A COMPARATIVE STUDY OF SUPERCRITICAL FLUID EXTRACTION AND ACCELERATED SOLVENT EXTRACTION OF LIPOPHILIC COMPOUNDS FROM LIGNOCELLULOSIC BIOMASS

This work is submitted in fulfilment of the requirements of the degree of Master of Applied Sciences: Chemistry in the Faculty of Applied Sciences at the Durban University of Technology.

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Declaration

The research contained in this thesis was completed by the candidate (Andile Khanyile) while based at the Department of Chemistry, Faculty of Applied Sciences, Durban University of Technology (DUT), Steve Biko Campus and at the Council for Scientific and Industrial Research, Biorefinery Industry Development Facility (CSIR-BIDF) Durban, South Africa, under the supervision of Prof. B.B Sithole, and co-supervision of Dr J.E Andrew and Dr V. Paul. The research was financially supported by the CSIR and the Department of Science and Innovation (DSI). The contents of this work have not been submitted in any form to another university. The results stated are the result of the candidate's studies, except when the work of others is acknowledged in the text.

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Dedication

This is dedicated to my family.

Ephesian 3vs 20

“Now all glory to God, who is able, through his mighty power at work within us, to accomplish infinitely more than we might ask or think.”

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List of Publications

The * indicates the corresponding author.

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Abstract

Lipophilic compounds are non-structural, heterogeneous compounds rich in terpenes, sterols, fatty acids, hydrocarbons, and glycerides. They have found widespread uses in different industries, such as the pharmaceutical, medical, cosmetic and nutraceutical sectors. They are typically extracted from wood using traditional techniques such as solvent extraction hydro- and steam- distillation. However, these techniques have several drawbacks such as long extraction times, high energy consumption, extensive solvent use and degradation of thermosensitive compounds, which are highly volatile. In this study, supercritical fluid extraction (SFE) and accelerated solvent extraction (ASE) were evaluated to extract lipophilic compounds from lignocellulosic biomass such as pinewood sawdust and Cannabis *Sativa* L. Their advantages of using low amounts of solvent, short extraction times and high selectivity allow them to be used as an alternative extraction technique to traditional methods. Moreover, SFE uses carbon dioxide, which is safe, cheap and readily available, and it does not alter the structure of the compounds. In contrast, ASE uses elevated temperatures and high pressures to prevent the evaporation of highly volatile compounds.

In order to solve challenges from both an economic and an environmental perspective, the interaction of process conditions on lipophilic compounds extraction efficiency was modelled and optimized using Response Surface Methodology (RSM) and Box-Behnken design (BBD). The extraction variables optimized for pinewood sawdust compounds were, SFE: co-solvent (ethanol) flow rate (1-2 ml/min), carbon dioxide (CO_2) flow rate (1-3 ml/min), Temperature (40-60 °C) and pressure (200-300 bar), and for ASE: static time (10-15 mins), static cycle (1-3) and temperature (80-160 °C).

The process parameters were optimized, and the experimental data was modelled using RSM for statistical analysis of the BBD extraction process. The experimental data's quadratic polynomial models gave a coefficient of determination (R^2) of 0.87 and 0.80 for ASE and SFE, respectively. The optimum conditions of ASE were temperature (160 °C), static time (12.5 mins), and static cycle (1), which resulted in a maximum yield of 4.2%. The optimum SFE conditions were temperature (50 °C), pressure (300 bar), CO_2 flow rate (3.2 ml/min), and a 2 ml/min co-solvent (ethanol)

flow rate that yielded 2.5% lipophilic compounds. The extraction efficiency of pinewood sawdust lipophilic compounds with ASE was higher compared to the SFE. Although ASE uses high temperatures that may degrade thermolabile compounds, the short extraction times may work in their favor since the extracts are not exposed to high temperatures for long periods. SFE uses low temperatures and long extraction times compared to ASE. Several properties affect the extraction efficiency, such as volatility, dissolving power, solubility, and fluid density of the extracting solvent. The extraction efficiency of lipophilic compounds by SFE may be affected by the supercritical fluid's solubility and differences in densities at different pressures. In ASE, the high yields were influenced by the high polarity of the solvent mixture and temperature with a short extraction time.

The extraction variables optimized using RSM for Cannabis *Sativa* L. for SFE were pressure (200-300 bar), co-solvent (ethanol) flow rate (1-2 ml/min) and CO_2 flow rate (1-2 ml/min). The R^2 was determined to be 0.9108. The optimum conditions were 300 bar pressure, 1 ml/min co-solvent (ethanol) flowrate, and 2 ml/min CO_2 flowrate, which gave a maximum yield of 88%. The high efficiency observed was brought by the increase in the flow rate of CO_2 at high pressures, which reduces the mass transfer resistance, while the cosolvent enhanced the solvating power of CO_2.

The ASE had a high extraction efficiency for the pinewood sawdust lipophilic compounds. However, the method's selectivity was very low according to the results obtained by pyrolysis gas chromatography-mass spectrometry (Py-GC/MS). The thermosensitive compounds, such as terpenes, decreased from 2.01% to 1.69% upon the addition of Tetramethylammonium hydroxide (TMAH). The initial concentration of terpenes was 7.21% in pinewood sawdust by SFE. Upon the addition of TMAH, the concentration of terpenes of the pinewood sawdust decreased to undetectable levels. The initial concentration of the terpenes of Cannabis *Sativa* L. was 14.29% and decreased in the presence of TMAH to 0.39%. The Fourier Transform Infrared Spectroscopy (FTIR) confirmed the presence of lipophilic compounds functional groups and a fingerprint region of lipophilic compounds of pinewood sawdust and Cannabis *Sativa* L. Thermogravimetric analysis (TGA), and differential scanning calorimetry (DSC) showed high thermal stability (250 – 400 ℃). This research demonstrated the ability of SFE to extract lipophilic compounds from pinewood sawdust Cannabis *Sativa L*.

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LIST OF SYMBOLS AND ACRONYMS

ABPR	Back pressure regulator
ANOVA	Analysis of variance
ASE	Accelerated solvent extraction
ATR	Attenuated total reflection
ATR	Total reflectance transmission
BBD	Box Behnken design
C.V %	Percentage of coefficient of variance.
CBD	Cannabidiol
CBDV	Cannabidivarin
CBN	Cannabinol
CO_2	Carbon dioxide
DSC	Differential scanning calorimetry
FTIR	Fourier transform infrared spectrometry
FTPP	Forestry, Timber, Pulp and Paper industry
GC/MS	Gas chromatography mass spectrometry
HD	Hydro-distillation
NIR	Near-infrared
Py(TMAH)-GC/MS	Pyrolysis(TMAH)-gas chromatography mass spectrometry

Py-GC/MS	Pyrolysis gas chromatography-mass spectrometry
R^2	Co-efficient of determination
RSM	Response surface methodology
SD	Steam distillation
SF-CO_2	Supercritical fluid carbon dioxide
SFE	Supercritical fluid extraction
STA	Simultaneous thermal analysis
Std. Dev	Standard Deviation
TGA	Thermogravimetric analysis
THC	Tetrahydrocannabinol
TMAH	Tetramethylammonium hydroxide
Δ-1(2)– THC	Delta-1(2)-tetrahydrocannabinol
Δ^8– THC	Delta-8-tetrahydrocannabinol
Δ^9– THC	Delta-9-tetrahydrocannabinol

CHAPTER 1

This chapter provides a brief insight into the waste disposal limitations and disadvantages of traditional extraction techniques for extracting lipophilic compounds from lignocellulosic biomass. The SFE and ASE techniques to overcome challenges brought by traditional extracting techniques are discussed. Further, both the aim and the study objectives are presented.

1.1 INTRODUCTION

Legislation to reduce environmentally harmful products has already begun to focus more resources on developing eco-friendly products based on natural resources due to increased energy consumption, disposal limitations, and building sustainability. Benefits of developing eco-friendly products based on natural resources, especially lignocellulosic biomass, include soil erosion reduction and higher overall land productivity.

The Forestry, Timber, Pulp and Paper (FTPP) industry contributes about 6% of the country's manufacturing GDP and is a major source of job growth, providing jobs to about 170000 people (Andrew et al., 2020b). The lumber and felling of wood produce tons of waste, leaving residues such as sawdust, wood chips, and logs unused. The disposal of waste has become a significant challenge confronting the forestry industry. This is primarily due to increased difficulty locating disposal sites and complying with waste management and disposal legislation's demanding environmental quality criteria (Andrew et al., 2020b).

Sawmills and pulp mills wood processing activities include chipping, screening, sawing, turning, drilling, or sanding, producing tons of sawdust waste. It was estimated that the amount of sawdust produced by South African sawmills in 2012 was projected to be roughly 376,000 tonnes if the proportion of sawdust generated from sawn timber averages around 20% (Olufemi et al., 2012). Compost companies and board manufacturers use this waste to produce compost and furniture; however, these activities are still not enough to manage the sawdust waste, leaving tons of the sawdust waste stockpiled in landfills. Significant expenditures are incurred to create

and upkeep landfill sites and transportation waste to these sites when landfilling is used as a waste disposal method.

Sawdust is an example of a lignocellulosic biomass waste that can be used to obtain high-value products through biorefinery technologies. Compared to petroleum resources, lignocellulosic biomass has a higher proportion of oxygen, lower portions of hydrogen, carbon, and classes of cellulose, hemicellulose, lignin, and non-structural extractives can be obtained by lignocellulosic feedstock biorefineries. Lignocellulosic feedstock biorefineries include the process of refining lignocellulosic biomass (wood, straw, etc.) into intermediate outputs (cellulose, hemicellulose, lignin, and extractives) that can be processed into value-added products, bio-materials, and fuel (de Jong and Jungmeier, 2015).

Cannabis growing has attracted disadvantaged or socially marginalized farmers who are already vulnerable and eager to take risks in exchange for a chance to profit. Cannabis has been produced by hiding the crop in difficult-to-access places or outback areas. The revenue from the cannabis crop has not been sufficient to alleviate poverty, and agricultural economics also disfavors people who lack capital. Moreover, many cannabis producers in South Africa live in areas where drug enforcement is more extensive than other government services.

The Southern Eye (2014) have reported that farmers in a district without access to modern healthcare tried unsuccessfully to legalize the cannabis they farmed for traditional therapeutic purposes. Even though the constitution has legalized cannabis to be planted for medicinal use and casual growing, possession for citizens, the farmers still do not benefit due to the lack of funds and resources to process cannabis for medicinal use. Even the alleged licensing rates, while low in contrast to the value of the Global Northern cannabis market, are far more than most South Africans could afford to pay for the opportunity to cultivate cannabis legally.

Since the Middle Ages, cannabis has been utilized to cure various diseases, and its medicinal value includes intoxicant, anti-inflammatory, and other therapeutic benefits. Cannabis constitutes cannabinoids, fatty acids, terpenes, and sterol that have been achieved through different extraction techniques over the past few years.

Over the years, traditional extraction techniques such as solvent extraction, hydro-distillation, steam distillation, maceration and enfleurage to obtain extracts processing

were of great significance. They have been used to obtain lipophilic compounds from sawdust (Gutiérrez et al., 1998a) and cannabis (Valizadehderakhshan et al., 2021). Lipophilic compounds are heterogeneous compounds consisting of fatty acids, fatty alcohols, resin acids, hydrocarbons, steroids, triterpenoids, and triglycerides. Most of these compounds are sensitive to heat and degrade when exposed to high temperatures. Employing traditional extraction methods to obtain lipophilic compounds from biomass has many disadvantages. These include the use of large amounts of toxic organic solvents that require disposal post-extraction, which is costly, long extraction times, and high temperatures which degrades thermolabile compounds and extracts unwanted compounds; also, the use of large amounts of organic solvents leads to large amounts of solvent waste residues in the final product that require removal and purification of the product which is time-consuming and costly (Talmaciu et al., 2016, Ház et al., 2016). The use of supercritical fluid and accelerated solvent extraction techniques offers an environmentally friendly and efficient alternative to high amounts of organic solvents as they require less solvent, has low-moderate extraction times, low energy required for extractions which are good for the protection of thermolabile compounds, and higher selectivity in the removal of wood components that add value to the extract, thus reducing the extraction of unwanted compounds (Garcia-Mendoza et al., 2017, Toubane et al., 2017).

Since sawdust and cannabis are composed of potentially high-value products that have found uses in a host of applications from cosmetic, pharmaceutical, lubricants, coatings, detergents to nutraceuticals as edible oils, phytochemicals, flavors, fragrances, and colors, using alternative and innovative extraction techniques for sawdust may transform the face of the FTPP industry, both economically and environmentally. Furthermore, cannabis can help marginalized farmers make a profit, reduce poverty and create job opportunities. This study focuses on optimizing the yield of lipophilic compounds from pinewood sawdust and cannabis by accelerated solvent extraction and supercritical fluid extraction. The extraction efficiency of these two techniques was compared according to their percentage yields.

Process optimization is a critical aspect in designing economically feasible processes. Process performance is influenced by many process parameters, including the temperature, pressure, time, and solvent amount. RSM has been used to model and optimize processes; it addresses all process parameters simultaneously, decreasing

computational complexity and simplifying the issue formulation (Shabaka and ElMaraghy, 2008). RSM is a method for improving and optimizing chemical processes that combine progressive mathematical and empirical methodologies. It assesses from experimental runs, targets response and experimental factors. Optimization is one of the most important techniques for developing a reliable chemical process for industrial applications. Equally, the model would provide valuable suggestions on the analysis, design and operation of the process that will be of enormous importance in scaling up the process. The BBD with RMS was used to optimize the experiments in this study.

1.2 Problem Statement

Demands to utilize environmentally friendly extraction techniques to produce high-value products from lignocellulosic biomass have increased worldwide due to large amounts of toxic organic solvents, long extraction times, and high temperatures that degrade thermal sensitive compounds, disposal issues of waste and poor selectivity associated with traditional techniques.

Due to the negative aspects of traditional methods, the application of supercritical fluid and accelerated solvent extraction provides an environmentally friendly and efficient alternative to using high amounts of organic solvent, long extraction times and high energy currently being used. The SFE and ASE use short extraction times, low temperatures, less solvent, and high selectivity.

Therefore, this project proposes to evaluate the efficiency of SFE and ASE as alternative green technologies to extract clean, high-quality lipophilic compounds from lignocellulosic biomass. SFE has advantages over traditional methods such as using carbon dioxide as a solvent due to its non-flammability, non-toxicity, and recyclability properties. It does not result in the extract's thermal degradation. No solvent residues on the final product remain. ASE has significant advantages over traditional techniques as it uses small amounts of solvents, is fast, and can use elevated temperatures over short periods.

1.3 Aim and Objectives of Study

1.3.1 Aim

The main aim of this study is to compare the extraction efficiency of SFE and ASE techniques to extract lipophilic compounds from lignocellulosic biomass such as pinewood sawdust and Cannabis *Sativa* L. to achieve high extraction yields

1.3.2 Objectives

Pinewood sawdust

- To optimize ASE extraction conditions: temperature, static time, and static cycle to obtain high yields of lipophilic compounds from pinewood sawdust and evaluate the effect of extraction conditions on lipophilic compounds.
- To optimize SFE conditions: temperature, pressure, co-solvent concentration to obtain high yields of lipophilic compounds from pinewood sawdust and evaluate the effect of extraction conditions on lipophilic compounds.
- To compare the extraction efficiency of accelerated solvent and supercritical fluid extraction techniques.

Cannabis *Sativa* L.

- Optimizing the SFE conditions: pressure, cosolvent flow rate and CO_2 flowrate to obtain high extraction efficiency from Cannabis *Sativa* L. and evaluate the effect of extraction conditions on the lipophilic compounds.

- To characterize the extracts (pine sawdust and Cannabis *Sativa* L) for chemical composition by Py-GC/MS and pyrolysis(TMAH)-gas chromatography/mass spectrometry (Py(TMAH)-GC/MS).
- To confirm the presence of functional groups of lipophilic compounds of sawdust and Cannabis *Sativa* L. by FTIR.
- To study the thermal stability of lipophilic compounds of sawdust using STA.

1.4 Thesis Statement

Lipophilic compounds can be found in large quantities in pine sawdust. As a result, pine sawdust may be used as a source of valuable products that can be used to make

lubricants or for oil production. The compounds of Cannabis *Sativa* L. extract can be used in pharmaceuticals or medicinal industries, which may profit the marginalized farmers.

1.5 Thesis Outline

This thesis is divided into 6 chapters as follows:

Chapter 1 introduces the availability of pinewood sawdust and Cannabis *Sativa* L. and provides the context for the study, giving the problem statement and providing the Aims and Objectives.

Chapter 2 Literature review on the chemistry and extraction of lipophilic compounds from lignocellulosic biomass such as pinewood sawdust lipophilic compounds and Cannabis *Sativa* L.

Chapter 3 Details the theoretical principles of supercritical fluid extraction, accelerated solvent extraction, Fourier transforms infrared spectroscopy, thermal analysis and pyrolysis-gas chromatography-mass spectrometry used to extract and characterize the lipophilic compounds.

Chapter 4 Describes the materials and methods applied to design and conduct the experiments in this work. This chapter includes using the response surface method and box-Behnken design to optimize the experiments for maximum yields.

Chapter 5 The results and discussions of findings obtained from implementing experimental designs and characterization techniques are entailed in this chapter.

Chapter 6 Outlines the overall conclusions of the studies. This chapter is inclusive of future recommendations.

References

Appendix

CHAPTER 2

2. LITERATURE REVIEW

This chapter focuses on the definition of lipophilic compounds from pinewood sawdust and Cannabis *Sativa* L. The chemistry of wood lipophilic compounds, followed by summary of the percentages of extractives found in different wood species, details on the distribution of lipophilic compounds mainly in sapwood and heartwood and a brief description of softwood and hardwood. The chemistry of the cannabis plant is described followed by the description of cannabis lipophilic compounds. Various extraction methods and other solvents for extracting lipophilic compounds are discussed briefly, followed by a description of different characterization techniques commonly used for qualitative and quantitative study of lipophilic compounds.

2.1 Pine Wood Sawdust

Pinewood sawdust is a timber and wood processing industry waste material containing chemical components, carbon (60.8%), hydrogen (5.2%), oxygen (33.8%) and nitrogen (0.9%) (Horisawa et al., 1999, Phonphuak and Chindaprasirt, 2015). Pinewood sawdust is widely used as a cooking fuel, packaging material and adsorption process because of its functional groups like carboxyl, hydroxyl, phenolic and amide groups (Zhou et al., 2015). However, a huge chunk of sawdust is disposed of in the landfills, a threat to the environment. It causes environmental degradation, loss of aesthetic value, and unpleasant odors resulting in air pollution. Also, being exposed to the sawdust causes respiratory threats to humankind (Owoyemi et al., 2016). Pine sawdust can be used as a source of valuable material as it contains a significant amount of lipophilic compounds that the pulp and paper industry have thoroughly studied (Zhou, 2011).

2.2 Wood Lipophilic Compounds

Lipophilic compounds are non-structural wood components of several different chemicals found in wood, such as fatty acids, resin acids, waxes, alcohols, terpenes, sterols, sterol esters, and glycerides (Akalın et al., 2017). They are primarily soluble in water and various organic solvents such as ethanol, benzene, dichloromethane, and chloroform (Kirker et al., 2013, Zasadowski et al., 2012, Eriksson et al., 2018, Yokoi et al., 2003, Soon and Chiang, 2012). Lipophilic compounds are commonly referred to as lipophilic extractives which cause sticky and pitch deposits in the pulp and paper industry, resulting in the pulp's poor quality giving rise to spots in the bleached pulp and paper (Sitholé et al., 2010, Sjöström, 1993).

Extractives are diverse compounds that make up 4-10% in most temperate wood species and 20% in tropical wood species. (Ashori and Nourbakhsh, 2010, Sjöström, 1993). These lipophilic compounds are volatile, nonpolar and low molecular weight molecules with various chemical properties (Golander, 2011). They contribute to the wood's color, basic density, and durability and defends the tree against insects, rotting, and fungus attacks. In addition, the amount and type of these compounds can alter the mechanical strength, color, and quality of wood (Sheshmani et al., 2012)

The number of lipophilic compounds differs with the species type species growth conditions, such as the geographical location, surrounding environment, and climatological. For instance, the quantity of extractives in Scots pine (*Pinus Sylvestris*) ranges between 2.5- 4.5%, Norway spruce (*Picea abies*) ranges between 1.0- 2.0%, and silver birch (*Betula pendula*) ranges between 1.0- 35% (Yualin Zhou,2011). Regardless of the species' growth conditions, the quantity of extractives in hardwood usually is high compared to softwoods (Demirbaş, 2004). The illustration of wood lipophilic compounds is shown in Figure 1.

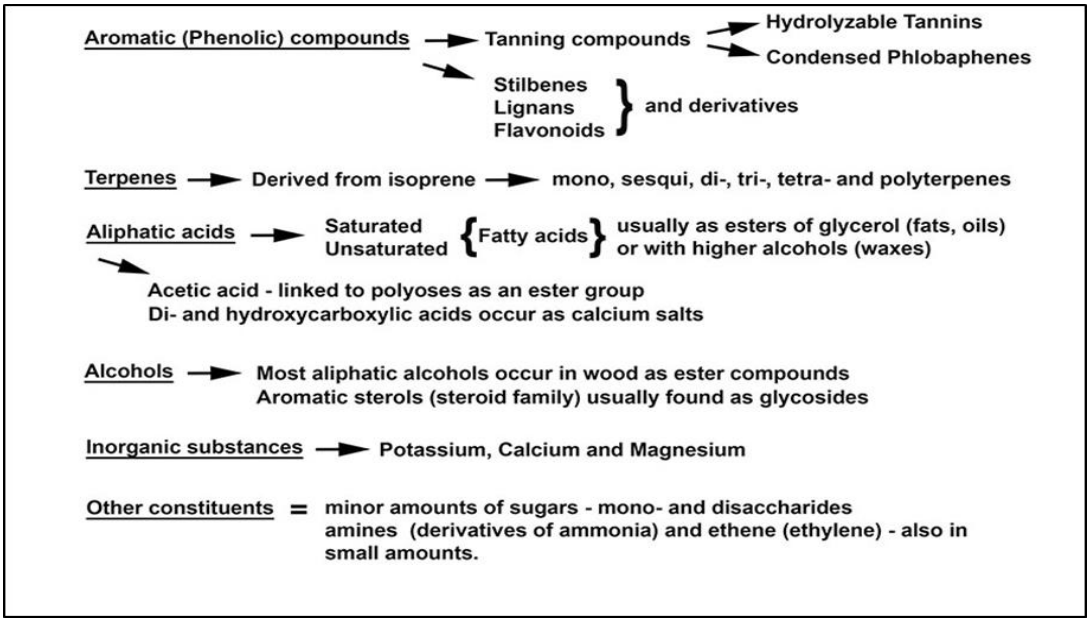

Figure 1: Illustration of wood extractives (Theander (1985).

2.3 Distribution of Lipophilic Compounds Constituents

Sapwood, heartwood, hardwood, and softwood pine contain lipophilic compounds components distributed in the parenchyma and epithelial cells, resin canals, intermediate lamellae, intercellular spaces, and the cell walls of tracheid, libriform fibers, and arteries, among other tree compartments (Myronycheva et al., 2018). Heartwood is a type of wood resistant to degradation caused by natural chemical transformations within the plant and contains many extractives. Sapwood is the outermost wood, and it is responsible for transporting water from the roots to the leaves (Gominho et al., 2015). The ray parenchyma resin and the resin canals both include softwood extractives (Wijayanto et al., 2015), while extractives dominate hardwoods due to the absence of resin canals (Sjöström and Westermark, 1999).

The chemical makeup of extractives and their content are not evenly distributed throughout the wood. The tree's morphological part and age, geographical patterns, and location of the tree in the forest determine their variation (Moodley, 2011, Terziev et al., 1997, Bikovens et al., 2013). The lipophilic compounds found from different tree parts are arranged from the low-molecular-mass resin and fatty acids to high-molecular-mass waxes, sterol esters, and triglycerides (Bikovens et al., 2013, Gutiérrez et al., 1998b).

2.3.1 Heartwood and sapwood

According to Eriksson et al. (2018), radial orientations in wood stems distinguish sapwood and heartwood extractives of the genus *Pinus*. The lipophilic extractive, especially fatty and resin acid, is richer in the sapwood and heartwood of the genus *Pinus*. However, heartwoods contain more lipophilic extractives than sapwood (Willför et al., 2003b). The position of heartwood and sapwood in a tree is shown in Figure 2.

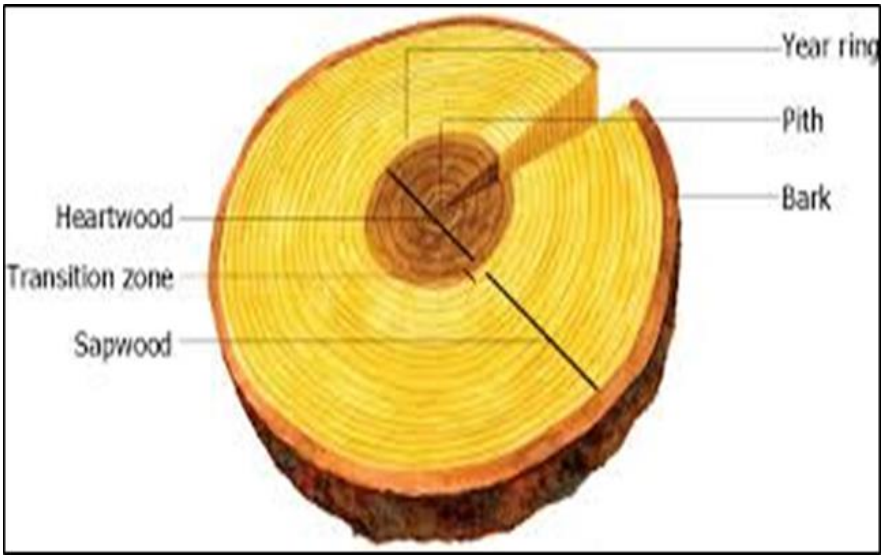

Figure 2: The composition of heartwood and sapwood in Scots pine wood (Lim, 2017)

Ekman and Holmbom (1989) highlighted that the amount of resin acids in heartwood is higher than in sapwood. In spruce and pine trees, heartwood contains fewer triglycerides and more free fatty acids than sapwood (Örså et al., 1997). Other compounds concentrated in the outer part of sapwood can be classified into sugars, starch, phenyl glycosides and certain phenols (Örså et al., 1997).

Heartwood consists of mainly phenolic extractives; when the sapwood is transformed into heartwood, the phenolic and other components are synthesized and deposited in the heartwood region (Sjöström and Westermark, 1999). The resin acids are highly concentrated above and underground of the heartwood, while the concentration of lipophilic extractives is high in the lower parts of the tree (Eriksson et al., 2018, Uusitalo, 2004, Koch, 1996). Table 1 shows the main compounds found in spruce and pine wood.

Table 1: Chemical composition and their approximate percentage in spruce and pine

Tree species	Spruce	Pine
Cellulose %	39.5	40.0
Hemicellulose %	30.6	40.0
Lignin %	27.5	27.7
Extractives %	2.1	3.5
Others %	0.3	0.3

2.3.2. Softwood and hardwood

Softwood is often white, non-durable, light, insect and fungal resistant. Hardwood is often robust, heavy, non-durable, fungal, and insect resistant. The significant elements of any form of wood, whether hardwood or softwood, are cellulose, hemicelluloses, lignin, and extractives (Silvy et al., 2018). According to Räisänen et al. (2009), softwood extractives are made of resin and fatty acids, which differ depending on the wood species, location, and growing conditions. Hardwood contains fatty acids, which vary depending on climates and seasons, inhibited in hot areas. However, the content of lipophilic extractives in hardwood is higher than that of extractives in softwood (Räisänen et al., 2009). The composition of lipophilic compounds is listed in Table 2 below

Willför et al. (2003a) compared lipophilic extractives of heartwood knots from seven Norway spruce trees. Five of the dead knots had higher resin acids and free diterpenyl alcohols in their lipophilic extractives compared to regular stem wood of the Norway spruce tree.

Table 2: Composition of the main lipid classes present in extractives of softwood and hardwood (Moodley, 2011).

