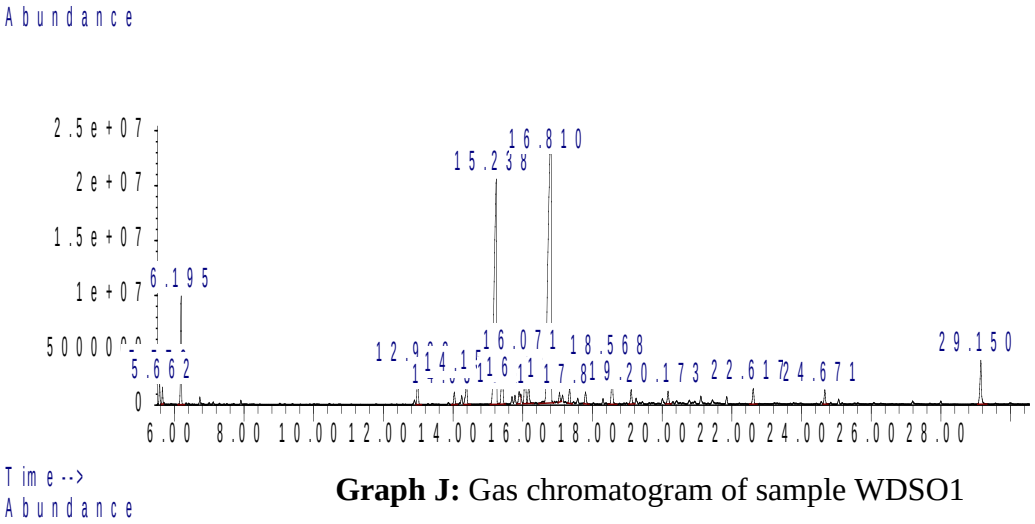
* The experiments shown in table 3.18 were all performed for 75 minutes using 0.4 ml xylene.

The gas chromatograms obtained for the experiments were shown in Figure 3.27.

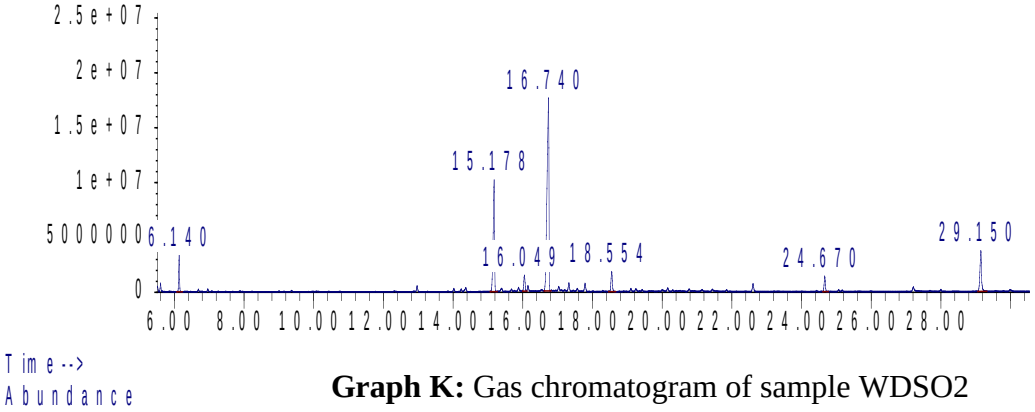
When the TIC of the salting out experiments were compared in Table 3.18. It can be noted that the greatest number of ions were seen in WDSO1 (4.16×10^9 ions) followed by WDSO2 (1.56×10^9 ions) and then WDSO3 (9.38×10^8 ions). The reason for this could be that the average leaf size used in the distillation was the greatest in WDSO1 (55.95mm) > WDSO2 (50.15mm) > WDSO3 (39.08mm). The more mature a leaf, the more oil it should contain, hence more ions should be collected. Also the amount of leaves used were slightly more in WDSO1 than in WDSO2 and WDSO3. This agrees with the graphs in Fig. 3.27 where Graph J (WDSO1) has the greatest amount of peaks then graph K (WDSO2) and then Graph L (WDSO3).

The peaks in the water distillation experiments with salt (WDSO1,WDSO2 and WDSO3) were then identified using the same method as explained in the steam distillation experiments (Table 3.19).

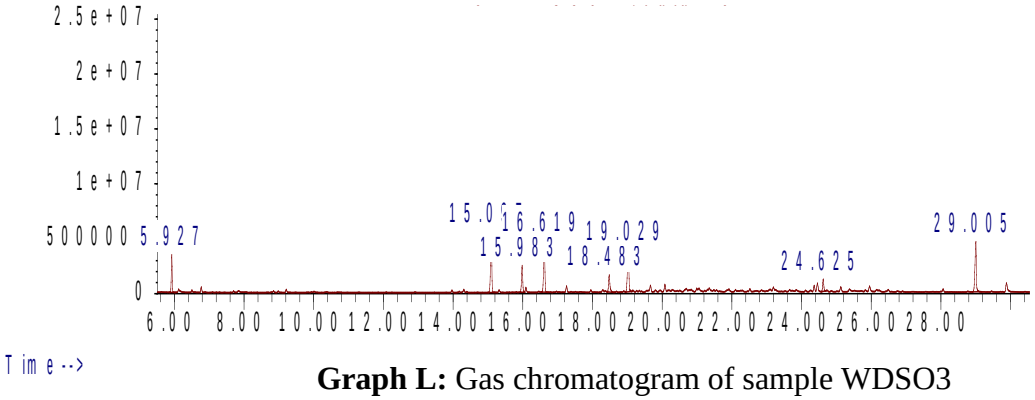
In Table 3.19, the constituents that were identified in each experiment (WDSO1, WDSO2,WDSO3) were compared. Fifty four constituents were identified from the three different oil samples, accounting for 96.17 % to 99.29 % oil content.



Graph J: Gas chromatogram of sample WDSO1



Graph K: Gas chromatogram of sample WDSO2



Graph L: Gas chromatogram of sample WDSO3

Figure 3.27: Comparison of the gas chromatograms of the Centella oil samples collected from water distillation experiments with salt, WDSO1, WDSO2 and WDSO3

The predominant constituents were be used for comparisons between experiments. In all the water distillation experiments with salt (WDSO1, WDSO2, WDSO3), the following constituents were the predominant ones: γ- terpenine, carophyllene ,γ-cadinene, α-carophyllene, germacrene D, germacrene B,caropphyllene oxide, decahydro 4,4,8,9,10 pentamethylnaphthalene,3,7,11,15-tetramethyl-2-hexadecen-1-ol and 2-naphthalenamine,n-methyl, phytol. (In blue in Table 3.19)

In the water distillation experiments with salting out the major constituent were observed to be germacrene D in WDSO1 (37.77%) and WDSO2 (43.15%) and then carophyllene at 22.50% and 18.53%. In WDOS3 it was observed that carophyllene was the major constituent at 14.55% and then germacrene D at 12.34% and also carophyllene oxide at 12.23%.

Carophyllene oxide was present in WDSO1 and WDSO2 samples but in small amounts and it was not a major constituent. The reason for the difference in constituents could be due to environmental factors such as life stage of plant,the soil quality, the amount of sunlight it received which all can affect the growth and metabolism of the plant. etc. hence altering the constituents of oil produced. It could also be possible that the smaller leaf size used in experiment WDSO3 (39.08 mm) contained more carophyllene than germacrene D and also carophyllene oxide.

When the amount of γ-cadinene in the samples were compared, the greatest amount was detected in WDSO1 (2.24%), followed by WDSO2 (0.62%) and none was detected in WDSO1. The reason it was detected in WDSO1 and WDSO2 could be because the samples have a higher TIC than WDSO3, and therefore more ions were present and a greater chance of containing γ-cadinene.

Table 3.19 Comparison of the different constituents present in the samples obtained from water distillation experiments WDSO1,WDSO2 and WDSO3 ([**bold**] - constituent present in significant value , [blue] - predominant constituent

	WDSO1		WDSO2		WDSO3		Averag
	Retention time [b]	**% Composition [c]**	**Retention time [b]**	**% Composition [c]**	**Retention time [b]**	**% Composition [c]**	**% Composition**
α-Terpinene	5.445	0.44	5.3836	0.37	-	-	0.40
ρ-Cymene	5.579	0.89	5.5247	0.47	-	-	0.68
Limonene	5.662	0.58	5.6094	0.73	5.368	0.95	0.75
γ-Terpinene	6.195	4.28	6.1385	3.59	5.927	6.19	4.69
Terpinolene	6.735	0.25	6.6959	0.24	-	-	0.25
Benzaldehyde, 2 methyl	**-**	**-**	**-**	**-**	**6.127**	**1.20**	**1.20**
Trans-3-Nonen-2-one	7.912	0.17	-	-	-	-	0.17
Linalol	**-**	**-**	**6.964**	**0.30**	**6.774**	**1.01**	**0.65**
Isoterpinolene	12.893	0.25	-	-	-	-	0.25
α-Terpineol	-	-	-	-	9.216	0.63	0.63
δ-Elemene	12.988	1.84	12.9681	0.81	-	-	1.33
Copaene	14.038	0.68	14.0264	0.44	-	-	0.56
β-Bourbonene	14.25	0.57	14.238	0.39	-	-	0.48

β-Elemene	**14.385**	**1.65**	**14.365**	**0.77**	**14.313**	**0.75**	**1.06**
Caryophyllene	15.238	22.50	15.1763	18.53	15.097	14.55	18.53
γ-cadinene	15.419	2.24	15.3951	0.62	-	-	1.43
Isoledene	15.694	0.47	15.6843	0.33	-	-	0.40
Bicyclo[4.4.0]dec-1-ene, 2-isopropyl-5-methyl-9-	15.778	0.52	-	-	-	-	0.52
Aromadendrene	15.898	0.81	15.8819	0.68	-	-	0.75
α-Caryophyllene	16.071	2.77	16.0512	2.35	15.983	6.21	3.78
***Allo*-Aromadendrene**	16.171	1.28	16.15	0.80	16.092	1.10	1.06
Naphthalene, 1,2,4a,5,6,8a-hexahydro-4,7-	16.577	0.43	16.5451	0.39	-	-	0.41
Germacrene D	16.81	37.77	16.7426	43.15	16.619	12.34	31.09
Bicyclogermacrene	17.056	0.72	17.0319	0.78	-	-	0.75
β-Cuvebene	17.145	0.64	-	-	-	-	0.64
Isocarophyllene	-	-	-	-	17.259	1.42	1.42
Germacrene A	17.345	1.03	17.3282	1.34	-	-	1.18
δ-cadinene	17.58	0.45	17.5681	0.56	-	-	0.50
(+)-4-Carene	17.804	0.81	17.7939	1.29	-	-	1.05
Heptasiloxane,hexadecamethyl	18.306	0.30	-	-	-	-	0.30
Germacrene B	18.568	2.43	18.5559	3.23	18.483	4.82	3.50
Carophyllene oxide	19.116	0.94	19.1062	0.50	19.029	12.23	4.56

1,7,7-Trimethyl-2-vinylbicyclo[2.2.1]hept-2-ene	19.255	0.39	19.2473	0.55	-	-	0.47
y-Gurjunene	-	-	19.4307	0.28	-	-	0.28
Humulene epoxide	-	-	-	-	19.666	1.68	1.68
Juniper camphor	20.009	0.42	20.0022	0.40	-	-	0.41
Spathulenol	20.173	0.82	20.1644	0.61	20.083	1.85	1.09
Ledene oxide	20.421	0.26	20.3056	0.19	-	-	0.22
α-Cadinol	20.784	0.27	20.7783	0.39	20.698	1.36	0.67
Isoaromadendrene epoxide	21.114	0.52	21.1522	0.39	20.998	0.83	0.58
Alpha-Farnesene	-	-	-	-	21.061	1.21	1.21
trans-z-.alpha.-bisabolene epoxide	21.448	0.28	21.4485	0.41	21.356	0.93	0.54
β-Ionone epoxide	22.617	0.92	22.6126	1.25	22.522	0.79	0.99
Aromadendrene oxide (2)	-	-	-	-	23.196	0.66	0.66
Isopropyl myristate	-	-	-	-	24.366	1.43	1.43
Decahydro 4,4,8,9,10 pentamethylnaphthalene	-	-	-	-	24.46	2.26	2.26
3,7,11,15-tetramethyl-2-hexadecen-1-ol	24.671	0.78	24.6728	2.16	24.625	2.70	1.88
Hexasiloxane, tetradecamethyl-	25.069	0.26	25.0679	0.29	25.13	1.41	0.65
Trans- chrysanthemal	-	-	25.1737	0.22	25.955	1.52	0.87
n-Hexadecanoic acid	27.195	0.19	27.2127	0.80	-	-	0.50
2 -Naphthalenamine, N -methyl	29.15	2.96	29.1529	7.60	29.005	13.29	7.95

Phytol	-	-	29.9925	0.46	29.884	2.98	1.72
Squalene	34.754	0.37	-	-	-	-	0.37
Eicosane	-	-	34.7831	0.63	-	-	0.63
Total		96.17		99.29		98.29	97.91

[a] Constituents are listed in order of elution time on the Zebron-ZB 274305 column

[b] Retention times calculated on Zebron-ZB 274305capillary column

[c] Relative area percentage: peak area relative to total peak area percent

Table 3.20 Summary and comparison of the major constituents present in the samples obtained from the water distillation experiments with salting out

Water distillation experiments with salting out							
	No. identified	WDSO1 (%)	No. identified	WDSO2 (%)	No. identified	WDSO3 (%)	Average salting out exp. % Composition
Monterpenoid hydrocarbons	7	7.51	6	6.69	2	7.13	7.11
Oxygenated monoterpenoids	1	0.17	1	0.30	3	2.84	1.10
Sesquiterpenoid hydrocarbons	18	78.80	17	75.46	8	42.39	65.55
Oxygenated sesquiterpenoids	7	3.51	7	2.88	7	19.55	8.65
Sulfidesesquiterpenoid	0	0.00	0	0.00	0	0.00	0.00
β-Ionone epoxide	1	0.92	1	1.25	1	0.79	0.99
Phytol	0	0.00	1	0.46	1	2.98	1.15
2-Naphthalenamine,N-methyl	1	2.96	1	7.60	1	13.29	7.95
Squalene	1	0.37	0	0.00	0	0.00	0.12
Other	5	1.93	5	4.65	5	9.32	5.30
Total	41	96.17	39	99.29	28	98.29	97.92

When the amount of γ-terpenine, α-carophyllene,germacrene B, decahydro 4,4,8,9,10 pentamethylnaphthalene, 3,7,11,15-tetramethyl-2-hexadecen-1-ol, 2-naphthalenamine,n-methyl and phytol were compared they were present in the greatest amounts in WDSO3 (6.19%, 2.77%, 2.43%, 0%, 0.78%, 2,96%, 0%), followed by WDSO2 (3.59%. 2.35%, 3.23%, 0.00%, 2.16%, 7.60%, 0.46%) and the lowest amount in WDSO1 (4.28%, 6.21%, 4.82%, 2.26%, 2.70%, 13.29%, 2.98%). However WDSO3 has the lowest TIC of 9.38 x 10^8 ions, but has the greatest amount of these constituents. The reason for this could be that the leaves harvested for WDSO3 were harvested from a more favourable area with good soil quality and enough light for photosynthesis. The variations in light conditions can affect the composition of essential oils collected for example α-carophyllene is produced in higher amounts in *Centella Asiatica* leaves when grown in an area with greater amounts of sunlight (Devkota et al., 2013). Therefore, WDSO3 leaves could have been harvested from plants in a higher light area than WDSO1 and WDSO2. WDSO3 was performed with smaller leaves, and this could also be the reason a difference in the composition of the oil between the three experiments is seen.

The other significant constituents identified in the water distillation experiments with salting out were benzaldehyde, 2 methyl, linalol, isocarophyllene, humulene epoxide, spathulenol, α-cadinol, alpha-farnesene, isopropylmyristate, hexasiloxane, tetradecamethyl- and trans-chrysanthemal which were all present in the greatest amounts in WDSO3. δ-Elemene, β-elemene, aromadendrene and allo-aromadendrene were all detected in the greatest amounts in WDSO1. In sample WDSO2 the following constituents were also identified in the greatest amounts germacrene A, (+)-4-carene and β-ionone epoxide.

From Table 3.20, the constituents that make up the greatest composition of the oil from *Centella Asiatica* leaves in the water distillation experiments with salting out were sesquiterpenoid hydrocarbons (42.39 - 78.80%) among which germacrene D at 12.34% (WDSO3), 37.77% (WDSO1), 43.15% (WDSO2) and carophyllene

22.50%, 18.53%, 14.55% in WDSO1, WDSO2 and WDSO3 respectively. Monoterpenoid hydrocarbons (6.69% - 97.51%) were also identified in the essential oil, such as γ- terpenine (3.59% - 6.19%) as well as oxygenated sesquiterpenoids (2.88% - 19.55%) such as carophyllene oxide (0.50% - 12.23%). Oxygenated monoterpenoids were also identified ranging from 0.17% to 2.84%. β-Ionone epoxide (0.79% - 1.25%), 2-naphthalenamine,n-methyl (2.96 - 13.29%), phytol (0.00 - 2.98%) and squalene (0.00 - 0.37%) were also identified.

The greatest amount of monoterpenoid hydrocarbons and sesquiterpenoid hydrocarbons observed in WDSO1 is probably because it had the highest total ion concentration and therefore it has a higher concentration of ions than the other two experiments. The oxygenated sesquiterpenoids and oxygenated monoterpenoids were present in the greatest amount in WDSO3 (19.55%, 2.84%). This could be due to environmental factors.

In all the experiments above there are a number of factors that can cause the variation seen in the oil collected. These factors can either be environmental, life stage of plant and experimental factors as explained in detail previously.

Comparison of water distillation with and without salt using fresh leaves

The average TIC of the water distillation experiments were compared with and without salt (Table 3.14) and it can be seen that water distillation with no salt (4.18×10^9 ions) on fresh leaves yields a higher TIC compared to distillation with salt (2.22×10^9 ions). Fifty four constituents were indentified in both the water distillation experiments with and without salt. However, some of the constituents were not the same. The reason the water distillation experiment without salt yields a higher amount of ions could be because even though salt should improve the separation of the polar compounds from the water, majority of the essential oil composition is made up from non-polar compounds.

When the average composition column of Tables 3.13 and Table 3.20 were compared, it can be seen that water distillation without salt yields a higher amount of sesquiterpenoids hydrocarbons (81.63%) and monoterpenoid hydrocarbons (8.62%) compared to the distillation with salt (65.55%, 7.11%). Water distillation with salt yielded a higher amount of oxygenated sesquiterpenoids (8.65%) as well as oxygenated monoterpenoids (1.10%) compared to distillation without salt with 2.49% and 0.00%, respectively. This can be explained by comparing the two water solutions. In a normal water distillation, the leaves were in contact with the water and any oil and its compounds that is distilled can react with the water molecules and form bonds. If the compounds in the oil are polar enough, it will be surrounded by water molecules and hence will dissolve in water.

When the salt concentration is increased, some of the water molecules are attracted by the salt ions, which decreases the number of water molecules available to interact with the polar compounds. As a result of the increased demand for solvent molecules, the polar compound interactions are stronger than the solvent-polar compound interactions. This process is known as salting out.

Therefore more oxygenated monoterpenoids and oxygenated sesquiterpenoids which are polar compounds are detected in salt water distillation because there are less solvent molecules available for them to dissolve in.

It can also be seen that in salting out distillation, phytol was detected and in the distillation with normal water there was none. This could be because phytol is an alcohol (i.e. has a polar group -OH) and will therefore not dissolve as much in salt water than normal water. 2-naphthalenamine,n-methyl was present in the salting out (7.95%) experiment in much higher amounts than normal water distillation (2.76%). This could also be due to the polarity of naphthalenamine, n-methyl (contains a -N-H-CH_3 group) and thus is more polar, hence more molecules were available for distillation with salt. β-Ionone epoxide was detected in slightly greater amounts in normal water distillation. This could be because it is a ketone

and is polar, but not as polar as an alcohol, so some is detected in normal water distillation and in salt water distillation.

The major constituents obtained in water distillation with and without salt (table 3.12 and table 3.19) were then compared. It can be seen that γ-terpenine, carophyllene, α-carophyllene, germacrene D, germacrene B and 2 -naphthalenamine,n-methyl were the major constituents in both sets of experiments. δ-elemene, β-elemene, γ-elemene, δ-cadinene were all major constituent of the normal water distillation experiments. However these compounds were present in small amounts in the salting out experiments. γ-elemene is however not present in salting out experiments. γ-Cadinene and carophyllene oxide were present in both sets of experiments, but in much greater amounts in the salting out experiments (1.43%, 4.56%) than in the normal water distillation experiments (0.29%, 0.52%). 3,7,11,15-tetramethyl-2-hexadecen-1-ol and phytol were present in the salting out experiments, however they were not present in the water distillation experiments. This is also due to the polarity of the molecules as they were both alcohols i.e. (both polar) so they will be detected in the salting out experiments, whereas in the normal water distillation experiments they would be dissolved in the water (polar) and therefore not detected. Decahydro 4,4,8,9,10 pentamethylnaphthalene is also only detected in the salting out experiments. The reason for the variation could be due to the salt stress, or due to environmental factors which would alter the composition in the leaves collected. It was only detected in WDSO3, so the leaves could have been harvested from an area which favoured the constituents production.

The main reason difference in composition between the water distillations with and without salt was observed is definitely due to the salt. The more salt in a solution, the less water molecules were present to dissolve a polar molecule, hence more polar molecules were distilled. This is confirmed by the greater amount of oxygenated compounds detected in the salting out experiments. There are many other factors that can affect the composition as mentioned earlier. One of the main reasons of performing the salting out experiment was to see if it

would increase the yield of the ions collected in the essential oil. When the TIC of the two sets of experiments were compared, it can be concluded that salting out did not increase the oil quality but it definitely does increase the amount of polar compounds such as oxygenated monoterpenoids and oxygenated sesquiterpenoids.

3.2.6 Comparison of the results obtained on using the BP apparatus and the Clevenger apparatus

In the initial results with the Clevenger apparatus on setup 1- sample SDP1 (steam distillation on fresh leaves) the TIC obtained was 1.29 x 10^9 ions. The experiment was performed with 42 g of leaves. The average TIC of steam distillation experiments using the BP apparatus was 4.61 g x 10^9 ions (Table 3.14); it can be seen that it is much larger than that of SDP-1. However the steam distillation experiments on the specialized apparatus was performed with 100 g of leaves. As proven in the initial optimisation results, the greater the amount of leaves the greater the amount of ions collected.

Table 3.21 Summary of the TIC obtained from the initial Clevenger apparatus experiments

Experiment name	TIC (ions)
SDP-1	1.29 X 10^9
SD-1	9.85 X 10^8
SD-2	1.17 X 10^9
SDS2W1	2.65 X 10^9
SDS2W2	3.11 X 10^9
SD-DRY	1.36 X 10^9
WD1	1.81 X 10^8

The initial results on the standard Clevenger apparatus were determined on much smaller amount of leaves and extracted under different conditions, the TIC cannot really be compared as every one of the TIC in the initial clevenger experiments would be much lower than the BP apparatus. Unfortunately the graphs of the initial results from the Clevenger apparatus and the BP apparatus could also not be compared directly because the same column that was used in the GC-MS during the initial sample analysis was not available. The major constituents identified in the different extraction methods can still be compared.

The predominant constituents identified from the initial Clevenger apparatus study using steam distillation was α-citral, alpha citral, carophyllene, α-carophyllene, alloaromadendrene, β-cuvebene, λ-gurjunene and juniper Camphor. From which carophyllene and β-cuvebene make up the greatest percentage composition.

The predominant constituents identified from the BP apparatus in all the steam distillation experiments (SQ1, SQ2, SQ3) were: γ- terpenine, δ-elemene, carophyllene, γ-cadinene, α-carophyllene, germacrene D, geramacrene A, germacrene B, 2-naphthalenamine, n- methyl and squalene. (In blue in Table 3.8) Carophyllene and germacrene D make up the greatest percentage composition (62.73 %).

The common predominant constituents in both experiments were carophyllene and α-carophyllene. Alloaromadendrene and β-cuvebene was present in the BP apparatus samples however in much small quantities than in the Clevenger experiments. λ-Gurjunene, citral and α-citral were all present in the steam distillation using the Clevenger apparatus, however it was not present in the BP apparatus samples. γ- terpenine,, γ-cadinene, germacrene D, geramacrene A, germacrene B, 2-naphthalenamine,n-methyl and squalene were all detected in the samples from the BP apparatus and not the Clevenger apparatus. δ-elemene and γ-elemene was present in both the clevenger and the BP apparatus samples.

The other constituents identified in significant quantities from the Clevenger apparatus steam distillation samples were myrcene, terpenine, linalol, citral ,neral, α-ciral, β-bisobolene, carophyllene oxide, 2-(4a8-Dimethyl-1,2,3,4,4a,5,6,8a-octahydro-2-napthalenyl-2-propanol), juniper Camphor, tau muurol, α-cadinol and phytol. The other significant constituents identified from the BP apparatus were β-elemene, aromadendrene, carophyllene oxide, isocarophyllene, bicylcogermacrene, +(-)-4-carene, β-ionone epoxide and phytol.

When the water distillation Clevenger apparatus results were compared, it can be seen that the major constituents were also carophyllene and α-carophyllene. In the water distillation experiments on the BP apparatus they were carophyllene and germacrene D.

The other constituents identified in the water distillation experiments using the clevenger apparatus were myrcene, terpenine, linalol, citral, β-bisobolene ,λ-gurjunene, juniper camphor, α-cadinol and phytol.

The other significant constituents identified from the BP apparatus were β-pinene, β-myrcene, γ-terpenine, δ-elemene ,β-elemene, γ-cadinene, aromadendrene, napthalene 1,2,4a,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-,δ-cadinene,germacrene B, carophyllene oxide, β-ionone epoxide and 2-naphthalenamine. In the clevenger apparatus setups and the BP apparatus γ-elemene and allo-aromadendrene were present.

The reason there were variations in the composition between the oil obtained from the BP apparatus and the initial Clevenger experiments can be due to the different extraction methods. Each different method of extraction has a different amount of heat and energy and this can cause bonds in the oil constituents to break and new ones form. It can cause rings to close or open and therefore changing the composition of the oil. This was most probably what happened in the case of the major constituents germacrene D and β-cuvebene. Also the plants used for the initial Clevenger apparatus were harvested from a different area than the ones

used for the BP apparatus. This could change the amount of constituents present as well as the type present depending on the sunlight, how much water they get and if the soil they grow in is not polluted etc. The other reason for the difference can that can be noted is that in the BP equipment experiments a large amount of leaves were used (about 100 g) whereas in Clevenger apparatus experiments it was a lot less (about 10 - 40 g). In the BP apparatus experiments, more components were identified than in the Clevenger apparatus experiments. The more leaves used, the greater chance of certain constituents being present. The design of apparatus can also be a factor, for example, if the equipment has large bends, or a longer path to travel before reaching the distillate flask, some oil components can get lost along the way. Another reason could be that the GC-MS column used for the BP apparatus was different from the one used initially, so some of the components with a short elution time might not be detected as clearly on the other column. There are various factors that can determine the composition of the essential oil. It is difficult to sometimes isolate these factors from each other, since many are interdependent and influence one another. These variables may include seasonal and maturity variation, geographical origin, genetic variation, part of the plant used, growth stages, and post-harvest drying and storage.

3.2.7 Comparison of the results obtained using the BP apparatus to previous similar studies

Table 3.22 shows the average percentage composition of the oil from the water and steam distillations and a comparison was made to similar studies that have been carried out in South Africa (Oyediji, 2004) and another study on C*entella Asiatica* in Nepal. (Devkota et al. , 2013)

The steam distillation results on fresh leaves was compared to the literature study done in South Africa and it can be seen that 14 constituents out of forty two constituents in steam distillation were the same. The predominant constituents in

the study by Oyediji (2004) were α-carophyllene (21.06%), carophyllene (19.08%), bicyclogermacrene (11.22%), germacrene B (6.29%) and myrcene (6.55%).

In this investigation the predominant constituents were determined to be germacrene D (40.59%), carophyllene (22.14%), α-carophyllene (3.96%) and germacrene B (3.36%). Only three of the five major constituents from the previous study were identified in this steam distillation study. Bicyclogermacrene was present in the steam distillation samples but in a small amount (1.13%). The constituents that were the same in both studies were carophyllene, α-carophyllene and germacrene B. Carophyllene was present in greater amounts in this steam distillation study. α-Carophyllene and germacrene B were however present in the South African study by Oyediji (2004) in much greater amounts.

In the water distillation study the predominant constituents were identified as germacrene D (34.82%), carophyllene (24.53%), α-carophyllene (3.09%), δ-elemene (2.98%) and germacrene B (2.25%). Only three of the five major constituents from the BP apparatus results were the same. Myrcene and bicyclogermacrene were identified in the water distillation samples but in small amounts of 0.59% and 0.14%, respectively, compared to the similar South African study by Oyediji (2004).

In the Nepal study the chemical composition of the essential oil from *Centella Asiatica* by hydrodistillation was investigated in three habitats namely shady grass land, open grass land and open agricultural land. The percentage composition of the oil from leaves grown in South Africa to the ones from Nepal. Both results from Nepal were shown to compare the composition of the oil in shady and open areas (Table 3.22).

Table 3.22 Summary of the average percentage composition of the C*entella Asiatica* oil present in the samples obtained from the steam distillation and water distillation experiments using fresh leaves and a comparison to a similar study conducted in South Africa and a study on *Centella Asiatica* outside South Africa

Constituents	Steam Distillation (AVG % composition)	Water Distillation (AVG % composition)	South African similar study (% composition)*	Nepal similar study on Centella asiatica (% composition)**	
				shady	open
β-Pinene	-	0.54	0.37	-	0.02
Myrcene	-	0.59	6.55	-	-
α-Phellandrene	-	-	0.19	-	-
α-Terpinene	0.09	0.55	0.47	-	-
ρ-Cymene	0.36	0.34	0.71	-	0.08
Limonene	0.08	0.24	1.00	-	-
γ-Terpinene	3.19	4.70	5.77	-	-
Terpinolene	0.17	0.34	-	-	-
Trans-3-Nonen-2-one	0.08	-	0.49	-	-
Isoterpinolene	0.35	0.24	-	-	-
δ-Elemene	2.37	2.98	-	-	-

Copaene	0.53	0.40	-	0.54	0.37
α-Cubebene	0.27	0.27	-	-	1.24
β-Bourbonene	0.41	0.45	-	-	-
β-Elemene	1.71	1.77	1.99	5.01	3.93
Caryophyllene	22.14	24.53	19.08	24.2	7.5
γ-Elemene	0.76	1.21	1.58	1.05	-
Isoledene	0.11	0.45	-	-	-
γ-Cadinene	1.69	0.71	-	-	-
Bicyclo[4.4.0]dec-1-ene, 2-isopropyl-5-methyl-9-methylene-	0.46	0.40	-	-	-
Aromadendrene	0.80	1.03	0.20	0.78	0.43
α-Caryophyllene	3.96	3.09	21.06	0.05	17.09
***Allo*-Aromadendrene**	1.35	1.28	0.45	0	0.73
Naphthalene, 1,2,4a,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-	0.13	0.83	-	-	-
Ocimene	-	0.10	-	-	-
Germacrene D	40.59	34.82	4.01	1.82	2.5
Bicyclogermacrene	1.13	0.14	11.22	-	-
β-Gurjunene	0.50	0.29	-	0.55	0.13
α-Elemene	0.11	0.45	-	-	-
β-Cuvebene	0.56	0.46	-	-	-

Isocarophyllene	0.69	0.22	-	3.01	5.24
Germacrene A	0.70	1.06	0.24	-	-
γ-Muurolene	0.09	0.36	-	-	-
β-cadinene	-	0.28	-	-	-
δ-cadinene	0.42	1.44	0.34	-	-
(+)-4-Carene	1.18	0.98	-	-	-
Germacrene B	3.36	2.25	6.29	-	-
α-Muurolene	-	0.12	-	-	-
Nerolidal	-	0.18	-	-	-
Carophyllene oxide	0.65	0.52	1.66	0.56	7.68
1,7,7-Trimethyl-2-vinylbicyclo[2.2.1]hept-2-ene	0.41	0.52	-	-	-
y-Gurjunene	-	0.16	-	-	-
Humulene epoxide	-	0.14	0.27	-	-
Juniper camphor	0.42	0.43	-	-	-
.-(-)Spathulenol	0.53	0.41	1.44	0.47	0.8
Allo-Aromadendrene oxide	-	0.08	-	-	-
Seychellene	-	0.18	-	-	-
α-Cadinol	0.10	0.31	-	-	-
Isoaromadendrene epoxide	-	0.12	-	0.2	1.46

Viridiflorol	0.21	0.30	0.26	-	0.56
β-Ionone epoxide	1.29	1.08	-	-	-
Phytol	0.79	-	-	-	-
Pinane	-	0.37	-	-	-
n-Hexadecanoic acid	0.12	-	-	-	-
2 -Naphthalenamine, N-methyl	1.94	2.76	-	-	-
Ethanone,1 -(2,5-dimethyl-4-phenylfuran-3-yl)-	-	0.38	-	-	-
Squalene	1.82	0.45	-	-	-
Total	98.60	98.29	85.64	38.24	49.76

* data obtained from Oyediji (2004)

** data obtained from Devkota et al. (2013)

[a] Constituents are listed in order of elution time on the Zebron-ZB 274305 column

[b] Retention times calculated on Zebron-ZB 274305capillary column

[c] Relative area percentage: peak area relative to total peak area percent

If the South African study was compared to the Nepal study it can be seen that α-carophyllene in the shady areas was found at 0.05% whereas in the open areas at 17.01%. This shows that α-carophyllene production was favoured in an area with high light intensity. When α-carophyllene was compared in the South African study (21.06%) to the steam distillation results of 3.96%, it can be seen that a possibility for this difference could be because the plant that was used for the distillation was from a more shady area whereas in the South African study could have been from a higher light intensity. Consequently having a greater amount of α-carophyllene. The same reason would also be for the water distillation study where α-carophyllene was present at lower values of 3.09% compared to 21.06%.

When the percentage composition of carophyllene was compared in the experiments, it was found in the greatest amounts in the water and steam distillation samples (24.53%, 22.14%) compared to the South African study (19.08%). When these results were compared to the Nepal study it can be noted that carophyllene production was favoured in shaded areas (24.2%) than open areas (7.5%).

Germacrene D is a major constituent of the water and steam distillation samples. Whereas in the South African study α-carophyllene was the major constituent and in the Nepal study carophyllene was the major constituent. When the amount of germacrene D in the water and steam distillations were compared, germacrene D was present at 34.83% and 40.59% respectively. However in the South African (4.01%) and Nepal (shade: 1.82% and open: 2.5 %) studies, it was present in much lower amounts.

When germacrene B and γ-terpenine from the similar study by Oyediji (6.29%, 5.77%) were compared to this study, the constituents were present in a greater percentage than in the steam distillation samples (3.36%, 4.70%) as well as the water distillation samples (2.25%, 3.19%).

β-Elemene and γ-elemene in the steam (1.71%, 0.76%) and water distillation experiments (1.77%, 1.21%) were then compared to the South African study (1.99%, 1.58%). There was a slightly greater amount in the South African study than in the water and steam distillation samples. When these constituents were compared to the ones observed in the results from Nepal, β-elemene and γ-elemene were found in greater amounts in shady areas (5.01%, 1.05%) than in open areas (3.93%, 0.00 %).

When δ-elemene, γ-cadinene, 2-napthalenamine,n-methyl and squalene was compared to the similar studies it was observed that these compounds were not detected in the similar studies. When δ-cadinene in the three experiments were compared, it was present in the smallest amounts in the South African study and in greatest amount in water distillation sample.

Out of forty two constituents identified in the South African study results only 14 constituents were the same as the steam distillation results. Both studies identified the following major constituents: carophyllene , α-carophyllene and germacrene B. However, although this study was done on *Centella Asiatica* leaves growing in South Africa, the composition of the oil differs greatly. The major constituent between the two studies were very different. Steam distillation had germacrene D as its major constituent and the South African similar study identified α-carophyllene. One of the reasons for this difference in the composition seen could be because the South African and Nepal study use hydrodistillation where steam distillation was used for the results. The difference in distillation methods can alter bonds, and form new ones to from different constituents.

The similarities in the results between water distillation and the South African study was compared and it can be seen that 21 constituents were identified from the fifty four constituents in the water distillation experiments. Both studies identified carophyllene, α-carophyllene and germacrene B as the same predominant constituents. Biyclogermacrene and myrcene were present in both water distillation and the South African study. The reason more constituents in the

water distillation sample were the same as the South African study could be because the same distillation type (i.e. water distillation) was used for both studies. However the major constituent of the water distillation experiment (germacrene D) varied from the South African study (α-carophyllene).

One of the major reasons that there was a difference observed in the major constituents in the experiments could be because of geographical variation. These differences could be due to varied soil textures and possible adaption response of different populations, resulting in different chemical products being formed, without morphological differences being observed in the plants. The difference in altitude between locations can affect the composition of the oil. (Schumacher et al., 1990) The other reason could be due to genetic variations between the plants. However, there were many other factors as well that can contribute to a difference in oil composition observed. The season in which the leaves were harvested and the life stage of the plant, length of exposure to sunlight , the availability of water and presence of fungal diseases and insects can also affect the composition of the oil.

In the South African similar study, 42 constituents were identified which contained high amounts of sesquiterpenoid hydrocarbons (68.80%), monoterepenoid hydrocarbons (20.20%), oxygenated monoterpenoids (5.36%), oxygenated sesquiterpenoids (3.90%) and sulfide sesquiterpenoids (0.76%).

When the average percentage composition column for the steam distillation experiments and the water distillation experiments were compared to the South African study (Tables 3.9 and 3.13). From the tables, it can be deduced that essential oil from the South African similar study had higher amounts of monoterpenoid hydrocarbons (20.20%), oxygenated monoterpenoids (5.46%) and oxygenated sesquiterpenoids (3.90%) present which was much higher than in the steam distillation (5.42%, 0.08%, 1.91%) and water distillation experiments (8.62%, 0.00%, 2.49%). However water (81.63%) and steam distillation (84.83%)

experiments had a greater amount of sesquiterpenoids compared to the South African study (68.80%).

The differences between the similar study in South Africa was probably mainly due to a different geographical location, as well as a number of factors which could all affect the composition of the oil collected. Such as maturity of the leaves, seasonal changes, day and time harvesting was done, genetic variation, insects, soil type, water availability and light intensity received by plant.

3.2.8 Commercial (synthetic) oil versus natural oil

A commercial product of the essential oil was obtained and also analysed by GC-MS and the chromatogram obtained was compared to the steam distillation on fresh leaves (42 g) (SDP1).

From figure 3.28, the commercial product (bottom graph) of the essential oil obtained was compared to the steam distillation with distilled water (45 g). The commercial product obtained was ordered from a company that usually uses the commercial product in the perfumery industry and it contains many alcohols which the natural product does not contain.

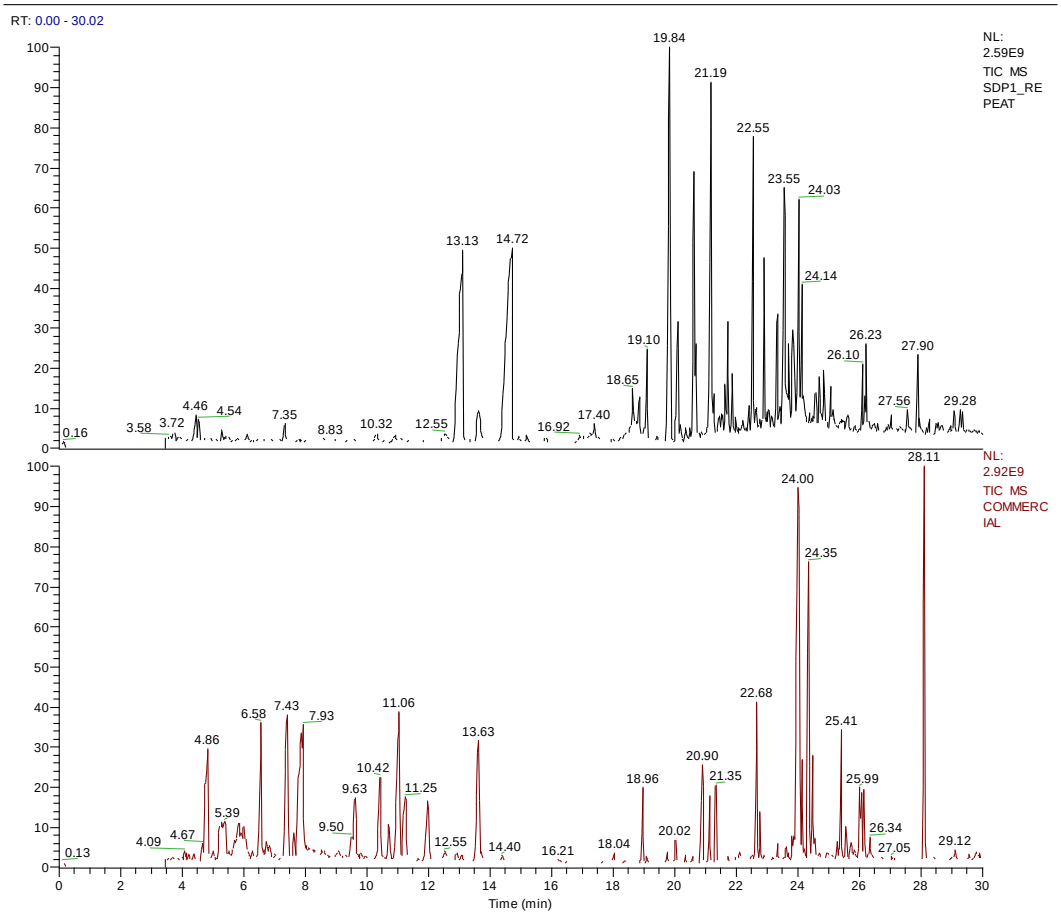


Figure 3.28 Comparison of the gas chromatogram of SDP1 (graph at the top) with a commercial *Centella Asiatica* oil.(graph at the bottom)

The only correlation between the two graphs were the peaks at 24.03 min in SDP-1 and 24.00 in the commercial sample, the rest of the retention times were completely different. The peak at 24.03 min represents alpha-cadinol. Since the height of the peaks and the retention times between the two graphs were very different, the commercial product was entirely synthetic and varies greatly from the natural product (top graph). The so-called 'natural identical' product and the naturally occurring essential oil were of an entirely different character as it can be seen by comparing the above graphs. This was reflected in their relative costs of the synthetic essential oils which are much cheaper to produce than the genuine ones.

The reason most essential oils are produced synthetically is because the perfumery and flavouring industries require continuity in their products and naturally occurring substances are always subject to change, due to seasonal changes etc. Even the extraction processes as shown in the table 3.22 vary the composition and concentration of constituents. Many aromatic oils like *Centella* contain a relatively small number of major constituents, several minor constituents and also a very large number of trace elements (top graph). To reconstruct such a complex combination of components including all trace elements would be virtually impossible.

It is the specific combination of constituents in a real essential oil, including the trace elements, which give its value therapeutically. The reason for this might be that these minute amounts of trace elements have synergistic or controlling effect on the main ones. Therefore the comparison of the graphs prove that synthetic oils cannot be used therapeutically as substitutes for the naturally occurring essential oils.

The steam distillation gas chromatograms obtained with the BP apparatus was not compared to the synthetic one. This was because the synthetic sample was performed using a different column on a different GC-MS. The retention times of the two graphs were as a result different and would not correlate. However, the components of the steam distillation study was compared to the initial SDP-1 constituents. There were some similarities between the SDP-1 and the steam distillation constituents, so if SDP-1 varies greatly from the synthetic one, and similarities were found between the steam distillation samples, it can be assumed that the steam distillation sample would also vary greatly from the synthetic oil.

3.3 The effect of heavy metals in the soil of *Centella Asiatica* plants

The soils of 12 *Centella asiatica* plants planted in separate pots were spiked with lead, mercury and chromium (VI) and three of them were the control pots. Three which were spiked with 10 ppm of lead (II), were named Pb1, Pb2 and Pb3. The other three were spiked with 10 ppm of mercury (II) and were named Hg1, Hg2 and Hg3. The final three were spiked with a 200 ppm of Cr(VI) and were named Cr1, Cr2 and Cr3.

After a week, the soil was collected using the conical method to achieve homogeneity in the sample. (Schumacher et al. ,1990) The soil samples were then digested and analysed using the ICP-OES. The results in tables below summarise the concentration of heavy metals detected in the soil samples. The results were analysed statistically using excel. Three soil samples from each pot were taken.

Table 3.23 The concentration of heavy metals: chromium, mercury and lead in the soil samples from the control pots

	Cr		Hg		Pb
Soil samples from control pots	mg kg^{-1}	Std. dev of sample	mg kg^{-1}	Std. dev of sample	mg kg^{-1}
Control 1	**68.02**	2.34	**nd**	-	**nd**
Control 2	**66.23**	0.18	**nd**	-	**nd**
Control 3	**70.60**	0.67	**nd**	-	**nd**

*nd - not detected *Std. dev - standard deviation

When the concentration of the chromium in Table 3.23 were compared in the control pots, it can be seen that even though this soil was not spiked, it already had a high concentration of chromium. The average concentration in the three pots was calculated to be 68.28 ± 1.41 mg kg^{-1} (Table 3.25). The natural concentration

of chromium in soil in the world was recorded to be between 10 - 50 mg kg^{-1}. (Shanker et al. ,2005) The reason for having a slightly higher concentration of chromium is due to the extensive use of chromium in the industrial industry, especially in South Africa possessing 90% of the world chromium ores. It can also be seen that no mercury and no lead were detected in the control samples.

