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Extraction of raw plant material using supercritical carbon dioxide

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Abstract

Many of the herbs and spices used by humans are important medicinal compounds. The aim of this work is to investigate the extraction mechanisms of plant materials by using supercritical fluid extraction (SFE). In order to do this, a supercritical extraction system was designed and set-up allowing to use supercritical CO_2 for the extraction of raw plant material, such as Sassafras albidum and Berberis vulgaris.

The planned work found difficulties due to legal problems and the objective plant material had to be changed for this reason. The first plant considered was Sassafras albidum which later on was changed to Berberis vulgaris. Supercritical extraction in dynamic and steady state were executed in order to study the extraction parameters and attempt to recover a compound called berberine.

The results of the experiments show that supercritical fluid extraction is a sufficient extraction method for the plant Berberis vulgaris. On the other hand, it was not possible to apply any analytical methods to prove that berberine was obtained since the amount of extract was not sufficient for the techniques available. Even so, physical properties such as smell and yellow color of the extract suggested that berberine might have been extracted. The whole designed supercritical extraction process needs improvements such as the introduction of organic solvents to increase the extraction yield and the introduction of an alternative analytical protocol such as High Performance Liquid Chromatography (HPLC).

Keywords: Supercritical Fluid Extraction, safrole, berberine ...

Resumo

Vária ervas e especiarias usadas por humanos contêm compostos com propriedades medicinais e em muitas partes do mundo acabam por ser a principal fonte de tratamento médico. O objetivo deste trabalho consiste em investigar os mecanismos de extração de plantas, usando para o efeito extração com fluidos supercríticos. Para esse efeito um sistema de extração foi desenvolvido e montado de forma a usar CO_2 supercrítico como solvente para a extração da planta.

A estrutura deste trabalho pode parecer irregular uma vez que a planta alvo teve de ser mudada por razões legais. A primeira planta a ser considerada foi Sassafras albidum e depois foi alterada para *Berberis vulgaris*. As extrações usando fluidos supercríticos em estado estacionário e dinâmico foram executadas de forma a estudar os parâmetros da extração e tentar obter o composto alvo berberine.

Os resultados experimentais mostram que extração usando fluidos supercríticos é efetiva na extração da planta *Berberis vulgaris*. Relativamente ao composto Berberine não foi possível provar a sua extração, mas propriedades físicas manifestadas no extrato como cheiro e cor amarela sugerem que o composto estava presente em pequena quantidade. É também sugerido que o processo seja alvo de um maior desenvolvimento nomeadamente introduzindo solventes orgânicos para aumentar o rendimento da extração ou introduzir um método alternativo para análise do composto alvo como por exemplo cromatografia líquida de alta eficiência.

Palavras-chave: Fluidos supercríticos, extração, berberine, safrole ...

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Chapter 1

Introduction

In this chapter, the motivation for realization of this work is presented and also the aims of the work are outlined.

This work was performed with collaboration with Warsaw University of Technology, Faculty of Chemical and Process Engineering, Warsaw, Poland, within the Erasmus program within the period of 25 of February 2016 to 16 of September 2016.

The primary subject described: "Valorisation of plant and oil rich in safrole in high pressure Carbon Dioxide (CO_2)" had to change during the stay due to a new law in Poland that forbids the use of the compound called safrole for academic investigation. The current subject of the work and thesis covers the extraction of the compound berberine from the raw plant material Berberis vulgaris.

1.1 Motivation

Many of the herbs and spices used by humans are important medicinal compounds. In fact, the World Health Organization (WHO) estimates that 80 percent of the World's population still uses traditional remedies, including plants, as their primary health care tools. Plants possess a large number of chemical compounds that are applied for biological functions like defense against insects, fungi and herbivorous mammals. The effects of this compounds on the human body are identical to those already well understood in conventional drugs. The know how reveals that the difference between herbal medicine and conventional drugs is insignificant considering their actions and potential for pharmacologic benefits.

Examples of medicinal plants are ginger, garlic, peppermint, lavender, sassafras, chamomile, barberry, aloe vera, among others. Even though these plants have pharmacological benefits by simple ingesting them, by knowing the active compounds which

provoke such effect it is possible to increase its properties by using it in different ways or compositions. For that reason, there's an increasing interest not only in identifying these active substances but also into isolate them from the raw plant material. When extracted from the plants, these compounds can be obtained in their pure form, as a solid or liquid, or in many cases through the plants essential oils.

Medicinal plants possess a wide range of applications, such as anxiety treatment, cough and fever diminishment, cholesterol reduction, anti-inflammatory actions, and so on.

Various methods can be used to extract these substances like cold pressing, steam distillation, organic solvent extraction, among others. In recent years, the interest in application of environmentally responsible processes has increased significantly including extraction methods for solid materials in order to separate active compounds from herbs and plants.

Supercritical fluid extraction (SFE), represents a valid and promising technique in many areas, including the extraction of solid materials. There are several references were such a method is used in a plant material in order to extract pure components and essential oils.

The extraction of active substances from medicinal plants seems to be a growing and important area since it allows to obtain compounds which further can be used in pharmaceutical research. New and more environmentally friendly processes allow an increase in purity of the substances obtained making them even more appropriate for pharmaceutical purposes.

Combining two growing areas, extraction of active substances from plants and the use of these compounds in pharmaceutical research creates a possibility to see that not only they complement each other but also represent an alternative thinking when it comes to cure or treat several diseases.

1.2 Aim of the work

The main focus of this work is to develop and investigate the extraction processes of raw plant materials using supercritical fluid extraction. More specifically, the aim is detailed as follows:

1. Design and set-up of a supercritical extraction system with the objective of obtaining compounds of interest from plant raw materials.
2. Performing experiments using supercritical CO_2 in order to test several process conditions in the extraction of plant materials such as *Sassafras albidum* and *Berberis vulgaris*.
3. Discussion of the results obtained and the effect of the various parameters on the course of the extraction process.

Chapter 2

Theoretical Background

In this chapter, the aim is to present the theoretical information that serves as background for the future work.

2.1 Medicinal Plants

Plants exist in a wide variety and all around, playing many roles in different cultures. There are thought to exist around 315 thousand species of plants being most of them seed plants (Tomatoes, Anise, Mimosa, among others). Plants provide most of the world's molecular oxygen and are the basis of most of earth's environments, especially on land. Plants are there for extremely important when it comes to animal life, supplying them food and shelter. Including the already referred most common plant group there are four main groups of plants that represent the entire diversity existent on the planet, being them the **Green algae**, **Bryophytes**, **Pteridophytes**, and **Seed plants**. [1]

Mankind has been using plants since its origin, but its uses change from culture to culture. One of the most basic uses of plants in human life's is has a food supply, by producing grains, fruits, and vegetables. They can also be used as ornaments decorating houses or streets, ceremonies, and has source of most medicines and drugs. When it comes to food applications it's important to understand that much of the human nutrition depends on plants, either directly through food and beverages or indirectly as food for animals or even to flavor food. As for the first case some important examples are rice, potatoes, vegetables, among others. In the case of beverages some common examples are coffee, tea, beer, wine, whisky, vodka, and others. When it comes to indirect nutrition some examples are in the feeding of animals like cows, sheep's, pigs, goats, and related to flavoring foods cases like rosemary, mint, cumin, cinnamon, among others. There are also by products of this plants which could have practical application when it comes to

food like in the case of fruits and flowers. [2]

Plants are also the source of many natural products such as essential oils, natural dyes, pigments, waxes, resins, tannins, amber, cork, among others. Other products which are manufactured but also derive from plants are soaps, perfumes, paint, rubber, latex, inks, and gums. Fuels, a very important factor in mankind's development, have also a vegetal origin like for example fossil fuels such as coal, firewood, petroleum and olive oil. Plants also have applications in construction, with wood being used in buildings, boats, furniture or even musical instruments and sports equipment. Materials like paper, cardboard, cotton, acetate and cellulose fibers are also obtained from various plants.

They are also involved in the origin of medicine, being the only way of treating or stabilizing certain conditions. Ancient civilizations like the Sumerians and the Egyptians had already a large knowledge in medicinal plants, including one like garlic, juniper, cannabis, mandrake, among others. The use of herbs to treat diseases is almost universal among non-industrialized societies and its use becomes a primary health source. One example is the use of different species of herbs and spices in cuisine, which is seen has an attempt to treat food-borne pathogens. [3]

Literature also suggests a relation between areas where the existence of pathogens is more common with the type of cuisine practiced in those areas. What was verified is that in those areas traditional cuisine is highly spiced, which supports the claim that this herbs and spices have an active effect against pathogens and several conditions.

It's also important to understand that even nowadays several countries still use plants with this purposes instead of the pharmaceutical alternative which in theory would be much more effective. This can be related with the ability of people in those areas to access advanced medical techniques, being the traditional methods the only medicinal possibility in some cases. Pharmaceutical products also cost a lot to develop and produce, having for that reason very high prices which can become a very large barrier in those areas with more difficulties.

Table 2.1: Examples of medicinal plants and their uses.

Plant	Plant Part	Location	Application
Polemonium reptans	Root	North America	Fever Inflammation Cough
Aloe vera	Leaf	Worldwide	Burns Wounds Skin conditions
Ambrosia hispida	Leaf	North America	Menstrual regulator Childbirth aid
Tetragastris balsamifera	Bark	South America	Aphrodisiac

Vaccinium macrocarpon	Berry	North America	Diarrhea Diabetes Liver problems
Eucalyptus globulus	Leaf	Worldwide	Cold medications Analgesic
Viscum album	Leaf/Berry	Europe and Asia	Seizures Headaches
Trigonella foenum-graecum	Seed/Leaf	India	Diabetes Menopause Loss of appetite
Equisetum arvense	Leaf/Bark	Worldwide	Tuberculosis Stop bleeding Kidney problems
Piper methysticum	Leaf	South Pacific	Sedative Anaesthetic
Amorphophallus konjac	Root	Eastern Asia	Obesity Reducing cholesterol Constipation
Curcuma longa	Root	Southern Asia	Aid digestion Relieve arthritis Menstruation
Azadirachta indica	Leaf	India	Treat worms Rheumatism Malaria
Verbena officinalis	Root	Americas and Asia	Sore throats Respiratory diseases
Triticum aestivum	Seeds	Worldwide	Antioxidant Anti-inflammatory

Even though the medicinal potential of this plants has been seen since the beginning by several societies, only when modern medicine was developed was possible to identify the plants compounds which are responsible for this medicinal applications and their effects in the human body. In fact, nowadays many of the available pharmaceuticals have its origins from medicinal plants, including aspirin, quinine, morphine, among others.

All plants produce chemical compounds as part of their normal metabolic activities. Besides the primary metabolites such as sugar and fats, there's a second type of metabolites which corresponds to compounds that serve a specific function and exist in smaller

amount within the plant. Examples of this metabolites are toxins directed to defend against predators and pheromones used to attract insects. This second type of metabolites, as it was mentioned before, can also have a therapeutic effect in humans or even be refined to produce drugs. Some examples of this are tobacco, cannabis, opium and cocaine.

These substances can be extracted from the plants using a wide range of methods, depending on the properties of the compound of interest. Some compounds are solid at normal conditions of pressure and temperature which makes it easy to separate it from the other compounds of the plant. Others, which in normal conditions of pressure and temperature are in liquid state, usually are extracted through the plants essential oil.

Essential oils correspond to oily aromatic liquids extracted from aromatic plant materials, being unstable and fragile volatile compounds. They could be biosynthesized in different plant organs as secondary metabolites such as flowers (jasmine, rose), herbs, buds, leaves, fruits, twigs, bark, seeds, wood, rhizome and roots. Due to their hydrophobic nature and their density often lower than that of water, they are generally lipophilic, soluble in organic solvents, immiscible with water. This oils have gained a renewed interest in several areas. As natural products, they have interesting physicochemical characteristics with high added values respecting the environment and also diverse biological activities. They are also used in order to provide a pleasant feeling of psychic comfort thanks to their pleasant odor. Thanks to their complex chemical composition, often composed of more than 100 different compounds, the essential oils have a broad biological and antimicrobial activity spectrum (antibacterial, antifungal, antiviral, pest control, insect repellents). In the pharmaceutical field, they're included in the composition of many dosage forms (capsules, ointments, creams, syrups, suppositories, aerosols and sprays).

Table 2.2: Examples of essential oils components and its medicinal properties.

EO components	Plant source	Medicinal properties
Sabinene	*Quercus ilex*	Antifungal
	Oenanthe crocata	Anti-Inflammatory
		Antioxidant
α-Pinène	*Pinus pinaster*	Anti-inflammatory
		Anti-oxidant
D-Limonène	*Citrus limon*	Antifungal
		Antioxidant
Myrcène	*Citrus aurantium*	Gastroprotective
γ-Terpinène	*Origanum vulgare*	Antioxidant
para-Cymène	*Cuminum cyminum*	Antifungal
Geraniol	*Pelargonium graveolens*	Insecticide
		Antimicrobial
		Anticancer

Linalool	*Lavandula officinalis*	Insect-repellent Anti-tumor Anti-inflammatory
Borneol	*Thymus satureioides*	Broad-spectrum Anti-microbial
Citral	*Aloysia citrodora*	Anti-tumor Antifungal Antibacterial Painkiller
Citronellal	*Cymbopogon citratus*	Insecticide Antifungal Antimicrobial
Camphor	*Lavendula stoechas*	Antispasmodic Sedative Anti-inflammatory Anti-anxiety
Carvone	*Mentha spicata*	Antispasmodic Antimicrobial
Thymol	*Thymus vulgaris*	Antiseptic Anti-inflammatory Cicatrizing
Carvacrol	*Thymus maroccamus*	Antimicrobial Anti-inflammatory
1,8-Cineole	*Eucalyptus polybractea*	Anti-inflammatory
Linalool Oxide	*Pelargonium graveolens*	Anxiolytic-like effects
Cis-Rose oxide	*Rosa damascena*	Anti-inflammatory Relaxant
β-Caryophyllene	*Rosmarinus oficialis*	Anti-inflammatory Antispasmodic Anticolitique
α-Bisabolol	*Matricaria recutita*	Anti-irritant Anti-inflammatory Antimicrobial
Caryophyllen oxide	*Chenopodium ambrosioides* *Psidium guajava*	Analgesic Anti-inflammatory Apoptosis inducer
Valerenic acid	*Valeriana officinalis*	Sedative Anti-anxiolytic
Eugenol	*Eugenia Caryophyllata*	Antifungal Antibacterial

Food industry also presents a growing demand for essential oils because of their important applications as food preservatives, innovation in food packaging and fighting against pathogens generating dangerous food poisoning like for example *Listeria monocytogenes* or *Salmonella typhimurium*. Various studies have demonstrated the efficiency of this type of oils in low doses in fighting against bacterial pathogens encountered in food industry and meat product. Essential oils can also be seen as an alternative to synthetic

antibiotics used in livestock since they have an effective antimicrobial activity. Other applications include medical and technical textiles, dyes, vitamins, phase-change materials, antibiotics, hormones and other drugs.

It's also important to understand that not all plants have a positive effect. Some of them, can become invasive damaging existing ecosystems by displacing native species which could cause crop losses. Animals and humans can also be harmed since several types of plants possess poisons and allergic compounds which could cause serious problems. When it comes to medicinal plants the same problem exists, since many plants are still being studied along with the compounds which make part of its composition. Sometimes the idea is that because something is from natural origin then is completely safe, which is not true. The same way pharmaceutical products possess a list of side effects, plants also may present side effects when taken in too much quantity. As it was mentioned before, a lot of the pharmaceutical products active substances come from medicinal plants which makes that some side effects might be same if the dosage of a certain substance is not controlled. [4]

Table 2.3: Examples of side effects associated with some medicinal plants.

Plant	Possible Side Effects
Curcuma longa	Stomach upset
	Nausea
	Dizziness
	Diarrhea
Aloe vera	Stomach pain
	Diarrhea
	Kidney problems
	Weight loss
Viscum album	Vomiting
	Diarrhea
	Cramping
	Liver damage
Eucalyptus globulus	Dizziness
	Muscle weakness
	Nausea
	Vomiting
Piper methysticum	Liver damage
	Fatigue
	Dark urine
	Yellowed Eyes
Polemonium reptans	Stomach upset
	Sneezing
Triticum aestivum	Flatulence
	Stomach discomfort
Verbena officinalis	Allergic reaction

It's important to refer that most of this side effects only happen when this plants are

used in high quantities or with high regularity.

2.2 Extraction Methods

2.2.1 Conventional Extraction

Since very early mankind started to try to extract the essential oils and compounds that derived from the many plants around them. The methods described show also an evolution in the methods used, some changes occur in the method itself and other from method to method.

2.2.1.1 Water Distillation

The Water Distillation extraction method consists in immersing the plant material in water and further bring it to boil. The water will evaporate, dragging with it volatile components which can be further collected. In a next stage, the vapors pass through a condenser which will allow them to become in liquid form and possible to collect. Condensers essentially make use of cold water passing around a main tube in order to lower the temperature. [5]

After the vapor being condensate into a vessel, the water and oil can be separated by simple decantation. It consists in a process for separation of mixtures, by removing a layer of liquid, generally one from which a precipitate has settled. For example, in the case of essential oils this are less dense that water allowing them to be on top of the water, separated by a layer which allows their separation simply by gravity.

The water obtain in the separation process is called hydrosol or sweet water. Examples of this products are rosewater, lavender water and orange water.

This process can be done at reduced pressure (under vacuum) in order to reduce the temperature to less than 100^oC, which is beneficial in protecting the plant material. According to the literature most processes are performed at atmospheric pressure and temperatures around 100^oC, which corresponds to the boiling point of water. Plants and products which are sensitive to high temperatures cannot be extracted by these methods since can be subjected to degradation.

Table 2.4: Literature on Water Distillation.

Plant	Temperature(^oC)	Pressure (bar)	Reference
Eucalyptus camadulensis	100	1	[6]
Syzygium aromaticum	100	1	[7]
Lavandula intermedia	100	1	[8]
Anethum sowa	100	1	[9]
Carum copticum	100	1	[10]
Citrus fruits	100	1	[11]

One of the main advantages of this method is the immiscibility between oil and water, being especially suitable for the extraction of petals and flowers since it doesn't involve compacting the plant. This method also protects the oils to a certain degree since the surrounding water acts as a barrier to prevent it from overheating. Never the less, when exposed to excessive temperatures or for too long it could cause degradation of the extracted compounds.

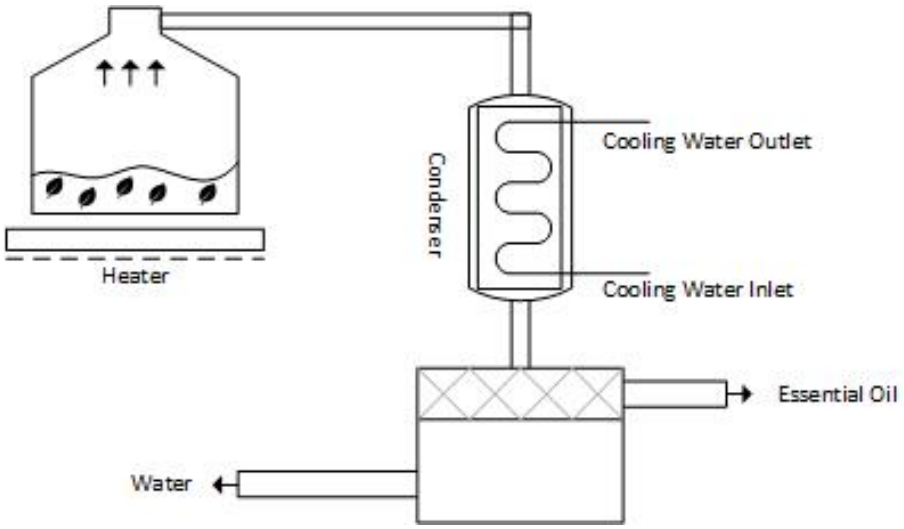


Figure 2.1: Water Distillation Apparatus

2.2.1.2 Steam Distillation

This technique consists in injecting water vapors through the plant matter which will carry in the process volatile materials. The water vapor moves from the bottom up and the boiling water is not in direct contact with the raw material which is suspended inside the vessel in a grid or perforated plate. The same principles can be applied here since water will be heated and turned into vapor, which will carry the volatile components. They further suffer condensation and are separated by decantation. [12]

Steam Distillation is usually used in conditions of atmospheric pressure and temperatures around 100^oC.

Table 2.5: Literature on Steam Distillation.

Plant	Temperature(^oC)	Pressure (bar)	Reference
Ephedra sinica	100	1	[13]
Origanum onites	100	1	[14]
Rosmarinus officinalis. L	100	1	[15]
Lavandula angustifolia	100	1	[16]
Pogostemon cablin	100	1	[17]
Mentha piperita	125	1	[18]
Nepeta persica	100	1	[19]

A big advantage of this process is the reduction of the thermal degradation of the plant material. Even so, the risk of degradations is not completely out since the plant material is still subjected to high temperatures. Steam distillation is a highly used technique and in the literature is often used has comparison to other innovative techniques that are being studied at that moment. [20]

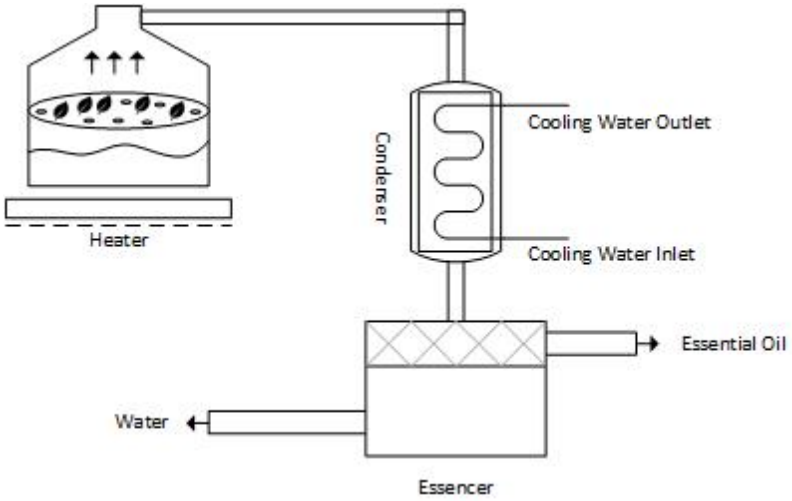


Figure 2.2: Steam Distillation Apparatus

There are alternative ways has how this method can be performed with changes in the configuration of the apparatus. One of them is called Vapor Distillation and differs from the previous one by having the vapor formation and the plant material in two different vessels. The two vessels are connected allowing the vapor generated in one to move to the other and get into contact with the plant material. The final steps are just the same with condensation of vapors and separation of the oil from the water.

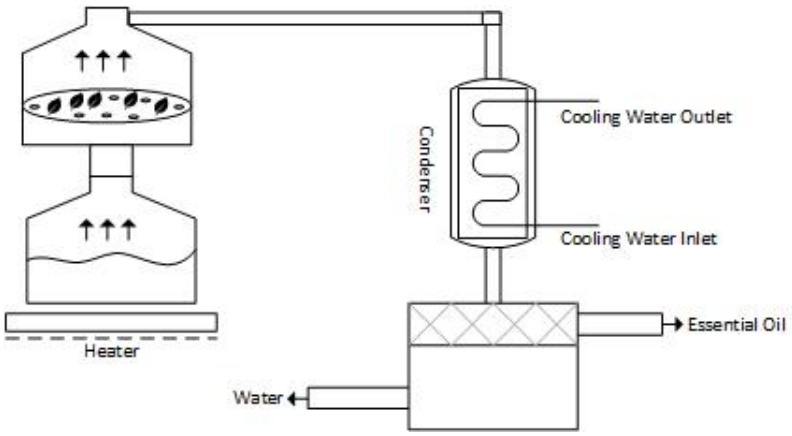


Figure 2.3: Vapor Distillation Apparatus

Another possible configuration is called Hydro Diffusion and consists on injecting

steam not from the bottom of the vessel but from the top making the steam move downward. The vapor mixture with essential oils is directly condensed below the plant support through a perforated tray. This method can reduce the steam consumption and the distillation tome, meanwhile, a better yield can be obtained in comparison with steam distillation.

The conditions in which both of this two different configurations operate are the same as in Steam Distillation with an atmospheric pressure and temperature close to water's boiling point.

2.2.1.3 Organic Solvent Extraction

This method consists on using organic solvents in order to separate two or more substances by dissolving the raw material and extracting the oil inside of it.

There are various types of possible apparatus in this technique being the most common the Sohxlet apparatus. This specific method uses a continuous circulation of organic solvent in order to extract several compounds from the plant material of interest. The solvent used is going to change from plant to plant being directly associated with the compound supposed to be extracted. [20]

The continuous recirculation is possible since the physical state of the organic solvent is changed first from liquid to vapor and later on again to liquid. The way the Soxhlet extractor is design is so that after the organic solvent been boiled, the vapor goes through a channel which leads to a condenser. In there, the organic solvent turns back into liquid and fills the recipient where the plant material is located, extracting the components from it. When the solvent reaches a certain level, it is sucked back to the beginning of the process starting it all over again. To each time the solvent is recirculated back into the heated vessel is called a cycle, being that denomination one of the most common ways to describe how long the process is occurring. The Soxhlet extractor is then composed by three main sections: A percolator which circulates the solvent, a thimble which retains the solid to be laved and a siphon mechanism which periodically empties the thimble. [21] [22]

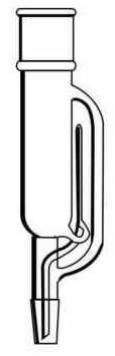


Figure 2.4: Soxhlet Extractor.

The operating conditions of this method are also going to depend on the solvent used for the extraction, performed at atmospheric pressure but with variable temperature since the boiling point is different from solvent to solvent.

Table 2.6: Boiling Point of most common solvents.

Solvent	Boiling Point (^oC)
Acetone	56
Dichloromethane	57,2
Hexane	68
Ethanol	78,37

There are numerous examples of the use of this process in the literature, in fact, it's used in many cases as a standard process for comparison between tradition and new developed extraction methods.

Table 2.7: Examples of solvent extraction in literature.

Plant	Temperature(^oC)	Pressure (bar)	Reference
Jasminum	78,4	1	[23]
Origanum onites	68	1	[14]
Eucalyptus loxophleba	90	1	[24]
Agaricus bisporus L.	80	1	[25]
Moringa oleifera	70	1	[26]
Ricinus communis L.	68	1	[27]

One of the main advantages of this method is that needs no control over it, meaning that the process can go through successive cycles without needing any outside action. Usually the extraction is over by controlling the color in the heated vessel, to see if it has high amount of the compound of interest.