Class of lipid in extractives	Pine sapwood (mg/100g wood)
Fatty Acids	4.0 ±1.0
Resin acid	3.9 ± 0.9
Sitosterol	0.2 ±0.02
Waxes	1.6 ± 0.3
Sterol esters	1.2 ± 0.2
Triglycerides	7.3 ± 2.0

2.4 Lipophilic Extractive Classes of Wood

2.4.1 Resin acids

Resin acid or rosin acids are wood lipophilic extractives that are insoluble in the water yet soluble in organic solvents such as dichloromethane, diethyl ether, ethanol, methanol, and hydrocarbon solvents like hexane (Willför et al., 2003c). They consist of mainly abietic, dehydroabietic and pimaric acid. Figure 3 illustrates distinct types of resin acid found in wood. The oleoresin fraction of coniferous wood species holds resin acids (Kim et al., 2019, Wiyono et al., 2006). Tall oil, a by-product of the kraft process, is collected commercially and used to manufacture resin acids. During the kraft process, fats and waxes in the lignocellulosic feedstock are transformed into soluble sodium salts of fatty and resin acids (Alén, 2000). Resin acids have been reported to be high in knots (4.5-30 % w/w) compared to stem wood (Willför et al., 2003c). As for heartwood, the variation in resin acids content can be explained by wood tissue type. Scots pine heartwood is better for resin extraction than lodgepole pine hearts because the extractives are evenly distributed for fatty- and resin acid extraction (Arshadi et al., 2013).

Softwood resin is commonly referred to as oleoresin located in resin canals. It constitutes tricyclic diterpenoic acids with similar structures (Jansson and Nilvebrant, 2009b). Resin acids in resin canals (oleoresin) have biological properties that protect the wood from deterioration. The physiological resin, which is found in the ray parenchyma cells and acts as a reserve food supply, is high in lipids, waxes, and

sterols. The softwood resin acids can be classified into three groups: aromatic, conjugated diene, and alkene (Gellerstedt et al., 2009). The carbon skeleton is lipophilic, whereas the carboxyl group is hydrophilic. Almost all acids have the same three-ring structure. The heartwood extractives' wood resin is deposited in the wood, and the sapwood-heartwood boundary is where the secondary phase of heartwood development occurs. Hardwood resin is the most common source of triterpenoids.

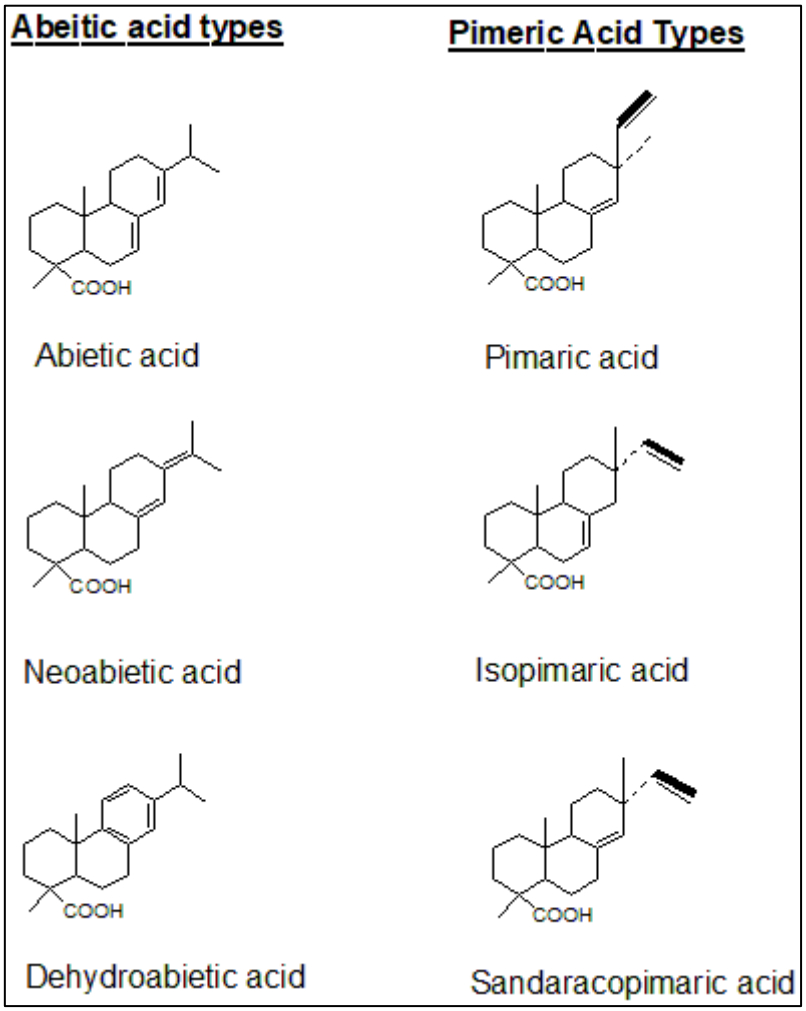

Figure 3: Pine and spruce resin acids classified as abietic and pimaric acids, adapted from (Jansson and Nilvebrant, 2009b).

It has been demonstrated that resin acids have different uses, from gasification to wax. Ajji (2006) prepared composites from pinewood resin using gamma radiation. Compounds such as butyl acrylate, butyl methacrylate, styrene, acrylamide, acrylonitrile, and unsaturated polyester styrene resin were used to produce the wood/polymer composites with pinewood. Pine resin was also used as the base for

the embalming fluid during mummification, adhesives, soaps, water-repellent surface coatings for ropes construction. However, one of its main applications is the manufacture of gum rosin with widespread use in the paper industry (Phun et al., 2017, Giuffra et al., 2011).

2.4.2 Terpenes

Terpenoids are defined as terpenes with different functional groups such as hydroxyl, carbonyl or carboxyl, and they exist in resin canals of conifers (Breitmaier, 2006). Terpenes are one of the primary compounds of lipophilic extractives. Terpenes and their derivatives are mainly cyclic compounds with a general appearance in the plant and animal kingdom (Breitmaier, 2006). Isoprene units are the basic structural unit of terpenes (5 carbons). They can also be classified into subgroups based on how many isoprene units are bound together in a terpene: monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), and triterpenes (C_{30}), as illustrated in Figure 4 below. The monoterpenes and sesquiterpenes are primary industrial importance (Jansson and Nilvebrant, 2009b).

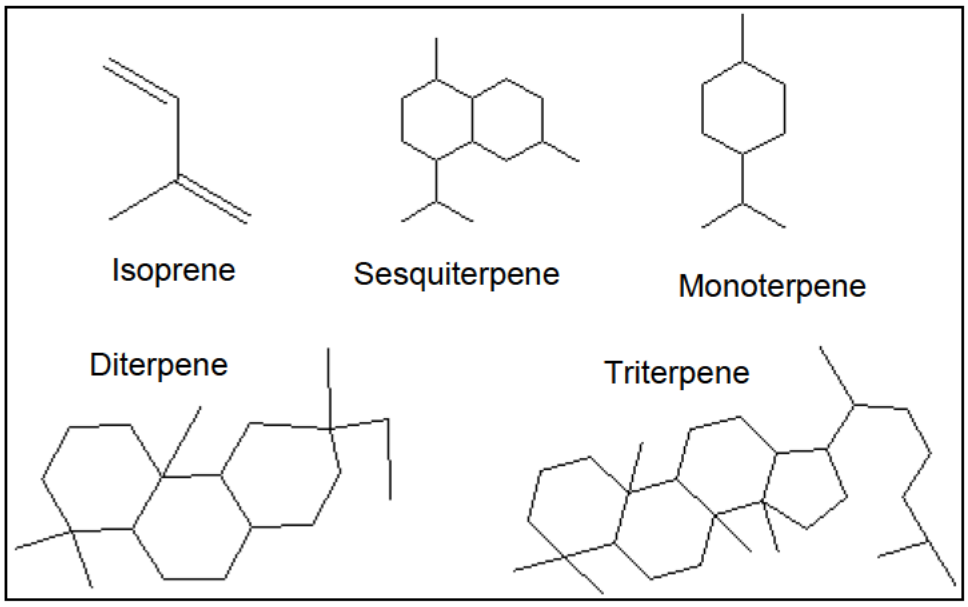

Figure 4: Isoprene units of different classes of terpenes,

adapted from (Jansson and Nilvebrant, 2009a)

The composition of terpenes in hardwoods and softwoods differs noticeably. Softwood terpenes include mono-, sesqui-, diterpenes, and sterols, whereas hardwood terpenes

mostly contain sterols, triterpenoids, and higher terpenes (Akay et al., 2011). The softwood resin terpenes give an aroma to the pine trees; such terpenes are classified as mono- and sesquiterpenes (Negi et al., 2020).

Monoterpenes are a class of highly volatile compounds consisting of acyclic, mono-, bi- and tricyclic compounds with different chemical properties, also known as secondary metabolites (Räisänen et al., 2009). The volatility of a monoterpene compound depends on its vapor pressure within the tissue where it is synthesized or stored, controlled by temperature and the compound concentration within the tissues (Räisänen et al., 2009, Jansson and Nilvebrant, 2009b). The concentration of volatile monoterpenes has been around 0.02-2%, emitted in the wood industry, particularly during softwood debarking, chipping, steaming, mechanical refinement, and chemical pulping (Bertaud et al., 2017). They're a source of compounds used in solvents, perfumes, and tastes, and they have shown promise as a biofuel and as solvents and synthetic precursors for flavors and fragrances (Harman-Ware et al., 2016). They also found pharmaceuticals, agrochemicals, food additives, and bioenergy uses (Panda, 2008). They are the major constituents of volatile oils and are widely used in industry as solvents for paints and varnishes. It can also be used as a cleaning solvent and a starting material for pharmaceuticals and other organic compounds, fragrances, aromas in perfumes, food flavourings, and the insecticide industry (Giuffra et al., 2011). Examples of common monoterpenes found in plants are illustrated in Figure 5.

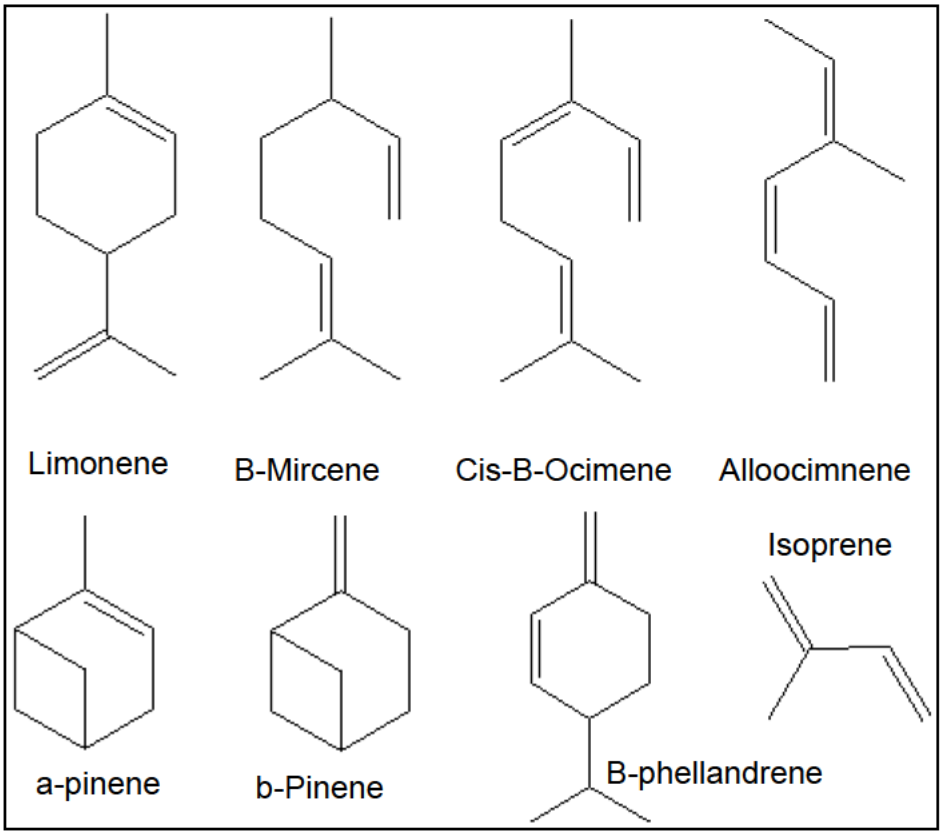

Figure 5: Illustration of monoterpenes structure,

adapted from (Mosquera et al., 2021).

With the molecular formula $C_{15}H_{24}$, Sesquiterpenes are substantially bigger molecules than monoterpenes, and they are also much more stable. Acyclic, monocyclic, bicyclic, tricyclic and tetracyclic sesquiterpenes are characterized by the number of carbon rings in their structure. Depending on their size, rings are divided into five, six, seven, and eleven membered rings (Chappell and Coates, 2010). It is simple to detect their physicochemical features and physiological activities if they are categorized according to the contained group in their structure, such as sesquiterpene alcohols, aldehydes, lactones. In addition, the sesquiterpenes are found in fungus, sponges, lichens, and protective waxes of insects (Akay et al., 2011). Anti-inflammatory, anticancer, antibacterial, and antifungal properties are biological properties present in these substances (Perveen and Al-Taweel, 2018). Sesquiterpenes also play an important part in plant growth hormones and signaling in response to their surroundings. Sesquiterpenes can be utilized as a natural insecticide and attract mates or mark territory for some insects and mammals, such as elephants (Cox-Georgian et al., 2019).

2.4.3 Fatty acids

Fatty acids are composed of a long chain of aliphatic mono-carbon acids with a carboxyl group at one end and a methyl (omega) group at the other. The number of carbon atoms in each differs in the saturation level (saturated, unsaturated, containing carbon-carbon double bond) (Golander, 2011). Saturated fatty acids are hydrogen-filled. Straight hydrocarbon chains with an even number of carbon atoms make up most saturated fatty acids. Fatty acids with 12–22 carbon atoms are the most frequent saturated fatty acids, as illustrated in Figure 6. Linoleic acid is one of the most abundant fatty acids in plant lipids. Demirbaş (1991) extracted fatty acids from beech wood by Soxhlet and SFE and analyzed them by chromatography. It was found that linoleic acid was the predominant fatty acid. Other fatty acids discovered were linolenic and palmitic acids.

Monounsaturated fatty acids with a single carbon-carbon double bond present in various places are known as unsaturated fatty acids (Kirk, 1998). The most common monounsaturated have a chain length of 16–22 carbon atoms with a cis-configured double bond, which means the hydrogen atoms on both sides of the double bond face the same direction. The structure of a fatty acid affects its melting point, with branched chains and cis double bonds having a lower melting point than saturated chains. Furthermore, whether the chain is even or odd determines the melting point of fatty acids, with the latter having higher melting points. Saturated fatty acids are highly stable, whereas unsaturated fatty acids are prone to oxidation: the higher the number of double bonds, the greater the oxidation vulnerability. Fatty acids derivatives are vital in cell signaling and act as a storage resource for cells as neutral lipids. As constituents of phospholipids, the "building blocks" of cell membranes, the fatty acids provide structural functions.

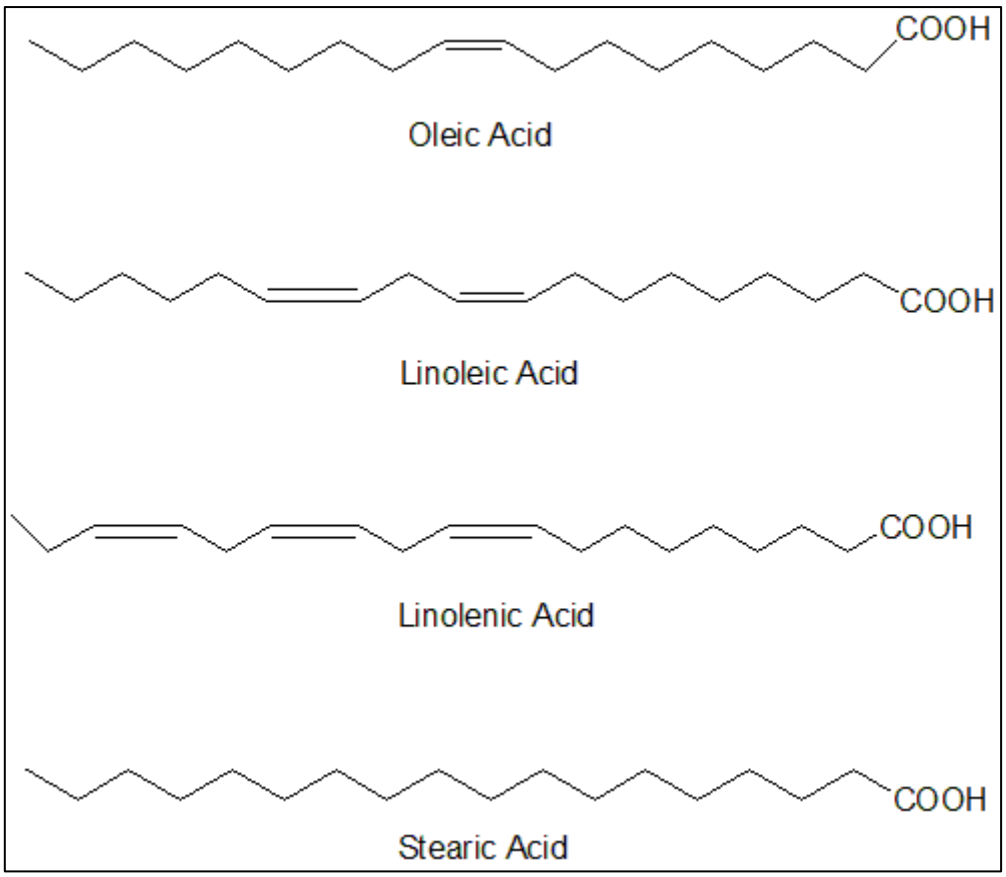

Figure 6: Structure of saturated and unsaturated fatty acids,

Adapted from (Bajpai, 2018)

2.4.4 Sterols and sterol esters

Sterols have a similar structure based on the cyclopentane-perhydrophenanthrene ring system, with a hydroxyl group and an 8–10 carbon lateral chain linked to carbon 17 (Ferrer et al., 2017). They are oxygenated compounds (Golander, 2011). Sterols are found in various forms, including free sterols (free alcohol), steryl esters, steryl glycosides, and acyl steryl glycosides, as illustrated in Figure 7 below. The hydroxyl group is esterified with a fatty acid in steryl esters. The presence of sugar connected to a hydroxyl group of the sterol molecule via a β-glycosidic bond distinguishes steryl glycosides (Ferrer et al., 2017).

Sterols possess β-sitosterol and methyl dehydro-abietate, which contain antibacterial, antiinflammation, anticancer, neuroprotection, and antioxidant properties (Burčová et al., 2018, He et al., 2018). Campesterol is a precursor for brassinosteroids, a family of hormones that regulate plant development and morphogenesis (Vriet et al., 2015,

Ferrer et al., 2017). However, plant sterols, frequently found as crystalline powder, have limited oil solubility and a higher melting point, limiting their use in food with a hydrophobic matrix. On the other hand, plant sterols are water-insoluble, making them difficult to use in nutrition with a hydrophilic matrix. The heartwood has a high content of sterols than sapwood (Saranpää and Nyberg, 1987), and in Norway spruce (Picea abies L. Karst) trunks, the number of free sterols and steryl esters increases in a centripetal direction (Höll and Lipp, 1987).

According to He et al. (2018), (Plat and Mensink, 2005), more than 250 plant sterol species have been discovered in plants and marine creatures. Plant sterols are further separated into 4,4-dimethyl sterols, 4-monomethyl sterols, and 4-desmethyl sterols based on the number of methyl groups on carbon-4. 4,4-dimethyl sterols and 4-monomethyl sterols are found in small amounts, whereas 4-desmethyl sterols are plentiful.

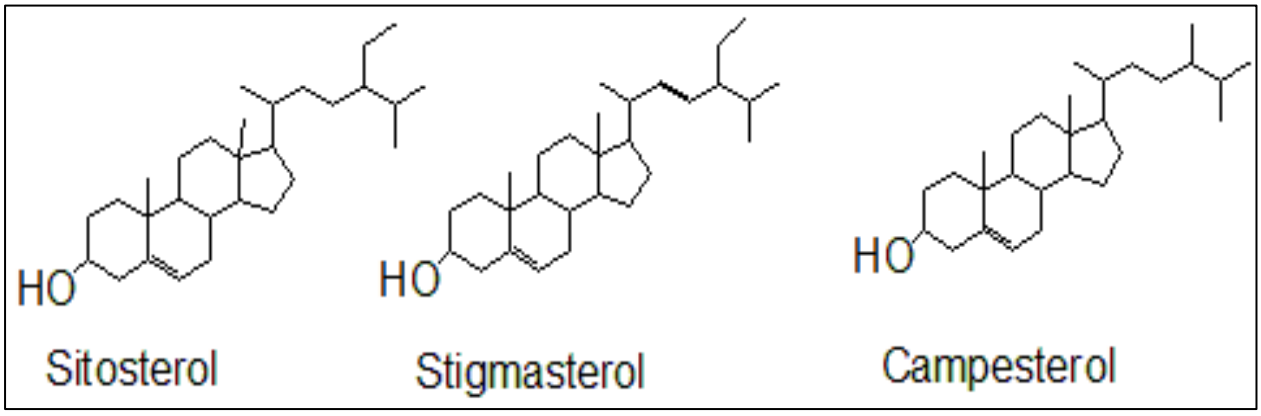

Figure 7: The chemical structure of sterols,

adapted from (Olivares et al., 2012).

2.5 Application of Lipophilic Extractives

Extractives are the predominant contributors to wood color, fragrance, and durability. Extractives also influence the pulping, drying, adhesion, hygroscopicity, and acoustic properties of wood (Kim et al., 2019, Wiyono et al., 2006). Many extractives have specific biological activities, and various woods have been used as a source of crude drugs and medicines for centuries. In recent studies, wood extractives have been employed for different purposes; Eriksson et al. (2018) extracted fatty and resin acids

from pine wood to manufacture tall oil diesel and resin acids, such as glues and inks (Harman-Ware et al., 2016).

2.6 Extraction Methods

Several extracting methods have been used to isolate lipophilic extractives from wood, sawdust, cones, barks, and knot wood of pine trees. Many researchers use traditional methods to separate lipophilic extractives from different species, such as distillation and solvent extraction techniques, as they are cheap and straightforward. These conventional techniques are associated with significant drawbacks, like using toxic solvents, leaving residues in the final product, large waste production, and high energy consumption (Yousefi et al., 2019). However, with the growing demand for safe, sufficient, clean, and environmentally friendly extracting techniques, different techniques such as accelerated solvent extraction, microwave digestion, and supercritical fluid extraction (Kilic et al. 2011; Mustafa and Turner 2011; Benouadah et al. 2019) are widely employed to reduce challenges faced with traditional methods. Different extraction techniques will be discussed for this study, with special emphasis on supercritical fluid extraction and accelerated solvent extraction.

2.6.1 Soxhlet extraction

In the presence of insoluble contaminants, Soxhlet extraction has historically been utilized for a solid material with limited solubility in a solvent. Simple extractions combine the homogenized mixture with a solvent that dissolves the desired components. The extraction solvent is continually cycled through the matrix by boiling and condensation, with the sample collected in the hot solvent (Luque de Castro and Priego-Capote, 2010). Different solvents extract different lipophilic constituents during extraction with solvent extraction. According to the Tappi T204 cm-97 method, dichloromethane extraction gives lower extractives than acetone or ethanol/benzene. Extraction with 1/3 ethanol and 2/3 benzene typically provides the highest extractives due to the further dissolution of low molecular weight carbohydrates and polyphenols. These generalizations do not apply to all wood species (Tappi T204 cm-97).

Mattos et al. (2016) confirmed that dichloromethane gives low extraction percentages. The amount of extractives removed by ethanol: toluene was three times more, at 2.85% compared to 0.65% extracted by dichloromethane. Apart from the low yield, dichloromethane has the problem of producing large amounts of dangerous chlorinated waste (Thurbide, 2000). In a study, hexane and petroleum ether extracted lipophilic extractives (Sun et al., 2002). Both solvents produced low extractives, with

hexane yielding 0.74% and petroleum ether yielding 0.55%. The polarity of solvents influences the amount of extractives extracted; the least polar solvent extracts a small amount of extractives.

Even though no solvent can altogether remove wood extractives, Soxhlet extraction with acetone, or even better, acetone: water (9:1), is a suitable solvent for standard wood resin components. It is harmless to human health and the environment because it is inert and stable. It can quickly be evaporated, leaving no water behind (Holmbom, 1999). However, Soxhlet is not different from other traditional methods. It also lacks selectivity, produces lower yields, and poses a safety and environmental risk due to the enormous amount of organic solvents and longer extraction times that result in compounds' disintegrating (Rasul, 2018).

2.6.2 Soxtec extraction

The Soxtec extraction process is a three-stage process. A sample containing a thimble is immersed in boiling solvent for around 60 min in the first stage. The sample-containing thimble is removed from the solvent in the second step, and the process is repeated as in the Soxhlet extraction method. This second stage can last up to 60 min. Solvent evaporation within the Soxtec apparatus happens in the final stage, lowering the final extract volume to 1-2 ml in 10–15 min. Soxtec extraction has several advantages over Soxhlet extraction, including faster extraction, less solvent usage, and sample concentration directly within the equipment. Refluxing the sample in a flask equipped with a condenser is a simple alternative to Soxtec (Sithole et al., 2013b).

Soon and Chiang (2012) used hexane, methanol and hot water to investigate the effects of extraction solvents on lipophilic extractives such as fatty acids, sterols, glycerides, and steryl esters. Due to volatile components, gas chromatography GC was used to analyze methanol and hexane extracts, while HPLC was used to analyze hot water extracts. Various polarity solvents extract different extractives, with hot water extracting the greatest total extractives from sapwood, and methanol extracting the most from heartwood. Sapwood extracted with hexane yielded 2.12% extractives, sapwood extracted with methanol yielded 4.09% extractives, and sapwood extracted with hot water yielded 5.15% extractives. Hexane-extracted heartwood had a 3.24%

yield, methanol-extracted heartwood had a 4.09% yield, and hot water-extracted heartwood had a 6.45 % yield.

Sithole (2010) extracted lipophilic extractives in sulphite pulps using Soxtec and Soxhlet with acetone. Both extraction methods can be used, but freeze-drying mode generates repeatable data. Analysis of the extracts by gas chromatography-mass spectrometry (GC/MS) to find the components present showed that only about 40% of the extracts were volatile enough to be analysed by the technique. The major compounds found were fatty acids, car-bamodithioic acid methyl ester, diethyldithiocarbamic acid, adipic dihydrazide and adipic acid. Soxtec extractions have several drawbacks, including a long extraction time, the possibility of target chemical degradation due to local warming effects, and a limited solvent selection (Arias et al., 2009).

2.6.3 Accelerated solvent extraction (ASE)

ASE operates at high temperatures and pressures, and tiny volumes of solvent are utilized to increase the sample matrices solubility and mass transfer (Yokoi et al., 2003). It is rapid and operates in most situations under an inert atmosphere of nitrogen gas, which allows the extraction to be completed quickly. Hot pressurized solvents are more effective at solubilizing extractives and penetrating the material (Willför et al., 2006). Different studies have optimized the extraction of lipophilic compounds from different tree parts. Little information has been published about optimizing the ASE to extract lipophilic compounds from pinewood sawdust.

Bikovens et al. (2013) studied the production of lipophilic extractives from grey alder bark, knot-wood and cones using ASE and compared the resulting lipophilic extractives' properties. ASE was used with n-hexane under a nitrogen atmosphere. The highest number of lipophilic extractives was obtained from cones, and it was found to be two times higher than in other parts. The stem wood contained fewer lipophilic extractives than knot wood, bark and cones.

Xu et al. (2010) also extracted lipophilic extractives with n-hexane from *Populus x euramericana* "Guatiento" stem-wood and bark. Glycerides were the most important component group of the lipophilic extractives, with 70% to 80% in stem wood and 60% to 70% in barks.