The mean of triplicate samples of each pot were then taken and summarised in table 3.24 (Appendix K)

Table 3.24 The average concentration of heavy metals: chromium, mercury and lead detected in the soil from each pot

	Chromium (mg kg^{-1})		Mercury (mg kg^{-1})		Lead (mg kg^{-1})	
Experiment names	Mean	Std. dev	Mean	Std. dev	Mean	Std. dev
Control	68.28	1.41	nd	-	nd	-
Soil Pb 1 experiments	21.84	1.10	0.00	-	nd	-
Soil Pb 2 experiments	28.53	1.07	0.00	-	nd	-
Soil Pb 3 experiments	21.00	1.94	0.00	-	nd	-
Soil Hg 1 experiments	82.09	0.94	3.80	3.44	nd	-
Soil Hg 2 experiments	49.37	2.14	18.02	0.63	nd	-
Soil Hg 3 experiments	71.22	0.53	66.5	1.91	nd	-
Soil Cr1 experiments	143.96	1.01	30.80	1.12	nd	-
Soil Cr 2 experiments	184.16	0.55	29.40	1.48	nd	-
Soil Cr 3 experiments	227.67	1.01	27.9	1.91	nd	-

*nd - not detected *Std. dev - standard deviation

The average concentration of lead, mercury and chromium detected in the soil in all the experiments was the calculated and summarised in table 3.25.

Table 3.25 The average concentration of heavy metals: chromium, mercury and lead detected in the soil from each pot

	Chromium (mg kg^{-1})		Mercury (mg kg^{-1})		Lead (mg kg^{-1})	
Experiment names	Mean	Std. dev	Mean	Std. dev	Mean	Std. dev
Control	68.28	1.41	nd	-	nd	-
Lead experiments	23.79	1.43	nd	-	nd	-
Mercury experiments	67.56	1.38	29.44	2.30	nd	-
Chromium experiments	185.27	0.88	29.37	1.54	nd	-

*nd - not detected *Std. dev - standard deviation

No lead in all the experiments was detected even in the lead experiments where the soil was spiked with lead. The obvious reason for this could be that some of the lead was taken up by the plant roots but this wasn't the case from the analysis of the leaves that was done (Table 3.26), it can be seen that no lead is detected in the leaves so it could not have been taken up into the leaves. The roots were unfortunately not analysed to find out if they could contain some lead. As it has been reported in previous studies, roots of *Centella asiatica* tend to bind more heavy metals followed by the stems and leaves. (Yap et al. , 2010) Lead is naturally present in soils generally in the range of 10 to 40 mg kg^{-1} (Herselman, 2007). However, even in the control soil sample no lead was detected. The soil was bought from a nursery and could have had the amount of heavy metals controlled. However in the soil that was spiked with 10 ppm of lead, no lead was also detected. Most lead is retained strongly in soil, preferring to form relatively stable organic-metal complexes or chelates with soil organic matter, or adsorbs onto mineral surfaces and lead would therefore be expected in the soil samples. These processes are however dependant on factors such as pH, soil type and particle size, organic matter content of soil and the amount of lead in the soil. (Papanikolaou et al. , 2005) In acidic conditions lead solubility increases and if the soil was acidic some could have been lost due to experimental error, such as if the leachate collected overflowed with water some lead could have been lost.

There could also have been an error in the sampling process of the soil, the conical method has been reported to have high standard deviations. (Schumacher, 1990) Therefore some of the lead could have accumulated in a certain part of the soil and because only such a small sample of soil is needed, even with the homogenization technique, some of the lead could have been in another part of the soil, or in such small quantities throughout the soil that it could not be detected. In the analyses of the leaves from the experiments (Table 3.26) it can be seen that no lead was detected in the leaf samples.

Table 3.26 The average concentration of chromium, mercury and lead detected in the leaves harvested from each experiment (Appendix L)

	Chromium (mg kg^{-1})		Mercury (mg kg^{-1})		Lead (mg kg^{-1})	
Experiment names	Mean	Std.dev	Mean	Std. dev	Mean	Std .dev
Control	16.67	3.94	nd	-	nd	-
Lead experiments	19.37	3.53	10.38	2.21	nd	-
Mercury experiments	26.48	2.55	12.15	2.70	nd	-
Chromium experiments	33.12	4.65	nd	-	nd	-

In the mercury experiments the soil was spiked with 10 ppm of Hg. Natural levels of mercury in soil is reported to be between 0.05 to 0.08 μg g^{-1}. (Fan, 1987) Mercury was not detected at all in the lead spiked pots as well as in the control samples which concentration are therefore below the natural limit found in soil. As expected the mercury concentration in the spiked pots is the highest, however the high mercury content in the chromium experiments is not expected. The mercury level in both the mercury experiments and the chromium experiments are much higher than the natural limit found in soil and also greater than the concentration of 10 ppm used to spike the soil. The reason for a high concentration of mercury in the chromium pots could be due to cross contamination of the soil. The pots were placed relatively close to each other and

because mercury can shift into the gaseous phase quite easily, some could have been airborne from the top soil and deposited in some of the chromium pots (Azevedo and Rodriguez, 2012). It is also highly soluble in water and therefore could have also contaminated the water of the neighbouring plants and deposited in the soil. The control pots and the lead pots were kept further away from the mercury pots and hence no increase in the mercury content was detected. The other reason for a higher mercury concentration could be that the pots were kept in an area where coal is often burnt. Coal is one of the main anthropogenic sources of mercury and therefore could have also deposited on the plant or dissolved in the water used. Once it is deposited in the soil, certain microorganisms can change it into methyl mercury. Hence increasing the concentration of mercury detected. However mercury in the air can also be present due to industrial activities such as mining, and can settle onto soil or dissolve in water.

When the amount of mercury in the leaves of *Centella Asiatica* (Table 3.26) was compared, it can be seen that mercury was detected in the leaves harvested from the mercury experiments, as expected, with a concentration of 12.15 ± 2.70 mg kg^{-1} and also in the lead experiments with a concentration of 10.38 ± 2.21 mg kg^{-1}. These values are much higher than limit of 0.5 µg g-1 (µg g^{-1} = mg kg^{-1}). (Caldas et al. , 2003)

When the amount of chromium in the soil of the experiments (Table 3.25) are compared it can be seen that the greatest amount of chromium, as expected was detected in the chromium experiments with a value of 185.27 ± 0.88 mg kg^{-1}, then in the mercury experiments with a concentration of 67.56 ± 1.38 mg kg^{-1} and the lowest amount was detected in the lead experiments 23.79 ± 1.43 mg kg^{-1}. The concentration of chromium in the spiked chromium experiments are much greater than the natural concentration. Depending on the oxidation state of the chromium either Cr(III) which is less mobile or Cr(VI) which is more mobile, will depend on how this high concentration of chromium in the soil will affect the plant. High concentrations of chromium in plants can alter plant metabolism, it can alter

germination processes as well as growth of leaves, roots and stems and alter essential oil content. (Shanker et al. , 2005)

The concentration of the chromium in the mercury experiments is slightly higher than the normal concentrations of chromium reported in soil of 10 to 50 mg kg^{-1}, but it is also very similar to the control chromium concentration of 68.28 ± 1.41 mg kg^{-1}. Which means no extra chromium was found in the soil than what the pot started off with.

When the amount of chromium in the lead experiments were compared it can be seen that it is within the natural concentration of chromium in soil with 23.79 ± 1.43 mg kg^{-1}. The reason this amount is less than the control could be due to a greater amount of mobile Cr(VI) was present and because it is more mobile it can be easily leached out and if over watered then it could be lost. There could also have been experimental error during sampling, where taking a completely accurate homogeneous sample was not entirely possible because of the small size of sample needed. Some chromium could have also been taken up by the plant.

When the concentration of chromium taken up by the *Centella asiatica* plants it compared (Table 3.26), it was observed that the greatest amount was detected in the chromium experiments as expected (33.12 ± 4.65 mg kg^{-1}), followed by the mercury experiments (26.48 ± 2.55 mg kg^{-1}) and then in the lead experiments (19.37 ± 3.53 mg kg^{-1}). This could be due to the greater amount of chromium present in the soils of the chromium experiments which were greater than the mercury experiments, which was greater than the lead experiments. The lowest concentration was however seen in the control leaves (16.67 ± 3.53 mg kg^{-1}), which had a greater chromium concentration in the soil than the lead experiments. A number of factors can affect the amount of chromium present in the leaves. Chromium is a toxic, nonessential element to plants. Plants therefore do not have specific mechanisms for its absorption. It is though that the plant takes up this heavy metal through carriers used for the absorbtion of essential metals for plant metabolism. The toxic effects of Cr are primarily dependent on the metal

speciation, which determines its uptake, translocation and accumulation. The use of metabolic inhibitors diminished Cr(VI) uptake whereas it did not affect Cr(III) uptake, indicating that Cr(VI) uptake depends on metabolic energy. (Shanker et al., 2005) The intracellular uptake in the leaves of *Centella asiatica* can also be limited by the nature of the metal ion, cell membrane permeability, and the concentration of extracellular ligands with an affinity for cations. (Mandiwana et al., 2006)

This analysis showed that the leaves of the different experiments took up some of the heavy metals, and therefore the leaves were harvested to see how the heavy metal content would affect the essential oil yield and composition.

3.3.1 The composition of the essential oil from the *Centella asiatica* leaves harvested from the pots spiked with heavy metals

As discussed in the earlier chapter, the steam distillation yield a better TIC. Therefore, this method was chosen to investigate how the heavy metal contamination in the soil of the *Centella asiatica* can affect the essential oil yield and composition.

All the steam distillation experiments on the leaves harvested from heavy metal contaminated soil were performed using setup 6 on fresh leaves at a distillation rate of 2/3 ml min^{-1} and 0.4 ml of initial xylene. Water distillation was performed for 30 minutes before leaves were added to false bottom extraction container. The conditions and TIC results from the chromium, mercury and lead spiked pots are shown in Table 3.27, Table 3.28 and Table 3.29, respectively.

Table 3.27 Steam distillation experiments using setup 6 on fresh leaves from Cr(VI) contaminated pots

Experiment name	Weight of leaves (g)	Average size of leaves (mm)	Distillation time (min)	TIC
Cr1	14.78	43.85	75	2.36E+09
Cr2	18.30	44.82	75	2.32E+09
Cr3	8.60	35.60	75	2.06E+09

Table 3.28 Steam distillation experiments using setup 6 on fresh leaves from Hg(II) contaminated pots

Experiment name	Weight of leaves (g)	Average size of leaves (mm)	Distillation time (min)	TIC
Hg1	11.54	36.35	75	3.69E+09
Hg2	13.68	45.31	75	2.01E+09
Hg3	14.80	35.71	75	1.70E+09

Table 3.29 Steam distillation experiments using setup 6 on fresh leaves from Pb(II) contaminated pots

Experiment name	Weight of leaves (g)	Average size of leaves (mm)	Distillation time (min)	TIC
Pb1	19.55	36.39	75	1.22E+09
Pb2	21.81	46.22	75	2.23E+09
Pb3	9.20	51.63	75	1.01E+09

Unfortunately in these experiments, the study was limited by the number of leaves available in one pot that was spiked, which was much smaller (8.6 - 21.81 g) compared to the average of around 100 g of leaves used in each of the steam distillation experiments using leaves from non-contaminated soil. In order to have

around 100 g of leaves whole flower beds would have to be spiked and that was not possible.

Chromium

Once steam distillation was performed on leaves from the chromium spiked pots, the oil collected was analysed and the graphs from the GC-MS are shown in Fig. 3.29. Visually all three graphs have a similar number of peaks. However, by comparing the total ion current (TIC) of the three experiments (Table 3.27), it can be seen that graph A has 2.36 x 10^9 ions which was greater than graph B and graph C with 2.32 x 10^9 ions and 2.06 x 10^9 ions, respectively. This shows that the greatest concentration of ions in the oil collected, was from experiment Cr1 than the concentration of ions collected in Cr2 and Cr3. Since the other parameters in the experiments were kept the same i.e. same distillation rate, amount of xylene and duration of experiment, they would not contribute to the variations observed in the results. Sample Cr3 had the smallest TIC, this was probably because only a small amount of leaves were available for extraction (8.60 g) compared to 14.78 g and 18.30 g used in Cr1 and Cr2 experiments, respectively. Some oil could also have volatised while the sample was collected from a beaker. If the TIC of the samples Cr1 and Cr2 are compared, the TIC of Cr1 was slightly greater than Cr2, however Cr2 sample used more leaves (18.30 g) than Cr1 (14.78 g). The size of the leaves used in Cr1 (43.85 mm) and Cr2 (44.82 mm) are very similar. The difference can therefore also be due to the amount of chromium present in the leaves (Table 3.30).

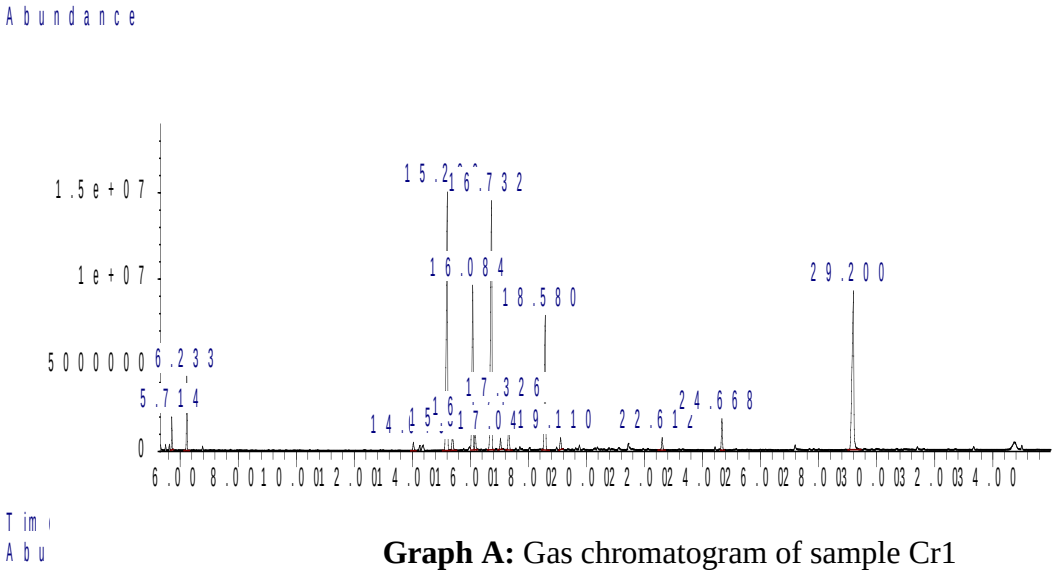
Table 3.30 Comparison of the concentration of chromium, mercury and lead found in the leaves from plants grown in the chromium spiked soil

	Cr		Hg	Pb
Leaves from Chromium pots	mg kg^{-1}	Std. dev of sample	mg kg^{-1}	mg kg^{-1}
Cr1	20.73	7.92	nd	nd
Cr2	31.65	1.15	nd	nd
Cr3	46.98	0.96	nd	nd

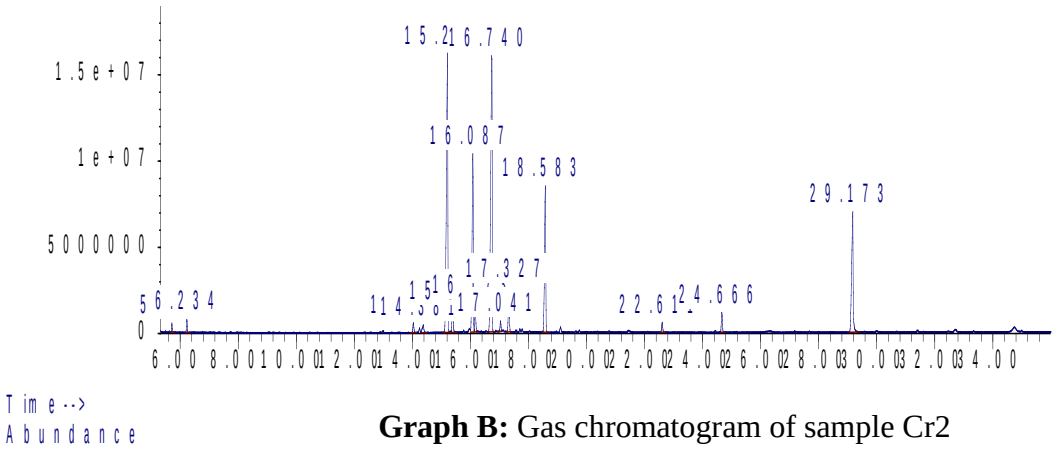
*nd - not detected *Std. dev - standard deviation

From Table 3.30 it can been observed that the highest concentration of chromium was present in the leaf Cr3 (46.98 ± 0.96 mg kg^{-1}) > Cr2 (31.65 ± 1.15 mg kg^{-1}) > Cr1 (20.73 ± 7.92 mg kg^{-1}). There was no mercury or lead detected in the leaves. This also makes sense since the soil in Cr1 had the lowest amount of chromium present in the soil, 143.96 ± 1.01 mg kg^{-1}, followed by Cr2 soil (184.16 ± 0.55 mg kg^{-1}) and then Cr3 soil with the greatest amount of chromium present (227.67 ± 1.01 mg kg^{-1}). This shows that the *Centella* plant does take up chromium into the leaves of the plant and the more chromium present in the soil, the greater the concentration of chromium in the leaves.

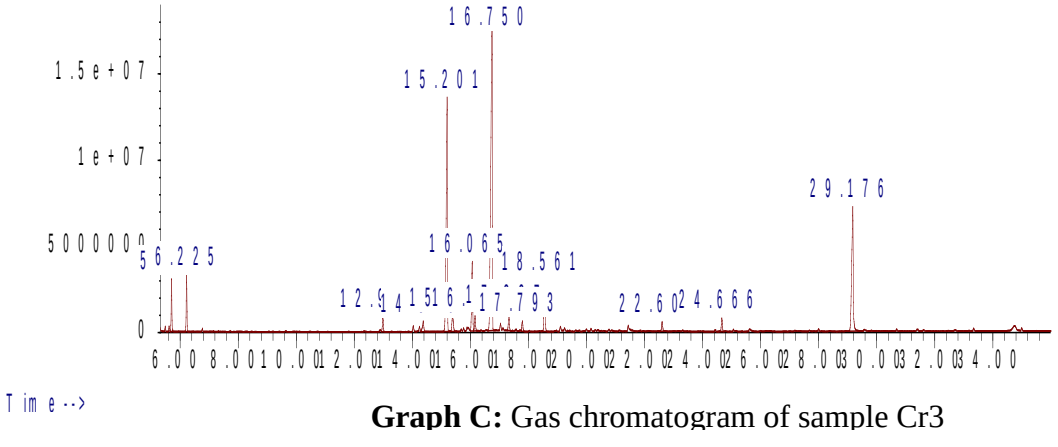
The TIC could have also been affected by the metal content in the *Centella Asiatica* plant and Cr1 with the lowest Cr contamination had the highest TIC, then Cr2 which had the second highest, and then Cr3 which had the highest Cr contamination and lowest TIC. Hence metal contamination could have affected the amount of ions collected. The greater the chromium contamination in the soil, the lower the TIC of the oil, and the hence the quality of the oil is negatively affected and the contamination could also affect the amount of essential oil produced by the plant.



Graph A: Gas chromatogram of sample Cr1



Graph B: Gas chromatogram of sample Cr2



Graph C: Gas chromatogram of sample Cr3

Figure 3.29 Comparison of the gas chromatograms of the Centella oil samples collected from steam distillation experiments Cr1, Cr2 and Cr3

Chromium can exist in 2 valence states, Cr(III) (less toxic) and Cr(VI) (more mobile and more toxic). Plants were spiked with Cr(VI). The toxic properties of Cr(VI) originated from the action of this form as an oxidising agent, as well as the formation of free radicals during the reduction of Cr(VI) to Cr(III) occurring inside the cell. Cr(III) can still be toxic in high concentrations by its ability to coordinate with various organic compounds resulting in inhibition of some metalloenzyme systems. (Shanker et al. , 2005) Chromium toxicity in plants can cause a low concentration of constituents in the oil and can affect the enzyme processes and reduce the growth of the plant. Therefore the difference in the TIC between Cr1 and Cr2 experiments can be due to the metal contamination. There are other factors such as favourable environmental factors for Cr1 and Cr2 plants and experimental errors. If for example, more xylene was collected in Cr2, it would decrease the concentration of oil collected. From Table 3.30 it can also be see that no mercury was present in the leaves of the chromium pots but there was mercury detected in the soil (Table 3.24). This can mean that the mercury was not taken up by the leaves of the *Centella Asiatica*. Mercury has been reported to accumulate in the roots of plants and sometimes in the shoots. In this study, focus was on the leaves and therefore samples of the roots and shoots were not taken, but it can be assumed that some mercury was taken up by the roots. (Azevedo and Rodriguez, 2012)

The mass spectrum for each major peak in the graphs were then analysed using the same method of comparison to the NIST library. Table 3.31 summarises the constituents present in the chromium spiked experiments.

From Table 3.32 the constituents that make up the greatest composition of the oil from *Centella asiatica* leaves in the steam distillation experiments using leaves harvested from plants grown in chromium spiked soil are sesquiterpenoid hydrocarbons (69.17 - 80.21%) among which germacrene D ranging from 20.80% (Cr1), 24.25% (Cr2), 35.06% (Cr3) and carophyllene 20.27%, 22.85%, 20.83% in Cr1, Cr2 and Cr3 respectively. Monoterpenoid hydrocarbons (1.22 -

6.26%) were also identified in the essential oil, such as γ- terpenine (0.59 - 3.10%) as well as oxygenated sesquiterpenoids (0.66 - 1.32%) such as carophyllene oxide (0.41 - 0.76%). β-ionone epoxide (0.66 - 0.82%), phytol (0.00 -0.15%) and 2-naphthalenamine,n-methyl (10.45 - 17.01%) were also identified.

The greatest amount of oxygenated sesquiterpenoids (2.26%) , 2-napthalenamine, n-methyl (17.01%) and β-ionone epoxide (0.82%) was observed in Cr1 oil samples (Table 3.32), followed by Cr3 (1.32%, 12.82%, 0.71%) and then by Cr2 (0.66%, 10.45%, 0.66%). Cr3 has the smallest average leaf size, the smallest amount of leaves used and the greatest chromium contamination but has a greater amount of these constituents than the Cr2 oil sample. This could be due to the smaller size of the leaves that could have a different composition than larger leaves. Cr3 pot could have had a more favourable environment than the Cr2 pot. The high chromium contamination could also alter the metabolism of the plant and therefore cause a higher amount of certain compounds to be present in the oil sample. Cr1 has a higher concentration of these constituents possibly because of the greater TIC.

When comparing the amount of sesquiterpenoids collected in each sample, the greatest amount was detected in Cr2 (80.21%) then in Cr3 (73.37%) and then in Cr1 (69.16%). The amount of monoterpenoid hydrocarbons were also compared and the greatest amount was present in oil sample Cr3 (6.26%) > Cr1(5.06%) > Cr2 (1.22%). Phytol was only present in Cr2 (0.15%). In all the steam distillations on leaves from chromium contaminated soils, no oxygenated monoterpenoids were detected. The reason for the differences could be due to the metabolism of the plant being affected by the heavy metal contamination but also could be due to factors such as environmental factors and any experimental errors discussed in previous chapter.

From Table 3.31, the constituents that were identified in each oil sample (Cr1, Cr2, Cr3) are compared. Forty three constituents were identified from the three different oil samples, accounting for 96.50% to 98.59% of the oil contents. In all the steam distillation experiments (Cr1, Cr2, Cr3) using leaves from plants grown in chromium contaminated soil the following constituents were the predominant ones: γ- terpenine, carophyllene, α-carophyllene, germacrene D, β-Bisobolene, germacrene B and 2-naphthalenamine,N-methyl. (In blue in Table 3.31)

When the percentage composition of α-carophyllene, β-Bisobolene and germacrene B are compared, it can be seen that these constituents are present in the highest concentrations in Cr2 oil samples (12.65%, 3.01%, 10.39%) followed by Cr1 (11.32%, 2.75%, 9.11%) and the least was detected in Cr3 oil samples (5.01%, 1.47% ,3.97%). The reason the least is detected in Cr3 oil samples could be due to the high chromium concentration in the leaves of Cr3 plant, which could alter the metabolism of the plant and it also has the lowest TIC, so therefore a much smaller amount of ions are available for analysis. The reason Cr2, with a lower TIC than Cr1, has a higher amount of these constituents than Cr1 could be due to the greater amount of leaves that were used for the Cr2 experiment (18.30 g) than the Cr1 (14.78 g) experiment. The greater number of leaves available the greater the chance of a higher amount of certain constituents being present.

Cr1 oil sample yielded a high percentage of γ- terpenine (3.10%) > Cr3 (2.72%) > Cr2 (0.59%). However carophyllene was detected at the highest percentage of 22.85% in Cr2 compared to Cr3 (20.83%) and Cr1 (20.27%). Germacrene D was found in the greatest amounts in the highest chromium contaminated leaves of Cr3 (35.06%), followed by Cr2 (24.25%) and then Cr1 (20.80%).

The other components that were identified in significant amounts were limonene and δ-elemene which were present in the greatest amounts in Cr3. γ-Elemene and *Allo*-aromadendrene were detected in the greatest amount in Cr2, and pinane was detected in Cr1.

Table 3.31 Comparison of the different constituents present in the samples obtained from steam distillation experiments using leaves from plants grown in chromium spiked soil Cr1, Cr 2 and Cr3 ([**bold**] - constituent present in significant value , [blue] - predominant constituent

	Cr 1		Cr 2		Cr 3		Average
Constituents [a]	**Retention time** [b]	**% Composition** [c]	**Retention time** [b]	**% Composition** [c]	**Retention time** [b]	**% Composition** [c]	**% Composition** [c]
α-Terpinene	5.50	0.22	-	-	-	-	0.07
β-Cymene	5.63	0.23	-	-	-	-	0.08
Limonene	5.72	1.32	5.72	0.36	5.71	2.54	1.41
γ-Terpinene	6.23	3.10	6.23	0.59	6.22	2.72	2.14
Terpinolene	6.77	0.19	-	-	6.77	0.17	0.12
δ-Elemene	-	-	12.99	0.10	12.99	1.04	0.38
Copaene	14.04	0.48	14.04	0.59	14.04	0.41	0.49
β-Bourbonene	14.25	0.31	14.25	0.31	14.25	0.36	0.33
β-Elemene	14.38	0.53	14.38	0.69	14.38	0.93	0.72
Caryophyllene	15.20	20.27	15.21	22.85	15.20	20.83	21.32
γ-Elemene	15.39	1.14	15.39	1.64	15.39	1.49	1.42
γ-Cadinene	-	-	-	-	15.77	0.22	0.07
Bicyclo[4.4.0]dec-1-ene, 2-isopropyl-5-	-	-	-	-	15.89	0.28	0.09

Aromadendrene	-	-	-	-	15.94	0.28	0.09
β-Farnesene	15.97	0.26	15.97	0.41	-	-	0.22
α-Caryophyllene	16.09	11.32	16.09	12.65	16.07	5.01	9.66
***Allo*-Aromadendrene**	16.17	1.40	16.17	1.65	16.16	1.04	1.36
Germacrene D	16.74	20.80	16.74	24.25	16.75	35.06	26.70
γ-Gurjunene	-	-	16.88	0.21	-	-	0.07
Bicyclogermacrene	17.04	0.65	17.04	0.78	17.04	0.58	0.67
β-cuvebene	-	-	17.13	0.23	17.13	0.30	0.18
β - Bisobolene	17.33	2.75	17.33	3.01	17.33	1.47	2.41
δ-Cadinene	-	-	17.57	0.21	17.58	0.23	0.15
β-Sesquiphellandrene	17.71	0.15	17.71	0.24	-	-	0.13
(+)-4-Carene	-	-	17.79	0.27	17.79	0.83	0.37
Germacrene B	18.58	9.11	18.58	10.39	18.56	3.97	7.82
Caryophyllene oxide	19.11	0.76	19.11	0.41	19.11	0.42	0.53
1,7,7-Trimethyl-2-vinylbicyclo[2.2.1]hept-	-	-	-	-	19.25	0.39	0.13
Humulene epoxide	19.76	0.32	-	-	-	-	0.11
Spathulenol	-	-	-	-	20.16	0.24	0.08
Isoaromadendrene epoxide	20.30	0.21	-	-	-	-	0.07
10,10-Dimethyl-2,6-	20.38	0.26	-	-	-	-	0.09

α-Cadinol	20.78	0.20	-	-	20.78	0.14	0.11
trans-z-alpha-bisabolene epoxide	21.44	0.51	21.44	0.25	21.44	0.52	0.42
β-Ionone epoxide	22.61	0.82	22.61	0.66	22.61	0.71	0.73
Pinane	24.67	1.84	24.67	1.24	24.67	0.92	1.33
1-Hexadecanol	-	-	-	-	25.63	0.36	0.12
Nonadecane	-	-	26.32	0.35	-	-	0.12
n-Hexadecanoic acid	27.19	0.48	27.18	0.11	-	-	0.20
2-Naphthalenamine, N-methyl-	29.20	17.01	29.17	10.45	29.17	12.82	13.42
Phytol	-	-	29.99	0.15	-	-	0.05
Eicosane	33.34	0.20	32.71	0.36	-	-	0.19
Tetracosane	34.73	1.76	34.75	1.09	34.74	1.42	1.42
Total		98.59		96.50		97.66	97.59

[a] Constituents are listed in order of elution time on the Zebron-ZB 274305 column

[b] Retention times calculated on Zebron-ZB 274305 capillary column

[c] Relative area percentage: peak area relative to total peak area percent

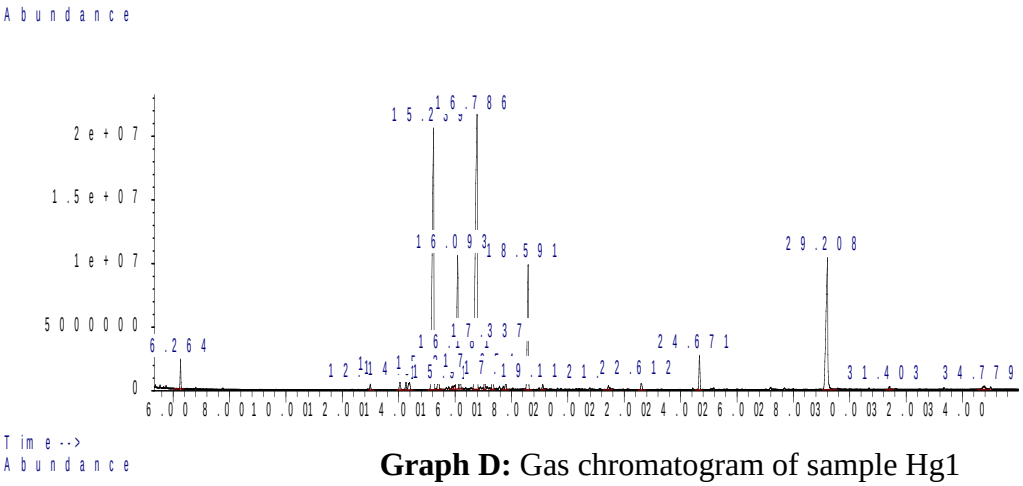
Table 3.32 Summary and comparison of the major constituents present in the samples obtained from the steam distillation experiments on leaves from chromium spiked soils

	Steam distillation experiments on leaves from chromium spiked soils						Average
	No. identified	Cr1 (%)	No. identified	Cr2 (%)	No. identified	Cr3 (%)	% composition
Monterpenoid hydrocarbons	5	5.06	3	1.22	4	6.26	4.18
Oxygenated monoterpenoids	0	0.00	0	0.00	0	0.00	0.00
Sesquiterpenoid hydrocarbons	13	69.16	17	80.21	17	73.47	74.28
Oxygenated sesquiterpenoids	6	2.26	2	0.66	3	1.32	1.41
β-Ionone epoxide	1	0.82	1	0.66	1	0.71	0.73
Phytol	0	0.00	1	0.15	0	0.00	0.05
2-Naphthalenamine, N-methyl	1	17.01	1	10.45	1	12.82	13.43
Other	4	4.28	5	3.15	3	3.08	3.50
Total	30	98.59	30	96.5	29	97.66	97.58

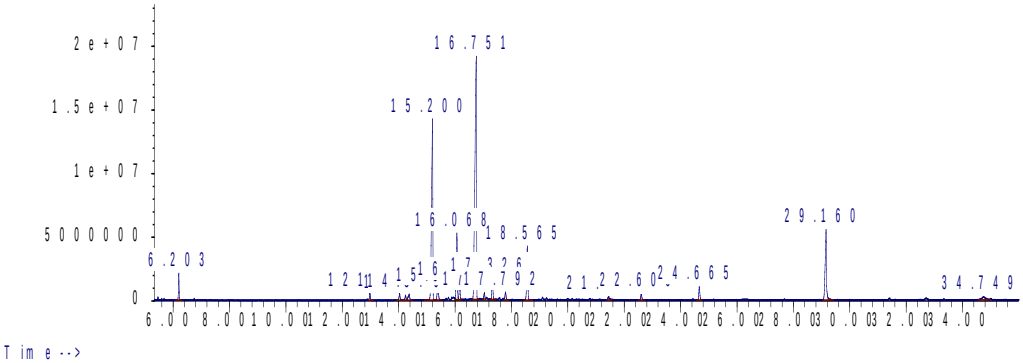
The reason for the difference in composition between the three experiments could be due to a higher than normal chromium concentrations which affect the plant by reducing photosynthesis, transpiration rate, and water uptake and chlorophyll synthesis. (Azevedo and Rodriguez, 2012) This in turn can affect the metabolism of the plant and normal essential oil production. There are also a number of other factors that could contribute to the difference in ratios in the essential oil constituents. For instance, if the chromium present in the plant is in the less toxic Cr(III) form or in the more mobile and toxic Cr(VI). Environmental factors can also affect the composition of the oil, for example soil quality, light intensity the plant gets, the water quality and the life stage of plant, which can affect the processes occurring in the plant and producing a different amount of constituents. There are also a number of experimental human errors that could have affected the concentration and composition, such as if an extra drop of xylene from the pipette was added, it would decrease the concentration of constituents, if some oil and xylene got stuck to the walls of the apparatus and were not collected and some oil could have vaporised. These factors would all lead to a decrease in amount of constituents present.

Mercury

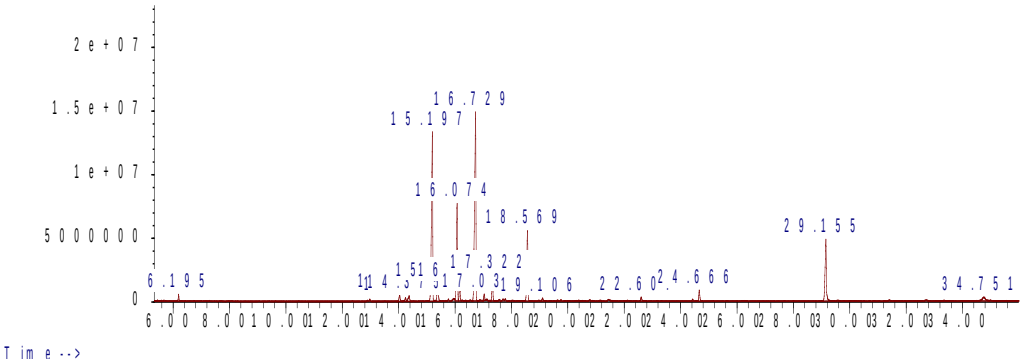
The chromatograms in Figure 3.30 and the TIC of the mercury samples (Table 3.28) are compared. The Hg1 oil sample had a greater number of ions detected with a TIC of 3.69×10^9 ions, followed by Hg2 with a TIC of 2.01×10^9 ions, then Hg3 with a TIC of 1.70×10^9 ions. This shows that the concentration of oil collected in experiment Hg1 was higher than the concentration of oil collected in Hg2 which was higher than Hg3. When the amount of leaves used in each experiment are compared (Table 3.28), it can actually be observed that Hg1 had the smallest amount of leaves (11.54 g) < Hg2 (13.68 g) < Hg3 (14.80 g). Also Hg1 and Hg3 had similar size leaves used (36.35 and 35.70 mm ,respectively) while Hg2 had a slightly larger average of leaves used (45.31 mm). None of these reasons would cause the Hg1 to have a higher TIC than Hg2. Therefore, the amounts of the



Graph D: Gas chromatogram of sample Hg1



Graph E: Gas chromatogram of sample Hg2



Graph F: Gas chromatogram of sample Hg3

Figure 3.30 Comparison of the gas chromatograms of the Centella oil samples collected from steam distillation experiments Hg1, Hg2 and Hg3

heavy metals chromium, mercury and lead in the leaves from the plants growing in the mercury contaminated pots were analysed.

Table 3.33 Comparison of the concentration of chromium, mercury and lead found in the leaves from plants grown in the mercury spiked soil

	Cr		Hg		Pb
Leaves from Mercury pots	mg kg^{-1}	Std. dev	mg kg^{-1}	Std. dev	mg kg^{-1}
Hg 1	**21.18**	2.82	**36.45**	4.67	nd
Hg 2	**19.40**	2.38	nd	-	nd
Hg 3	**38.86**	2.44	nd	-	nd

*nd - not detected *Std. dev - standard deviation

When the amount of chromium in the leaves harvested from the mercury spiked pots are compared (Table 3.33), the greatest amount was seen in leaf Hg3, 38.86 ± 2.44 mg kg^{-1}, followed by Hg1 with 21.18 ± 2.82 mg kg^{-1}, and the lowest amount in Hg2 with 19.40 ± 2.38 mg kg^{-1}.

When the concentration of mercury in the leaves from the mercury spiked pots are compared, mercury was only present in the Hg1 leaf with a concentration of 36.45 ± 4.67 mg kg^{-1}.

When this was compared to the amount of heavy metals present in the mercury spiked soils (Table 3.24), it can be seen that chromium concentrations were 82.09 ± 0.94 mg kg^{-1}, 49.37 ± 2.14 mg kg^{-1} and 71.22 ± 0.53 mg kg^{-1} in soil samples Hg1, Hg2 and Hg3, respectively. The lowest amount of chromium was in the Hg2 soil and the lowest amount of chromium detected in the leaves was in Hg2. Hg3 had the highest chromium concentration in the leaves, but Hg1 had the highest concentration of chromium in the soil. Therefore a higher amount of chromium would be expected in the Hg1 leaves. The reason that Hg1 could have a slightly smaller amount in the leaves could be that some of the chromium (Cr(VI)) in the soil of the Hg1 pot could have been reduced to the more immobile and less toxic Cr(III). Cr(VI) is rapidly reduced to Cr(III) when Fe(II) or Mn(II) occur in

groundwater. Cr (III) is thought to be taken up by cation exchange sites and a metabolic driven process takes up Cr(VI). Cr(VI) competes with various elements of similar electronic structure, therefore it seems that Cr(VI) has the benefit at the entry level into the plant system. (Shanker et al. , 2005) Hence in the Hg1 pot, less of the more mobile Cr(VI) could have been available for being absorbed by roots of the plant and translocated to the leaves. There are a number of factors that can affect the uptake of heavy metals into plants, including pH of the soil, salinity and presence of humic (organic) matter.

The amount of mercury detected in the soil (Table 3.24) was 3.80 ± 3.44 mg kg^{-1}, 18.02 ± 0.63 mg kg^{-1} and 66.5 ± 01.91 mg kg^{-1} in the Hg1, Hg2 and Hg3 samples, respectively. When this was compared to the amount of mercury present in the leaves it can be seen that only the leaf Hg1 had mercury present. The Hg1 soil sample has however the smallest amount of mercury present and the leaves from the pots with the greater amounts of mercury Hg2 and Hg3 had no mercury detected. The dynamics between the amount of Hg that exist in the soil and its uptake by plants is not linear and depends on several variables. These include cation exchange capacity, soil aeration, the pH of the soil and the plant species. (Azevedo and Rodriguez, 2012) The absorbtion can be reduced when the soils pH is high or there is a large quantity of lime or salts. (Abu- Darwish et al. , 2009) Most plants that tend to uptake Hg tend to accumulate it in the roots. Mercury released to the soil mostly remains in solid phase through adsorption onto clay particles, sulfides and organic matters. (Yadav, 2010) This could be the reason why there was mercury present in all the soil samples and in only one of the leaf samples. The reason mercury was detected in Hg1 leaf and not the other two leaves could be due to the pH of the soils and the lime and salt content. If the pH of the soil was higher in Hg2 and Hg3 and it had more lime and salts present than Hg1, the mercury would react with the lime and salts and decrease its availability for uptake in the plants. Depending on the redox potential of the soil, mercury can also be oxidised and later complex with major ligands (e.g. Cl^-,OH^-,S^{2-}) and dissolved organic carbon thus increasing the metal solubility. (Canela and Jardim,

1997) Consequently being available to be taken up by the plants. In the Hg1 leaf, mercury could have also been directly adsorbed onto the leaf from the air.

The difference in the TIC between the experiments can be therefore due to the metal contamination. Hg3 had the lowest TIC which could be due to the high chromium concentration in the leaves (38.86 ± 2.44 mg kg-1) and the high mercury concentration in the soil (66.5 ± 01.91 mg kg^{-1}). Mercury can specifically cause phyotoxicity in plants by a number of mechanisms such as affecting the permeability of cells membrane, its ability to favour reactions with phosphate and sulphydryl (SH) groups, by substituting the plants essential ions with mercury and its ability to disrupt functions involving important proteins. (Azevedo and Rodriguez, 2012) It can then disrupt essential oil production and alter the constituents present. Hg1 oil sample had a higher TIC than Hg2, even though no mercury was found in the Hg2 leaves. The reason for this could be that the mercury concentration in the Hg2 soil (18.02 ± 0.63 mg kg^{-1}) was higher than Hg1 (3.80 ± 3.44 mg kg^{-1}). As a result mercury seems to have a greater effect on the plants oil production if present in high amounts in the soil than in the leaves.

There are other factors such as environmental conditions that could have caused Hg1 to produce more oil than Hg2. Such as environmental factors, if the plant was grown in more sun and had more favourable environment for photosynthesis and oil production. All these factors are interdependent and can't be isolated from the other.

The mass spectrums for each major peak in the Figure 3.30 were then analysed using the same method of comparison to the NIST library. Table 3.34 summarises the constituents present in the mercury spiked experiments. Table 3.35 is a summary of the different classes of compounds present in the oil samples from leaves harvested from *Centella* grown in mercury contaminated soil.

From Table 3.35 the constituents that make up the greatest composition of the oil from *Centella asiatica* leaves in the steam distillation experiments using leaves

harvested from plants grown in mercury spiked soil are sesquiterpenoid hydrocarbons (80.27% - 86.27%) among which germacrene D ranging from 28.91% (Hg3), 31.47% (Hg1), 38.20% (Hg2) and carophyllene 22.72%, 22.98%, 24.79% in Hg2, Hg1 and Hg3 respectively. The other major constituent present is 2 Naphthalenamine, N-methyl with a percentage composition of 9.13%, 9.20% and 12.77% in oil samples Hg3, Hg2 and Hg1 respectively. These 3 constituents alone account for 62.83% (Hg3), 67.22% (Hg1) and 70.12% (Hg2) of the percentage oil composition. Monoterpenoid hydrocarbons (0.79 - 2.60%) were also identified in the essential oil, such as γ- terpenine (0.53 - 1.79%) as well as oxygenated sesquiterpenoids (0.56 - 0.75%) such as carophyllene oxide (0.23 - 0.35%). β-ionone epoxide (0.5 - 0.67%) and phytol (1.23 - 1.80%) were also identified.

The greatest amount of monterpenoid hydrocarbons (2.60%), oxygenated sesquiterpenoids (0.75%) and β-ionone epoxide (0.67%) was observed in Hg2 oil samples (Table 3.35), then in Hg1 oil samples (1.62%, 0.63%, 0.50%) and then in Hg3 (0.79%, 0.56%, 0.50%). It can be seen that the same percentage composition was detected for β-ionone epoxide in Hg1 and Hg3 oil samples.

The reason that greater amounts of these constituents are present in Hg2 could be due to the slightly smaller concentration of the chromium present in the leaves (19.403 ± 2.384 mg kg^{-1}) than in Hg1 and Hg2 (Table 3.33). Hence the production and composition of the oil could have been disrupted by the metal contamination in the soil and the leaves. However, Hg concentrations in leaf sample Hg1 had mercury present and the soil samples also contained mercury, which could have altered some processes in the plant and hence produce a different amount of oil and different composition of the plant.