2.2.1.4 Cold Pressing

Cold Pressing is a method which is based on machine squeezing the raw material at room temperature for the release of essential oils, which are washed in cold running water. The oil is then separated from the water by centrifugation or by simple decantation. Even though the end result and theory involved are the same, there are several types of machines with different configurations, using from plates to screws to perform the extraction. The oils originated from this method are mainly used in cooking applications, like for example oils from olive and sunflower. Since the process itself is mechanic the operating conditions are usually at normal pressure and temperature, which makes the raw material not has exposed to big sources of heat, making possible less degradation of the oil to occur. Other big example of this method is the extraction from Citrus peel since not only there's a large quantity of oil in this material but it has also a very low cost to grow and harvest the raw materials. [28]

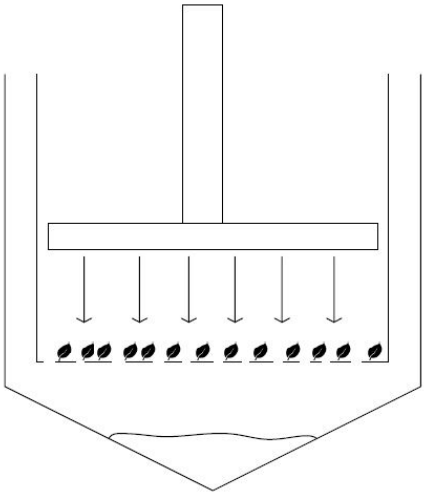


Figure 2.5: Cold Pressing apparatus.

Even though is not has popular has the other methods, cold pressing is also referred in literature as a comparison method.

Table 2.8: Examples of cold pressing extraction in literature.

Plant	Temperature(^oC)	Pressure (bar)	Reference
Citrus fruits	Normal Temperature	1	[11]
Camelina sativa	Normal Temperature	1	[29]
Rheum rhaponticum L.	Normal Temperature	1	[30]
Juglans regia L. var. Franquette	25/50/70	1	[31]
Canola seeds	Normal Temperature	100	[32]
Vitis vinífera L.	Normal Temperature	1	[33]

As it was mentioned before, one of main advantages of this type of extraction rests on the fact that it doesn't use high temperatures avoiding oil degradation. Another major advantage of this method is the fact that it doesn't use organic solvents, turning this process not only much safer, but also simpler since no solvent separation processes are needed prior the extraction.

Through this method is also possible to retain bioactive compounds such as essential fatty acids, phenolics, flavonoids and tocopherols in the oils. In some cases, after the centrifugation part of the process some other products besides the oil might be used to other applications like proteins, minerals, and other substances.

2.2.2 Green Extraction with Innovative Methods

In modern industrial production the words eco-friendly, sustainability, high efficiency and quality become more and more common. By applying the concepts of green extraction in creating processes which would allow reducing operation units, energy consumption, CO_2 emissions, among other factors new methods were developed has alternatives to the most traditional ones. The focus of this new processes is not just to improve the

One of main disadvantages of conventional techniques is the fact that essential oils components are highly sensitive to heat and can easily suffer chemical alterations (isomerization, oxidation) due to the high applied temperatures.

2.2.2.1 Subcritical Extraction

Subcritical Fluid Extraction is a method in which the solvent substance is operated in conditions close of critical pressure (P_c) and temperature (T_c). In simple terms, the subcritical conditions are achieved by changing these parameters along the critical point. From the literature is possible to see that the most common fluids used in this type of processes are water and carbon dioxide (CO_2).

Table 2.9: Literature examples of subcritical extraction.

Plant	Solvent	Temperature(^oC)	Pressure (bar)	Reference
Contaminated Soil	Water	250 - 300	50	[34]
Coriandrum sativum L.	Water	100 - 175	20	[35]
Curcuma longa L.	Water	120 - 160	10	[36]
Red Paprika	CO_2	6	56	[37]
Coriandrum sativum L.	Water	350	200	[38]
Thymus mastichina	Water	100 - 175	20 - 200	[39]

- **Subcritical Water Extraction (SWE)**

Subcritical Water Extraction it's a technique which operates at temperatures between 100 and 374^oC and pressure high enough to maintain the liquid state. Usually the temperature used in this type of processes is 200^oC. It can be performed in batch or continue systems, being the last the most common.

One possible apparatus configuration for this type of process is for the case of subcritical extraction using water as solvent. This system uses tanks, pumps, extraction vessels, oven for the heating of the extraction vessel, heat exchangers for cooling of extract and a sample collection system. The pump is essentially employed for pumping the water (and extract) through the system. In some other cases, another pump can be employed for flushing the tubing's. Also, one extra tank can be employed to contain an organic solvent which would act as a co-solvent (modifier), being necessary to use another pump in order to introduce it into the system. A pressure restrictor is needed to maintain the appropriate pressure in the equipment. [20]

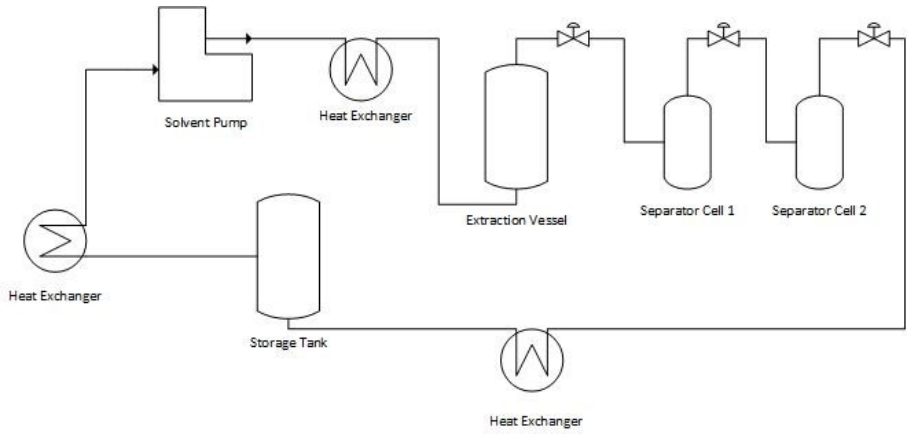


Figure 2.6: Subcritical Water Extraction apparatus example has described above.

The unique properties of water are namely its disproportionately high boiling point for its mass, a high dielectric constant and high polarity. As the temperature rises, there is a systematic decrease in permittivity, an increase in the diffusion rate and a decrease in the viscosity and surface tension. This way, more polar target materials with high solubility in water at ambient conditions are extracted more efficiently at lower temperatures, whereas moderately polar and non-polar targets require a less polar medium induced by elevated temperature. The most important parameters in this type of process are then temperature, particle size and flow rate.

This method also functions has a powerful alternative to more traditional extraction methods since it uses low working temperatures and enables a rapid extraction. Other positive aspects in using this technique are its simplicity, low cost, and favorable environmental impact. Also when compared to the various traditional methods, Subcritical Water Extraction presents several advantages like shorter extraction times, higher quality of the extract, lower costs of the extracting agent, an environmentally compatible technique and low solvent consumption.

In the literature comparisons can be found between supercritical CO_2 and SWE, concluding that even though the extraction conditions are softer and less expensive to run that supercritical CO_2 the last still becomes cheaper to implement on an industrial level, since SWE requires specific equipment.

- **Extraction with Subcritical** CO_2

The subcritical state of carbon dioxide (CO_2) is obtained when the temperature is between 31^oC and 55^oC and pressure between 0.5 MPa and 7.4 MPa. Under these conditions the CO_2 behaves as a non-polar solvent. The apparatus for this type of process is similar to the configuration presented on the Subcritical Water Extraction process, changing only on the extracting solvent. This type of process can also incorporate various types of modifiers in order to improve the extraction results.

This method avoids the degradations observed in the steam distillation or entrainment by vapor due to the high temperatures and the presence of water. According to the literature, extracts obtained by this technique present flavors very similar to those of fresh vegetable raw materials. Also, when compared to SWE its shown to have a superior quality of extract. [20]

2.2.2.2 Ultrasound Assisted Extraction

This method uses ultrasounds in order to allow the intensification of the extraction process from the plant raw material when used in combination with other techniques like hydrodistillation and solvent extraction. Ultrasounds are essentially sound waves with frequencies higher than the upper audible limit of the human hearing. [40]

The plant raw material is immersed in water or solvent and at the same time it is subjected to the action of the ultrasound. This technique was developed especially for the extraction of certain molecules of therapeutic interest even though nowadays it its used for large number of other applications.

Commonly, the used ultrasonic waves have a frequency of 20 kHz – 1MH. This waves will induce mechanical vibrations on the walls and membranes of the plants allowing a faster extraction. A very important parameter in this type of processes is the size of the particles, or in other words, the milling degree of the plant material. The smallest the size the more particles will be subjected to the action of the ultrasound waves. [20]

In terms of equipment necessary, it's very simple since it consists in only adding an ultrasound generator to an already existing extraction process. This makes its implementation not only very simple but also very inexpensive.

When compared with the more traditional methods, it presents advantages such as improving extraction efficiency and rate, a reduction in the extraction temperature, and increasing the selection range of solvents that can be used. Finally, other advantages of this method consist in the properties of ultrasound which can be an intensification of the mass transfer, cell disruption, improvement of solvent penetration and capillary effect.

Ultrasound Assisted Extraction has been growing in terms of applications since it's a simple way to improve already establish extraction methods.

Table 2.10: Literature Examples of Ultrasound Assisted Extraction.

Plant	Assisted Process	Reference
Lavandula intermedia	Steam Distillation	[8]
Artemisia annua L.	Hot Maceration	[41]
Olea europaea	Maceration	[42]
Artocarpus heterophyllus	Solvent Extraction	[43]
Limonium sinuatum	Solvent Extraction	[44]
Allium tuberosum	Solvent Extraction	[45]

2.2.2.3 Microwave Assisted Extraction

Microwave Assisted Extraction also becomes an innovative technique by allowing to improve methods already existent. The use of this technique evolved with the development of the green extraction method concept and the need for new energy saving extraction methods.

Microwaves are electromagnetic based waves with a frequency between 300 MHz and 30 GHz and a wavelength between 1 cm and 1 m. The commonly used frequency is 2450 MHz which corresponds to wavelength of 12,2 cm. [20]

In recent times, this process has developed several variants such as microwave assisted hydrodistillation, solvent free microwave extraction, microwave -accelerated steam distillation, microwave steam distillation, microwave hydrodiffusion and gravity and portable microwave assisted extraction. It consists on one of the most promising techniques, presenting advantages such has high reproducibility in shorter times, simplified manipulation, reduced solvent consumption and lower energy output.

Table 2.11: Literature examples on Microwave Assisted Extraction.

Plant	Assisted Process	Reference
Citrus fruits	Distillation	[11]
Soil	Solvent Extraction	[46]
Lavandula angustifolia	Steam Distillation	[16]
Aromatic herbs	Hydro-distillation	[47]
Thymus vulgaris L.	Hydro-distillation	[48]
Curcuma longa	Solvent Extraction	[49]

From all the variants there were some worth specifying:

- **Solvent free microwave extraction (SFME)**

This variant is based on the combination of microwave heating energy and dry distillation, in order to achieve at atmospheric pressure, the extraction of a fresh plant material without the need of adding any water or organic solvents.

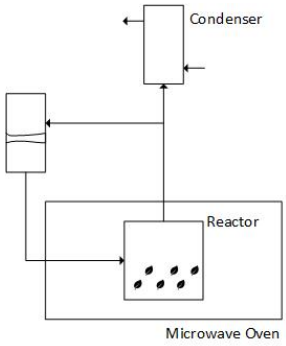


Figure 2.7: SFME apparatus.

- **Microwave hydrodiffusion and gravity (MHG)**

This method consists in placing the plant material in a reversed microwave reactor without any added water or solvent. It combines microwave heating of a reversed alembic with the earth gravity at atmospheric pressure.

When the water inside the plant is heated by the microwaves, causes the rupture of the material allowing to obtain the extracts. Under gravity, these extracts fall to the bottom out of the microwave reactor and in direction to the cooling system.

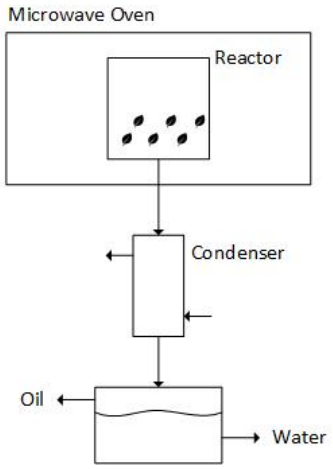


Figure 2.8: MHG apparatus.

From the literature is possible to conclude that this method not is power saving, but reduces extraction time and environmental impact.

- **Microwave steam distillation (MSD)**

In this case the same principles regarding the effect of the microwaves on the plant material apply. It results from a modification of the traditional steam distillation method in which by action of the microwaves the plant material will release its components in a faster way being then carried by the steam in a condenser so the extract can be collected.

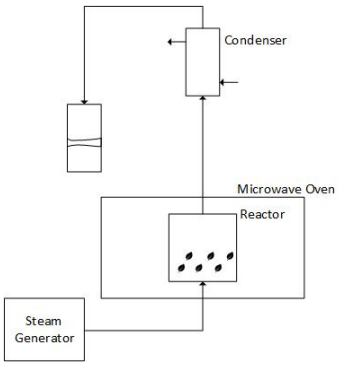


Figure 2.9: MSD apparatus.

In comparison to the traditional methods it was proven to reduce the extraction time and better sensory properties by allowing the high production of fresh oil without causing significant changes in its composition.

- **Microwave steam diffusion (MSDf)**

It is based on the same principles as for the Microwave Steam Distillation (MSD) except that vapors flow through the plant material down.

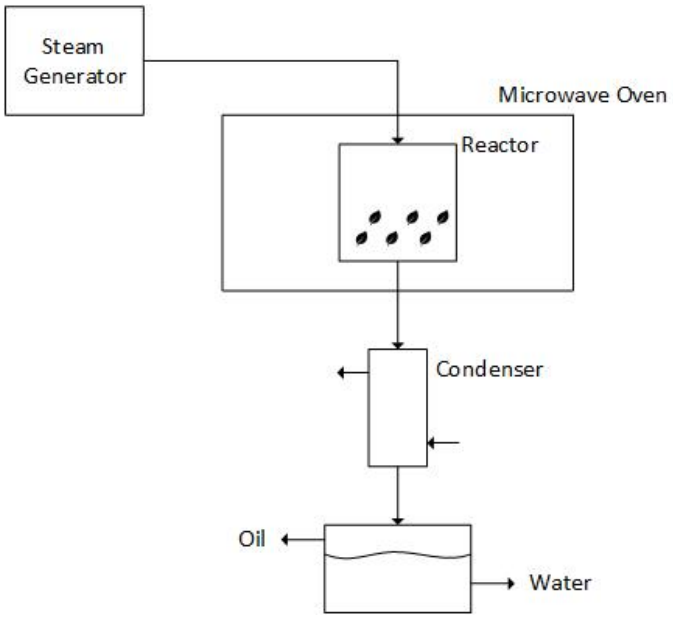


Figure 2.10: MSDf apparatus.

Microwave steam diffusion is a green cleaner, environmentally friendly and an economic procedure.

2.2.2.4 Supercritical Fluid Extraction

Supercritical Fluid Extraction has emerged as an attractive separation technique for the food and pharmaceutical industries due to a growing demand for natural processes that do not introduce any residual organic chemicals. It uses fluids in well-defined conditions of pressure and temperature and when those conditions are reached, the fluids manifest very interesting properties like high diffusivity, low viscosity, and a density close to that of liquids. [20]

Supercritical fluid extraction can be accomplished using a static, dynamic, or coupled static/dynamic mode. In static extraction, a fixed amount of supercritical fluid interacts with the plant material. The extraction vessel containing the plant material is pressurized with the chosen fluid at a certain temperature. The high diffusivity of the supercritical fluid allows it to go through the plant material removing the components of interest. After the extraction is completed, a valve is open allowing the extract to be removed

by decompression into a trap. Usually, a static extraction is followed by some time of dynamic extraction in order to improve the extraction efficiency. In some cases, the supercritical fluid can be recovered in order to introduce it into the process again.

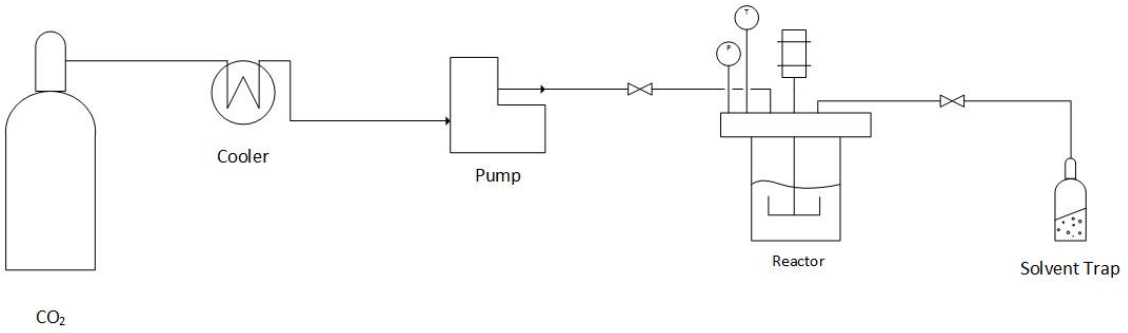


Figure 2.11: Static Supercritical Extraction apparatus.

When it comes to dynamic extraction, this uses fresh supercritical fluid which is continuously passed through the plant material. A big concern of this type of processes is that impurities in supercritical fluids become a concern when using large amounts of fluid during an extraction. Other problems of this type of extraction are the fact that a larger amount of supercritical fluid will eventually extract non wanted components and also cause motion of the plant material which could cause clogged problems. In spite of these problems, dynamic extraction is the favored strategy for at least 90% of all reported applications of supercritical fluid extraction.

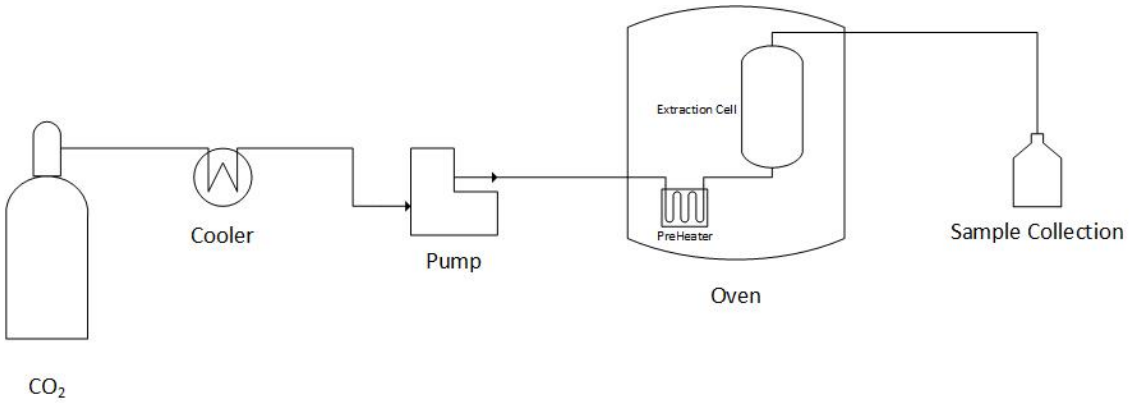


Figure 2.12: Dynamic Supercritical Extraction apparatus.

A combination of an initial static period followed by a dynamic one is gaining popularity, especially for situations where solvated analyte must diffuse to the matrix surface to be extracted. The extraction starts in a static mode with no net flow through the system. When the extraction has proceeded for a given amount of time, the system is put into a dynamic mode.

Supercritical Fluid Extraction can be influence by several parameters:

- Particle size and shape;
- Surface area and porosity;
- Moisture content;
- Changes in morphology;
- Sample size.

Carbon dioxide (CO_2) is generally the most widely used solvent for extraction because of its numerous advantages. The most important ones are having a critical point easy to reach (a critical pressure of 73,9 bar and a critical temperature of $31{,}2^oC$), being unaggressive for thermolabile molecules of the plant essence, being chemically inert and non-toxic, non-flammable, available in high purity at relatively low cost, and allows easy elimination of its traces from the obtained extract by simple depression.

Table 2.12: Literature examples on Supercritical Extraction.

Plant	Solvent	Process type	Reference
Eucalyptus loxophleba	CO_2	Dynamic	[24]
Syzygium aromaticum	CO_2	Stationary	[50]
Leptocarpha rivularis	CO_2	Stationary/Dynamic	[51]
Anethum sowa	CO_2	Stationary	[9]
Myristica fragrans	CO_2	Dynamic	[52]
Carum copticum	CO_2	Static/Dynamic	[10]
Mentha pulegium L.	CO_2	Static/Dynamic	[53]
Foeniculum vulgare	CO_2	Static	[54]
Marchantia convoluta	CO_2	Static/Dynamic	[55]
Lippia alba	CO_2	Dynamic	[56]
Rosmarinus officinalis	CO_2	Static	[57]
Rose geranium	CO_2	Dynamic	[58]
Matricaria chamomilla	CO_2	Static	[59]
Cuminum cyminum L.	CO_2	Dynamic	[60]

Essentially this is a process which is based on the use and recycling of fluid in repeated steps of compression and depression. The use of this technique in extraction has grown in the most recent years, being one of biggest obstacles to its development the high cost in equipment, installations and maintenance operations.

Also, extracts from this technique presented a higher quality, possessing better functional and biological activities in comparison with extracts produced by some more traditional techniques. Studies also showed a better antibacterial and antifungal properties in supercritical products.

The major limitations of this process as mentioned before have to do with the cost associated, which increases as the pressure conditions considered also become higher. Also already mentioned, CO_2 is the main solvent used in this type of processes, which

also brings limitations to the process since its non-polarity limits the dissolving power of this solvent. This fact makes so that CO_2 can't be used on it's on in a wide range of processes, especially in the extraction of most polar compounds. To solve this problem, organic solvents are added to the process in order to improve the extraction capabilities of the solvent to those compounds of interest.

The organic solvents are usually called modifiers and the most common used in this type of processes are ethanol, methanol, hexane, water, dichloromethane, chloroform, formic acid, among others. They have influence in a large number of properties like increase and decrease of polarity, chirality, and the ability to further complex metal-organic compounds. The use of modifiers may create the idea that the process is not as environmental friendly has previously thought but the reality is that these organic solvents are used in very small amounts and when the yields are compared, what comes across in most cases is an improvement of the extraction process which makes its use worth it.

Compared with traditional methods, supercritical extraction presents several advantages like allowing a faster extraction process, being suitable for extraction and purification of compounds having low volatility present in solid or liquid, minimizes the risk of thermal degradation, and becomes a versatile and efficient process with multiple configurations. The limitations of this type of process are an inaccurate modeling, the impossibility to scale, its consistency and reproducibility may vary in continuous production, and as it was mentioned before it becomes an expensive process to assemble because of the special high pressure equipment necessary.

2.3 Supercritical Fluids

It is now 186 years since Baron Charles Cagniard de la Tour discovered that, above a certain temperature, single substances do not condense or evaporate, but exist only as fluids. He noted visually that the gas-liquid boundary disappeared when the temperature of certain materials was increased by heating each of them in a closed glass container.

A fluid is characterized has a substance that continually deforms under an applied shear stress. They are a subset of the phases of matter and include liquids, gases, plasmas and also plastic solids. In the following years, the concept of "critical point" was studied and characterized by the parameters of critical pressure (P_c) and critical temperature (T_C). Studies were then carried around this area, above and below, in order to see the behavior of this fluids by changing the conditions of pressure and temperature. [61]

A critical point of a substance is characterized by the point which represents the end of the phase equilibrium curve. As it was mentioned before, this point is achieved when specific conditions of temperature and pressure are reached, which are designated as critical temperature (T_c) and critical pressure (P_c). The value of this point change from substance to substance and causes changes in its physical properties.

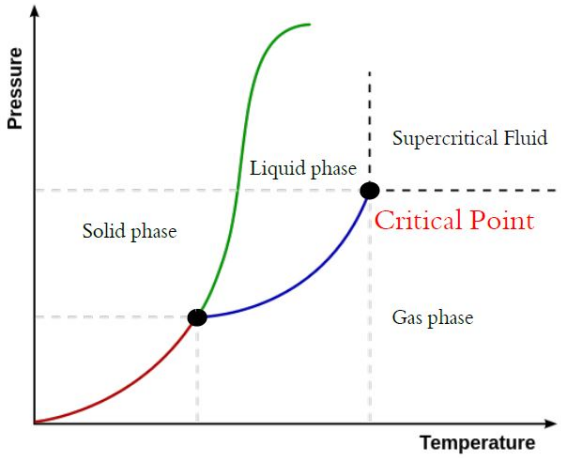


Figure 2.13: Illustration of the critical point.

Table 2.13: Critical Conditions for most common solvents.

Solvent	Critical Temperature (^{o}C)	Critical Pressure (atm)	Critical Density (g/cm^3)
Carbon Dioxide	30,95	72,8	0,469
Water	373,95	217,8	0,322
Methane	-82,75	45,4	0,162
Ethane	32,15	48,1	0,203
Propane	96,65	41,9	0,217
Ethylene	9,25	49,7	0,215
Propylene	91,75	45,4	0,232
Methanol	239,45	79,8	0,272
Ethanol	240,75	60,6	0,276
Acetone	234,95	46,4	0,278
Nitrous Oxide	33,42	72,5	0,452

There are two main fluid states around the critical point, the subcritical and the supercritical state. The subcritical state is reached when a substance is subjected to a pressure higher than the critical pressure (P_c) and a temperature bellow the critical temperature (T_c), or vice versa. In this state, fluids present a wide range of properties like low viscosity, density close to that of liquids, and the diffusivity between that of a gas and a liquid. Subcritical fluids are not as studied has supercritical ones and the level of applications is not as big. On the other hand, in most cases they become much more easy to achieve the conditions necessary for this state, allowing a certain variability between pressure and temperature. In some cases, like extraction what happens is that the temperature can be low so no thermal degradation occurs and the pressure can be risen above the critical point, achieving the subcritical state anyway.

Supercritical fluids are essentially any substance at a temperature and pressure above its critical point, where distinct liquid and gas phases do not exist. It can effuse through

solids like a gas, and dissolve materials like a liquid. In the supercritical region the fluid presents specific properties like an intermediate behavior between that of a liquid and of a gas, high diffusivity and low viscosity.

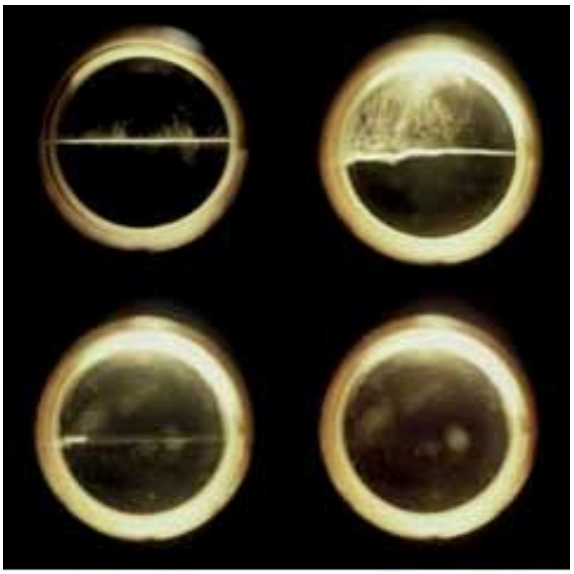


Figure 2.14: Representation of the various states until achieving a supercritical fluid.