Benouadah et al. (2019) highlighted the chemical composition of lipophilic extractives from sapwood and heartwood of *Pinus halepensis* Mill stem. The extraction was carried out under operating conditions of 90°C, 138 MPa, and static cycle 3 × 5 min with n-hexane for lipophilic extractive. The heartwood was found to have a high concentration of lipophilic extractives of 1.6% compared to sapwood with 1.1%. The lipophilic extractives consisted mainly of oleoresin (resin acids, terpenes), fats (fatty acids, glycerides, steryl esters, sterols) and waxes (fatty alcohols) in both sapwood and heartwood.

Kilic et al. (2011) found *Pinus brutia* to have the highest amount of lipophilic extractives (35.1 mg/g) compared to *Pinus halepensis* (31.3 mg/g) and *Pinus sylvestris* (9.0 mg/g) had the lowest amount. Resin acids were the major lipophilic group in all pinus trees.

Thurbide (2000) used acetone to isolate extractives from wood pulp. The extraction process involved flushing the sample compartments with three volumes of fresh acetone at the same pressure (150 atm) and temperature (100 ºC). They concluded that the ASE was 30 times faster and used 75% less solvent than Soxhlet extraction. ASE can be performed with simple pumping and heating equipment, is compatible with numerous organic solvents of varying polarity, and can provide an efficient alternative to using a Soxhlet apparatus for extracting wood pulp.

2.6.4 Extraction parameters for ASE.

2.6.4.1 Temperature

One of the most important parameters affecting the efficiency and selectivity of ASE is the temperature during the extraction. High temperatures aid in the disruption of the analyte-sample matrix connections generated by van der Waals forces, hydrogen bonding, and dipole attraction, which improves extraction efficiency (Moreau et al., 2003). The surface tension of the solvent, solutes, and matrix is reduced as the temperature rises, which improves the sample's solvent wetting. Solvent cavities can be produced more easily if the surface tension of the solvent is reduced, allowing analytes to dissolve more quickly. However, high temperatures result in the coextraction of unwanted compounds and affect thermosensitive compounds when extracting lipophilic compounds even though high temperatures and a polar solvent enhanced the extraction yield (Moreau et al., 2003).

2.6.4.2 Static extraction time and the number of cycles

The static extraction time and the number of cycles is two related parameters. The number of cycles refers to how many times a new solvent enters the cell and comes into touch with the sample, whilst static time is how long the analyte and solvent should be in contact. Furthermore, longer exposure to the solvent causes the matrix to swell, allowing more solvent to penetrate the sample interstices and improve the interaction between the solvent and the analyte (Sun et al., 2012). However, long extraction times and static cycles with higher temperatures is not recommended since compounds degrade after being heated for a long time (Moreau et al., 2003).

2.7 Supercritical Fluid Extraction (SFE)

As a gas, supercritical fluids have a low viscosity, a high density as a liquid, and a level of diffusion between gases and liquids that varies with density (Akalın et al., 2017). When pressure and temperature values are adjusted, the density of supercritical fluids changes and a slight increase in pressure can result in a large rise in fluid density (Yousefi et al., 2019). CO_2 is one of the most used supercritical fluids. It is an ideal solvent because of its qualities such as non-toxicity, non-flammability, low cost, chemical stability, and the fact that it is a gas at room temperature and pressure. However, the extraction relies on the supercritical fluid's solvating characteristics (Akalın et al., 2017).

Several research groups have observed higher selectivity when extracting lipophilic extractives with supercritical fluid- carbon dioxide(SF-CO_2). As a result, cosolvents and modifiers have increased the yields and efficiency of SF-CO_2 extraction (Lee 1992). Cosolvents are solvents that improve SFE extraction performance by enhancing solute solubility (Machado et al., 2015). SFE has been combined with methanol, water, chloroform, acetone, and ethylic ether. These solvents, on the other hand, are directed at select groups. Methanol, for example, boosts extraction efficiency due to cosolvent interaction with matrix fibres rather than a contribution to CO_2 solvent capacity (Machado et al., 2015). Acetone and dichloromethane can effectively remove phytosterols and lipophilic extractives from wood, leaving a significant quantity of fatty acid esters behind (M'esz'aros, 2007). Petroleum ether is enough to extract ketones and waxes (Lu et al., 2017).

Ethanol is the cosolvent of choice for this study due to its affinity for the matrice's chemical characteristics. When ethanol is added in small volumes, it increases the molecular interactions between the supercritical solvent and the polar molecules, resulting in faster extraction (Martins et al., 2016). Below is an overview of important parameters to consider when using SFE.

2.7.1 Extraction parameters of SFE

2.7.1.1 Effect of cosolvent on extraction

As a nonpolar solvent, SF-CO_2 is less successful in extracting polar solutes. It is, however, a good solvent for extracting nonpolar, low-molecular-weight and volatile chemicals. Adding an organic cosolvent to SF-CO_2 can improve its solvating power (Herrero et al., 2005). Further, using cosolvents lowers the temperature required for extraction. Adding cosolvents causes biomass to swell, increasing the internal volume and surface area of contact and mass transfer by forming hydrogen bonds with intracellular biomass compounds (Yousefi et al., 2019).

Martins et al. (2016) highlighted that employing maximum pressure of 300 bar and ethanol range of 2.5-5.0 wt% are recommended globally. However, the amount of cosolvent used varies depending on the chemicals extracted and the degree of selectivity. The extraction of low molecular weight phenols and lipophilic chemicals from *Pinus pinaster* was optimized by Conde et al. (2013b). The optimum conditions were 10 MPa, 30 °C, 1% ethanol, and a yield of 4.1 wt% was obtained. According to the results obtained, these conditions were preferential for natural resin and fatty acids, which were the primary components of lipophilic compounds. Further, the triterpenoids were enhanced by adding 8% ethanol at 160 bar and 40 °C in a study by Domingues et al. (2012).

Ethanol is the cosolvent of choice for this study due to its affinity for the matrices chemical characteristics. When ethanol is added in small volumes, it increases the molecular interactions between the supercritical solvent and the polar molecules, resulting in faster extraction (Martins et al., 2016).

2.7.1.2 Flow rate

The supercritical fluid's flow rate influences the extraction efficiency. As the CO_2 flow rate increases, the residence duration decreases, and the solute disintegration decreases. Further, a high flow rate may increase the intermolecular connection and power between the solute and the solvent; this could decrease intra-molecule dispersion and be particularly important in extraction (Rozzi et al., 2002b). The extraction efficiency and selectivity of lipophilic compounds are influenced by the flow rate of CO_2, the concentration of co-solvents, and the extraction period. The flow rate influences the residence time for the contact between the solute and the solvent. An increase in CO_2 flow rate, for example, reduces residence time and, as a result, solute dissolution. When the CO_2 flow rate was evaluated without the cosolvent, the total extract increased, whereas the target analyte in the extract almost remained unchanged; upon adding cosolvent (Machmudah et al., 2006, Molino et al., 2020).

2.7.1.3. Extraction time and sample size

Short extraction time is one of the advantages that SFE has over traditional methods. Pre-treatment of the sample is crucial since the disruption of the cells decreases the mass transfer resistance and enhances the extraction efficiency by increasing the surface area. However, over grinding the sample is not recommended as it can cause re-adsorption of the sample on the matrix surface. Extraction time has been optimized in a study by Akalın et al. (2013), where a short extraction time of 123.67 min was optimum, and the yield was 15.95 wt% at a temperature of 315.81 °C.

2.7.1.4 Temperature and pressure

An increase in temperature enhances the solubility and increases the solute's vapour pressure whilst decreasing the density of the CO_2 and its solvating power at constant pressure (Akay et al., 2011). However, the temperature ranges of 40 to 60 °C were found to affect the extraction efficiency as the yield increased positively. While an increase in temperature from 60 to 80 °C decreased the extraction efficiency due to a decrease in density and the high temperatures degrading thermosensitive compounds (Zhao and Zhang, 2013). On the other hand, high pressures increase mass transfer, solvent power and extraction efficiency. Further, the high pressures decrease the extraction selectivity because of the co-extraction of unwanted compounds (Glisic et al., 2010).

2.8 Cannabis Plant

Cannabis is an annual flowering plant in the *Cannabaceae* family cultivated in many parts, primarily in Asia (Andre et al., 2016). It contains several chemically active compounds such as cannabinoids, terpenoids, flavonoids, and alkaloids, with cannabinoids as the most active compounds (Bonini et al., 2018). The different compounds of the plant have been used for therapeutic and recreational purposes over time, such as the extraction of curative oils from seeds or the hallucinogenic effects of inflorescences. The ancient Greeks and Romans later acknowledged the therapeutic properties of Cannabis. Further, during the Industrial Revolution, marijuana became a popular item for economic and therapeutic purposes (Andre et al., 2016, Bonini et al., 2018).

2.8.1 Chemistry of Cannabis *Sativa* L.

Despite being one of the most studied plants, the chemistry of Cannabis remains complex. Among the more than 480 chemical components found in cannabis, cannabinoids are the most distinctive and unique to the Cannabis plant (ElSohly and Slade, 2005, Isidore et al., 2021). There are already over 100 cannabinoids identified, which are divided into ten subclasses, including Tetrahydrocannabinol (THC), Cannabinol (CBN), Cannabidiol (CBD) (Isidore et al., 2021). The THC subclass without nitrogen, but Δ^9– THC) is the most active, causing the physiological effects of marijuana usage (ElSohly and Slade, 2005). However, Δ^9-THC is a non-psychotropic compound and is well-tolerated, enabling it to treat numerous disorders. CBD has low toxicity, which has allowed for clinical-level studies on its therapeutic efficacy. CBD exhibits high anti-inflammatory properties, which could be used to treat neuro-inflammatory disorders (Attard et al., 2018). Freshly dried marijuana has very little THC and CBN, and the age of marijuana samples can be determined based on the THC and CBN concentration, depending on storage circumstances. As a result, analyzing the amounts of THC, CBD, and CBN in cannabis product samples is critical for determining their quality (ElSohly and Slade, 2005, Brenneisen, 2007). This study will focus on the chemical constituents of solvents extracted from cannabis.

2.8.2 Chemical constituents of Cannabis *Sativa* L.

The family *Cannabaceae* includes Cannabis *Sativa L*. composed primarily of terpenes, oxygenated terpenes, sesquiterpenes, and oxygenated sesquiterpenes (Andre et al., 2016). At the same time, its seed oil is high in linoleic acid -linolenic acid, stearidonic acid, terpenoids, sitosterol, and methyl salicylate (Kitrytė et al., 2018). Cannabis contains cannabinoids (more than 104 have been identified) and alcohols, aldehydes, n-alkanes, wax esters, and sterols (Andre et al., 2016, Baldino et al., 2020a, Attard et al., 2018). Cannabinoids are terpenophenolic chemicals made by alkylating an alkyl-resorcinol with a monoterpene unit. They have a C_{21}-skeleton and are nitrogen-free lipophilic, and phenolic (Stearn, 1970); the illustration of the molecular structures of cannabinoids detected in Cannabis *Sativa* L. are shown in Figure 8 below. The characteristic smell of the cannabis plant is caused by an essential oil produced by the circular glands. Also, it contains phenylpropane derivatives (e.g., eugenol, cis and trans-anethol) and mono- and sesquiterpenes (e.g., humulene, a- and b-pinene, limonene, b-caryophyllene, caryophyllene oxide) (Pellati et al., 2018c). Furthermore, it contains small amounts of other phenolic compounds (e.g., dihydrophenanthrene derivatives, spiroindane, dihydrostilbene), flavonoids, amino acids, sugars and nitrogen-containing compounds (amines, amides) (Bonini et al., 2018).

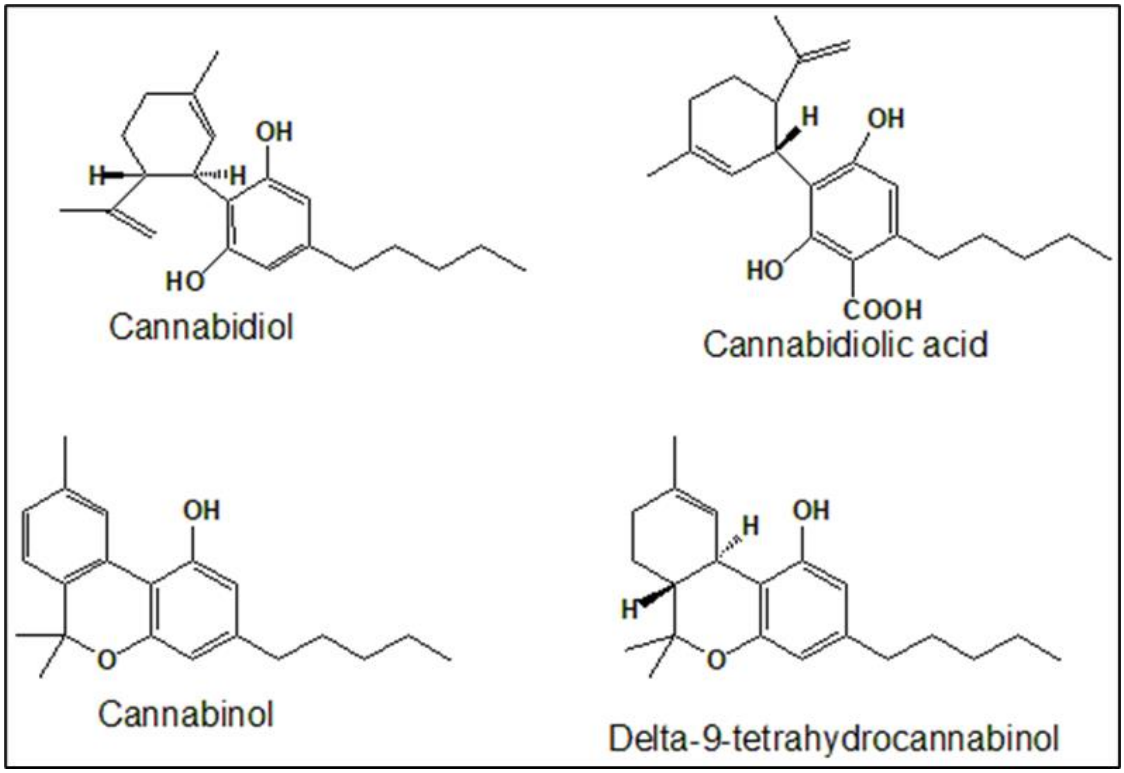

Figure 8: Molecular structures of cannabinoids detected in Cannabis *Sativa L.*

Adapted from (Lelario et al., 2018)

Karğılı and Aytaç (2022) highlighted the cannabinoid content of different varieties of Cannabis plants grown in various climates and extracted under various supercritical settings. Pressure, cosolvent, climatic parameters, and strain significantly impacted extraction yield. The Narl strain produced the highest CBD (7.70%), While the Papatya strain and Elnur stain had the highest amount of THC, 90.82% and 58.22%, respectively. The lowest amount of CBD was identified in the Elnur strain of 3.29%. They concluded that cannabis grown in areas with more rain, lower temperatures, and higher relative humidity yielded higher yields. However, cannabis can adapt to a wide range of climate conditions. As a result, it is a plant found in both warm and subtropical climate zones. Pre-planting rainfall is significant. It grows well in humid climates, although it may also be cultivated in desert climates if adequately irrigated. Cannabis plant development is accelerated by drought and high humidity (Abdollahi et al., 2020). Also, Cannabinoids are biosynthesized, collected, and decarboxylated in the plant. In most cases, decarboxylation occurs spontaneously in plant tissues, but it is possible

to speed up the process by heating, exposing it to light, storing it longer, or oxidizing it (Brighenti et al., 2017, Booth et al., 2017).

Terpene compounds are a family of cannabis components that are primarily volatile such as limonene, myrcene, and pinene (Booth and Bohlmann, 2019b). They define some of the unique organoleptic aspects of different cannabis types and may also impact its medical qualities. While terpene content does not always reveal where the cannabis plants came from, it is a phenotypic feature with a lot of variation across different cannabis cultivars and between specimens of the same variety subjected to varied environmental conditions, as seen with cannabinoids (Booth and Bohlmann, 2019a). A study by (Namdar et al., 2018) investigated the effects of three different extraction solvents on the chemical composition of terpenes. Results show that the mixture of ethanol and hexane (7:3, v/v) provided the best extraction yield. According to Farag and Kayser (2017), the generation of terpenoid compounds varies according to the environment. Terpenoids are created as a defense mechanism, same as phytocannabinoids are, and the amount of terpenoids produced rises with sun exposure; however, it falls with soil fertility. Different terpenes commonly found in cannabis plants are displayed with their structures in Figure 9 below.

Naz et al. (2017) demonstrated the main compounds extracted from leaves of Cannabis *Sativa L.* and Cannabis *Indica* by hydrodistillation (HD), steam distillation (SD) and SFE, were caryophyllene (40.6-50.0%), humulene (9.51-16.0%), *trans*-α-bergamotene (4.42-6.31%), *cis*-β-farnesene (8.63-9.01%) and δ-limonene (5.13-8.19%) respectively. The main components of Cannabis *Indica* were caryophyllene (21.1-25.1%), carophyllene oxide (4.13-5.02%), linalool (20.8-22.1%), trans-α-bergamotene (3.23-5.16%), cis-β-farnesene (2.10-3.68%), menthol (7.20-9.43%), δlimonene (6.13-7.19%), eucalyptol (9.67-12.10%), and carvone (2.11-5.13%) respectively (Naz et al., 2017). The essential oil yields obtained using the SFE technique were higher than those obtained using the HD and extraction methods. There was a difference in the chemical contents of the investigated Cannabis *Sativa L.* and Cannabis *Indica* essential oil samples, which might be attributed to the plant's chemotype and biotype, geographical cultivation, climatic conditions, and extraction procedure.

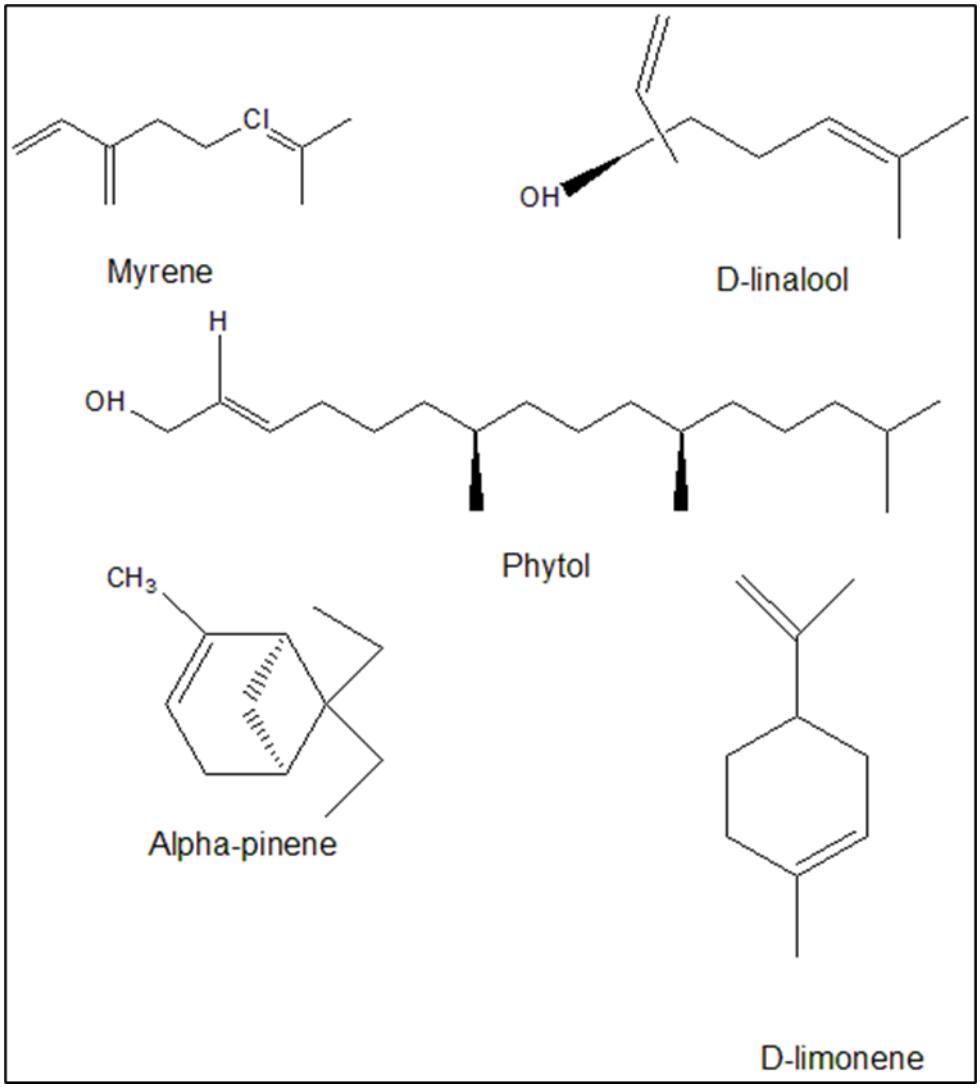

Figure 9: Illustration of terpenoids found in Cannabis *Sativa* L. plant drawing inspired by (Bonini et al., 2018)

2.9 Extraction Procedures for Cannabis Compounds

Lipophilic compound's sensitivity to various process parameters such as temperature makes extracting these compounds from the plant matrix complicated. Therefore, selecting the optimal extraction procedure for the preparation of plant extracts is critical. Extraction procedures that have been in use, such as Soxhlet extraction, maceration and HD, are time-consuming, energy-intensive, require large amounts of solvents, co-extract other undesirable components, and environmental pollution, must be considered in the overall evaluation of these techniques. The low extraction selectivity caused by the co-extraction of several undesirable compounds results in

low percentages of the compounds of interest in the final product and clashes with the simplicity of these procedures.

Winterization has been applied to overcome this problem (Grijó et al., 2018). This process involves combining the extract with ethanol and then cooling the combination to shallow temperatures (down to -40 °C) to precipitate the waxes (paraffinic chemicals) that are ubiquitously present on the surface of plant materials. However, winterization, on the other hand, is a time-consuming procedure (24 to 48 hours) that results in an ethanolic extract solution (Grijó et al., 2018, Marzorati et al., 2020).

In addition, the disadvantages of traditional methods can be overcome by using advanced extraction techniques such as SFE and ASE, which allows the target compounds to dissolve in the solvent by destroying the cell wall, resulting in a higher extraction yield in a shorter amount of time (Rozzi et al., 2002b). Also, these processes limit the use of synthetic and organic solvents, reduce operational time, and provide a higher-yielding, higher-quality extract.

When choosing a method, the product characteristics must be considered. Cannabinoids, for example, can be extracted in either an acidic or neutral state, depending on the application. The extraction of acidic cannabinoids must be done at room temperature to ensure their preservation. According to Fathordoobady et al. (2019), high temperatures are suitable for extracting decarboxylate acidic cannabinoids, although a higher temperature may cause certain terpenes and minor components to be lost. The extraction parameters and their effect using supercritical fluid extraction will be discussed below.

2.9.1 Supercritical fluid extraction (SFE)

Decaffeination of coffee using SFE brought attention to supercritical fluid, which has become popular since the 1980s (Baldino et al., 2020b). Several industries have been using this technique primarily to extract valuable compounds from plants. Today, studies show that extracting valuable chemicals from plant materials depends on numerous SFE parameters, the most essential of which are programmable. The primary factors determining extraction efficiency are the process parameters such as temperature, pressure, particle size and moisture content of plant material, extraction time, CO_2 flow rate, and solvent-to-feed ratio (Ibañez et al., 2012).

Since the supercritical fluid has a higher diffusion coefficient, lower viscosity, and lower surface tension than a liquid solvent, it penetrates the sample matrix more profoundly and allows better mass transfer. When compared to traditional procedures, SFE can significantly cut extraction time. Its solvation power may be modified by changing temperature and pressure, and supercritical fluid has better selectivity than liquid solvent (Azmir et al., 2013).

Grijó et al. (2018) presented the results of cannabinoid extraction from two types of Cannabis flowers utilizing pressured fluids. The extractions were carried out with pure SF-CO_2 and ethanol as a cosolvent, with decarboxylation and winterization procedures being compared. The decarboxylation method used increased the amounts of the desired cannabinoids. The two primary bioactive chemicals in this category with therapeutic promise were CBD and Δ^9– THC. On the other hand, fresh flowers had minimal quantities of these chemicals.

Rovetto and Aieta (2017) investigated the concentration of cannabinoids under different process parameters with multistage pressure increments and the constant pressure of 17, 24 and 34 MPa and 328 K with a flow rate of 200 g/min of CO_2. The extraction efficiency of 92% was achieved, and the extraction yield was highly dependent on pressure and plant material starting composition. The effect of ethanol as a co-solvent was investigated by two approaches: the constant co-solvent flow and by applying pulses of ethanol at different times throughout the extraction procedure. They concluded that the pulse regime was suitable for the extraction of cannabinoids.

2.9.1.1 Effect of temperature

SFE efficiency does not necessarily have a direct relationship with temperature. Depending on the type of material, targeted chemicals, and pressure, the temperature impacts SFE yield two-fold (Montañés et al., 2018). The SFE efficiency can be improved by increasing the extraction pressure and temperature. The yield of extracts increases with increasing temperature at high pressures and then remains constant, whereas, at low extraction pressures, the yield initially increases with increasing temperature and then decreases. When employing high extraction temperatures, several heat-sensitive components may break down or oxidize, causing them to lose compounds with biological activity (El-Ananya and Ali, 2018).

Furthermore, the density of CO_2 drops as the temperature rises, resulting in a decrease in oil solubility in SF-CO_2 up to the cross-over point due to an increase in the extract's vapor pressure. Kitrytė et al. (2018) employed a temperature range of 35-70 °C, and 70 °C was the optimal temperature for cannabinoids. While Gallo-Molina et al. (2019) used a temperature range of 40-80 °C, the optimal temperature was 60 °C.

2.9.1.2 Pressure

Generally, the higher the pressure, the larger the CO_2's solvent power and, thus, extraction efficacy, which is frequently countered by a poorer extraction selectivity. According to numerous studies, CO_2 extraction pressure is one of the most important characteristics of the SFE process (Vági et al., 2019). Increased pressure increases the density of supercritical CO_2, which improves oil solubility and thus the seed oil recovery. However, increased extraction pressure reduces solvent diffusivity and thus mass transfer, resulting in reduced oil recovery. In the optimization range from 100-500 bar, 450 bar was the optimal pressure giving high extraction efficiency (Kitrytė et al., 2018). The extraction yields increased when the pressure was increased from 250 to 450 bar at a constant temperature of 45 °C, but the yield of cannabinoids did not improve with the increase in pressure. High pressures increase the extraction efficiency but decrease the selectivity of the SFE as the cannabinoids concentration did not improve with the increase of pressure, but other compounds were dominant (Vági et al., 2019). High pressures increase the density, and lipophilic compounds extractions are favored when higher pressures are employed. Attard et al. (2018) reported lipophilic compounds like long-chain fatty alcohols, aldehydes, n-alkanes, wax esters and sterols when high pressures were employed.

2.9.1.3 CO_2 flow rate

The flow rate of the supercritical fluid can also affect the extraction yield as it passes through the plant matrix. As the fluid flow rate increases, the resistance to mass transfer falls until the outgoing fluid becomes saturated, resulting in the solute solubility equilibrium and a maximum yield. The thickness of the diffusion film layer around the solid particles is lowered as the CO_2 flow rate increases, resulting in a reduction in the mass transfer resistance surrounding the solid particles and thus an increase in extraction yield (Attard et al., 2018).

2.9.1.4 Co-solvent

Since CO_2 is non-polar and cannot extract high polar compounds, adding a polar solvent improves the extraction efficiency. Still, it enhances the selectivity of the extraction and the solubility of these polar chemicals (Yousefi et al., 2019).

2.10 Other Uses of Cannabis *Sativa* L.

Medicinal and therapeutic applications for Cannabis have been popular for many years. Cannabis *Sativa* L. (hemp) is a source of various biologically active compounds such as cannabinoids, terpenes, and phenolic compounds, which exhibit antibacterial, antifungal, anti-inflammatory and anticancer properties (De Petrocellis et al., 2011). Singh et al. (2018) employed Cannabis extract as a reducing agent for reducing the salts to nanoparticles and formed core-AuNPs (C-AuNPs) and core-AgNPs (C-AgNPs). Cannabis *Sativa* L. has also been used for fiber, oil production, and simply as an additive for food products (Baldino et al., 2020b).