The sesquiterpenoid hydrocarbons were detected in the greatest amounts in the Hg3 oil sample. Hg3 oil had the smallest TIC and the greatest chromium concentration in the leaves. It also had the greatest mercury contamination in the

Table 3.34 Comparison of the different constituents present in the samples obtained from steam distillation experiments using leaves from plants grown in mercury spiked soil Hg1, Hg2 and Hg3 ([**bold**] - constituent present in significant value , [blue] - predominant constituent

	Hg 1		Hg 2		Hg 3		Averag
Constituents [a]	**Retention time** [b]	**% Composition** [c]	**Retention time** [b]	**% Composition** [c]	**Retention time** [b]	**% Composition** [c]	**% Composition** [c]
γ-Terpinene	6.27	1.11	6.20	1.79	6.20	0.53	1.14
δ-Elemene	13.00	0.29	12.98	0.65	12.98	0.21	0.39
Copaene	14.05	0.68	14.03	0.63	14.03	0.75	0.69
β-Bourbonene	14.26	0.48	14.25	0.51	14.24	0.43	0.47
β-Elemene	14.39	0.69	14.38	0.85	14.38	0.88	0.80
Caryophyllene	15.24	22.98	15.20	22.72	15.20	24.79	23.50
γ-Elemene	15.40	1.07	15.39	1.53	15.38	2.55	1.72
γ-Cadinene	-	0.00	15.77	0.22	15.77	0.22	0.15
Bicyclo[4.4.0]dec-1-ene, 2-isopropyl-5-	-	0.00	15.89	0.25	-	0.00	0.08
Aromadendrene	15.90	0.23	15.94	0.35	-	0.00	0.19
β-Farnesene	15.99	0.33	-	0.00	15.95	0.54	0.29
α-Caryophyllene	16.09	8.50	16.07	6.85	16.07	12.01	9.12
***Allo*-Aromadendrene**	16.18	1.79	16.16	1.63	16.17	1.89	1.77
Germacrene D	16.79	31.47	16.75	38.30	16.73	28.91	32.89

Bicyclogermacrene	17.05	0.86	17.04	0.61	17.04	0.76	0.74
β-cuvebene	17.14	0.23	-	0.00	17.12	0.20	0.14
β - Bisobolene	17.34	2.59	17.33	2.12	17.32	2.86	2.52
δ-Cadinene	17.58	0.23	17.57	0.19	17.57	0.18	0.20
β-Sesquiphellandrene	17.71	0.19	-	0.00	17.71	0.21	0.13
(+)-4-Carene	17.80	0.51	17.79	0.81	17.79	0.26	0.52
Germacrene B	18.59	7.68	18.56	5.75	18.57	8.89	7.44
Caryophyllene oxide	19.11	0.35	19.11	0.23	19.11	0.34	0.31
1,7,7-Trimethyl-2-vinylbicyclo[2.2.1]hept-	-	0.00	19.25	0.19	-	0.00	0.06
Spathulenol	-	0.00	20.16	0.15	-	0.00	0.05
trans-z-alpha-bisabolene epoxide	21.44	0.28	21.44	0.37	21.44	0.22	0.29
β-Ionone epoxide	22.61	0.50	22.61	0.67	22.61	0.50	0.56
Isopropyl myristate	-	0.00	-	0.00	24.43	0.21	0.07
Phytol	24.67	1.80	24.67	1.27	24.67	1.23	1.43
n-Hexadecanoic acid	27.19	0.15	-	0.00	0.00	0.00	0.05
2-Naphthalenamine, N-methyl-	29.21	12.77	29.16	9.20	29.16	9.13	10.37
Eicosane	34.78	0.55	34.75	0.97	34.75	1.32	0.95
Total		98.30		98.84		100.00	99.05

Table 3.35 Summary and comparison of the major constituents present in the samples obtained from the steam distillation experiments on leaves from mercury spiked soils

	Steam distillation experiments on leaves from mercury spiked soils						Average
	No. identified	Hg1 (%)	No. identified	Hg2 (%)	No. identified	Hg3 (%)	% Composition
Monterpenoid hydrocarbons	2	1.62	2	2.60	2	0.79	1.67
Oxygenated monoterpenoids	0	0.00	0	0.00	0	0.00	0.00
Sesquiterpenoid hydrocarbons	17	80.27	16	83.18	17	86.27	83.24
Oxygenated sesquiterpenoids	2	0.63	3	0.75	2	0.56	0.65
β-Ionone epoxide	1	0.50	1	0.67	1	0.50	0.56
Phytol	1	1.80	1	1.27	1	1.23	1.43
2-Naphthalenamine,N-methyl	1	12.77	1	9.20	1	9.13	10.37
Other	2	0.71	2	1.17	2	1.52	1.13
Total		98.30		98.84		100.00	99.05

soil. The reason for observing a higher concentration of sesquiterpenoids could be that the smaller the amount of ions present (TIC), the lower the concentration of each constituent present.

However because some of the constituents are found in very minute amounts, mainly the main components will be identified in a sample of low TIC and the others will either have very small values or don't exist. The high concentration of heavy metals can also affect the yield of the oil available to extract as well as the metabolic processes in the leaves that can affect essential oil production and change the composition of the oil.

When the amount of 2-Naphthalenamine,n-methyl and phytol are compared in the oil samples, we see they are present in the greatest amounts in Hg1 (12.77%, 1.80%) followed by Hg2 (9.20%, 1.27%) and Hg3 (9.13%, 1.23%). The reason for this could be due to the higher ion concentration in Hg1 > Hg2 > Hg3. The more ions present for analysis and a greater chance of detecting a higher amount of each constituent.

From Table 3.34 the constituents that were identified in each oil sample (Hg1, Hg2 ,Hg3) are compared. Thirty one constituents were identified from the three different oil samples, accounting for 98.30% to 99.05% of the oil contents. The predominant constituents will however be used for comparisons between experiments. In all the steam distillation experiments (Hg1, Hg2, Hg3) using leaves from plants grown in mercury contaminated soil the following constituents were the predominant ones : carophyllene, α-carophyllene, germacrene D, β-bisabolene, germacrene B and 2-naphthalenamine,N-methyl. (In blue in Table 3.34)

When the percentage composition of carophyllene, α-carophyllene, β-Bisobolene, and germacrene B are compared in the three mercury experiments, it can be seen that the greatest amount of these constituents are present in Hg3 (24.79%,

12.01%, 2.86%, 8.89%), followed by Hg1 (22.98%, 8.50%, 2.59%, 7.68%) and then Hg2 oil samples (22.72%, 6.85%, 2.12%, 5.75%). Hg3 has however the smallest TIC and the greatest chromium contamination in the leaves and the greatest mercury contamination in the soil. The high heavy metal contamination could be a reason to why a difference in oil composition is observed. Since the speciation of a metal in the soil relies on a number of factors, such as pH of soil, moisture content and amount of organic matter present. The conditions of the pots could have varied and a difference in composition was observed. There are many other reasons why there was a difference in composition observed such as the amount of sunlight a plant gets, the water pH and the genetic makeup of a plant.

Germacrene D was detected with a percentage composition of 28.91%, 31.47% and 38.30% in the Hg2, Hg1 and Hg3, respectively. The reason more germacrene D was seen in Hg2 could be due to the slightly larger leaves (45.31 mm) used than Hg1 (36.35 mm) and Hg3 (35.71 mm), which could contain more oil sacs and hence have a greater amount of constituents present than smaller leaves. Also smaller less mature leaves could have a different oil composition than larger leaves. Hg2 also had no mercury and the smallest concentration of chromium present in the leaves.

There are many reasons for the differences seen in the ratios of essential oils constituents. Such as experimental factors, for example the xylene used to collect the oil is volatile, so one cannot control the amounts of xylene. In some experiments more xylene could have been present than in others and even a drop of xylene can change the concentration of a constituents present in the oil sample, because of such small quantities collected. Some of the oil constituents can be also lost on the walls of the apparatus.

The heavy metal contamination of mercury in the soils seems to affect the amount of total ions detected. However the mercury present in the leaves seems not to affected the TIC as much.

Lead

Once steam distillation was performed on fresh leaves from the lead spiked pots, the essential oils were analysed on a GC-MS and the graphs in figure 3.31 were compared as well as the TIC of the lead samples (Table 3.29). The Pb2 oil sample had a greater number of ions detected with a TIC of 2.23 x 10^9, followed by Pb1 with a TIC of 1.22 x 10^9 ions, then Pb3 with a TIC of 1.01 x 10^9. The number of constituents collected in experiment Pb2 > Pb1 > Pb3. When the amount of leaves used in each experiment are compared (table 3.29), it can actually be observed that Pb2 had the greatest amount of leaves (21.81 g) > Pb1 (19.55 g) > Pb3 (9.20 g). The larger the average leaf sized used, the greater the amount of oil collected and therefore the greater number of ions are detected.

When the concentration of chromium in the soil of the lead spiked pots are compared (Table 3.24), the greatest amount is present in Pb2 (28.53 ± 1.07 mg kg^{-1}) , folowed by Pb1 (21.84 ± 1.10 mg kg^{-1}), then Pb3 (21.00 ± 1.94 mg kg^{-1}).

Table 3.36 Comparison of the concentration of chromium, mercury and lead found in the leaves from plants grown in the lead spiked soil

	Cr		Hg		Pb
Leaves from Lead pots	mg kg^{-1}	Std. Dev	mg kg^{-1}	Std. dev	mg kg^{-1}
Pb 1	**21.37**	1.43	**31.14**	3.83	nd
Pb 2	**17.09**	1.46	nd	-	nd
Pb 3	**19.67**	5.76	nd	-	nd

*nd - not detected *Std. dev - standard deviation

When the concentration of chromium in the leaves from the lead spiked pots are compared the greatest amount was detected in Pb1 (21.37 ± 1.43 mg kg^{-1}) > Pb3 (19.67 ± 5.76 mg kg^{-1}) > Pb2 (17.09 ± 1.46 mg kg^{-1}) (Table 3.36). Since chromium was detected in the control sample of the soil with an average of 68.28 ± 1.41 mg kg^{-1}. It shows that chromium was already present in the soil, even

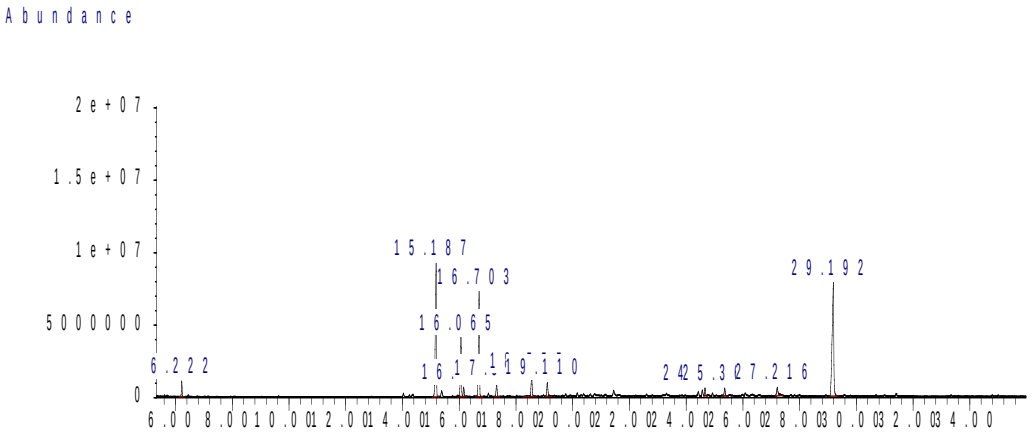
before it got spiked with chromium. The natural concentration of chromium in soil is around 10 to 50 mg kg^{-1}. The amounts of chromium detected in this study, was within the natural limits of chromium (Table 3.24). No chromium was used to spike the soil of the lead experiments, only lead was used. This shows that the chromium present within natural levels should not affect the essential oil composition and production as much as if higher concentrations of chromium were added.

The amount of mercury in the lead spiked soils are compared (Table 3.24) and no mercury was present but when amount of mercury present in the leaves are compared it can be seen (table 3.36) that mercury was present in Pb1 (31.14 ±3.83 mg kg^{-1}) and not in Pb2 or Pb3. Since no mercury was detected in the lead experiment soils, the mercury could not be taken up by the plants from the soil, but rather from the adsorption of mercury from air, directly onto the plant.

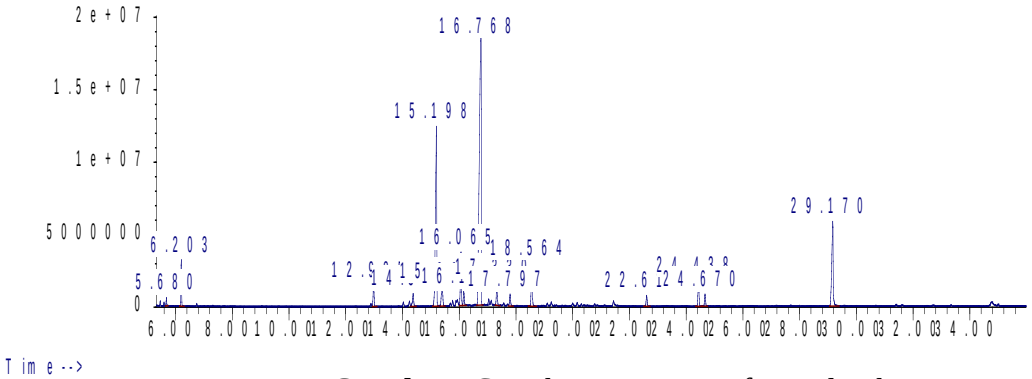
The concentration of the lead present in the lead spiked soils are compared and no lead was detected. It can also be seen that that when we compare the amount of lead in the leaves, no lead was present as well.

However the soil was spiked with 10 ppm of lead. The reason no lead was detected could be due to the the pH of the water and the dissolved salt content. If the pH >5.4 , the total solubility of lead is approximately 30 μg L^{-1} in hard water and approximately 500 μg L^{-1} in soft water. Formation of lead sulphate in soft water also limits the lead concentration in solution. Above pH 5.4, the lead carbonates limit the amount of soluble lead. At low pH, dissolved lead in ionic form (Pb^{2+}) are the predominant species. (Papanikolaou et al., 2005)

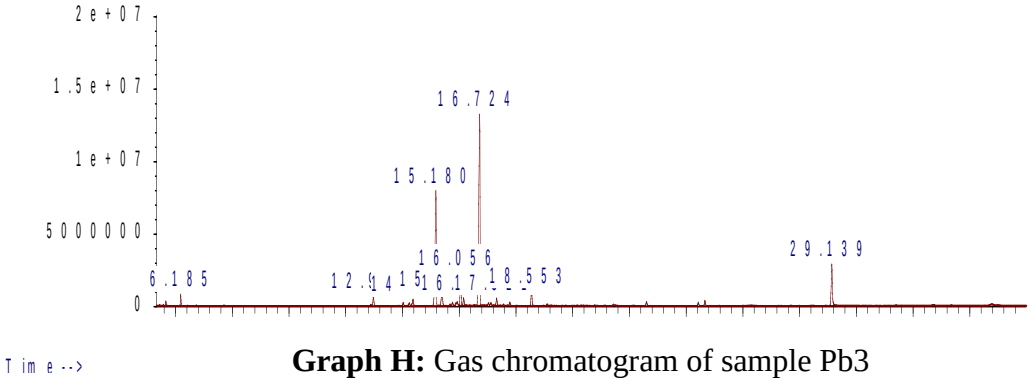
Therefore the mobility of lead will increase in environments having low pH due to the enhanced solubility of lead under acidic conditions. This could be the reason why no lead was present in the soil, if the water used to water the plants was more acidic, the more mobile form of lead is the predominant ion and therefore could have washed



Graph G: Gas chromatogram of sample Pb1



Graph H: Gas chromatogram of sample Pb2



Graph H: Gas chromatogram of sample Pb3

Figure 3.31: Comparison of the gas chromatograms of the *Centella asiatica* oil samples collected from steam distillation experiments Pb1, Pb2 and Pb3

out of the leachate bowl if overfilled or some lead could have been taken up by the roots. Most lead is retained strongly in soil, preferring to form relatively stable organic-metal complexes or chelates with soil organic matter, or adsorbs onto mineral surfaces and we would therefore expect to see lead in the soil samples. However these processes are dependent a member of factors such as soil type, soil pH, particle size, the amount of humic matter and iron oxides present as well as the cation exchange capacity (CEC) and the amount of lead in soil. (Papanikolaou et al., 2005)

The mass spectrums for each major peak in the graphs were then analysed using the same method of comparison to the NIST library. Table 3.37 summarises the constituents present in the lead spiked experiments.

From Table 3.38 the constituents that make up the greatest composition of the oil from *Centella asiatica* leaves in the steam distillation experiments using leaves harvested from plants grown in lead spiked soil are sesquiterpenoid hydrocarbons (55.85 - 86.79%) among which germacrene D (16.15% - 42.04%) as well as carophyllene (18.04% - 23.61%). Oxygenated monoterpenoids were also identified in the essential oil (0.24 - 6.59%), such as carophyllene oxide (0.24% - 2.55%) as well as monoterpenoid hydrocarbons (1.54 - 4.43%) such as γ-terpenine (1.54 - 2.41%). β-ionone epoxide (0.43 - 0.89%), phytol (0.82 -1.15%) and 2-naphthalenamine, N-methyl (8.44 - 26.44%) were also identified.

The greatest amount of monterpenoid hydrocarbons (4.43%) and β-ionone epoxide (0.89%) was observed in Pb2 oil samples (Table 3.38), then in Pb3 oil samples (2.44%, 0.73%) and then in Pb1 (1.54%, 0.43%). The reason that greater amounts of these constituents are present in Pb2 could be due to the slightly greater amount of leaves used, as well as the greater number of ions present in Pb2 compared to Pb1 and Pb3 (Table 3.29). As mentioned earlier the more oil sacs are present the greater the chance of more constituents being present.

Table 3.37 Comparison of the different constituents present in the samples obtained from steam distillation experiments using leaves from plants grown in lead spiked soil Pb1, Pb2 and Pb3 ([**bold**] - constituent present in significant value , [blue] - predominant constituent

	Pb 1		Pb 2		Pb 3		Averag
Constituents [a]	**Retention time** [b]	**% Composition** [c]	**Retention time** [b]	**% Composition** [c]	**Retention time** [b]	**% Composition** [c]	**% Composition** [c]
α-Terpinene	-	-	5.46	0.23	-	-	0.08
β-Cymene	-	-	5.60	0.17	-	-	0.06
Limonene	-	-	5.68	0.39	5.67	0.46	0.28
γ-Terpinene	6.22	1.54	6.20	2.41	6.19	1.30	1.75
Isoterpinolene	-	-	12.90	0.21	-	-	0.07
δ-Elemene	-	-	12.98	1.59	12.98	1.56	1.05
Copaene	14.04	0.48	14.03	0.32	14.03	0.53	0.44
β-Bourbonene	14.25	0.28	14.25	0.45	14.25	0.56	0.43
β-Elemene	**14.38**	**0.54**	**14.38**	**1.15**	**14.37**	**1.36**	**1.02**
Caryophyllene	15.18	21.33	15.20	18.04	15.18	23.61	20.99
γ-Elemene	15.38	1.10	15.39	2.22	15.39	2.82	2.05
α-Cuvebene	-	-	15.69	0.23	-	-	0.08
β-Gurjunene	-	-	15.77	0.44	15.77	0.60	0.35

Bicyclo[4.4.0]dec-1-ene, 2-isopropyl-5-	-	-	15.89	0.43	15.89	0.46	0.30
Aromadendrene	-	-	15.94	0.61	15.93	0.79	0.47
α-Caryophyllene	16.07	8.56	16.07	4.37	16.06	5.71	6.21
***Allo*-Aromadendrene**	16.16	1.24	16.16	1.23	16.16	1.49	1.32
Germacrene D	16.70	16.15	16.77	40.77	16.72	42.04	32.99
Bicyclogermacrene	17.03	0.49	17.05	0.63	17.03	0.55	0.56
β-cuvebene	-	-	17.13	0.54	17.12	0.64	0.39
β - Bisobolene	17.32	1.92	17.33	2.06	17.32	1.38	1.79
δ-Cadinene	-	-	17.58	0.36	-	-	0.12
(+)-4-Carene	-	-	17.79	1.01	17.79	0.67	0.56
Germacrene B	18.56	3.33	18.56	3.68	18.56	2.71	3.24
Isolongifolene,7,8-dehydro-8a-hydroxy	18.98	0.27	-	-	-	-	0.09
Caryophyllene oxide	**19.11**	**2.55**	**19.11**	**0.28**	**19.11**	**0.24**	**1.02**
1,7,7-Trimethyl-2-vinylbicyclo[2.2.1]hept-	-	-	19.25	0.49	-	-	0.16
γ-Gurjunene	-	-	20.00	0.34	-	-	0.11
Humulene epoxide	19.76	0.38	-	0.00	-	-	0.13
Spathulenol	20.16	0.52	20.16	0.35	-	-	0.29
10,10-Dimethyl-2,6-	20.39	0.50	-	-	-	-	0.17
Hedione	20.62	0.44	-	-	-	-	0.15

α-Cadinol	20.78	0.54	20.78	0.16	-	-	0.23
α-Farnesene	21.15	0.43	-	-	-	-	0.14
trans-z-alpha-bisabolene epoxide	21.45	1.09	21.44	0.48	-	-	0.52
β-Ionone epoxide	22.61	0.43	22.61	0.89	22.61	0.73	0.68
Aromadendrene oxide (2)	23.31	0.27	-	-	-	-	0.09
Isopropyl myristate	24.43	0.81	24.44	2.07	24.43	0.53	1.14
Decahydro 4,4,8,9,10	24.57	0.96	-	-	-	-	0.32
Phytol	24.67	1.15	24.67	0.90	24.67	0.82	0.96
ledene oxide	24.93	0.47	-	-	-	-	0.16
Salycilic acid	25.36	1.29	-	-	-	-	0.43
Pinane	26.09	0.79	-	-	-	-	0.26
Trans chrysthemal	26.34	0.67	-	-	-	-	0.22
n-Hexadecanoic acid	27.21	2.42	-	-	-	-	0.81
2-Naphthalenamine, N-methyl-	29.20	26.44	29.17	9.03	29.14	8.44	14.64
Ethanone, 1-(2,5-dimethyl-4-phenylfuran-3-	31.40	0.43	-	-	-	-	0.14
Squalene	-	-	34.78	0.95	-	-	0.32
Tetracosane	-	-	35.00	0.15	-	-	0.05
Total		99.80		99.67		100.00	99.82

Table 3.38 Summary and comparison of the major constituents present in the samples obtained from the steam distillation experiments on leaves from lead spiked soils

	Steam distillation experiments on leaves from lead spiked soils						Average
	No. identified	Pb1 (%)	No.	Pb2 (%)	No. identified	Pb3 (%)	%
Monterpenoid hydrocarbons	1	1.54	6	4.43	3	2.44	2.80
Oxygenated monoterpenoids	0	0.00	0	0.00	0	0.00	0.00
Sesquiterpenoid hydrocarbons	12	55.85	19	79.48	16	86.79	74.04
Oxygenated sesquiterpenoids	9	6.59	4	1.27	1	0.24	2.70
β-Ionone epoxide	1	0.43	1	0.89	1	0.73	0.68
Phytol	1	1.15	1	0.90	1	0.82	0.96
2-Naphthalenamine,N -methyl	1	26.44	1	9.03	1	8.44	14.64
Other	8	7.80	4	3.67	1	0.54	4.00
Total		99.80		99.67		100.00	99.82

Pb1 had mercury present in the leaves while Pb2 and Pb3 had none present in the leaves (Table 3.36). This could have affected the yield of oil produced and the ratio of constituents present. Pb2 had the lowest metal concentration in the leaves and this could have also contributed the ratio of constituents detected.

The greatest amount of oxygenated sesquiterpenoids (6.59%), 2-napthalenamine, N-methyl (26.44%) and phytol (1.15%) were detected in Pb1 oil samples, followed by Pb2 oil samples (1.27%, 9.03%, 0.90%) and the least in Pb3 oil samples (0.24%, 8.44%, 0.82%). When the following constituents are compared γ-terpenine, β-bisabolene and germacrene B are in the oil samples. It can be seen that these constituents are present in the greatest amounts in Pb2 oil samples (2.41%, 2.06%, 3.68%) > Pb1 (1.54%, 1.92%, 3.33%) > Pb3 (1.30%, 1.38%, 2.71%) as well (table 3.37). The reason for Pb1 and Pb2 to have a greater amount of these constituents compared to Pb3 could be because of the smallest ion concentration detected in Pb3 (1.01×10^{9}). The reason Pb1 had a greater number of these constituents than Pb2 could be because of the high concentration of chromium present in the Pb2 (28.53 ± 1.07 mg kg^{-1}) pots and this could have also affected the metabolism of the plant and hence the oil production and composition. Since the speciation of a metal in the soil relies on a number of factors, such as pH of soil, moisture content and amount of organic matter present. The amount of mobile chromium Cr(VI) to Cr(III) could have also been a contributing factor to the difference seen in the constituents detected. If one pot contains more mobile and toxic Cr(VI) it can affect the plant more than if a pot contains more Cr(III).

The constituents that were identified in each oil sample (Pb1, Pb2, Pb3) are compared in table 3.37. Forty nine constituents were identified from the three different oil samples, accounting for 98.80% to 100% of the oil contents. The predominant constituents will mainly be used for comparisons between the experiments. In all the steam distillation experiments (Pb1, Pb2, Pb3) using leaves

from plants grown in mercury contaminated soil the following constituents were the predominant ones: γ-terpenine, carophyllene, γ-elemene, α-carophyllene, germacrene D, β-bisobolene, germacrene B and 2-naphthalenamine,N-methyl. (In blue in Table 3.37)

The greatest amount of sesquiterpenoid hydrocarbons are detected in Pb3 > Pb2 > Pb1. This includes the major constituent germacrene D which was detected at 42.04%, 40.77% and 16.15% in Pb3, Pb2 and Pb1, respectively. As well as γ-elemene detected at 2.82%, 2.22% and 1.10%, respectively. The reason for this could be due to the larger leaves used (51.63 mm) in Pb3 > Pb2 (46.22 mm) > Pb1 (36.39 mm). As mentioned earlier the larger the leaves the more oil sacs they contain and because they are more mature, they can have a different ratio of constituents present than younger smaller leaves. The heavy metal contamination present in the soil and in the leaves could have also affected the ratio of constituents present.

Carophyllene was detected in the greatest amounts in Pb3 (23.61%) > Pb1 (21.33%) > Pb2 (18.04%). α-Carophyllene was detected in the greatest amounts in Pb1 (8.56%) followed by Pb3 (5.71%) then Pb2 (4.37%). The reason for the differences in the ratios of the constituents detected could be due to environmental factors such as the amount of sunlight a plant receives. As explained before the better the conditions for plant growth and photosynthesis, the greater the production of the essential oil, and the different conditions for growth such as the amount of sunlight and soil quality would affect which constituents are produced in the oil.

There are many reasons for the differences we see in the ratios of essential oils constituents between the three experiments. Environmental factors as explained earlier can affect the amount of oil produced in the plant. Experimental factors could also have been a contributing factor such as if extra xylene was collected the oil, which would decrease the concentration of compounds present. Some of

the oil constituents could have vaporised during the extraction and are lost on the walls of the apparatus. The heavy metal contamination is another factor that could have altered the ratio of constituents present. The heavy metals can affect the plants depending on their metal speciation. Toxic heavy metal ions are thought to enter the plant cells by the same uptake process as micronutrients, competing with these elements for adsorption. (Azevedo and Rodriguez, 2012) All these factors are interdependent and cannot be isolated from each other.

Comparison of the essential oil yield and composition of the steam distillation extraction on leaves from non-contaminated and heavy metal contaminated soil

The results from the steam distillation done on leaves from non-contaminated soils were then compared to the leaves from chromium, mercury and lead contaminated soils. Due to the small amounts of oil collected, the total number of ions collected are used for comparison as mentioned earlier. The greater amount of oil collected, the greater number of ions should be present.

The average TIC between the essential oils from steam distillation extractions on fresh leaves from non-contaminated soils were compared to the ones on leaves from heavy metal contaminated soil in Table 3.39. The greatest amount of ions can be seen in the oil sample from steam distillation experiments (4.61×10^9 ions). The steam extraction was run with 100 g of leaves from non-contaminated soil whereas less leaves were used in the extractions on leaves from contaminated soils (8.60 g - 21.81 g) (Tables 3.27 - 3.29). As explained earlier, the more leaves used for extraction the greater number of oil sacs present, and hence the greater number of ions are detected in the experiments.

The number of leaves available for extraction in the heavy metal contaminated pots were limited because of the pot size, in order to get 100 g of leaves of each contaminated plant, a very large flower bed would need to be spiked with chromium, mercury and lead. This was not possible and could have also been a risk of heavy metal pollution if not controlled properly. Since there is such a big

difference in the amount of leaves used in the non-contaminated soil, it was not possible to deduce if the heavy metal contamination affected the amount of ions detected in the essential oils extracted from *Centella asiatica*.

Table 3.39 Comparison of the total ion current (TIC) between steam distillation with leaves from non-contaminated soil to leaves from heavy metal contaminated soil

Experiment	Average TIC concentration	Std.dev.
Non-contaminated pots (steam)	4.61E+09	3.74E+09
Chromium spike pots	2.25E+09	1.62E+08
Mercury spiked pots	2.46E+09	1.07E+09
Lead spiked pots	1.49E+09	6.55E+08

However the TIC of the leaves used from the plants grown in the heavy metal contaminated soil can be compared because of the similar amount of leaves used; on average 16.85 g from the lead contaminated pots, 13.89 g from the mercury contaminated pots and 13.34 g from the chromium contaminated pots. When the TIC is compared (table 3.39) it can be seen that the highest TIC is detected in oil samples from mercury contaminated pots (2.46E+09 ions) then in chromium pots (2.25E+09 ions) and the least in lead contaminated pots (1.49E+09 ions). In the sample of oil from leaves grown in lead contaminated pots, on average, a greater number of leaves were used than in the other extractions but the smallest amount of ions are detected. Slightly more leaves and hence a greater TIC in the leaf extractions from mercury contaminated soil than in the chromium contaminated soil.

When the average heavy metal contamination in the soil (table 3.25) is compared to the amount of ions detected, it can be observed that there is no pattern. Lead contaminated pots have the least amount of heavy metal contamination however the smallest TIC. The chromium contaminated pots had the largest heavy metal

contamination but a in between TIC and mercury contaminated pots had a in between average heavy metal concentration but a higher TIC.

The heavy contamination in the leaves (table 3.26) were also compared to the TIC and it was observed that the greatest concentration of heavy metals was observed in the leaves from chromium contaminated soil, which had a TIC of 2.25E+09 ions. The smallest concentration of heavy metals was observed in the leaves from the lead experiments, which had the smallest TIC of 1.49E+09. The leaves from the mercury experiments had the highest TIC of 2.46E+09.

The average percentage composition of each major compound class from table 3.40 are compared, it was observed that steam distillation on leaves from non-contaminated soil yielded a greater amount of sesquiterpenoid hydrocarbons (84.83%), monoterpenoid hydrocarbons (5.42%), β-inone epoxide (1.29) and squalene (1.82%) than the oil samples from the leaves harvested from plants in heavy metal contaminated soil. The reason the greatest amount of these constituents are seen in the oil from the leaves from non-contaminated pots could be because of its' higher TIC compared to the TIC from the oil obtained from the leaves from the heavy metal contaminated pots. The other reason could be that the heavy metal presence can alter different metabolisms of the plant and cause a different amount and composition of essential oils produced.

The greatest number of oxygenated sesquiterpenoids was observed in the oil from the leaves harvested from the lead pots. 2-Naphthenamine,n methyl is also present in much higher amounts in leaves harvested from the lead spiked pots (14.64%) than the ones from non-contaminated pots (1.94%). The greatest amount of phytol was observed in the leaves harvested from the mercury spiked pots. The reason for these constituents having greater percentage compositions could be due to the heavy metal contamination and the processes that it interferes with in the plant, which would cause a different ratio of ions present. For example β-bisobolene was present in significant amounts in the extractions from leaves from heavy metal

contaminated soils and none was detected in the non-contaminated soils. (Table 3.41).

γ-Terpenine, δ-elemene and germacrene D are present in the oil obtained from leaves of non-contaminated pots and yielded 3.19%, 2.37% and 40.59%, respectively, which was greater than the yield from oil obtained from heavy metal contaminated pots which yielded (1.75 - 2.14%), (0.38 - 1.05%) and (26.70 - 32.99%). This could be due to the higher TIC of the oil from the steam distillation extraction, however the heavy metal concentration could also have affected the TIC of the other oils from the leaves of heavy metal contaminated soils. There are many other factors that can affect the amount of oil collected. As discussed in detail before such as environmental factors and experimental factors.

Carophyllene in the oil from the leaves harvested from plants grown in non-contaminated soil have a slightly greater amount present (22.14%) compared to the oils samples from leaves grown in lead spiked pots (20.99%) and chromium spiked pots (21.32%), however it is slightly less than the oil sample from leaves grown in mercury spiked soil (23.50%). α-Carophyllene, germacrene B and 2-Naphthalenamine,N-methyl are all present in the greatest amounts in the oil samples from leaves harvested from plants grown in heavy metal contaminated soil with a range of 6.21 - 9.66%, 3.24 - 7.82% and 10.37 - 14.64%, repectively. This shows that even though steam distillation on leaves from non-contaminated pots have a higher TIC and a greater number of leaves used for extraction, the heavy metal contamination does affect the plant and the processes in production of the essential oil and its constituents.

The level of essential elements in a plant is conditional and can be affected by the ability of the plant to selectively accumulate some of these elements. (Abu-Darwish , 2009) From the extraction on *Centella Asiatica* leaves from heavy metal contaminated soils it can be seen that *Centella* does take up chromium and mercury into its leaves, however lead is not detected in the soil or in the leaves.

Table 3.40 Summary and comparison of the average percentage composition of the major classes of compounds

Pots	non-contaminated	Chromium	Mercury	Lead
		% Composition	% Composition	% Composition
Monterpenoid hydrocarbons	5.42	4.18	1.67	2.80
Oxygenated monoterpenoids	0.08	0.00	0.00	0.00
Sesquiterpenoid hydrocarbons	84.83	74.28	83.24	74.04
Oxygenated sesquiterpenoids	1.91	1.41	0.65	2.70
β-Ionone epoxide	1.29	0.73	0.56	0.68
Phytol	0.79	0.05	1.43	0.96
2-Naphthalenamine,N-methyl	1.94	13.43	10.37	14.64
Squalene	1.82	0.00	0.00	0.32
Other	0.53	3.50	1.13	3.69
Total	98.60	97.58	99.05	99.82

Table 3.41 Summary of the average percentage composition of the C*entella asiatica* oil present in the oil samples obtained from the steam distillation on leaves from non-contaminated soil and from heavy metal contaminated soil.

Constituents [a]	Non-contaminated leaves (Avg. % composition)	Chromium spiked pots (Avg. % composition)	Mercury spiked pots (Avg. % composition)	Lead spiked pots (Avg. % composition)
α-Terpinene	0.09	0.07	1.14	0.08
β-Cymene	0.36	0.08	-	0.06
Limonene	0.08	1.41	-	0.28
γ-Terpinene	3.19	2.14	-	1.75
Terpinolene	0.17	0.12	-	-
Trans-3-nonene-2-one	0.08	-	-	-
Isoterpinolene	0.35	-	-	0.07
δ-Elemene	2.37	0.38	0.39	1.05
Copaene	0.53	0.49	0.69	0.44
β-Bourbonene	0.41	0.33	0.47	0.43
β-Elemene	1.71	0.72	0.80	1.02
Caryophyllene	22.14	21.32	23.50	20.99
γ-Elemene	0.76	1.42	1.72	2.05
Isoledene	0.11	-	-	-

γ-Cadinene	1.69	0.07	0.15	-
α-Cuvebene	0.27	-	-	0.08
β-Gurjunene	0.50	-	-	0.35
Bicyclo[4.4.0]dec-1-ene, 2-isopropyl-5-methyl-9-methylene-	0.46	0.09	0.08	0.30
Aromadendrene	0.80	0.09	0.19	0.47
β-Farnesene	-	0.22	0.29	-
α-Caryophyllene	3.96	9.66	9.12	6.21
***Allo*-Aromadendrene**	1.35	1.36	1.77	1.32
γ-Muurolene	0.09	-	-	-
Naphthalene, 1,2,4a,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-	0.13	-	-	-
Germacrene D	40.59	26.70	32.89	32.99
Bicyclogermacrene	1.13	0.67	0.74	0.56
β-cuvebene	0.56	0.18	0.14	0.39
α-Elemene	0.11	-	-	-
Isocarophyllene	0.69	-	-	-
Germacrene A	0.70	-	-	-
β - Bisobolene	-	2.41	2.52	1.79
δ-Cadinene	0.42	0.15	0.20	0.12
β-Sesquiphellandrene	-	0.13	0.13	-

(+)-4-Carene	1.18	0.37	0.52	0.56
Germacrene B	3.36	7.82	7.44	3.24
Isolongifolene,7,8-dehydro-8a-hydroxy	-	-	-	0.09
Caryophyllene oxide	0.65	0.53	0.31	1.02
1,7,7-Trimethyl-2-vinylbicyclo[2.2.1]hept-2-ene	0.41	0.13	0.06	0.16
Juniper camphor	0.42	-	-	-
γ-Gurjunene	-	0.07	-	0.11
Humulene epoxide	-	0.11	-	0.13
Spathulenol	0.53	0.08	0.05	0.29
Isoaromadendrene epoxide	-	0.07	-	-
10,10-Dimethyl-2,6-dimethylenebicyclo[7.2.0]undecan-5.beta.-ol	-	0.09	-	0.17
Hedione	-	-	-	0.15
α-Cadinol	0.10	0.11	-	0.23
α-Farnesene	-	-	-	0.14
trans-z-alpha-bisabolene epoxide	-	0.42	0.29	0.52
Viridiflorol	0.21	-	-	-
β-Ionone epoxide	1.29	0.73	0.56	0.68
pinane	-	1.33	-	-
1-Hexadecanol	-	0.12	-	-

Nonadecane	-	0.12	-	-
Aromadendrene oxide (2)	-	-	-	0.09
Isopropyl myristate	-	-	0.07	1.14
Decahydro 4,4,8,9,10 pentamethylnaphthalene	-	-	-	0.32
Phytol	0.79	0.05	1.43	0.96
ledene oxide	-	-	-	0.16
Salycilic acid	-	-	-	0.43
Pinane	-	-	-	0.26
Trans chrysthemal	-	-	-	0.22
n-Hexadecanoic acid	0.12	0.20	0.05	0.81
2-Naphthalenamine, N-methyl-	1.94	13.42	10.37	14.64
Ethanone, 1-(2,5-dimethyl-4-phenylfuran-3-yl)-	-	-	-	0.14
Squalene	1.82	-	-	0.32
Eicosane	-	0.19	0.95	-
Tetracosane	-	1.42	-	0.05
Total	98.60	97.59	99.05	99.82

roots of *Centella* were not analysed. However it has been reported that the highest levels of heavy metals were generally found in the roots of *Centella asiatica* (Yap et al., 2010). The reasons for this could be due to the fact that root hairs contain higher surface area for adsorption and absorption of heavy metals when compared to stems and roots, the roots are the first organ to take up all the heavy metals from the soils before distributing to other parts of the plant and the root is the only organ covered with soils when compared to stems and leaves. (Yap et al., 2010)

Plants lack the ability to escape from contaminated sites and therefore have evolved mechanisms to handle exposure to toxicants. Certain species have evolved to regulate the amount of pollutants which are taken from the environment, usually by sequestration and inactivation in subcellular compartments, or tolerate the deleterious effects of heavy metals. (Azevedo and Rodriguez, 2012) Lead could have therefore been taken up by the roots of the plant and due to some mechanism it did not transport it further into the leaves. However there are a number of factors that could have affected the absorption and accumulation of the heavy metal into the plant. These include soil aeration, cation-exchange capacity (Azevedo and Rodriguez, 2012), moisture, temperature, pH of soil, organic matter and nutrient availability. (Yap et al. , 2010) These factors also affect the metal speciation in the soil, for example Cr(VI) which is highly mobile and toxic, present in acidic conditions, compared to less mobile Cr(III) which is less toxic.

4 Conclusions

One of the aims of the study was to determine the yield of essential oil and optimize and compare the different extraction methods. In the initial experiments using the clevenger and variation of the clevenger apparatus it was concluded that the amount of oil obtained from the leaves is very small and difficult to quantify. Methods of extraction for these experiments were therefore optimized to produce an oil that is concentrated enough for analysis. The method of extraction to get a sufficient concentration of oil for analysis from the Clevenger and variation of the Clevenger apparatus was to use 45 g of leaves (minimum) in all the extractions and distillations should run for 3 hours. 0.7 ml of hexane should be used for separating the oil and water and a very small amount of sodium sulphate should be used when drying. The total number of ions present in the oil samples that were extracted using the clevenger and variation of the Clevenger apparatus were compared and it was evident that the variation of the Clevenger apparatus was more efficient in yielding a higher concentration of ions.

The predominant constituents obtained from these initial experiments using the variation of the clevenger apparatus were citral, α–citral, β-carophyllene, α-carophyllene, allo-aromadendrene, β-cuvebene, λ-gurjunene and juniper campher. The constituents that were found in the samples of all the essential oils from the water and steam distillations using the Clevenger, variation of the Clevenger and solvent extraction were myrcene, linalool, β-carophyllene, λ-elemene, α-carophyllene, allo-aromadendrene, β-cuvebene, β-bisobolene and λ-gurjunene.

If one of the simpler extraction method were to be used to extract some specific constituent like myrcene from *Centella Asiatica* leaves, water distillation using the variation of the Clevenger apparatus should be used since it had the greatest abundance of myrcene. If an extraction method were to be used to extract delta-elemene, solvent extraction should be used. However if an extraction method is to be used to get the most constituents for therapeutic uses of the oil, steam distillation using the variation of the Clevenger apparatus should be used on a

greater number of leaves. Soxhlet extraction should not be used for extracting volatile compounds like essential oils. Vinegar was used instead of distilled water in the steam extraction distillation using the variation of the Clevenger apparatus and from the results it showed that distilled water is more effective in extracting the most constituents of oil from the leaves than vinegar. When using dry leaves in the Clevenger variation apparatus, the extraction process seemed to yield less oil than in the extraction on fresh leaves, however it was more effective than fresh leaves by having a greater amount of constituents present than fresh leaves. Therefore when the leaves dry the oil sacs are not affected. It was confirmed later in the study when extracting essential oils from dry leaves using the BP apparatus.

After these initial findings using the basic variation of the Clevenger apparatus, it was decided to investigate the apparatus described by the British Pharmacopedia IV (BP apparatus) in the hope that the amount of oil collected from *Centella asiatica* could be quantified, which was not successfully determined in the initial experiments run using the variation of the Clevenger apparatus. The conditions for extraction using this apparatus were optimised and it was found that for the extraction of essential oils from *Centella asiatica* the best parameters to use were 100 g of leaves at a distillation rate of 2/3 ml min^{-1} for 75 minutes using 0.4 ml of xylene initially. It was also necessary to run the 30 minute distillation with no plant material as well. Unfortunately the amount of essential oil could not be quantified even using a larger number of leaves, because of the very small amount of oil that *Centella Asiatica* leaves contain. To obtain more leaves per experiment (± 500g) was not possible as a field of *Centella Asiatica* would be needed and was not available. The total number of ions detected in the oil samples were then therefore used to compare the extraction method, the more ions present, the greater the concentration of oil collected and a better quality oil is collected.

The solvents used to collect the oil were also compared and the solvent with the lowest vapour pressure was found to be the best collecting medium for the essential oil. Xylene was thus the better collecting medium because it does not vaporize as readily as hexane.

These optimised conditions were then used to determine if water or steam distillation is more effective for the extraction of the essential oil from the leaves using the BP apparatus. The steam distillation extraction yielded a greater number of ions compared to water distillation on the same apparatus using the same parameters and the same amount of leaves. It can therefore be concluded that steam distillation yielded a more concentrated and better quality essential oil than water distillation. In steam distillation using the BP apparatus, 43 compounds were identified which on average represents 98.60% of the composition of the oil. In water distillation 54 compounds were identified which on average represents 98.29 % of the composition of the oil. Hence more constituents were identified in water distillation than in steam distillation.

If an extraction method was to be used to collect the greatest amount of ions, steam distillation would be a more effective method in collecting the essential oil from *Centella asiati*ca leaves than water distillation. If an extraction method were to be chosen to extract the greatest amount of compounds then water distillation would be more effective.

The essential oil extracted using steam distillation yielded a greater amount of sesquiterpenoid hydrocarbons, β-ionone epoxide and squalene. Oxygenated monoterpenoids and phytol were only present in steam distillation not water distillation. Monoterpenoid hydrocarbons, oxygenated sesquiterpenoids and 2-naphthalenamine,n-methyl were present in greater amounts in oil samples from water distillation.

In all the steam distillation experiments (SQ1, SQ2, SQ3) the following constituents were found to be the predominant ones : γ- terpenine, δ-elemene, carophyllene, γ-cadinene, α-carophyllene, germacrene D, germacrene A, germacrene B, 2-naphthalenamine,n-methyl and squalene.

In all the water distillation experiments (WDQ1, WDQ2, WDQ3) the following constituents were found to be the predominant ones: γ-terpenine, δ-elemene, β-

elemene, carophyllene, γ-elemene, α-carophyllene, germacrene D, δ-cadinene, germacrene B and 2-naphthalenamine,n-methyl.

Since steam distillation was found to be more effective by yielding a better quality oil, this method was used to determine the constituents present in dry leaves. Steam distillation on fresh leaves yielded a greater number of ions compared to dry leaves. Forty two constituents were indentified in the steam distillation experiments on fresh leaves and forty five constituents identified using dry leaves. Hence the extraction of essential oils using steam distillation on dry leaves yielded a slightly greater number of constituents than with fresh leaves. Steam distillation on fresh leaves was found to contain a higher amount of sesquiterpenoids hydrocarbons and monoterpenoid hydrocarbons compared to the ones on dry leaves. Steam distillation on dry leaves still yielded a higher amount of oxygenated sesquiterpenoids as well as oxygenated monoterpenoids compared to fresh leaves. Squalene was only detected in fresh leaves.

Water distillation using salting-out was also investigated to see if the yield of the essential oil collected would increase. Water distillation with no salt on fresh leaves were found to have a higher ion concentration, and hence a higher oil concentration, compared to the salting-out experiment. Water distillation without salt yielded a higher amount of sesquiterpenoids hydrocarbons and monoterpenoid hydrocarbons compared to the distillation with salt. However the oil collected from the salting-out experiment yielded a greater amount of polar compounds, such as oxygenated sesquiterpenoids as well as oxygenated monoterpenoids, compared to distillation without salt.

In all the water distillation experiments with salt (WDSO1, WDSO2, WDSO3) the following constituents were the predominant ones: γ-terpenine, carophyllene, γ-cadinene, α-carophyllene, germacrene D, germacrene B, carophyllene oxide, decahydro 4,4,8,9,10 pentamethylnaphthalene,3,7,11,15-tetramethyl-2-hexadecen-1-ol, 2-naphthalenamine,n-methyl and phytol.

The major constituents that were detected as common constituents in all the extractions on the BP apparatus were γ-terpenine, carophyllene, γ-elemene, α-carophyllene, germacrene D, germacrene B and 2-naphthalenamine,n-methyl. Germacrene D and α-carophyllene were present in the greatest amounts in steam distillation on fresh leaves. Carophyllene and γ-terpenine was found in the greatest amounts in water distillation on fresh leaves.

The similarities in the results between water distillation and the other studies in South Africa were compared. Twenty-one constituents were identified from the fifty-four constituents in the water distillation experiments. Both studies identified carophyllene, α-carophyllene and germacrene B as the same predominant constituents. Biyclogermacrene and myrcene are present in both water distillation and the other study. However the major constituent of the water distillation experiment (germacrene D) varied (α-carophyllene). In the other similar study greater amounts of monoterpenoid hydrocarbons (20.20%), oxygenated monoterpenoids (5.46%) and oxygenated sesquiterpenoids (3.90%) were found than in the steam distillation (5.42%, 0.08%, 1.91%) and water distillation experiments (8.62%, 0.00%, 2.49%) in this study. However water (81.63%) and steam distillation (84.83%) experiments in this investigation had a greater amount of sesquiterpenoids compared to the other SA study (68.80%).