From the image above it's possible to see that in a first stage there's a clear separation between gas and liquid phases. With the increase in pressure and temperature it's possible to see that the separation gradually vanishes becoming more and more like a one phase system. [62]

As it was previously said, the supercritical state can be achieved when a specific set of conditions are surpassed (critical pressure (P_c) and critical temperature(T_c)). Different compounds possess different values of critical pressure and temperature, so even if a substance can be putted in a supercritical state it might not be worth to introduce it into a process.

It's clearly possible to see that from all the compounds presented above that CO_2 is the one with the easiest conditions to reach the critical point, being that one of the reasons for it to be the most common supercritical solvent. Other advantages of supercritical CO_2 are:

- Safe and environmentally friendly;
- Recyclable, in a sense that after its use on a certain process in can be recapture and used again in the process;
- Inexpensive and easily available;
- No residue is left after its use;

- On contrary to other popular solvents it has zero surface tension. This factor can be compared with other existent solvents in the table presented below.

Table 2.14: Viscosity, Surface Tension and Density comparison between solvents.

Solvent	Viscosity (cP)	Surface Tension (dyne/cm)
Liquid Carbon Dioxide	0,08	1,5
Supercritical Carbon Dioxide	0,03	0
Methanol	0,54	22,1
1,1,1-Trichloroethane	0,81	25,2
Water	1,0	72,0
Isopropyl Alcohol	2,4	21,8

Supercritical fluids can occur on the nature in cases like hydrothermal circulation and planetary atmospheres. In the bottom of the ocean certain areas possess heat spots which in combination with the pressure in the bottom of the ocean might create these type fluids. In the case of planetary atmospheres such fluids can be found in planets like Venus, Jupiter, and Saturn. This type of planets, are mainly composed by gases like carbon dioxide, hydrogen, helium and nitrogen.

Due to its many advantages, supercritical CO_2 is used in a large range of applications. Supercritical fluids in general first started only being used on analytical chemistry, and not much support was given to its application. A significant change happened when a patent was released reporting the decaffeination of green coffee using supercritical CO_2. From that moment on a significant development on the use of supercritical fluids started, especially when referring to supercritical fluid extraction (SFE).

The supercritical fluid extraction can be applied in the extraction of hops, cholesterol from butter, perfumes and flavors from natural products, and unsaturated fatty acids from fish oils. Other more specific applications can be impregnation and in situ deposition of chemicals, cleaning of electronic parts, nucleation and particle size regulation, chemical reaction and synthesis of organic chemicals and remediation of soil. There are so many applications that supercritical fluid technology has become an interdisciplinary field, utilized by chemical engineers, chemists, food scientists, materials scientists, agronomists, and researchers in biotechnology and environmental control.

In the last ten years, the range of supercritical fluids has expanded from commodity chemicals and synthetic fuels toward more complex, highly specialized, and more valuable substances. In addition to applying the technology for the purpose of new product isolation and purification, considerable effort has been devoted to gaining a better fundamental understanding of molecular structure, phase behavior, solvation processes between solute and fluid phase, and transport properties of supercritical fluids.

The disadvantages of supercritical fluids are that high pressures and sometimes temperatures are involved, and, in the case of water, there are corrosion problems. As the

technology to overcome them is available, these disadvantages become cost and convenience factors to weight against potential advantages.

Table 2.15: Main applications for supercritical fluids

Pharmaceuticals: Extraction of biologically active Ingredients; Fermentation broth extraction; Protein purification.	**Foods:** Flavor Extracting and concentration Extraction of flagrance Processing essential oils Flavor and fragrance infusion
Nutraceuticals: Vitamin Extraction; Concentration of active ingredients; Anti-oxidant extraction.	**Polymers:** Renewal of monomers an oligomers; Infusion of components; Removal of binder from powered metals.
Cleaning: Precision machined components; Silicon wafers; Medical Implants and electronic components.	**Reaction Chemistry:** Reactions and organic product synthesis; Hydrogenation Reaction; Polymerization reactions and synthesis.

2.4 Analytical Methods

The analytical methods are an essential part of any project of this nature. Even though the aim of the work is focus in extraction and not in analytical methods, these are the ones who allow us to understand what was obtained and in certain cases in how much was actually extracted. When it comes to the analyzes of plant materials there are several methods found in the literature which are constantly used. Some of the most referenced are Gas Chromatography copulated with a Mass Spectrometer (GC/MS), High Performance Liquid Chromatography (HPLC), Thin-layer chromatography (TLC) or simply Gas Chromatography (GC).

Table 2.16: Literature analytical methods examples.

Plant	Analytical Method	Reference
Asarum species	GC	[63]
Lavandula species	GC, GC/MS	[64]
Berberis tinctoria Lesch.	GC/MS	[65]
Berberis vulgaris L.	HPLC	[66]
Berberis croatica/vulgaris	HPLC	[67]
Eucalyptus loxophleba	GC/MS	[24]
Anethum sowa	GC, TLC	[9]
Carum copticum	GC, GC/MS	[10]
Mentha pulegium L.	GC, GC/MS	[53]
Foeniculum vulgare	GC	[54]
Marchantia convoluta	GC/MS	[55]
Lippia alba	TLC, GC	[56]

Rosmarinus officinalis	GC, TLC	[57]
Matricaria chamomilla	HPLC	[59]
Ilex paraguariensis	GC/MS	[68]
Ocotea odorifera	GC/MS	[69]
Origanum onites	GC/MS	[14]
Rosmarinus officinalis. L	GC/MS	[15]
Matricaria chamomilla	GC/MS, HPLC	[70]
Pogostemon cablin	GC/MS	[17]
Ocimum basilicum L.	GC, GC/MS	[71]
Mentha piperita	GC/MS	[18]
Nepeta cataria L.	GC, GC/MS	[19]
Artemisia annua	GC/MS	[72]

The technique used depends from the objectives of the experiments, essentially of what is intended from the sample. For example, in the case of plant extraction, most authors choose to use HPLC or GC/MS since these techniques allow an analytical and quantitative analyzes. Other methods like GC on the other hand, only allow a simpler representation which ends up not being sufficient to prove that one or more compounds are present in the analyzed sample.

- **Gas Chromatography (GC)**

This technique allows to separate all of the components in a sample and provides a representative spectral output. The sample is injected into the injection port while the device vaporizes it for later separate and analyze the various components. The temperature of the injection port must be high enough to vaporize a liquid specimen instantaneously. [73]

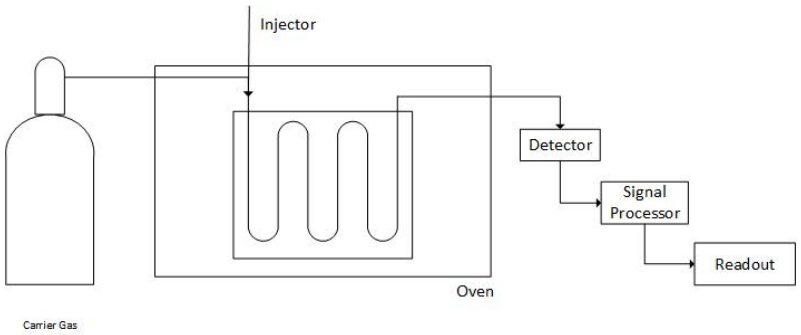


Figure 2.15: Gas Chromatography (GC) apparatus.

The time between injection and elution is called the "retention time". The size of the peaks is proportional to the quantity of the corresponding compound in the substance analyzed. The retention time for a compound is not fixed as many factors can influence it even if the same GC and column are used. These factors include the gas flow rate, temperature differences in the oven and column, column degradation, and column length. These factors can make it difficult to compare retention times since that even if you use

the same GC just a few days apart, there could be a small difference in the retention time of a compound.

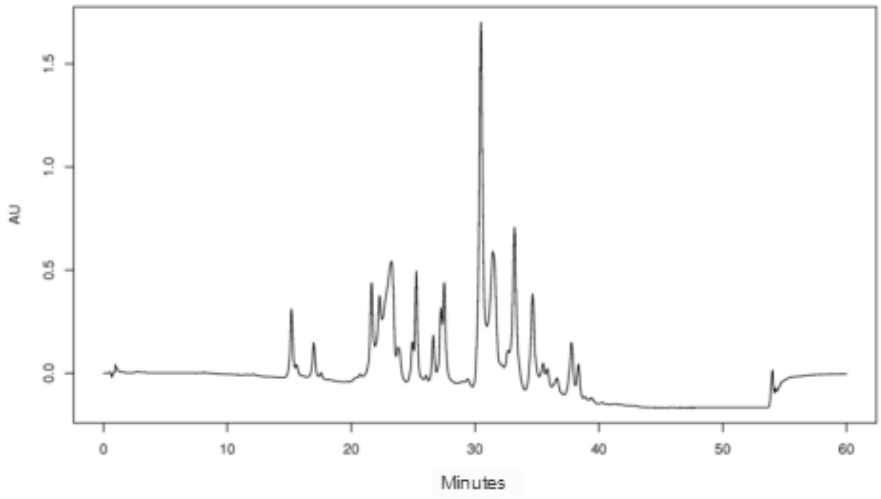


Figure 2.16: Example of GC output.

There are several types of detectors used in Gas Chromatography,

- Argon Ionization Detector;
- Flame Ionization Detector;
- Flame Emission Detector;
- Cross Section Detector;
- Thermal Conductivity Detector;
- Electron Capture Detector.

The type of detector used will depend on the properties of the compounds of interest since that the wrong choice could cause their degradation influencing the end results.

- **GC/MS**

This method represents a combination of the GC method explained above with Mass Spectrometry (MS). This technique allows to identify substances by electrically charging the specimen molecules and accelerating them through a magnetic field. This allows to break the molecules into charged fragments and detecting the different charges. [74]

A spectral plot displays the mass of each fragment and through the small masses presented is possible to reach the value of the "parental" mass. This value when compared with the literature allows the identification of a compound.

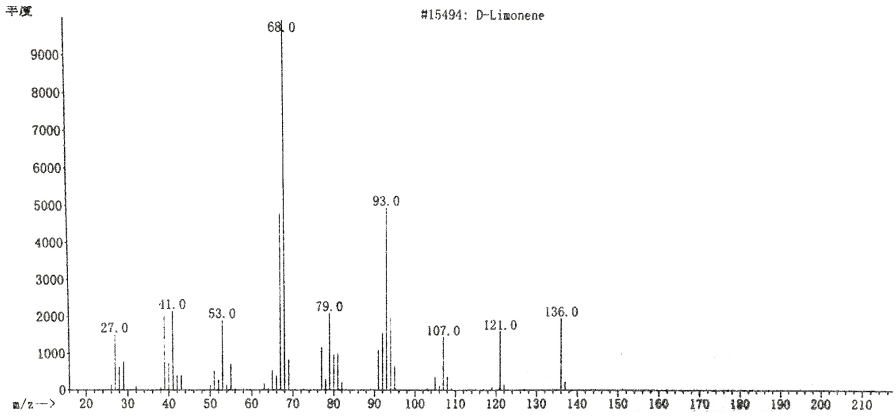


Figure 2.17: Example of GC/MS output.

Even though this method seems to be more effective and precise when it comes to the identification of the various compounds, the spectral output is more difficult to read and requires more training and experience in order to make a good analysis. On the other hand, GC alone is far simpler to use and to interpret the output data.

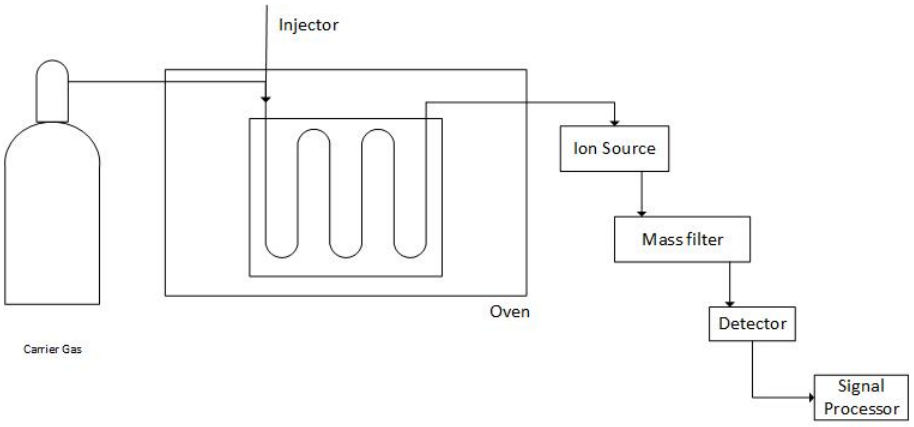


Figure 2.18: GC/MS apparatus

- **High Performance Liquid Chromatography (HPLC)**

High Performance Liquid Chromatography is basically a highly improved from of column liquid chromatography. What happens is that instead of a solvent being allowed to drip through a column under gravity, it is forced through under high pressures. HPLC works on the same principles than the rest of the chromatographic separations which consists on separating a sample into its constituent parts because of the difference in the relative affinities of different molecules for the mobile phase and the stationary phase used in the separation. [75]

There are several variants of this technique:

- Normal phase HPLC;

- Reverse phase HPLC;
- Size exclusion HPLC;
- Ion exchange HPLC;

HPLC results can be influenced by parameters such as the Internal diameter of the column, particle size, pore size, pump pressure, and by the type of detectors used. There are some different types of detectors such as UV-spectroscopy, fluorescence, mass-spectrometric and electrochemical detectors.

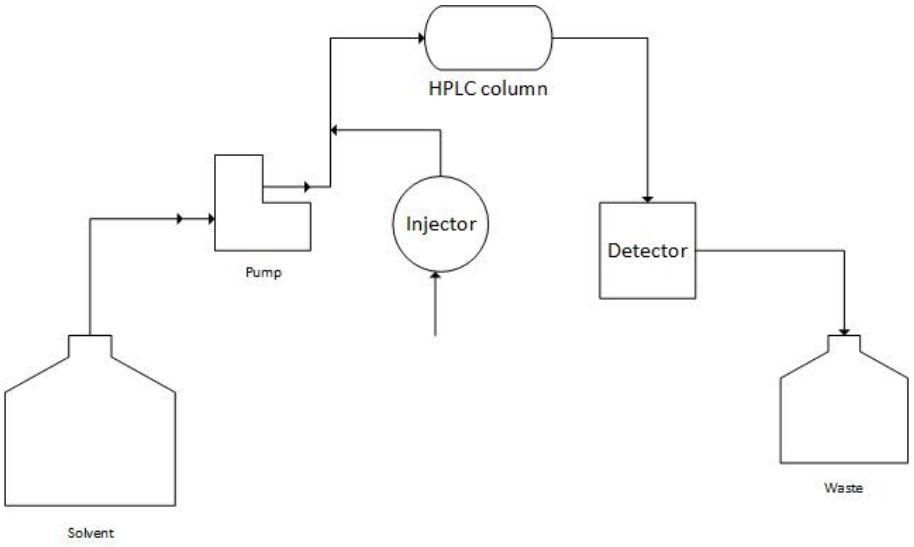


Figure 2.19: Example of an HPLC apparatus

- **Differential Scanning Calorimetry (DSC)**

Differential Scanning Calorimetry (DSC) consists on a technique in which the difference in the amount of heat required to increase the temperature of a sample and reference is measured as a function of temperature. Both the sample and reference are maintained at nearly the same temperature throughout the experiment. Two types of DSC:

- Power compensated DSC, in which the power supply is kept constant.
- Heat flux DSC, which keeps constant the heat flux.

A basic principle underlying this technique is that when the sample undergoes a physical transformation such as phase transitions, more or less heat will need to flow to it then the reference to maintain both the same temperature. Whether less or more heat must flow to the sample depends on whether the process is exothermic or endothermic. If a process is exothermic then the system will need to transfer less heat to match the

reference temperature, while the opposite happens if the process is endothermic since it's removing heat from the system so it takes more heat to increase the temperature. [76]

As it was mentioned before there are several techniques used for analyses of extracts but only this were quickly described since they will serve as based for the future work.

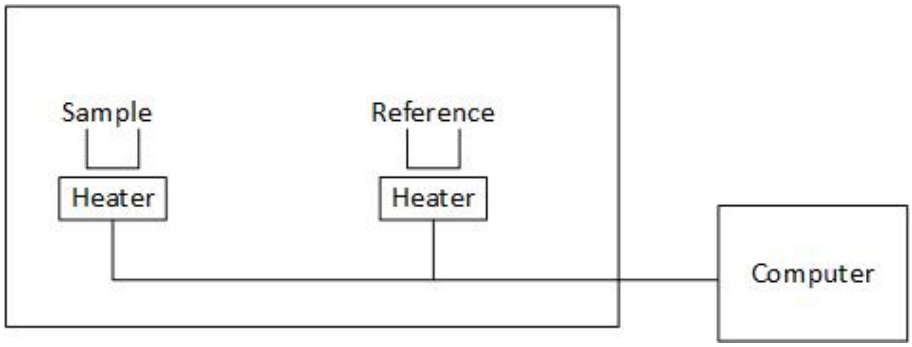


Figure 2.20: Example of an DSC apparatus

Chapter 3

Safrole - Extraction and Reaction

This chapter is dedicated to show the work developed on the substance safrole and the research performed on its extraction and epoxidation reaction.

3.1 Sassafras

3.1.1 Sassafras Plant

Sassafras is a genus in the family *Lauraceae*, native from eastern North America and eastern Asia. Its trees grow from 9 to 35 meters tall and usually present an orange-brown or yellow bark. The *Sassafras* genus contains three surviving and one extinct species, which are *Sassafras albidum*, *Sassafras tzumu*, *Sassafras randaiense*, and *Sassafras hesperia* respectively.

Native Americans have used Sassafras for centuries by rubbing the leaves directly into a wound, and using different parts of the plant for many medicinal purposes such as treating acne, urinary disorders, and high fevers. *Sassafras* has also been applied to insect bites and stings to relieve symptoms, and is also known to be used as a sudorific agent in eye inflammation treatment, rheumatism, gout, swelling, and cutaneous eruptions. Before the twentieth century, *Sassafras* enjoyed a great reputation in the medical literature and became valued for its power to improve the flavor of other medicines. It also represents a food source for many types of animals. Leaves, bark, stems, and fruits are often eaten by birds and mammals like porcupines, groundhogs, marsh rabbits, and American black bears in small quantities. When it comes to humans this plants are mostly used for culinary, medicinal, and aromatic purposes. [77]

The main extract in this type of plants is the essential oil with the amount extracted depending on the section of the plant considered, and varying from plant to plant. In the case of *Sassafras albidum* the highest amount of oil that can be obtain comes from the

root bark and corresponds to about 10%. Its other constituents are camphorous matter, resins, wax (decomposition product of Sassafrid), tannic acid, gum, albumen (Egg White), starch, lignin and salts. [78]

Figure 3.1: Sassafras albidum tree.

Has it been mentioned before the *Sassafras* family is composed by four main types of plants. Of this four, *Sassafras hesperia* is extinct and the plants of interest in terms of safrole content are *Sassafras albidum* and *Sassafras tzumu*. *Sassafras albidum* is native to eastern North America and usually grows at altitudes up to 1500 meters, achieving a height of 15 to 20 meters. The leaves are alternate, green to yellow green and its fruit is characteristically dark-blue. From medical proposes to culinary applications the range of uses of this plant is enormous, being now a day extremely related to drug production since its safrole content is around 88%.

Table 3.1: *Sassafras albidum* oil composition.

Compound	Composition
Safrole	82,8% - 88,8%
Camphor	1,2% - 6,8%
Methyl Eugenol	1,4% - 2,3%
Eucalyptol (1,8-cineole)	1,1%

Sassafras tzumu is native to China and grows at altitudes from 100 to 1900 meters reaching heights up to 35 meters. Its wood is mainly used for shipbuilding and furniture making because of its durability. Essential oils can be extracted from barks, roots, or fruit having a safrole content of 97%.

Table 3.2: Examples of other plants with high content in Safrole.

Plant	Plant Part	Safrole Content
Cinnamomum burmanni	Leaf	97 – 99%
Cinnamomum camphora	Root bark	50 – 80%
Cinnamomum micranthum	Wood	95%
Cinnamomum parthenoxylon	Wood	80%
Cinnamomum paucifolium	Leaf	68 – 90%
Cinnamomum petrophilum	Leaf	97%
Curcuma amada	Rhizome	9.3%
Octea cymbarum	Wood	84 – 93%
Piper callosum	Leaf	70%
Piper hispidinervium	Leaf	81 – 88%

3.1.2 Sassafras Oil

Sassafras oil is a yellow liquid which results of applying extraction methods on *Sassafras* genus plants. This oil can be extracted using multiple processes like Steam distillation, Organic Solvent extraction, Supercritical Fluid Extraction, among others. This oil is characterized by being mainly composed by safrole, however, its content and form the other compounds which also make part of the oils composition, end up variating with the type of plant from where they're extracted from. [79]

The oil has been applied through the history in multiple areas like food and medicine. It was believed to hold the cure from problems like hangovers to serious conditions like syphilis. The Europeans first discover the properties of this oils when arrived to America, and didn't waste time in starting ship it to Europe. Since that, *Sassafras* oil has been used in a huge variety of foods, medicines, and other products.

Figure 3.2: Sassafras oil.

One example of this applications is root beer, being extremely popular in places like the United States of America. This component was later prohibited due to its high content

in safrole which was proven to have carcinogenic properties. The recently discovered properties of the oil cause many applications to be dropt or in research to find substitutes with less health impact.

Due to this, the market of this oil is today very reduced and highly controlled especially because of safrole, making its isolation the main application of the oil.

Figure 3.3: Root beer, one of the past main applications for Sassafras oil.

3.2 Safrole

Safrole is a naturally occurring substance which exists at room temperature as a colorless or pale yellow liquid with an odor of sassafras. This compound is highly soluble in alcohol, miscible with chloroform and ether, and practically insoluble in water. [79]

Table 3.3: Safrole Properties.

Properties	**Data**
Molecular weight	162,2 g/mol
Density	1,1 g/cm^3 at 20°C
Melting point	11,2°C
Boiling point	234,5°C
Vapor pressure	0,0618 $mmHg$ at 25°C

Safrole has been used as a flavoring agent in drugs and in the manufacture of heliotropin, perfumes, soaps, and piperonyl butoxide (a compound used in insecticides to enhance the its properties).

It is also a substance highly controlled since not only the raw material is used for the illicit production of the drug 3,4 – methylenedioxymethamphetamine (MDMA, or ecstasy) but also it was proven to be highly carcinogenic. For that reason, besides the many applications, any addition of safrole or oil of *Sassafras* to food is extremely forbidden.

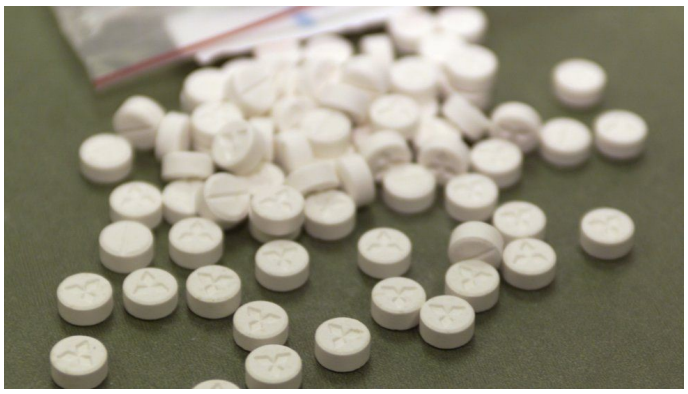


Figure 3.4: Main safrole application today: Ecstasy

Safrole is on the lists for controlled substances as a drug precursor by organizations like the European Union, The United Nations, FDA, among others. The common daily intake of this substance, according to the literature, is around 0,3 mg. Even with strict control, safrole is naturally present in natural products like *Sassafras* tree and in edible spices like cinnamon, nutmeg, ginger, and black and white pepper.

3.2.1 Safrole Oxide

In more recent years, the epoxidation reaction of safrole has been studied in order to obtain has product safrole oxide. This compound doesn't exist naturally and can only be obtain by the reaction of safrole. Safrole oxide has a chemical formula of $C_{10}H_{10}O_3$ and at normal temperature can be found as a solid.

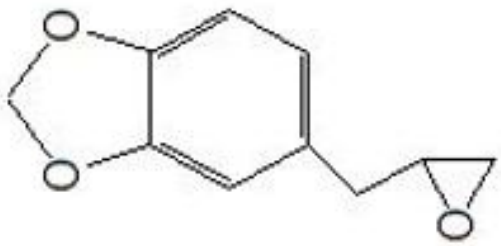


Figure 3.5: Safrole Oxide Structure

By its structure it's possible to observe that it differs from safrole by possessing one more oxygen (O) atom. The double bound at the end of the safrole molecule allows for that extra Oxygen to bind forming a new molecule with different properties associated to it.

Table 3.4: Safrole Oxide Properties.

Properties	Data
Molecular Weight	178,2 *g/mol*
Melting point	61,23 °C
Boiling point	265,82 °C
Vapor pressure	0,00911 *mmHg* at 25°C

The traditional way of obtaining safrole oxide by epoxidation of safrole is done by reacting it with m-chloroperbenzoic acid (mCPBA).

Figure 3.6: Epoxidation of safrole using MCPBA has an oxidizing agent.

The compound m-chloroperbenzoic acid (mCPBA) is a peroxycarboxylic acid used widely as an oxidant in organic synthesis and it's preferred to other peroxy acids because its relatively easy to handle. The main applications for this compound can be found in processes where oxidation of ketones, sulfides, amines, and also alkenes occurs. Even then it's relatively stable still presents some risks like causing fire upon contact with flammable materials.

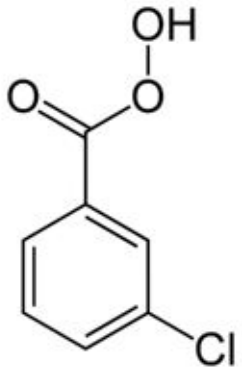


Figure 3.7: mCPBA Structure.

The recent interest in safrole oxide has to do with its anti-angiogenesis and apoptosis-inducing activity in the in vitro therapy. Apoptosis refers to a programmed cell death, and represents a fundamental activity in order to maintain the balance of the organism by eliminating unwanted cells. It has been shown that safrole oxide can attach itself to

the DNA strings allowing it, in the right composition, to induce cell death or even induce certain differentiation in cells.

From the literature is shown that safrole oxide in concentrations of 10 μgmL^{-1}, induced in the human umbilical vein, caused cell transdifferentiation to neuron-like cells, has that with a concentration of 20 μgmL^{-1} it was induced apoptosis in human oral cancer HSC3. The ability of this compound to affect cell behavior makes it possible to use in therapy and treatments of diseases like cancer, explaining the rise in interest on this compound in the most recent years.

3.3 Experimental Part

Sassafras albidum was from the beginning the plant of interest and safrole the target compound. Has it been mentioned before, safrole has carcinogenic properties which makes its used highly controlled but the legislation on how buy and operate this compound depends from country to country. Due to legal complications it was not possible to continue with the experimental part of this work since it would be necessary to get a special license, and the waiting time to get that authorization would surpass the time available for the execution of this project. Even though the experimental part cannot be performed, it's important to show all the planning already made for that stage of the work both for the extraction process but also for the epoxidation reactions.