2.11 Techniques to Characterize Lipophilic Compounds

Several techniques are available to characterize lipophilic compounds. These include GC (Plat and Mensink, 2005, Uçar and Fengel, 1995), Py-GC/MS (Prinsen et al., 2012b), GC/MS (Arisandi et al., 2020), FTIR and TA (Xiao et al., 2001). In this study, three analytical techniques will characterize lipophilic compounds in pinewood sawdust: Py-GC/MS& Py-TMAH-GC/MS, FTIR and STA. Two techniques will be used to characterize Cannabis *Sativa L.* lipophilic compounds, Py-GC/MS& Py-TMAH-GC/MS and STA.

2.11.1 Gas Chromatography-Mass Spectrometry (GC-MS)

Kilic et al. (2011) analyzed the composition of lipophilic extractives with gas chromatography-flame ionization detector and gas chromatography-mass spectrometry. Both were coupled with a medium-high capillary column with temperature programmed at 120 °C/min, which increased incrementally by 6 °C/min to 320 °C. This study reported resin acids as the main components of the five Pinus cones studied and constituted more than 50% of all the identified lipophilic extractives. Some hydroxy resin acids were determined in all cones within the range of 1.1–2.4

mg/g and were named "modified resin acids". Modified resin acids could be the relevant compounds of crystalline resins found in the cones. Short-chain acids were found in very low amounts. The identification of the constituents was based on mass spectra and comparison with compounds found in the spectral library (Kilic et al., 2011).

Arisandi et al. (2020) first evaporated samples with dichloromethane before analysis, which converted the extractives into their volatiles. This study kept the mass range between 50 to 800 amu, and GC-MS detected free fatty acids, sterols-steroids, and triterpenoids. The highest extractive content was measured in the bark of the top part, *Eucalyptus pellita*. Free fatty acids, sterols-steroids, triterpenoids, and other compounds were detected in all parts of *Eucalyptus pellita*. Palmitic, oleic and linoleic acids, as well as β-sitosterol, were dominant lipophilic extractives. Long-chain fatty acids were the major lipophilic groups in the heartwood. Sterols-steroids were the major components in the bottom and top part of the bark.

Eriksson et al. (2018) used GC/MS with an auto-sampler operating in electron-impact mode (EI 70eV) to determine lipophilic extractives' amount and composition, especially the fatty and resin acids in Scots pine trees strands one due for cutting and one due for thinning. The capillary column was coated with cross-linked 5% phenyl methyl siloxane. For separation, the temperature program of the column was set at 100 °C isothermals for 0 min, rising to 220 °C at 10 °C/min then to 235 °C at 1 °C/min and finally to 260 °C at 10 °C/min. The system was maintained at 260 °C for 5.5 min. This study highlighted a difference in concentration and distribution of extractives due to the differences in the composition of the wood tissue, age and size of the species analyzed. The strand due for cutting had higher lipophilic extractives yields than the strand due to thinning.

Kebbi-Benkeder et al. (2015) analyzed knot wood extractives of 12 European softwood and hardwood species. Knot wood presented a higher concentration of extractives than heartwood. The main compounds were lignans, stilbenes and flavonoids, lignans being present only in softwood species. Apart from oak, knot wood in softwood species contained more significant amounts of extractives than that in hardwood species.

2.11.2 Pyrolysis Gas-Chromatography-Mass Spectrometry (Py-GC/MS)

Py-GC/MS is an analytical method to identify compounds by studying the resulting molecular fragments. It involves heating the biomass sample at a specific rate to high temperatures and holding it there for a time in an inert atmosphere (Mészáros et al., 2007a). The large high-molecular-weight molecules of biomass are thermally decomposed by heat mediated cleavage in an inert atmosphere such as helium to create a suite of smaller low molecular weight samples before they are analyzed for their composition by mass spectrometry (Crawford and Quinn 2017).

Pyrolysis is the first stage of combustion and gasification. During the pyrolytic process, long chains of carbon and high molecular weight compounds are broken down into smaller and simpler molecules, providing the three main products: gas, condensable vapor, and solid residue (Renzi, 2017). The proportion of these three products can vary depending on the pyrolysis process and conditions such as rate of degradation and temperature ranges (Renzi, 2017).

The pyrolysis temperature affects both the composition and yield of the product (Basu, 2010). High yields are obtained with low temperatures, while low results are observed with high temperatures. Volatiles and liquids are found with rapid heating to a moderate temperature of 400–600 °C. However, the heating rate is not enough to define the final product. The residence time of the product in the reactor is also essential, which allows the sample to be heated in a controlled and reproducible manner. The sample will be decomposed over the same temperature range, hence major control of pyrolytic behavior.

The coupling of the pyrolytic unit with appropriate analytical devices is fundamental for obtaining comprehensive qualitative and quantitative results. Combining pyrolysis with modern analytical techniques, such as high-resolution capillary GC and mass spectrometry (MS), results in a powerful tool for investigating complex organic materials. For this study, a pyrolytic unit will be coupled with gas chromatography-mass spectrometry.

Py-GC/MS permits the study of a broader range of molecules compared to the ones that can be detected by simple GC/MS, especially in the case of intractable, non-volatile, and high boiling point components (Mészáros et al., 2007b). Py-GC/MS allows direct analysis of the original natural material since the pyrolytic unit can produce

volatile fragments that can be analyzed using GC/MS. A pyrolysis unit is shown in Figure 10 below.

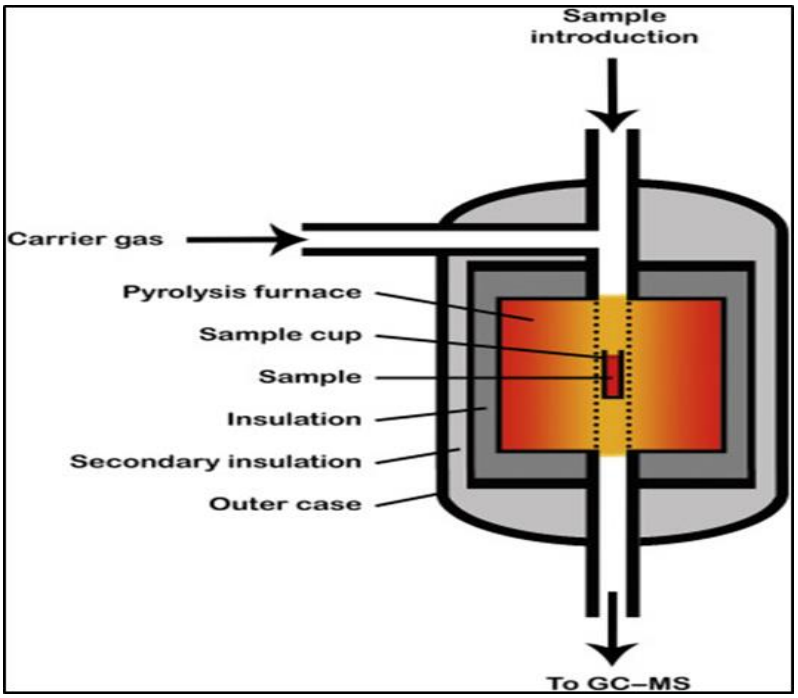

Figure 10: Schematic diagram of Gas chromatography-mass spectrometry coupled with pyrolysis unit

(Crawford and Quinn, 2017)

Mattos et al. (2016) used the Py-GC/MS technique to identify wood extractives. The extractives were inserted in a pyro-probe heated from room temperature to a high temperature of 1200 ºC for the total combustion and volatilization of the compounds before injecting the sample to GC/MS. The GC/MS oven temperature started from 50 ºC, which was kept for 2 min and programmed to heat up to 120 ºC at a heating rate of 5 ºC/min for 5 min to 300 ºC at 5 ºC/min. In this study, 61 compounds of extractives were identified by the fragmentation pattern of molecules in the mass spectra. The predominant extractives were fatty acids, with the main compound n-hexadecenoic acids extracted by ethanol: toluene.

Gutiérrez et al. (2006), (Jose et al., 2007) employed Py-GC/MS to characterize lipid compounds in industrial hemp (Cannabis *Sativa* L.). The compounds identified were n-alkanes, free and esterified sterols and triterpenols, waxes, long-chain n-fatty acids,

n-aldehydes, n-fatty alcohols, steroid hydrocarbons, and steroid and triterpenoid ketones, as well as steryl glycosides.

2.12 Thermal Analysis (TA)

TGA is the branch of thermal analysis dedicated to understanding the mass change of a sample in one of two modes, as a function of temperature in the scanning mode or as a function of time in the isothermal mode. Desorption, absorption, sublimation, vaporization, oxidation, reduction, and decomposition are thermal, which result in changes in sample mass under investigation (Poletto et al., 2012). TGA is extensively used to characterize the decomposition and thermal stability of materials under various conditions and to examine the kinetics of the physicochemical processes occurring in the sample (Perrotin-Brunel et al., 2010). For this study, the TA will assess the thermal degradation of changes of the wood extracts after extraction by ASE and SFE.

TGA has been widely used to evaluate the thermal decomposition process of solid-state materials such as lignocellulosic materials (Tenorio et al. 2013, Pétrissans et al. 2014, Toscano et al. 2015). The thermal decomposition of wood and other natural fibers involves many competitive and consecutive reactions because of the chemical complexity of lignocellulosic materials (Poletto 2016). The results suggest that more substantial holocellulose and lignin with lower extractive contents give the wood more excellent thermal stability.

Mattos et al. (2016) analysed a sample with a weight range of 5 to 10 mg. The temperature ranged between 25 and 600 ºC with a heating rate of 10 ºC /min under an inert nitrogen atmosphere. Gao (2012) highlighted the three weight-loss stages of pine sawdust. The main pine sawdust thermal decomposition compounds were small molar gases, acetaldehyde, acetic acid, and anhydride with formic acetic anhydride. Figure 11 Shows the thermogravimetric spectrum obtained from TGA of lipophilic extractives extracted with acetone.

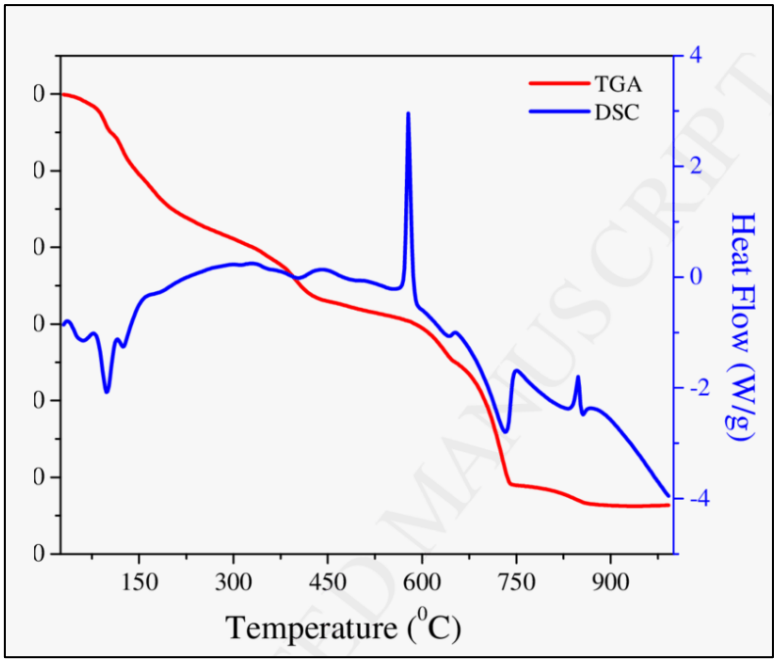

Figure 11: Thermogravimetric analysis spectrum of lipophilic extractives of the pine tree

(Mattos et al., 2016)

2.13 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR is an analytical technique used to obtain vibrational information of the wood's chemical structure and compositional changes due to various extractions (Mattos et al., 2016). Different studies have employed FTIR to analyze Cannabis terpenes. For example, Callado et al. (2018) compared the FTIR to other dispersive instruments. The present research aimed to develop a fast, economical, robust, and environmentally friendly method based on near-infrared technology that quantifies the main cannabinoids present in Cannabis *Sativa* L. samples.

These vibrations are quantized, and as they occur, the compounds absorb IR energy regions of the IR portion of the spectrum. (Berben et al. 1987; Pandey and Theagarajan 1997; Oliveira et al. 2020), have used FTIR for wood surface characterization, lignin estimation, and carbohydrate content in wood lignocellulosic and lipophilic extractives. The functional groups attributed to corresponding wavelengths for lipophilic compounds of wood are listed In Table 3 below.

Table 3: Summary of lipophilic extractives spectral bands reported by Berben et al. 1987; Pandey and Theagarajan 1997; Mattos et al. 2016; Oliveira et al. 2020.

Frequency (cm^{-1})	Assignment	Functional groups
34000	O-H stretch (hydrogen bond)	Hydroxyl function
2984 – 2823	C-H stretch	Aliphatic groups: fatty esters, free fatty acids, and lipophilic alcohols.
1745 – 1710	C=O carbonyl stretch	Non-conjugate ketones and conjugated carboxyl acids: Free fatty acids, aldehydes, and fatty esters
1690	COOR carbonyl stretch mode	Carboxylic function
1510	C=C	Aromatic compounds
1800 – 800	C-C, C-H	Fingerprint region of wood

CHAPTER 3

3.1 THEORY OF INSTRUMENTS

The principles and operational procedures of instruments employed in this work are described in detail. Table 4 below summarizes the equipment used and its purpose.

Table 4: Summary of equipment used in research and their purposes.

	Equipment used	Purpose
1	Grounding and measuring samples of pine wood sawdust	
1,1	Wiley miler	Grinding sawdust.
	Mechanical shaker	Measuring particle size of pinewood sawdust and Cannabis flowers.
	Blender	Grinding Cannabis *Sativa* L. flowers.
2	Extraction of lipophilic compounds	
2,1	ASE	Extract lipophilic compounds at elevated temperatures.
	SFE	Extract lipophilic compounds with CO_2 and cosolvent: ethanol.
	Rotary vapor	Remove solvent from extracts.
	Scale	Measure the initial mass and extracts.
3	Characterization	
3,1	TGA	Thermal analysis.
	FTIR	Functional groups.
	Py-GC-MS/Py(TMAH)-GC/MS	Identification of compounds.

3.2 Description of Extractors and Characterization Equipment

3.2.1 Accelerated solvent extractor (ASE)

ASE uses organic solvents above their boiling point at elevated temperatures and pressures to achieve a short extraction time, decrease solvent consumption, increase the extraction yield, and successfully remove high-quality analytes from different matrices (Van der Esch et al., 1997). These conditions cause the extraction solvent to be quickly diffused across the sample matrix and result in the analyte being dissolved and recovered more fully. To remove the analyte from the extraction vessel must first be desorbed from its original site in the sample matrix and then diffused across the organic section of the matrix to reach the matrix–fluid interface. The analyte is dispersed into the extraction phase at this point. It diffuses through the extraction phase within the pore, and finally, it reaches the convection-affected part of the extraction phase (Mustafa and Turner 2011; Richter and Raynie 2012).

The sample is placed in a stainless-steel cell with filter paper at the bottom of the cell during the extraction process. The oven temperature is adjusted to ensure that the cell contents reach the predetermined point at the start of the static cycle. The cell is placed into a heated oven chamber. An auto seal arm grabs the cell holding the sample into an oven and then fill the cell with the extraction solvent until the desired pressure is reached (1500 psi), as illustrated in the extraction process scheme in Figure 12. The extraction cell is then pressurized to raise the extraction solvent's boiling point and solubilize the analyte at a higher than necessary atmospheric pressure. The static valve is used to maintain the pressure. The cell is heated through the repeated static cycles, and the sample is then extracted, then collected by the cell's automatic filling and voiding (Mustafa and Turner 2011; Richter and Raynie 2012).

The use of ASE provides many benefits, including more excellent reproducibility and less discrimination in extraction, the possibility of changing the extraction temperature from room temperature to 300 °C, shorter extraction times and line extract purification (Toubane et al., 2017). ASE requires less time, consumes less solvent during the extraction, and has proven effective for several environmental solid samples with the added benefit of automation. The extraction parameters of ASE are listed below.

3.2.1.1 Solvent

The solvent must solubilize the target analytes for an effective extraction while leaving the sample matrix intact. The polarity should be closely matched to that of the target compounds of the extraction solvent. For extracting a wide variety of compound groups, combining solvents of various polarities may be used (Mattos et al., 2016). Solvents such as methanol, ethanol and water, toluene and ethanol, water, dichloromethane, and acetone are widely used. When extracting analytes at low concentrations, the rate of extraction is not affected by the analyte concentration but rather by the mass transfer rate; therefore, the suitable solvent chemical properties should be chosen to ensure solvation and the release of the analyte (Maran et al., 2014, Pourmortazavi and Hajimirsadeghi, 2007).

3.2.1.2 Temperature

The solvent's viscosity decreases as the temperature rises, boosting its capacity to permeate the matrix and solubilize the target analytes. The increased thermal energy also benefits the breaking of analyte matrix linkages and enhances analyte diffusion to the matrix's surface (Luthria et al., 2019). Elevated temperatures aid in the disruption of analyte-sample matrix connections mediated by van der Waals forces, hydrogen bonding, and dipole attraction, which improves extraction efficiency. By lowering the activation energy required for the desorption process, thermal energy can help overcome cohesive (molecule–molecule) and adhesive contacts between different molecules, the analyte, and the sample matrix (Luthria et al., 2019). Additionally, the increased temperature lowers the surface tension of the solvent, solutes, and matrix, which improves the sample's solvent wetting (Richter et al., 1996). Reduced solvent surface tension allows for the easier formation of solvent cavities, allowing analytes to dissolve more quickly in the solvent. Increased temperature reduces the viscosity of a liquid solvent, allowing it to penetrate deeper into the matrix particle, resulting in a better extraction process (Richter et al., 1996).

3.2.1.3 Pressure

The impact of pressure keeps the solvents as liquids above their boiling temperatures in the atmosphere. Because the pressures employed in rapid solvent extraction are substantially over the limitations required to keep the solvents in a liquid condition, no pressure changes are necessary to change the solvents. The recommended extraction pressure is 1500 psi. Using high pressure during the extraction prevents air

bubbles in the matrix from preventing the solvent from reaching the analyte. Under these conditions, the analyte's solubility and analyte desorption kinetics from the sample matrix is improved. The impact of pressure on most substance recovery is usually insignificant (Rostagno et al., 2009).

3.2.1.4 Cycles

Static cycles have been incorporated during the extraction process to provide new solvents, which helps maintain and improve extraction quality. This effectively approximates difficult extraction circumstances without using problematic flow restrictors to maintain pressure. When more than one flush cycle is employed in a system, the flush volume is divided by the number of flushes. The sample cell is purged with inert gas at the end of the last extraction cycle to remove the solvent from the cell and the tubing into the collecting vial, avoiding any loss or memory effects (Yang et al., 2014). Nitrogen is generally used as the purge gas after the final static cycle has been completed. Since the initial flush volume has been broken, no additional solvent is used in the extraction. Static cycles have shown effective for sample forms with high analyte concentrations or samples with a difficult-to-penetrate matrix (Yang et al., 2014).

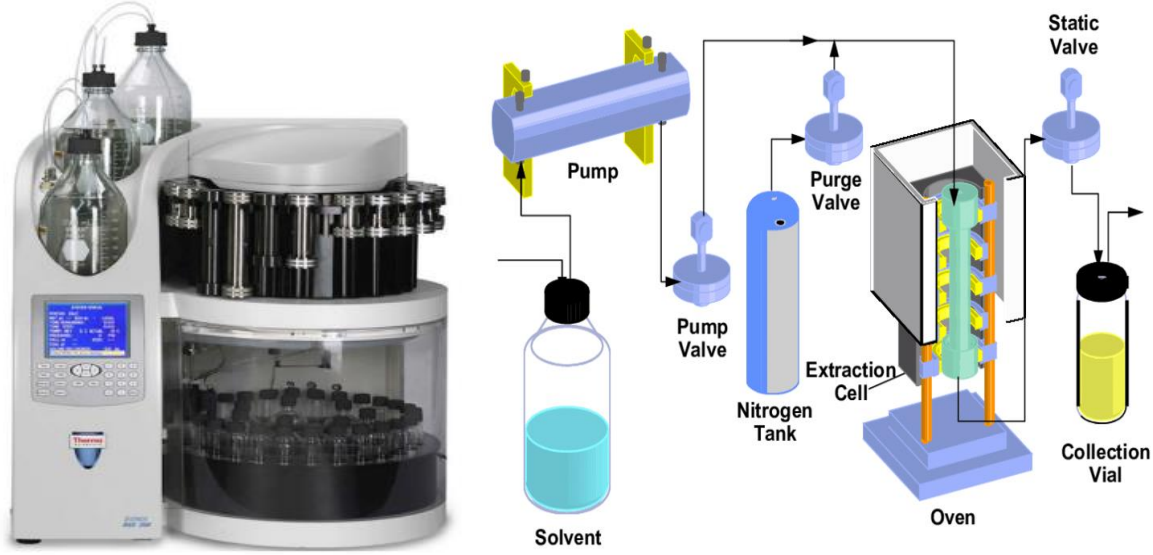

Figure 12: Illustration of ASE Dionex ASE 350 system (Yang et al., 2014) and a schematic flow of ASE (Van der Esch et al., 1997)

3.2.2 Supercritical fluid extraction (SFE)

The extraction method is based on the interaction between a solid raw material and a pressurized solvent, which extracts the interesting compounds from the solid phase (Mezzomo et al., 2009). Time is given for the equilibrium and the target component to be transferred from the solid phase to the liquid phase. Then the solid and liquid phases are physically separated. The solubility of the target component in the chosen solvent, determined by the interactions between the solvent and the solute, is the driving force behind any extraction procedure. By functioning at a pressure and temperature near its critical stage, the process improves the behavior of the solvents (Ferreira-Santos et al., 2020). Supercritical fluids have a high mass transfer rate compared to liquid solvents. The non-toxic nature of the fluid and the ability to change solvating power through density changes make supercritical fluids preferable to organic solvents.

A supercritical fluid is a fluid that surpasses the critical temperature and pressure where the liquid and gaseous phases are indistinguishable, while all phases have properties simultaneously (Chai et al., 2020). The fluid density is like a liquid and is proportional to its solvating strength. The diffusion and viscosity properties of supercritical fluids are of those of gases; nevertheless, supercritical fluids exist in a single phase and have properties like both gases and liquids. Regardless of how much the pressure increases, a liquid phase will not appear above the critical temperature. At the critical temperature, the critical pressure is the pressure that causes the gas to turn into a liquid.

In comparison to an analogue, CO_2 is selected as a fluid of choice, instead of an organic solvent due to it being non-toxic, non-flammable, non-corrosive, has a low critical stage of 73.8 bar and 31.1°C, which favors thermally sensitive compounds, does not leave residues in the extracts and results in high purity at low cost. One of the strong benefits of using it as a solvent is the absence of adverse health effects (Ferreira-Santos et al., 2020). Further, the advantage of CO_2 over organic solvents is that CO_2 rapidly evaporates from the extract at room temperature, but an organic solvent will leave a residue at room temperature.

The solvating properties of a supercritical fluid, created by using pressure and temperature above a compound, mixture, or element's critical point, are the basis for

SFE. Extraction by supercritical fluid depends on some intrinsic adjustable properties of supercritical fluid, such as temperature and pressure, and some irrelevant qualities, such as sample matrix characteristics, interaction with specific analysts, and various ambient conditions (Santos et al., 2012).

Two pumps, a back-pressure regulator, a heat exchanger, numerous ball valves, and four pressure vessels make up the unit. The storage tank, extractor, and two separators are all pressure vessels. The storage tank functions as a capacitor to counteract the pump's cyclic activity. The lignocellulosic biomass sample is placed into the extractor for extraction. The extracted material is precipitated from the CO_2 depressurization in the two separators. The scheme diagram showing the connection of these components is shown in Figure 13 below.

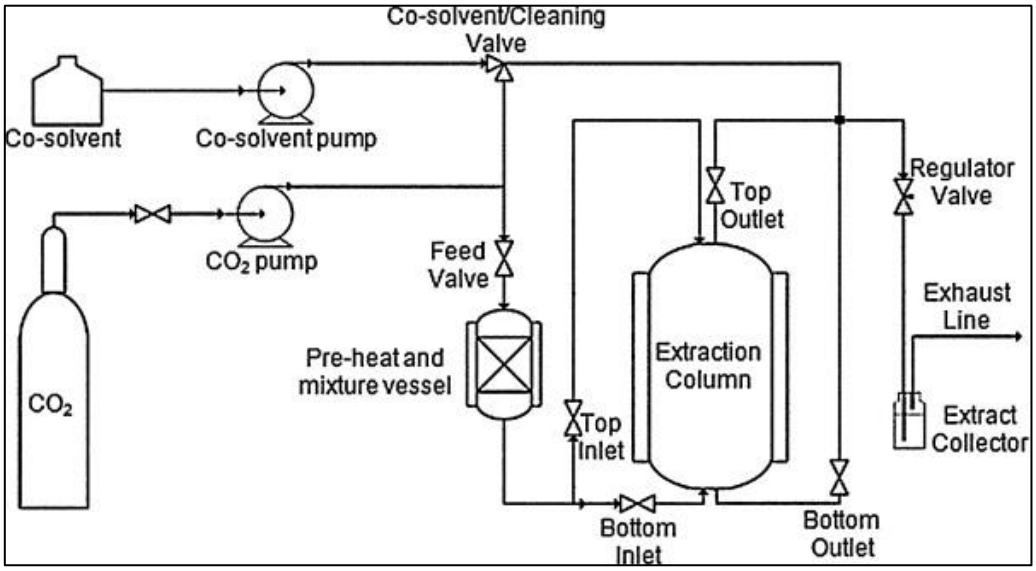

Figure 13: illustration of a schematic diagram of SFE

(Santos et al., 2012)

SFE of targeted components from a plant matrix is governed by various parameters, including pretreatment of plant material, particle size, temperature, pressure, time, solvent flow rate, and solvent-to-feed ratio, similar to traditional extraction procedures. These variables impact extraction efficiency, measured in yield and recovery of the desired components. The yield refers to the total extract collected per unit mass of starting feed material. In contrast, recovery refers to the proportion of the targeted component recovered in the extract originally contained in the feed material.

3.2.2.1 Supercritical fluid carbon dioxide (SF-CO_2) flow rate

Because it is non-explosive, non-toxic, and affordable and can solubilize lipophilic compounds and be easily removed from finished products, SF-CO_2 is an appealing alternative to organic solvents. Another benefit is gaseous CO_2 at room temperature and pressure, which simplifies compound recovery and allows solvent-free extraction (Chai et al., 2020).

The extraction yield can also be affected by the flow rate of supercritical fluid going through the sample matrix. As the flow velocity of the fluid increases, the barrier to mass transfer reduces until the outgoing fluid gets saturated; as a result, the solute solubility equilibrium is achieved, resulting in a maximum yield (Ferreira-Santos et al., 2020). The resistance to mass transfer diminishes as the SF-CO_2 flow rate increases, and as a result, the extraction yield begins to increase. In investigations, CO_2 flow rates range from 1 to 10 L/min, depending on the solubility of seed oil in CO_2. Both high and low flow rates reduce extraction yield. Using an ABPR (backpressure regulator) and a gas flow meter, the SF-CO_2 flow rate can be controlled. A restrictor with varied internal diameters might be easily modified to change the flow rate (Santos et al., 2012).

3.2.2.2 Pressure

In several studies, CO_2 extraction pressure is one of the most important parameters in the SFE process. Generally, the higher the pressure, the larger the CO_2's solvent power and, thus, extraction efficiency, frequently compensated by a reduced extraction selectivity. Increasing the pressure increases the density of supercritical CO_2, which enhances the solubility of the analyte and, as a result, increases lipophilic compounds recovery. On the other hand, decreasing the extraction pressure reduces the solvent diffusivity and thus the convective mass transfer associated with the

extraction process, resulting in lower recovery of compounds. The pressure in SFE is controlled by an ABPR, which maintains the CO_2 pressure at the desired level (Zeković et al., 2016, Santos et al., 2020, Duba and Fiori, 2015).