There were a number of factors found to contribute to the difference in the chemical composition between the essential oil samples collected. It is difficult to segregate these factors from each other, since many are interdependent and influence one another. Factors that affected the amount of oil collected and the amounts of chemical constituents present in an oil sample were the amount of leaves used, the size of the leaves used and the life stage of the plant. There are a number of other factors that could have affected the difference in composition observed between the oils. The different extraction methods can cause molecular rearrangement, hydrolysis of double bonds, and in general will produce substances not generally found in the plant (Bowles, 2003). This can cause a difference in the number of chemical constituents detected and the ratios of these

constituents. Other factors included geographical variation, altitude (Hussain, 2009), genetic variations between the plants, seasonal changes, light variation, water availability and soil quality.

Pollution can also affect the chemical composition of the oil since the soil quality of where the *Centella asiatica* plants were growing was important. The plant likes to grow in damp environments, therefore they are extremely prone to pollution. This was investigated by spiking the soil of some *Centella asiatica* plants with chromium(VI), mercury(II) and lead(II).

The greatest chromium and mercury concentrations in the soils after spiking were found as expected in the chromium and mercury spiked soils, respectively. No lead was detected in the soil and leaf samples not even in the lead experiments, where the soil was spiked with lead. It was hypothesised that some of the lead could have been taken up by the plant roots and into the plant, however the leaves of *Centella Asiatica* were analysed and no lead was detected, which could mean that the lead could be in the plant root or stems, or the plant has a process of decreasing the effect of this heavy metal, however stems or roots were not investigated in this study Some lead could also been lost from the pots when watered. A future study on the lead present in roots and stems of *Centella* should also be analysed. So a conclusion could not be raised on how lead affects the *Centella Asiatica* plant and should be further investigated.

It was determined that *Centella Asiatica* can store heavy metals in the leaves. Since it is a medical plant with many therapeutic uses, this finding should arise a great concern about heavy metal contamination of herbal raw materials of *Centella Asiatica*. It also highlights the importance of good quality control on *Centella Asiatica*, so that heavy metals are not ingested. The people in the rural areas who use it as a raw plant for herbal preparations could be at risk of ingesting heavy metals if grown in a polluted area.

It was found that even if a plant was contaminated with heavy metals, the essential oil from *Centella Asiatica* can be used safely with no heavy metal contamination. This is because steam distillation causes the volatile constituents to vaporise and the heavy metals in the leaves are too heavy to be vaporised. The chemical constituents of the essential oil, which could affect the therapeutic properties was found to be affected by the heavy metal contamination in the plants. Plants lack the ability to escape from contaminated sites and therefore have evolved mechanisms to handle exposure to toxicants. Certain species can regulate the amount of pollutants which are taken from the surroundings. This could be investigated for *Centella asiatica* in a future study.

Steam distillation on leaves from non-contaminated soil found to yield a greater amount of sesquiterpenoid hydrocarbons (84.83%), monoterpenoid hydrocarbons (5.42%), β-ionone epoxide (1.29) and squalene (1.82%) than the oil samples from the leaves harvested from plants in heavy metal contaminated soil. The affect of heavy metals on plants are dependent on the amount of heavy metals present and the metal speciation. These can be affected by water quality, soil aeration, pH of soil and the amount of organic matter present.

Overall the composition of the essential oil varies in constituents (slightly since there is some similarity) and quite significantly in the abundance of the constituents, depending on the extraction methods used. The more leaves used in the extraction process, the greater number of constituents are present. There are more studies conducted on the tincture and the tincture is used more extensively in certain therapeutic products than the oil, this is probably because the yield of the oil from *Centella* leaves is so low. The tincture has therapeutic uses as well as the oil but a much higher yield of tincture is obtained in extraction methods, making it more economical to produce the tincture than the oil where tons of leaves would be needed.

The commercial product (synthetic oil) of *Centella Asiatica* oil was found to be completely different from the natural oil and therefore synthetic oils cannot be

used therapeutically as substitutes for the naturally occurring essential oils. It could possibly be used in the fragrance industry but not therapeutically.

The method of optimization of extraction in this study refers to the essential oil of *Centella asiatica* specifically. It could be used and optimised for other therapeutic plants in future studies. The extraction method that would be the most effective in yielding the greatest amount of ions, would be steam distillation of fresh leaves using the BP apparatus and 100 g of leaves.

In future the following studies could be done on *Centella asiatica*. There have been no studies done on the length of time for significant degradation of constituents, or the effects of such degradation on the therapeutic qualities of oils. Investigations can be done to determine how different environmental factors affect the essential oil composition and the yield of oil obtained. New equipment could be developed with Teflon surfaces to prevent any oil from adhering to the side of the equipment to maximize the essential oil yield when extraction is carried out with small amounts of plant material.

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6 Appendices

APPENDIX A

Heavy metal solution calculations

Lead Nitrate ($Pb(NO_3)_2$) - ion Pb^{2+}- 10 ppm solution

10 ppm = 10mg L^{-1} x 1g/1000 mg

= 0.01 g L^{-1}

c = n/v

g/L = (mol x g mol^{-1}) / L

MM (Pb^{2+}) = 207.2 g mol^{-1}

mol = (g L^{-1} x L) / g mol^{-1}

= (0.01 g L^{-1} x 1L) / 207.2 g mol^{-1}

= 0.0000482 mol

MM ($Pb(NO_3)_2$) = MM (Pb) + 2(MM (N) + 3 (MM(O)))

= 207.2 g + 2 (14 + 3(16))

= 207.2 g + 124

= 331.2 g mol^{-1}

n = m/MM

m = n x MM

= (0.0000482 mol)(331.2 g mol^{-1})

= 0.01596 g

Therefore we must dissolve 0.01596 g of $Pb(NO_3)_2$ in 1 L of distilled water.

This was done using a 1 L volumetric flask and the amount of the lead nitrate needed was weighed on a analytical scale.

Mercurous Nitrate ($HgNO_3.H_2O$) - ion Hg^{2+} - 10 ppm solution

10 ppm = 10mg L^{-1} x 1g/1000 mg

= 0.01 g L^{-1}

c = n/v

g/L = (mol x g mol^{-1}) / L

MM (Hg^{2+}) = 200.59 g mol^{-1}

mol = (g L^{-1} x L) / g mol^{-1}

= (0.01 g L^{-1} x 0.5 L) / 200.59 g mol^{-1}

= 0.0000249 mol

MM ($HgNO_3.H_2O$) = MM (Hg) + MM (N) + 3 (MM(O)) + 2(MM(H)) + MM(O)

= 200.59g + 14 + 3(16) + 2(1) + 16

= 280.6 g mol^{-1}

n = m/MM

m = n x MM

= (0.0000249 mol)(280.6 g mol^{-1})

= 0.007 g

Therefore we must dissolve 0.007 g of $HgNO_3$. H_2O in 500 ml of distilled water.

This was done using a 0.5 L volumetric flask and the amount of the mercurous nitrate needed was weighed on a analytical scale - First a small amount if the distilled water must be heated and the mercurous chloride added a little by little until dissolved, then the solution must be cooled and poured into the 500 ml volumetric flask and topped up with distilled water to the 500 ml mark.

Sodium Dichromate ($Na_2Cr_2O_7.2H_2O$) - ion Cr^{6+} - 200 ppm solution

200 ppm = 200 mg L^{-1} x 1g/1000 mg

= 0.2 g L^{-1}

c = n/v

g/L = (mol x g/mol) / L

mol = (g L^{-1} x L) / g/mol

= (0.2 g L^{-1} x 1L) / 104 g mol^{-1}

= 0.001923 mol

MM (Cr) = 52 g/mol

But since there are 2 molecules of Cr in $Cr_2O_7^{2-}$ = 104g mol^{-1}

MM ($Na_2Cr_2O_7.2H_2O$) = 2(MM(Na)) + 2(MM(Cr)) + 7 (MM(O)) + 2(2(MM(H)) +MM(O)

= 2 (22.98) + 2(52) + 7(16) + 2(2(1) + 16)

= 45.96 + 104 + 112 + 36

= 297.96 g mol^{-1}

n = m/MM

m = n x MM

= (0.001923 mol)(297.96 g mol^{-1})

= 0.573 g

Therefore we must dissolve 0.573 g of $Na_2Cr_2O_7.2H_2O$ in 1L of distilled water.

This was done using a 1 L volumetric flask and adding the amount of sodium dichromate necessary which was weighed on a analytical scale.

APPENDIX B

B.1 Scanning electron micrographs of the Centella leaf before extraction and after extraction

Fresh leaf before extraction

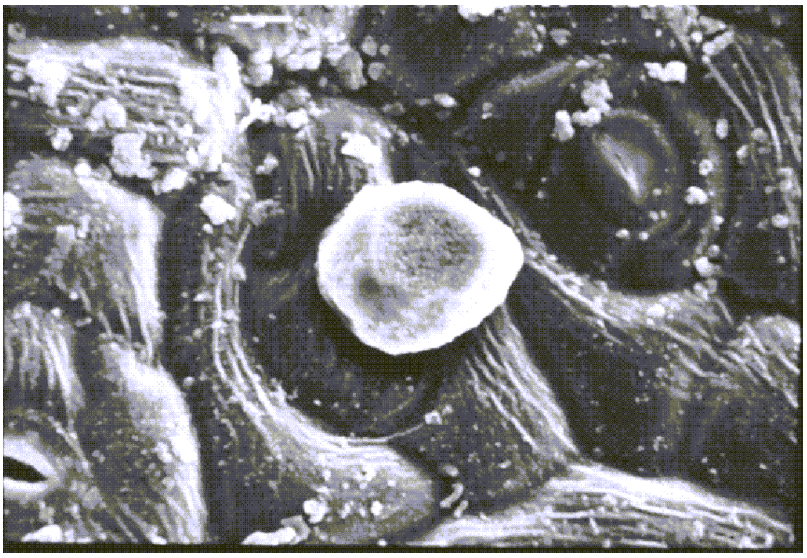


Figure B1 Scanning electron Micrograph of a fresh leaf before extraction (1100x, 20keV) where an essential oil sac can be seen near the stomata

Leaf after steam distillation

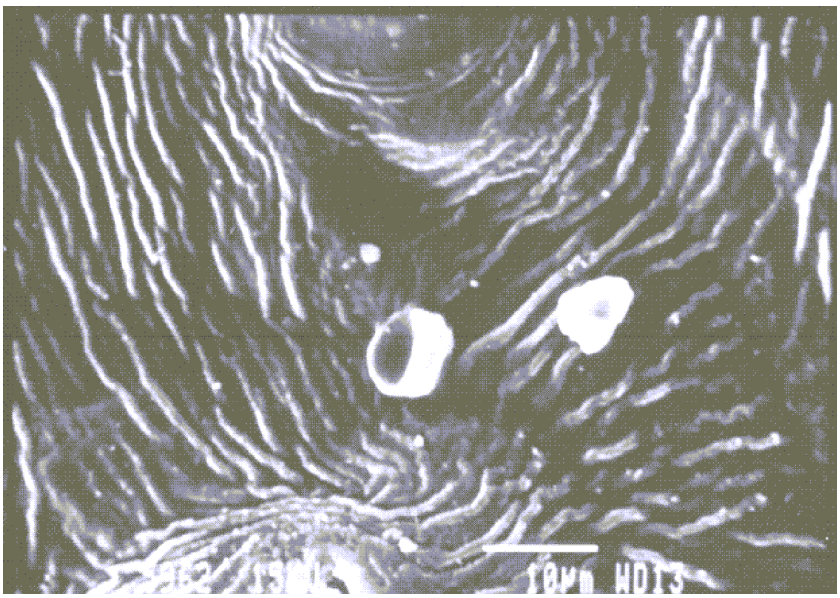


Figure B2 Scanning electron Micrograph of a distilled leaf after extraction (2000x, 15keV) where the essential oil sac can be seen collapsed after extraction.

B.2 Scanning electron micrograph of the Lavender leaf

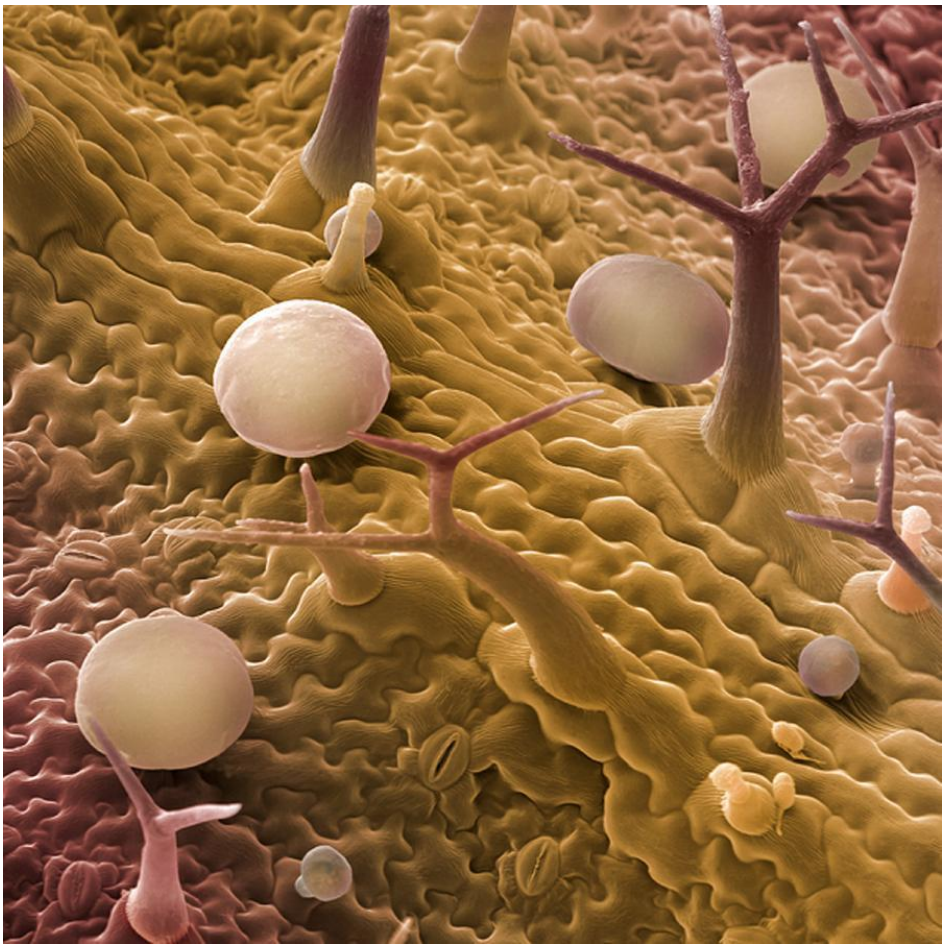


Figure B3 French lavender leaf surface. Coloured scanning electron micrograph (SEM) of a section of French lavender leaf, showing the tooth-like structures (trichomes, spikes). A number of oil glands (spheres) can also be seen. When the leaf is touched or damaged, the glands rupture and release the oil that gives lavender its distinctive fragrance. Magnification: x284 when printed 10 centimeters wide. (Lavender. http://sciencephotolibrary.tumblr.com/page/19 (accessed 01 December 2013)