3.3.1 Solubility Parameters

Before start both the reaction and epoxidation stages it is important to understand the solubility of certain compounds in the supercritical fluid to be used as a solvent. In this case the chosen supercritical fluid was CO_2 and so it is important to understand if the solubility of the target compounds is favorable in this solvent. Unfortunately, solubility in supercritical fluids is not a very developed area so it's hard to find stablish values for most components. Most of the existent values are not even measured ones but yes deduced from proposed models which allow approximations to the behavior of certain substances in supercritical fluids. Most of this models are complicated and hard to apply, being necessary a lot of specific data, which for compounds not so studied like safrole, becomes very hard to obtain them. In the case of this work these parameters would be important to have an idea if both extraction and epoxidation reaction would even be possible to perform in supercritical CO_2. In the case of the extraction the solubility would have to be checked for the essential oil, or at least for its main components which would give the safrole solubility that is extremely important for the epoxidation stage.

Since these type of values are extremely rare even on the literature, such parameter had to be assumed by attempting to calculate solubility parameters in supercritical fluids. Like it was said before some existing models can predict with some degree of

certainty this values but are very restricted on the conditions in which they can be calculated. The model chosen comes from the concept of solubility parameter (Hildebrand and Scott, 1950), which has been known for liquids and used in interpreting and predicting thermodynamic behavior of solutions.

The model found in the literature has for parameters the reduced temperature (T_r), reduced pressure (P_r), compressibility factor (Z), and the gas constant (R). The way this results would be interpreted follows the same logic that in the case of solubility parameters, which essentially consists in comparing the values and the closest they are the most soluble with each other. [80]

The calculations were made for the conditions of 55^oC and 122 bar, comparing both safrole and other essential oil components with CO_2. The same conditions were used also to calculate this parameter for Hydrogen Peroxide (H_2O_2), in order to see if this was also soluble in CO_2. Due to the lack of literature regarding reactions with safrole, including epoxidation, the conditions presented came from reactions of this type but where other components were used. Never the less, since that by those cases those reactions worked, it was decided to test this same conditions in proposed case.

Table 3.5: Solubility Parameters.

Component	**Solubility Parameter Value**
CO_2	3,52
Safrole	3,96
Camphor	3,66
Methyleugenol	3,68
1,8-Cineole	3,68
H_2O_2	3,33

One important aspect to have into account is that this values give only an approximation of what could happen in reality, even the models found in this part of the research suggested that further investigation was needed in order to come closer and closer to what is verified in experimental work. The type of calculations made in this stage even that simple, represent the closest way available in order to have an idea of the process behavior.

Thus, the table above shows very close values in all the compounds, especially when it comes to safrole, camphor, methyl eugenol, and 1,8-Cineole which makes sense considering that this four compounds exist together in oil. Their value is also close to CO_2 which leads to believe that the extraction phase would be possible and that the oil will interact well with the supercritical CO_2. Hydrogen peroxide also presents a very close value to this compounds and CO_2, which leads to believe that this compounds can have a good solubility between them allowing the epoxidation reaction to happen.

3.3.2 Extraction

The extraction of safrole was planned to be made from the raw material of *Sassafras albidum*, specifically from its root bark. The plant material would be bought and then milled in order to have a specific particle size or at least a controlled range of particle size inside the reactor. The *Sassafras* plant root bark was actually already bought before it was discovered the legal issues with operating it. It was also planned to buy references to main essential oil components so than later a comparison could be made by analytical methods, since it's not possible to extract safrole by himself but only within the essential oil.

Table 3.6: List of compounds planned for the extraction stage.

Compound	Unit	Unit Size	Company
Sassafras Root Bark	10	50 g	Nanga
Camphor	1	100 g	Sigma Aldrich
Methyl eugenol	1	Not Specified	Sigma Aldrich
1,8 - Cineole	1	100 mL	Sigma Aldrich
Safrole	1	500 mL	Sigma Aldrich

Even though the extraction stage was mostly theoretical, the plant material was still subjected to a first extraction by a more traditional method in order to see how much oil would be possible to extract that way.

3.3.2.1 Steam Distillation

The steam distillation apparatus was composed by a heater, two vessels and a condenser. As it was previously said the purpose of this first experiment was to take advantage of the fact that the plant material was already bought and use a simple traditional extraction process in order to have an idea of how much oil it would be possible to extract and further on compare to what was recovered by supercritical extraction.

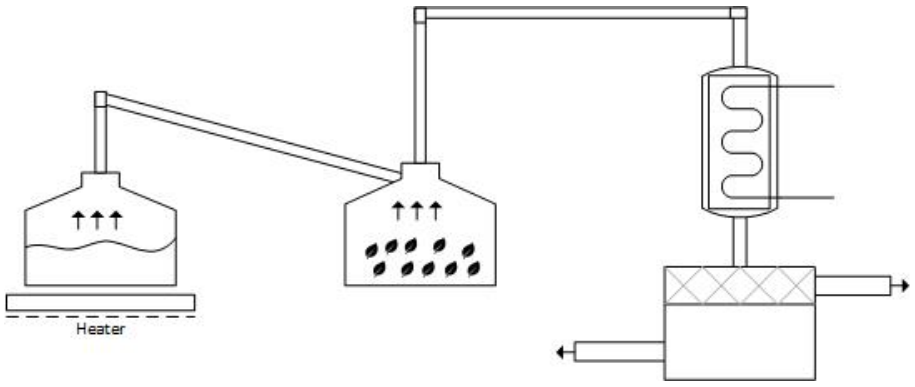


Figure 3.8: Scheme of the apparatus used for the steam distillation.

The *Sassafras* root bark was put inside of one of the vessels in an amount of 50 grams. The water was put inside the other vessel and then heated until it boils. The vapor goes

though the plant material and goes back into the liquid state when passing through the condenser.

Figure 3.9: Sassafras albidum root bark.

After the extraction is possible to see a minor change in color of the plant material which could indicate that something was actually extracted. It's possible to see that the extracted material is more brown and without that yellowish bits seen in the material before extraction.

Figure 3.10: Sassafras albidum root bark after steam distillation.

Unfortunately, the results of this extraction were not the best, only being able to get from 50 grams of material an amount of oil in the order of the 35 mg which corresponds to only 0,07%. For a material which, according to the literature, would possess around 10% of oil it was a really low value and it was though that it has to be a problem with the supplier. Later it was possible to understand that the supplier probably removed most traces of the oil because it contained large amounts of safrole.

Has it's possible to see the amount of oil is very low and nothing compared to what was expected in theory.

Figure 3.11: Oil obtained by steam distillation.

3.3.2.2 Supercritical Extraction

In the case of the supercritical extraction this is essentially theoretical because the issues with safrole ended up being discovered before this stage. In the literature there are no examples of *Sassafras albidum* extraction by using supercritical CO_2 so all the steps to perform this extraction were deduced from other articles on other types of plants. The general apparatus would be a dynamic extraction system with a CO_2 pump, an extraction vessel, heater and a pressure control valve.

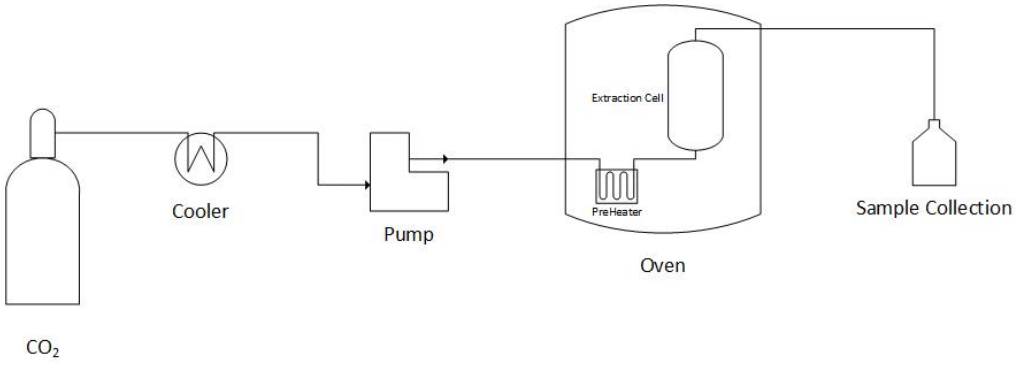


Figure 3.12: Proposed apparatus for supercritical extraction.

The plant material after being milled would be introduced into the extraction vessel and then the oil would be collected in a vessel by depressurization at normal temperature and pressure. The CO_2 supply would be done by tanks containing the solvent in liquid state. The pump would possess a refrigeration system allowing it to take the CO_2 in

liquid state and turn the pumping operation more effective. In most systems, when the tanks possess the solvent in gas state there's a need to low down the temperature in order to turn that gas into the liquid state so then it can be pumped.

To end this first stage, the oil would be collected so a part of it would be analyzed to see its safrole content and the rest would be used for the next stage on the epoxidation reaction.

3.3.3 Epoxidation Reaction

The epoxidation reaction was planned to use the *Sassafras* oil obtained in the extraction stage and then use a supercritical steady system in order to perform the epoxidation reaction. The essential oil contains over 80% of safrole so it's plausible to assume that using the essential oil will allow the epoxidation reaction of safrole. For this stage were also acquired other compounds which were considered to use further on.

Table 3.7: Compounds considered to buy for the epoxidation reaction using safrole.

Compound	Unit	Unit Size	Company
Cyclohexene	1	100 mL	Sigma Aldrich
Sodium Bicarbonate	1	500 g	Sigma Aldrich
Hydrogen Peroxide	1	500 mL	Sigma Aldrich
Dimethylformamide	1	250 mL	Sigma Aldrich
Cyclohexene Oxide	1	100 mL	Sigma Aldrich
Safrole Oxide	1	100 g	Leancare Ltd.

An epoxidation reaction is characterized by both carbons of a double bond becoming bonded to the same oxygen atom. One of the most common methods for preparing epoxides is by reaction with peracids, RCO3H. The epoxidation reaction is believed to occur in a single step and the epoxides produced on a larger scale are ethylene oxide and propylene oxide.

When it comes to the epoxidation reaction of safrole has its been mentioned before the most common method uses m-chloroperbenzoic acid (mCPBA) has an oxidizing agent instead of the peracids mentioned above. Another alternative could be the use of catalysts in the reaction process, existing in this pathway many types of epoxidation processes like in the examples of Jacobsen-Katsuki and Shi Epoxidation. By using a catalyst, reactions are able to occur faster and require less activation energy. Another advantage is that catalysts are not consumed in the in the catalyzed reaction, therefore continuing to catalyze the reaction with small quantities of reactant. Catalysts are often specific for one particular reaction and this is particularly so for enzymes which catalyze biological reactions. Examples of the use of catalysts on an industrial level are the production of ammonia, cracking of gas oil, sulfuric acid, nitric acid, and of synthesis gas (carbon monoxide and hydrogen).

Regarding the epoxidation of safrole, catalysts are shown to be involved in its isomerization reaction. This mechanism allows to obtain two different molecules by simply

subjecting Safrole to the action of a catalyst.

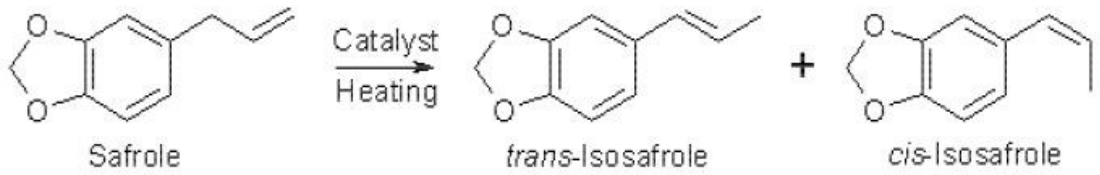


Figure 3.13: Isomerization reaction of safrole by catalyst action.

Besides the most common methods of epoxidation already presented, other methods of safrole epoxidation exist in the literature. An example of this is when carbon tetrachloride (CCl_4) is used in a solution with mCPBA and safrole at a temperature of 0^oC, with the reaction solution stirred at the same temperature for 8 hours. The solution was then washed, dried, and analyzed obtaining in this particular case a yield of 89%. The scheme of this reaction is shown below.

As it was mentioned, the most common method for safrole epoxidation uses meta-Chloroperoxybenzoic acid (mCPBA), which is characterized to be a strong oxidizing agent. From the literature it's possible to take an example where carbon tetrachloride (CCl_4) is used in a solution with mCPBA and safrole at a temperature of 0^oC, with the reaction solution stirred at the same temperature for 8 hours. The solution was then washed, dried, and analyzed obtaining in this particular case a yield of 89%. The scheme of this reaction is shown below. [81]

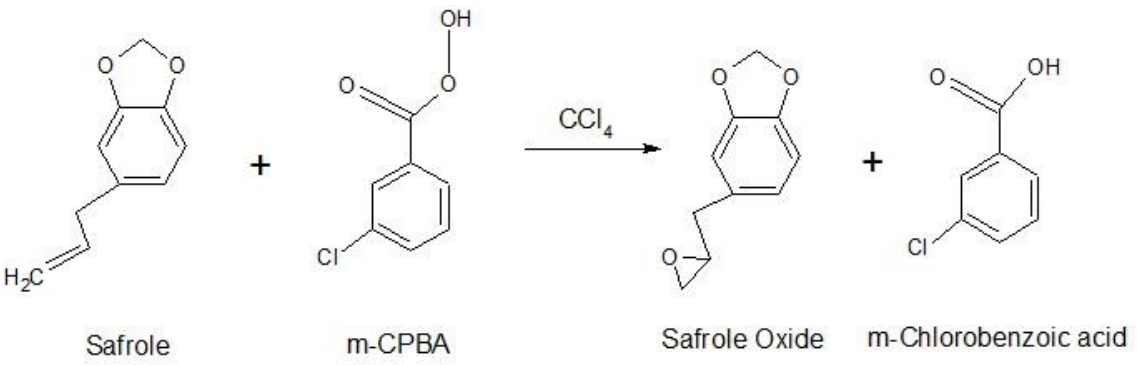


Figure 3.14: Safrole epoxidation reaction.

As its been mentioned before, the original purpose of this work was that after the extraction process using supercritical carbon dioxide, an epoxidation reaction would also be performed in supercritical conditions with the objective of obtaining and transforming safrole into safrole oxide.

The first line of thought involves considering the use of the same oxidizing agent, mCPBA, in a hope that with supercritical carbon dioxide as a solvent the epoxidation reaction would still be able to occur. Literature review has shown no examples of such

type of reaction in this type conditions. However, by searching in a more general matter with focus only on epoxidation reactions in supercritical conditions, an interesting article appeared where epoxidation reactions were performed in supercritical conditions by using hydrogen peroxide has an oxidizing agent. This method not only turned the process more environmentally friendly but also much cheaper that the one planed before, since hydrogen peroxide is relatively cheap and easy to obtain.

Because there was no literature reporting epoxidation reaction of safrole in this conditions it was decided to perform this new method in two different stages. At first, it would be performed a "test" reaction to see if the epoxidation was possible with these parameters. Another advantage of this approach is the fact that the test reaction is far simpler and less expensive to perform, allowing to get viable data without much time of investment which would be an advantage when start using safrole. By using the same conditions in the paper and by selecting one of the papers compounds, cyclohexene, it would be possible to compare the yield obtained and check if such reaction was actually possible.

As shown on the table above, the compounds necessary for this first stage besides cyclohexene would be according to the literature Sodium Bicarbonate ($NaHCO_3$), Hydrogen Peroxide (H_2O_2) and Dimethyl Formamide. The necessary equipment would be a Stainless Steel Parr High Pressure/High Temperature Stirred Autoclave, temperature controller, agitation impeller, and solvent traps (possibly filled with acetone).

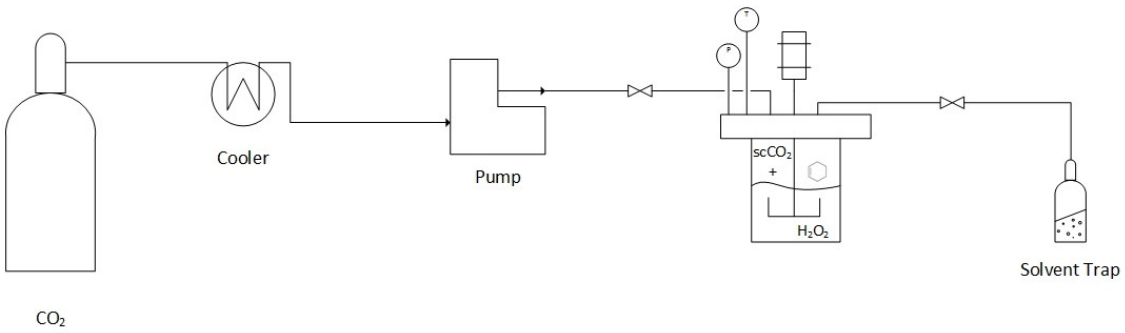


Figure 3.15: Proposed epoxidation apparatus.

The epoxidation reaction would be performed in a biphasic system composed of a CO_2/Olefin phase and an aqueous H_2O_2 phase. According to the literature, CO_2 and cyclohexene become completely miscible above 82 bar at a temperature of 40^oC. The reaction is possible due to the in situ formation of peroxycarbonic acid, from the interaction of CO_2 and H_2O_2. This acid is going to be responsible for the oxidation of cyclohexene.

The epoxidation reaction occurs in an aqueous H_2O_2 phase. In order to improve the effectiveness of peroxycarbonic acid as an oxidizer, other compounds can be added to the system. In this case, $NaHCO_3$ can be added in order to improve the concentration of peroxycarbonic acid as also the co-solvent dimethylformamide in order to improve cyclohexene solubility in the aqueous phase.

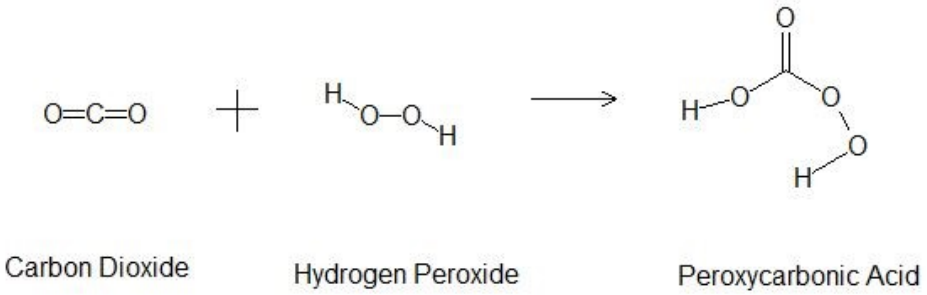


Figure 3.16: Peroxycarbonic Acid Formation.

Table 3.8: Reactant Molar Ratios.

	C_6H_{10}	H_2O_2	H_2O	$NaHCO_3$	CO_2 or N_2
No additives	1	2,6	11,3	0	23,6
$NaHCO_3$	1	2,6	11,3	0,04	23,6

Relatively to the co-solvent added, as it was previously said, the choice is dimethylformamide (12 wt %). With the components being H_2O_2, dimethylformamide, CO_2 and 0,1 mol % $NaHCO_3$, on the conditions of 40^oC and 120 bar, the reaction time was 20 hours.

Table 3.9: Reactant Molar Ratios of second reaction.

C_6H_{10}	H_2O_2	H_2O	$NaHCO_3$	Co-solvent	CO_2 or N_2
1	2,6	11,3	0,04	5	18

In a second stage, using the same apparatus and conditions, cyclohexene would be replaced by safrole in order to perform again the epoxidation reaction. In principle, such apparatus and conditions would work in performing this reaction, being the biggest uncertainty the solubility of safrole in supercritical carbon dioxide.

On both cases it would be necessary to compare the results obtain through analytical methods and that's why safrole oxide and cyclohexene also appear on the considered products list in order to have a reference from which is possible to compare if the epoxidation reaction was successful.

3.4 Proposed Analysis

A literature search on analytical methods for safrole was also done in order to have a viable way to understand if the results supposedly to be obtained were the ones pretended from the beginning. When it comes to the analysis of safrole after extraction there

were two main methods presented in the literature, Gas Chromatography (GC) and Gas Chromatography/Mass spectrometry (GC/MS).

Table 3.10: Literature on safrole analysis.

Method	Reference
GC	[82]
GC	[63]
GC/MS	[83]
GC/MS	[69]
GC	[84]

The type of analysis used will depend on the conditions of the experiment and the level of detail necessary. As it was mentioned before, in a scenario were the presence of a certain compound is known, analytical data from GC can be used to support that claim and have an idea of the quantitative weight of that compound in the sample. On the other hand, if there's no idea on which components exist in a sample, then a method such as GC/MS will be more appropriate.

In both techniques there's parameters which are important to control in order to have the most viable analyzes possible. One of those parameters is the retention time, which essentially represents the time between injection and elution of the sample. Each component possesses a different retention time since they all have different properties which affect their speed in the column ending up leaving in different moments. In the case of safrole, this compound belongs to the composition of an essential oil. When the oil is injected the different compounds including safrole will interact with the column in a different way, possessing has it was already said different elution time (retention time).

Table 3.11: Retention Times.

Component	Retention Time (min)
Safrole	13,22
Camphor	4,79
Methyleugenol	18,8
1,8-Cineole	16,22

There are several types of detectors used in Gas Chromatography. According to the literature, in the case of safrole analyses the most common type is the flame ionization detector. Essentially this detector uses a hydrogen flame in order to burn the organic compounds and from the forming ions it is then possible to understand from the proportion in the gas stream the amount of a certain compound in the sample. According to the literature the most common carrier gas used is Helium and systems operating on temperatures in a range of 240^oC to 290^oC.

Chapter 4

Berberine - Sources and Extraction

In this chapter, the aim is to present the compound that was selected has an alternative subject and describe the experimental work performed on it.

4.1 Sources and Origins

As mentioned before, there are multiple medicinal plants and which one possesses different applications. In this part of the work the focus was on the *Berberis vulgaris* plant, which belongs to the *Berberis genus*. This genus is characterized by possessing around 450 species, possessing plants with deciduous and evergreen shrubs from 1 to 5 meters tall. They can usually be found throughout the temperate and subtropical regions of the world, existing a large diversity of species in South America, Africa, Asia, Europe, and North America. Other examples of plants belonging to this genus are *Berberis aggregate*, *Berberis aristata*, *Berberis valdiviana*, *Berberis verruculosa*, among others. [85]

Berberis vulgaris is the most common species in the genus *Berberis*, and is native from places like central and southern Europe, northwest Africa and western Asia. It's believed to possess a name that derives from the Arabic language signifying shell, which comes from the fact of its leaves being glossy like the inside of an oyster shell. *Berberis vulgaris* is also known has European barberry, Jaundice berry, Pepperidge, Sowberry, Barberry, among others.

It gives origin to a fruit, a red berry which appears in late summer or autumn. This berries are edible and rich in vitamin C, though with a very sharp flavor. They are not widely consumed since harvesting them is very difficult, resulting in a very important food supply for many small birds. It was traditionally used as an ingredient in making jams, jellies, and juices in Europe, as currant in rice in southwestern Asia, and as a flavoring agent in candy on Russia. [86]

Figure 4.1: Berberis vulgaris tree.

This plant has become popular not only in food but also medicinal applications. Barberry has played an important role in herbal healing for more than 2500 years, from the ancient Egyptians to the European herbalists and Russian healers. Applications through the centuries went from preventing plagues to treat liver problems, inflammation, and high blood pressure. Also, American Indians used it to improve appetite and treat stomach problems.

Nowadays it continues to be extremely relevant in the medicinal area, being the bark of stem and roots the parts of the plant most used in this areas. It continues to be seen has an effective treatment for fevers, gout, renal and biliary diseases, rheumatic symptoms, diarrhea, gastric indigestion, among others.

Table 4.1: *Berberis vulgaris* medicinal applications.

System	Plant part	Effect
Cardiovascular	Dried leaf	Antiedema
	Stem bark	Antihypertensive
Blood gastrointestinal	Root	Intestinal ulcers
	Dried plant	Choleretic
	Root	Cholagogue
	Dried root	Laxative
	Fruit	Stomachic
	Root	Diarrhea
Endocrine	Fruit	Painful menstruation
	Root	Inhibit pregnancy
	Root	Promote fertility-male
Immune system	Dried root	Anti-inflammatory
	Dried root bark	Rheumatoid arthritis
	Flowers	Gout
Central nervous system	Dried root cortex	Antipyretic
	Dried root	Sedative
Respiratory	Fruit	Whooping cough
	Dried leaf	Cold symptoms
Skin	Dried leaf	Wounds or ulcers
	Dried root cortex	Disinfectant

The medicinal applications of this plant comes from the compound berberine. A quaternary ammonium salt from the protoberberine group of benzylisoquinoline alkaloids with a characteristic strong yellow color and given by the chemical formula $C_{20}H_{18}NO_4^+$.

Table 4.2: Berberine Properties.

Properties	Data
Molecular Weight	336,7 *g/mol*
Melting point	145 °C

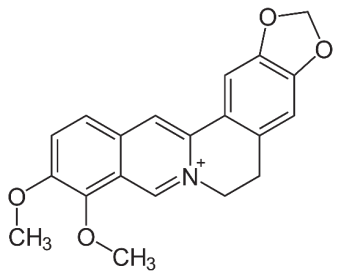


Figure 4.2: Chemical Structure of Berberine.

It's widely used in dying wool, leather and wood. Under ultraviolet light, shows a strong fluorescence, being used in histology for staining heparin in mast cells. It's also considered to have anti-inflammatory and anti-diabetic properties being under investigation in order to determine whether it may have applications as a drug in treating conditions like diabetes, hyperlipidemia, MRSA infection (Methicillin-resistant Staphylococcus aureus), lower cholesterol and even cancer.

Other applications are found in reducing glucose production in the liver, synergize with anti-depressant medication, help with body fat loss, treating diarrhea by bacteria, and as a stimulator of the immune system.

Berberine can be synthetized by multiple chemical processes but the most common way to obtain it is through extraction of raw plant materials. Examples of plants on which this compound can be found are *Tinospora cordifolia*, *Argemone Mexicana*, *Eschscholzia californica*, *Coptis chinensis* (Chinese Goldthread), *Xanthorhiza simplicissima* (Yellowroot), *Phellodendron amurense*, *Berberis aristata* (Tree Turmeric), *Berberis vulgaris* (Barberry), *Berberis aquifolium* (Oregon grape), and *Hydrastis Canadensis* (Goldenseal). Berberine can be found not only in various plants but also in almost all sections of this plants like roots, rhizomes, stems, and bark. Because of this and the large distribution of this plants around the world, this compound also presents a big availability around the world.

Table 4.3: Berberine content in different plants.