3.2.2.3 Temperature

SFE efficiency does not necessarily have a direct relationship with temperature. In addition, the temperature has a twofold impact on SFE yield depending on the type of targeted chemicals and pressure. Due to an increase in the vapor pressure of the oil, increasing the temperature decreases the CO_2 density, resulting in a decrease in the solubility of lipophilic compounds in SF-CO_2. When using higher extraction temperatures, many heat-sensitive compounds present in lipids, such as unsaturated fatty acids and similar bioactive compounds, may degrade or oxidize, compromising their and losing their biological activity (Hatami et al., 2019, Montañés et al., 2018).

The SFE efficiency can be improved by increasing the extraction pressure and temperature. The extracted oil yield grows with the temperature at higher pressures and remains constant. The extracted oil yield initially increases and drops at lower extraction pressures (Hatami et al., 2019, Montañés et al., 2018).

3.3 Thermal analysis (TA)

TA is a technique for describing material degradation and thermal stability. It is also used to the change in mass of a sample as a function of time, and DSC measures the differential heat flow at a constant temperature. TGA and DSC were two procedures employed to analyze the samples using a STA.

The TGA depicts thermal events in the sample being analyzed, such as desorption, absorption, sublimation, flammability, oxidation, reduction, and breakdown. On the other hand, DSC displays melting and crystallization behavior, glass transition temperature, and kinetic studies of the sample under consideration. The simultaneous thermal analyzer has a horizontal dual beam arrangement with automatic beam growth compensation and analyzes two TGA samples simultaneously. (https://www.netzsch-thermal-analysis.com/Accessed:12/02/2021).

Figure 14 shows the STA apparatus, including a furnace thermocouple, balance system, heating element, and gas. The sample and reference balances are both kept in the furnace. The sensitivity-calibrated driving coils measure the masses of the

sample and the reference independently. A TGA signal is sent because of the mass difference. The effects of beam expansion, convection flow, and buoyant force are balanced in the differential mass measurement. As a result, thermogravimetry measurement is susceptible. The independent driving coils measure the mass of the sample and the reference, allowing for straightforward electrical modification of the TGA baseline drift (https://www.netzsch-thermal-analysis.com/Accessed:12/02/2021)

In the furnace, there is a microbalance for the sample and a reference sample. A sample is weighed on a microbalance after being placed in a ceramic crucible. A computer-controlled oven surrounds the crucible. A flowing stream of gas can control the background atmosphere. To avoid oxidation of the material, nitrogen (N_2) is employed as a gas. The development of gas or the volatilization of a liquid breakdown product reduces the bulk of the sample (https://www.netzsch-thermal-analysis.com/Accessed:12/02/2021). As each subsequent breakdown stage happens, a typical thermogram consists of a series of plateaus at diminishing mass. The characteristics of the recorded TGA& DSC curve are influenced by several elements, including sample mass, volume, physicochemical properties of the sample, and atmospheric pressure in the sample chamber. The scanning rate has a major impact on the characteristics of the recorded TGA& DSC curve.

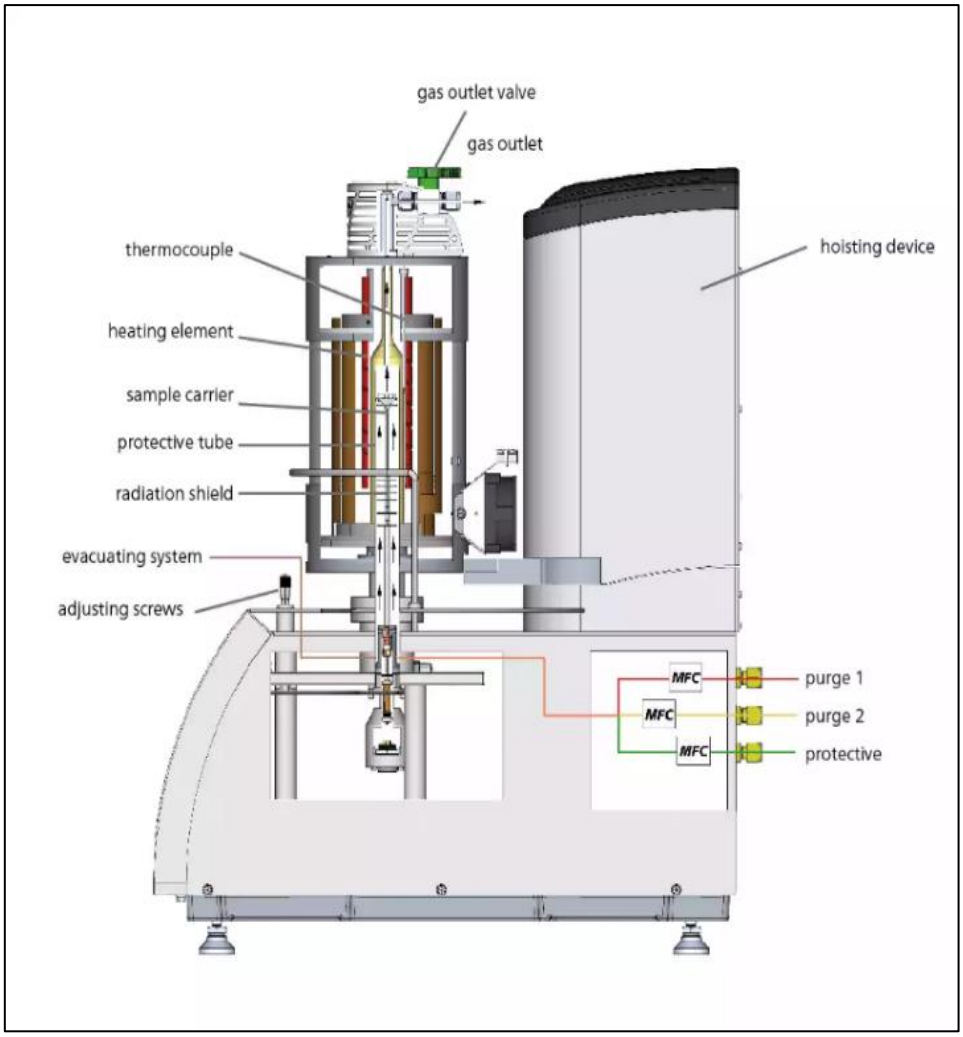

Figure 14: Illustration of a simultaneous thermal analyzer (STA)

(https://www.netzsch-thermal-analysis.com/Accessed:12/02/2021)

3.4 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR theory is that two beams interact to produce an interferogram. The interferogram is a signal generated as a function of the route length difference between the two beams. The mathematical method of Fourier-transformation can interconvert the two realms of distance and frequency. Before reaching a detector, the radiation from the source passes through an interferometer and hits the sample. The data is transformed to digital form by an analogue-to-digital converter and transmitted to the computer for Fourier-transformation after the amplified signal. A filter has removed high-frequency contributions (Stuart 2005).

The interaction of molecules or atoms with electromagnetic radiation is the basis of infrared spectroscopy. Organic compound atoms and groups of atoms vibrate with

enhanced amplitude around the covalent bonds that bind them when exposed to infrared radiation. An organic molecule's absorption of infrared radiation will occur at certain frequencies characteristic of the types of bonds and atoms present in the specific functional groups of that molecule because the functional groups of organic molecules comprise specific configurations of bonded atoms. These vibrations are quantized, and the compounds absorb infrared energy in particular parts of the infrared spectrum as they occur. The FTIR spectroscopy diagram with its components is illustrated in Figure 15 below.

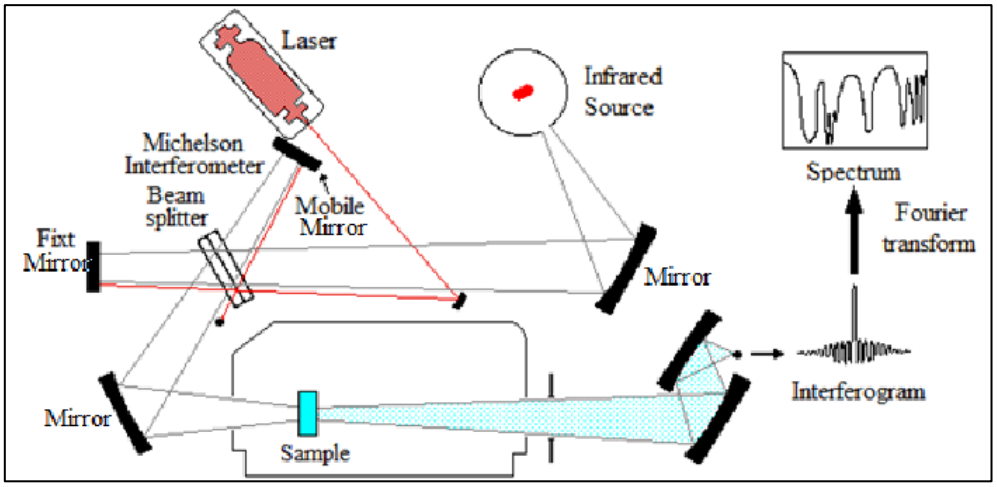

Figure 15: Illustration of an FTIR spectrometer

(Theophanides, 2012)

3.5 Rotary Evaporator

The Rotary Evaporator illustrated in Figure 16 below uses a spinning motion above a hot water bath to separate a blend of solvent and extracts under a vacuum. The mixture is poured into a flask with a round bottom connected to the condenser and chiller. The chiller is set to 10 °C to match the cooling temperature of the solvent. The vacuum is activated before placing the round bottom flask with the mixture in the hot water bath. The water bath is heated to a temperature near the solvent used to extract the components from the sample. The manufacturer determines all other specifications, like the condenser's speed. The rotary evaporator is turned on to start the separation process. If the separation is successful, the higher boiling point substance will be kept in the original round bottom flask. The lower boiling point substance will be collected in the collection flask (https://engineering.purdue.edu/ Accessed:11/02/2021).

Figure 16: Diagram of rotation evaporator Bucchi RE rotary vaporator (https://engineering.purdue.edu/ Accessed:11/02/2021)

3.6 Pyrolysis Gas Chromatography-Mass Spectroscopy (Py-GC/MS)

Pyrolysis is described as the heat energy-induced decomposition and removal of chemical bonds of a molecule in an inert atmosphere. The molecules in the initial sample are broken down into primary low-molecular-weight fragments that can be analyzed more quickly and secondary high-molecular-weight fragments. The pyrolysis circumstances (time, temperature, and heating rate) and the nature of the sample content determine the kind and distribution of the produced degradation products. Samples of the same substance pyrolyzed under the same conditions deteriorate in the same way as samples of the same degradation products repeatedly. Samples from other sources, on the other hand, behave differently. As a result, investigating the degradation products and the thermal degradation process can reveal the type and origin of the initial sample content (Ohra-aho, 2017).

In combination with contemporary analytical techniques such as high-resolution capillary GC and mass spectrometry (MS), pyrolysis is useful for analyzing complex organic compounds MS. Py-GC/MS allows the investigation of a greater spectrum of compounds than ordinary GC/MS can detect, particularly in complex, non-volatile, and high boiling point components. Py-GC/MS allows direct examination of the original natural component, which may subsequently be analyzed using GC/MS, thanks to the pyrolytic unit's ability to create volatile fragments (Renzi, 2017).

The Py-GC/MS schematic diagram in Figure 17 consists of a pyrolyzer, gas stream, chromatographic column, oven, and a spectrometric detector. The sample is injected into a hot interface, and the sample degradation begins as soon as the sample is in contact with a hot zone. The volatiles are performed into a gas simultaneously. The volatile products are swept by an inert gas stream, normally helium, into the gas chromatographic column separated by oven temperature programming. Finally, a spectrometric detector allows for identification with the help of a spectral library for the qualitative assessment of the fragments.

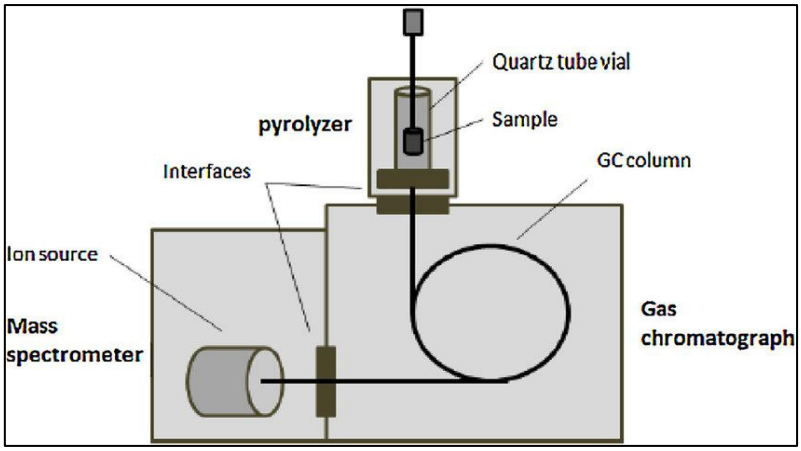

Figure 17: Schematic diagram of Py-GC/MS (Girardin et al., 2017)

The preferred method for drying pine sawdust is freeze-drying the sample. This method sublimates water out of a frozen sample at low pressure. Freeze-drying provides a couple of benefits. The first is to create a very porous sample with a high surface area to volume ratio, generally increasing extraction efficiency. The second benefit is that hydrolysis of the glycerides can be avoided by removing water at a low temperature.

CHAPTER 4

4. MATERIALS AND METHODS

The methodology followed in this study is the emphasis of the chapter. The sample collection and reagents are described first, followed by sample preparation used in this work. The experimental design (BBD, RSM) used to model and optimize lipophilic compounds is described below. The sections below describe the extraction of lipophilic compounds from lignocellulosic biomass by SFE and ASE. The characterization techniques were used to confirm the presence of lipophilic compounds in extracts. FTIR was used to verify functional groups, a STA to assess thermal stability, and Py-GC/MS was used for the identification of lipophilic composition.

4.1. Sample and Reagents.

4.1.1 Samples

Pinus patula sawdust sample was obtained from a sawmill in South Africa, stored at 4 °C.

Cannabis *Sativa* L. samples were airdried and stored at 4 °C.

4.1.2 Reagents and gases.

Toluene (95% purity) and ethanol (99% purity) were obtained from Sigma Aldrich (South Africa). Ethanol: toluene(1:2) was used to extract lipophilic compounds by ASE, while ethanol was used to extract lipophilic compounds by SFE and Soxhlet.

CO_2, helium (H_2) and N_2 gases were obtained from Afrox, South Africa. CO_2 was used in the SFE as the main extraction solvent. Helium and nitrogen are inert and were used as purge gasses for Py-GC/MS and STA.

4.2. Sample Preparation

4.2.1 Sawdust

The *Pinus patula* sawdust was ground using a Willey mill and sieved to a particle size of 425 µm using a mechanical sieve shaker with standardized sieves. A Kett FD610 infrared moisture balance (Kett, USA) was used to measure the moisture content of the sawdust, which was then stored at 4°C for further use to avoid loss of volatile compounds.

4.2.2 Cannabis *Sativa*.

Cannabis *Sativa* L. flowers samples were obtained from a farm in South Africa. Drying samples before extraction reduces their water content, which can function as a barrier to extracting the compounds of interest. The samples were ground using a laboratory blender to minimize particle size; this helps to enhance the extraction efficiency. The moisture content of the Cannabis *Sativa* L. was measured using Kett FD610. The ground Cannabis was stored at 4 °C until further use.

4.3 Application of RSM and BBD optimize the SFE and ASE of lipophilic compounds from pinewood sawdust and Cannabis *Sativa* L.

RSM develops a polynomial predictive model that relates the response of the process to the independent process variables. Combinations of experimental parameters such as temperature, pressure, temperature, cosolvent flow rate, CO_2 flow rate, static cycle, and static time were screened for this study. BBD was employed to investigate the effect of process parameters on the efficiency of SFE and ASE. The BBD uses ANOVA to examine significant impacts on the response in BBD. The regression model can display the optimal results by evaluating the model derivatives (Onoji et al., 2019, Kaiser et al., 2013).

4.3.1 Optimisation using RSM for Pinewood sawdust

4.3.1.1 Optimization of Accelerated solvent extraction (ASE)

The RSM and BBD was used to model and optimize lipophilic compounds extraction by ASE. The three independent variables, temperature (A), static cycle (B), and static time (C), were the input parameters, while the yield of the lipophilic compound was the response output. The input parameters were varied within the range of temperature (80 and 160 °C), static cycle (1 and 3) and static time (10 and 15 min) to investigate the effect of input parameters on the yield of lipophilic compounds from the pinewood sawdust. Input parameters and ranges were chosen based on previous work (Jablonsky, 2015, Jablonsky et al., 2015, Andrew et al., 2020a). A three-factor BBD of the RMS was used to generate 17 experimental runs, as illustrated in Table 5 below.

Table 5: BBD of process parameters (temperature, static time, and static cycle) for ASE of lipophilic compounds from pinewood sawdust.

Run	Input			Run	Input		
	A: Temperature (C°)	B: Static time (min)	C: Static cycle		A: Temperature (C°)	B: Static time (min)	C: Static cycle
1	120	10	1	9	160	12,5	1
2	120	12,5	2	10	160	12,5	3
3	160	15	2	11	120	15	1
4	120	12,5	2	12	120	12,5	2
5	120	15	3	13	120	10	3
6	80	12,5	1	14	80	12,5	3
7	120	12,5	2	15	80	15	2
8	120	12,5	2	16	80	10	2
				17	160	10	2

4.3.2 Optimization of Supercritical fluid extraction (SFE)

RSM was applied to SFE to identify the most significant process parameters affecting the extraction yield. The input parameters were pressure (varied between 200 and 300 bar), temperature (varied between 40 and 60 ºC), CO_2 flow rate (varied between 2 and 5 ml/min), and cosolvent flow rate (varied between 1 and 2 ml/min), with the yield of lipophilic compounds (%) as the output parameter. Here, a five-factor BBD experimental was used to generate a total of 29 experimental runs, as illustrated in Table 6 below. These SFE experimental conditions were chosen based on literature (Rozzi et al. 2002b; Pourmortazavi and Hajimirsadeghi, 2007; Martins et al. 2016)

Table 6: BBD of process parameter (temperature, pressure, cosolvent flow rate and CO_2 low rate) of SFE of lipophilic compound from pinewood sawdust.

	Input					Input			
Run	A: Temp (°C)	B: Pressure (bar)	C: flow rate (ml/min)	D: co-solvent flow rate (ml/min)	Run	A: Temp (°C)	B: Pressure (bar)	C: flow rate (ml/min)	D: co-solvent flow rate (ml/min)
1	50	250	3,2	1,5	10	60	300	3,2	1,5
2	40	250	5	1,5	11	50	250	5	2
3	50	250	3,2	1,5	12	50	300	3,2	1
4	50	200	2	1,5	13	40	200	3,2	1,5
5	50	250	2	1	14	50	250	3,2	1,5
6	50	300	5	1,5	15	50	200	3,2	1
7	40	250	2	1,5	16	60	250	5	1,5
8	50	200	3,2	2	17	50	300	3,2	2
9	50	250	2	2	18	60	200	3,2	1,5
	Input					Input			
Run	A: Temp (°C)	B: Pressure (bar)	C: flow rate (ml/min)	D: co-solvent flow rate (ml/min)	Run	A: Temp (°C)	B: Pressure (bar)	C: flow rate (ml/min)	D: co-solvent flow rate (ml/min)
19	60	250	3,2	1	25	40	250	3,2	2
20	50	250	3,2	1,5	26	60	250	3,2	2
21	60	250	2	1,5	27	50	200	5	1,5
22	50	250	3,2	1,5	28	50	250	5	1
23	50	300	2	1,5	29	40	300	3,2	1,5
24	40	250	3,2	1					

4.4. Modelling of the ASE and SFE for lipophilic compounds from pine sawdust

The extraction characteristics are determined by basic mathematical formula with no physical interpretation or estimates of mass transfer coefficients and hence provide minimal insight into the complete extraction process. The experimental data obtained from the experimental parameters (Table 5 (ASE) & 6 (SFE)) were fitted in the quadratic polynomial model equation by RMS, relating the input parameters to the lipophilic compounds yield with the input parameters, according to Equation 1 for ASE and Equation 2 for SFE using Design-Expert software (Stat-Ease Inc., USA).

$$Y= \beta_0 +\beta_1X_1 +\beta_2X_2 +\beta_3X_3 + \beta_{11}X_1^2 + \beta_{22}X_2^2 + \beta_{33}X_3^2 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{23}X_2X \quad (1)$$

Where Y is the lipophilic compounds yield response, β_0 is the intercept, β_1X_1 to β_3X_3 represents the linear blending portion, $\beta_{11}X_1^2$ to $\beta_{22}X_2^2$ are quadratic coefficients, and $\beta_{12}X_1X_2$ to $\beta_{23}X_2X_3$ are the interaction coefficients. The significance of the model was assessed by the ANOVA using Design-Expert software (Stat-Ease Inc., USA). Three-dimensional plots were obtained to study the interaction of one parameter with another.

$$Y_{SFE} = þ_0 + þ_1X_1 + þ_2X_2 + þ_3X_3 + þ_4X_4 + þ_{12}X_1X_2 + þ_{13}X_1X_3 + þ_{14}X_1X_4 + þ_{15}X_2X_3 + þ_{23}X_2X_4 + þ_{24}X_3X_4 + þ_{11}X_1^2 + þ_{22}X_2^2 + þ_{33}X_3^2 + þ_{44}X_4^2 \quad (2)$$

Where Y_{SFE} represents the process output, $þ_0$ is the model constant. The linear coefficients are $þ_0$, $þ_1X_1$, $þ_2X_2$, $þ_3X_3$, and $þ_4X_4$ while $þ_{12}X_1X_2$, $þ_{13}X_1X_3$, $þ_{14}X_1X_4$, $þ_{15}X_2X_3$, $þ_{23}X_2X_4$ and $þ_{24}X_3X_4$, $þ_{11}X_1^2$, $þ_{22}X_2^2$, $þ_{33}X_3^2$, and $þ_{44}X_4^2$ are the quadratic and the interactive coefficients, respectively. The model's significance was assessed by the ANOVA using Design-Expert software (Stat-Ease Inc., USA), as shown in Tables 11 and 12. Three-dimensional plots were obtained to study the interaction of one parameter with another.

4.5 Extraction Process of Lipophilic Compounds by ASE and SFE from Pine Sawdust

4.5.1 ASE of lipophilic compounds from pinewood sawdust

A Dionex ASE 350 (Dionex Corp, Sunnyvale, CA) extraction system, as shown in Figure 18, was employed to extract lipophilic compounds from pinewood sawdust with ethanol: toluene (1:2) mixture as a solvent. A stainless steel Dionex cell of 66 ml was used. A glass fibre filter was placed at the bottom of the cell before filling the cell with 6 g of pinewood sawdust to prevent unwanted particles from passing through into the collection vessel.

The cell was placed in the cell tray of the automated extraction system following BBD extraction conditions defined in Table 5. The extraction solvent (ethanol: toluene (1:2v/v)) pressured at 1500 Psi was filled into a cell containing pinewood sawdust, 5 min of heating before static extraction time was conducted to ensure that the sample was at the equilibrium temperature. The extractions were performed at different static cycles of 1 and 3, the temperature of 80 and160 °C and static time of 10 and15 min was followed according to experimental design in Table 5. Before each experiment, the cell is heated f or a specific time, which varies according to extraction temperature. At 80 °C, heat for 5 min, when extraction temperature was set at 120 ºC, it was heated for 6 min, and at 160 ºC, it was heated for 8 min. After static extraction time, the cell was flushed with 50% of extraction solvent ethanol: toluene and extracts were collected in 250 ml bottles.

Figure 18: Accelerated solvent extractor system used for extraction of lipophilic compounds

4.5.2 Rotary evaporator

The extracts were transferred into a 500 ml round bottom flask and a Bucchi RE rotary evaporator (displayed in Figure 19) at 55 °C and 250 bar was used to evaporate the extract to dryness. The dried extract was weighed by subtracting the mass of an empty round bottom flask from the mass of the flask with extract post drying. The extraction yield was as expressed as the percentage of the extract weight to the sawdust (6 g).

$$\text{Extraction yield\%} = \frac{\text{Mass of extract}}{\text{Mass of raw material}} \times 100$$

Figure 19: Rotary vapor system used to evaporate the solvent out of the extract.

4.5.3 SFE of lipophilic compounds from pinewood sawdust

SFE were conducted using an MV-10 ASFE extraction system (Supercritical Fluid Technologies, Waters, USA) consisting of a CO_2 cylinder, cool water circulator, column thermostat, solvent pump, and backpressure regulator. A mass of 3 g of sawdust was packed into an extraction vessel with an internal volume of 10 ml and placed in an oven. The pressure was adjusted at the ABPR and introduced into the extraction vessel with the pump's adjustment and the flow rate of CO_2 and co-solvent set according to the BBD experiments defined in Table 6. All extractions involved an initial dynamic extraction phase of 15 min followed by a static extraction phase of 10 min and a dynamic extraction phase of 15 min at a flow of 1 ml/min of the makeup solvent (ethanol). Upon completion of the extraction, the extraction vessel was depressurized, and the extract was collected in a sample vial connected to an ABPR. The system was flushed with the co-solvent, and the washings were collected and added to the extracts. The extract was dried at 105 °C, and the extraction yield was expressed as a percentage of the extract weight to the initial weight of sawdust used. The extract was stored at 4 °C until required for further analysis.

4.6. Extraction of Cannabis *Sativa* L. Lipophilic Compounds

4.6.1 Application of RSM and BBD to optimize the SFE of lipophilic compounds from Cannabis *Sativa* L.

4.6.1.1 Optimization of SFE for Cannabis extraction

The RSM was used to determine the conditions for the optimal extraction of Cannabis *Sativa* L. lipophilic compounds by supercritical CO_2 with ethanol as co-solvent. RSM was used to identify the most significant process parameters affecting the extraction yield. The input parameters were pressure (varied between 200 and 300 bar), CO_2 flow rate (varied between 0 and 5 ml/min), and co-solvent flow rate (varied between 0 and 2 ml/min), with the yield of lipophilic compounds (%) as the output parameter. Box-Behnken experimental design was used to generate a total of 10 experimental runs, as illustrated in Table 7 below. The extraction ranges were chosen based on previous studies (Baldino et al., 2020a).

Table 7: BBD of process parameter (pressure, cosolvent flow rate and CO_2 low rate) of SFE of lipophilic compound from Cannabis *Sativa* L.

Input			
Run	A: Cosolvent (ml/min)	B: Pressure (bar)	C: CO_2 (ml/min)
1	0	300	2,5
2	1	250	2,5
3	0	250	5
4	2	200	2,5
5	1	200	0
6	1	300	5
7	0	250	2
8	0	200	2,5
9	2	250	5
10	2	300	2.5

4.7 Modelling of the SFE for lipophilic compounds from Cannabis *Sativa* L.

The experimental data were fitted in the polynomial model equation, relating the input parameters to the yield of lipophilic compounds to the input parameters, according to Equation (3) using Design-Expert software (Stat-Ease Inc., USA).

$$Y= \omega_0 +\omega_{1X1} +\omega_{2X2} +\omega_{3X3} \quad (3)$$

where Y is the response of the lipophilic compound, ω_0 is the intercept, ω_{1X1} to ω_{3X3} represents the linear blending portion. The significance of the model was assessed by the ANOVA.

Yield calculation

The yield of Cannabis lipophilic compounds extracted was calculated, the weight of the ground Cannabis *Sativa* L. flowers was used as the initial weight. The ground

Cannabis *Sativa* L. was used in determining the yield of lipophilic compounds recovered after the solvent was removed from the extract as follows

$$\text{Yield} = \frac{weight\ of\ the\ \ extracted}{weight\ of\ the\ ground\ Cannabis\ flowers}\ \text{compounds}\ x\ 100$$

4.8 Characterization of Lipophilic Compounds

The presence of lipophilic compounds in the pinewood extracts of ASE and SFE was analyzed by FTIR, STA and Py-GC/MS.

4.8.1 Fourier Transform Infrared Spectroscopy (FTIR)

The functional groups of lipophilic compounds were analyzed using a Frontier Universal ATR-FTIR Fourier transform infrared spectrophotometer (PerkinElmer, USA). Universal attenuated total reflectance transmission (ATR) mode was used for obtaining the spectra over the wavenumber range between 4000 cm^{-1} and 600 cm^{-1}. Each spectrum was created using an average of 4 scans with a resolution of 4 cm^{-1}.