APPENDIX C

Standards analysed by the GC

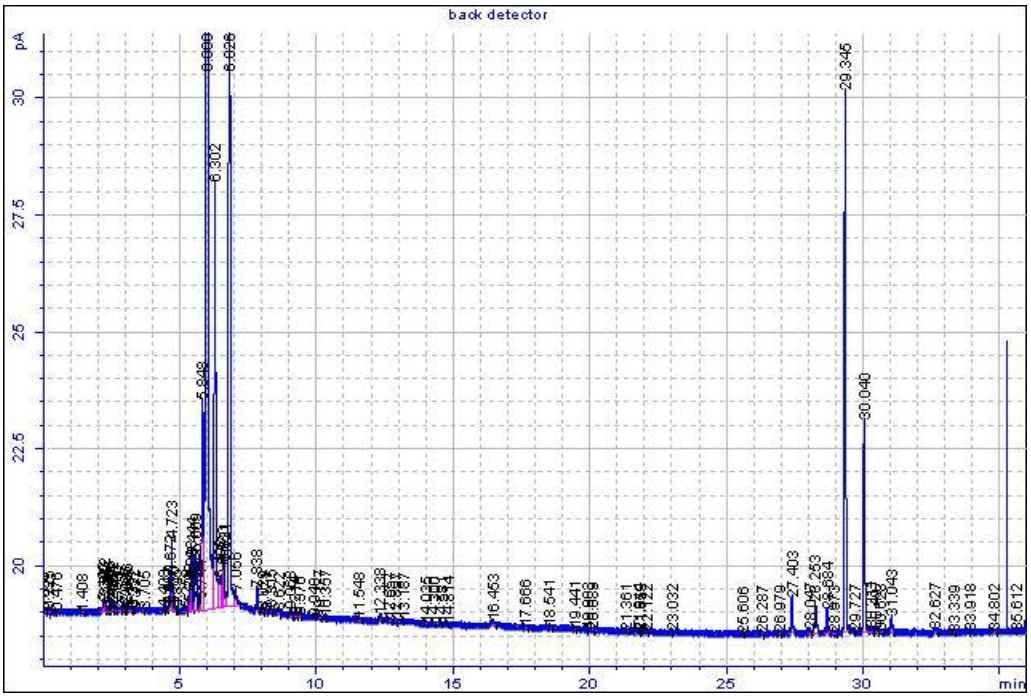


Figure C1 Gas Chromatogram of myrcene

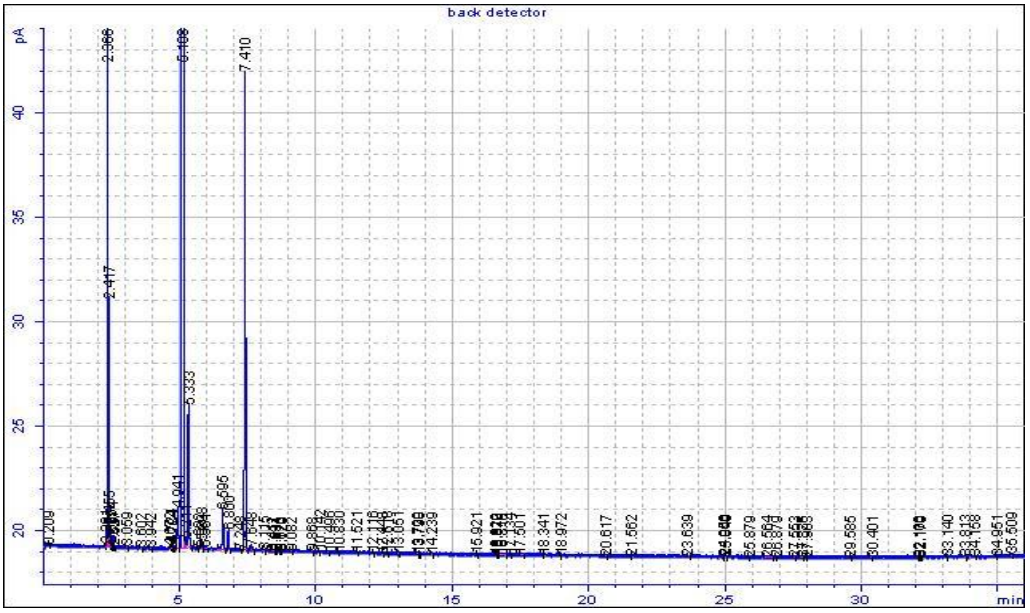


Figure C2 Gas Chromatogram of alpha-pinene

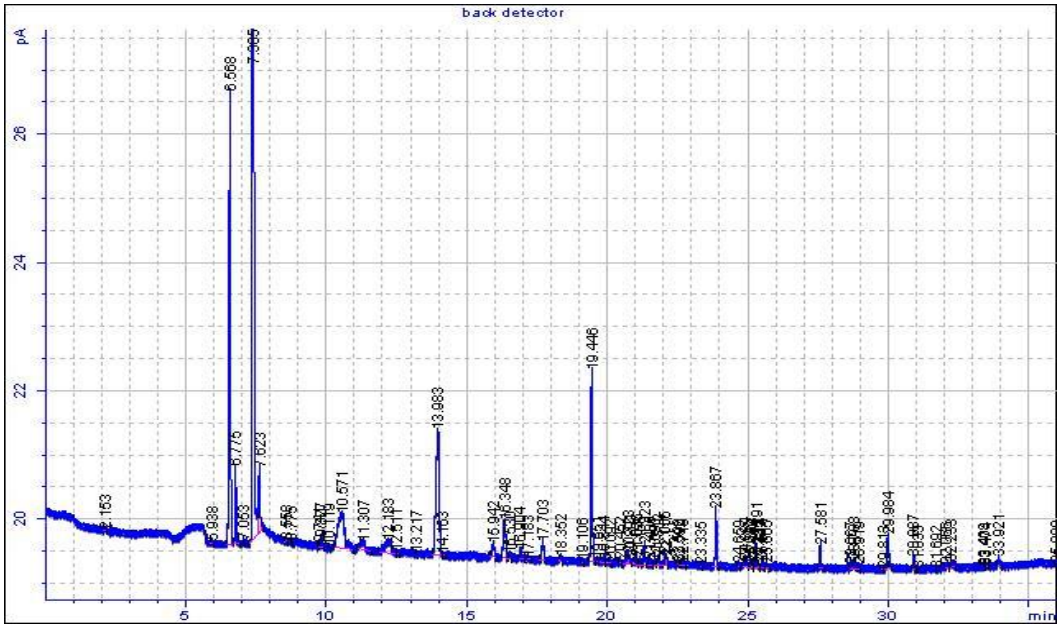


Figure C3 Gas Chromatogram of y-terpinene

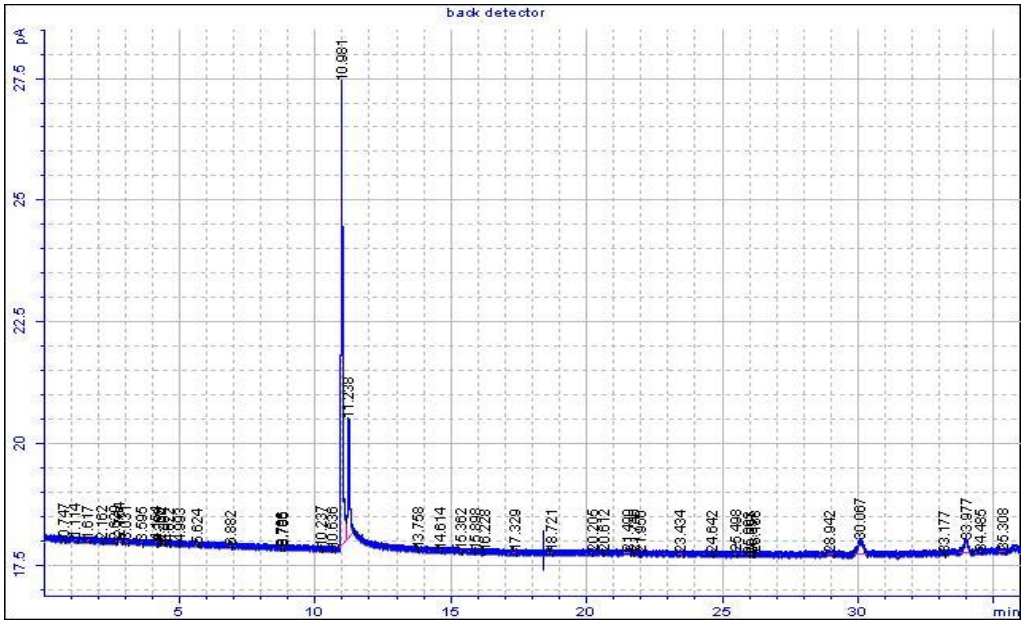


Figure C4 Gas Chromatogram of menthone

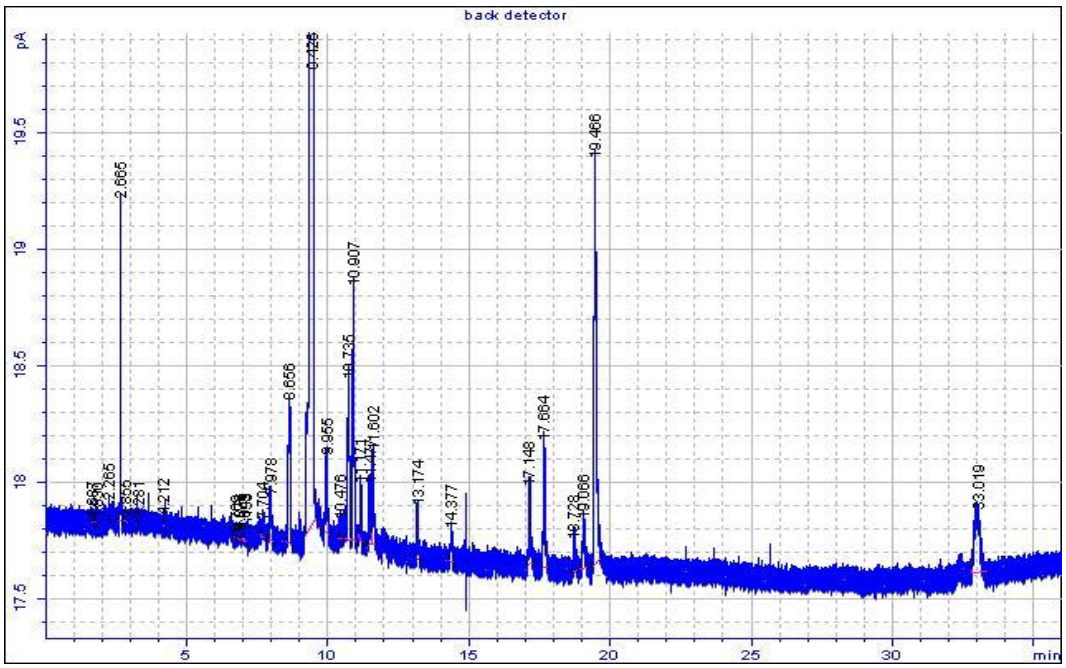


Figure C5 Gas chromatogram of terpinolene

APPENDIX D

Gas chromatograms and mass spectra of The standards run on the GC-MS

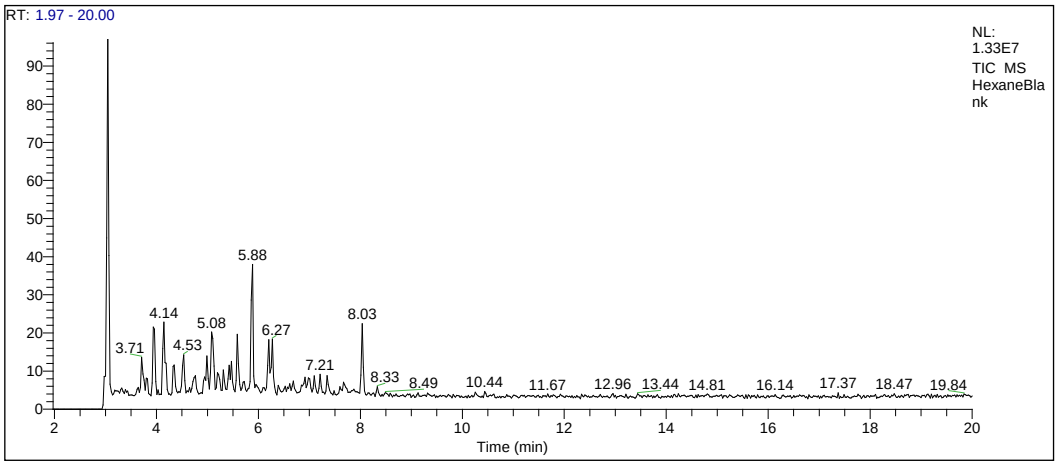


Figure D1 Blank - hexane background graph

α – pinene

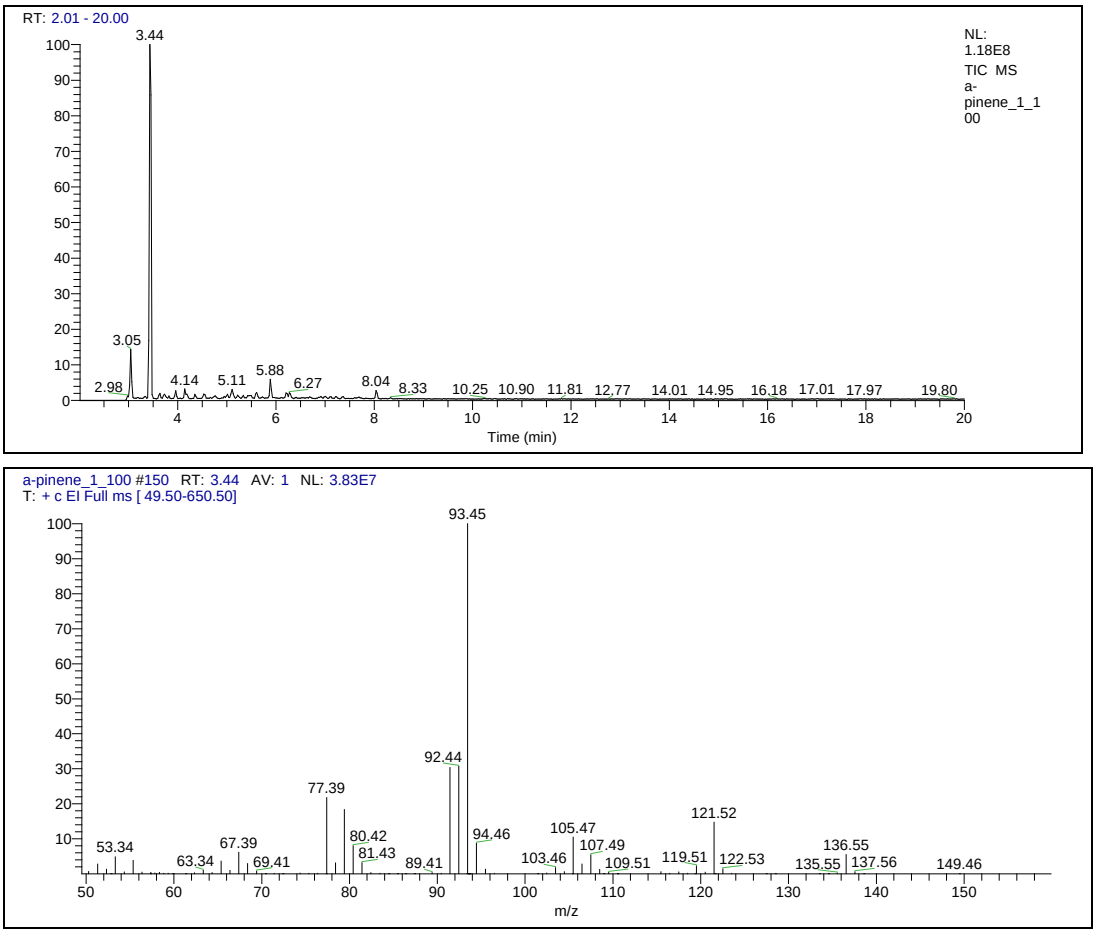


Figure D2 Gas chromatogram (top) and mass spectrum (bottom) of α – pinene

β – pinene

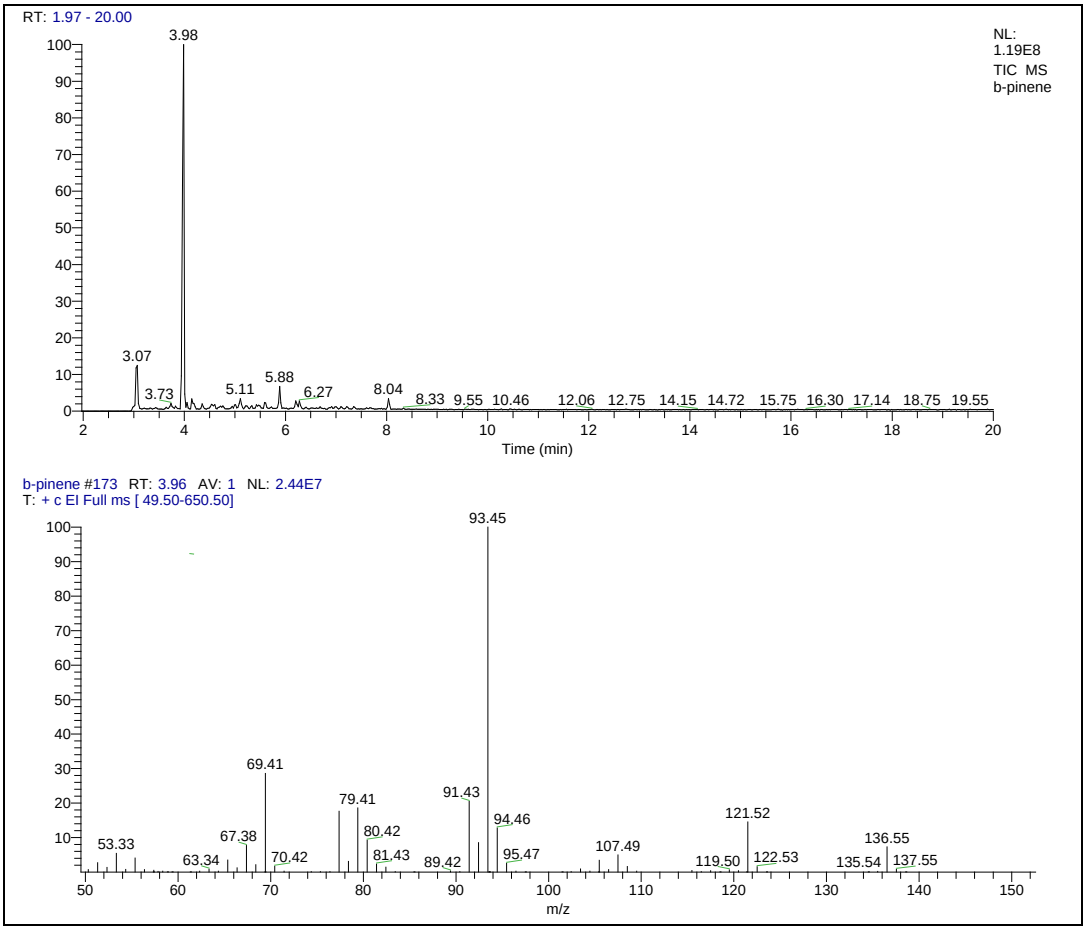


Figure D3 Gas chromatogram (top) and mass spectrum (bottom) of β – pinene

Myrcene

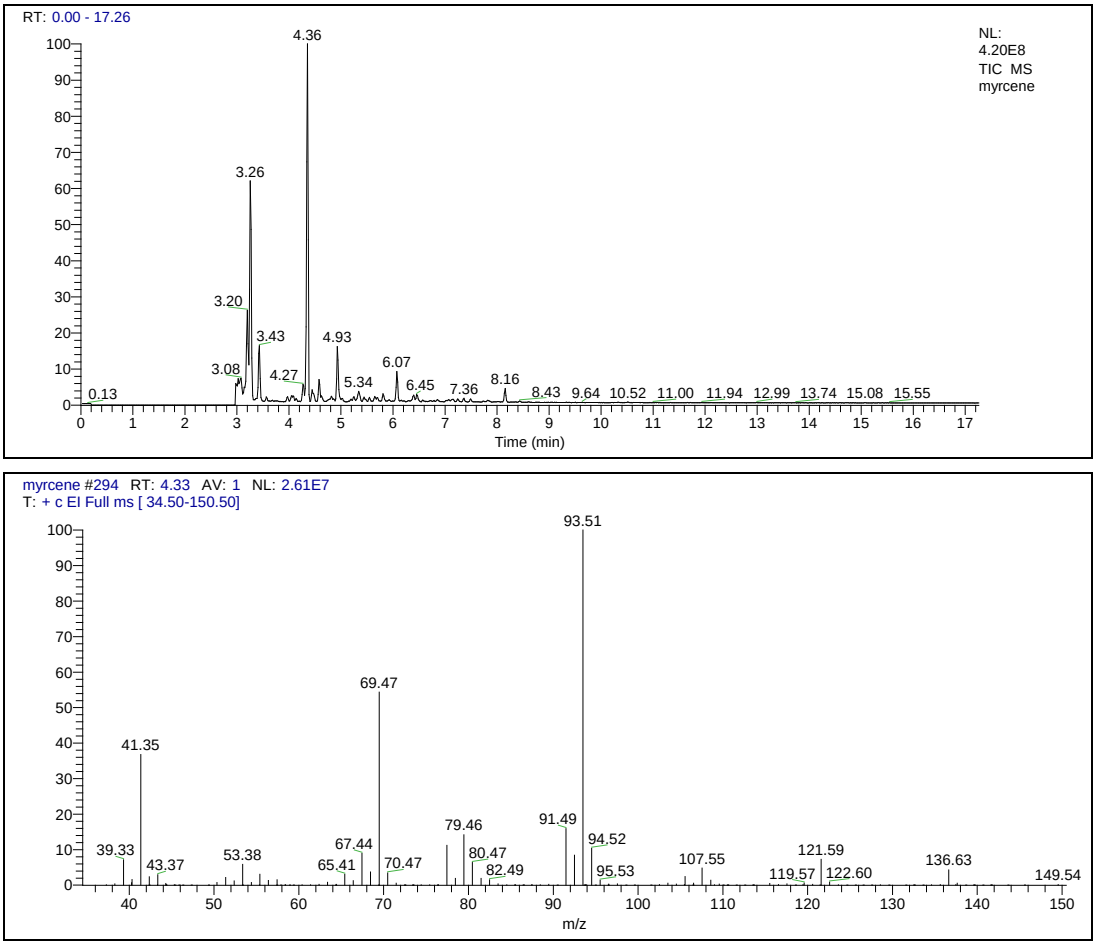


Figure D4 Gas chromatogram (top) and mass spectrum (bottom) of myrcene

Phellandrene

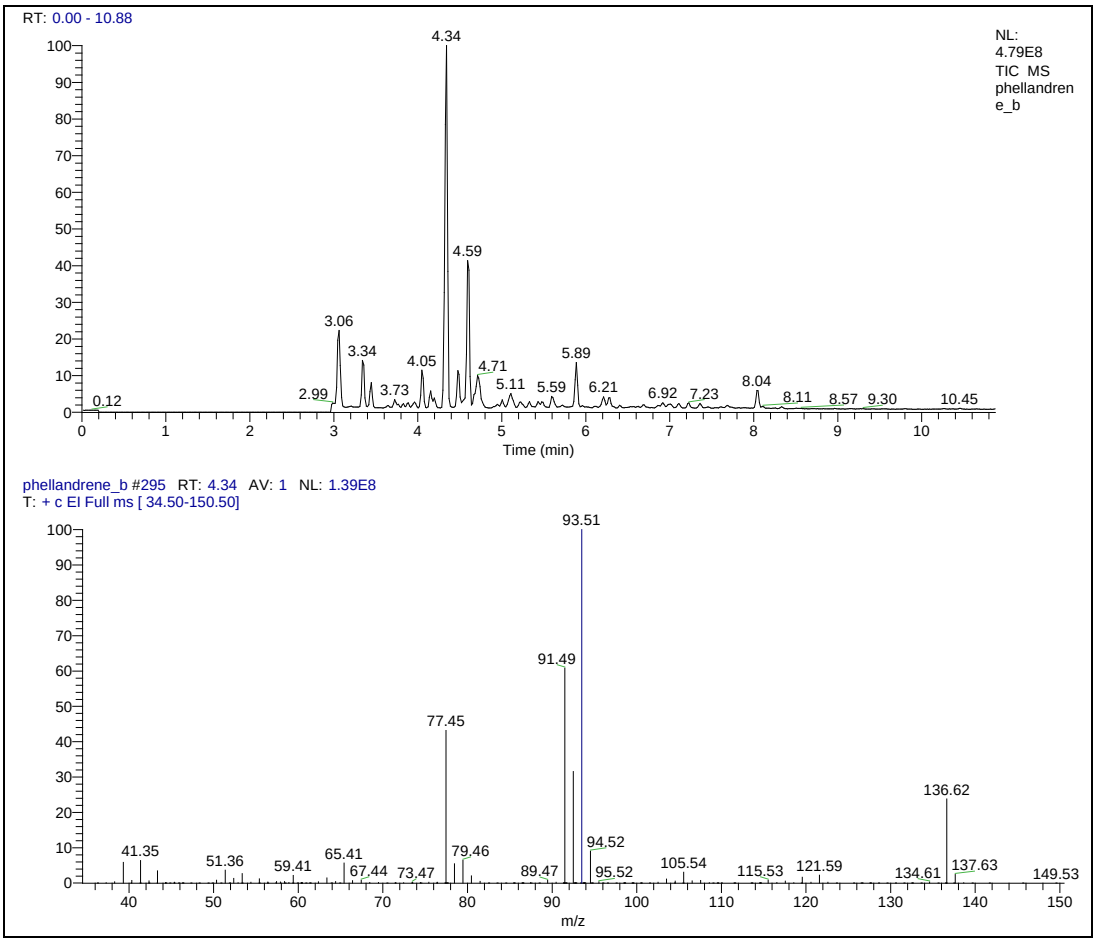


Figure D5 Gas chromatogram (top) and mass spectrum (bottom) of Phellandrene

Menthone

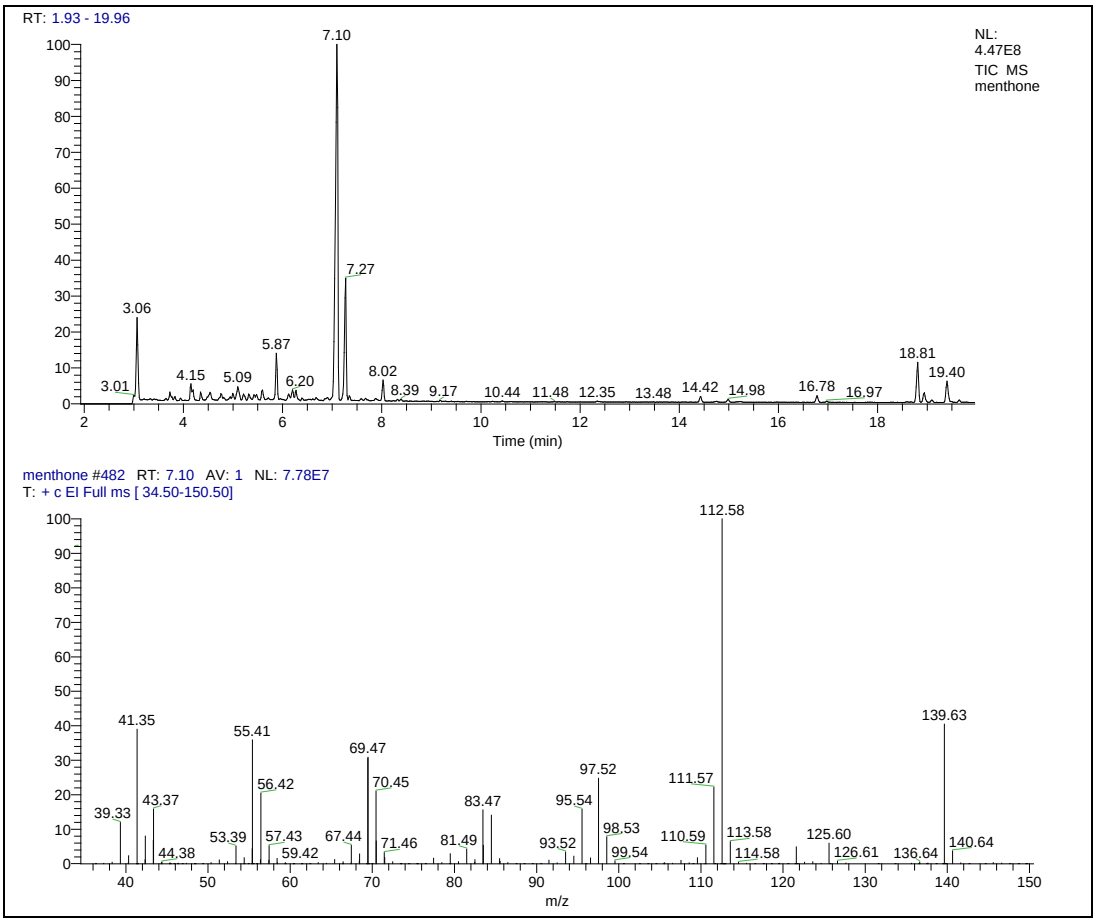


Figure D6 Gas chromatogram (top) and mass spectrum (bottom) of Menthone

X- terpinene

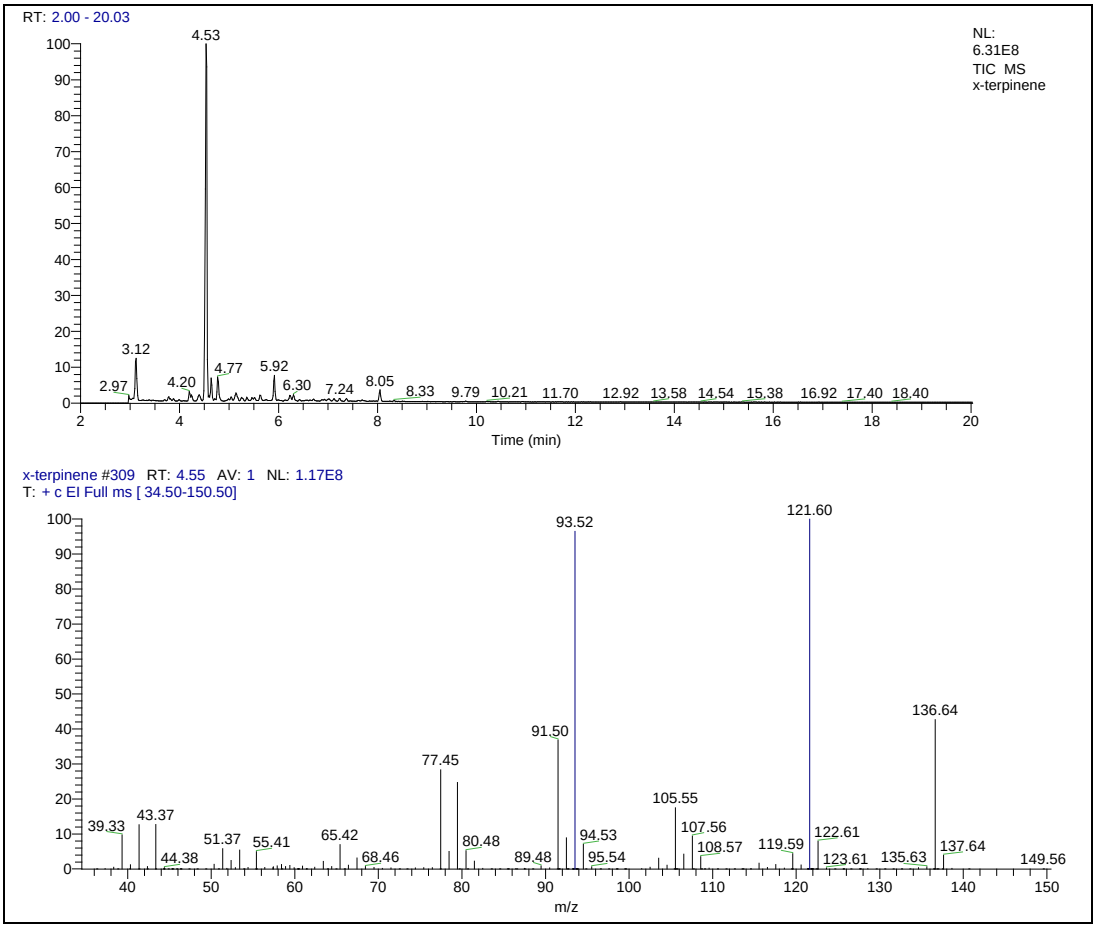


Figure D7 Gas chromatogram (top) and mass spectrum (bottom) of x- terpinene

γ-terpinene

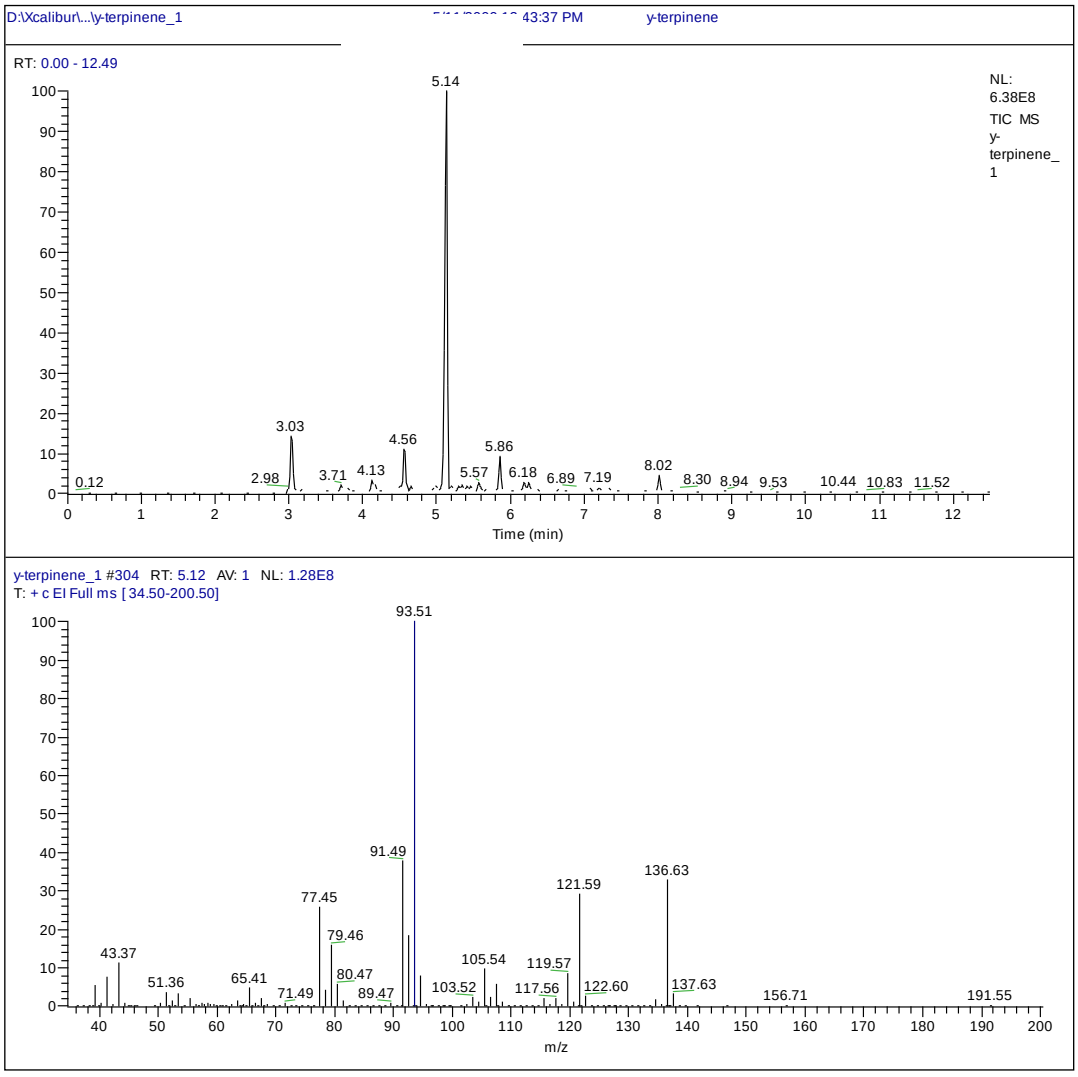


Figure D8 Gas chromatogram (top) and mass spectrum (bottom) of y-terpinene

Terpinen-4-ol

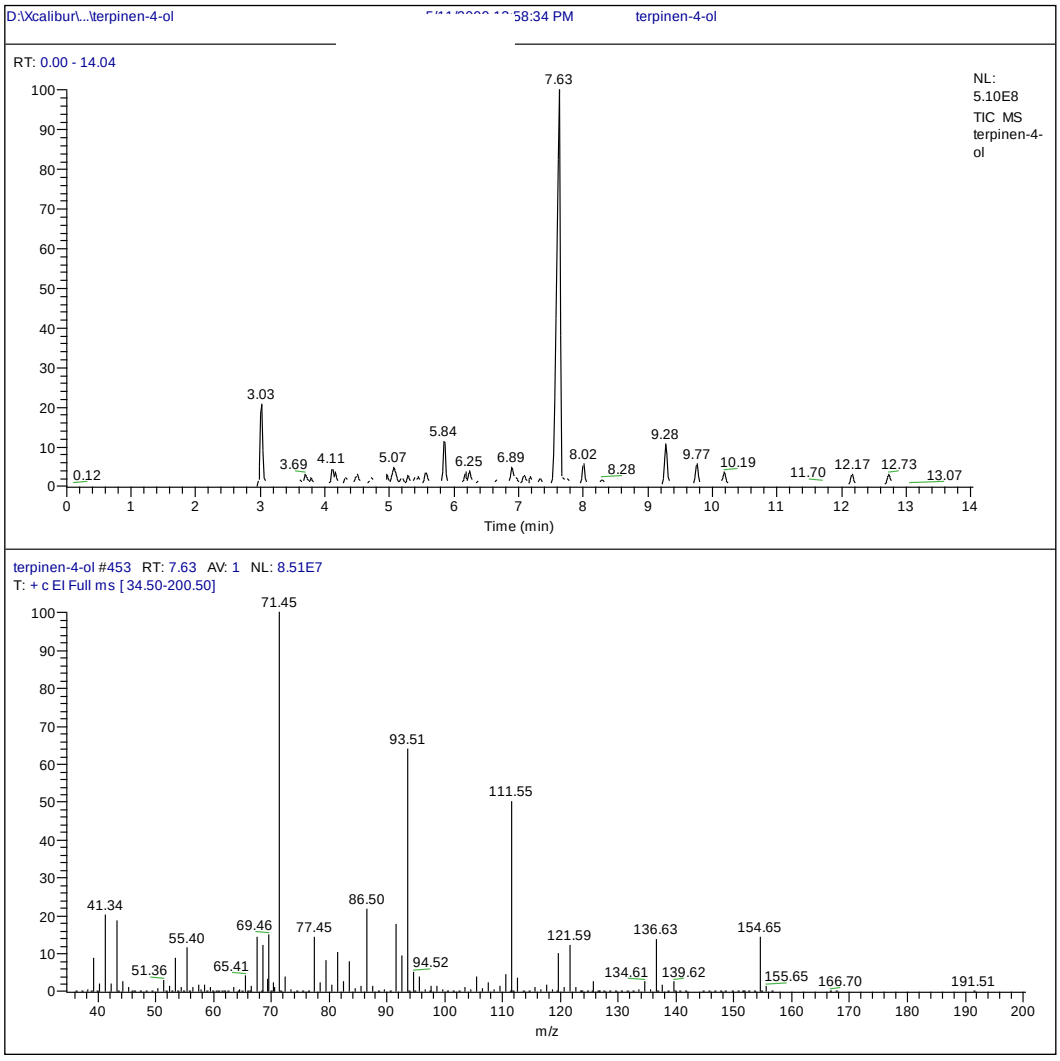


Figure D9 Gas chromatogram (top) and mass spectrum (bottom) of Terpinen-4-ol

Terpinolene

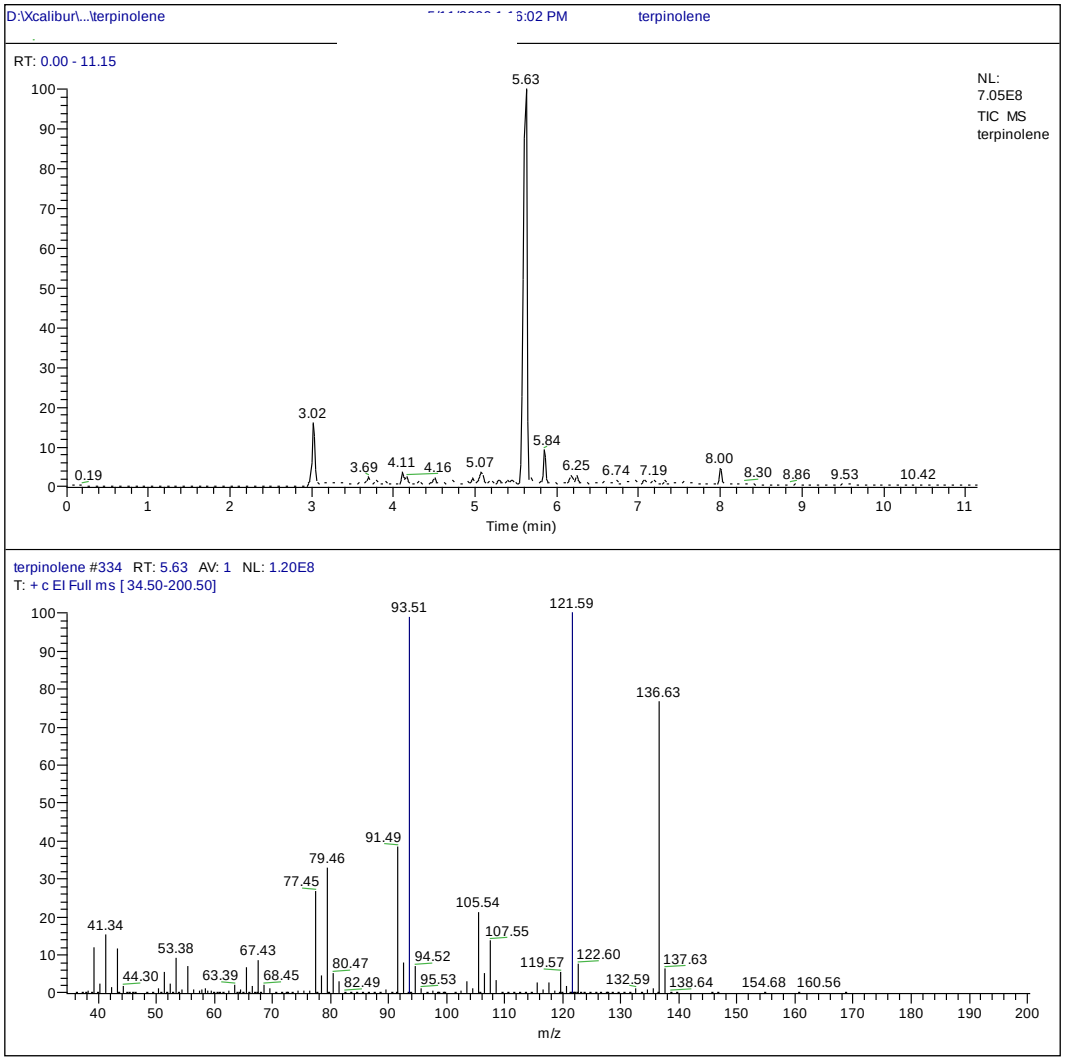


Figure D10 Gas chromatogram (top) and mass spectrum (bottom) of Terpinolene

Camphene

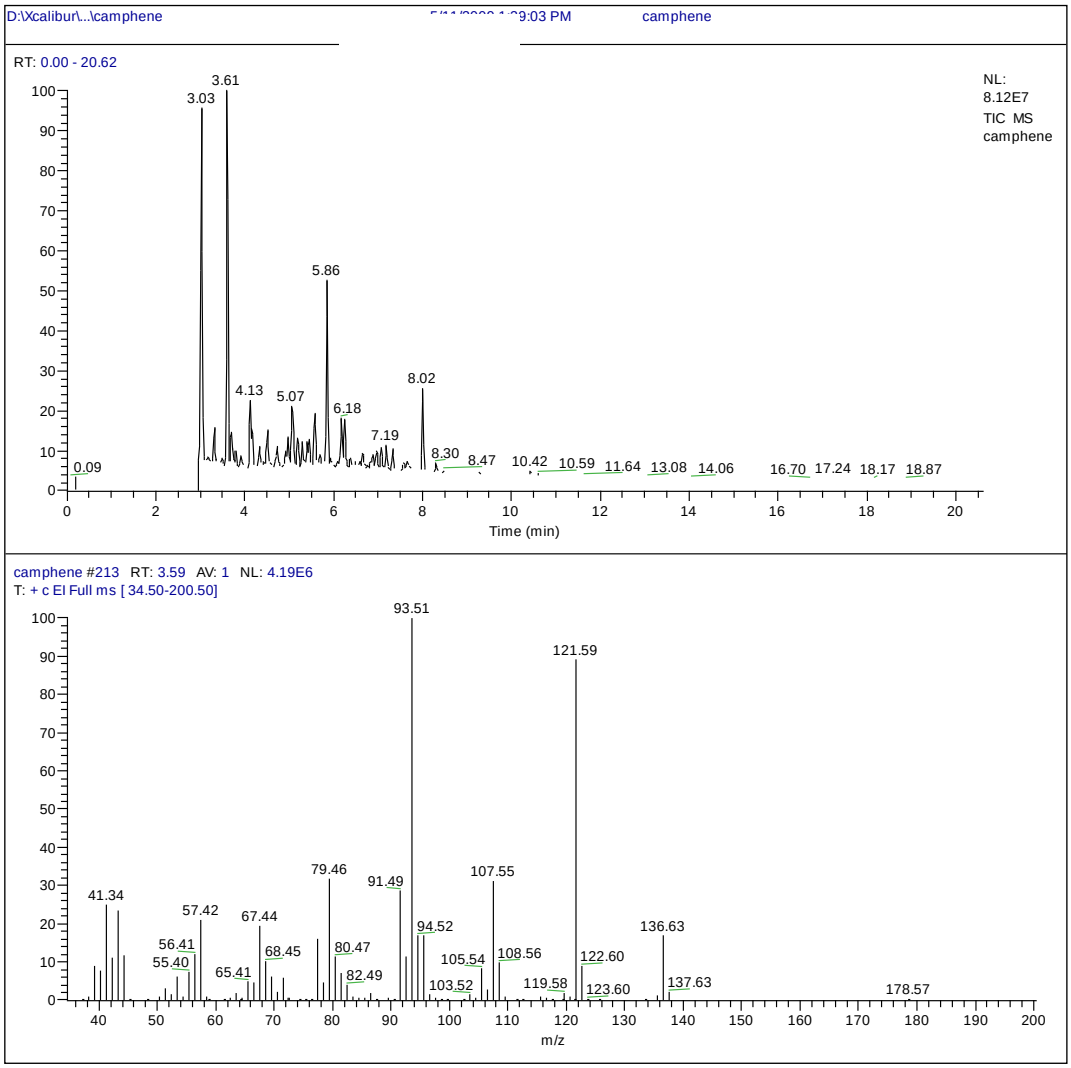


Figure D11 Gas chromatogram (top) and mass spectrum (bottom) of Camphene

Mix of standards

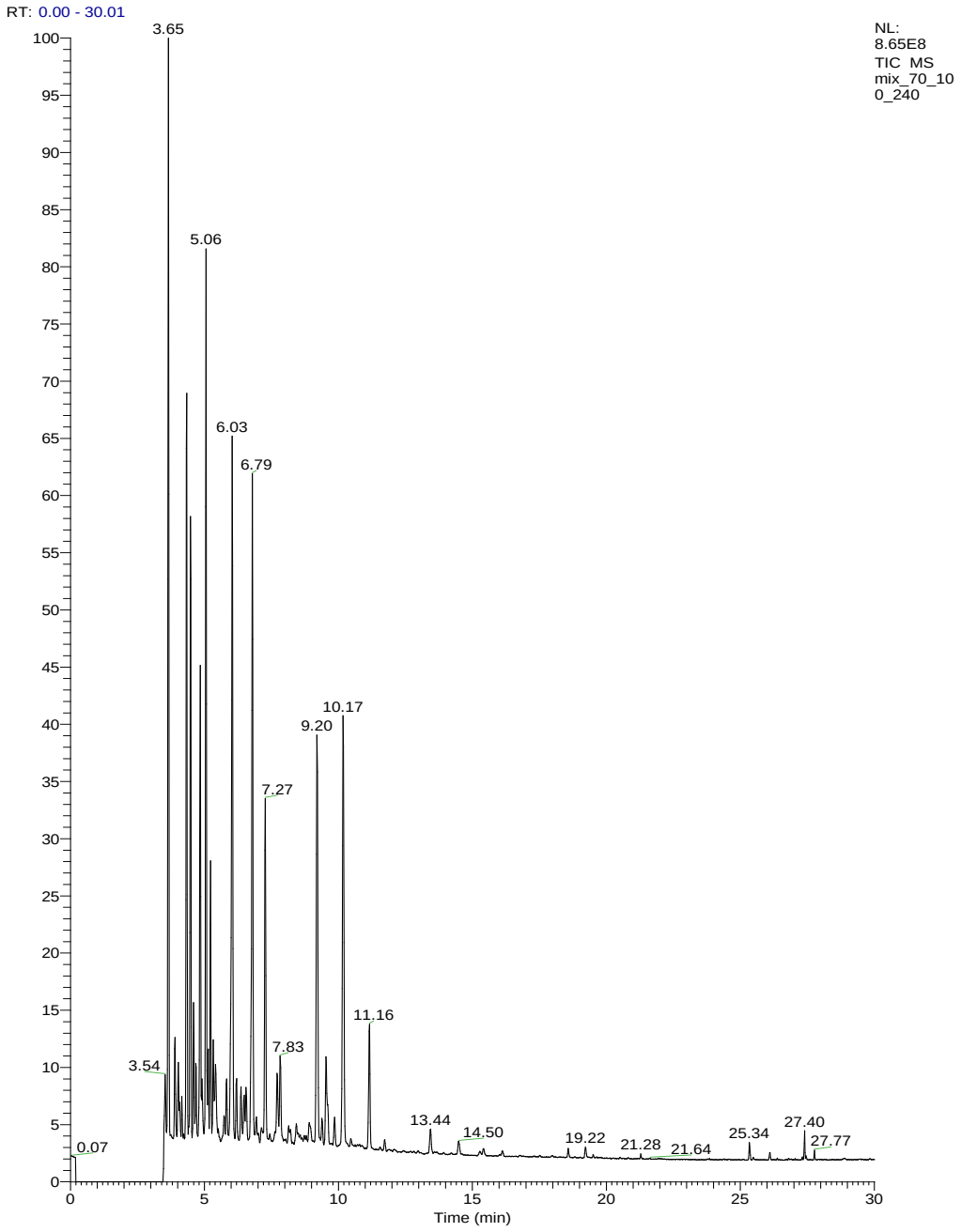


Figure D12 Gas chromatogram of the mixture of standards

APPENDIX E

Identification of constituents in steam distillation setup 1 (SDP1 sample) using the standards and the NIST library

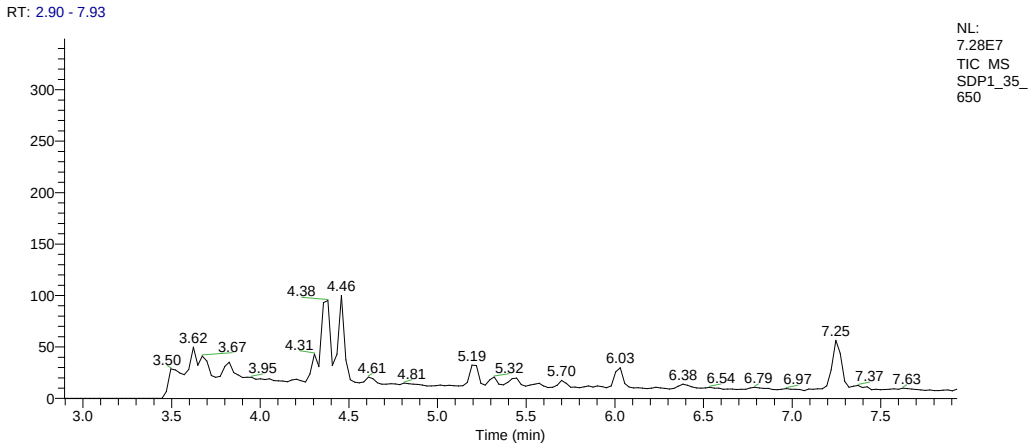


Figure E1 Gas chromatogram of SDP1 between retention times of 3.0 - 7.5 minutes

The peak at 7.25 minutes was then examined and the Mass spectrum obtained look as follows:

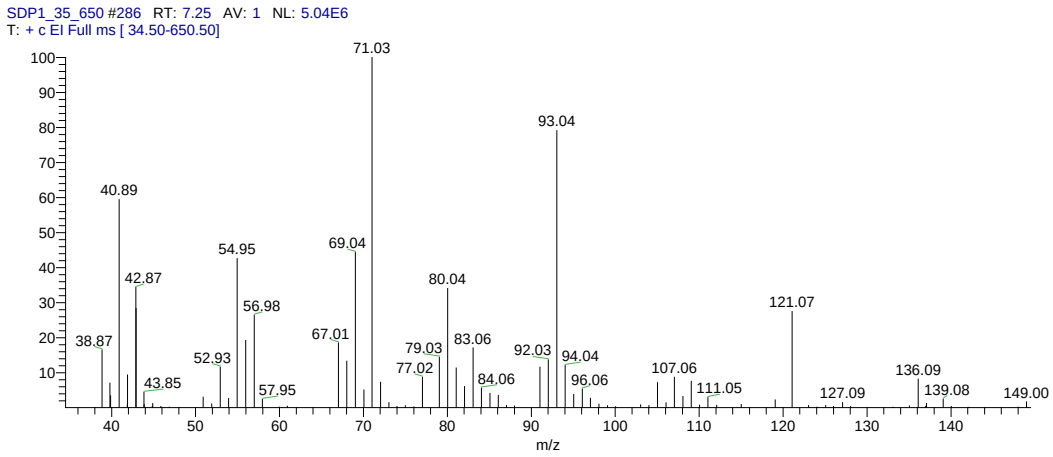


Figure E2 Mass spectrum of peak at 7.25 min in the gas chromatogram of SDP1

Using the NIST library it was found that it correlates to linalool with a similar mass spectrum.

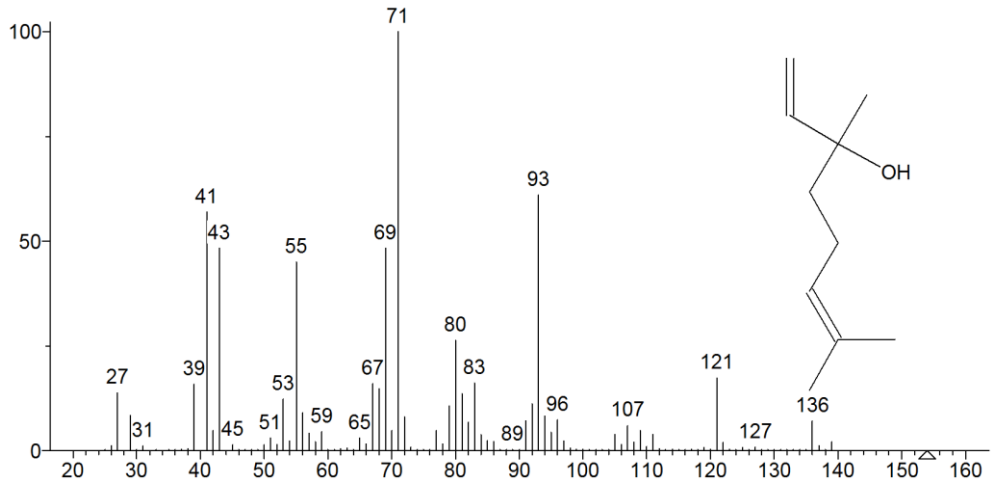


Figure E3 Mass spectrum of Linalol

The next part of the chromatogram was then examined:

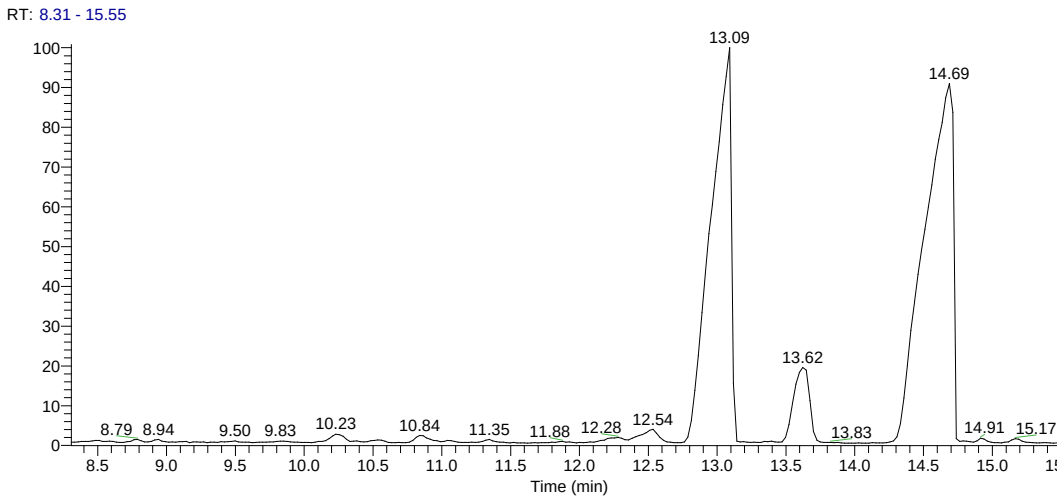


Figure E4 Gas chromatogram of SDP1 between retention times of 8.5 – 15.5 minutes

The peak at 13.09 minutes has a mass spectrum as follows:

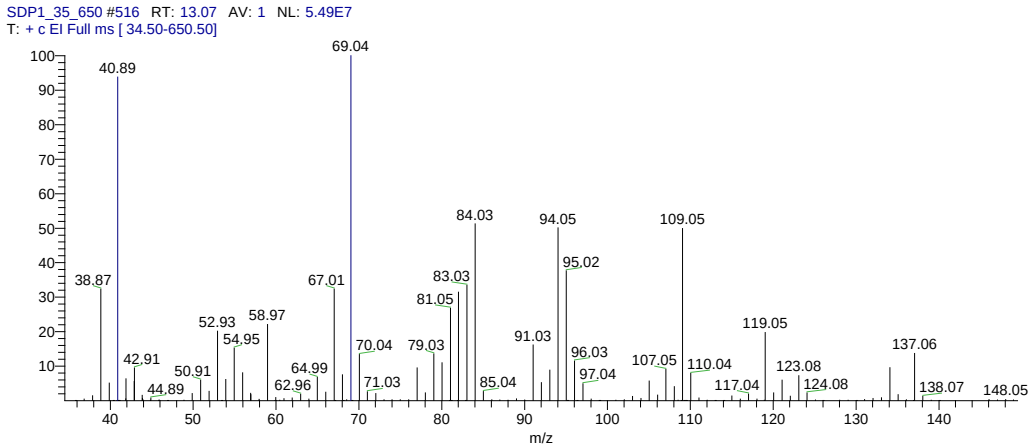


Figure E5 Mass spectrum of peak at 13.09 min in the gas chromatogram of SDP1

This was found to correlate to citral with a similar Mass spectrum.

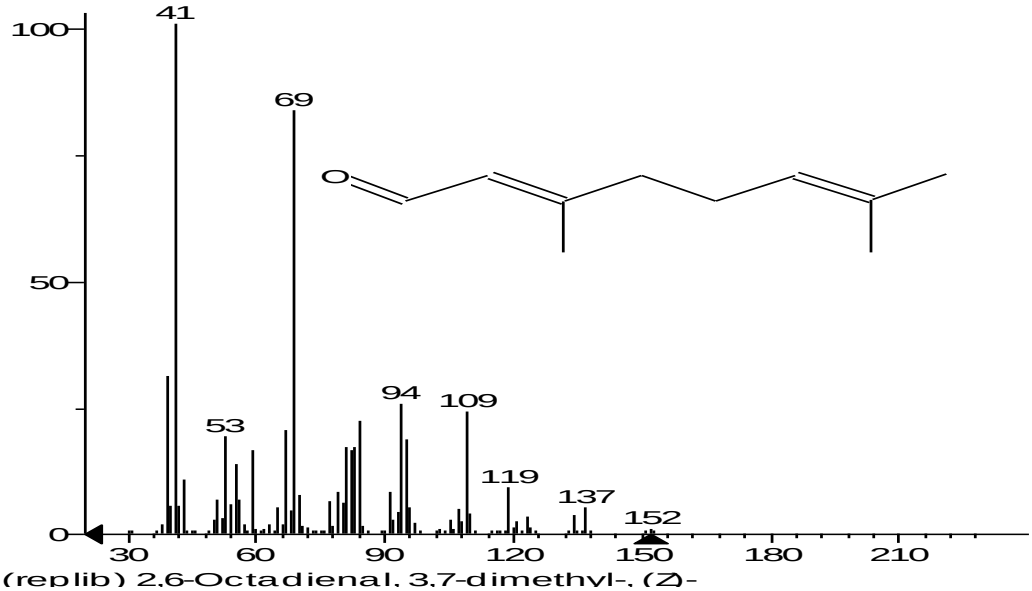


Figure E6 Mass spectrum of Citral

The peak at 13.62 minutes has a Mass spectrum as follows:

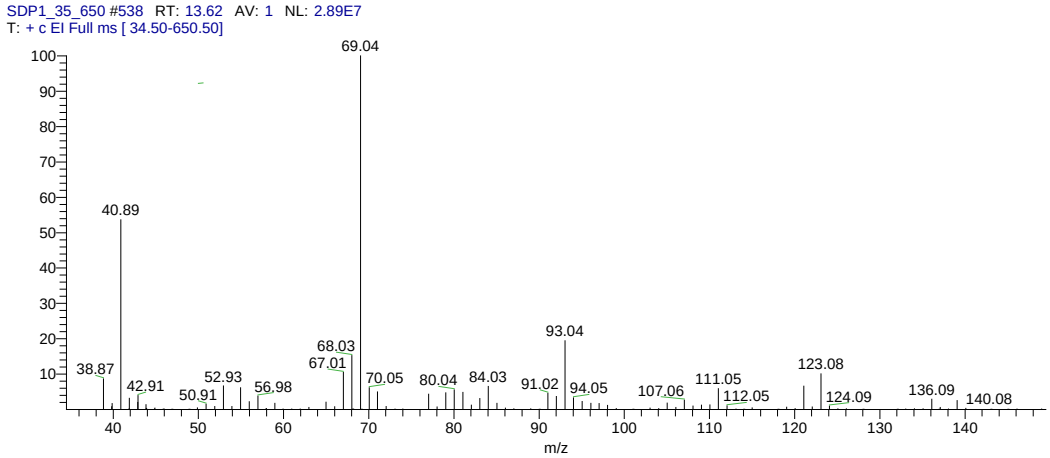
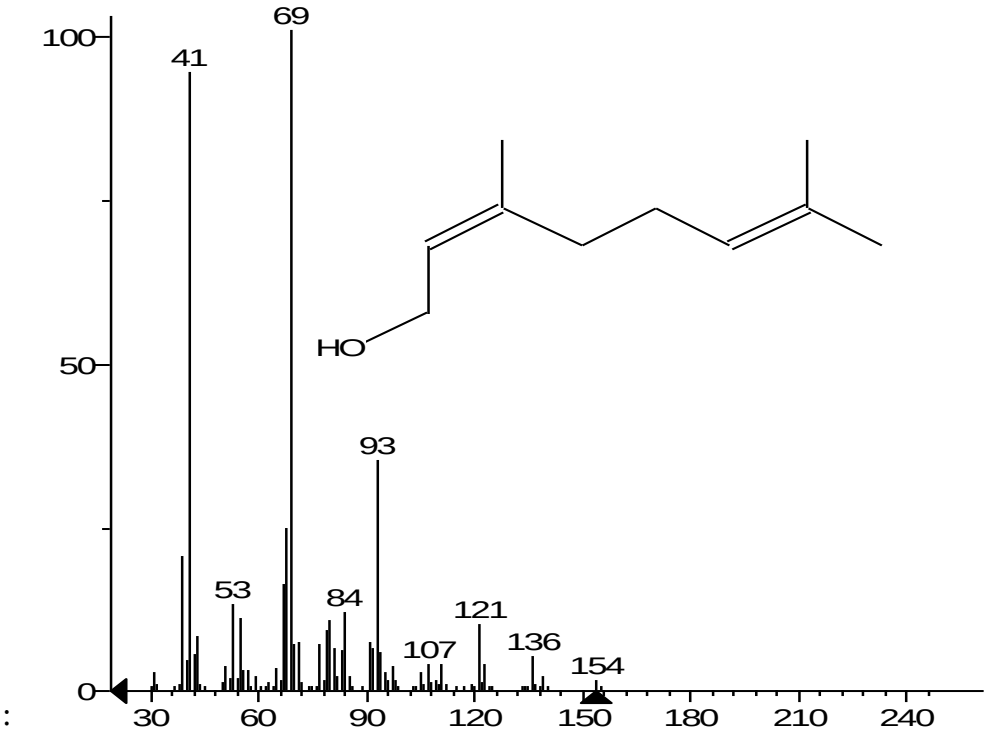


Figure E7 Mass spectrum of peak at 13.62 min in the gas chromatogram of SDP1

This mass spectrum was found to correlate to Neral by using the NIST library.



:

Figure E8 Mass spectrum of peak of Neral

The next peak was then examined. The peak at 14.69 minutes has a Mass spectrum as follows:

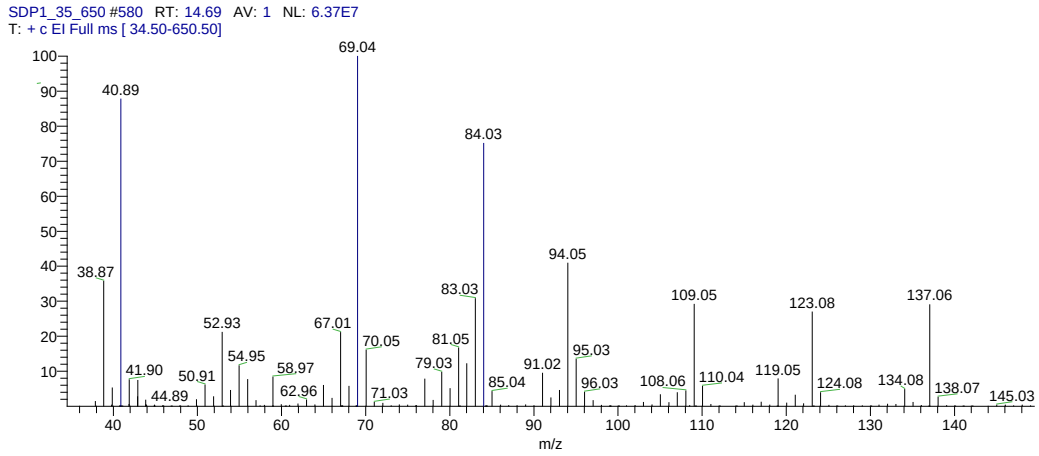


Figure E9 Mass spectrum of peak at 14.69 min in the gas chromatogram of SDP1

This mass spectrum was found to correlate to alpha-citral.

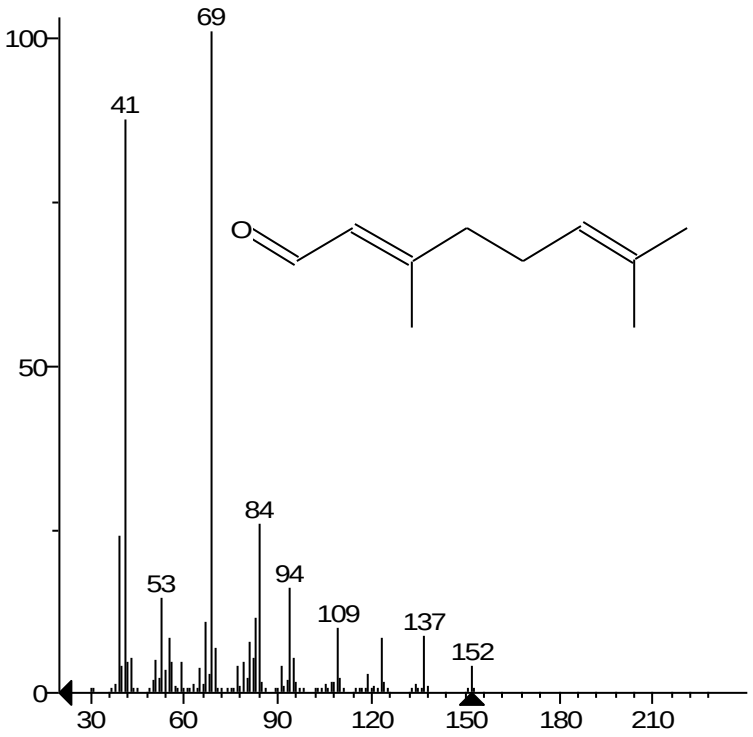


Figure E10 Mass spectrum of alpha-citral

The next part of the chromatogram from 15.5 - 21.5 min was examined

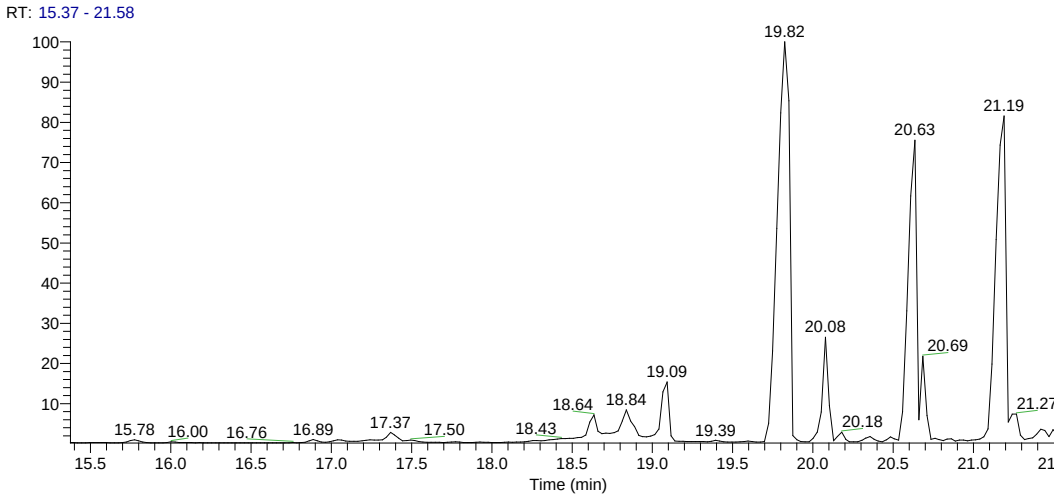


Figure E11 Gas chromatogram of SDP1 between retentions time of 15.5 – 21. 5 minutes

The peak at 17.37 minutes was examined and had a Mass spectrum of:

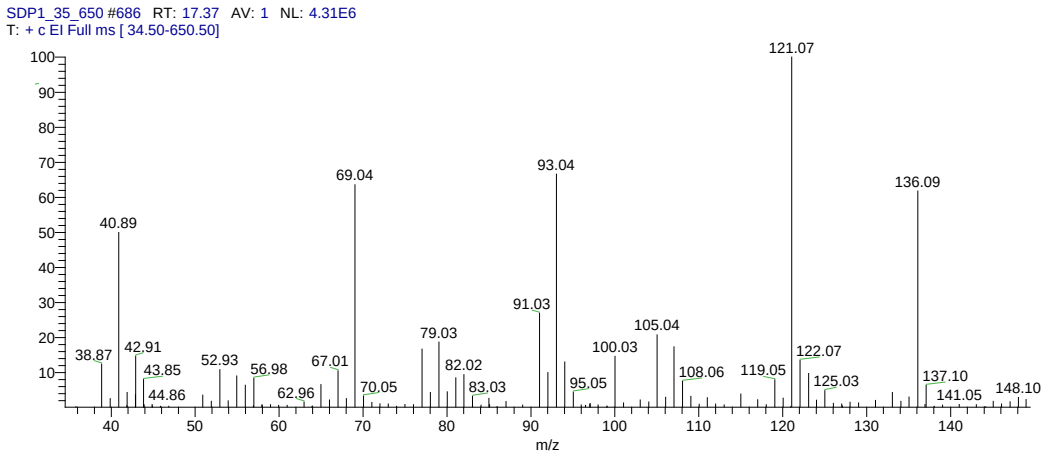


Figure E12 Mass spectrum of peak at 17.37 min in the gas chromatogram of SDP1

Which correlates to delta- Elemene:

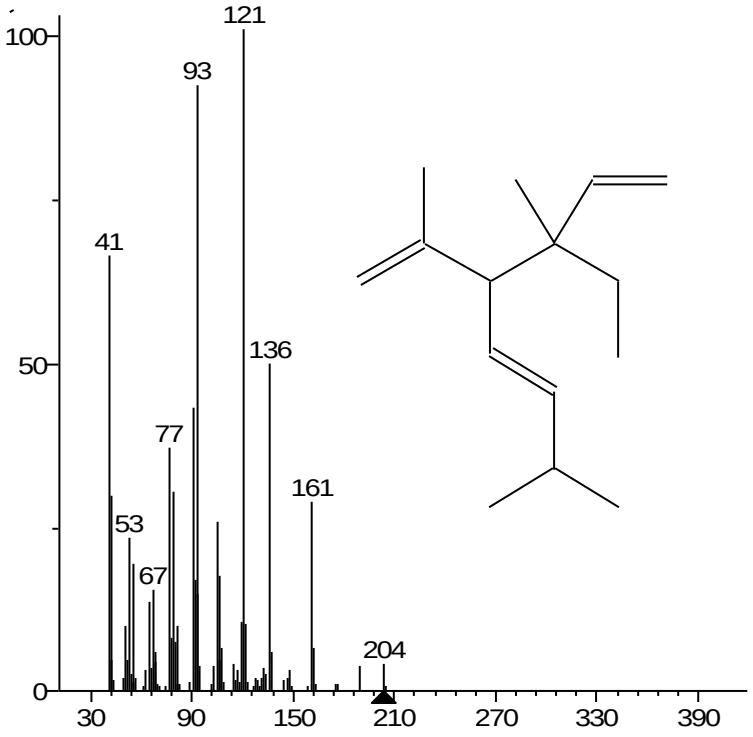


Figure E13 Mass spectrum of delta-elemene

The peak at 19.82 minutes were then examined and was found to have a mass spectrum as follows:

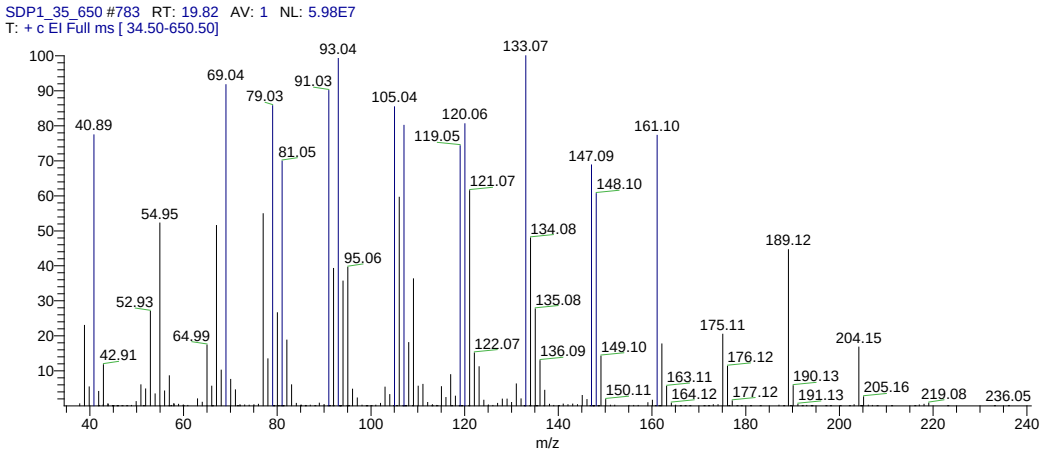


Figure E14 Mass spectrum of peak at 19.82 min in the gas chromatogram of SDP1

This mass spectrum was identified as B-Carophyllene with mass spectrum of:

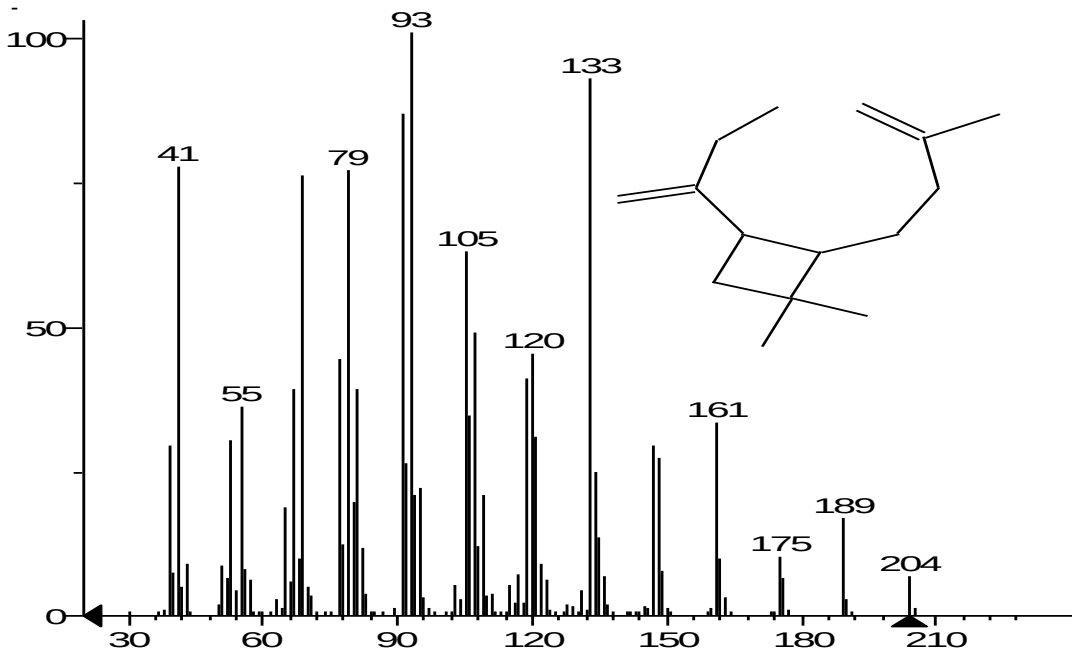


Figure E15 Mass spectrum of B-Carophyllene

The peak at 20.08 minutes is then examined and has a Mass spectrum as follows:

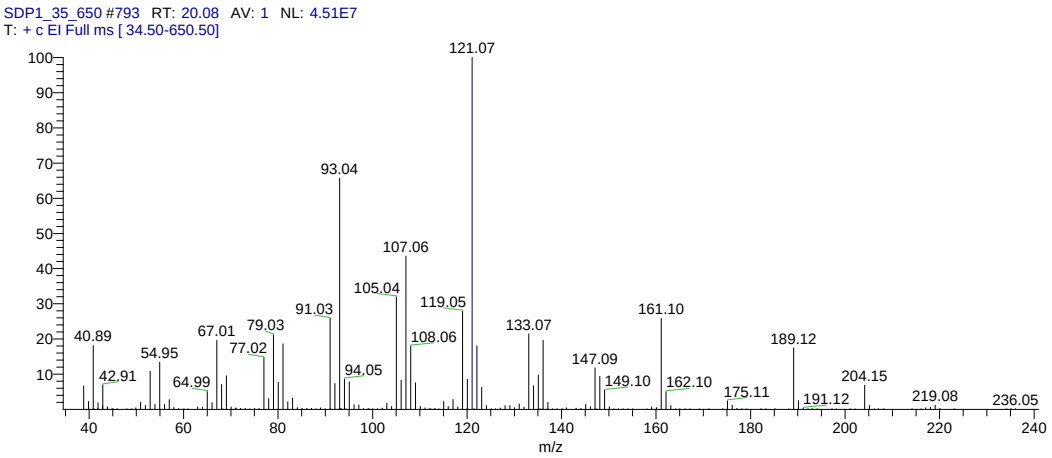


Figure E16 Mass spectrum of peak at 20.08 min in the gas chromatogram of SDP1

It was identified as y-elemene.