Plant	Origin	Berberine content
Berberis aquifolium	Wertern North America	0,5 - 6,0 %

Hydrastis canadensis	North America	0,5 – 6,0 %
Berberis aristata	India/Nepal	3,18 %
Berberis Tinctoria	India	1,46 %
Phellodendron amurense	Asia	0,65 %
Tinospora cordifolia	India	0,035 %
Argemone mexicana	Mexico	0,05 %

As it's possible to understand by the table above, even though berberine can be found and obtain in almost all over the world, when it comes to pharmaceutical and industrial applications the amount of this compound that can be found in most plants is very small limiting the types of processes that can be used on the industrial scale due to the investment necessary and amount of raw material necessary in order to obtain a significant amount of this compound. As it was mentioned before, the most common plant possessing this type of compound is *Berberis vulgaris*, having some studies revealed an amount of berberine in this plant in the order of 6%. This means that not only is a plant that exists with a high distribution but also the amount of berberine discovered in the literature suggests that could be close of some practical industrial applications.

4.2 Experimental Part

4.2.1 Materials

The root bark of *Berberis vulgaris* was obtained from a polish company called Nanga, and the material had its origin from Pakistan. Berberine chloride form was purchased from Sigma Aldrich. CO_2 in its liquid form was bought from The Linde Group (Purity: 99.995%).

Table 4.4: Materials used.

Name of Reagent	Units	Unit Size	Company
Berberis vulgaris	10	50 g	Nanga
Berberine chloride	1	5 g	Sigma Aldrich
Carbon dioxide	2	20 kg	The Linde Group

4.2.2 Supercritical Fluid Extraction Systems

The experimental part was divided into the use of two different supercritical extraction systems which were chosen based on the objective for each stage of the work. The systems considered were a dynamic and static extraction, in which the first would be used to study the process parameters and the second one to attempt extracting the target compound.

4.2.2.1 Dynamic System

A dynamic extraction system consists on a constant flow of fresh solvent going through the plant material extracting the compounds of interest. This system was mainly used for studying the effects of the different process parameters.

This system operates with a pump for CO_2 which is possesses an internal cooling system, an extractor, a heater, a pressure control valve and two CO_2 supply tanks. The amount of CO_2 used during the several experiments is controlled in order to make an estimate on when to change to the second cylinder. When it comes to the extractor this is divided into two main sections: an exterior metal vessel and an inner grid extraction vessel.

The exterior vessel possesses the dimensions of 250 mm in length and 10,2 mm in diameter. It possesses a thicker wall in order to sustain the high pressures which is exposed, allowing the extraction of the raw material which is put inside. This exterior vessel is submersed in a water bath which is warmed to the desired temperature by a heater.

Figure 4.3: Exterior extraction vessel.

As for the inner vessel, the dimensions are 150 mm in length and 8 mm in diameter with a grid of mesh 100 (0,15 mm). This inner vessel will contain the milled plant material and then will be sealed using cotton wool.

Figure 4.4: Inner extraction vessel.

A back pressure regulator valve is used to control the pressure in the system. The collection is made at room temperature and atmospheric pressure by simple system

depressurization. The valve sits on top of a heater in order to avoid a clog caused by the depressurization of the CO_2.

Figure 4.5: Pressure control valve.

Temperature and pressure are controlled through two digital screens which are connected to two sensors. The pressure sensor is connected to the main tubes were the supercritical CO_2 passes and the temperature sensor is put inside of the water bath.

Figure 4.6: Digital screens giving the values of temperature (^oC) and pressure (bar).

The apparatus was design in order to perform extraction reactions using only CO_2 as solvent. If co-solvents wanted to be used the system could be adapted by simply introducing a second pump which would be responsible for introducing the organic solvents into the extraction system.

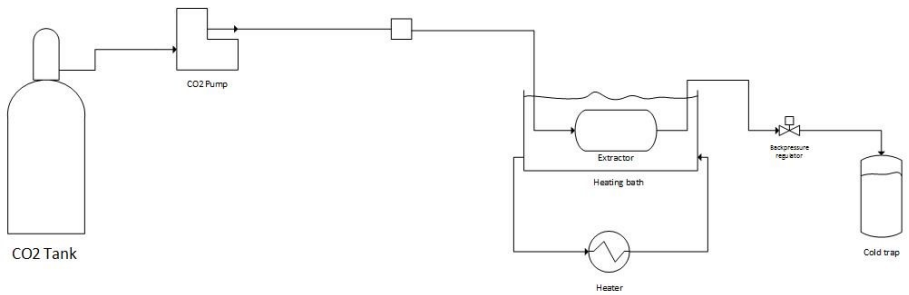


Figure 4.7: Design Dynamic Supercritical Extraction System.

4.2.2.2 Static System

This type of system was selected in order to collect as much powder of the target compound as possible. The main advantage of this type of system is the fact it possesses a bigger volume (V = 1,2 L) allowing the use of more plant material which would translate in a higher amount of extract obtained.

This system is composed by a steel reactor, an oven, an agitation system and a system of valves for both pressurization and depressurization of the system. The steel reactor has a cylindrical shape and also possesses a thick wall in order to sustain the high pressure inside.

Figure 4.8: Type of reactor used in static extraction.

This reactor is put inside an oven which will control the temperature inside the reactor. The oven has also a cylindrical shape and the reactor is put through a hole that exists in the center of it. The temperature is indicated through digital monitors and it presents the values of temperature inside and outside of the reactor.

Figure 4.9: Oven for reactor temperature control.

The pressure is indicated through an analogic indicator. The depressurization is made at normal temperature and pressure and can be done by using organic solvents or not.

Figure 4.10: Analogic pressure indicator.

The system apparatus was also design to only operate with supercritical CO_2 but it would be possible to also operate using co-solvents as long another pump was added.

4.2.3 Extraction Process

4.2.3.1 Extraction in Dynamic Mode

The extraction process using a dynamic system was performed using several types of conditions, having pressures in the range of 70 to 250 bar and testing at temperatures of 40^oC and 60^oC. The extraction process starts by first milling the plant material. The root bark of *Berberis vulgaris* is characterized by its yellowish color and strong smell.

Figure 4.11: Example of non milled root bark.

The milling process was done using a coffee grinder (Sencor SCG 1050WH / SCG 1050BK). After being milled the material is then sieved in order to ensure that only a specific material size would go into the extractor. The sieve was made using a net with

the size of mesh 22 (0,8 mm). Usually the sample would have to go more than one time through the sieve in order to guarantee that almost no dust was introduced into the extractor.

Figure 4.12: Example of milled material.

The material is put inside the inner vessel which acts as a "basket" for the sieved plant material in an amount of around 2 grams. The fact that the inner vessel is composed by a net of a smaller size also helps to make a second barrier in which the dust particles that stay in the plant material can be removed. The way the inner vessel is filled goes through three main stages:

1. Weight the vessel alone without any plant material.
2. Weight the vessel now with the plant material in it.
3. Weight the full vessel already sealed with the cotton wool.

By the end of this stage it's possible to know the size of the vessel, the amount of material inside of the vessel and the weight of the cotton wool. Another important factor is that because this inner vessel was made using a net material, there were some difficulties in its construction. After some extraction the vessel started to give in so by using tape the integrity of the inner vessel was maintained.

Then, after all the valves being closed and checked, the CO_2 tank is open and the pump activated right after that. The CO_2 in the tank is in its liquid form (under pressure), and when the valve is open the liquid is sucked into the pump and introduced into the system. As it was mentioned before, the pump possesses a cooling system that allow the solvent to remain liquid turning the pumping more effective. The pump used was a metering one, allowing this way to establish a constant flow of 10 ml/min, which is the equivalent of 5 g/min. The limitations of this pump are present mostly bellow and at the critical point, where the gas and liquid CO_2 do not allow it to be 100% efficient. Only

after the critical point, at supercritical state, the pump can be efficient allowing a steady rise in pressure.

After leaving the pump and achieve the required pressure, the fluid goes through a serpentine which was also immersed in water so that when the fluid reaches the extractor it has the pressure and temperature pretended.

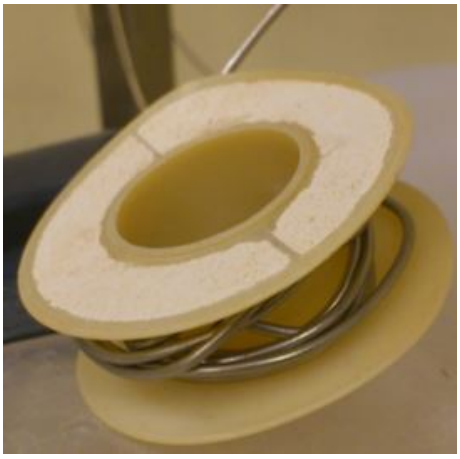


Figure 4.13: Serpentine used.

Now in the supercritical state, CO_2 goes through the plant material inside the extractor allowing the extraction of certain compounds. The temperature in the extractor is controlled by the temperature in the hot water bath.

The pressure of the entire system is controlled by a back pressure valve which further allows the collection of the extract by depressurization at normal temperature and pressure. Actually, every time there was a change in the pressure used for the extraction the valve had to be regulated prior in order to keep that specific pressure along the entire extraction time. The measurements were made at the end each hour by stopping the process and remove the sample. Then, the sample would be introduced again into the extractor and the all process restarted with a total extraction time of around four hours.

Figure 4.14: Apparatus used for dynamic extraction.

4.2.3.2 Extraction in Static Mode

As it was already mentioned, the propose of using this extraction system was to use more plant material in a hope to obtain a larger quantity of extract. The first thing to do also passes by milling the plant material using the same sieve has before with a grid size of mesh 22 (0,8 mm). The amount of material which was used for this type of extraction was around 22 grams and the vessel in which the material was introduced was also different from the one used in the dynamic extraction.

In this case, the vessel was more like a small bag made of an elastic nylon net with a net size of DEN 20, which still allowed some residual dust to escape. The bag was closed by making a node and making sure that all the material was packed.

Figure 4.15: Bag containing plant material.

Then, the bag full of plant material was suspended inside the reactor vessel and completely sealed. The CO_2 tank is open and the pump is turn on right after it allowing the pressure to build up inside the reactor. Then the stirring system was turn on to 100 rpm allowing to steer the fluid inside and help with the extraction process. The pressure and temperature conditions used were 130 bar and 70^oC respectively. The low conditions applied have to do with the limitations imposed by the equipment. At the time of the experiments the pump was constantly malfunctioning and barley rising the pressure in the system. A solution found to get more pressure was to stop the pump and increase the temperature, allowing an increase of pressure as the temperature also increases.

The extraction occurred for two straight hours and the collection was made by depressurization at normal temperature and pressure. It's possible to see that when there's a depressurization of CO_2 that the temperature goes down and it looks like the vessel freezes. A cotton wool is put on the entry of the vessel so if any residual splash happen it still gets retained inside the vessel.

The collection vessel and the reactor itself were then rinsed with acetone in order to collect any residual extract that was not removed by the depressurization. The process of depressurization has to be done slowly in order to control the flow of CO_2 leaving the system and not lose any extract in the process.

Figure 4.16: Apparatus used for static extraction.

Figure 4.17: Collection by depressurization.

4.2.4 Water content

The water content of the *Berberis vulgaris* was investigated in order to see if moisture was an important factor when it came to the extraction of this plant material. The root bark was divided into two samples of 2 grams each, being one of them milled and the other just like it came from the supplier. Both samples were put inside an oven at $120^{o}C$ for two hours. When it comes to the milled sample the water content was found to be 0,20%. This shows that the influence of this parameter in the extraction is not significant. For the not milled sample the water content was 0,21% which also seems to be non-significant.

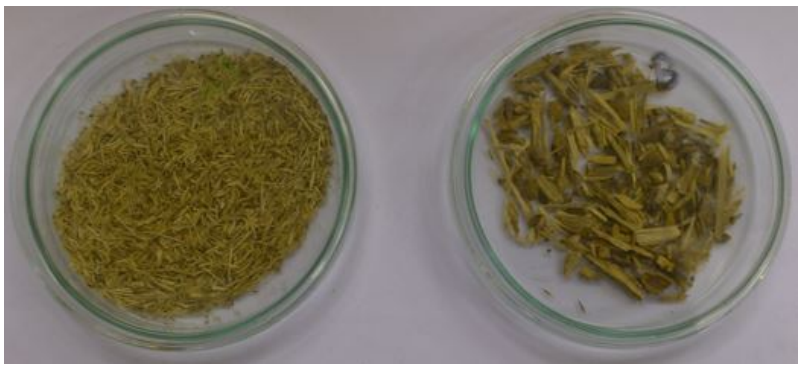


Figure 4.18: Samples for analyzes of water content.

4.3 Analytical Method

The analytical stage allows to guarantee if an extraction process was successful in removing from the plant material a certain compound by identify it in the extract and in what quantity. As it already been mentioned, there are several methods of analyzing extraction products from GC/MS to HPLC, depending on the objective of the work itself. In the case of berberine the first option to analyze it was to use HPLC in the first place. Some literature was found were berberine analyses is described and from there a protocol could be developed with that objective.

Table 4.5: Literature data on berberine analytical methods.

Method	Reference
HPLC	[87]
HPLC	[88]
HPLC	[89]
HPLC	[66]
HPLC	[67]
HPLC	[90]
HPLC	[91]

With the shortage of time available to perform more experiments, it was thought that a simpler analysis could be made in order to prove that berberine was in fact extracted. With that in mind, the proposed technique was differential scanning calorimetry (DSC) which would allow to compare the extract with a reference in terms of how much heat is necessary to raise the temperature of each of them.

Due to a very low amount of extract obtained even this method wasn't possible to be performed leaving only certain properties like color and smell suggesting the presence of berberine in the extract.

Chapter 5

Results and Discussion

In this chapter, the aim is to present the results obtained in the experimental part and make a discussion of those results.

For the experimental part, parameters like Temperature, Pressure and Extraction Time were tested.The range of pressures tested was from 70 to 250 bar and the temperatures applied were 40^oC and 60^oC.

The reaction time was always kept on 4 hours and the measurements were made at each hour.

The first set of conditions considered were a pressure of 100 bar, a temperature of 40^oC, and an extraction time of 4 hours.

Table 5.1: Cumulative Yield values for the conditions of 100 bar and 40^oC (first experiment).

Time (Hours)	Cumulative Yield (%)
1	3,71
2	4,59
3	5,12
4	5,34

The yield was calculated at each hour and has time would pass it would be added to the previous yield. The extraction yield at the first hour was of 3,71%. The gradual increase on the overall cumulative yield is consistent with the expected results since the yield value is higher and higher in each hour.

This cumulative yield can be obtained by constantly adding the yields calculated for each hour. In this case the system seems to be progressing into a “step” which could mean that after 4 hours of extraction there is not much left to extract. This "step" consists on a stabilization of the extraction yield after some time.

A clearer vision of the overall extraction process can be obtained by showing the curve resulting from the calculated extraction yields. From this, it will then be possible to better understand if such stabilization was already reached or if it's very close to do so.

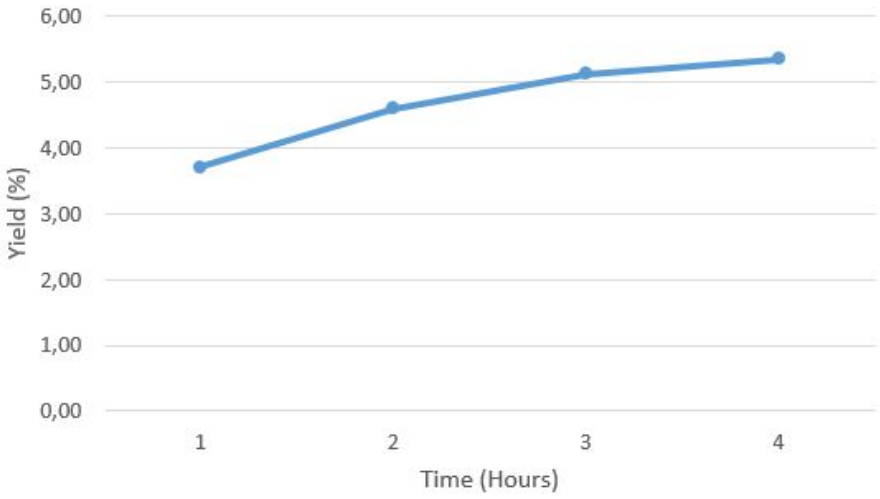


Figure 5.1: Cumulative Yield at 100 bar at 40^oC (first experiment).

The experiment was performed one more time using the same conditions in order to see if the behavior was the same that in the first one. It's possible to see that the yield registered in the first hour was of 2,62% and in the following hours the extraction yield continues to increase suggesting a similar behavior when compared with the first try.

Table 5.2: Cumulative Yield values for the conditions of 100 bar and 40^oC (second experiment).

Time (Hours)	Cumulative Yield (%)
1	2,62
2	3,78
3	4,41
4	4,72

Not only the behavior of the cumulative yield seems to be the same, but through the representation of the curve it's possible to see that it almost goes into a "step" after 4 hours of extraction. This could suggest that some amount could still be extracted from the raw plant material and that the "step" would probably be achieved if the extraction process would be continued for a couple more hours.

The final value after 4 hours is different but since the initial value was also smaller than in the first experiment is normal that with the same behavior the final value would also be smaller.

Overall this second experiment shows a similar behavior when it comes to the extraction curve only with different initial and final values.

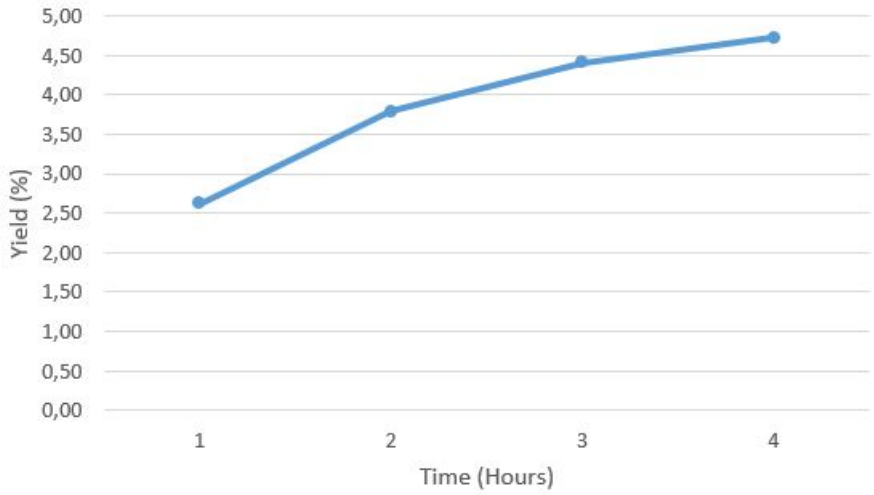


Figure 5.2: Cumulative Yield at 100 bar at 40^oC (second experiment).

The next experiment was performed by keeping the temperature at 40^oC and the extraction time at 4 hours. The only condition which changed was the pressure to 150 bar. Just like the experiment before, the first hour records a superior extraction yield being in this case of 2,57%. Again the behavior seems to be consistent with the previous experiments.

Table 5.3: Data from 150 bar and 40^oC (first experiment).

Time (Hours)	Cumulative Yield (%)
1	2,57
2	3,87
3	5,03
4	6,10

When presenting the cumulative yield curve is possible to see a completely different behavior from the other cases since it doesn't suggest to be even close to a step.

It behaves more like a straight line suggesting that it could be extracted much more from the raw plant material. If this was an isolated case, than it would be a plausible explanation but when looking at the previous cases and what would be expected theoretically, other scenarios could be drawn from this data.

One plausible alternative would be of experimental error. Extraction from raw plant materials is a difficult system being difficult to maintain the same conditions since some plant material can be lost even by just introducing or removing the extraction vessel.

Other possible explanation has to do with the supercritical system in itself. Supercritical systems are only now being more studied and so solid models don't exist yet.

This causes to be hard to obtain the exact same values for all experiments and that even a small change in the process conditions could cause a large difference in the end results.

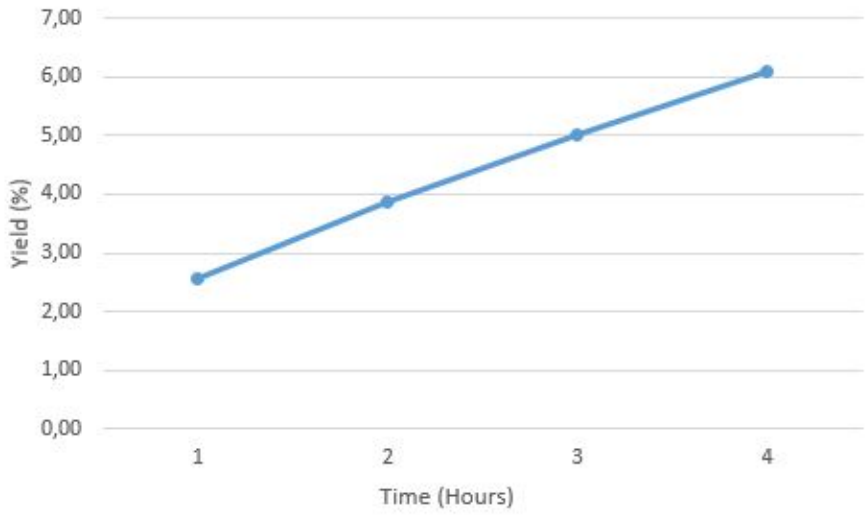


Figure 5.3: Cumulative Yield at the conditions of 150 bar and 40^oC (first experiment).

The second experiment, performed with the same conditions, showed a calculated yield at the first hour of 2,69%. This value, even that slightly higher that the previous one, makes that the curve starts around the same range of values allowing to, considering a similar behavior, to obtain a final value after 4 hours within the same range of values as the previous one.

In reality what happens is a final value considerably smaller that it the previous case. This might suggest that something went wrong in the first experiment using this conditions, resulting in a significantly increase of the calculated yield.

Table 5.4: Data from 150 bar and 40^oC (second experiment).

Time (Hours)	Cumulative Yield (%)
1	2,69
2	3,57
3	4,08
4	4,46

The cumulative yield curve shows in this case a behavior a little different since it tends more into a step that in the first case. Like it was previously suggested, this fact might indicate a less significant amount existing to be extract after the 4 hours of extraction.

Even so, the duration of the extraction process is still proving to be insufficient for the temperature of 40^oC.

In the end is possible to see that the overall behavior still shows a tendency to go up instead of stabilizing within the 4 hours. On the other hand, the final yield obtained for this particular case is much lower than in the first experiment, what can be explained by the different behavior of the cumulative curve.

It would be expected that if the extraction would continue for more than 4 hours that it would eventually come to stabilize.

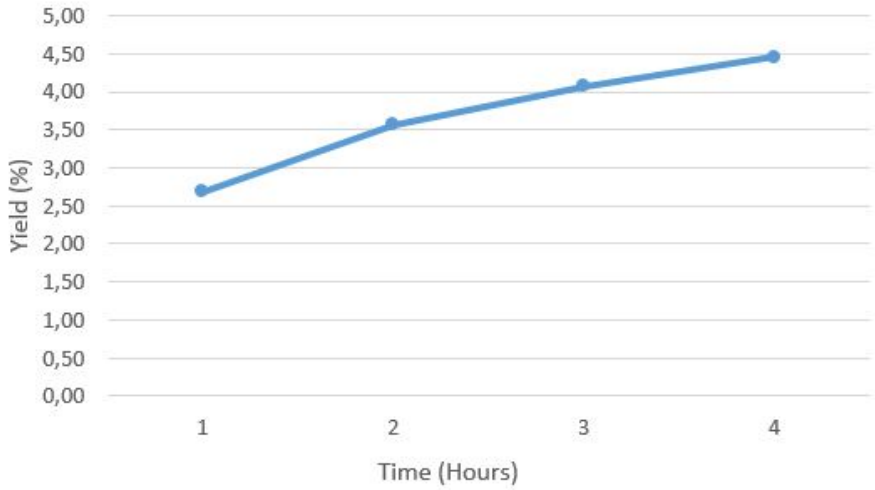


Figure 5.4: Cumulative Yield at the conditions of 150 bar and 40^oC (second experiment).

In the next set of conditions, the temperature and extraction time were kept the same changing only the pressure to 200 bar. In this experiment the behavior of the obtained yield started the same as the others but after the third hour it completely changed.

The large increase on the calculated yield could have been caused by some experimental issue as its been mentioned before.

The extractions performed so far clearly suggest that it's very difficult to completely control the experimental factors such as the distribution of the material. In fact, because the inner vessel was composed by a grid, there was the possibility that the smallest particles would leave the vessel when this was introduced or removed from the exterior vessel.

Table 5.5: Data from 200 bar and 40^oC (first experiment).

Time (Hours)	Cumulative Yield (%)
1	3,53
2	4,59
3	4,78
4	5,34

When it comes to the cumulative yield curve, it's possible to see that in the first 3 hours the behavior suggests that it's tending to a step but after the fourth hour it rises again. This could be explained by some loss in material when the inner vessel was removed causing a higher increase in weight loss of the sample.

Experimental issues can only be solved by the acquirement of experience by the operator and so the development of a method which allows to minimize such mistakes. The familiarity of the operator with the equipments is also an important factor which could cause divergences on the final results.

For this reason is clear that the first experiments need to be looked at with care since

those experiments are the ones on which the operator had less experience.

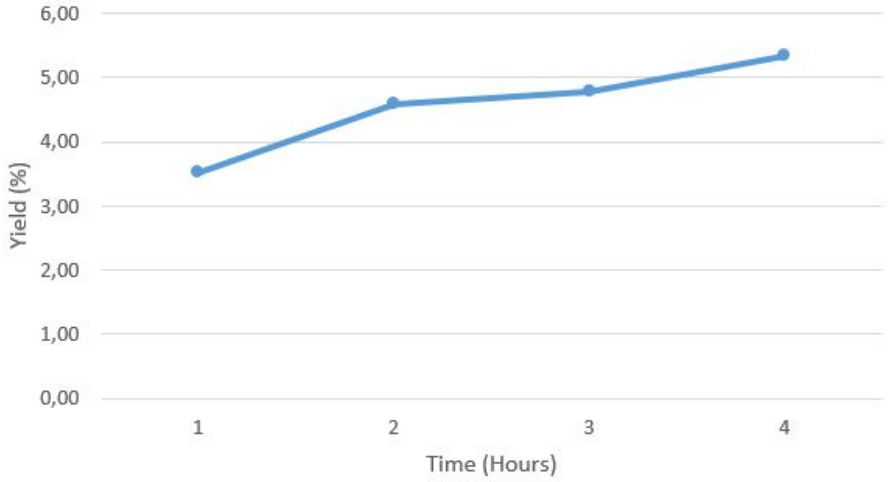


Figure 5.5: Cumulative Yield at the conditions of 200 bar and 40^oC (first experiment).

On the second try with this conditions it is possible to see that the values obtained make more sense than in the previous experiment. The yield obtained at the first hour was 2,73%.

The yield in each hour continuously increases and there was no sudden increase of yield which could suggest that differently from the previous experiment there was no significant loss of weight by the material sample.

After the 4 hours of extraction the yield final value obtained was 4,62%. This value was considerably smaller when compared to the previous yield obtained on the last extraction process hour.

This low value can be explained not only in terms of the weight loss of the sample but also in terms of the behavior of the curve and its initial value. The initial value in this case is much smaller as well which leads to a curve with an similar behavior to have a smaller value on the final value as well.

Table 5.6: Data from 200 bar and 40^oC (second experiment).