4.8.2 Thermal Analysis (TA)

Thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC) of the lipophilic compounds were performed with a simultaneous thermal analyzer (NETZSCH STA-409). The temperature varies, and the mass of a sample is measured over time. The weight difference between the sample's initial and final mass is used to calculate the sample's deterioration and determine thermal stability. The apparatus was continually flushed with nitrogen. The sample weight was weighed to be 10 mg. Each sample was heated from 30 ° to 600 °C at a flow rate of 10 °C min^{-1}. Nitrogen flow rate at 20 ml/min.

4.8.3 Pyrolysis-Gas Chromatography-Mass Spectrometry (Py-GC/MS)

Py-GC/MS method for analyzing and identifying lipophilic compounds of pinewood sawdust and Cannabis *Sativa L*. TMAH was used as a derivatizing agent because derivatizing agents makes it possible to detect compounds in a sample that would otherwise be overlooked or underestimated. TMAH is used in the methylation of carboxyl and hydroxyl groups in controlled heating conditions. Since direct pyrolysis produces a weak chromatographic profile, it is impossible to detect some pyrolysis products. Also, Derivatizing completely transforms the chromatographic profile,

increasing the resolution and degree of separation. Challinor reaction mechanism involves first heat hydrolysis of the original molecule's ester bond or ether by a highly basic alkylating agent like TMAH, resulting in salts. The methyl groups of the tetramethylammonium cation attack carboxylate and alcoholate anions nucleophilically at high temperatures, resulting in the production of methyl derivatives (Sithole et al., 2013a, Mészáros et al., 2007a, Renzi, 2017).

A single-short GA/PY-3030 D (Frontier Lab, Japan), attached to an ultra-alloy capillary column (30 m x 0.25 mm, 0.25 µm), was used for chromatographic separation. Samples were weighed (approximately 100 µg) into a pyrolysis sample holder, and some samples were combined with 0.5 ml of 25% TMAH. The sample holder was placed in an autosampler (*AS-1020E/ET)* tray attached to a pyrolyzer. The samples were automatically transferred into a pyrolysis chamber. The samples were pyrolyzed at 550 °C for 2 min, while the interface temperature to the analytical column was set at 350 °C*.* The Py-GC/MS interface was kept at 250 °C and the injection port at 250 °C*.* Helium was the inert gas flow used to move pyrolysis products from the pyrolysis chamber to the injector of the GC using a split ratio of 50:1. The injection temperature was set to 280 °C and the column flow rate to 1.0 ml/min with helium as a carrier gas.

The GC/MS oven started at 50 °C kept for 2 min during pyrolysis. The temperature was programmed after the pyrolysis products were injected, it was programmed at 120 °C with a heating rate of 5 °C/min and kept for 5 min, heat to 280 °C with the rate of 5 °C/min and kept for 8 min. Finally, heated to 300 °C with a heating rate of 5 °C/min and kept for 5 min. The mass spectrometer's ion source and interface temperatures were set to 200 °C and 300 °C, respectively. The scan range used for the mass selective detector was 40-650 m/z at 1 scan s^{-1} under electron impact at 70 eV. Shimadzu post-run analysis software was used to analyze the data. The pyrolysis products were identified by comparing their mass spectra with the mass spectrum NIST library and WR10 attached to the instrument (Coulier et al., 2005, Marques et al., 2007, Prinsen et al., 2012a)

CHAPTER 5

5. RESULTS AND DISCUSSION

The experimental results and findings are discussed in this chapter. The optimization of SFE and ASE by response surface methodology is discussed, and results are compared. The Py-GC/MS in the presence and absence of TMAH used to identify lipophilic compounds is discussed and compared. The TA and functional group confirmation by FTIR are also discussed.

5.1 Optimization of Lipophilic Compounds Yield and Development of the RMS Model of Lipophilic Compounds of Pinewood Sawdust.

5.1.1 Accelerated solvent extraction (ASE)

The experimental conditions with the yields of lipophilic compounds extracted from pinewood sawdust are presented in Table 8. The experimental data were used to develop a quadratic polynomial equation showing the relationship between the yield as an output response with the input variables, A-temperature, B-static time, and C-static cycle, as shown in Equation 3.

Lipophilic compounds Yield for ASE (%) = +1.90 +0.4750A + 0.0750B + 0.0250C + 0.0750AB - P0.7250AC -0.1750BC + 0.8125A2 +0.0625B2 + 0.0125C2 (3)

Table 8: BBD of process parameters (temperature, static time, and static cycle) for ASE of lipophilic compounds from pinewood sawdust.

Run	Input			Output	Run	Input			Output
	A: Temperature (C°)	B: Static time (min)	C: Static cycle	Yield (%)		A: Temperature (C°)	B: Static time (min)	C: Static cycle	Yield (%)
1	120	10	1	1,4	9	160	12,5	1	4,2
2	120	12,5	2	1,6	10	160	12,5	3	2,1
3	160	15	2	3,5	11	120	15	1	1,8
4	120	12,5	2	1,6	12	120	12,5	2	2
5	120	15	3	2,2	13	120	10	3	2,5
6	80	12,5	1	1,9	14	80	12,5	3	2,7
7	120	12,5	2	2	15	80	15	2	2,3
8	120	12,5	2	2,3	16	80	10	2	2,2
					17	160	10	2	3,1

5.1.2 Supercritical fluid extraction (SFE)

The experimental conditions with the yields of lipophilic compounds extracted from pinewood sawdust are presented in Table 9. The experimental data were used to develop a quadratic polynomial equation showing the relationship between the yield as an output response with the input variables as shown in equation 4.

Lipophilic compounds for SFE yield (%) = -0.5217 +0.1486 + 0.04401 - 4.9894 - 0.000855 + 0.007143 - 0.02250- 0.002357 + 0.0171 - 0.07533 (4)

Table 9: BBD of process parameter (temperature, pressure, cosolvent flow rate and CO_2 low rate) of SFE of lipophilic compound from pinewood sawdust.

	Input				Output		Input				Output
Run	A: Temp (°C)	B: Pressure (bar)	C: flow rate (ml/min)	D: co-solvent flow rate (ml/min)	Yield (%)	Run	A: Temp (°C)	B: Pressure (bar)	C: flow rate (ml/min)	D: co-solvent flow rate (ml/min)	Yield (%)
1	50	250	3,2	1,5	0.8	10	60	300	3,2	1,5	0.36
2	40	250	5	1,5	0.73	11	50	250	5	2	0.62
3	50	250	3,2	1,5	0.77	12	50	300	3,2	1	0.79
4	50	200	2	1,5	0.02	13	40	200	3,2	1,5	0.54
5	50	250	2	1	1.38	14	50	250	3,2	1,5	0.63
6	50	300	5	1,5	0.81	15	50	200	3,2	1	0.25
7	40	250	2	1,5	1.3	16	60	250	5	1,5	0.4
8	50	200	3,2	2	0.25	17	50	300	3,2	2	2.5
9	50	250	2	2	1.5	18	60	200	3,2	1,5	0.6
	Input				Output		Input				Output
Run	A: Temp (°C)	B: Pressure (bar)	C: flow rate (ml/min)	D: co-solvent flow rate (ml/min)	Yield (%)	Run	A: Temp (°C)	B: Pressure (bar)	C: flow rate (ml/min)	D: co-solvent flow rate (ml/min)	Yield (%)
19	60	250	3,2	1	0.5	25	40	250	3,2	2	1.6
20	50	250	3,2	1,5	0.89	26	60	250	3,2	2	0.55
21	60	250	2	1,5	0.61	27	50	200	5	1,5	0.61
22	50	250	3,2	1,5	0.87	28	50	250	5	1	0.6
23	50	300	2	1,5	0.81	29	40	300	3,2	1,5	2.01
24	40	250	3,2	1	1.1						

5.2 Response Surface Regression (RSM)

The ANOVA of the quadratic regression models obtained from the experimental values was used to evaluate the authenticity of the fitted models displayed in Tables 10 ASE and 11 SFE. The yield model of lipophilic compounds displayed high F-values of 3.91 and 3.93 with low p-values of 0.0428 and 0.0076, respectively. The high F values and low p-values (<0.05) indicate significant polynomial models. The value of the R^2 of the polynomial model shown in Table 12 for the two models were 0.8242 (percentage yield of lipophilic compounds by ASE) and 0.7970 (percentage yield of lipophilic compounds by SFE), specifying that the model can explicate for 82% of the variations in the data shown for ASE models and 80% of the variation in the data shown for SFE.

Table 10: ANOVA of a full polynomial model for extracting lipophilic compounds from pinewood sawdust extracted by ASE.

Source	Sum of Squares	Df	Mean Square	F-value	p-value	
Model	6.94	9	0.7716	3.91	0.0428	Significant
A-Temperature	1.8	1	1.8	9.16	0.0192	
B-Static time	0.045	1	0.045	0.2283	0.6474	
C-Static Cycle	0.005	1	0.005	0.0254	0.878	
AB	0.0225	1	0.0225	0.1141	0.7454	
AC	2.1	1	2,1	10,66	0,0138	
BC	0.1225	1	0.1225	0.6214	0.4564	
A^2	2.78	1	2.78	14.1	0.0071	
B^2	0.0164	1	0.0164	0.0834	0.7811	
C^2	0.0007	1	0.0007	0.0033	0.9555	

Residual	1.38	7	0,1971			
Lack of Fit	1.02	3	0.34	3.78	0.1159	not significant
Pure Error	0.36	4	0.09			
Cor Total	8.32	16				

Table 11: ANOVA of a full polynomial model the developed model of lipophilic compounds extracted by SFE.

Source	Sum of Squares	Df	Mean Square	F-value	p-value	
Model	6.48	14	0.4629	3.93	0.0076	Significant
A-Temp	1.32	1	1.32	11.23	0.0047	
B-Pressure	1.77	1	1.77	15.02	0.0017	
C-flow rate	0.2852	1	0.2852	2.42	0.1421	
D-cosolvent flow rate	0.4226	1	0.4226	3.58	0.0792	
AB	0.731	1	0.731	6.2	0.0259	
AC	0.0471	1	0.0471	0.3999	0.5373	
AD	0.0506	1	0.0506	0.4295	0.5229	
BC	0.1283	1	0.1283	1.09	0.3144	
BD	0.731	1	0.731	6.2	0.0259	
CD	0.0131	1	0.0131	0.1112	0.7437	
A^2	0.0103	1	0.0103	0.0873	0.772	
B^2	0.0189	1	0.0189	0.16	0.6952	
C^2	0.0118	1	0.0118	0.1	0.7565	
D^2	0.2788	1	0.2788	2.37	0.1463	
Residual	1.65	14	0.1179			
Lack of Fit	1.61	10	0.1608	15.14	0.0093	Significant
Pure Error	0.0425	4	0.0106			
Cor Total	8.13	28				

Table 12: The statistical ANOVA analysis of the ASE and SFE extract.

	ASE	SFE
Std. Dev.	0.4436	0.3433
Mean	1.98	0.8414
C.V. %	22.43	40.81
R^2	0.8863	0.797
Adjusted R^2	0.7401	0.5941
Predicted R^2	0.2866	-0.0719
Adeq Precision	8.1152	7.121

5.3 Interactive Effect of the Input Parameters on the yield of Lipophilic Compounds of Pinewood Sawdust

The main effects of the three ASE parameters (temperature, static cycle, and static time) and four SFE parameters (temperature, pressure, cosolvent flow rate, and CO_2 flow rate) and their interactions on the lipophilic extraction yields are shown in the 3D RMS plots in Fig. 20a-c and Fig. 21a-f for ASE and SFE, respectively.

5.3.1 Accelerated solvent extraction

5.3.1.1 Effect of temperature ASE

The RSM plot illustrating temperature with static time and the static cycle is shown in Figures 20a and 20b below. The results show that the maximum yield of 42% was obtained when the lowest number of static cycles and the highest temperature was implemented (160 °C, 1 static cycle, and 12.5 min). This trend corresponds well with Vek et al. (2018) study, which found almost all lipophilic compounds were extracted using 1 static cycle. The static extraction time did not significantly affect the extraction yield at high temperatures (160 °C). Also, the solute and solvent had a minimal contact

time; the dominant factor determining extraction efficiency is the solute dissolving in the solvent at a high temperature (Jentzer et al., 2015). Figure 20a shows the interaction between static time and temperature at a constant static cycle of 3 cycles. At 80 °C, the yield was 2.7% higher than at 160 °C of 2.5% with a 12.5 min extraction time. Low temperatures and long extraction cycles give high yields.

In contrast, high temperatures and long extraction cycles give low yields due to the degradation of thermosensitive compounds. Low temperatures are more suited for extraction when a long extraction time is employed. At 120 °C, the yield increased with time from 1.13% to 2.3% at 15%. This outcome is contrary since a long extraction time negatively affected the yield by degrading some compounds. However, the extraction yield at mild temperature was predominantly affected by solubility and solvent contact with the sample.

When the static cycle was increased at a high temperature, the yield decreased drastically from 4.2% to 2.5% (160 °C, 12.5 min, and 3 static cycles). This may be due to the degradation of thermolabile compounds. It has been reported in previous studies that using a long extraction time with corresponding high temperatures increases the degradation rate of thermolabile compounds, resulting in a decrease in the extraction yield (Aka et al., 2013). Increasing temperature reduces the solvent intermolecular forces and consequently the viscosity, thereby increasing the solvent's ability to solubilize the target analyte and wet the matrix by reducing the surface tension allowing the solvent to better coat the sample (Mustafa and Turner 2011; Richter and Raynie 2012). This makes it easier to form cavities, and lipid molecules are more readily dissolved in the solvent. A high temperature was also the main contributor to lutein extraction's high yield (Kang, Kim, and Moon 2016). It was also found that increasing temperature increased the extra-activity of substances in all evaluated samples in a study (Jablonský et al. 2016). Breaking analyte–matrix linkages requires more energy, increasing temperature makes the diffusion of analytes easier (Jentzer et al., 2015). In addition, using low static cycles reduces the risk of losing thermolabile compounds at high temperatures. Figure 20b illustrates the interaction of temperature and static cycle at a constant time of 15 min. The yield was constant when the temperature was 80 °C and 120 °C with different static cycles; this demonstrates that the extraction temperature was no longer effective at 15 min with 3 static cycles. While

at 120 °C, 1 static cycle, the yield was lower than when 3 static cycles were employed at this same temperature.

5.3.1.2 Effect of static time

The response surface graph illustrating the interactive effects of static time on the yield of lipophilic compounds is shown in Figures 20a and 20c below. It was observed that increasing static time from 10-15 min with simultaneous increase of the static cycle favored low to moderate temperatures to extract lipophilic compounds. Maximum static time and static cycle at high temperatures had a negative impact on the yield. Long static cycles favor low extraction temperature, as long static cycles and high temperatures degrade thermolabile compounds, resulting in a decrease in yield. Increasing the extraction time from 12.5 to 15 min at 120 ºC increased the yield of extracts significantly. High extraction time favored the use of moderate temperatures. However, low static times and moderate static cycles did not improve the extraction yield. As stated above, the temperature at a static time of 15 min and 3 static cycles is not effective because the contact time was longer, dissolution was no longer a limiting factor, and the extraction temperature was no longer a factor (Jentzer et al., 2015).

5.3.1.3 Effect of static cycle

The response surface graph illustrating the interactive effects of static time on the yield of lipophilic compounds is shown in Figures 20b and 20c. The results show that an increase in the static cycle from 1-3 at a high temperature of 160 °C resulted in a decrease in the yield of lipophilic compounds. When the extraction temperature was like the extraction solvent's boiling points, the yields did not differ significantly and varied between 2% - 2.3%. However, positive effects were noted with a variation of the number of static cycles at mild temperatures. For instance, a significant increase in the extraction yield at a temperature of 120 °C was observed when a long extraction cycle (3 cycles) was implemented; the yield increased from 2% to 2.3%. This result indicates that the variation in the number of static cycles influences the extraction yield by increasing it at moderate temperatures. This could be due to the increased contact period between the sample and solvent. In this study it was evident that varying extraction cycles affected the extraction yield at low to moderate temperatures, and this is similar to the findings from other studies where the variation in the number of cycles increased the yield (Toubane et al., 2017).

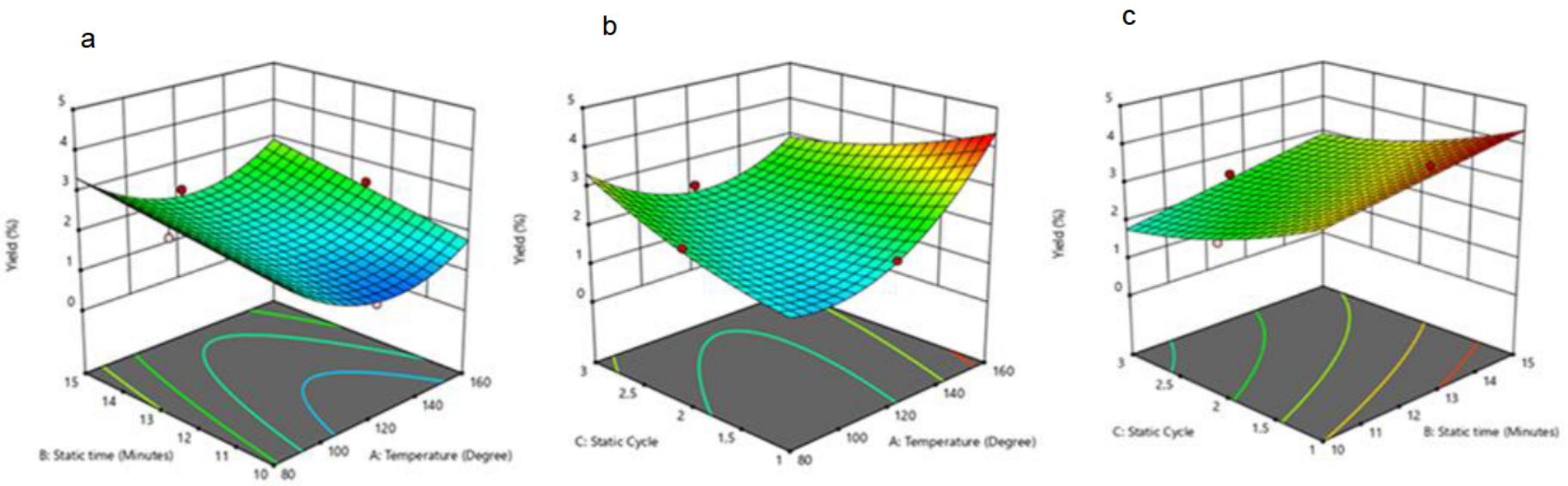

Figure 20: 3-D RSM plot showing extraction parameters' interaction with the yield. A: showing the interaction of static time and temperature. b: showing the interaction of the number of static cycles and temperature, and c: showing the interaction of static cycle and static time on the extraction yield of lipophilic compounds.

5.3.2 Supercritical fluid extraction (SFE)

5.3.2.1 Effect of the flow rate of co-solvent

The effect of the co-solvent flow rate on the yield of lipophilic compounds is shown in Figures 21, 21e & 21f. The addition of cosolvent increases the CO_2 solvent power and polarity and enhances extracts' solubility (Pourmortazavi and Hajimirsadeghi, 2007). Conde et al. (2013b) added that the ratio of target solute solubilities in the presence and absence of cosolvent CO_2 could also be used to calculate the co-solvent effect. The yield of lipophilic compounds was observed to increase with an increase in the cosolvent flow rate from 1 to 2 ml/min and reached the maximum extraction yield at 2 ml/min. The flow rate increment might have enhanced the extract's solubility since polar cosolvents actuate structural changes, and extraction efficiency improves due to hydrogen bonding and dipole-dipole interaction between the cosolvent and sample (Esquivel-Hernández et al., 2016). A flow rate of cosolvent has been reported to improve the extraction yield of plant extract with an increase in the amount of cosolvent as more polar constituents are extracted with a more polar solvent such as ethanol (Maran et al., 2014, Pourmortazavi and Hajimirsadeghi, 2007).

Furthermore, the increase in the cosolvent flow rate from 1-2 ml/min at a temperature of 50 °C resulted in the maximum lipophilic compounds yield of 2.5%. This may be due to the effect of heat on the solvent's solubility and the increase in solubility brought by the addition of the cosolvent since the solubility enhancement of the cosolvent is attributed to the increase in solvent density or intermolecular forces (Güçlü-Üstündağ and Temelli, 2005). However, increasing the cosolvent flow rate to 2 ml/min at a temperature above 50 °C resulted in a decrease in the extraction yield. A possible reason for this decrease may be the solute volatility being the dominant factor over low fluid density (Rozzi et al., 2002). Increasing the pressure to 300 bar at high temperatures resulted in low yields at a maximum cosolvent flow rate (2 ml/min). This may be explained by the extract’s volatility and polarity decrease. The unique dipole-dipole and hydrogen bonding interactions established between solutes and the alcohol, which increase the solubility of polar molecules in the supercritical fluid, may explain the dominance of ethanol concentration in proportion to pressure (Martins et al., 2016). Increasing the cosolvent flow rate at a low temperature of 40 °C from 1 to 2 ml/min decreased the yield to 0.73%, while the highest yield achieved at 40 °C was 2.01% when the cosolvent flow rate was 1.5 ml/min.

5.3.2.2 Effect of supercritical fluid carbon dioxide (CO_2)

The CO_2 flow rate on the yield of lipophilic compounds is shown in Figures 21b, 21d, and 21f below. The yield of lipophilic compounds decreased with an increase in CO_2 flow rate between 3.2 – 5 ml/min. This may be associated with a decrease in contact time between the solvent and sample (Rozzi et al., 2002). Using high flow rates forces the solvent through the sample, passing it only around the sample matrix, not diffusing it through the sample pores restricting the movement of CO_2 into and out of the sample (Rozzi et al., 2002). Furthermore, using low flow rates improves the analyte's trapping ability and increases the extraction efficiency due to the intraparticle diffusion resistance (Papamichail et al., 2000). A low flow rate improves the mass transfer, thus increasing the contact time between solvent and sample to be extracted. Thus, the fluid's slow movement promotes deeper fluid penetration into the solute matrix and reduces the linear velocity, resulting in high extraction efficiency (Yi et al., 2009). The yield of lipophilic compounds increased from 0.54 to 2.5% at a flow rate of 3.2 ml/min of CO_2 when the temperature was increased from 40 – 50 °C at different pressures. An increase in the fluid flow rate and temperature resulted in low yields, while mild flow rates and high pressures increased the extraction yield. The maximum yield was obtained when a low CO_2 flow rate was used; this might be due to enough contact time between the solvent and sample matrix.

5.3.2.3 Effect of pressure

The interaction of the pressure on the yield of lipophilic compounds is shown in Figures 21a, 21d, & 21e. The increase in supercritical fluid carbon dioxide pressure results in low volatility, high polarity, and extraction of high molecular weight compounds (Wang et al., 2008, Brunner, 2005). In this study, the increase in pressure from 200 to 300 bar at a temperature of 50 °C resulted in an increased yield of lipophilic compounds from 1.38 to 2.5%. This may be due to increased solvent density and a decreased distance between the molecules that enable increased interaction between the samples and fluid CO_2, resulting in enhanced solubility of the solute and enhanced mass transfer and diffusion of the solvent into the sample (Papamichail et al., 2000, Lang and Wai, 2001, Maran et al., 2014). An increase in the yield of lipophilic compounds was also observed with increasing pressure in a study by Talmaciu et al. (2016), (Conde et al., 2013a), where the solubility was also reported to be enhanced with the increase in pressure. An analysis of the recovery of lipophilic compounds from

spruce bark showed that high pressures are suitable for extracting lipophilic compounds. The yield improved with increasing pressure to a maximum of 2.08% at 200 bar due to the increase in solvent density (Talmaciu et al., 2016).

The results obtained in this study showed that using high pressure improves the yield of lipophilic compounds due to greater solvating power and high densities of the fluid at different temperatures. However, a decrease in the yield of lipophilic compounds was observed with an increase in temperature at 60 °C at high pressures. This may be due to decreased fluid density, increase in volatility, and polarity of the extracts (Wagner et al., 2013). Low yields at 200-250 bar pressures may be associated with vapor pressure on the solubility of the lipophilic compounds. Supercritical CO_2 behaves like an ideal gas that does not have unique dissolvable attributes at low pressures (de Castro et al., 1994, Rajaei et al., 2005).

5.3.2.4 Effect of temperature

Increasing temperature decreases supercritical fluid density while increasing the solute's volatility (Pourmortazavi and Hajimirsadeghi, 2007, Rozzi et al., 2002b). Thus extraction capability decreases with a decrease in the density of the SF-CO_2, and the amount of extract increases with the increase in solute volatility (Taylor, 1996). The effect of temperature on the extraction yield of lipophilic compounds is observed in Figures 21a, 21b & 21c. The increase in extraction temperature from 40 to 50 °C at a constant pressure of 300 bar and maximum cosolvent flow rate of 2 ml/min (Figure 21c) resulted in a maximum yield of lipophilic compounds of 2.5%. This observation may be explained by the "crossover point", which is a parameter that is dependent on temperature when the pressure is constant and the solubility changes, increase in temperature has a positive effect on the yield of lipophilic compounds regardless of the decrease in density of the fluid since the vapor pressure of the analyte increases.

The solubility is governed by a balance between the solvent density and the change in the solute vapor pressure with increasing temperature, which results in this cross-over pressure (Akay et al., 2011). Also, the density of SF-CO_2 drops more slowly at higher pressures as temperature rises than at lower pressures. The increase in vapor pressure of the solute that happens with rising temperature from 40 to 50°C is able to overcome the SF-diminished CO_2's solvating capability with this higher pressure setting (Wagner et al., 2013).

Similar results were observed in a study by Conde et al. (2013b), where 50 °C and 10% co-solvent gave a high yield of lipophilic extractives extracted from spruce bark. In this study, the increase in temperature to 60 °C and CO_2 flow rate of 5 ml/min, as shown in Figure 21b, decreased the extraction yield to 0.4%, even though the lipophilic compounds' solubility may still increase. Mass transfer accelerated with increased temperature (Turner et al., 2001, Esquivel-Hernández et al., 2016). However, the decrease in the yield may be associated with a low fluid density being the dominant factor. At pressures in the critical range, the influence of temperature on solute solubility is different and fluid density is very sensitive to temperature in the critical range (Wang et al., 2008). In addition, some lipophilic compounds are thermolabile compounds and using high temperatures may degrade some of them.