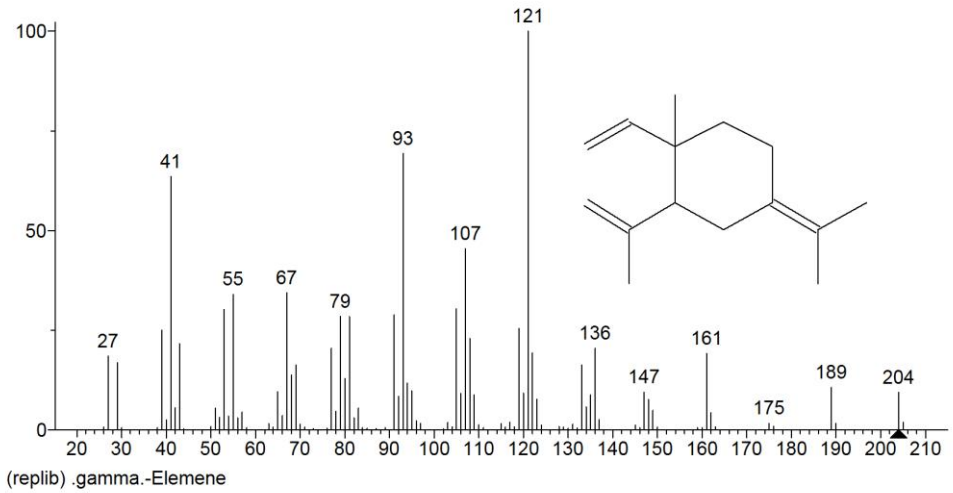


Figure E17 Mass spectrum of y-elemene

The peak at 20.63 minutes was then identified and had a mass spectrum of:

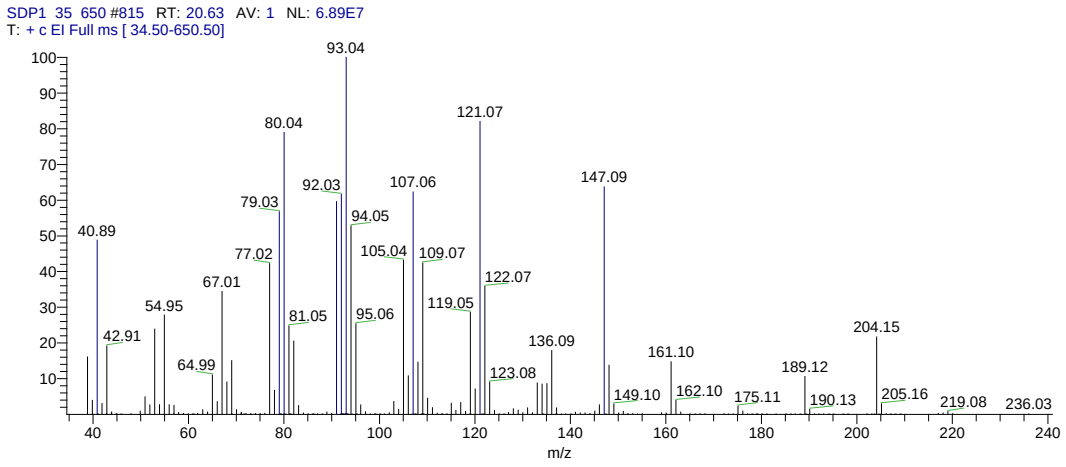


Figure E18 Mass spectrum of peak at 20.63 min in the gas chromatogram of SDP1

And it was identified as alpha-Humulene.

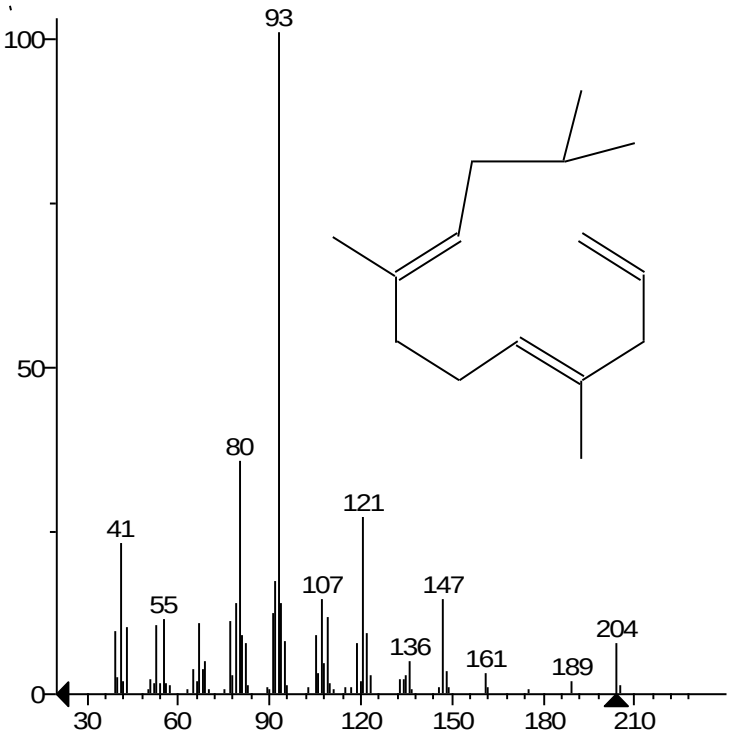


Figure E19 Mass spectrum of alpha- Humulene

The peak at 20.69 minutes was examined and it was found to have a mass spectrum of:

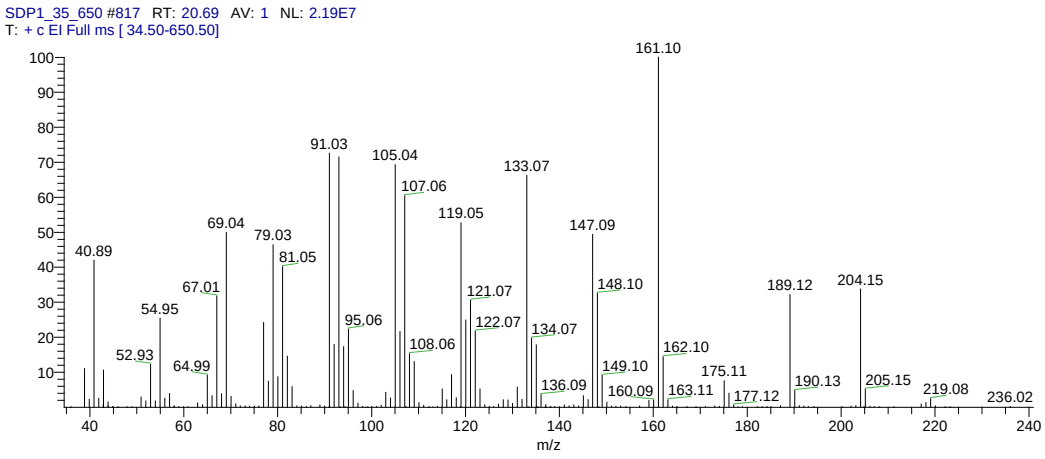


Figure E20 Mass spectrum of peak at 20.96 min in the gas chromatogram of SDP1

This was identified as allo-aromadendrene.

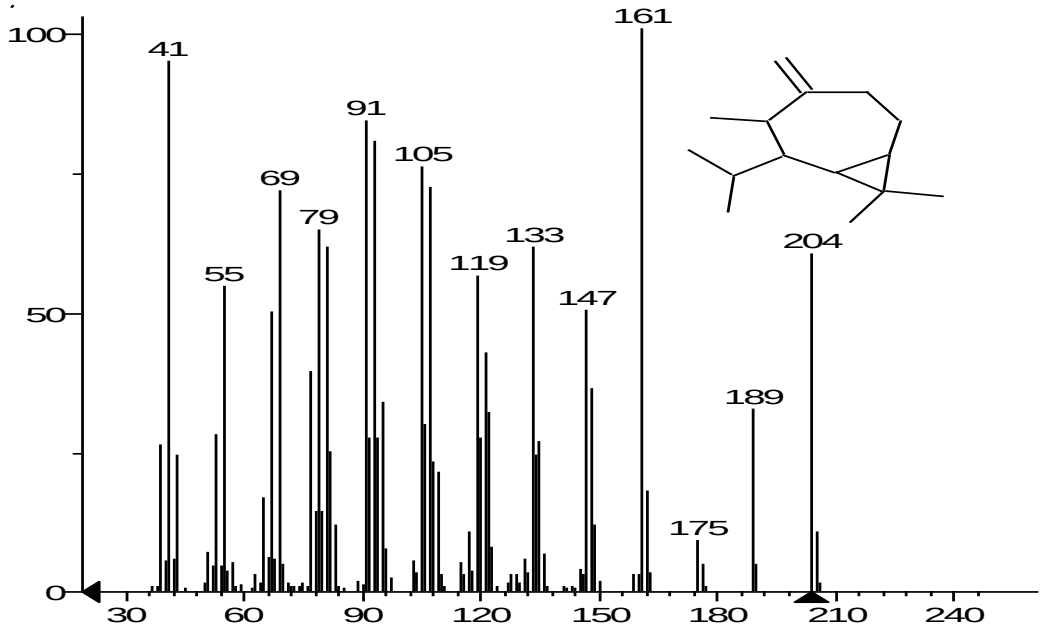


Figure E21 Mass spectrum of peak of allo-aromadendrene.

The peak at 21.19 min was then examined and it was found to have a mass spectrum

of:

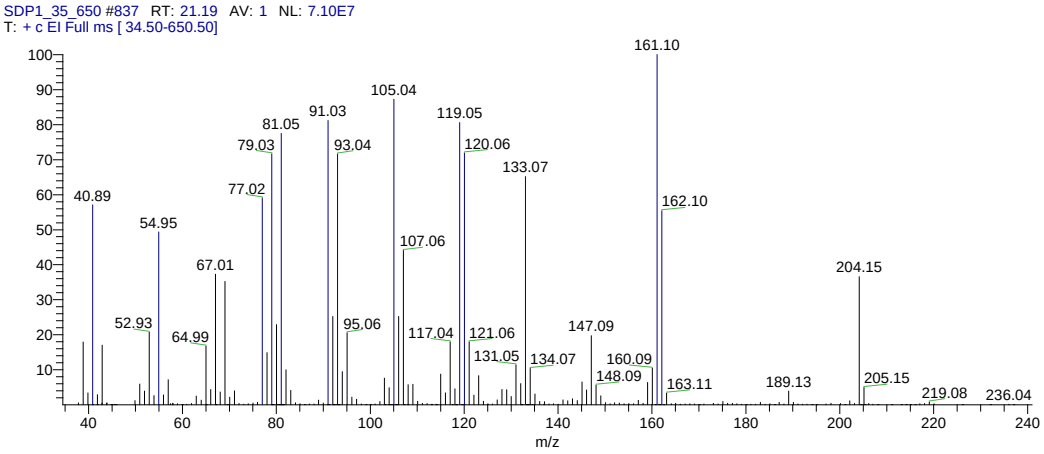
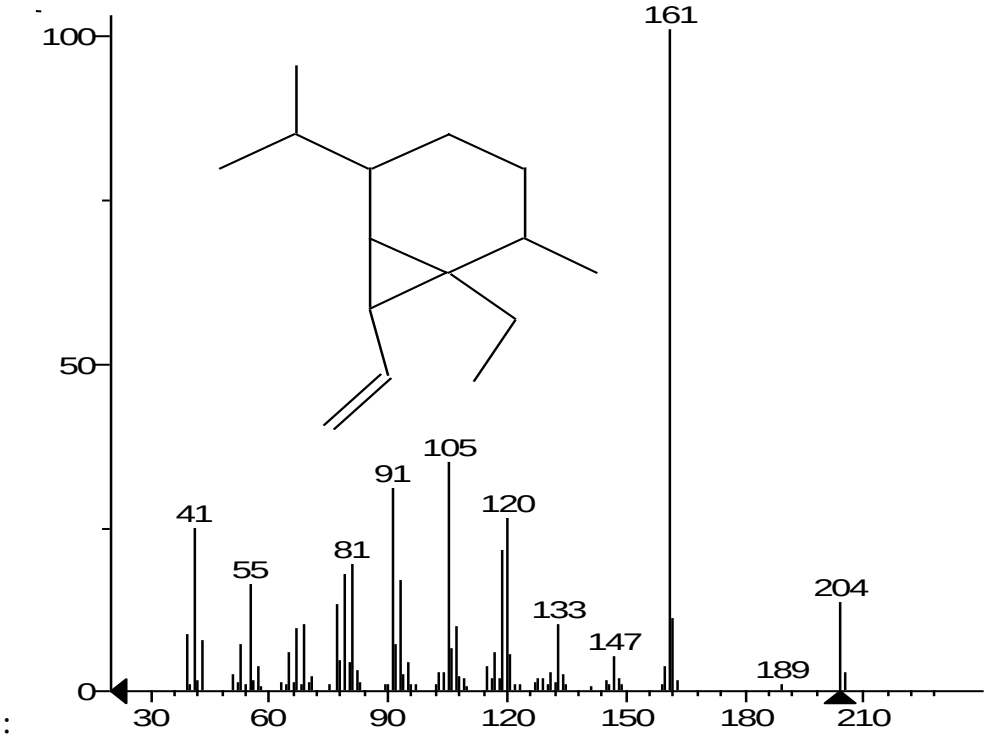


Figure E22 Mass spectrum of peak at 21.19 min in the gas chromatogram of SDP1

This was identified as B-Cuvebene.



:

Figure E23 Mass spectrum of B-Cuvebene

The next part of the chromatogram was then examined:

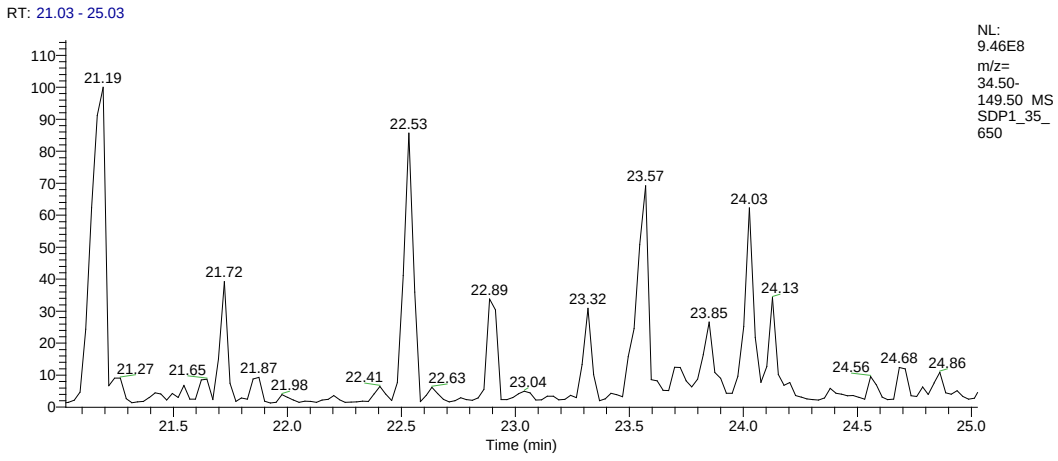


Figure E24 Gas chromatogram of SDP1 between retention times of 21.5 – 25.8 minutes

The peak at 21.72 minutes was found to have a mass spectrum of:

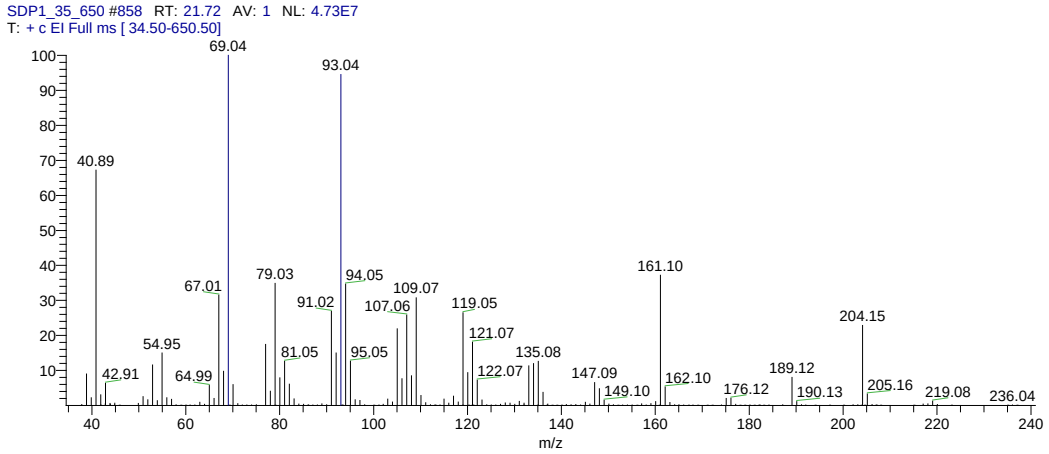


Figure E25 Mass spectrum of peak at 21.72 min in the gas chromatogram of SDP1

This was identified as B-Bisobolene.

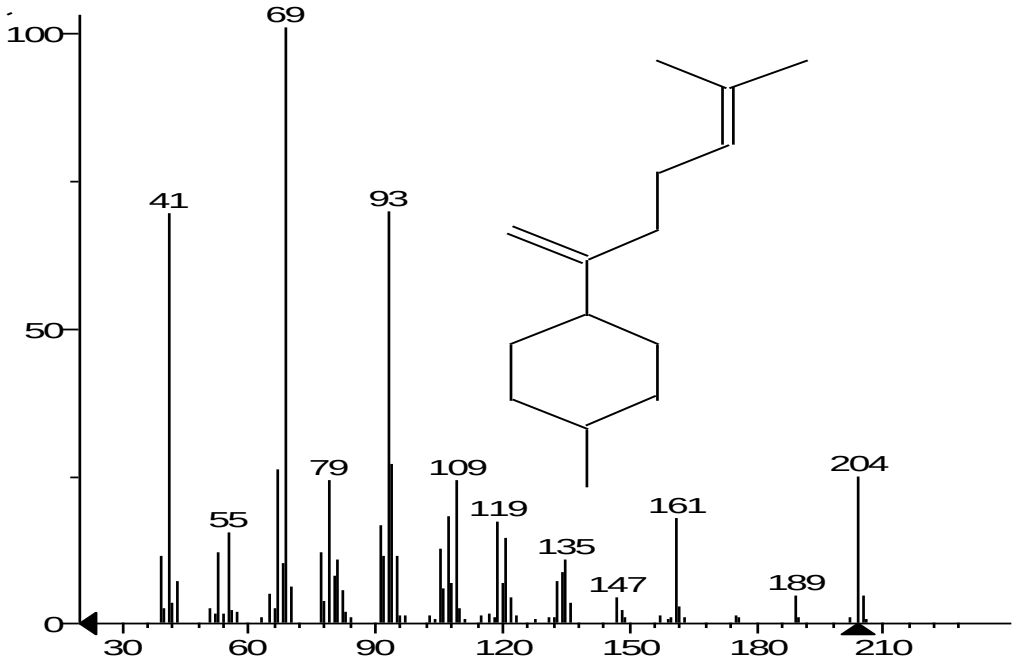


Figure E26 Mass spectrum of B-Bisobolene

The peak at 22.53 minutes was then examined and the mass spectrum was obtained:

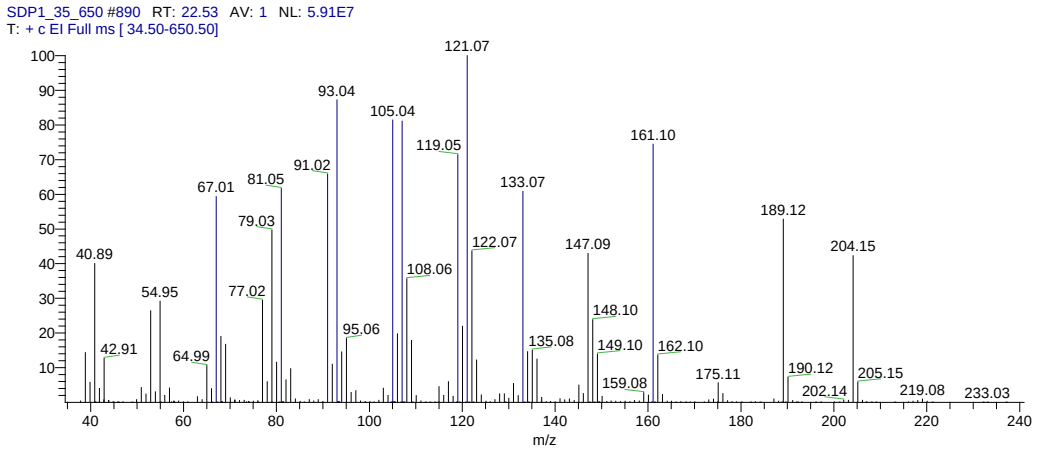


Figure E27 Mass spectrum of peak at 22.53 min in the gas chromatogram of SDP1

This was identified as λ-Gurjunene.

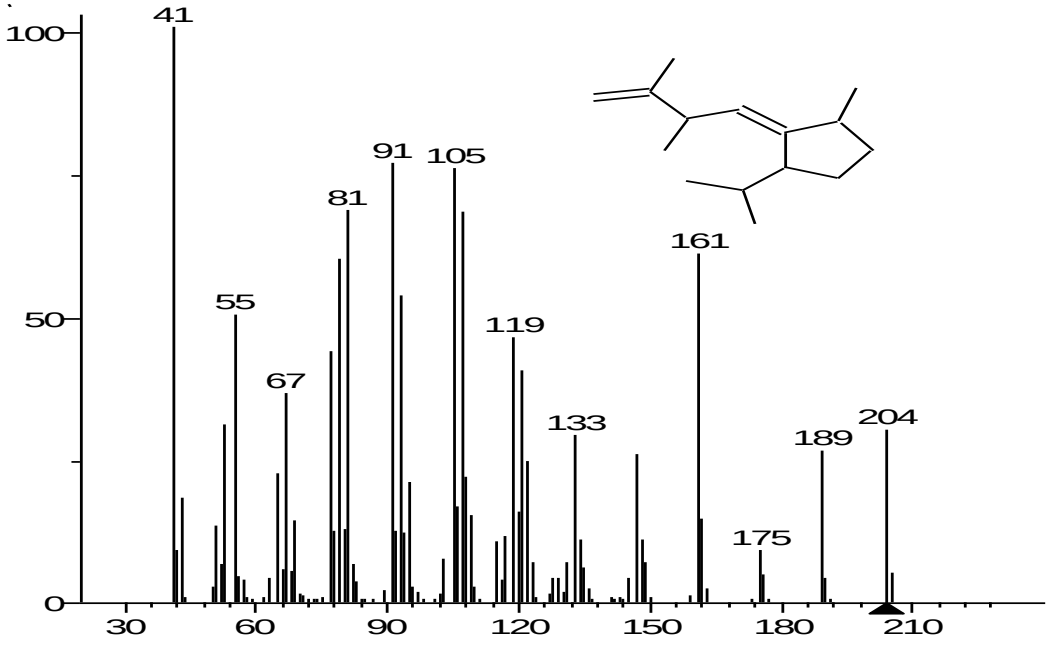


Figure E28 Mass spectrum of λ-Gurjunene.

The peak at 22.89 minutes had a mass spectrum of:

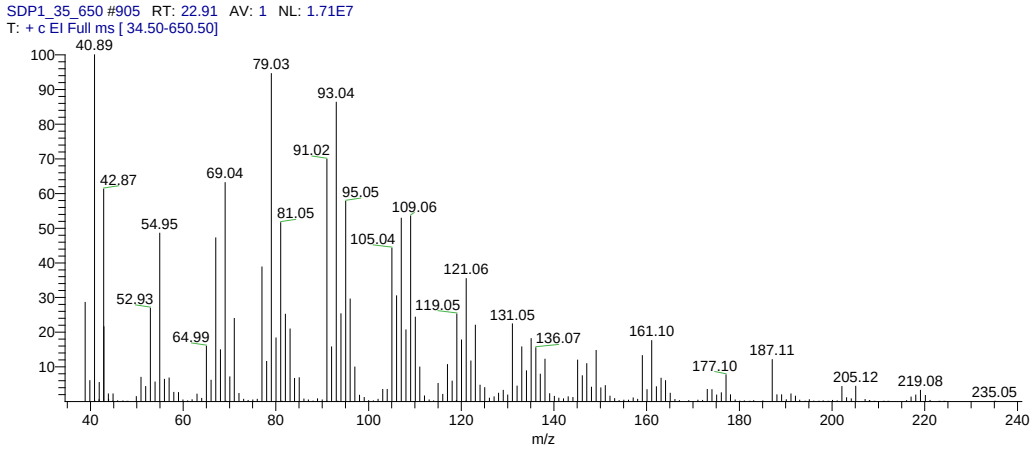


Figure E29 Mass spectrum of peak at 22.89 min in the gas chromatogram of SDP1

It was identified as Carophyllene oxide.

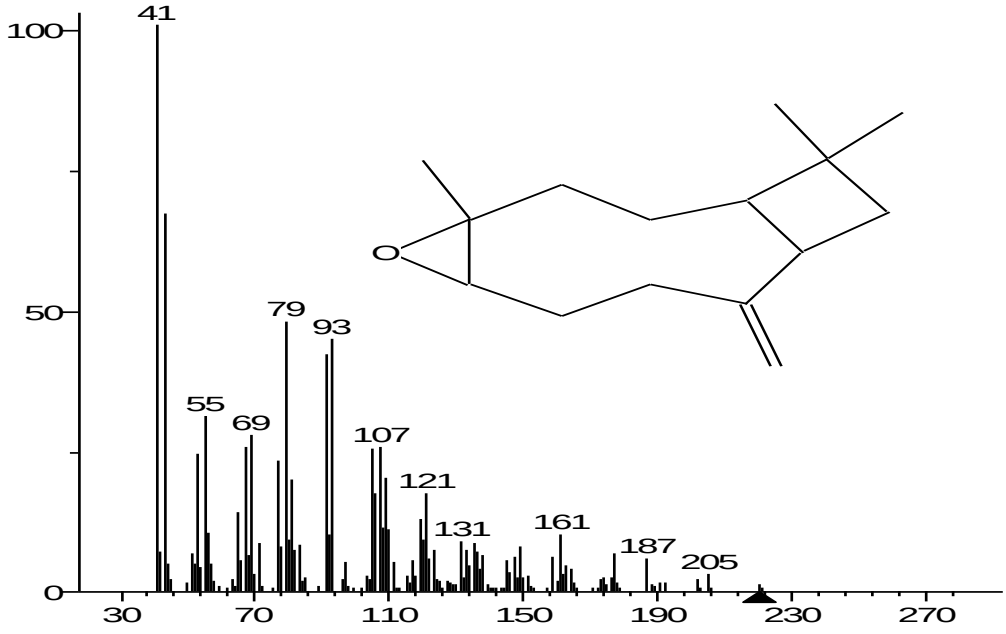


Figure E30 Mass spectrum of carophyllene oxide

The peak at 23.32 minutes was then examined and the mass spectrum obtained:

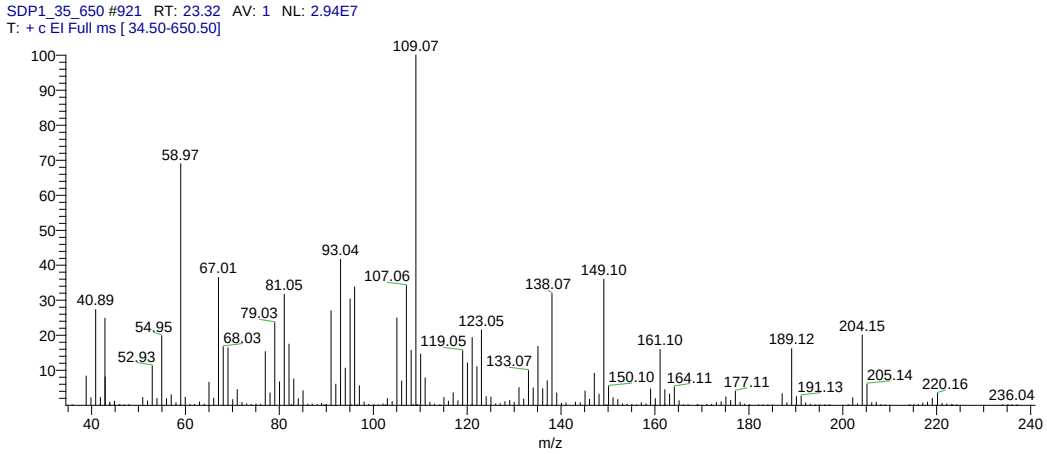


Figure E31 Mass spectrum of peak at 23.32 min in the gas chromatogram of SDP1

This was identified as 2 – (4a8-Dimethyl-1, 2,3,4,4a, 5,6,8a-octahydro-2-napphthalenyl-2-propanol.

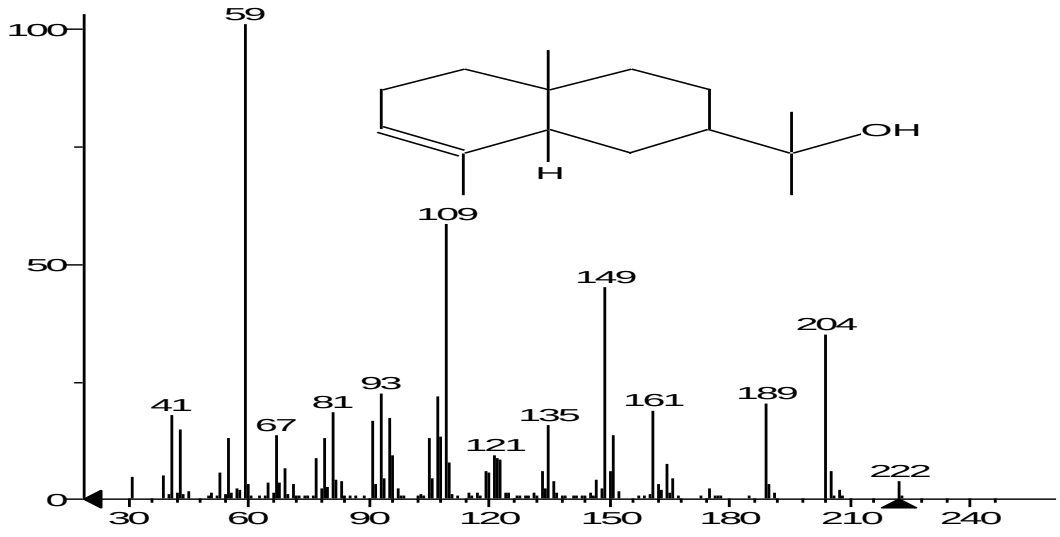


Figure E32 Mass spectrum of 2 – (4a8-Dimethyl-1, 2,3,4,4a, 5,6,8a-octahydro-2-napphthalenyl-2-propanol

The peak at 23.57 minutes was examined and the mass spectrum obtained:

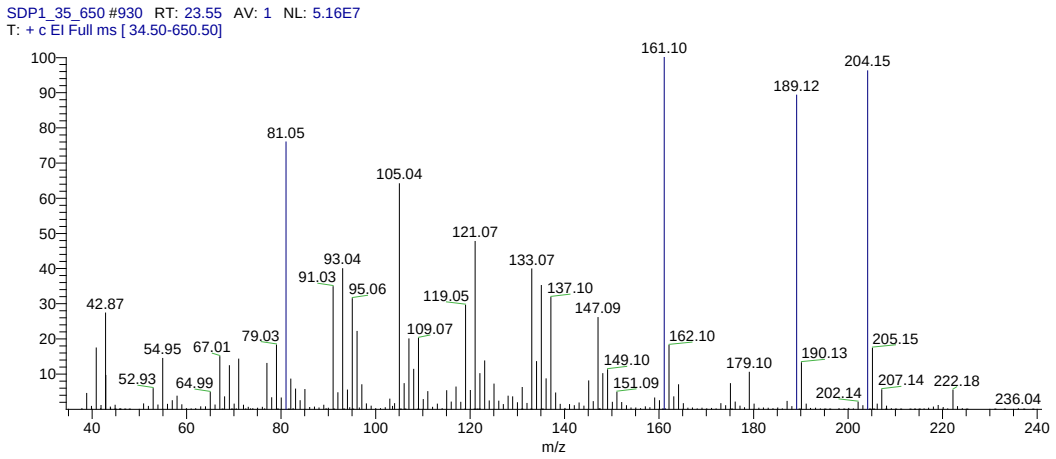


Figure E33 Mass spectrum of peak at 23.57 min in the gas chromatogram of SDP1

This was identified as Juniper Camphor.

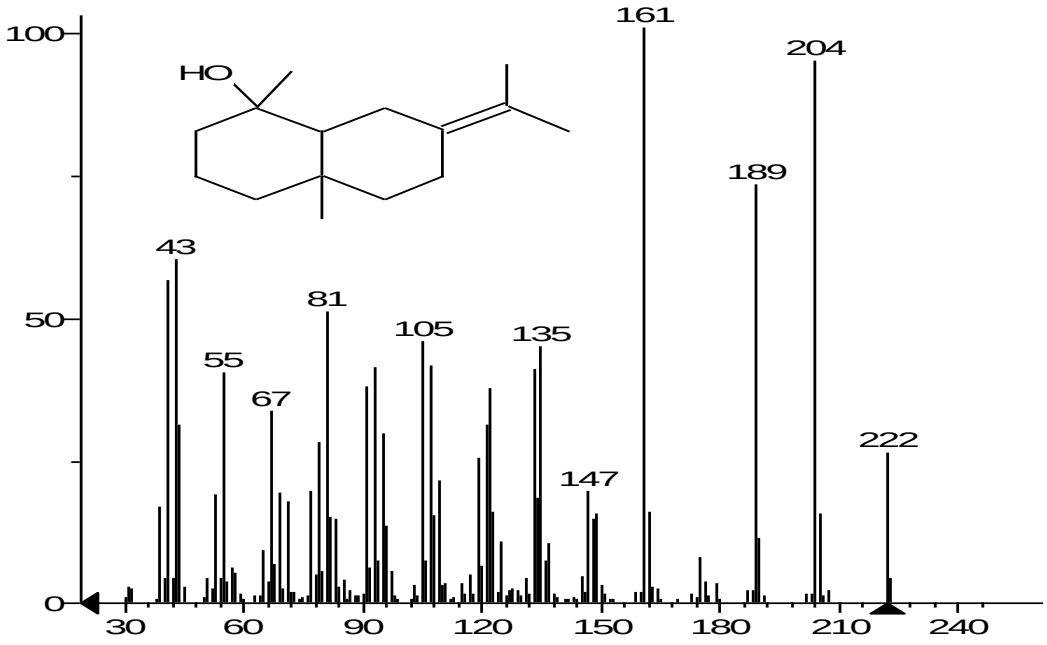


Figure E34 Mass spectrum of Juniper Campher

The peak at 23.85 minutes was examined and the mass spectrum obtained is as follows:

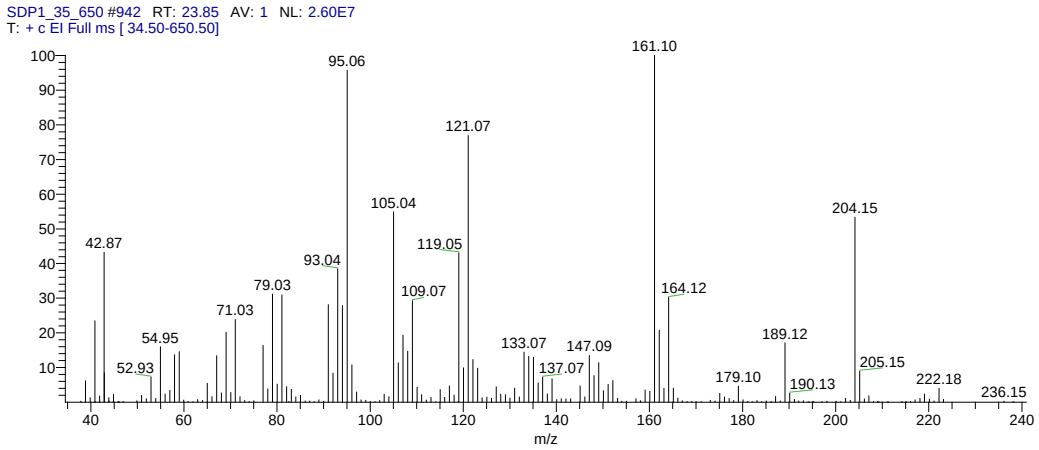


Figure E35 Mass spectrum of peak at 23.85min in the gas chromatogram of SDP1

This was identified as tau-Muurolol.

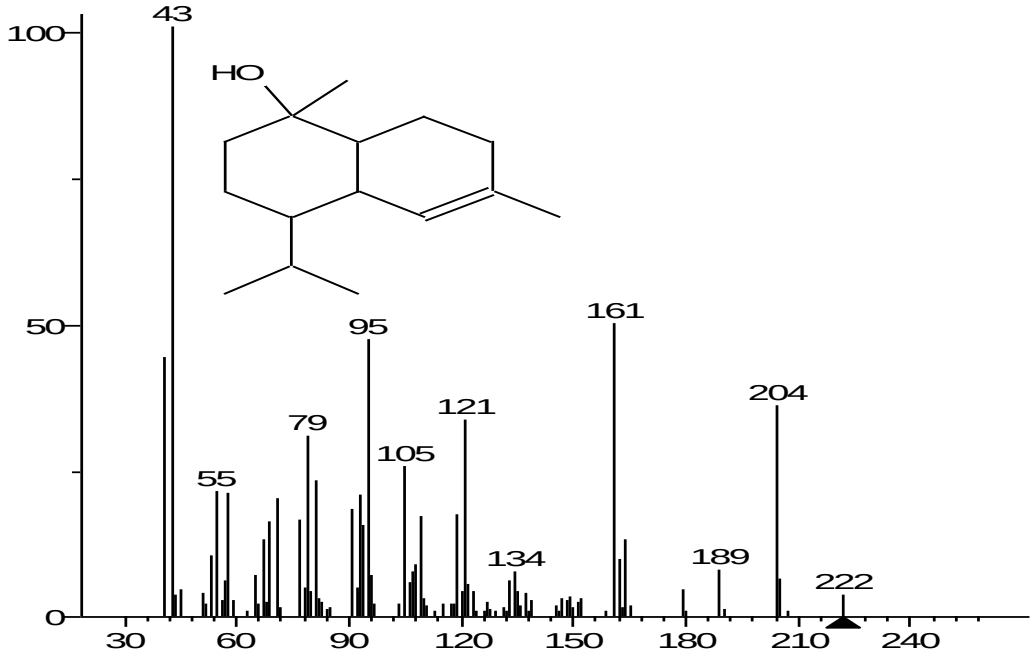


Figure E36 Mass spectrum of tau-Muurolol

The peak at 24.03 minutes was then examined.

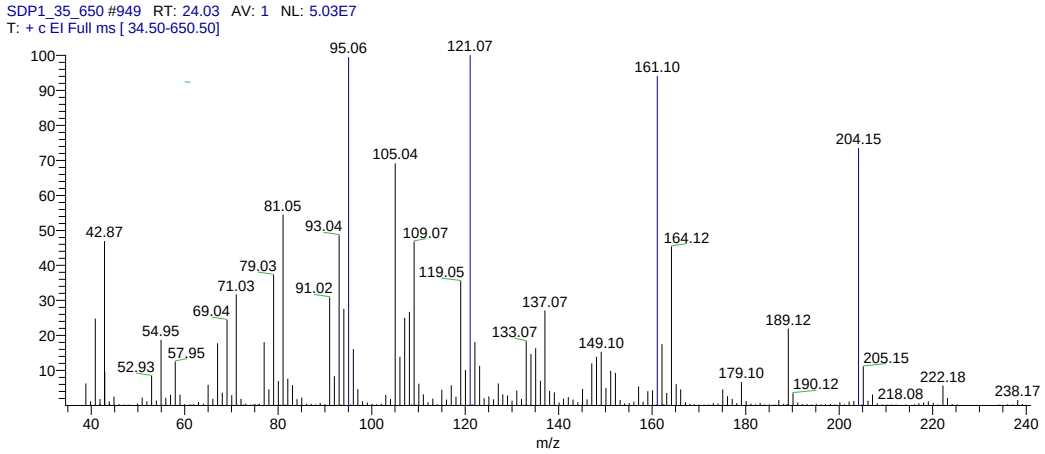


Figure E37 Mass spectrum of peak at 24.03min in the gas chromatogram of SDP1

The peak was identified as alpha-cadinol.

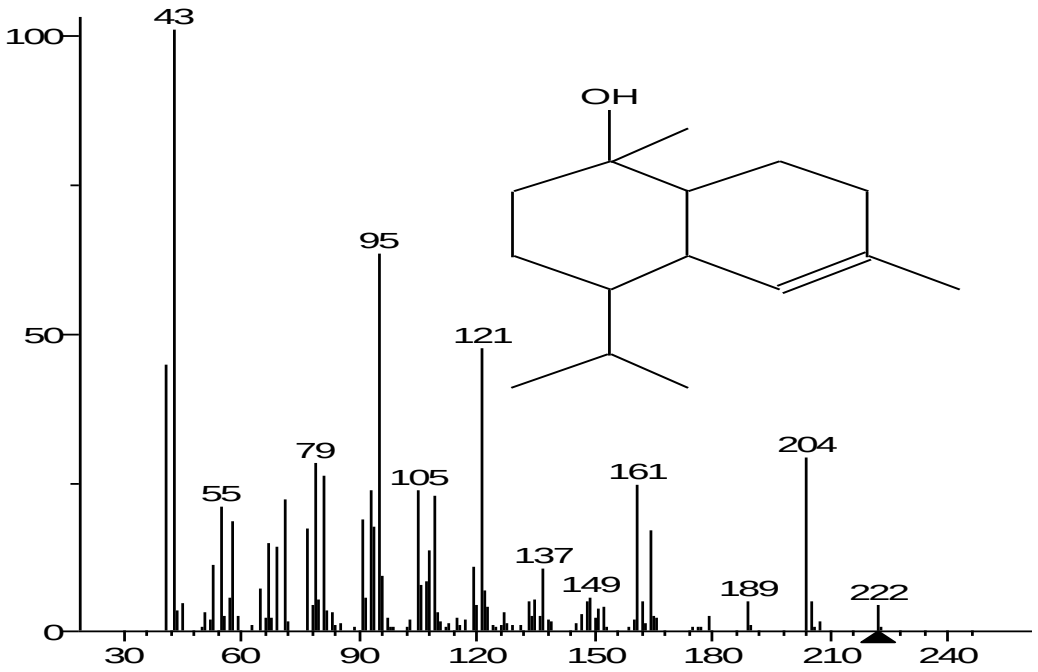


Figure E38 Mass spectrum of peak of alpha-Cadinol

The peak at 26.23 minutes was examined and it had a mass spectrum as follows:

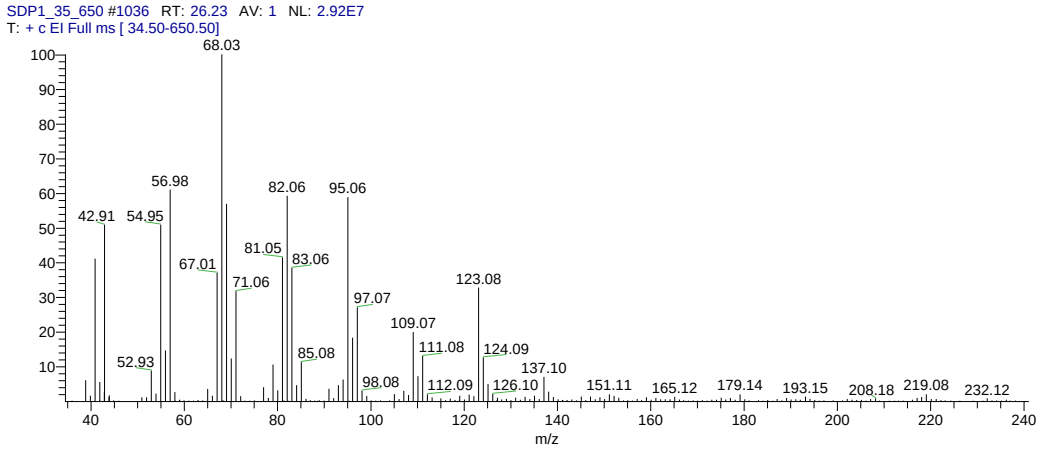


Figure E39 Mass spectrum of peak at 26.23min in the gas chromatogram of SDP1

This was identified as phytol.