Time (Hours)	Cumulative Yield (%)
1	2,73
2	3,76
3	4,28
4	4,62

As for the cumulative curve, it's clear that the behavior tends almost into a step. This result comparing to the one got in the previous experiments makes more sense and gives a better explanation for the process behavior in this conditions. It's possible to see a curve being formed as an alternative to the weird behavior shown at the previous experiment.

Again it's possible to see that at the temperature of 40^oC, 4 hours are not enough to achieve the "step" and to extract everything that is possible from the plant material.

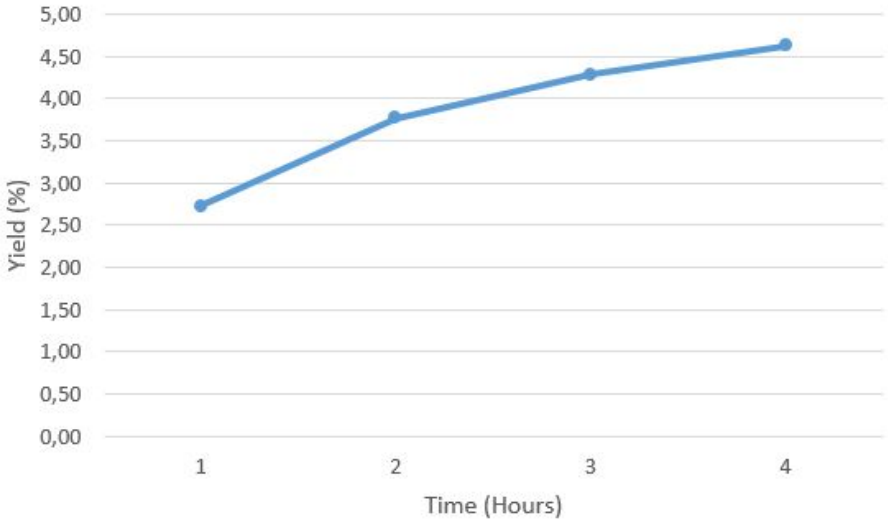


Figure 5.6: Cumulative Yield at the conditions of 200 bar and 40^oC (second experiment).

On the set of conditions of 250 bar and 40^oC, the experiment was performed for 4 hours presenting different results that the previous experiments. The yield after the first hour is consistent with the values obtained before, possessing a behavior very similar except at the third hour were the yield increased instead of being smaller than the previous one. Again this increase in yield could have resulted of a mass lost when introducing or removing the inner vessel from the extractor. This would result in a larger mass difference and by consequence a bigger yield than expected.

Table 5.7: Data from 250 bar and 40^oC (first experiment).

Time (Hours)	Cumulative Yield (%)
1	2,83
2	4,08
3	5,33
4	5,62

When it comes to the cumulative graph is possible to see that the behavior is mostly a constant growth of the extraction yield presenting a straight line. This behavior last for the first 3 hours tending closer to a step on the fourth hour.

It's clear that the shape of the curve is not similar to the curves obtained so far. Until now, the curves have displayed a gradual increase of yield resulting on an actual curve tending into a step.

This case on the other hand, presents a rapid and constant increase of the yield, being represented by a straight line as it was mentioned before.

Although, after the fourth hour the behavior seems to be quite similar to what was seen before, suggesting that after the 4 hours there was not much left to extract just like in the previous cases.

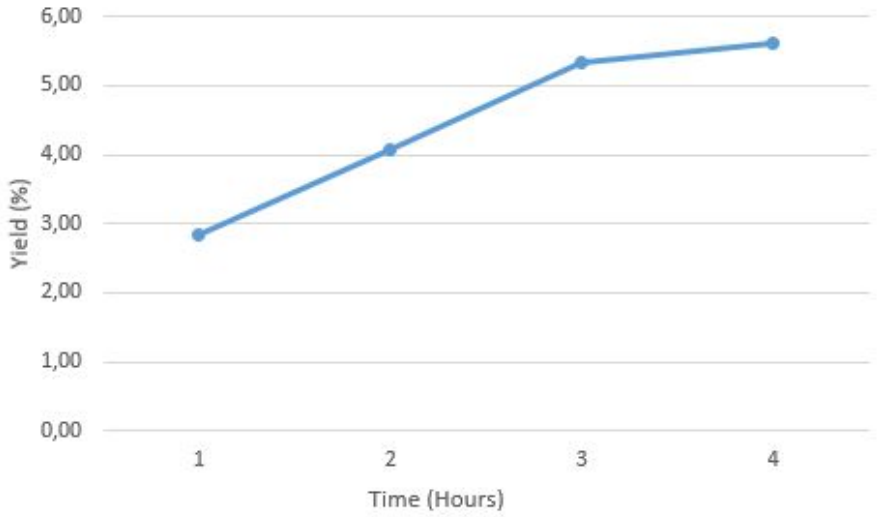


Figure 5.7: Cumulative Yield at the conditions of 250 bar and 40^oC (first experiment).

The experiment was done one more time in order to check the first results obtained. The yield obtained in the first hour is slightly higher than in the first experiment presenting a value of 3,43%. The general behavior is more consistent with all previous experiments on other conditions with a very steep fall in yield between the first and second hour, followed by a constant low in yield on the course of the 4 hours.

Table 5.8: Data from 250 bar and 40^oC (second experiment).

Time (Hours)	Cumulative Yield (%)
1	3,43
2	4,42
3	4,81
4	5,07

Regarding the cumulative graph is possible to see that the behavior is no longer a constant rise in yield but instead it tends closer to step has times passes. The final yield is also lower than in the first experiment even if the first value was higher what can be explained by the curve shape.

In this case it's possible to see a clear improvement when compared to the first experiment using this conditions. The behavior of the curve looks consistent to the previous experiments and after 4 hours seems to be around the same range of values.

There's a gradual increase of the yield but its still possible to see a straight line behavior between the second and fourth hour of experiment.

It's also important to mention the value obtained after the first hour its superior to the one obtained at the first experiment with this conditions. On the other hand the final value after four hours is smaller what goes against what would be expected since that if the initial value is high then the final one would be expected to be higher than in the previous cases.

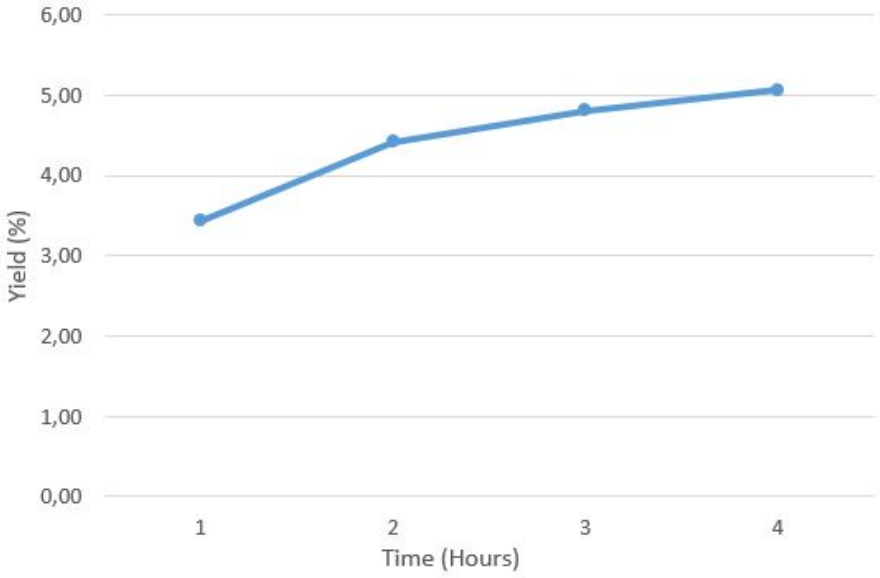
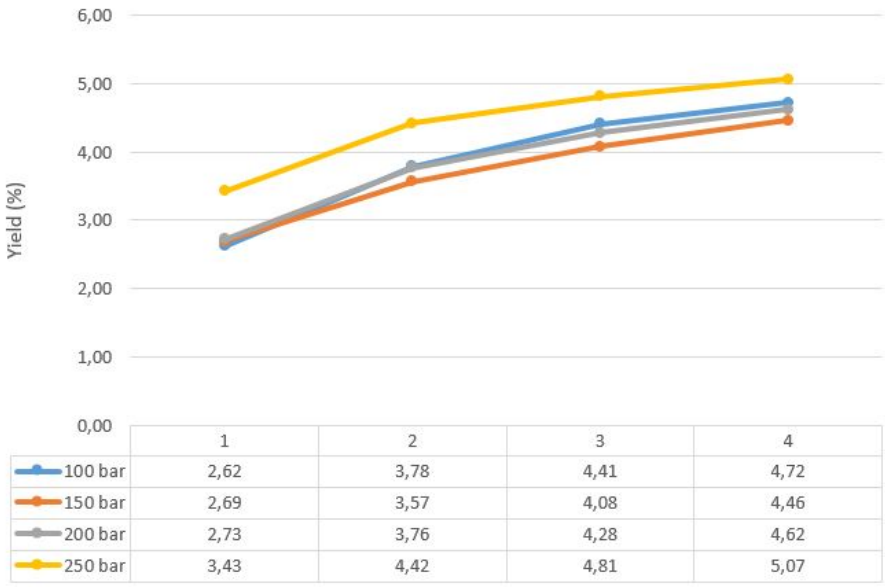


Figure 5.8: Cumulative Yield at the conditions of 250 bar and 40^oC (second experiment).

Having all the experimental data at 40^oC presented, it's important to compare all the results obtained at different pressures. It's possible to see that there's no clear effect of the pressure on the process when temperature is kept constant at 40^oC. At the pressure of 250 bar the extraction yield is higher but the value is still not as significant to consider a real influence of pressure since the values are very close to each other.

It's also possible to see that the curves have mostly the same behavior, with the yields in each hour practically similar.



	1	2	3	4
100 bar	2,62	3,78	4,41	4,72
150 bar	2,69	3,57	4,08	4,46
200 bar	2,73	3,76	4,28	4,62
250 bar	3,43	4,42	4,81	5,07

Figure 5.9: Comparison of the effect of different pressures at the temperature of 40^oC.

For the next set of experiments, the temperature was changed from 40^oC to 60^oC and the pressure was again changed with a range of 70 to 250 bar. The experiments were started with a pressure of 70 bar during a time of 4 hours. This conditions are particularly interesting since they are on the border of the supercritical state, so it would be interesting to see how the process behaves on a subcritical area.

The behavior of the yield curve obtained in each hour presents a similar behave than the ones at 40^oC. It started with a yield of 5,84% and kept going doing in the following hours. The negative value recorded on the fourth hour results on an increase of mass which led to the difference being negative.

One possibility which could explain this phenomenon is the plant having nothing else to extract which could lead to an increase of weight by the action of other external elements.

Table 5.9: Data from 70 bar and 60^oC (first experiment).

Time (Hours)	Cumulative Yield (%)
1	5,84
2	6,94
3	7,66
4	7,52

When it comes to the cumulative yield curve it is possible to see an almost constant growing curve that suddenly leads to a step. The final yield obtained is close to 8% which could be considered a high value but the problem might have not to do with the curve but with the high initial value on the first hour of extraction.

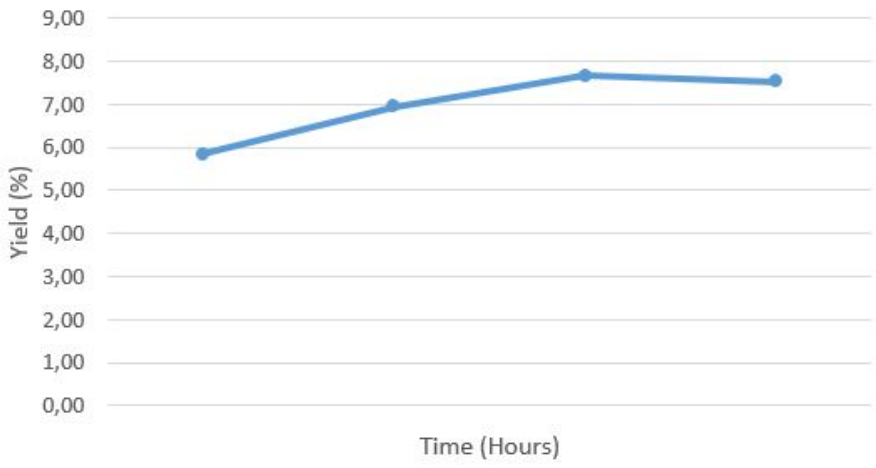


Figure 5.10: Cumulative Yield at the conditions of 70 bar and 60^oC (first experiment).

The experiment was performed one more time in order to compare the results obtained. In this second experiment the yield on the first hour was lower with the value of 4,30%. The general behavior of the curve seems to be more similar to what was expected with a lowering of the extraction yield on each hour.

Table 5.10: Data from 70 bar and 60^oC (second experiment).

Time (Hours)	Cumulative Yield (%)
1	4,30
2	5,74
3	6,26
4	6,33

When it comes to the cumulative extraction curve it's possible to see that it tends into a step and with a final yield much lower than in the first experiment.

The fact that it tends into a step goes against what was expected since this is the lowest pressure tested.

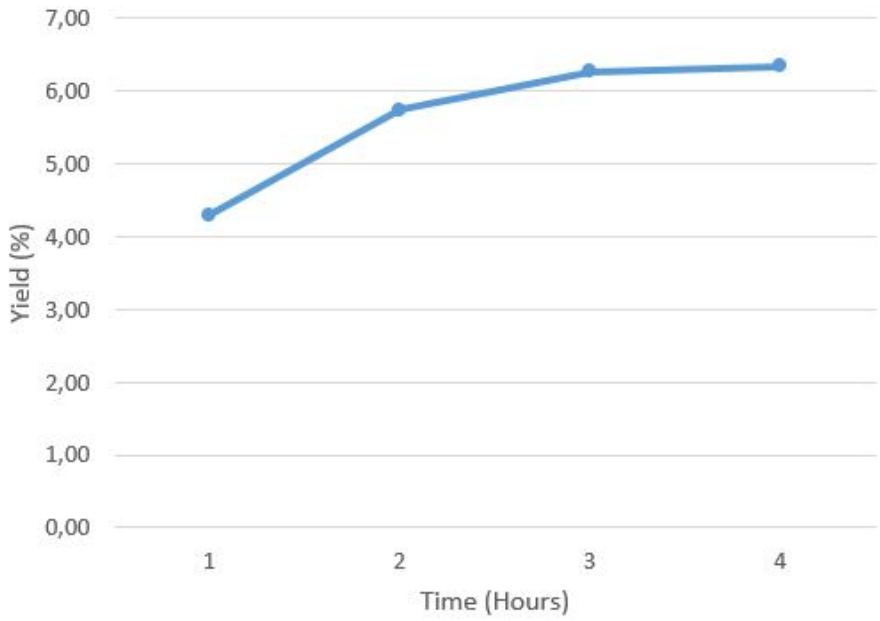


Figure 5.11: Cumulative Yield at the conditions of 70 bar and 60^oC (second experiment).

In the next experiment the pressure was changed to 100 bar keeping the temperature and extraction time constant. The yield at the first hour was 5,10%, keeping to go down until the third hour. At the fourth hour there's an increase in yield which could mean that in some way an amount of material was lost from the inner extractor, causing a larger mass difference.

Table 5.11: Data from 100 bar and 60^oC (first experiment).

Time (Hours)	Cumulative Yield (%)
1	5,10
2	7,20
3	7,37
4	8,23

The cumulative yield shows a weird behavior since that at the second hour a step

seemed to be forming, and then with the increase in yield that same step disappeared on the fourth hour of extraction. It also achieves large final yields which could be influenced by some experimental failure.

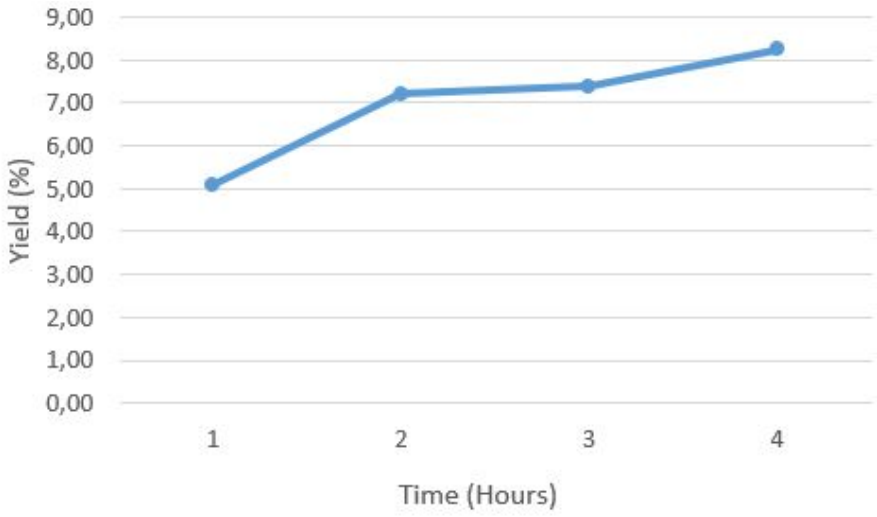


Figure 5.12: Cumulative Yield at the conditions of 100 bar and 60^oC (first experiment).

The results obtained in the first experiment are considered weird when compared with the previous experiences which made that the experiment had to be performed one more time in order to compare the results obtained.

Although in this second experiment things became even more incoherent since the behavior of the curves was even more different than usual with a massive increase of yield in just 2 hours of extraction. After that, the mass increased in very small percentages making this behavior completely different from anything that was observed until now.

Table 5.12: Data from 100 bar and 60^oC (second experiment).

Time (Hours)	Cumulative Yield (%)
1	6,51
2	6,89
3	6,62
4	6,38

Regarding the cumulative yield, it's clear that the behavior is completely different from what usually seen, with the yield actually going down instead of the normal behavior.

In fact, is possible to see that none of the values make any sense. From the start the yield obtained at the first hour is just too high when compared to all the other experiments.

Also, the fact that it goes up and then constantly down also presents a major difference to the previous experiments.

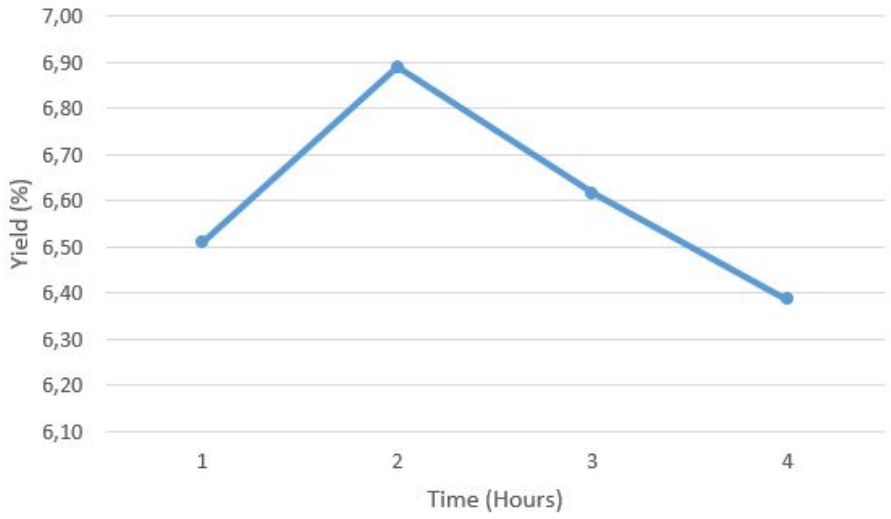


Figure 5.13: Cumulative Yield at the conditions of 100 bar and 60^oC (second experiment).

Since the results obtained were again so strange, it was decided to perform this experiment one more time, but unfortunately it was only possible to do so for 3 hours since the equipment did not allow for the usual 4 hours of extraction to be performed.

In this 3 hours experiment it's possible to see a more general behavior in which the yield goes up more at each hour of extraction and it seems that there were no major difficulties with the extraction process.

Table 5.13: Data from 100 bar and 60^oC (third experiment).

Time (Hours)	Cumulative Yield (%)
1	4,25
2	5,72
3	6,40

When it comes to the cumulative yield is possible to see also a tendency to stabilize even though only 3 hours have passed. By the behavior of the curve it's possible to assume that it would be even closer to a step closer to 7%. The initial yield value also presents the smallest of all obtained in these conditions and the closest one to what would be the common values for the yield at the first hour of extraction.

Like it was mentioned before, there was no time to perform this third try since the equipment didn't allow the conditions to be achieve in a faster way so these results even that are not perfect are an approximation of what could be expected at this operating conditions.

It is very interesting to see how can similar experiments present such different results.This shows how hard it is to control the behavior of these systems and how the search for new models is important for a better understanding and application of supercritical fluids.

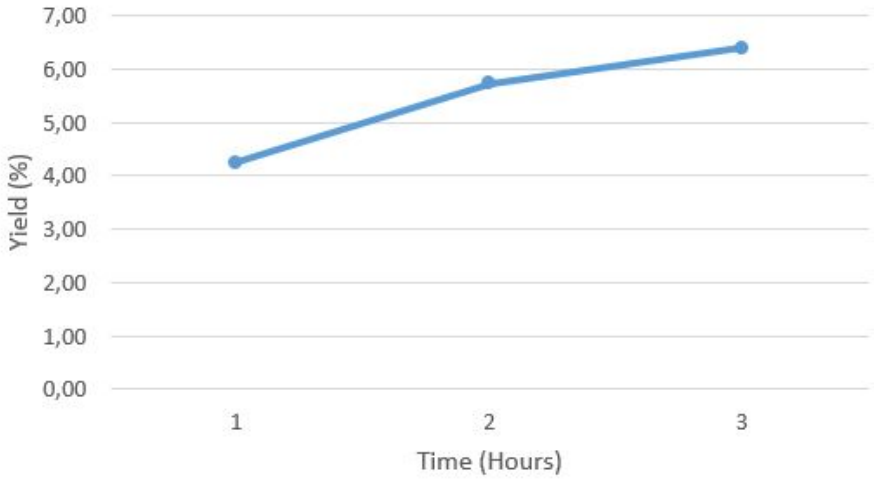


Figure 5.14: Cumulative Yield at the conditions of 100 bar and 60^oC (third experiment).

New conditions were then applied by changing the pressure to 150 bar. Temperature and extraction time were kept the same. In a first experiment using these conditions it's possible to see an expected behavior and also a lower value after 1 hour of extraction (3,54%).

The final value, 6,42%, also seems to be consistent with the general data obtained so far for this temperature.

Even if it was not very clear when using a pressure of 100 bar, when considering all the data obtained so far at the temperature of 60^oC, it is possible to see that in the 4 hours of extraction time there is a tendency to achieve the "step". In this case, that tendency to a stabilization is also quite clear.

Table 5.14: Data from 150 bar and 60^oC (first experiment).

Time (Hours)	Cumulative Yield (%)
1	3,54
2	5,43
3	6,12
4	6,42

As it was mentioned before, the cumulative curve doesn't present much surprises since its behavior is within the expected and tending to a step after 4 hours extraction.

The gradual growth of the yield obtained and the small initial value may suggest that in this case everything went well and that there were no significant influences, which would destabilize the overall extraction system.

Also, as it was mentioned above, a stabilization of the extraction yield after 4 hours would suggest a tendency for the extraction to be faster at this temperature causing in the various pressures a similar behavior in only 4 hours of running. On the other hand, at 40^oC it was clear that four hours was not enough to achieve that step.

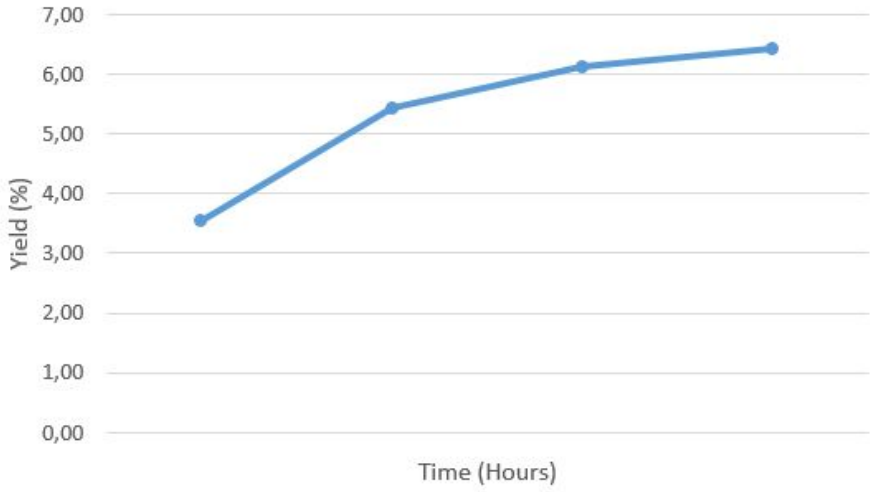


Figure 5.15: Cumulative Yield at the conditions of 150 bar and 60^oC (first experiment).

As a normal procedure, in order to confirm the values, the experiment was performed once more. In this second experiment is possible to see that again at the fourth hour there's a slight increase in the extraction yield witch causes a deviation of the curves behavior. Again this can be explained by some sudden mass lost which could cause these abnormalities.

Both initial and final value can be considered within range when compared with the other experiments at 60^oC.

Table 5.15: Data from 150 bar and 60^oC (second experiment).

Time (Hours)	Cumulative Yield (%)
1	5,12
2	6,05
3	6,23
4	6,55

When it comes to the cumulative yield is possible to see the change on the normal curve behavior at the fourth hour, causing a final yield of close to 7%. The curve seems to tend into a stabilization between the second and third hour which would suggest a very quick extraction.

The problem itself might not be on the value obtained at the fourth hour of extraction but yes on the third one, causing a slight depression and by result the different curve behavior presented.

Again, a plausible explanation for this is the inner vessel letting some of the smaller particles get way by the friction between the inner and exterior vessel. Even being very careful in the stages of milling, sieving, filling the inner vessel with material, and also taking and introducing it into the extraction vessel, a small complication or mass lost could cause a massive change in the curves behavior.

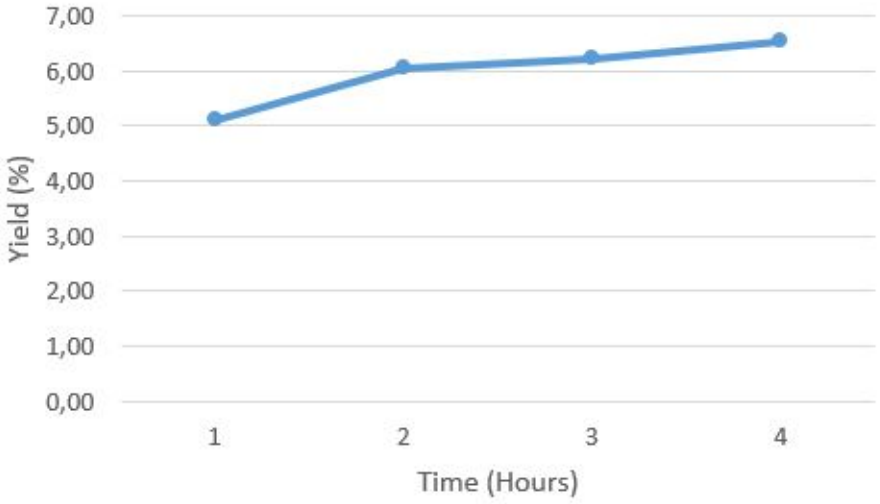


Figure 5.16: Cumulative Yield at the conditions of 150 bar and 60^oC (second experiment).