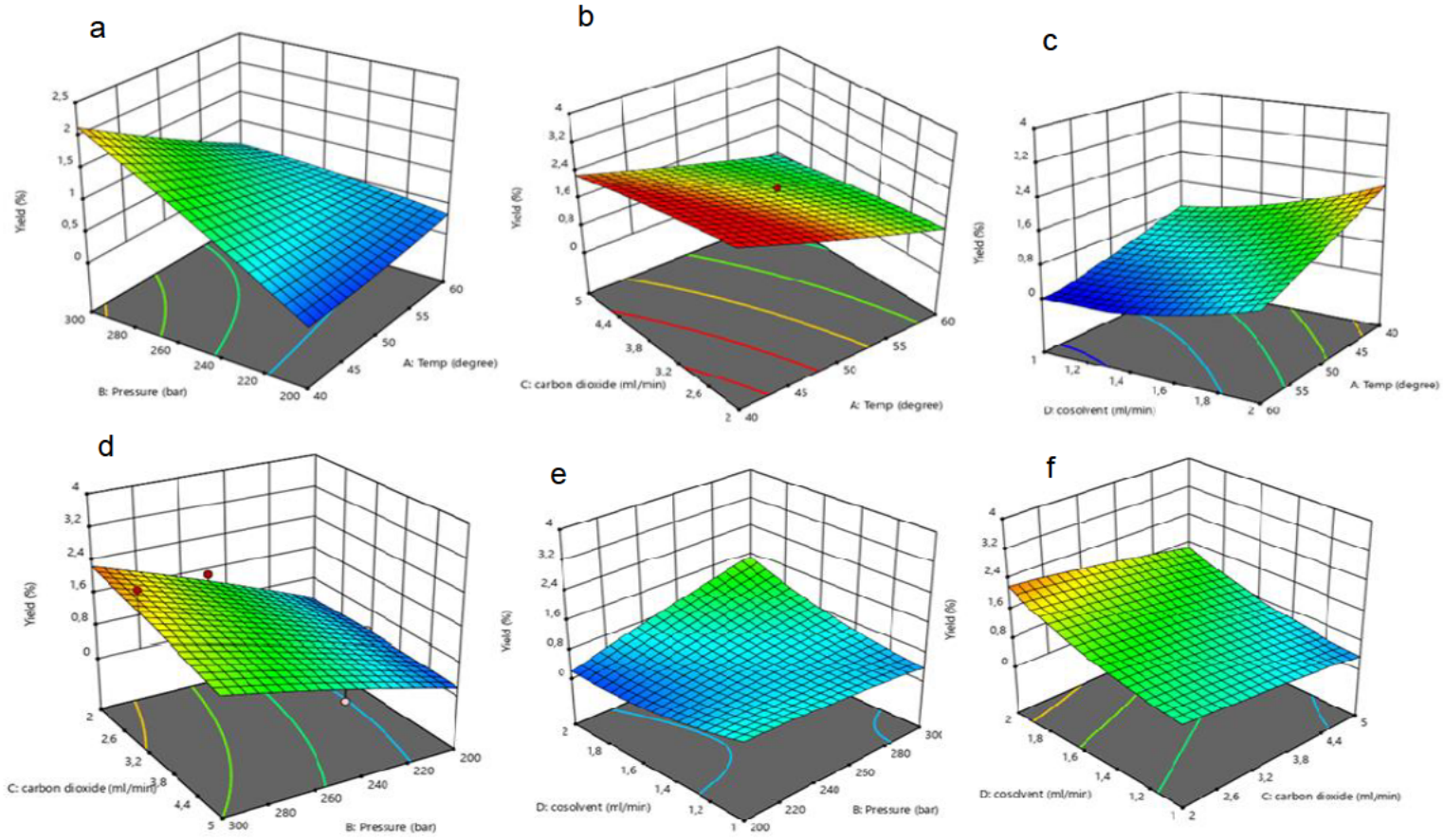

Figure 21: 3-D RSM plot showing the interaction of input parameter on the extraction yield, a: shows the interaction of pressure and temperature, b: shows CO_2 flow rate and temperature, c: shows cosolvent flow rate and temperature. d: shows CO_2 flow rate and pressure, e: shows cosolvent flow rate and pressure and f: shows cosolvent and CO_2 flow rate interaction.

5.4 Comparison of SFE and ASE efficiency of lipophilic compounds from Pinewood sawdust

Using the optimal conditions of SFE and ASE, the yield of lipophilic compounds from pine sawdust was 2.5% and 4.2%, respectively. Although ASE uses high temperatures that may degrade thermolabile compounds, the short extraction times may work in their favour since the extracts are not exposed to high temperatures for long periods. SFE uses low temperatures and long extraction times, and several properties affect the extraction efficiency. These include volatility, dissolving power, solubility and density of the extracting solvent. The extraction efficiency of lipophilic compounds by SFE may be affected by the supercritical fluid's solubility and differences in densities at different pressures. In ASE, the high yields were influenced by the high polarity of the solvent mixture and temperature with a short extraction time.

5.5 Optimization of lipophilic compounds yield and development of the RSM model of lipophilic compounds of Cannabis *Sativa* L.

The experimental conditions with the lipophilic compound yield are presented in Table 14. The developed polynomial model for lipophilic compounds yields optimization is represented in equation 5 shows the interaction between pressure, CO_2 flow rate, and cosolvent flow rate for the SFE.

$$Y= 6,585577 + 10,59738 + 0.083990 + 2,03059 \quad (5)$$

where Y represents the lipophilic compounds yields of Cannabis, A, B, and C represent cosolvent flow rate, pressure, and CO_2 flow rate. ANOVA of the regression model gave an F-value of 20.43 and a P-value of 0.0015, demonstrating the significance of the model (Table 13). The model had R^2 of 0,9108 thus could account for 91% of the variability observed in the response. The result shows that cosolvent flow rate, pressure, and CO_2 flow rate significantly affected the lipophilic compounds released, as evidenced by their P-values ($P < 0.0015$).

Table 13: ANOVA of the lipophilic compounds Cannabis *Sativa* L. SFE

Source	Sum of Squares	Df	Mean Square	F-value	p-value	
Model	622.32	3	207.44	20.43	0.0015	Significant
A-Cosolvent	148.15	1	148.15	14.59	0.0088	
B-Pressure	317.78	1	317.78	31.3	0.0014	
C-CO_2	11.91	1	11.91	1.17	0.3203	
Residual	60.93	6	10.15			
Cor Total	683.24	9				

Std. Dev.	4,15	R^2	0,9144
Mean	43,11	Adjusted R^2	0,8717
C.V. %	9,62	Predicted R^2	0,7449
		Adeq Precision	11,6192

Table 14: BBD of the process parameter and the lipophilic compounds output of SFE.

Input				Output
Run	A: Cosolvent (ml/min)	B: Pressure (Bar)	C: CO_2 (ml/min)	Yield (%)
1	0	300	2.5	43.2
2	1	250	2.5	42.3
3	0	250	5	36.1
4	2	200	2.5	35.1
5	1	200	0	32.9
6	1	300	5	55
7	0	250	2	31.36
8	0	200	2.5	30
9	2	250	5	46
10	2	300	2.5	52

5.6 Interactive effects of the process parameters on lipophilic compounds yield from Cannabis *Sativa* L.

The main effects of the three SFE parameters (pressure, cosolvent and CO_2 flow rate) and their effect on the yield of the lipophilic compound are shown in the 3D response surface plot in Figure 22.

The supercritical fluid has a good solvent power at high pressures, and the presence of a co-solvent enhances the solvation power of SF-CO_2 (Gallo-Molina et al., 2019). The maximum extraction yield was 55%, as shown in Table 14 which corresponds to run 6 at 300 bar, 1 ml/min cosolvent, and 5 ml/min CO_2. A maximum extraction yield was obtained at 34 MPa, and Da Porto et al. (2012b) achieved a maximum yield at 30 MPa. However, their yields were lower compared to the maximum yield obtained when 300 bar was employed. It was noted that the increase in CO_2 flow rate had a

positive effect: when the flow rate was increased from 2.5 to 5ml/min, a maximum yield was obtained. This can be explained by a mass transfer phenomenon, where the increase in flow rate of CO_2 at high pressures reduces the mass transfer resistance (Akanda et al., 2015). However, using high flow rates is not recommended as a high flow rate may reduce the solute-solvent contact. The cosolvent flow rate is observed to have a minimal effect on the yield of lipophilic compounds. At a constant pressure of 300 bar, the cosolvent flow rate was 1 ml/min, which was higher compared to the maximum cosolvent flow rate (2 ml/min), which gave an extraction efficiency of 52%. However, it can be concluded that the presence of the cosolvent enhances the solvating power of CO_2, which increases the efficiency since the increase of the cosolvent flow rate from 0-2 ml/min increased the yield drastically from 43.2- 55%.

Because of its hydroxyl group, adding ethanol with CO_2 significantly impacts fatty acids (OH). Because of its strong negative charge, oxygen is prone to hydrogen bonding with other positively charged polar groups in fatty acids. It improves the solute-solvent interaction (CO_2 and ethanol) (fatty acids). Furthermore, as the flow rate of the co-solvent increases, the solubility drops, which could be owing to the non-polar hydrocarbon chain (Devi and Khanam, 2019b).

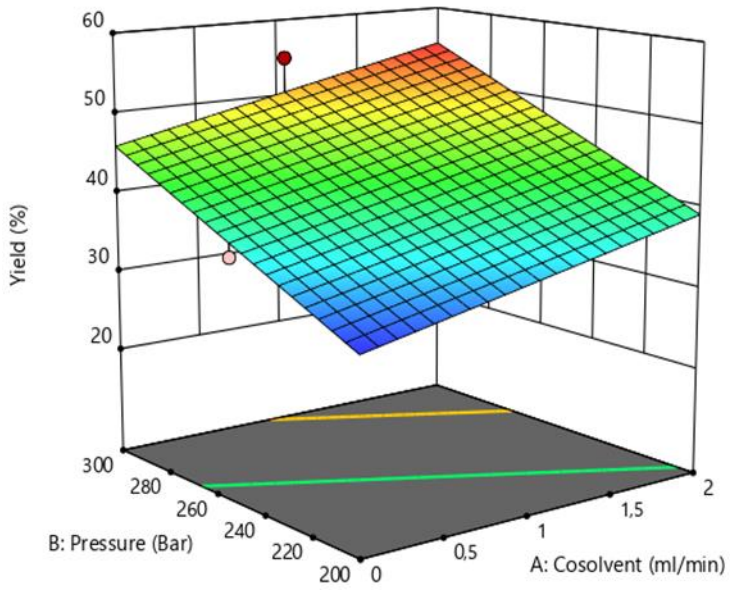

Figure 22: 3-D RSM plot showing the interaction of pressure and cosolvent flow rate.

5.7 Characterization of Lipophilic Compounds

5.7.1 Thermal analysis (TA)

TGA and DSC results of the lipophilic extracts extracted from pinewood sawdust are shown in Figures 23 and 24.

The thermogram displayed in Figure 23 shows the percentage of weight loss as a function of temperature. A minimum weight loss was observed before 150°C, attributed to high volatiles. When the temperature was increased to 450°C, the weight loss rate increased significantly to over 50%. This degradation may be associated with medium volatiles and lipophilic compounds (Shebani, van Reenen and Meincken 2008; Poletto et al. 2012). The TGA plot for both techniques had a slightly different slope, which may be due to the chemical variation not showing any significant differences in thermal degradation. While heat flow (H.F.) measurements performed by DSC in Figure 24 displayed a significant difference in the curves. The small exothermic peak observed at 150°C displayed by the SFE curve was not observed with ASE, indicating that each sample's lipophilic compounds differed significantly. The DSC curve of ASE shows a complete decomposition of compounds starting from a low temperature, representing a high content of lipophilic compounds present in the sample that may be degraded quickly.

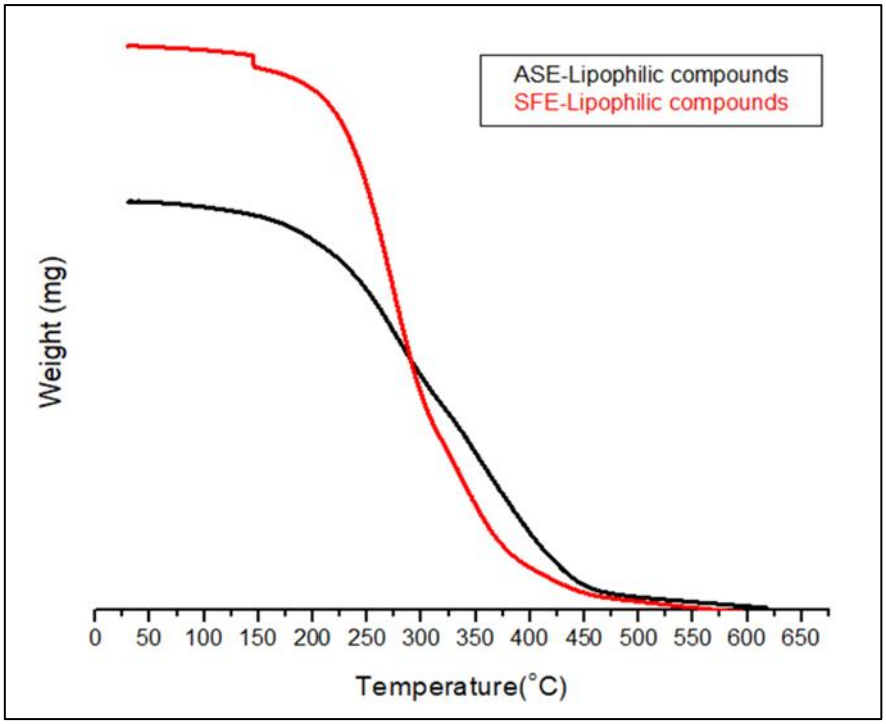

Figure 23: Thermogram of lipophilic compounds extracted from pinewood sawdust using ASE and SFE.

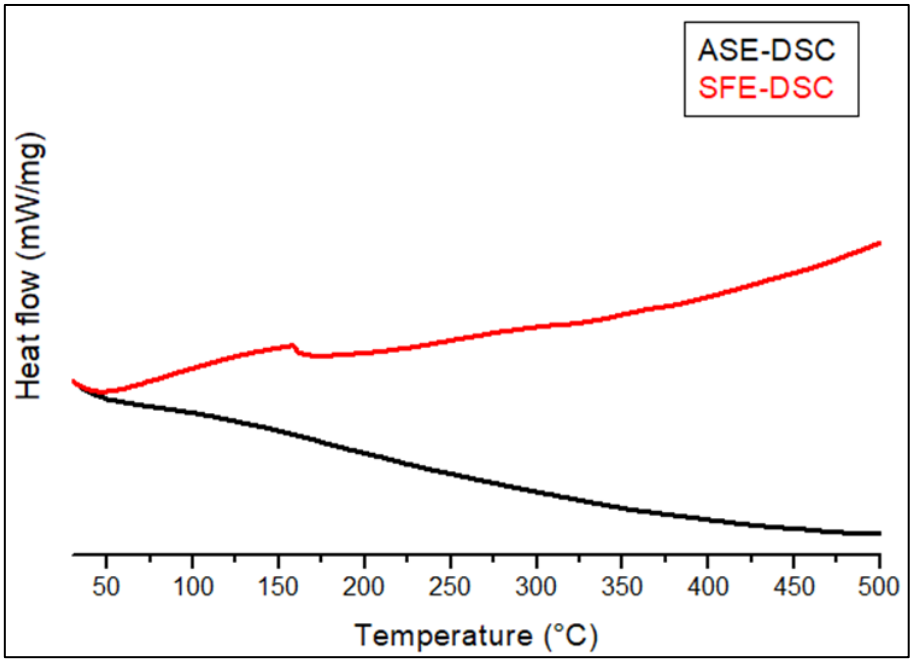

Figure 24: DSC curve of lipophilic compounds extracted from pinewood sawdust by ASE and SFE.

5.7.2 Fourier transform infrared (FTIR) spectroscopy of lipophilic compounds

The FTIR spectra of the lipophilic compounds extracted with ASE and SFE are shown in Figure 25; a broad and medium intensity band was observed at 3385 cm^{-1}, corresponding to O-H groups. The bands at 2925 cm^{-1} and 2854cm^{-1} correspond to asymmetric and symmetric vibration stretching of aliphatic C-H. The band at 1705 cm^{-1} is associated with the C=O stretching vibration of conjugated and non-conjugated acids, and CH_2 groups are more intense in the compounds extracted with SFE. The band at 1514 cm^{-1} corresponds to the symmetrical stretching (benzene ring) and symmetrical stretching of the C=C aromatic ring. The sharp bands at 1456 cm^{-1} and 1378 cm^{-1} -CH_2 and -CH_3, indicative of bending vibrations of aliphatic groups, are observed in the compounds extracted by SFE but not in the compounds extracted by ASE. This indicates a variation in the lipophilic compounds present in each extract. The bands at 1160 cm^{-1} and 1032 cm^{-1} (C-O) correspond to the stretching of trans, and cis ester groups are more intense for compounds extracted by ASE. This band indicates the interaction between carbon single-bonded oxygen stretching and in-plane carbon single-bonded hydroxyl bending in carboxylic acids (Ajuong and Breese, 1998, Sun and Sun, 2001). Wavenumber 1800 cm-1 to 600 cm^{-1} is considered the fingerprint region of wood (Mattos et al. 2016). Previous studies have shown that bands related to wood extractives occur at 1705cm^{-1}, 1640cm^{-1}, 1514cm^{-1} and 1237cm^{-1} (Poletto et al. 2012; Mattos et al. 2016).

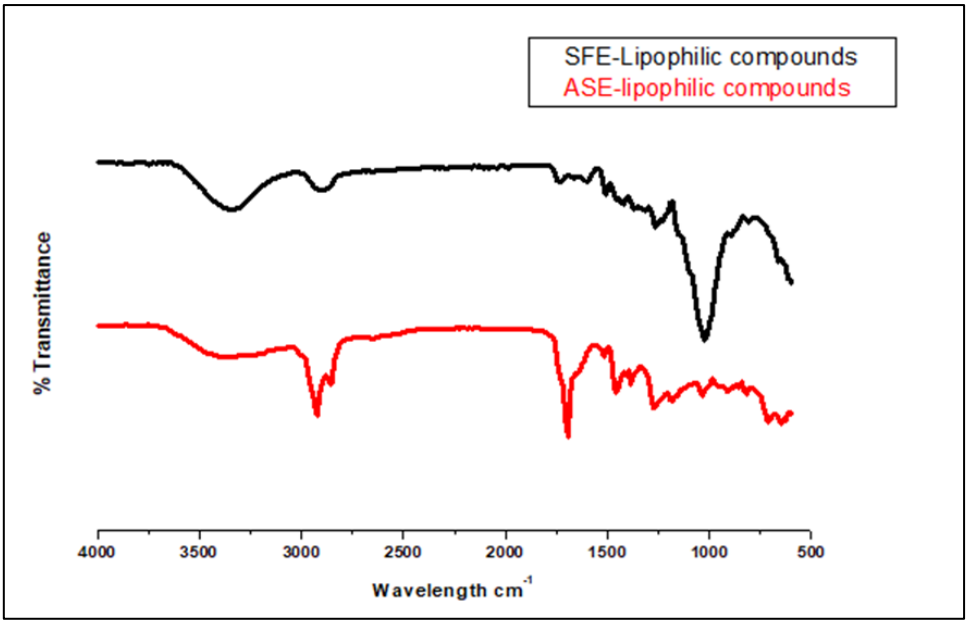

Figure 25: FTIR of lipophilic compounds extracted by ASE and SFE of pine sawdust.

The FTIR analysis of extracted lipophilic compounds of Cannabis *Sativa* L. revealed absorbance peaks corresponding to several bands. Figure 26 depicts the spectrum of these analyses.

FTIR was used to identify functional groups of lipophilic compounds of Cannabis *Sativa* L. The broad absorption bands at 3500 to 3330 cm^{-1} were assigned to -O-H stretching vibration from the aromatic C-OH group situated in the para-position of the carboxylic group fragment; this absorption band can be classified as a hydroxyl group of a crystal form of cannabinoids formed from a solid. The crystal form of CBD has its hydroxyl group with various vibrational energies, which are indicated by two signals at roughly 3500 cm^{-1} (Galvão, 2019, Dorado et al., 2001, Geskovski et al., 2021, Güler et al., 2016, Devi and Khanam, 2019a). The two bands at 3000 and 2886 cm^{-1} represent stretching vibrations more specific for carboxyl groups and the stretching of the C-H bond of the CH_2 (sp^2) group. The bands at 3000 cm^{-1} may be due to (or attributed to) the aromatic component as well as the presence of fatty acids. At the same time, the bands at 2886 cm^{-1} are attributed to the respective asymmetric and symmetric stretching of the C-H bond of the CH_3(sp^3) group of fatty acids (Galvão, 2019, Dorado et al., 2001, Geskovski et al., 2021, Güler et al., 2016, Devi and Khanam, 2019a). The 1600 cm^{-1} represents C=C unsaturated acyl groups originating from the carbonyl (C=O) stretching vibration associated with unsaturated

hydrocarbons. The -OH stretching assigned to 1521 cm^{-1} are overtones of the −OH group, most probably from water, and symmetric and asymmetric stretching of N=O bond in the aliphatic nitro group, which indicated the presence of the nitrogenous compounds. The bands at 1451-1273 cm^{-1} correspond to bending vibrations of the CH_2 and CH_3 aliphatic groups and are mainly attributed to alkyl bending in carbohydrate and N-H and C-N stretching vibrations (Galvão, 2019, Dorado et al., 2001, Geskovski et al., 2021, Güler et al., 2016, Devi and Khanam, 2019a). The bands in the region 1100-1093 cm^{-1} are due to stretching vibrations of C-O-C and C-O, representing the presence of ester groups (antisymmetric axial stretching and asymmetric axial stretching). The strong 880 cm^{-1} bands were created by the CH_2 wiggling vibrations from the CH_2 group and overlapping of the CH_2 rocking vibrations and out-of-plane CH vibrations in an open and long chain of fatty acid (Galvão, 2019, Dorado et al., 2001, Geskovski et al., 2021, Güler et al., 2016, Devi and Khanam, 2019a).

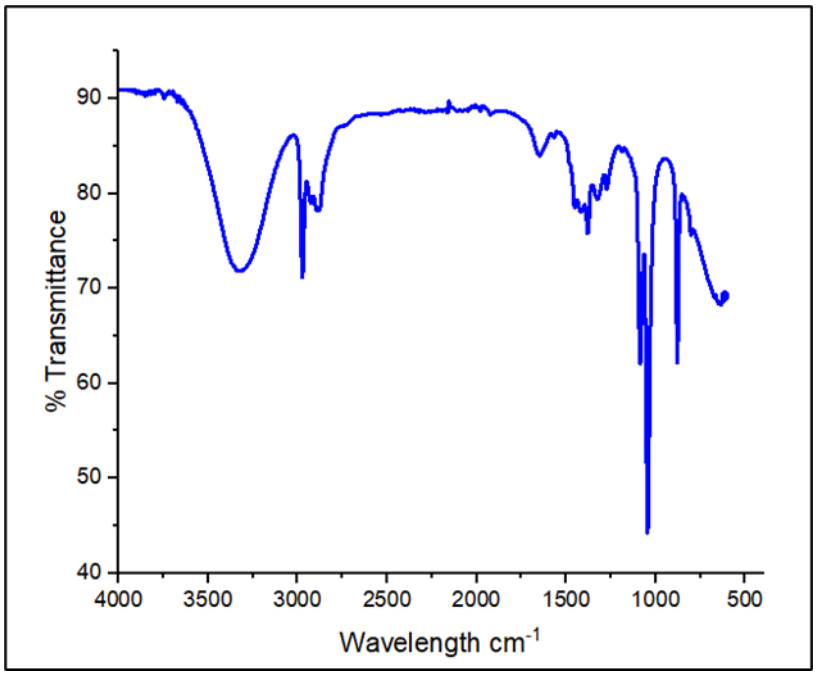

Figure 26: FTIR of lipophilic compounds extracted by SFE from Cannabis flowers.

5.7.3 Pyrolysis-Gas Chromatography/ Mass Spectrometry (Py-GC/MS) of lipophilic compounds from pinewood sawdust

Py-GC-MS Under inert conditions, the samples are degraded. After that, the breakdown products are separated using a chromatographic column, and their mass patterns are detected using a mass spectrometer (Dümichen et al., 2015). Lipid is broken down into long-chain fatty acids and long-chain alcohols during pyrolysis. Secondary cracking occurs at greater temperatures, resulting in additional short-chain molecules (Li et al., 2017, Maher and Bressler, 2007). The identified lipophilic compounds and their concentrations are listed in Table 15 and their pyrograms are shown in the appendix below as Figure A and B. The total lipophilic compounds concentration was 80.76% and 77.79% by ASE and SFE, respectively.

5.7.3.1 Fatty acids by Py-GC/MS

The concentration of the main compounds identified was slightly different for both extracts. The fatty acids percentage identified in the extract by ASE were higher than in the SFE extract. However, most of the compounds identified in both extracts were similar compounds, the 9-octadecenoic acid was the most dominant fatty acid in the SFE extract. In contrast, decanoic acid was the most dominant fatty acid for ASE extract. The results (Table 15) indicate that glutaric acid and butyric acid were not identified in the extract in the case of ASE. While 9-hexadecenoic acid, succinic acid, 9,12-octadecenoic acid and Isopropyl palmitate were not identified in the extract by SFE. Unlike other acids, formic acid and octadecenoic acid which were detected in both extracts, the formic acid was of low percentage in the SFE extract while octadecenoic acid was of low percentage in the ASE extract. However, the retention times were similar for both fatty acids detected in the extracts.

The variety of fatty acids identified in each extract could be influenced by the polarity of extraction solvents, the difference in equipment used for extraction, and the methods' selectivity. According to the research, genetic factors, growing location, climatic circumstances, and tree growth rate can influence the amount and composition of extractives produced by trees of the same species (Willför et al., 2003c). Extractive occurrence and specified extractive contents also differ between tree age classes/felling kinds, heartwood/sapwood, forest site types, sawmill residues and standing trees (Nastić et al., 2020). Furthermore, the coextraction of 9-

octadecenoic acid was only observed with SFE. The results show that SFE is selective for 9-octadecenoic acid. Due to the hydroxyl group on fatty acids, adding ethanol with CO_2 has a significant impact. Because of its strong negative charge, oxygen is prone to hydrogen bonding with other positively charged polar groups in fatty acids. It boosts the solute-solvent interaction (CO_2 and ethanol) (fatty acids). Furthermore, as the co-solvent flow rate increases, the solubility drops, possibly due to the non-polar hydrocarbon chain.

5.7.3.2 Aromatic compounds by Py-GC/MS

The identified aromatic compounds were only detected in the extract by ASE. The concentration ranged from 0.94-3.29%; vanillin, vanillin acetate, and vanillin acid hydrazine were the only aromatic compounds. In the SFE, aromatic compounds were not detected. The vanillin identified in this study followed the findings in Mattos (2016) study where vanillin was detected around the same retention time, however at a low percentage compared to the amount detected in this study. In a study by Masendra et al. (2020), vanillin acetate and vanillin hydrazine were identified in Pinus merkusii bark with a lower percentage than the percentage detected in this study.

5.7.3.3 Long-chain aliphatic alcohols by Py-GC/MS

p-Cymen-7-ol and isoeugenol 1 identified long-chain aliphatic alcohols in the SFE and ASE extract. The concentration was slightly high for both compounds of the ASE extract compared to SFE extract, while 1-nonanol, Retinol was only detected in the ASE extract.

5.7.3.4 Monoterpenes by Py-GC/MS

The most important monoterpene identified in the extracts was α-pinene, with SFE having a slightly high percentage compared to ASE extract. α-pinene is present in many pine species. It is known to increase the economic value of pine extract because of its industrial importance to produce pharmaceuticals, insecticides and repellents, solvents, and antimicrobials (Mattos et al., 2016). In addition, the percentage of α-pinene was lower than the content of α-pinene (6.43%) determined in the study by (Graikou et al., 2012, Ulukanli et al., 2014). Further, o-Cymene, Cetene, and 1-Decene were other compounds detected in the SFE extract. Mattos (2016) showed that 1-decene was detected at 9.66min with 0.256%. This differs from the findings presented here. The percentage of 1-decene was 1.48% at 28.792 min. The results (Table15)

show that the SFE was more selective for monoterpenes than ASE. The most interesting finding was that α-pinene was detected in both extracts. o-Cymene with 1.05% detected in the SFE extract has been identified in a previous study of pine oil extracted from *Pinus sylvestris* (needles, twigs and cone) with 4.3% (Kamkaen, 2015).

5.7.3.5 Hydrocarbons by Py-GC/MS

In the SFE extract, o-xylene, 1-nonene, 4-dodecene, naphthalene, decahydro-, trans-, 1-pentadecene, cyclodecene, phenanthrene, 2,3,4, naphthalene, 2,6-bis (1,1-dimethyl ethyl)- were the predominant hydrocarbons. In the ASE, oxirane, octyl-, 1-pentadecene, cyclodecene, phenanthrene, 7, and naphthalene, 2,6-bis (1,1-dimethyl ethyl)- were the predominant compounds identified. It is difficult to explain this result as both extracts contained different hydrocarbon compounds. There are, however, other possible explanations, such as the selectivity and the effect of process parameters. Phenanthrene, 1,2, 3, is the only common hydrocarbon between the two extracts detected, with SFE showing a high percentage than ASE extract. The yield of naphthalene detected in the SFE extract might result from resin acids arising from the cleavage in the A ring of the parent molecule before complete aromatization. This cleavage is likely facilitated by the ease at which the resin acids undergo decarboxylation (Kan et al., 2016, Renzi, 2017).