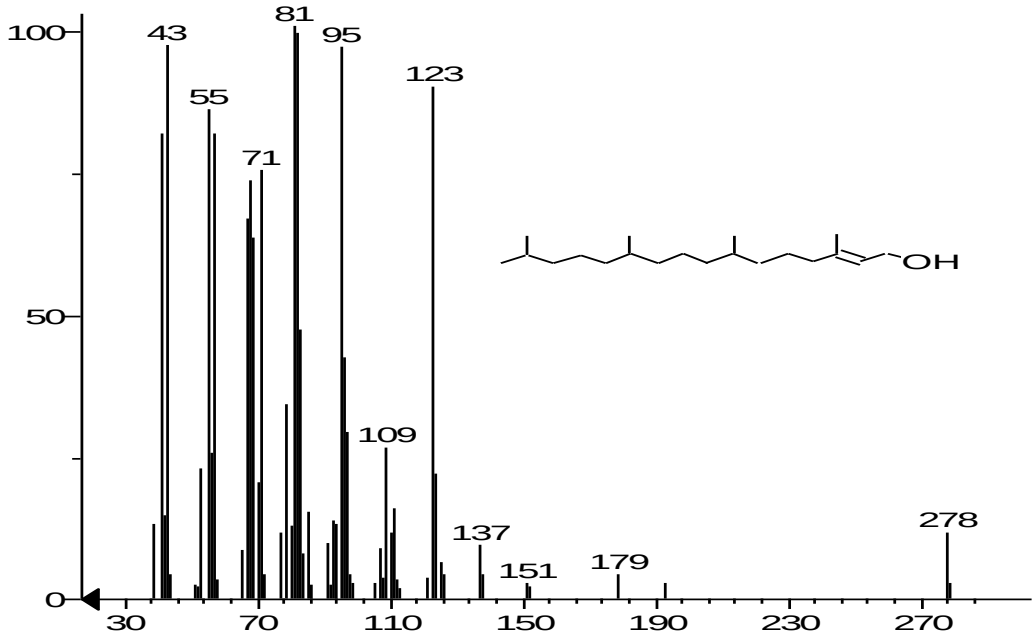


Figure E40 Mass spectrum of phytol

APPENDIX F

The separate graphs of the different extractions run on the GC-MS

Steam distillation

Setup 1

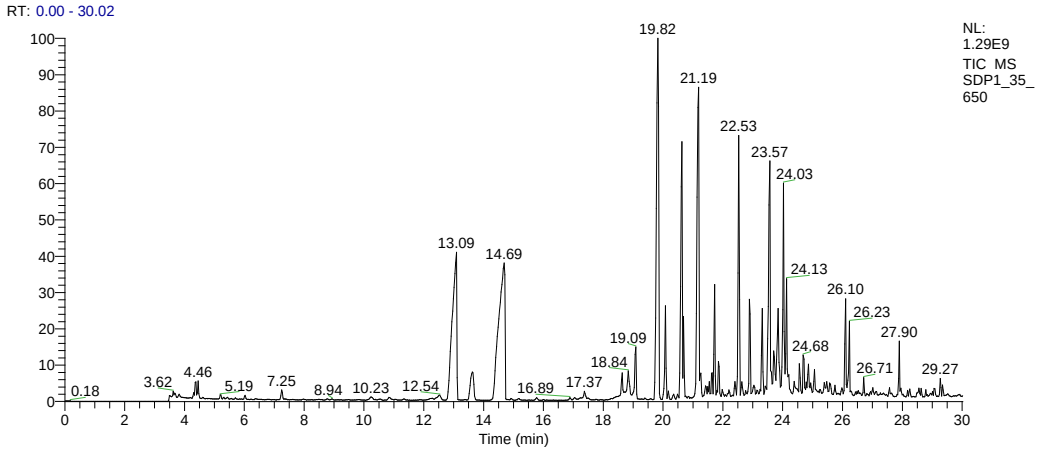


Figure F1 Gas chromatogram of steam distillation setup 1 with fresh leaves (45g) using distilled water. (SDP1)

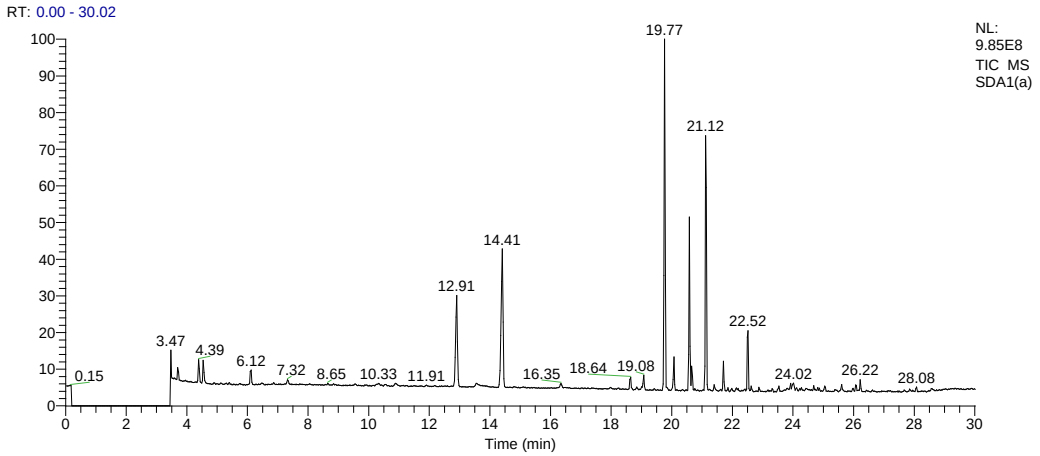


Figure F2 Gas chromatogram of steam distillation setup 1 with fresh leaves (10g) using distilled water. (SD-1)

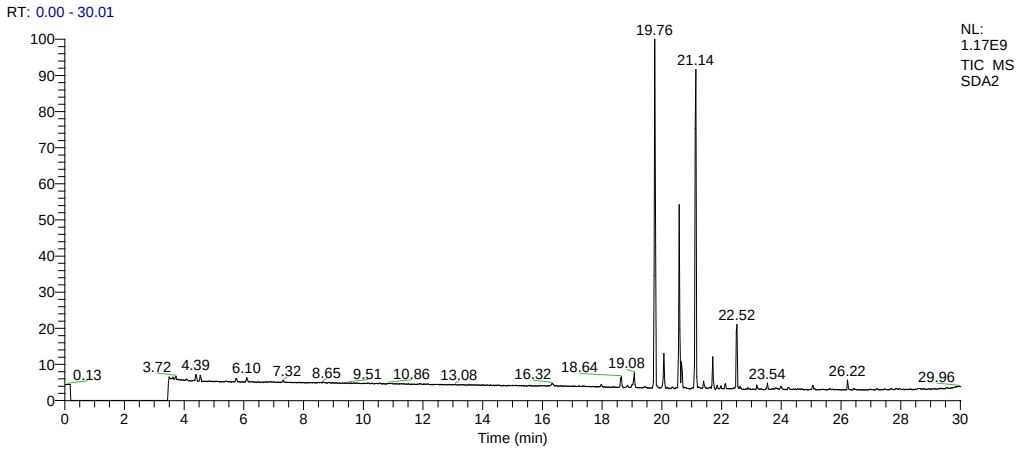


Figure F3 Gas chromatogram of steam distillation setup 1 with fresh leaves (10 g) using distilled water. (SD-2)

Setup 2

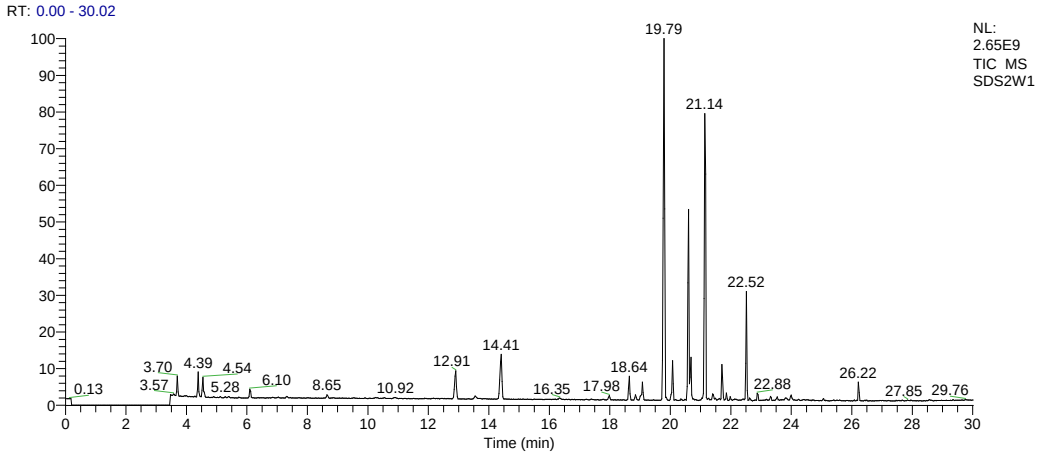


Figure F4 Gas chromatogram of steam distillation setup 2 with fresh leaves (10g) using distilled water. (SDS2W1)

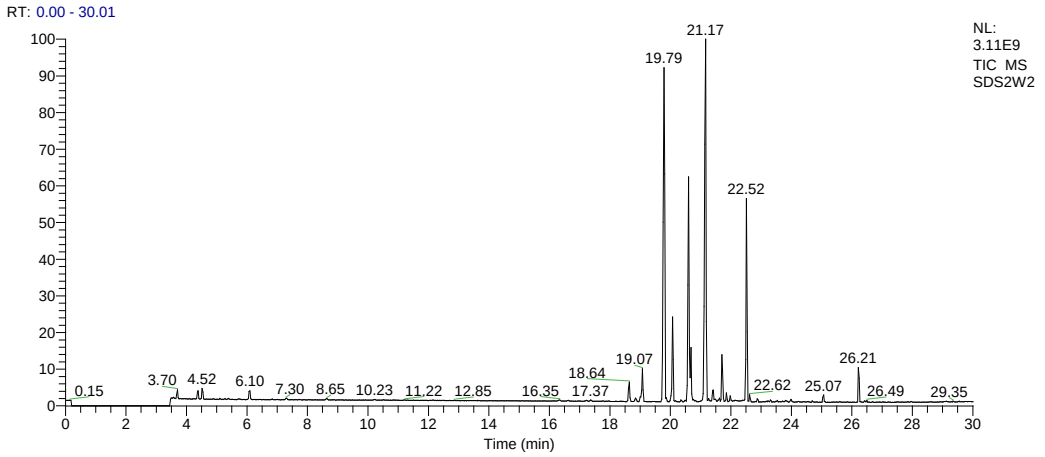


Figure F5 Gas chromatogram of steam distillation setup 2 with fresh leaves (10 g) using distilled water. (SDS2W2)

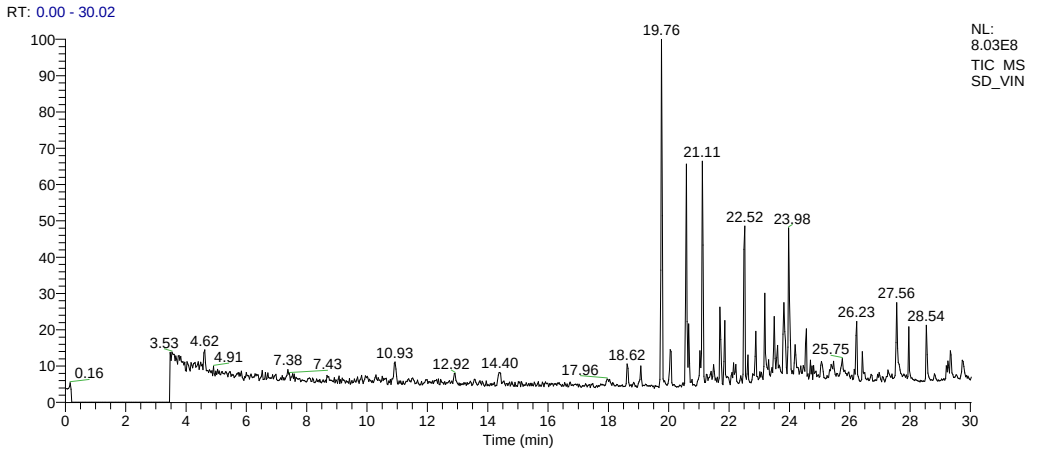


Figure F6 Gas chromatogram of steam distillation setup 2 with fresh leaves (25 g) using vinegar.(SD-VIN)

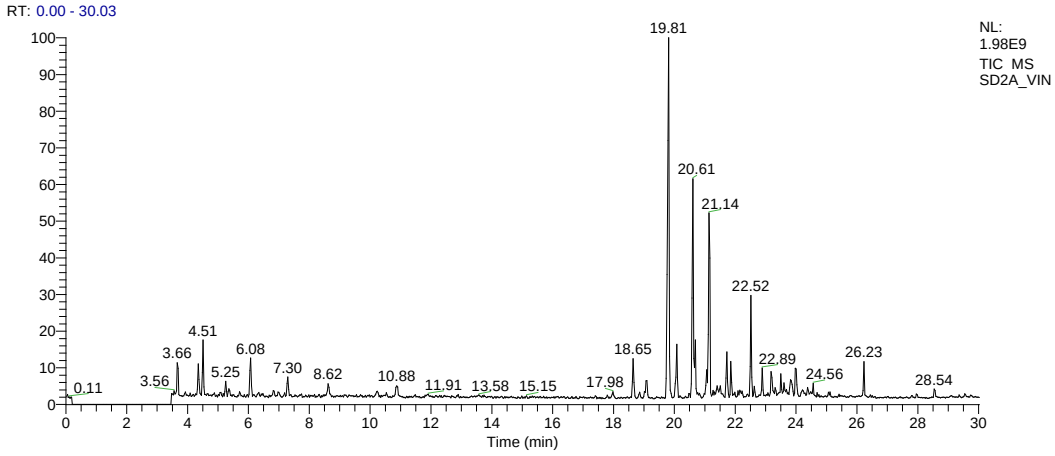


Figure F7 Gas chromatogram of steam distillation setup 2 with fresh leaves (25g) using vinegar.(SD-VIN-2)

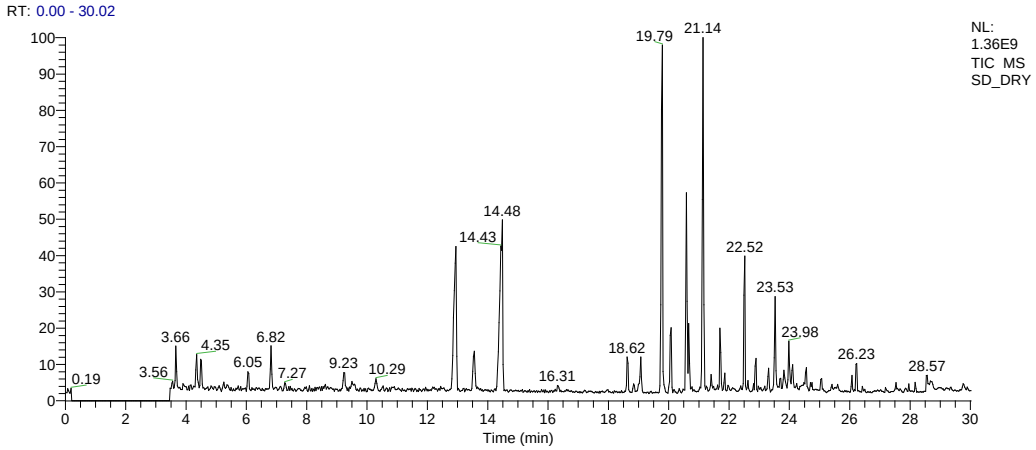


Figure F8 Gas chromatogram of steam distillation setup 2 with dry leaves (5g) using distilled water. (SD-DRY1)

Water distillation

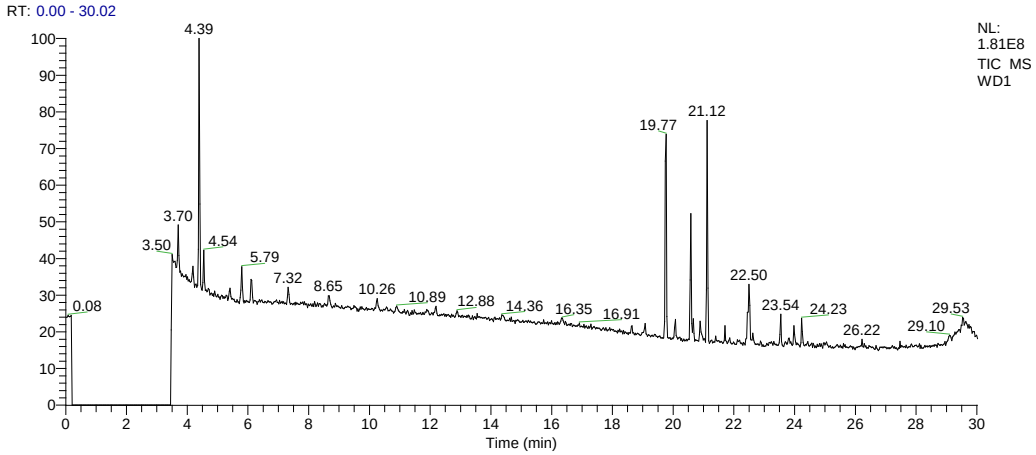


Figure F9 Gas chromatogram of water distillation with fresh leaves (40g) using. (WD1)

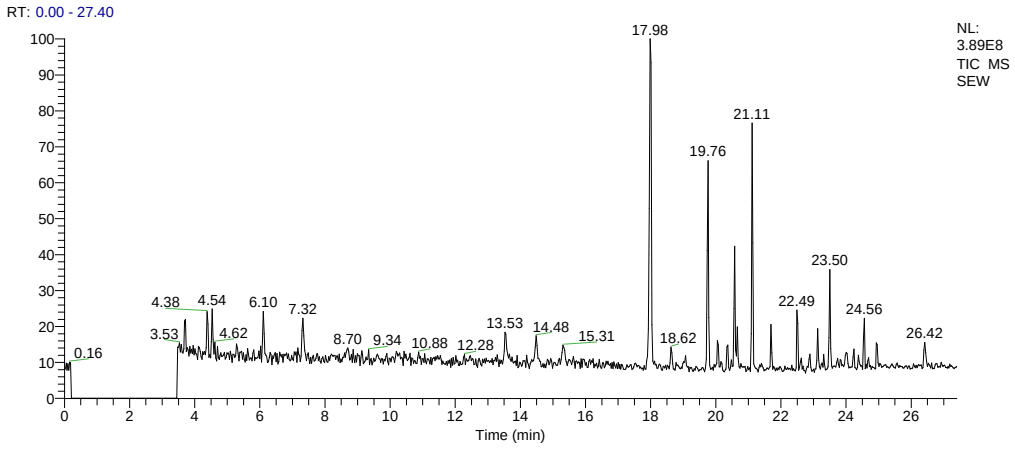


Figure F10 Gas chromatogram of solvent extraction using distilled water. (SEW)

APPENDIX G

GC results run on the samples of C*entella* essential oil from the different extraction methods

Steam distillation – Setup 1

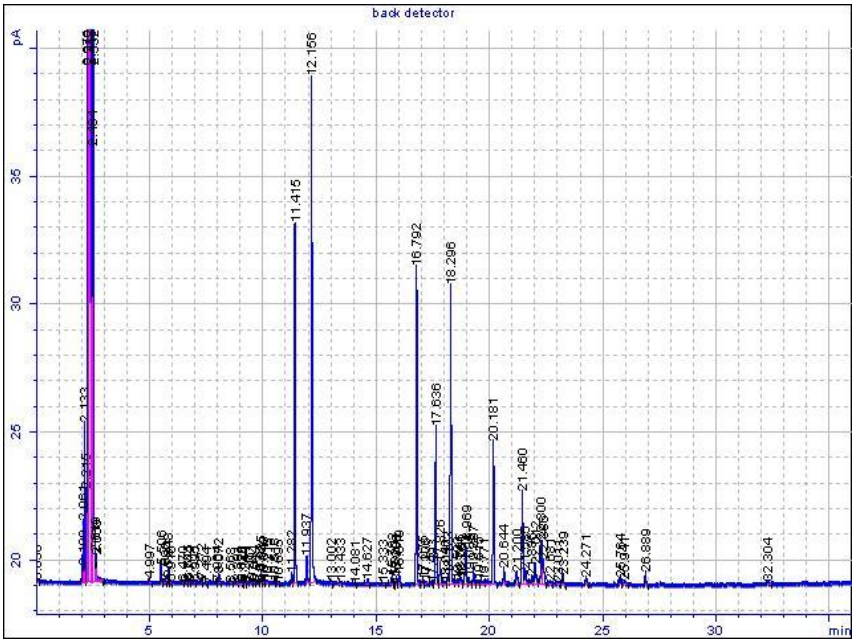


Figure G1 Gas chromatogram of steam distillation setup 1 with fresh leaves (45 g) using distilled water. (SDP1)

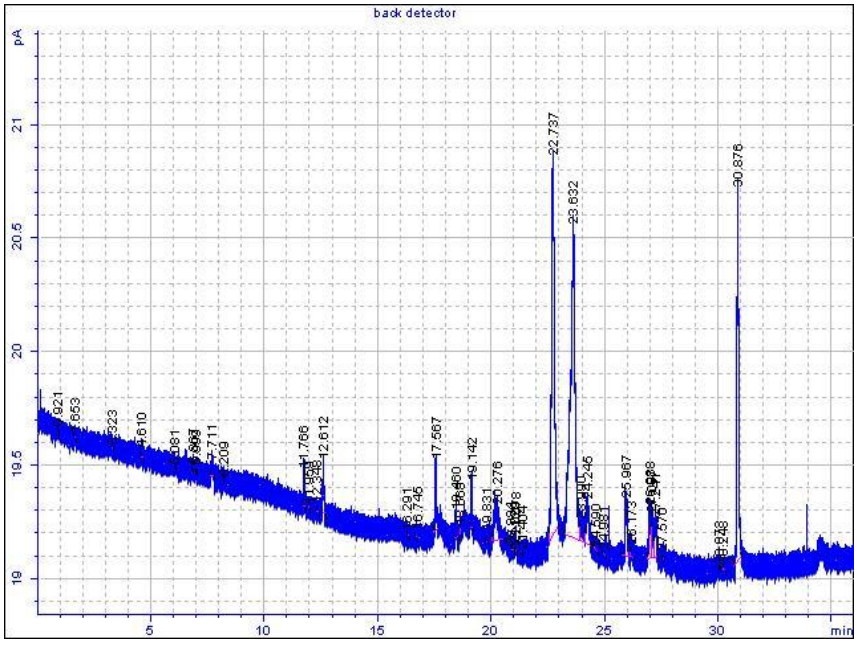


Figure G2 Gas chromatogram of steam distillation setup 1 with fresh leaves (10 g) using distilled water. (SD-1)

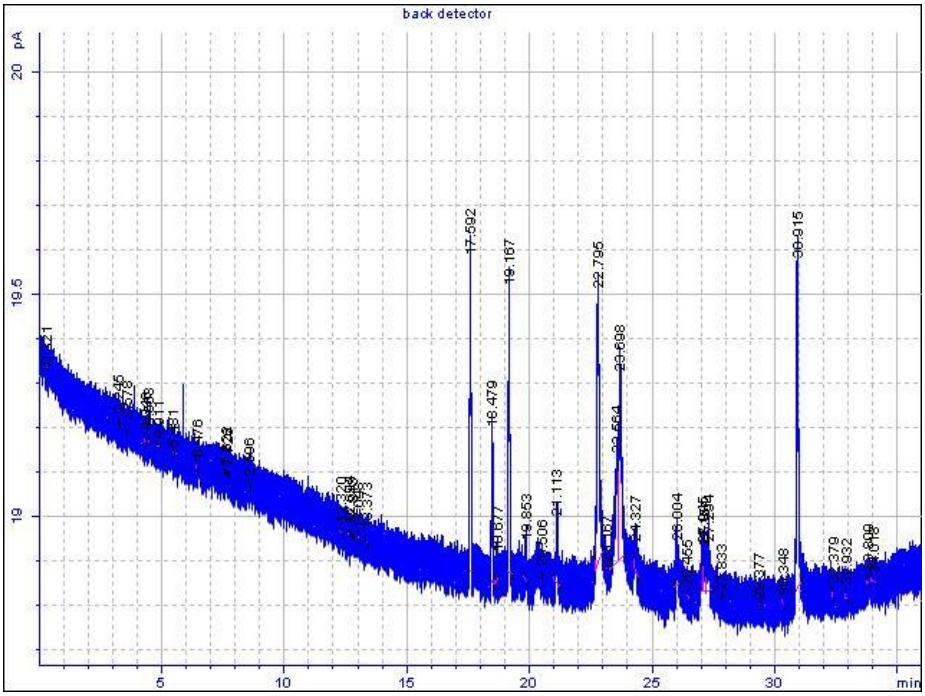


Figure G3 Gas chromatogram of steam distillation setup 1 with fresh leaves (10 g) using distilled water. (SD-2)

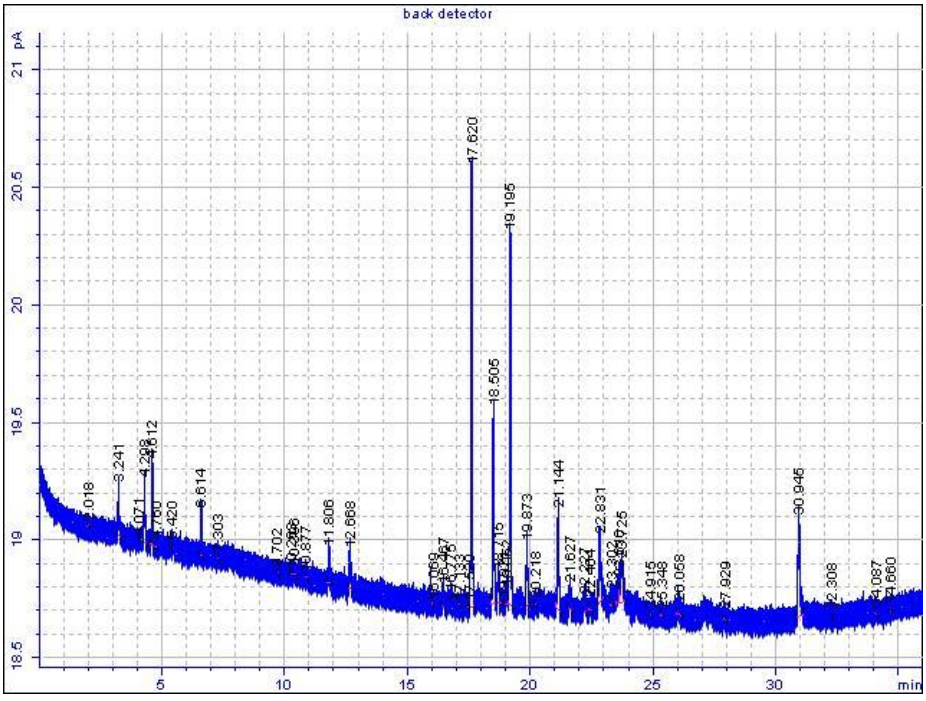


Figure G4 Gas chromatogram of steam distillation setup 1 with fresh leaves (10 g) using distilled water. (SD-3)

STEAM DISTILLATION SETUP 2

Setup 2

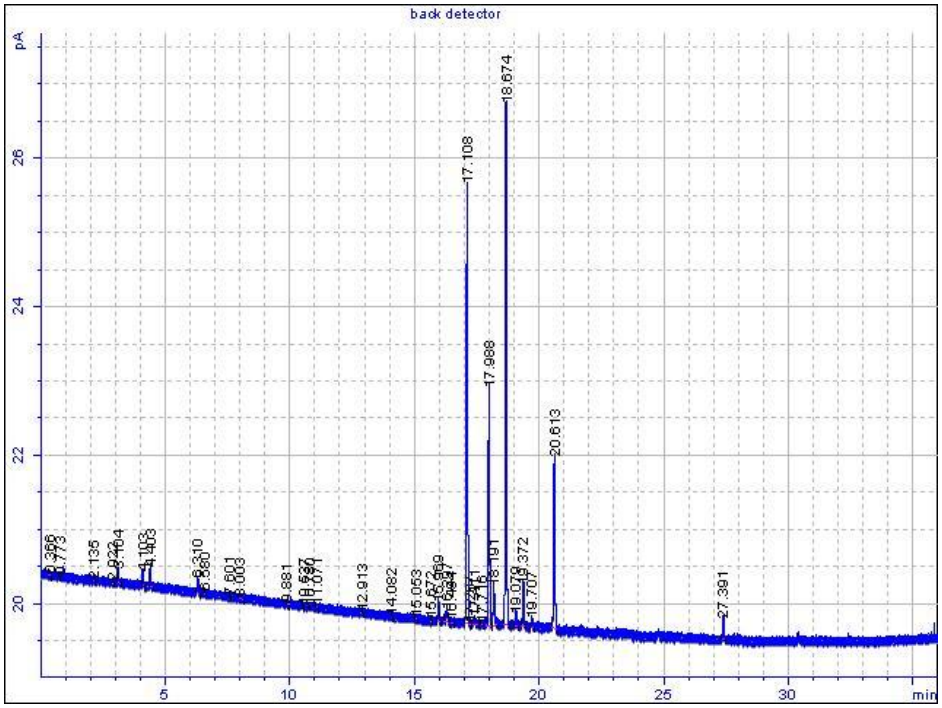


Figure G5 Gas chromatogram of steam distillation setup 2 with fresh leaves (10 g) using distilled water. (SDS2W1)

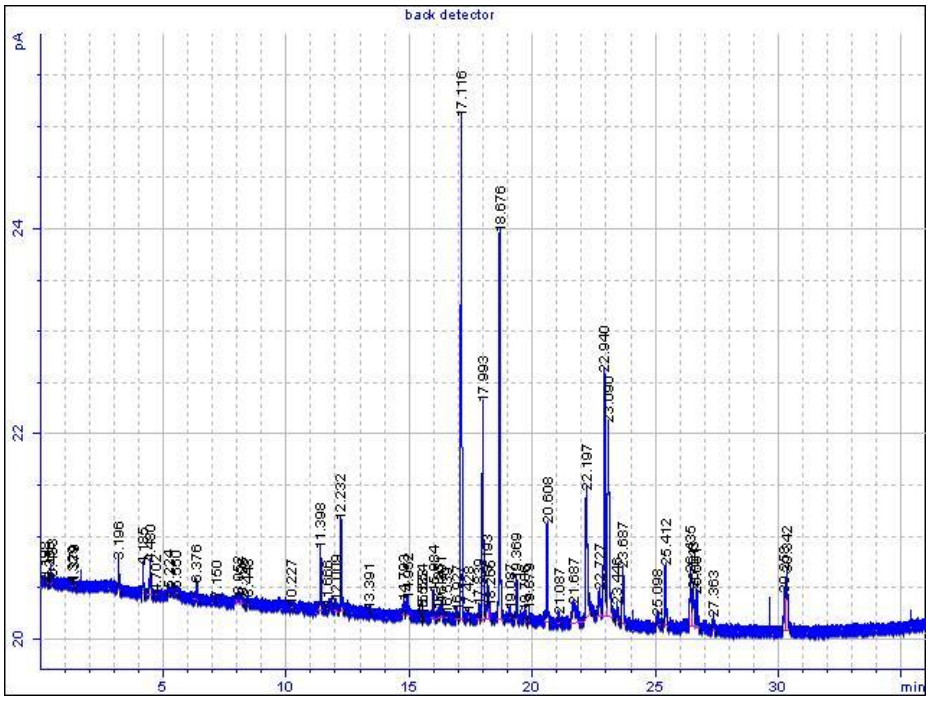


Figure G6 Gas chromatogram of steam distillation setup 2 with fresh leaves (10 g) using distilled water. (SDS2W2)

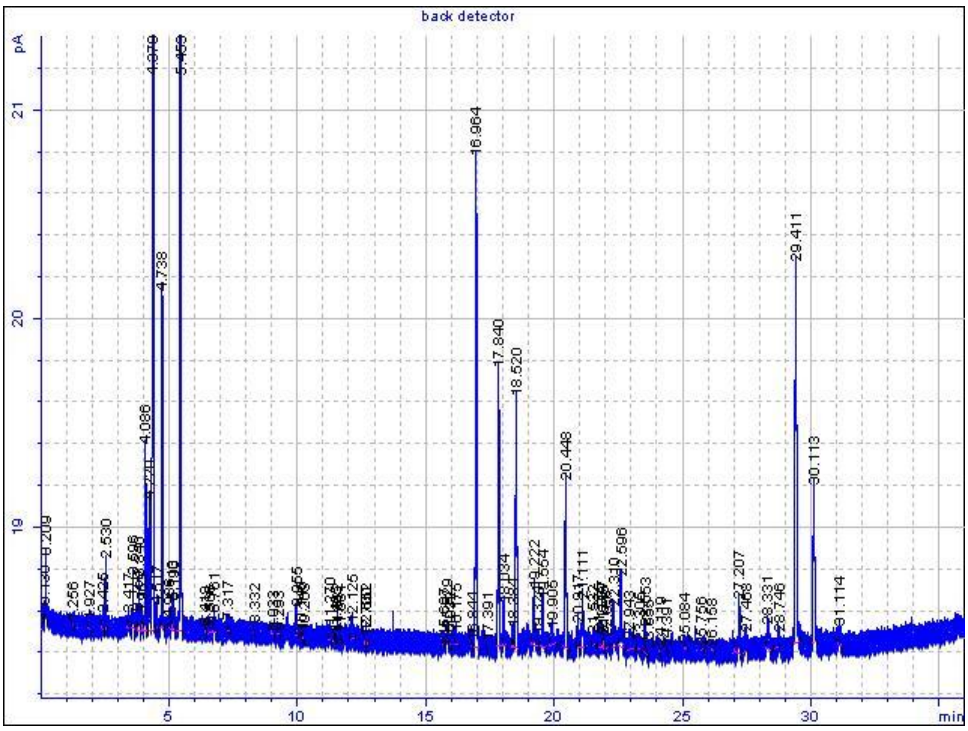


Figure G7 Gas chromatogram of steam distillation setup 2 with fresh leaves (25 g) using vinegar. (SD-VIN)

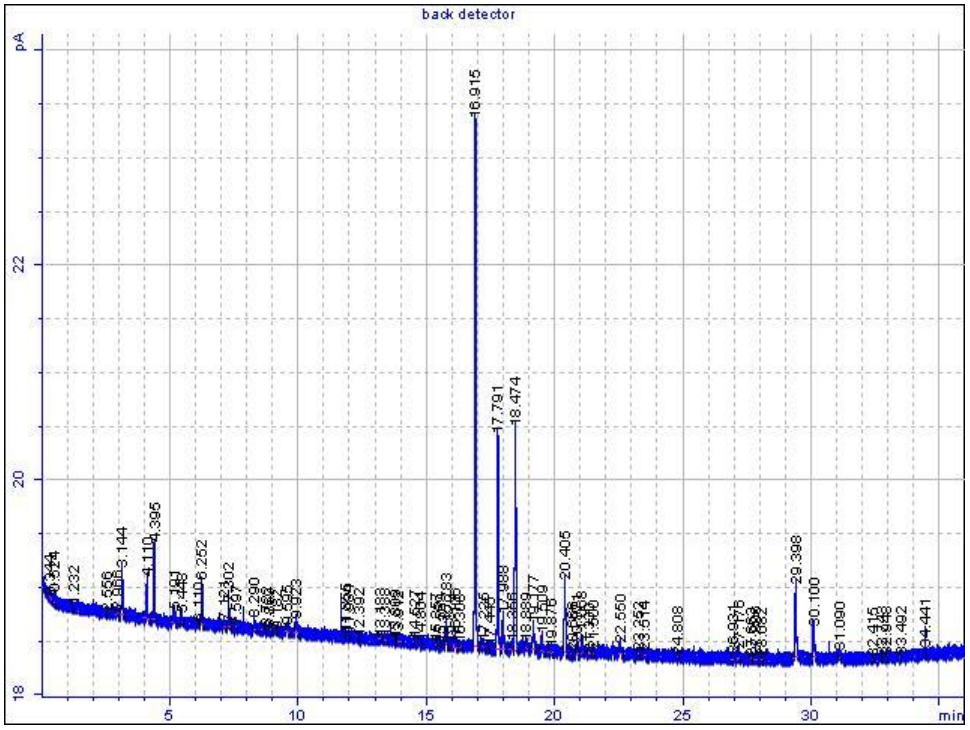


Figure G8 Gas chromatogram of steam distillation setup 2 with fresh leaves (25 g) using vinegar. (SD-VIN-2)

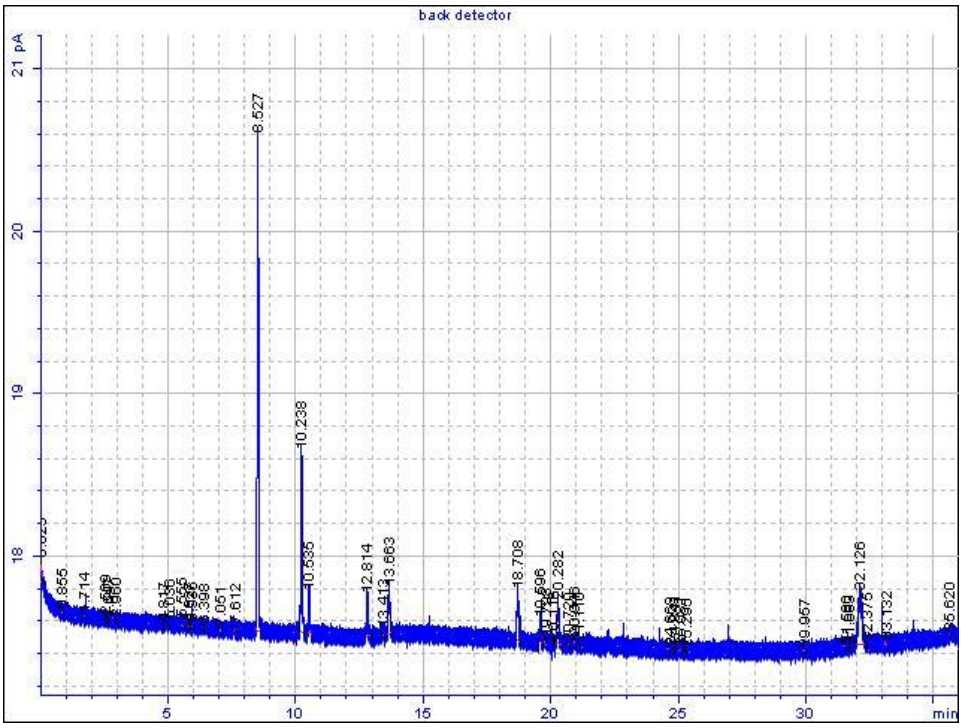


Figure G9 Gas chromatogram of steam distillation setup 2 with dry leaves (5 g) using distilled water. (SD-DRY1)

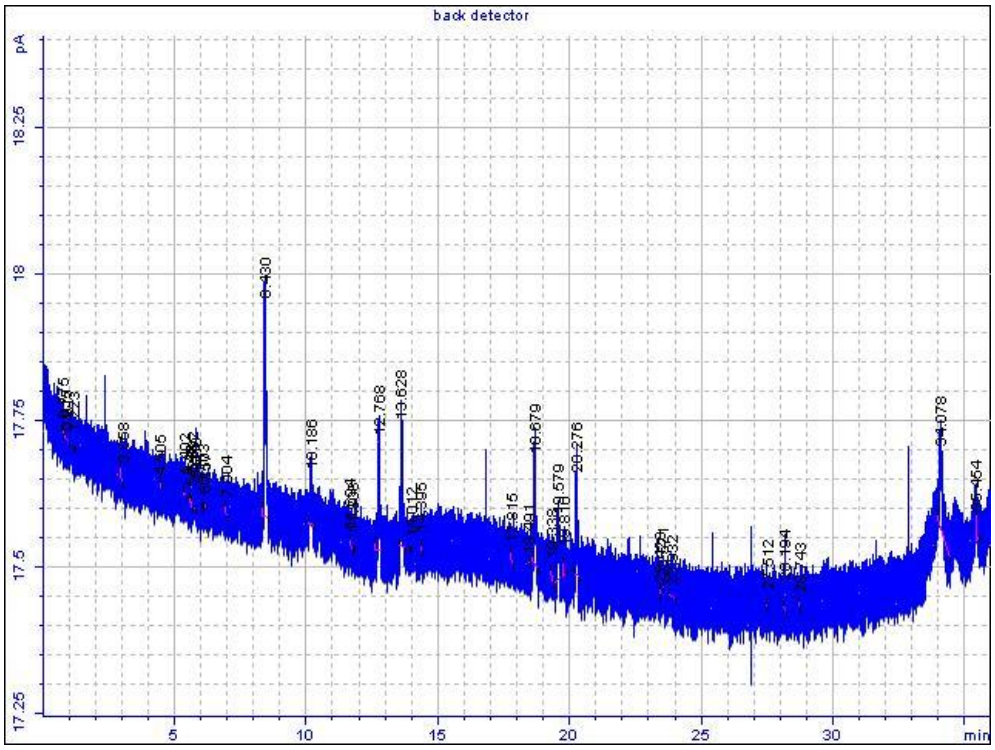


Figure G10 Gas chromatogram of steam distillation setup 2 with dry leaves (5 g) using distilled water. (SD-DRY2)

Water distillation

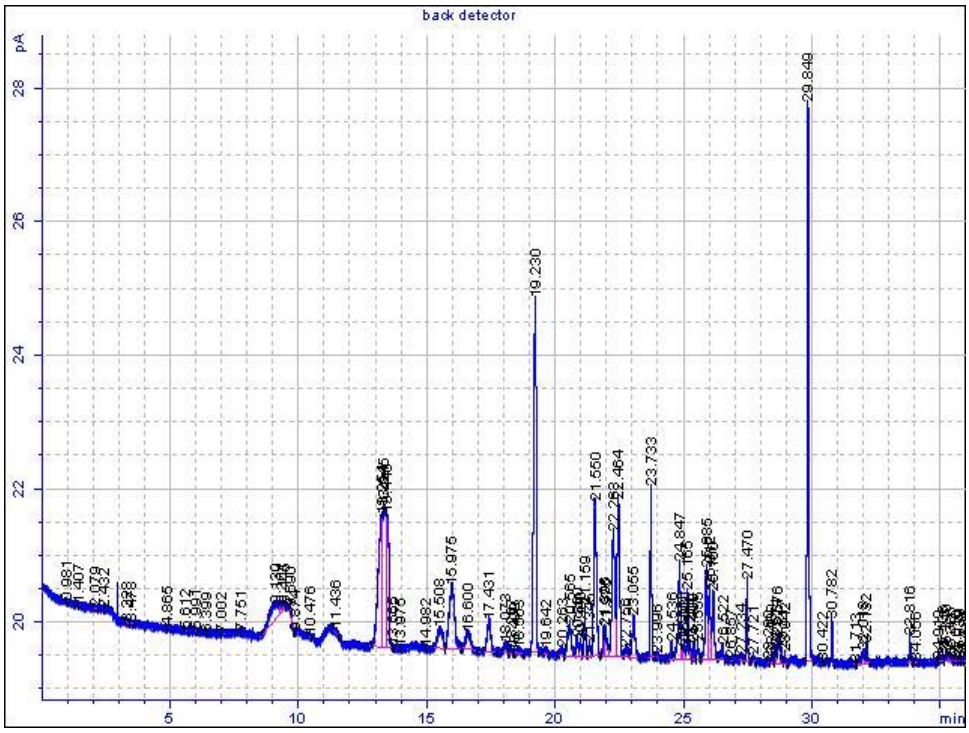


Figure G11 Gas chromatogram of water distillation with fresh leaves (40 g) (WD1)