Since the results of the second experiment were not very reliable, a third experiment using the same conditions was performed. In this case the same problem appears, having a high initial yield and a strange curve behavior with a high yield increase at the fourth hour.

Table 5.16: Data from 150 bar and 60^oC (third experiment).

Time (Hours)	Cumulative Yield (%)
1	5,36
2	5,94
3	6,10
4	6,32

The cumulative curve presents an even weirder behavior by continuing to go up for the entire 4 hours. There are two main section which deserve a more careful analysis being one of them between the first and second hour, and the other between the third and fourth hour. As it was mentioned before, on the fourth hour there is a significant increase of the yield obtained which could be related to some kind of problem at that moment or be result of a problem at the third hour, causing a depression and a weird behavior of the curve.

Between the first and second hour of extraction there is also a big increase but when looking at the previous experiments is possible to see that such behavior is normal. Even that slightly high, the first value is within range when compared with the previous experiments.

Extraction using a continuous supercritical system is difficult in a sense that's a complex system in which small alteration in the parameters could cause major differences and so it becomes difficult to achieve replicated results. Also, as it was mentioned before, the material itself is hard to control so it could also cause some differences in the final

results.

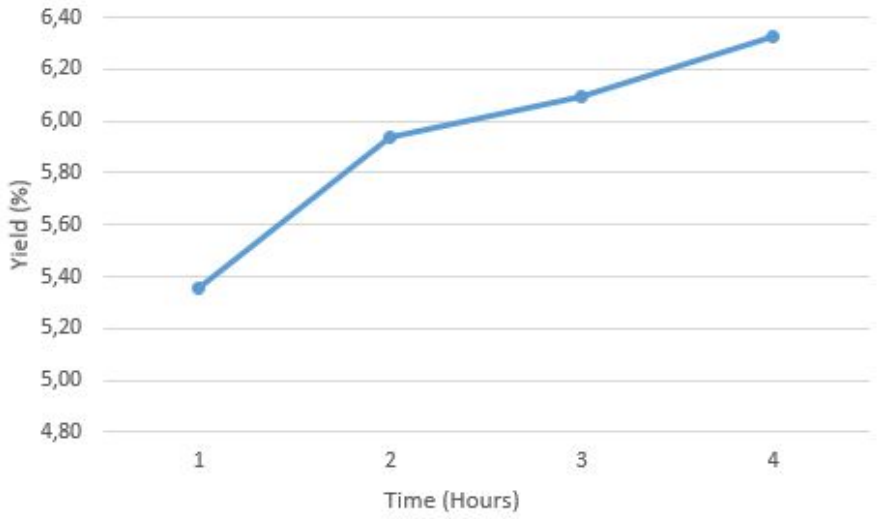


Figure 5.17: Cumulative Yield at the conditions of 150 bar and 60^oC (third experiment).

The conditions were then changed again to a pressure of 200 bar keeping everything else constant. Over the extraction time, in each hour the yield values increase at different rates becoming in line with the previous experiments for the first 3 hours.

Again, in the fourth hour, the extraction yield becomes negative, causing the value of cumulative yield to go down. This negative value might imply that the sample gained weight.

Even though the values are in the same range as the ones in previous experiments, they are still considered low values. The suppose weight gain could be caused by the influence of the humidity in the air, which could enter the raw material and cause an increase of mass.

Table 5.17: Data from 200 bar and 60^oC (first experiment).

Time (Hours)	Cumulative Yield (%)
1	4,44
2	5,86
3	6,12
4	6,06

The cumulative yield curve shows a behavior similar to previous experiments ending up achieving a step.

This experiment just like the others before, was also done one more time but the results can't be shown since this experiment has a methodology error. The sample was left at the open the entire night which was seen later that would affect the end results. Since the methodology is not corrected that second experiment shouldn't be shown since it's not in conformity with all the others.

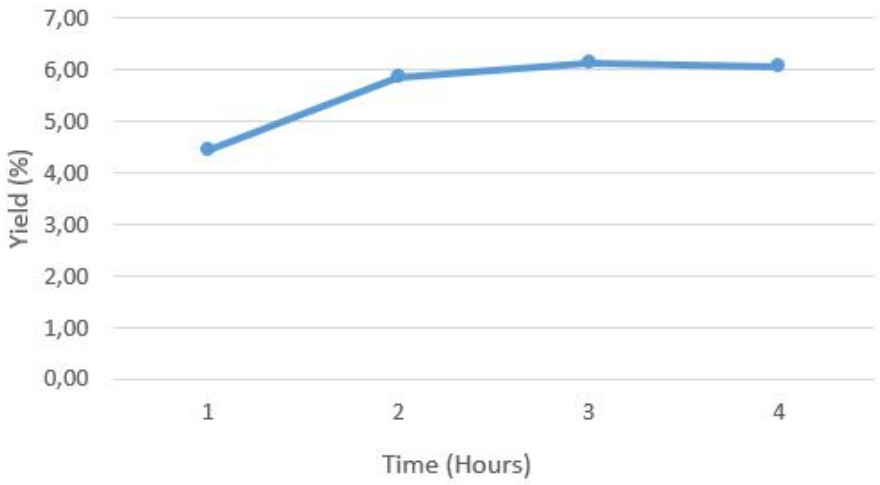


Figure 5.18: Cumulative Yield at the conditions of 200 bar and 60^oC (first experiment).

In the final set of conditions, the pressure was turn to 250 bar and all the other parameters were kept constant. The yield obtained in each hour shows an increase at different rates which, considering the previous experiences, it was expected. After the first hour the extraction yield starts at a low value of 4,12% and after 4 hours it achieves a yield close to 7%.

Simply by looking at the values is possible to see that a "step" was not achieved since the difference between the third and fourth hour yields is too great.

On the other hand, it does not seem to exist any disperse values which would lead into a weird looking extraction yield curve.

Table 5.18: Data from 250 bar and 60^oC (first experiment).

Time (Hours)	Cumulative Yield (%)
1	4,12
2	5,44
3	6,28
4	6,57

Has for the cumulative curve is possible to see an expected behavior where it tends into a step after 4 hours, achieving a yield closer to 7%.

Even though the behavior is according to what was expected the final yield seems to be high when compared to the literature. This could also be caused by the yield obtain in the first hour which was also higher than expected.

Theoretically, the higher the pressure, the higher would the extraction yield be. With this in mind, it is possible to see that the experimental data do not support such claim, and at some point, actually goes against it.

Further experiments need then to be made in order to understand how reliable are these values.

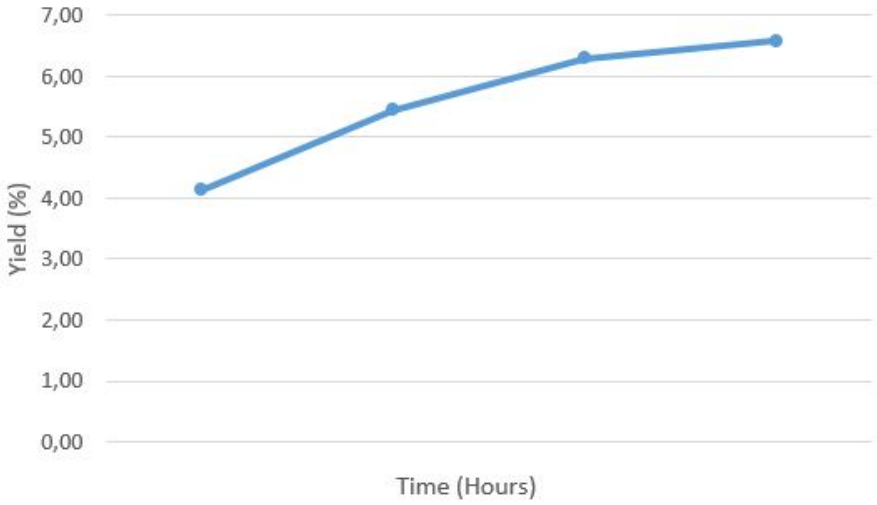


Figure 5.19: Cumulative Yield at the conditions of 250 bar and $60^{o}C$ (first experiment).

In order to compare the obtained values, the experiment was performed once more. Analyzing the yields obtained in each hour of extraction it's possible to confirm a similar behavior of the curve which tends into a "step".

When it comes to the values obtained it is possible to see that it starts with a slightly higher value, 4,53%, and curiously ends with a much smaller value, 5,87%, when compared with the previous experiments.

By looking at the values it does not seem to exist anything out of the ordinary for the first 3 hours, with a steady increase of the extraction yield.

On the other hand, this happens only in those first 3 hours since that at the fourth hour there's a very small increase of mass which leads to the value of the yield to be negative. This results in a decrease of the cumulative extraction yield.

Table 5.19: Data from 250 bar and $60^{o}C$ (second experiment).

Time (Hours)	Cumulative Yield (%)
1	4,53
2	5,64
3	5,89
4	5,87

The cumulative yield curve presents an expected shape, tending into a step very quickly. Even with the small mass increase, graphically it still looks like a step was formed in a range closer to 6%.

As it was mentioned before, theoretically these were the best operating conditions for extraction since they are a combination of highest temperature and pressure, so the fact that it tends into a step in only 4 hours goes into that expectation. But we can forget the fact that the extraction yield obtained after those 4 hours does not relate to that perspective when compared with other pressures at the same temperature.

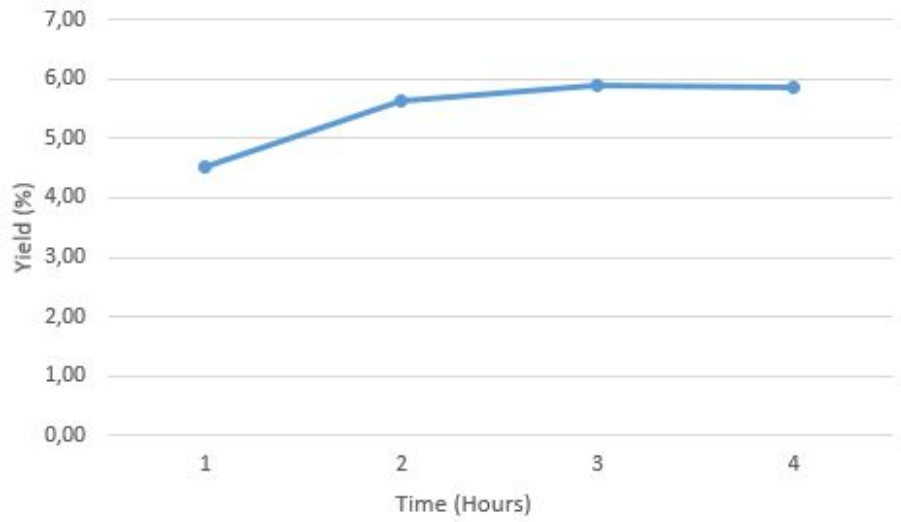
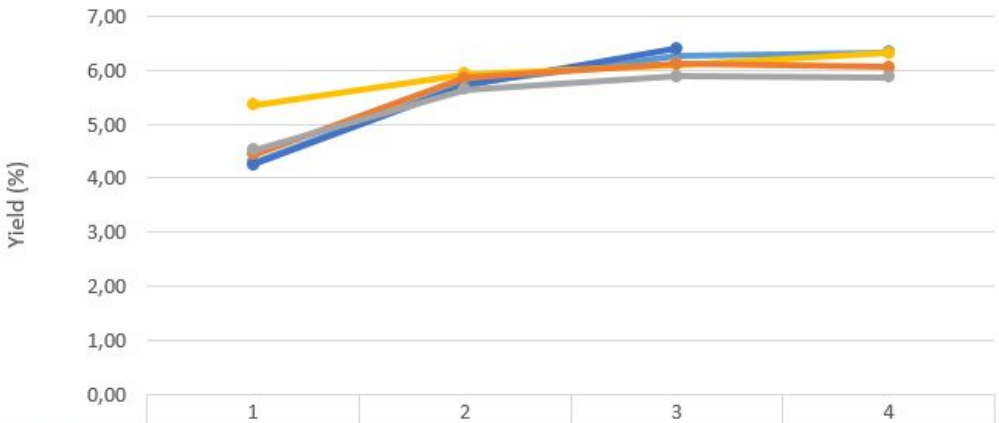


Figure 5.20: Cumulative Yield at the conditions of 250 bar and 60^oC (second experiment).

The values obtained for the different pressures were then organized into one graph so they could be compared and analyzed. Looking at the data is possible to see that all the curves have almost the same behavior and that tend to very close values which leads to think that there's no significant influence of pressure in this type of process and rest of set of conditions. The value of 100 bar was used at only three hours since that from all the experiments performed with those conditions is the only one that makes sense or allows a clear comparison with the other sets of pressure.



	1	2	3	4
70 bar	4,30	5,74	6,26	6,33
100 bar	4,25	5,72	6,40	
150 bar	5,36	5,94	6,10	6,32
200 bar	4,44	5,86	6,12	6,06
250 bar	4,53	5,64	5,89	5,87

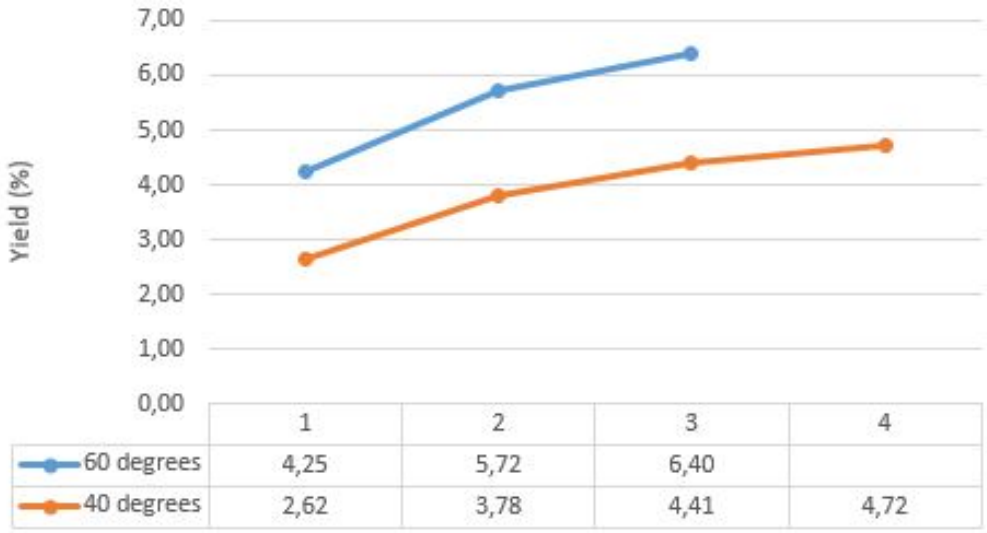
Figure 5.21: Comparison of different pressures at the temperature of 60^oC.

Like it was mentioned before, one of the main ideas taken from this work is that

temperature might have a strong influence on the extraction process.

With this in mind, the temperatures of 40^oC and 60^oC were compared for each pressure value considered.

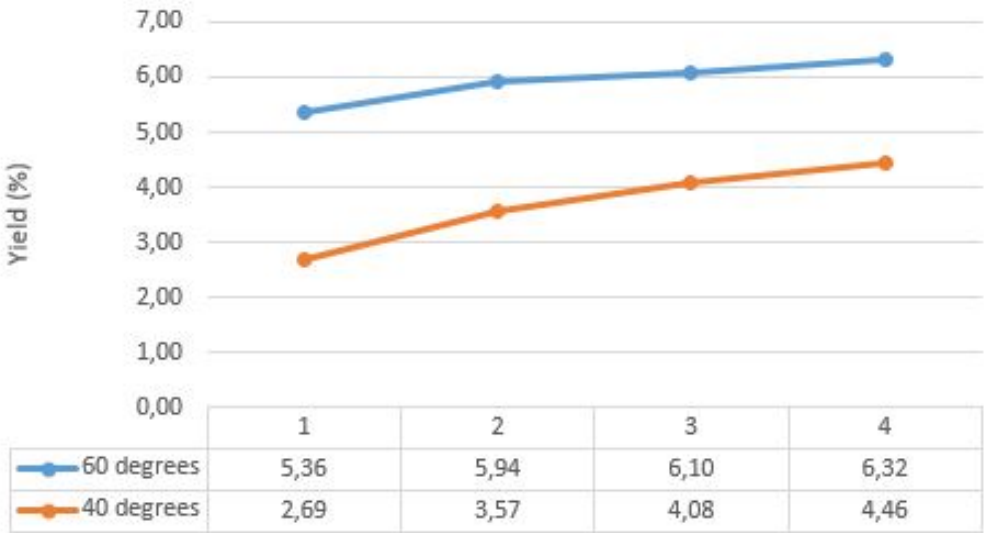
In the case of 100 bar, is clear that at 60^oC the cumulative yield shown suggest a better extraction, achieving higher values.



	1	2	3	4
60 degrees	4,25	5,72	6,40	
40 degrees	2,62	3,78	4,41	4,72

Figure 5.22: Comparison of different temperatures at the pressure of 100 bar.

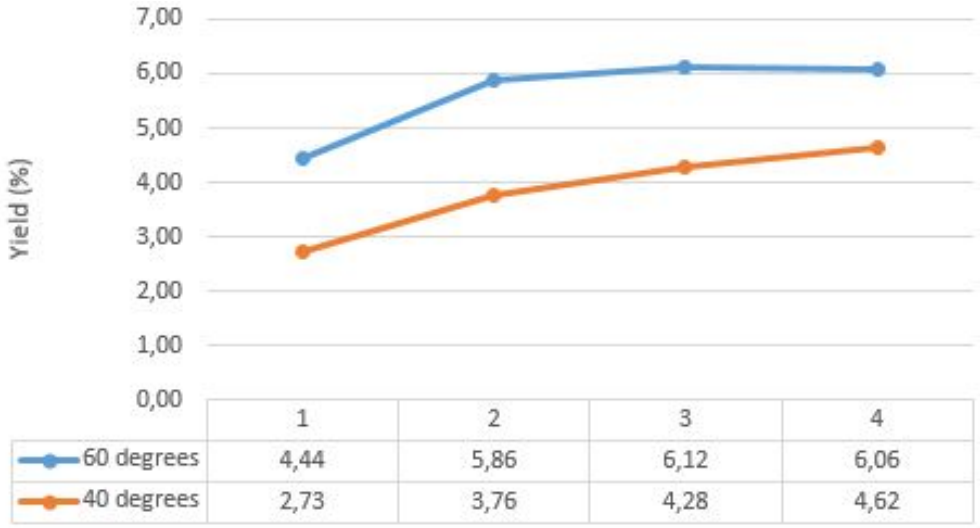
In the case of 150 bar the same relation is shown with a "step" being achieved much faster and the yield values being much higher for the temperature of 60^oC.



	1	2	3	4
60 degrees	5,36	5,94	6,10	6,32
40 degrees	2,69	3,57	4,08	4,46

Figure 5.23: Comparison of different temperatures at the pressure of 150 bar.

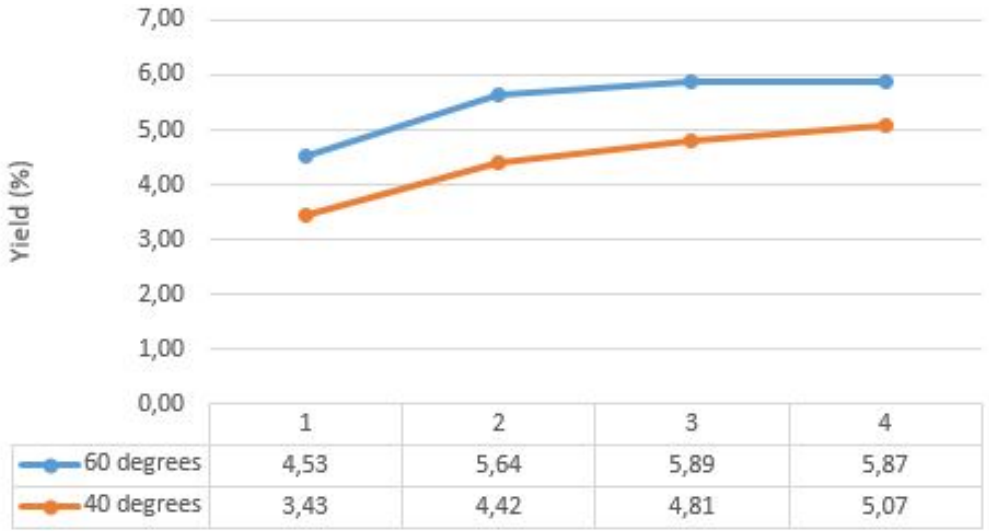
Again for 200 bar is clear that such "step" is achieved much faster at 60^oC. In fact, at 40^oC the step is not even achieved within the four hours of extraction.



	1	2	3	4
60 degrees	4,44	5,86	6,12	6,06
40 degrees	2,73	3,76	4,28	4,62

Figure 5.24: Comparison of different temperatures at the pressure of 200 bar.

In the case of 250 bar, again is possible to see this effect of the temperature on the overall process. It's probably one of the best cases to see the formation of the so called "step", when compared with the slight increase of the yield at $40^{o}C$.



	1	2	3	4
60 degrees	4,53	5,64	5,89	5,87
40 degrees	3,43	4,42	4,81	5,07

Figure 5.25: Comparison of different temperatures at the pressure of 250 bar.

This way, the effect of the temperature on the overall extraction process, suggests to be highly significant considering all the data presented.

Chapter 6

Conclusions

In this chapter, the aim is to present the main conclusions of the performed work.

Medicinal plants possess many compounds which can be used for pharmaceutical applications. Traditional extraction techniques were focused on using organic solvents or apply conditions that would degrade compounds being of interest. For that reason, this work was focused in application of supercritical fluid extraction to extract the medicinal based compounds from raw materials. This innovative method allows a more environmentally friendly extraction, which uses mild conditions to avoid compound degradation. By using CO_2 as solvent it is possible to remove its traces from the extract allowing to obtain a pure product. A great interest was demonstrated to study supercritical fluid extraction applied to raw plant materials because high quality extracts could be captured from them to apply those compounds in multiple areas. From the presented study, the following conclusions can be drawn:

1. The investigation was first focused on the plant Sassafras albidum with the main purpose of safrole extraction. Due to legal reasons, it became impossible to acquire the necessary compounds or even the raw plant material with a high content of safrole since a license is required in order to work with these type of compounds. Being impossible to proceed with the experimental part with this plant, provided us to search for an alternative subject. There it was made a decision to focus on investigation on another plant called Berberis vulgaris, with the objective of obtaining the compound berberine. This required an additional time for ordering and delivery of Berberis vulgaris, but our main purpose was to select this raw material to shorten the time of ordering and delivery to minimum. Several experiments

were performed in order to understand if the technique is valuable in this cases and identify the influence of the process parameters.

2. The study focused on supercritical extraction allow to state that is a valuable method for the extraction of the plant materials. According to mass differentiation the extraction yields were calculated as to be from 4,46% to 8,23%, guarantying that the extraction not only is possible but sufficient.

3. The influence of temperature in the supercritical fluid extraction process is visible because higher temperature increased the extraction yield within the same extraction time. The investigated temperatures were 40^oC and 60^oC. The same extraction time of four hours, at 60^oC made the extraction process faster achieving higher yields compared with the same conditions at 40^oC.

4. The influence of pressure in the extraction process was found not to be significant. At the temperature of 60^oC, the experimental extraction curves tend mostly to the same "step" values and present the same kind of behavior witch could indicate that the change in pressure does not affect the extraction process. At 40^oC on the other hand, it seems to have a small influence on the extraction yields obtained but the values are so close to each other that loses relevance. The influence of the extraction time is clearly visible since, in all experiments, it was possible to see that the longer the extraction process occurred, the more the curve would tend to a stabilization of the extracted amount. The typical behavior observed is, has time passes, that the differential yield presents a lower and lower value causing the cumulative yield curve to progress into a "step". This simply means that there is a maximum amount of extract can be obtained and that the longer the extraction process occurs the more the curve tends into that "step". It can also suggest that the process is controlled by the solute diffusion in the individual particles of the plant raw material and that an increase in the extraction time corresponds to more mass transfer resistance.

5. The obtained yields are not referent to berberine in specific but to the overall yield obtained by mass differentiation. Due to the high yield values obtained in the first extraction hour, the content of water in the plant material was considered to be significant. Importance of raw material studied as received or milled was investigated as well. The water contents observed in both milled and received materials were 0,20% and 0,21% respectively which leads to conclude that a large portion of what was extracted in the first hour is related to plant compounds other than water.The investigation was focused as well on recovering the powder. It is possible to conclude that even using a static extraction system instead of a dynamic one, containing a bigger volume (V = 1,2 L) and a larger amount of plant material as a sample, the extraction was possible allowing through mass difference a yield close to 3%. Powder was not obtained. Collection in acetone allowed to obtain a yellowish liquid and a strong smell, the same like in the plant. These physical properties don't

prove that berberine was present, but suggest that some residual amount might exist. Differential scanning calorimetry (DSC) was selected to analyze the obtained extract, by comparing the amount of heat necessary to increase the temperature of the extract with a reference of pure berberine. Unfortunately, sufficient amount of extract was not obtained in order to use this analytical method.

6. The application of supercritical extraction on the plant Berberis vulgaris showed good results and high prospects for future studies. From the experiments performed, it is clearly noticed that supercritical CO_2 could be a good option for the extraction of berberine from this plant. Nevertheless, this method will require more development and improvement with the possible introduction of co-solvents in the extraction process or develop an alternative analytical protocol such as with HPLC.

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Appendix A

Table Results

Table A.1: Data from 100 bar and 40^oC (first experiment).

Time (Hours)	Yield (%)	Cumulative Yield (%)
1	3,71	3,71
2	0,88	4,59
3	0,54	5,12
4	0,22	5,34

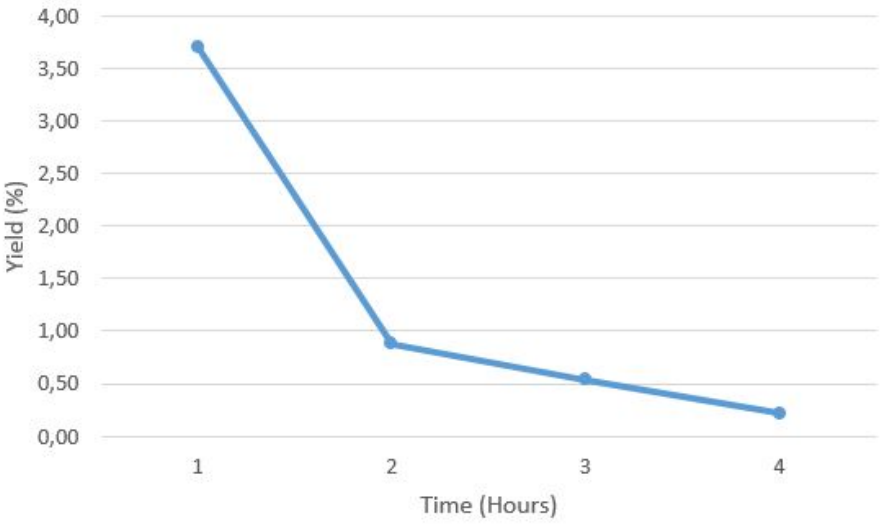

Figure A.1: Extraction Yield at 100 bar and 40^oC (first experiment).