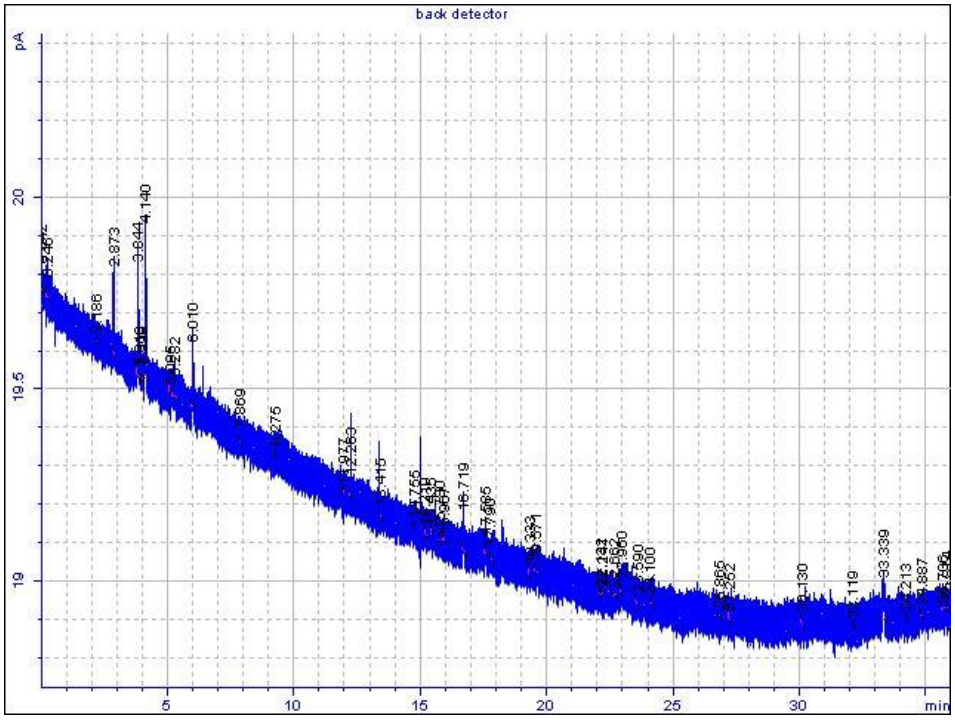


Figure G12 Gas chromatogram of water distillation with fresh leaves (40 g) (WD2)

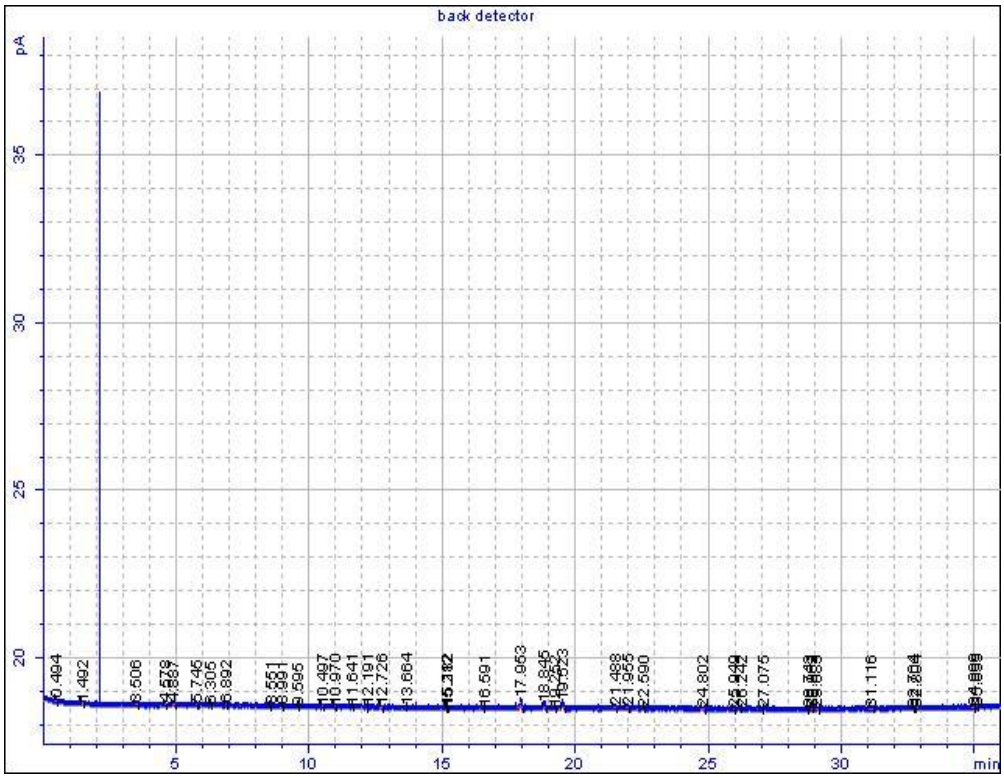


Figure G13 Gas chromatogram of water distillation with dry leaves (4g). ($WD_{DRY}1$)

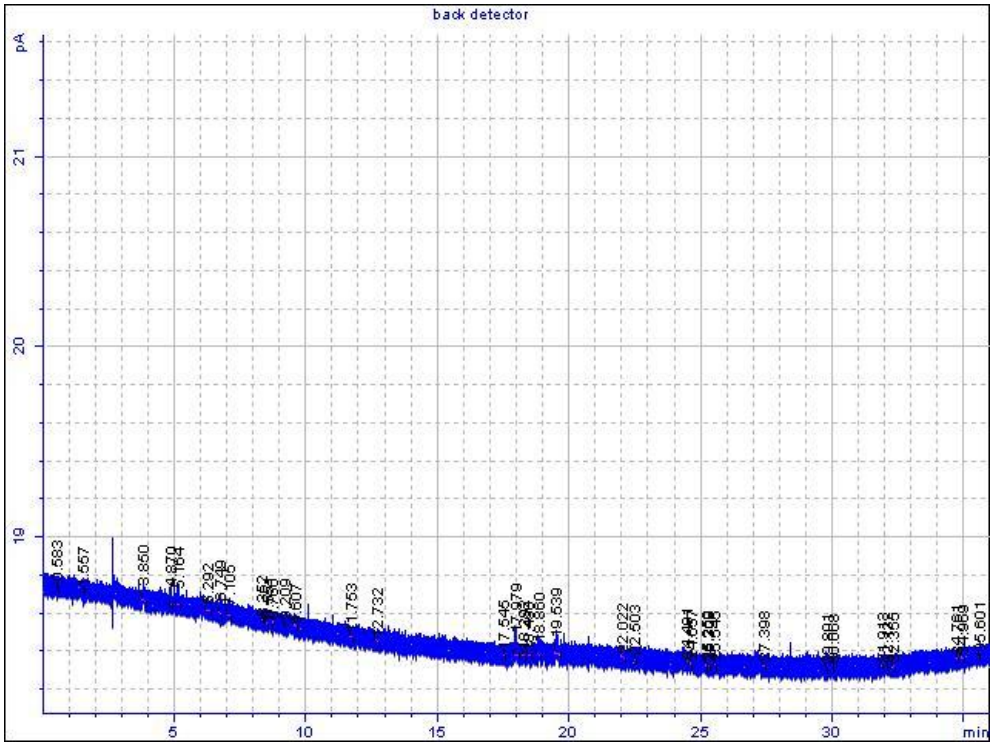


Figure G14 Gas chromatogram of water distillation with dry leaves (4g) using. (WDDRY2)

Solvent extraction

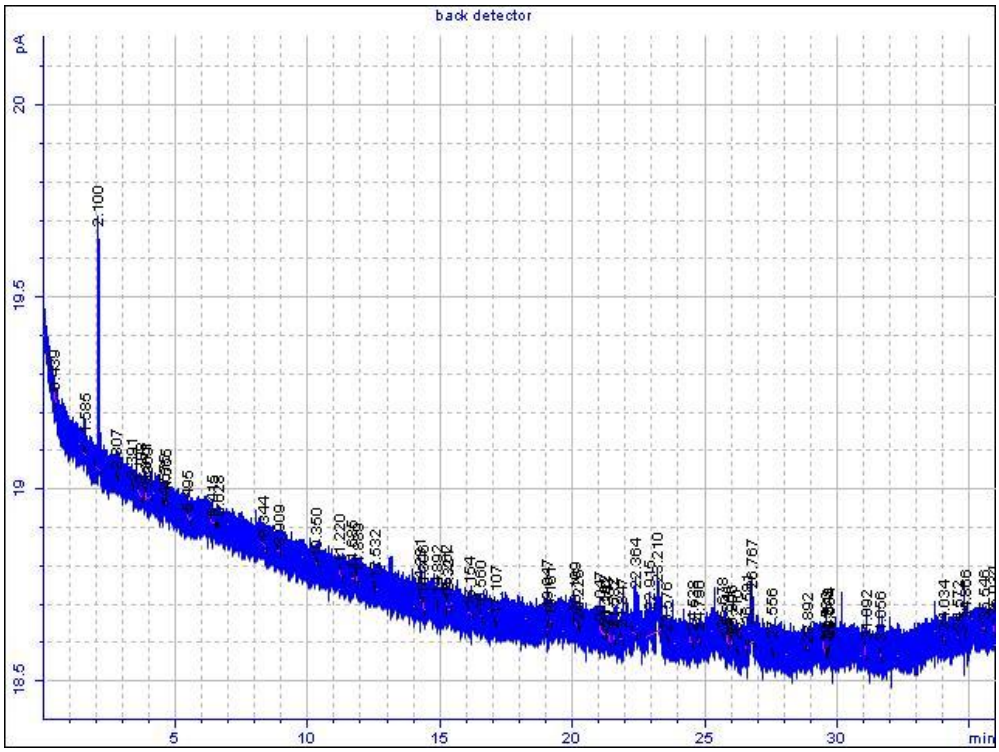


Figure G15 Gas chromatogram of solvent extraction with glacial acetic acid (SEA)

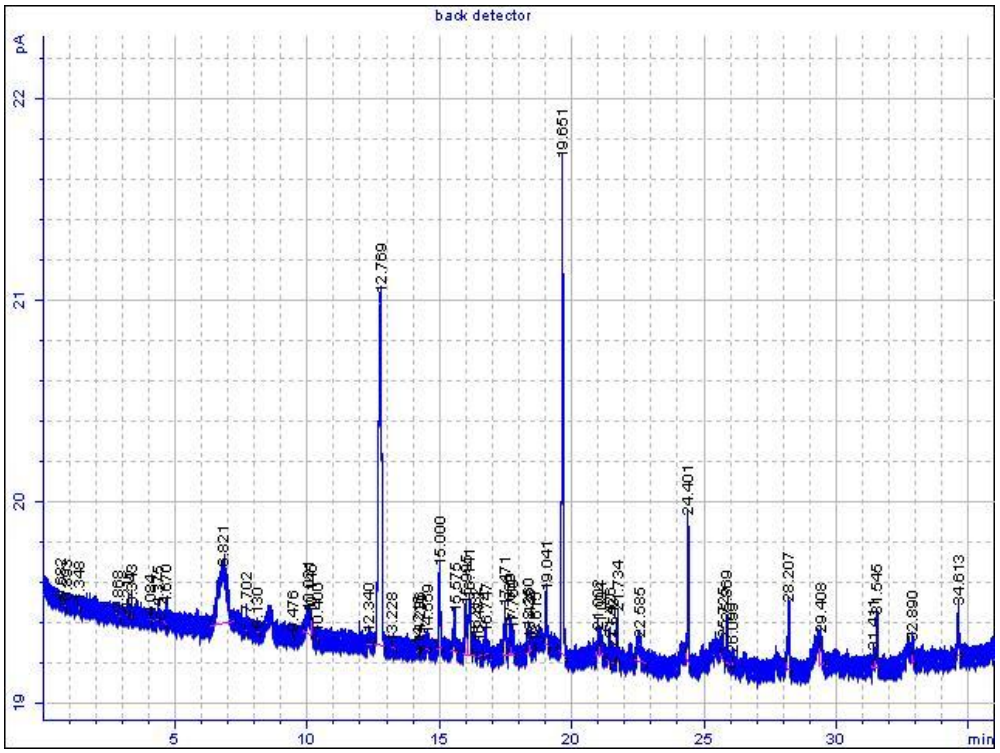


Figure G16 Gas chromatogram of solvent extraction with distilled water (SEW)

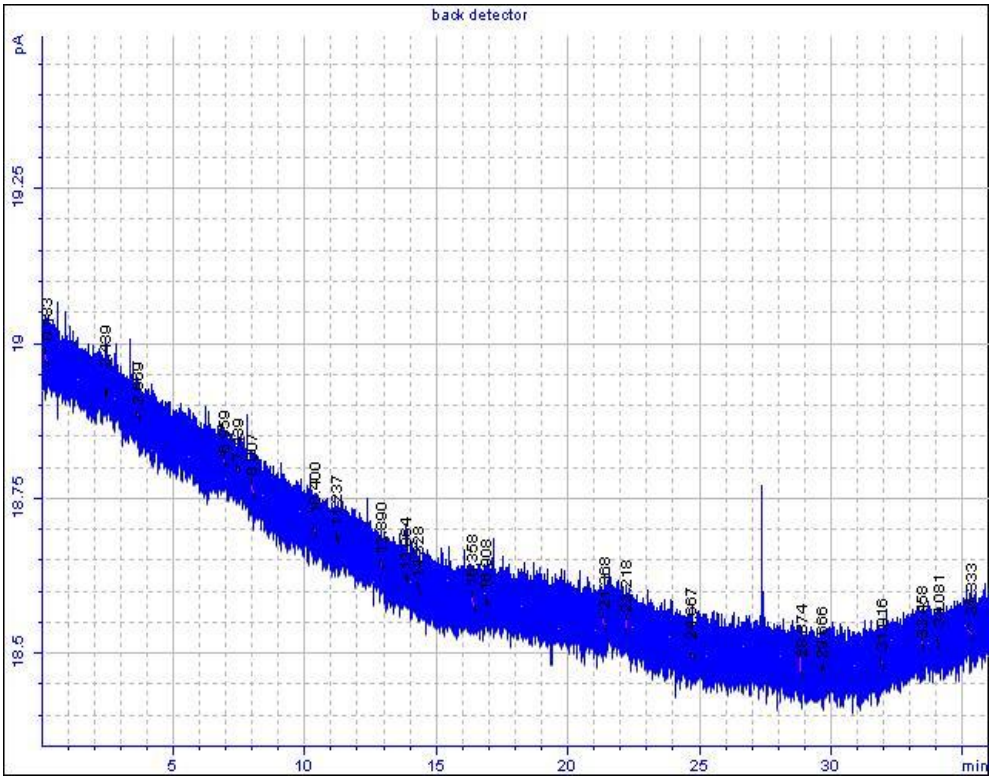


Figure G17 Gas chromatogram of solvent extraction with vinegar (SEV)

SOXHLET EXTRACTION

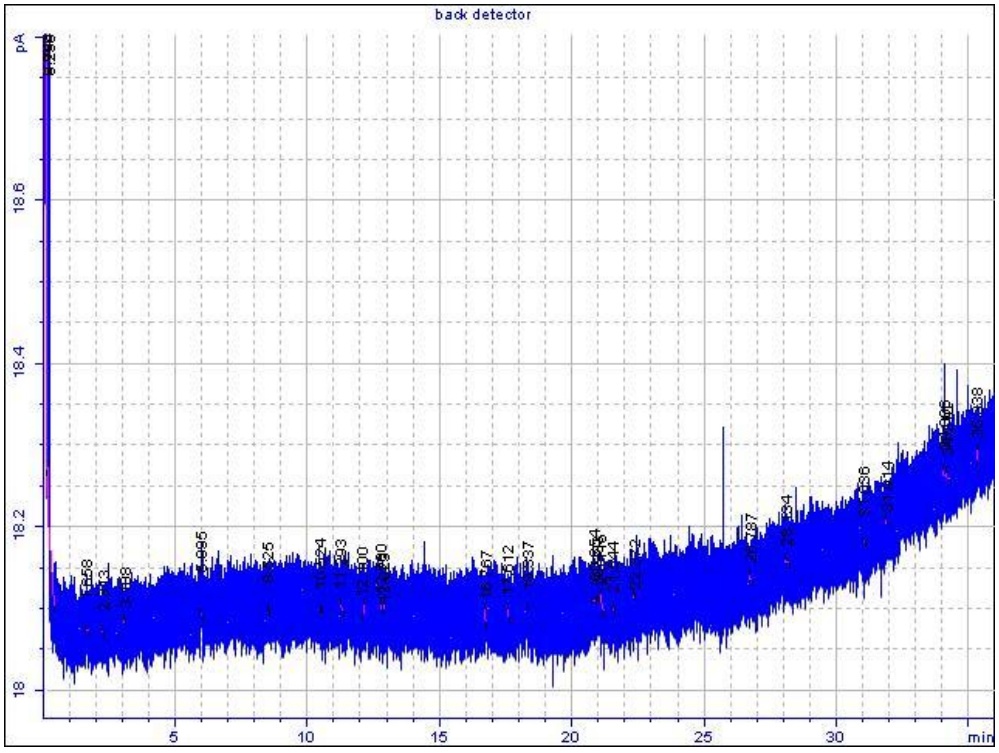


Figure G18 Gas chromatogram obtained after soxhlet extraction conducted with distilled water (SOX1)

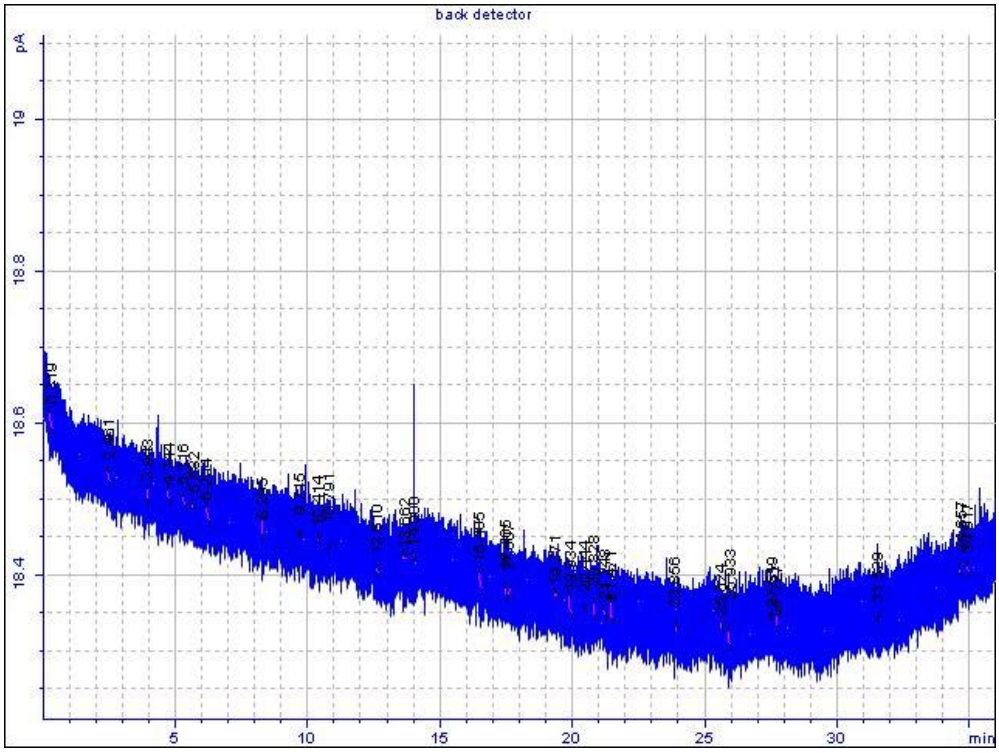


Figure G19: Gas chromatogram obtained after soxhlet extraction conducted with distilled water (SOX1)

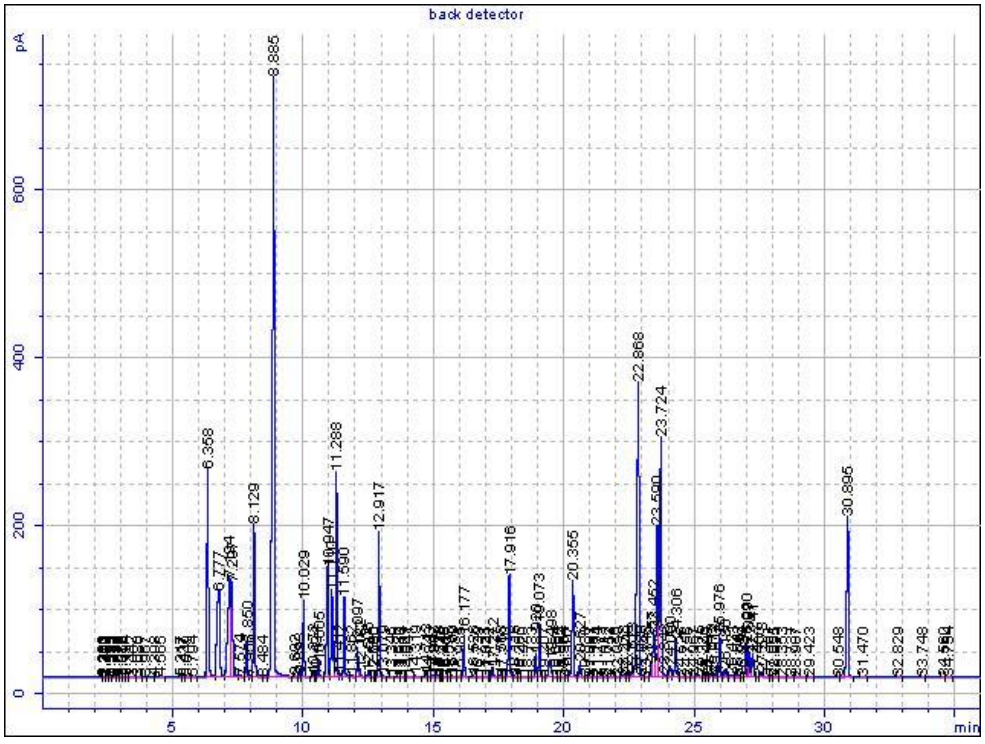


Figure G20: Gas Chromatogram of the commercial synthetic oil of C*entella Asiatica*

APPENDIX H

Summary of the TIC and the time of extraction needed to optimise the extraction

Table I1 The TIC in the oil samples at different times of extraction

Experiment name	TIC (ions)	TIME (min)
TM 1	4.40E+08	35
TM 2	3.29E+09	60
TM 3	4.15E+09	75
TM 4	3.69E+09	90

APPENDIX I

Summary of the TIC and the amount of leaves used for essential oil extraction to optimise the leaves needed for the experiments

Table I1 The TIC in the oil samples at different times of extraction

Experiment name	amount of leaves (g)	TIC (ions)
Leaf 1	30.65	4.45E+09
Leaf 2	50.04	4.50E+09
Leaf 3	100.11	4.61E+09

APPENDIX J

Identification of constituents present in the SQ1 chromatogram by using the NIST-MS library

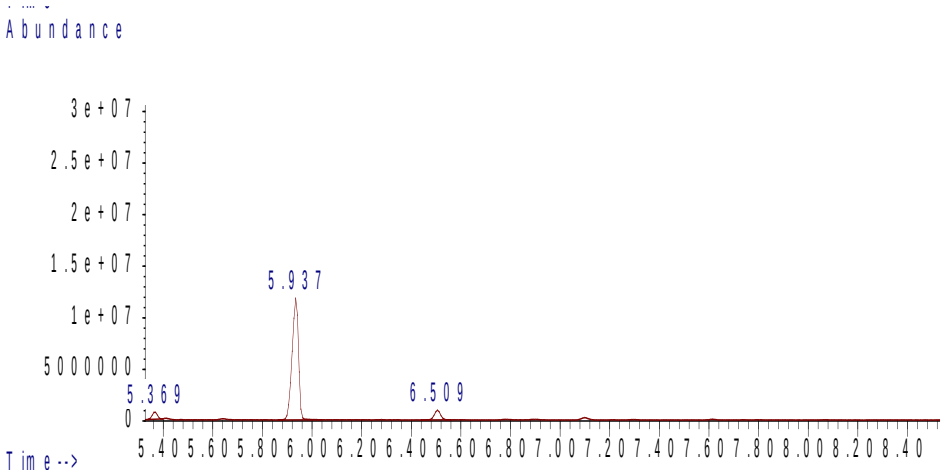


Figure J1 Gas chromatogram of experiment SQ1 between the retention times from 5.4 - 8.4 minutes

The peak at 6.509 had the following mass spectrum:

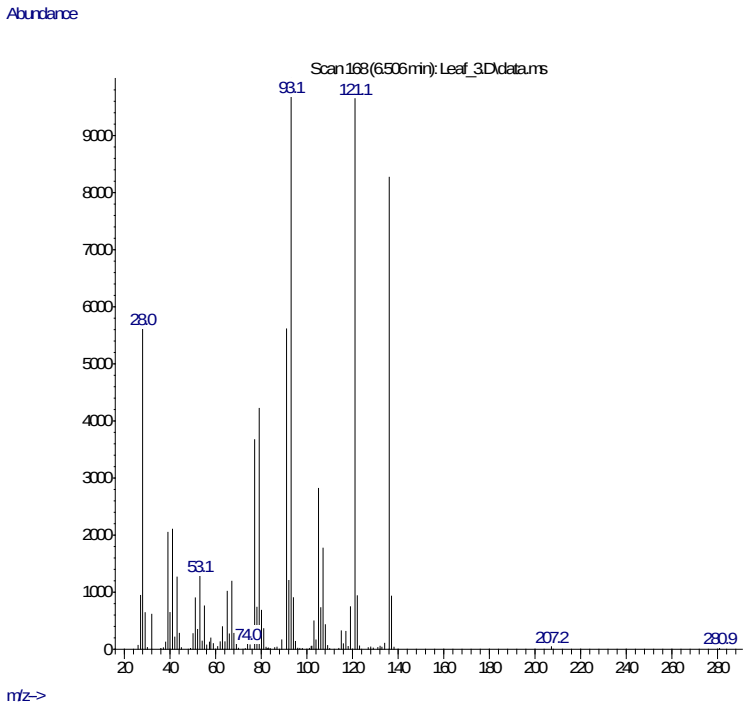


Figure J2 Mass spectrum of peak at 6.509 minutes in gas chromatogram SQ1

This mass spectrum (Fig. J2) was then compared to our standards and NIST-MS library and it was found to correlate to terpenoline.

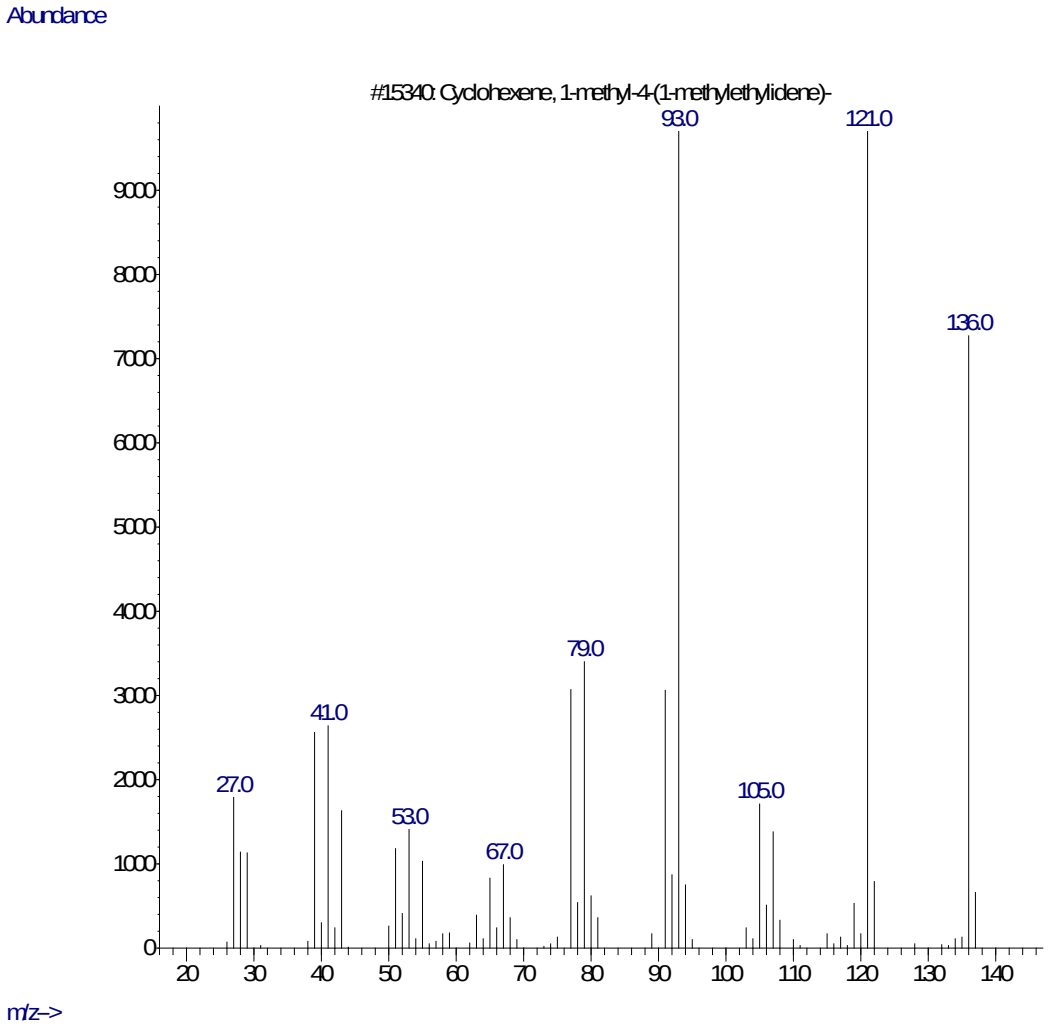


Figure J3 Mass spectrum of terpenoline

The peak at 6.509 minutes therefore correlates to Terpenoline

The second part of the chromatogram was examined between the retention times of 12.00 - 21.00 min.

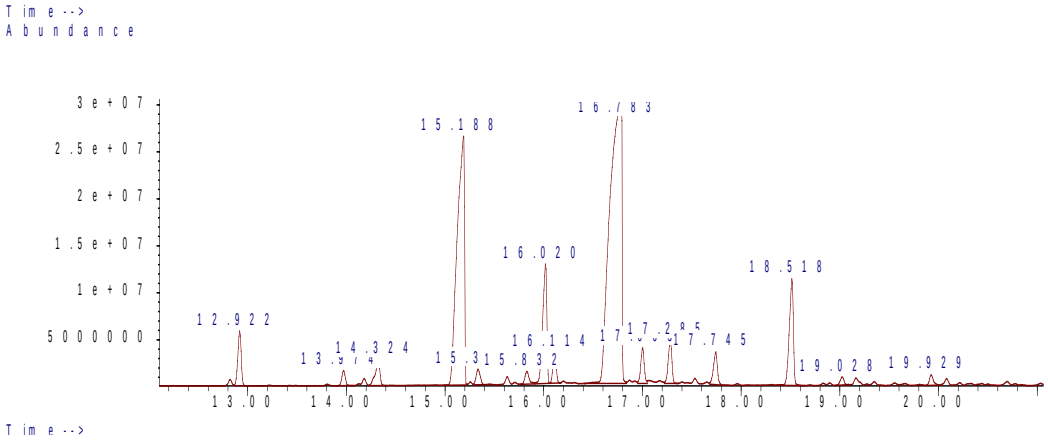


Figure J4 Gas chromatogram of experiment SQ1 between the retention times from 12-21 minutes

If the peak at 15.188 min is examined. The following mass spectrum was obtained:

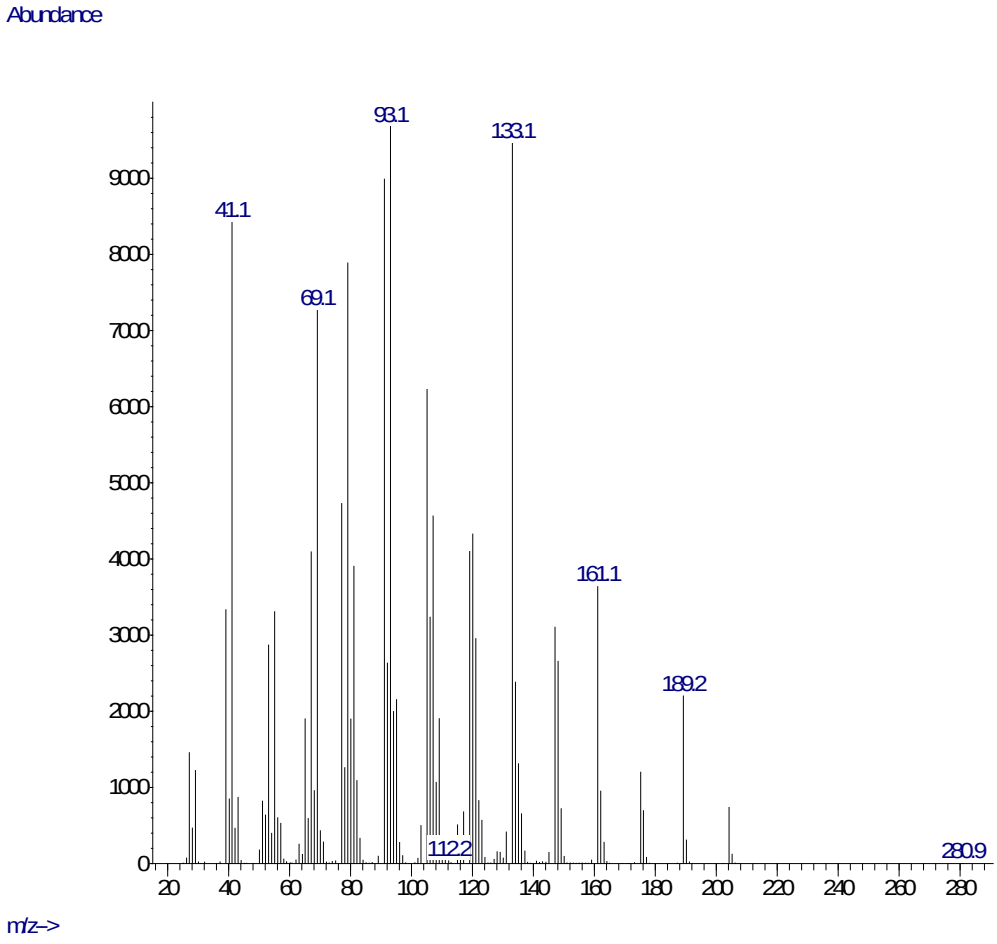


Figure J5 Mass spectrum of peak at 15.188 minutes in gas chromatogram SQ1

This mass spectrum (Fig. J5) was then compared to our standards and NIST-MS library and it was found to correlate to carophyllene.

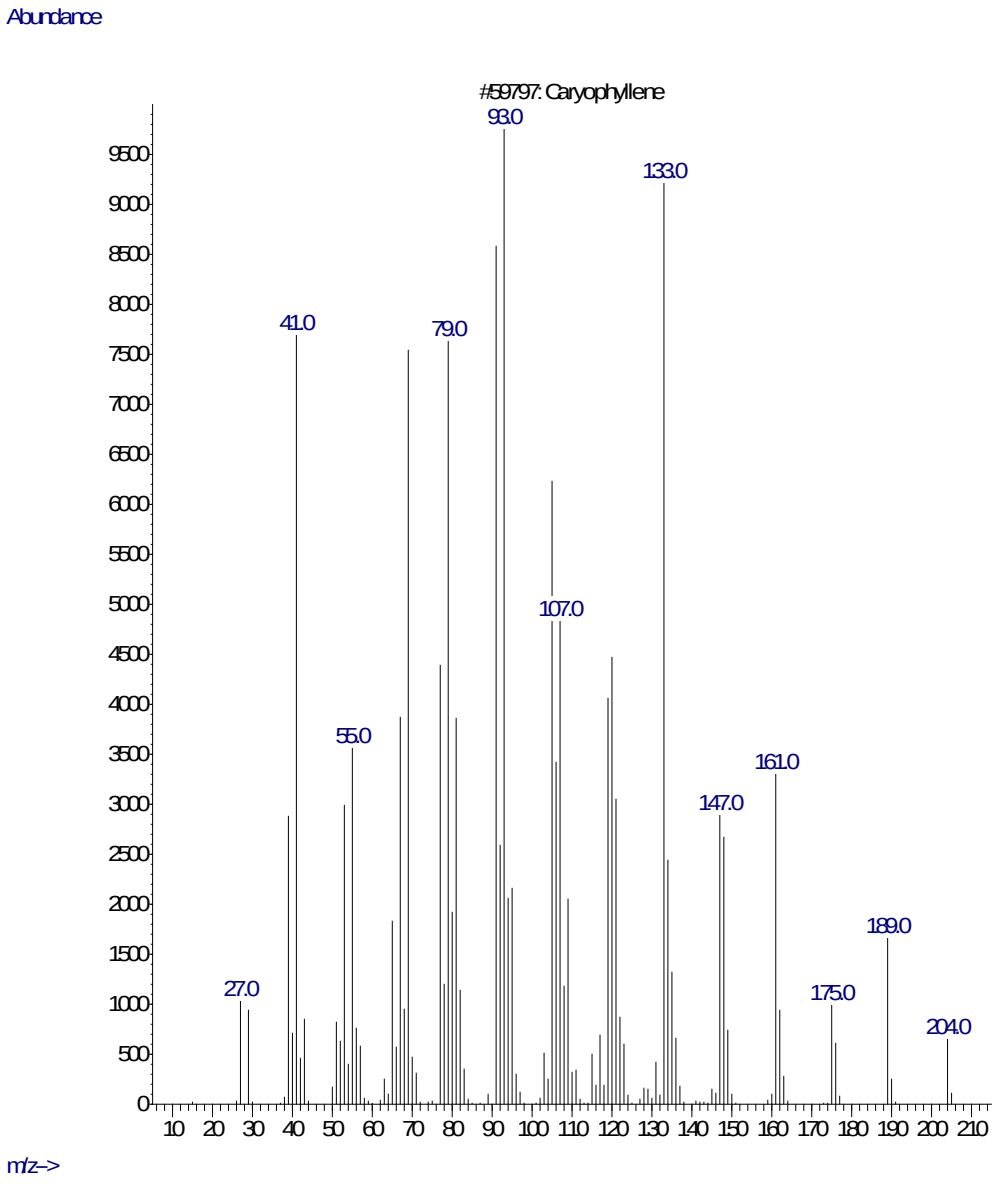


Figure J6 Mass spectrum of carophyllene

This process was then continued to identify all the peaks in the chromatogram.

Since in every experiment about 40 constituents are identified, to show all the identifications would be not be practical. Hence an example of the process used for identification is show here and only the summaries of the constituents identified were shown in the main study.

APPENDIX K

Calculations for the average heavy metal concentration in each pot.

Chromium spiked pots

Table K1 The calculations for average concentrations of heavy metals in Cr1 pot (Statistics determined on excel)

	Cr		Hg		Pb
Soil from Cr 1 pot	mg kg^{-1}	std dev of sample	mg kg^{-1}	std dev of sample	mg kg^{-1}
Cr 1 sample 1	**186.376**	0.099	**0.000**	-	nd
Cr 1 sample 2	**101.945**	1.486	0.00	-	nd
Cr 1 sample 3	**143.566**	0.911	92.39	1.94	nd
Total	431.887		92.392		

Average Cr in experiments	143.96	Average Hg in experiment	30.80
Variance of population	1.02	Variance of population	1.25
Std deviation of population	1.01	Std deviation of population	1.12

Table K2 The calculations for average concentrations of heavy metals in Cr2 pot (Statistics determined on excel)

	Cr		Hg		Pb
Soil from Cr 2 pot	mg kg^{-1}	std dev of sample	mg kg^{-1}	std dev of sample	mg kg^{-1}
Cr 2 sample 1	**208.083**	0.583	**51.421**	2.319	nd
Cr 2 sample 2	**182.434**	0.623	0.00	-	nd
Cr 2 sample 3	**161.974**	0.434	36.78	1.10	nd
Total	552.491		88.197		

Average Cr in experiments	184.16	Average Hg in experiment	29.40
Variance of population	0.31	Variance of population	2.19
Std deviation of population	0.55	Std deviation of population	1.48

Table K3 The calculations for average concentrations of heavy metals in Cr3 pot (Statistics determined on excel)

	Cr		Hg		Pb
Soil from Cr 3 pot	mg kg^{-1}	std dev of sample	mg kg^{-1}	std dev of sample	mg kg^{-1}
Cr 3 sample 1	**246.704**	0.720	**81.103**	2.407	nd
Cr 3 sample 2	**306.509**	1.383	2.60	2.27	nd
Cr 3 sample 3	**129.783**	0.808	0.00	-	nd
Total	682.996		83.699		

Average Cr in experiments	227.67	Average Hg in experiment	27.90
Variance of population	1.03	Variance of population	3.65
Std deviation of population	1.01	Std deviation of population	1.91

Mercury spiked pots

Table K4 The calculations for average concentrations of heavy metals in Hg1 pot (Statistics determined on excel)

	Cr		Hg		Pb
Soil from Hg1 pot	mg kg^{-1}	std dev of sample	mg kg^{-1}	std dev of sample	mg kg^{-1}
Hg 1 sample 1	**73.830**	1.390	**11.405**	5.635	nd
Hg 1 sample 2	**105.547**	0.405	0.00	-	nd
Hg 1 sample 3	**66.895**	0.745	0.00	1.94	nd
Total	246.272		11.405		

Average Cr in experiments	82.09	Average Hg in experiments	3.80
Variance of population	0.88	Variance of population	11.83
Std deviation of population	0.94	Std deviation of population	3.44

Table K5 The calculations for average concentrations of heavy metals in Hg2 pot (Statistics determined on excel)

	Cr		Hg		Pb
Soil from Hg2 pot	mg kg^{-1}	std dev of sample	mg kg^{-1}	std dev of sample	mg kg^{-1}
Hg 2 sample 1	**22.943**	2.404	**54.065**	1.073	nd
Hg 2 sample 2	**95.000**	2.671	0.00	-	nd
Hg 2 sample 3	**30.176**	0.915	0.00	0.20	nd
Total	148.119		54.065		

Average Cr in experiments	49.37	Average Hg in experiments	18.02
Variance of population	4.58	Variance of population	0.40
Std deviation of population	2.14	Std deviation of population	0.63

Table K6 The calculations for average concentrations of heavy metals in Hg3 pot (Statistics determined on excel)

	Cr		Hg		Pb
Soil from Hg3 pot	mg kg^{-1}	std dev of sample	mg kg^{-1}	std dev of sample	mg kg^{-1}
Hg 3 sample 1	**89.313**	0.065	**76.554**	1.973	nd
Hg 3 sample 2	**60.080**	0.595	56.69	0.62	nd
Hg 3 sample 3	**64.271**	0.689	66.27	2.58	nd
Total	213.664		199.507		

Average Cr in experiments	71.22	Average Hg in experiments	66.50
Variance of population	0.28	Variance of population	3.64
Std deviation of population	0.53	Std deviation of population	1.91

Lead spiked pots

Table K7 The calculations for average concentrations of heavy metals in Pb1 pot (Statistics determined on excel)

	Cr		Hg		Pb
Soil from Pb 1 pot	mg kg^{-1}	std dev of sample	mg kg^{-1}	std dev of sample	mg kg^{-1}
Pb 1 sample 1	**22.663**	0.423	**0.000**	-	nd
Pb 1 sample 2	**22.773**	0.797	0.00	-	nd
Pb 1 sample 3	**20.088**	1.672	0.00	0.00	nd
Total	65.524		0.000		
Average Cr in experiments	21.84	Average Hg in experiments	0.00		
Variance of population	1.20	Variance of population	0.00		
Std deviation of population	1.10	Std deviation of population	0.00		

Table K8 The calculations for average concentrations of heavy metals in Pb2 pot (Statistics determined on excel)

	Cr		Hg		Pb
Soil from Pb 2 pot	mg kg^{-1}	std dev of sample	mg kg^{-1}	std dev of sample	mg kg^{-1}
Pb 2 sample 1	**22.447**	1.219	**0.000**	0	nd
Pb 2 sample 2	**17.131**	1.111	0.00	-	nd
Pb 2 sample 3	**46.013**	0.842	0.00	0.00	nd
Total	85.591		0.000		

Average Cr in experiments	28.53	Average Hg in experiments	0.00
Variance of population	1.14	Variance of population	0.00
Std deviation of population	1.07	Std deviation of population	0.00

Table K9 The calculations for average concentrations of heavy metals in Pb3 pot (Statistics determined on excel)

	Cr		Hg		Pb
Soil from Pb 3 pot	mg kg^{-1}	std dev of sample	mg kg^{-1}	std dev of sample	mg kg^{-1}
Pb 3 sample 1	**22.519**	1.246	**0.000**	0	nd
Pb 3 sample 2	**18.953**	2.556	0.00	0.00	nd
Pb 3 sample 3	**21.540**	1.778	0.00	-	nd
Total	63.012		0.000		

Average Cr in experiments	21.00	Average Hg in experiments	0.00
Variance of population	3.75	Variance of population	0.00
Std deviation of population	1.94	Std deviation of population	0.00

APPENDIX L

The calculations for the average heavy metal concentration in the leaves from Centella Asiatica

Table L1 The calculations for the average concentration of heavy metals in Cr1,Cr2 and Cr3

	Cr		Hg	Pb
Leaves from Chromium pots	mg kg^{-1}	std dev of sample	mg kg^{-1}	mg kg^{-1}
Cr 1	20.73	7.92	nd	nd
Cr 2	31.65	1.15	nd	nd
Cr 3	46.98	0.96	nd	nd
Total	99.36			

Average Cr conc in Cr experiments	33.12
Variance of population	21.63
Std deviation of population	4.65

Table L2 The calculations for the average concentration of heavy metals in Hg1, Hg2 and Hg3

	Cr		Hg		Pb
Leaves from Mercury pots	mg kg^{-1}	Std. dev	mg kg^{-1}	Std. dev	mg kg^{-1}
Hg 1	**21.18**	2.82	**36.45**	4.67	nd
Hg 2	**19.40**	2.38	nd	-	nd
Hg 3	**38.86**	2.44	nd	-	nd
Total			36.453		
Average Cr in Hg experiments	26.48	Average Hg in experiment	36.45		
Variance of population	6.51	Variance of population	7.27		
Std deviation of population	2.55	Std deviation of population	2.70		

Table L3 The calculations for the average concentration of heavy metals in Pb1, Pb2 and Pb3

	Cr		Hg		Pb
Leaves from Lead pots	mg kg^{-1}	Std. Dev	mg kg^{-1}	Std. dev	mg kg^{-1}
Pb 1	**21.37**	1.43	**31.14**	3.83	nd
Pb 2	**17.09**	1.46	nd	-	nd
Pb 3	**19.67**	5.76	nd	-	nd
Total	58.123		31.136		

Average Cr in Pb experiments	19.37	Average Hg in experiment	31.136
Variance of population	12.45	Variance of population	4.879425333
Std deviation of population	3.53	Std deviation of population	2.20894213