Table A.2: Data from 100 bar and 40^oC (second experiment).

Time (Hours)	Yield (%)	Cumulative Yield (%)
1	2,62	2,62
2	1,16	3,78
3	0,62	4,41
4	0,31	4,72

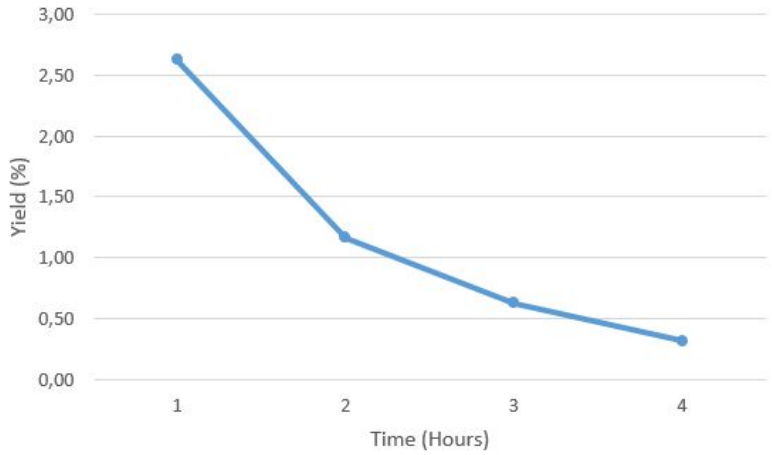

Figure A.2: Extraction Yield at 100 bar and 40^oC (second experiment).

Table A.3: Data from 150 bar and 40^oC (first experiment).

Time (Hours)	Yield (%)	Cumulative Yield (%)
1	2,57	2,57
2	1,30	3,87
3	1,16	5,03
4	1,07	6,10

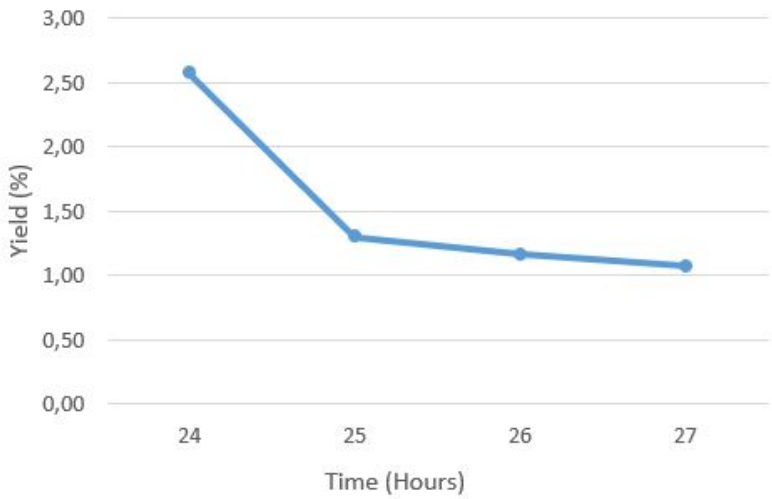

Figure A.3: Yield obtained at the conditions of 150 bar and 40^oC (first experiment).

Table A.4: Data from 150 bar and 40^oC (second experiment).

Time (Hours)	Yield (%)	Cumulative Yield (%)
1	2,69	2,69
2	0,88	3,57
3	0,51	4,08
4	0,38	4,46

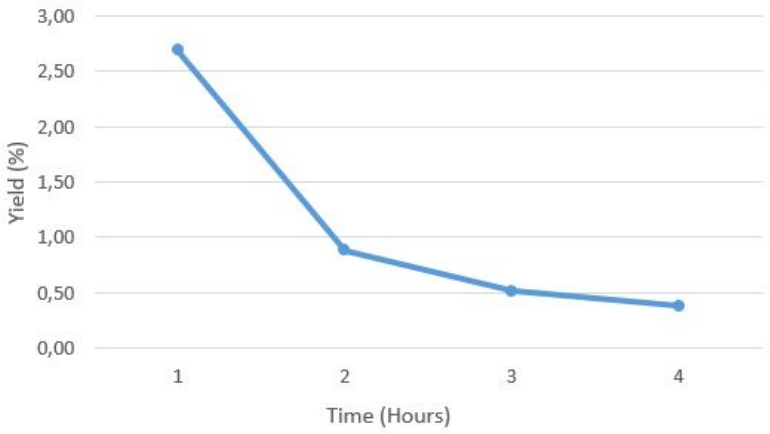

Figure A.4: Yield obtained at the conditions of 150 bar and 40^oC (second experiment).

Table A.5: Data from 200 bar and 40^oC (first experiment).

Time (Hours)	Yield (%)	Cumulative Yield (%)
1	3,53	3,53
2	1,06	4,59
3	0,20	4,78
4	0,55	5,34

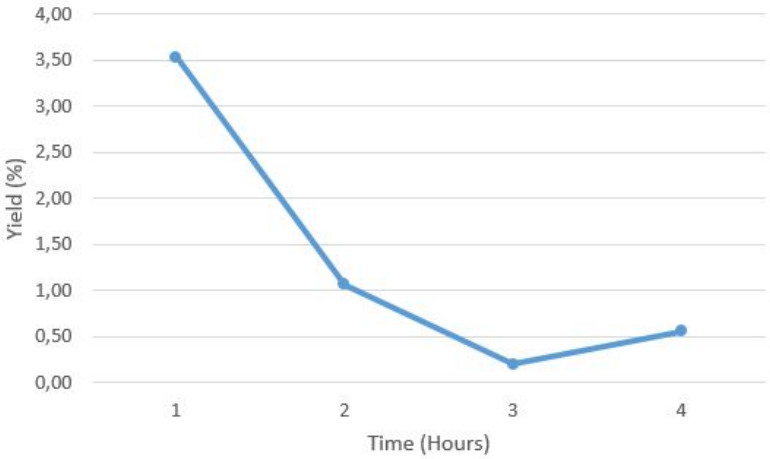

Figure A.5: Yield obtained at the conditions of 200 bar and 40^oC (first experiment).

Table A.6: Data from 200 bar and 40^oC (second experiment).

Time (Hours)	Yield (%)	Cumulative Yield (%)
1	2,73	2,73
2	1,04	3,76
3	0,52	4,28
4	0,34	4,62

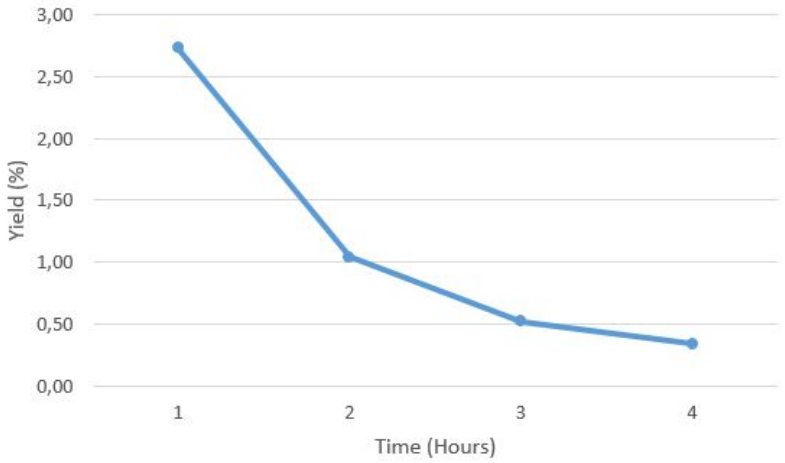

Figure A.6: Yield obtained at the conditions of 200 bar and 40^oC (second experiment).

Table A.7: Data from 250 bar and 40^oC (first experiment).

Time (Hours)	Yield (%)	Cumulative Yield (%)
1	2,83	2,83
2	1,24	4,08
3	1,25	5,33
4	0,29	5,62

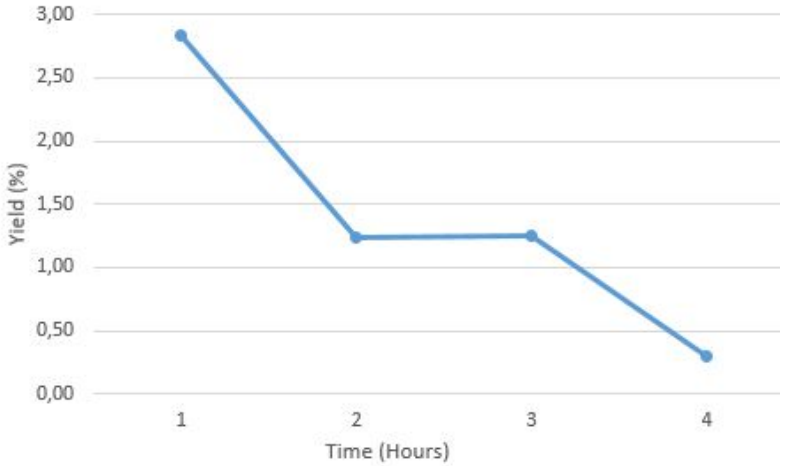

Figure A.7: Yield obtained at the conditions of 250 bar and 40^oC (first experiment).

Table A.8: Data from 250 bar and 40^oC (second experiment).

Time (Hours)	Yield (%)	Cumulative Yield (%)
1	3,43	3,43
2	0,99	4,42
3	0,39	4,81
4	0,26	5,07

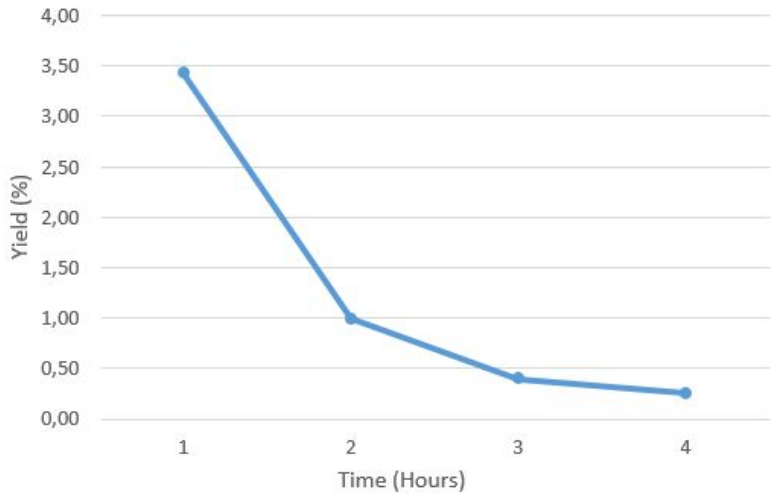

Figure A.8: Yield obtained at the conditions of 250 bar and 40^oC (second experiment).

Table A.9: Data from 70 bar and 60^oC (first experiment).

Time (Hours)	Yield (%)	Cumulative Yield (%)
1	5,84	5,84
2	1,10	6,94
3	0,72	7,66
4	-0,14	7,52

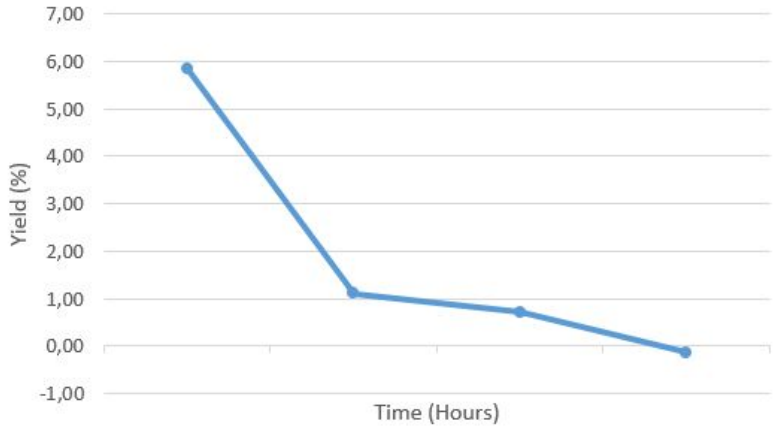

Figure A.9: Yield obtained at the conditions of 70 bar and 60^oC (first experiment).

Table A.10: Data from 70 bar and 60^oC (second experiment).

Time (Hours)	Yield (%)	Cumulative Yield (%)
1	4,30	4,30
2	1,44	5,74
3	0,52	6,26
4	0,07	6,33

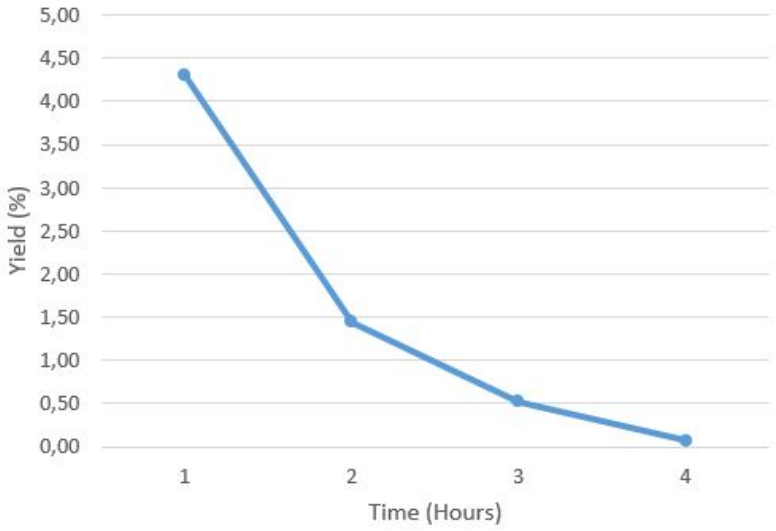

Figure A.10: Yield obtained at the conditions of 70 bar and 60^oC (second experiment).

Table A.11: Data from 100 bar and 60^oC (first experiment).

Time (Hours)	Yield (%)	Cumulative Yield (%)
1	5,10	5,10
2	2,10	7,20
3	0,18	7,37
4	0,86	8,23

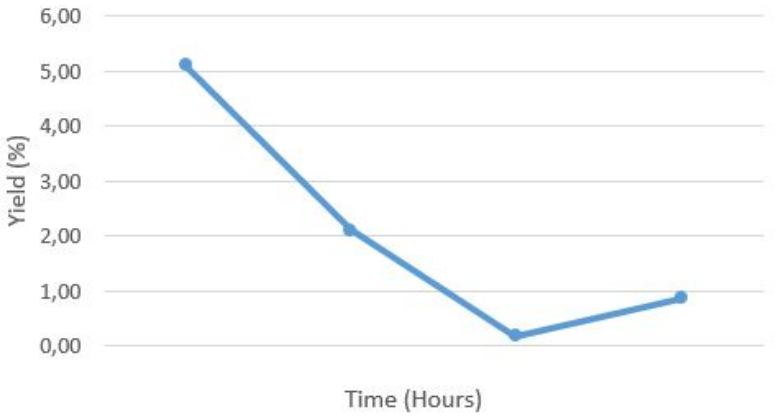

Figure A.11: Yield obtained at the conditions of 100 bar and 60^oC (first experiment).

Table A.12: Data from 100 bar and 60^oC (second experiment).

Time (Hours)	Yield (%)	Cumulative Yield (%)
1	6,51	6,51
2	0,38	6,89
3	-0,27	6,62
4	-0,23	6,38

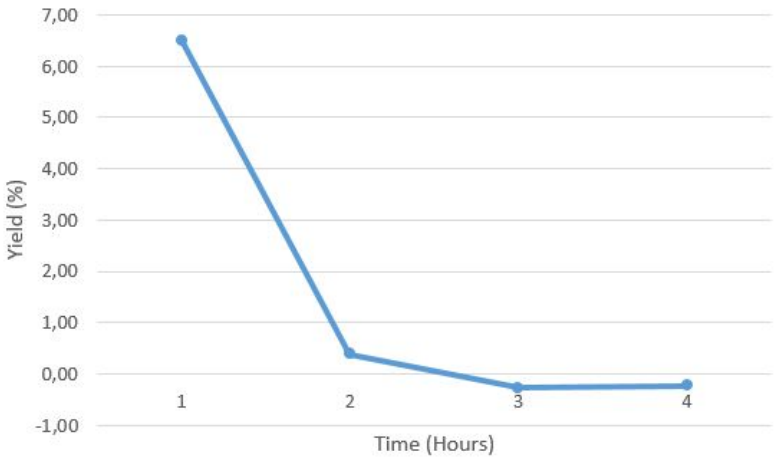

Figure A.12: Yield obtained at the conditions of 100 bar and 60^oC (second experiment).

Table A.13: Data from 100 bar and 60^oC (third experiment).

Time (Hours)	Yield (%)	Cumulative Yield (%)
1	4,25	4,25
2	1,42	5,72
3	0,66	6,40

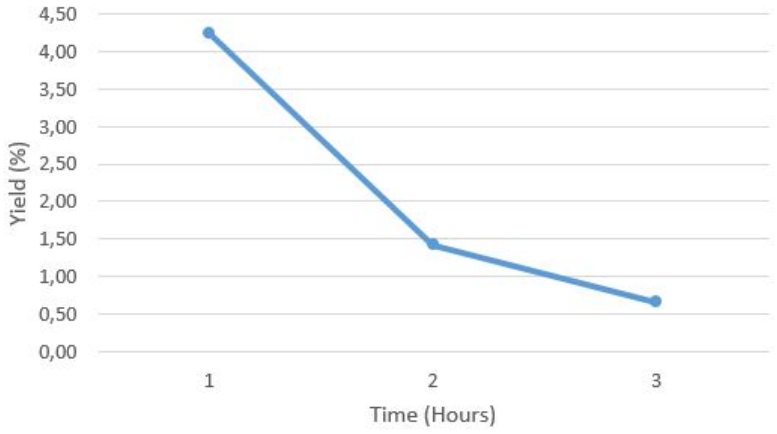

Figure A.13: Yield obtained at the conditions of 100 bar and 60^oC (third experiment).

Table A.14: Data from 150 bar and 60^oC (first experiment).

Time (Hours)	Yield (%)	Cumulative Yield (%)
1	3,54	3,54
2	1,89	5,43
3	0,69	6,12
4	0,30	6,42

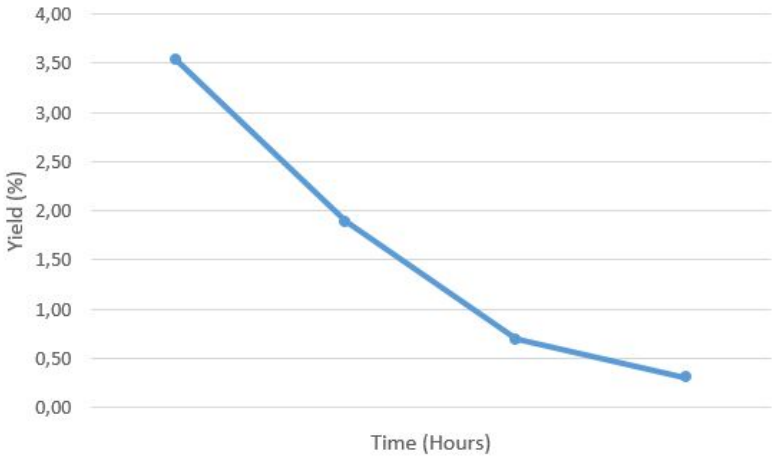

Figure A.14: Yield obtained at the conditions of 150 bar and 60^oC (first experiment).

Table A.15: Data from 150 bar and 60^oC (second experiment).

Time (Hours)	Yield (%)	Cumulative Yield (%)
1	5,12	5,12
2	0,94	6,05
3	0,17	6,23
4	0,32	6,55

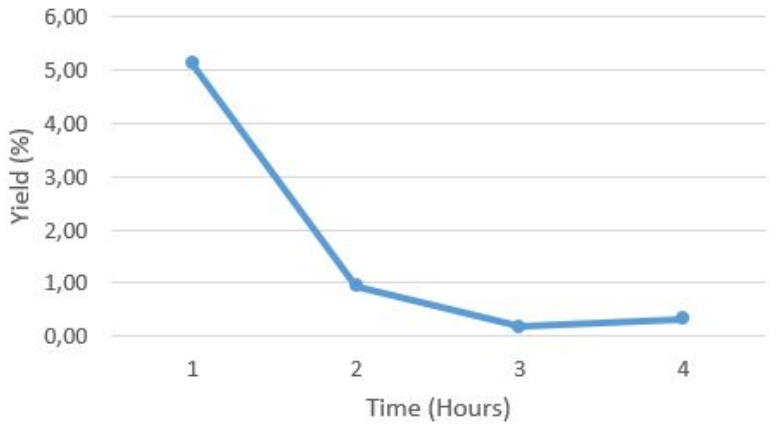

Figure A.15: Yield obtained at the conditions of 150 bar and 60^oC (second experiment).

Table A.16: Data from 150 bar and 60^oC (third experiment).

Time (Hours)	Yield (%)	Cumulative Yield (%)
1	5,36	5,36
2	0,58	5,94
3	0,16	6,10
4	0,22	6,32

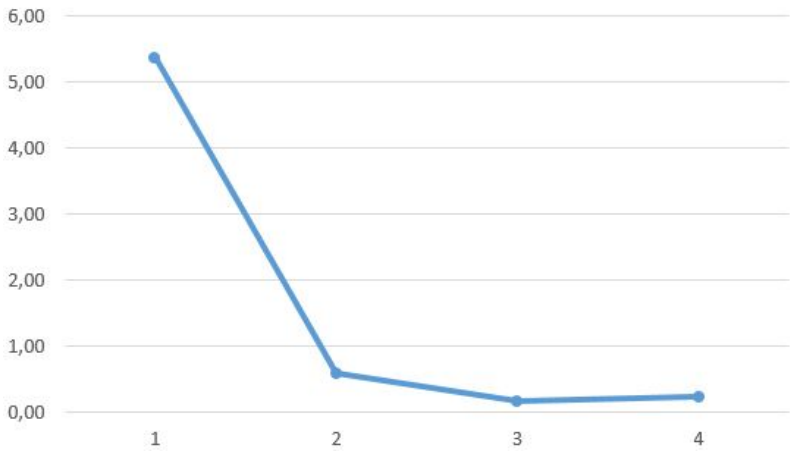

Figure A.16: Yield obtained at the conditions of 150 bar and 60^oC (third experiment).

Table A.17: Data from 200 bar and 60^oC (first experiment).

Time (Hours)	Yield (%)	Cumulative Yield (%)
1	4,44	4,44
2	1,42	5,86
3	0,26	6,12
4	-0,06	6,06

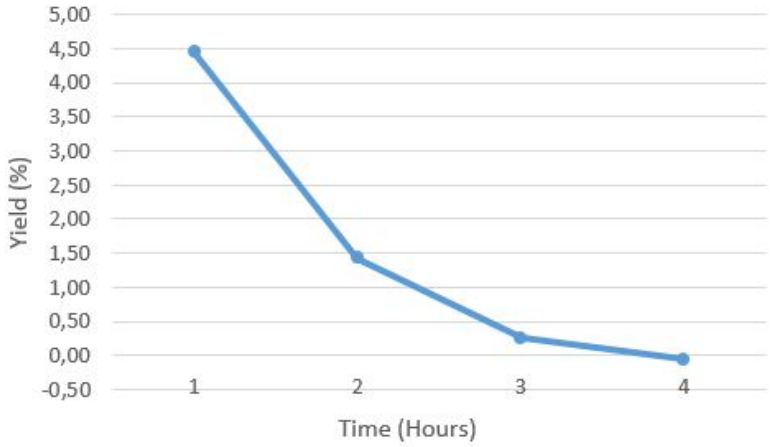

Figure A.17: Yield obtained at the conditions of 200 bar and 60^oC (first experiment).

Table A.18: Data from 250 bar and 60^oC (first experiment).

Time (Hours)	Yield (%)	Cumulative Yield (%)
1	4,12	4,12
2	1,32	5,44
3	0,84	6,28
4	0,29	6,57

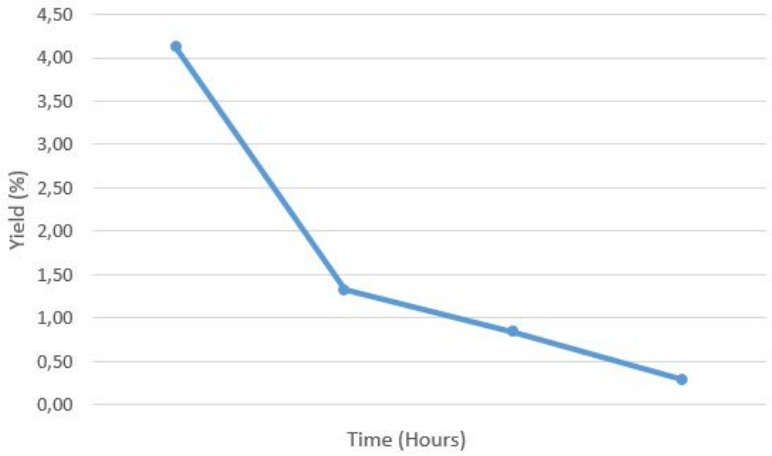

Figure A.18: Yield obtained at the conditions of 250 bar and 60^oC (first experiment).

Table A.19: Data from 250 bar and 60^oC (second experiment).

Time (Hours)	Yield (%)	Cumulative Yield (%)
1	4,53	4,53
2	1,12	5,64
3	0,25	5,89
4	-0,02	5,87

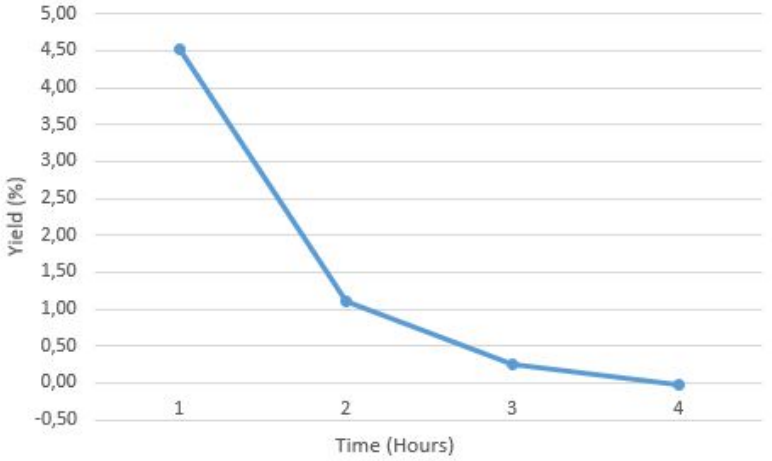

Figure A.19: Yield obtained at the conditions of 250 bar and 60^oC (second experiment).