5.7.3.6 Resin acids by Py-GC/MS

Methyl abietate and Abietic acid were the resin group compounds detected in the SFE, while methyl abietate was the only resin compounds detected in ASE. The amount of resin acid ranged from 2.44- 20.1%, which is relatively high compared to the range of resin acids that have been detected in previous studies where 1.1 - 4.4% was reported in heartwood and 0.3 -1.1% in sapwood (Hovelstad et al., 2006). The abietic acid was identified as the major resin acid with a high content of 20.1%. Abietic acid was identified as the main resin acid group in sapwood with 2.44% and heartwood with 3.83% from *pinus halepensis* extracted by ASE using n-hexane (Benouadah et al., 2018), which was relatively lower than the amount of abietic acid identified in this study extracted by SFE. These results corroborate a great deal of the previous work in a study by Conde (2013), where the major resin acid was abietic acid. The high percentage of abietic acid observed in the SFE extract maybe be due to the effect brought by the increase in temperature at constant pressure (Ritter and Campbell, 1991). However, the previously described results show that ASE is selective for abietic

acid, and this study could not demonstrate that. Verkasalo et al., (2021) reported that the amount of resin acids is influenced by environmental factors and tree and wood age and size. However, their genetics is the main factor in the amount of resin acids detected. Methyl abietate with 2.04% was only detected in the extract by SFE and hardly in the extract by ASE. This finding was in contradiction with the findings in the study by (Ház et al., 2016), where methyl abietate (3.01%) was detected in the extract by ASE with n-Hexane. In the literature, it has been reported that the bark constitutes high amounts of lipophilic compounds; this explains the high content of methyl abietate detected in spruce bark than the amount detected in this study from sawdust. Dehydroabietic acid was identified in both extracts, with SFE showing a higher content than ASE. Dehydroabietic acid's high boiling point property allows the resin acid to be volatilized at 500°C (Kim et al., 2019). SFE is highly selective for resin acids compared to ASE. The results show that the extraction method is also another factor contributing to the compounds' content. In addition, dehydroabietic acid results from the dehydration of abietic acid during pyrolysis (Kim et al., 2019). No abietic acid was detected in the ASE extract; hence no dehydroabietic acid was identified.

5.7.3.7 Terpenoids by Py-GC/MS

Terpenoids are known for adding flavor and perfume to oil. Light and heat also easily destroy them (Renzi, 2017). Hydroquinone was the dominant terpenoid identified in the ASE extract. Benzoic acid acetate was another compound identified in the ASE extract. No terpenoids were identified In the SFE extract. It can be assumed that the amount of temperature and time that the sample was exposed to during the extraction process destroyed the terpenoids. This finding was unexpected and suggested that ASE parameters are suitable for extracting the identified terpenoids, which contradicts the fact that terpenoids are sensitive to heat as the high temperature was employed.

5.7.3.8 Other compounds by Py-GC/MS

o-Cresol, benzoic acid, acetate, formamide, camphor-10-sulfonamide, 2-methylresorcinol, acetate, furan, 2-(2-furanylmethyl)-5-methyl-, 1H-Indene, 3-ethyl-1-(1-methylethyl)-, benzenepropanol, 4-hydroxy-3-methoxy-, coniferyl aldehyde, 3,5-decadiyne, 2,2-dimethyl-, 2,6-dimethyl-1,3,5,7-octatetraene, E, E-, and 1,3,5,7,11,13-Tridecanehexol, 2,6,10-trimethyl detected in ASE. Aniline-1-(13)C, octanal, resorcinol monoacetate, phenol, 4-(ethoxymethyl)-2-methoxy-, E-14-hexadecenal, and 2(1H)-phenanthrenone, 3,4,4a,9,10,10a-hexahydro-1,1,4a-trimethyl- detected in SFE. Most

of these compounds have been identified as phenol compounds as both methods have been demonstrated to be selective for extracting phenols in previous studies.

Table 15: Lipophilic compounds and Concentration of different compounds identified in ASE and SFE by Py-GC/MS.

Compound	Retention time (min)	% Area ASE	Area SFE
Fatty acids			
Formic acid	11.419	3,67	0,81
1-nonanol	15.268	0,11	-
9-Octadecenoic acid	17.286	0,62	-
9-Octadecenoic acid	19.811	-	1,56
9-Octadecenoic acid	29.148	-	0,77
9-Octadecenoic acid	33.553	-	11,55
9-Hexadecenoic acid	28.319	1,21	-
9-Hexadecenoic acid	34.089	10,13	-
Succinic acid	30.986	1,74	-
Glutaric acid	31.376	-	0,72
9,12-Octadecenoic acid	32.445	0,75	-
Isopropyl palmitate	33.255	6,12	-
Butyric acid	35,13	-	2,31
Decanoic acid	35,405	11,69	
Terpenoids			
Benzoic acid	14,208	1,09	-
Hydroquinone, acetate	14,975	1,57	-

Compound	Retention time (min)	% Area ASE	Area SFE
Aromatic compounds			
Vanillin, acetate	19,822	3,29	-
Vanillin	22,101	2,89	-
Vanilic acid hydrazide	24,474	0,94	-
Aliphatic alcohols			
1-nonanol	15,268	0,11	-
p-Cymen-7-ol	17,97	3,17	1,42
ISOEUGENOL 1	19,171	1,04	-
ISOEUGENOL 1	21,535	1,76	0,54
Retinol	31,686	0,74	-
Monoterpenes			
o-Cymene	9,785	-	1,05
α-Pinene	10,112	2,01	2,32
Cetene	20,765	-	1
Cetene	23,276	-	0,73
Cetene	25,651	-	0,63
1-Decene	28,792	-	1,48
Other compounds			

Compound	retention time	% Area ASE	Area SFE
Hydrocarbons			
o-Xylene	5,285	-	0,77
m - xylene AND p – xylene	5,063	0,56	-
1-Nonene	6,167	-	0,55
4-Dodecene, (Zhen et al.)-	12,276	-	1,08
Oxirane, octyl-	12,113	1,17	-
Naphthalene, decahydro-, trans-	17,081	-	0,76
1-Pentadecene	20,761	1,14	-
	23,86	-	0,61
	24,935	-	0,57
Cyclodecene	25,137	1,12	-
Phenanthrene, 7	32,674	1,06	-
	32,722	-	6,69
Phenanthrene, 2,3,4	33,76	-	1,11
Phenanthrene, 1,2,3,	33,929	1,42	-
	34,004	-	3,57
Naphthalene, 2,6-bis(1,1-dimethylethyl)-	35,495	-	2,76

Resin acids				ANILINE-1-(13)C	8,436	-	1,23	Hydroquinone, acetate	14,975	1,57	-
Methyl abietate	33,678	-	2,04	Octanal	8,967	-	1,06	Resorcinol monoacetate	14,987	-	0,64
	34,737	1,48	-	o-Cresol	10,601	1	-	Formamide	15,411	0,63	-
Dehydroabietic acid	34,408	1,08	4,27		11,235	1,17	-	Camphor-10-sulfonamide	16,427	0,55	-
Abietic acid, TMS derivative	34,871	-	20,1	Benzoic acid	14,208	1,09	-	2-Methylresorcinol, acetate	17,496	0,89	-
other compounds				3,5-Decadiyne, 2,2-dimethyl-		27,912	1,43	-	22,3	0,97	-
1H-Indene, 3-ethyl-1-(1-methylethyl)-	23,853	1,11	-	2,6-Dimethyl-1,3,5,7-octatetraene, E,E-	28,736	1,83	-				
Benzenepropanol, 4-hydroxy-3-methoxy-	26,022	0,96	-	E-14-Hexadecenal	30,034	-	0,7				
Phenol, 4-(ethoxymethyl)-2-methoxy-	26,067	-	1,36	1,3,5,7,11,13-Tridecanehexol, 2,6,10-trimethyl-	30,133	1,24	-				
Coniferyl aldehyde	27,713	1,98	-	2(1H)-Phenanthrenone, 3,4,4a,9,10,10a-hexahydro-1,1,4a-trimethyl-	30,146	-	1,03				

The identified lipophilic compound in the presence of TMAH and their peak areas are listed in Table 16 and their pyrograms are shown in the appendix below as Figure C and D. The total lipophilic compounds concentrations were 99.2% and 70.37% by ASE and SFE, respectively. Excluding solvents.

5.7.3.9 Fatty acids with TMAH

The fatty acids ranged from 0.15 to 38.6% area and 0.11 to 25.61% area for ASE and SFE, respectively, as shown in Table 16. The extract by ASE extract had a large amount of fatty acids compared to those detected in the SFE extract. 10-Octadecenoic acid, methyl ester was detected as a predominant fatty acid in the ASE extract. The major fatty acid in the SFE extract was 9-Octadecenoic acid, methyl ester with a concentration of 25.61%. However, both these compounds of high concentration were only detected for each method, not in both extracts. Nonanedioic acid, dimethyl ester, hexadecanoic acid, and methyl ester were identified in both extracts around the same retention time. However, both fatty acids identified in the ASE extract had slightly higher percentage areas than those identified in the SFE extract. Fatty acid esters are claimed to be substantially more abundant in pine extractives than free fatty acids, accounting for 30–50 wt%. Up to 4% of total extracted components, respectively (Hemingway and Hillis, 1971, Korus et al., 2019) and the concentration of fatty acids identified in this study was in range.

5.7.3.10 Monoterpenes with TMAH

1-Decene and α-pinene are the only monoterpenes detected by Py (TMAH)-GC/MS, and these two compounds were only identified in the ASE extract. The concentration of α-pinene detected in this study was higher (0.94%) than the amount detected in *Pinus* species in (Kim, Lee and Yun, 2013) study. They found a low amount of 0.68% in *P. thunbergii* leaves which were relatively higher than one detected in the leaves of *P. rigida (0.13%)* by GC-MS. The needles of two chemotypes of *Pinus pinaster Ait*. Contained a high level of -pinene (Arrabal et al., 2014).

5.7.3.11 Terpenoids with TMAH

Thermal decomposition of resin acids may have resulted in the detection of terpene-derived compounds (Pinto et al., 2018). The most important terpenoid detected in this study was terpineol, with ASE having a slightly higher percentage than the amount identified in SFE. Retinol acetate was another compound detected in the SFE extract,

concluding that SFE is more selective to terpenoids than ASE**.** Other parts of the tree, such as pine *Pinea* needles, have been found to contain α-terpineol (0.35%) (de Simón et al., 2001b); this amount is lower than the amount of terpineol detected in this study.

5.7.3.12 Hydrocarbon with TMAH

Phenanthrene and Stigmasta-3,5-diene were identified in both extracts. The phenanthrene had a high percentage in the ASE extract, while the Stigmasta-3,5-diene was high in the SFE extract. Phenanthrene, 7-ethenyl and phenanthrene, 1,2,3,4,4a,9,10,10a-octahydro were other hydrocarbons detected in the SFE extract. The concentration of Stigmasta-3,5-diene (1.17%) extracted with ASE using toluene: ethanol is higher than the concentration (0.508%) detected in a previous study by Mattos (2016) using the same solvent and with dichloromethane.Stigmasta-3,5-diene is a sterol hydrocarbon produced from the fragmentation of the sterol molecule β-sitosterol (Aued-Pimentel et al., 2013, Mattos et al., 2016).

5.7.3.13 Resin acid with TMAH

Methyl abietate was the only resin compound detected in the presence of TMAH. ASE had a high concentration of methyl abietate (7.81%) compared to the amount detected in the SFE extract (7.39%). The amount of methyl abietate increased in the presence of TMAH; the increase could have been brought by isomerization or degradation of dehydroabietic and abietic acid to form more methyl abietate (Kim et al., 2019). In addition, high temperatures above 500°C degrade some resin acids by losing the carboxylate moiety; this includes compounds with high boiling points such as dehydroabietic acid (Kim et al., 2019).

5.7.3.14 Sterols with TMAH

(3methyl, 24S)- stigmast-5-en-3-ol was the only sterol identified in the SFE extract, and no sterols were detected in the ASE extract. This finding contrasted with results when no TMAH was added; sterols were not detected in both extracts; this might be due to the Py-GC/MS not being amenable to detecting sterols. Also, in the SFE, when CO_2 is combined with ethanol, it enhances the solubilization of more polar compounds and is not favorable for extracting sterols (Martins et al., 2016).

Table 16: Composition of lipophilic compounds from pinewood sawdust by Py-GC/MS in the presence of TMAH.

Compound	Retention time (min)	% Area ASE	Area SFE	Compound	Retention time (min)	% Area ASE	Area SFE
Fatty acids				**Terpenoids**			
Nonanoic acid, methyl ester	6,772	1,16	-	Terpineol	14,827	2,06	-
4-Decenoic acid, methyl ester	9,435	0,81	-	Terpineol	14,836	-	0,96
Octanoic acid, methyl ester	12,894	1,71	-	Retinol, acetate	43,269	-	0,8
Octanoic acid, methyl ester	15,85	1,67	-	**Hydrocarbon**			
10-Undecenoic acid, methyl ester	23,327	-	4,67	Phenanthrene,7-ethenyl-1,2,3,4,4a,4b,5,6,7,8,10,10a-dodecahydro-4a,7-dimethyl-1-methylene-, [4aS-(4a.alpha.,4a.beta.,7.beta.,1	44,079	-	7,85
10-Undecenoic acid, methyl ester	24,199	-	1,31	Phenanthrene, 2,3,4,4a,9,10-hexahydro-1,4a-dimethyl-7-(1-methylethyl)-, (Goldberg and Rokem)-	44,607	15,6	-
9-Dodecenoic acid, methyl ester, (Goldberg and Rokem)-	23,987	1,21	-	Phenanthrene, 2,3,4,4a,9,10-hexahydro-1,4a-dimethyl-7-(1-methylethyl)-, (Goldberg and Rokem)-	44,641	-	6,52
Nonanedioic acid, dimethyl ester	27,536	-	0,11	4a(2H)-Phenanthrenecarboxylic acid, 1,3,4,9,10,10a-hexahydro-6-methoxy-1,1-dimethyl-7-(1-methylethenyl)-, methyl ester, (4aR-tr	46,262	0,95	-
Nonanedioic acid, dimethyl ester	27,554	4,02	-	Phenanthrene, 1,2,3,4,4a,9,10,10a-octahydro-1,1,4a-trimethyl-7-(1-methylethyl)-, (4aS-trans)-	47,167	-	0,83
Hexadecanoic acid, methyl ester	32,849	0,36	-	Stigmasta-3,5-diene	56,159	-	0,41

Hexadecanoic acid, methyl ester	37,301	-	2,68	Stigmasta-3,5-diene	56,18	1,71	-
Hexadecanoic acid, methyl ester	37,326	13,8	-	**Resin acid**			
Hexadecanoic acid, 14-methyl-, methyl ester	38,742	1,38	-	Methyl abietate	44,023	5,4	-
Linoelaidic acid	40,449	-	0,44	Methyl abietate	45,425	2,41	-
Linoelaidic acid	40,457	1,47	-	Methyl abietate	45,464	-	7,39
10-Octadecenoic acid, methyl ester	40,687	38,6	-	**Sterols**			
9-Octadecenoic acid (Zhen et al.)-, methyl ester	40,714	-	25,61	(3methyl, 24S)- stigmast-5-en-3-ol	59,495	-	1,19
Eicosanoic acid, methyl ester	41,175	3,04	-	(3methyl, 24S)- stigmast-5-en-3-ol	60,645	-	0,84
9,11-Octadecadienoic acid, methyl ester, (E,E)-	41,353	-	8,2	**Monoterpenes**			
9,12-Octadecadienoic acid, methyl ester, (E,E)-	44,46	-	0,56	1-Decene	3,641	0,75	-
Heptadecanoic acid, methyl ester	50,77	0,15	-	α-Pinene	10,128	0,94	-

5.8 Comparison of lipophilic compounds identified by Py-GC/MS and Py(TMAH)-GC/MS extracted from pinewood sawdust by ASE and SFE

The lipophilic compound identified in SFE, and ASE extracts are generally identical to those previously reported in extracted pinewood, pine needles, and pine bark studies (Mattos et al., 2016, de Simón et al., 2001a, Arrabal et al., 2014). Many fatty acids were identified in the presence of TMAH compared to the absence of TMAH. However, 1-decene was identified in the ASE extract with Py-GC/MS without the TMAH, while in the SFE extract, it was identified after the addition of derivative TMAH. 9-octadecanoic acid was the only fatty acid methylated in the presence of TMAH. TMAH derives compounds from methylated compounds that are readily volatilized (Kim et al., 2019). Hydrocarbons were the most common groups identified in extracts with and without TMAH. Phenanthrene was high in extracts with TMAH; the results are contradictory as phenanthrene,1.2.3 was only identified in extracts by ASE; however, with TMAH, it is identified in the extract by SFE. Phenanthrene, 2.3.4 was only identified in the extract by SFE when TMAH was not added. Upon the addition of TMAH, the compound is detected to be high in the ASE extract compared to the amount detected in the SFE extract. Phenanthrene, 7 was only identified in ASE without TMAH and in the SFE in the presence of TMAH. Alpha-pinene was detected in all extracts of ASE both with and without TMAH; the alpha-pinene detected in the extract with TMAH was relatively lower than the amount detected in the extract without TMAH. The SFE extract without TMAH had a slightly higher alpha-pinene percentage than the ASE extract; however, the alpha-pinene was not detected in the SFE extract with TMAH. The identified 1-decene in both extraction methods in the presence and absence of TMAH is contradicting. It was not found in the SFE extract with TMAH; however, it was detected when TMAH was not used, while in the ASE extract, the compound was detected in the absence of TMAH. Resin acids are non-structural biomass constituents existing in the resin canals (Kim et al., 2019). The resin acid identified in ASE and SFE extract with and without TMAH was methyl abietate. The concentration detected in the presence of TMAH for both extracts was high compared to the percentage are detected in the absence of TMAH. Terpenoids identified with Py-GC/MS are entirely different from terpenes identified in the presence of TMAH.

Terpineol was identified in both extracts, with ASE extract having a slightly high (2.06%) compared to the concentration (0.96%) identified in SFE extract. Sterols and hydrocarbons were detected in the presence of TMAH, while aromatic compounds and long-chain aliphatic alcohol were detected in the absence of TMAH. The percentages of all the compound groups identified are summarized in Figure 27 below.

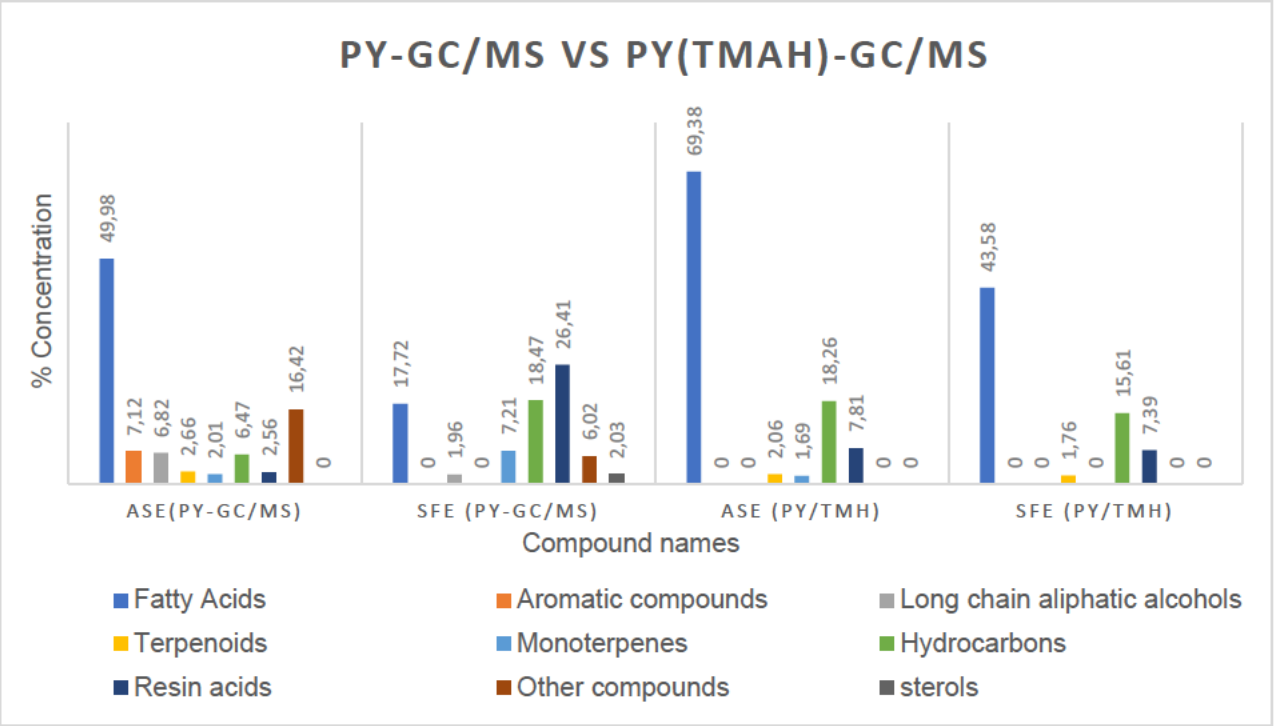

Figure 27: Summary of lipophilic compounds identified by Py-GC/MS and Py(TMAH)-GC/MS extracted by ASE and SFE.

The ASE is more selective for fatty acids as the percentage of fatty acids was high with and without the derivative. It increased from 49.98 to 69.38% upon the addition of TMAH derivatives. A similar trend was noted for the SFE extract, where the fatty acids increased from 17.72 to 43.58% upon the TMAH derivative. However, the ASE is more selective for the fatty acids as the percentage of fatty acids identified by both methods remained higher than the amount of fatty acids identified in the SFE. The amount of terpenes and terpenoids in both identification methods showed that the ASE is selective for the terpenes as it was detected in both methods; however, the addition of TMAH for determining terpenes is not necessary the percentage of terpenes decreased with the addition of the derivative from 2.01 to 1.69%. This might be due to the evaporation of the compound during storage since the compounds change with

time. Also, the decrease in this volatile compound contradicts the literature, as it is said that the addition of TMAH improves the identification of volatile compounds. In the SFE extract, the terpenes were not detected in the presence of TMAH, which might be due to the compound evaporating during storage as it is readily volatile. The SFE (7.21%) is more selective for terpenes than ASE (2.01%). However, the addition of TMAH did not show any significant impact on the amount of terpenes, sterols, aliphatic alcohols, and other compounds detected in the SFE extract when TMAH was added.

A decrease in the percentage of resin acids for the SFE extract was noted with the addition of TMAH, from 26,41 to 7.39%. While it was observed to increase for the ASE extract with the addition of TMAH, it increased from 2.56% to 7.81%. Resin acids are prone to adsorption on the walls of the pyrolyzer, the injection system, and the analyte column, which causes tailing and peak loss making them unidentifiable on the MS. The addition of TMAH increases the amount of compounds detected by reacting the polar reactive compounds with the methyl carbonium ion from TMAH; this helps the compounds be more detectable and eliminates peak loss and tailing. Hence the increase in the amount of resin acid compounds detected for the ASE extracts with the addition of TMAH. The percentage of hydrocarbons increased for ASE extract with TMAH, while for SFE extract, there was a slight decrease from 18.47 to 18,26%. The SFE was more selective for hydrocarbons with and without the TMAH.

5.9 Pyrolysis-Gas Chromatography/Mass Spectrometry of lipophilic compounds of Cannabis *Sativa* L.

The Py-GC/MS results of the Cannabis *Sativa* L. lipophilic compounds obtained with the SFE contained fatty acids, sterols and steroids, hydrocarbons, terpinols, terpenes and resin acids. The lipophilic constituents are listed in Table 17 below and the pyrogram is shown in the appendix as Figure E.

5.9.1 Fatty acids

Fatty acids are natural esterified fatty acids in triglycerides that originate from parenchyma cells in wood. Formic acid and pentanoic acid were the most predominant fatty acids of the Cannabis *Sativa* L. with varying concentrations of 0.73% and 0.66%, respectively. These essential fatty acids have anti-inflammatory, anti-hypertension, anti-vasoconstrictor, anti-cancer, and anti-thrombotic properties (Yousefi et al., 2019). Formic and pentanoic acid are both classified as saturated short-chain carboxylic acids. However, formic acid lacks the hydrophobic properties associated with lipids. Pentanoic acid also known as valeric acid, is a straight, saturated chain, alkyl carboxylic acid. It is a white, greasy liquid with an overpowering stale cheese stench. Valeric acid and its esters are mostly utilized in perfumes and cosmetics, food additives (due to the fruity flavor of the esters), plasticizers, and medications (Goldberg and Rokem, 2009). Pentadecanoic acid, also known as myristic acid, is a saturated straight-chain fatty acid classified as a long-chain fatty acid, identified with the lowest concentration of 0.3%. The concentration of octadecanoic acid was detected as 0.57%, known for its physiological activities. It is a long-chain saturated fatty acid with no effect on lipoprotein cholesterol concentrations in men or women. The octadecanoic acid is also known as stearic acid. Octadecanoic acid/stearic acid is commonly used in detergents, soaps, and cosmetics, such as shampoos and shaving cream products, because it has a polar head group that can bind with metal cations (Zhen et al., 2015). This non-polar chain confers solubility in organic solvents. The amount of stearic acid identified in this study was lower than detected in the hemp seed oil, 1.57% extracted by SFE, using the same pressure rate (300 bar) with a slightly low temperature of 40°C (Da Porto et al., 2012a).

5.9.2 Terpenoids and cannabinoids

Terpenes are not only responsible for the characteristic aroma of the plant. However, terpenes can increase blood-brain barrier permeability and interact with neurotransmitter receptors, thus contributing to cannabinoid-mediated analgesic and psychotic effects (Pellati et al., 2018b). Compounds with an OH group, such as terpenes, disintegrate at the conventional EI ion source, making analysis difficult without derivatization (Amirav et al., 2021). It is worth noting that some terpenes, particularly sesquiterpenes, are difficult to distinguish due to a lack of reliable standards for many of these chemicals. As a result, descriptions of Cannabis terpene

profiles may contain unknown chemicals, rely on tentative identification, or give partial profiles of selected compounds. α-Pinene, α-cymene, caryophyllene, globulol, isocaryophillene, citronellyl 2-methylpropanoate, and citronellol were the volatile terpenes detected in the Cannabis extracts without derivatives as shown in Table 17. Globulol was the most abundant terpene determined with a concentration of 4.5% at the retention of 29,729 min. The content of α-Pinene was determined to be 0.64% at the retention of 12.7 min, caryophyllene was determined to 2.66% at a retention 23,49 to 25,405 min, α-cymene was determined 0.38% at the retention of 12.18min, L-Linalool was determined to be 0,88% at the retention of 14.287min, isocaryophillene was determined to be 3.17% at the retention of 25,986 to 26,395 min, citronellyl 2-methylpropanoate was determined to be 0.77% at the retention of 33,945min, and geranyl linalool isomer determined to be 0.52% at a retention time of 34,934min.

In the extraction of neutral cannabinoids, only the ethanol content and its quadratic influence were significant; acid forms, on the other hand, were not measured. Only two cannabinoids were classified, cannabinol and cannabidiol, where ethanol was used as a cosolvent on undercarboxylated Cannabis flower (Omar et al., 2013). In a study by Ciolino et al. (2018), they determined cannabichromene at the retention of 16.6 min using GC/MS TMS derivatives, which is earlier than the retention of the cannabichromene detected in our study without the derivative at 30.595 min. Cannabichromene is a decarboxylated form of cannabichromenic acid (CBCA), a neutral form found naturally in plants, as shown in Figure 29. CBDA is synthesized through a solid-state reaction accelerated by environmental conditions such as temperature, light, and oxygen. The cyclization reaction produces cannabichromene, more evident in the inert atmosphere. The cyclization is technically defined as an intramolecular Markovnikov addition of the phenolic OH to the isopropenyl group's double bond. Cannabichromene possesses anti-inflammatory, antifungal, and analgesic properties. The amount of cannabichromene ranges between 0.2—0.9% identified by GC/MS (Nagy et al., 2019).

Analysis of cannabinoids without derivatization shows that compounds with free one or two groups of OH tend to react with the standard EI metallic ion source surface and decompose, plus they exhibit compound-dependent nonlinear response. However, the carboxylated cannabidiol was detected without a derivative in this study. The concentration of cannabidiol was determined to be 32.3% at different retention times

ranging from 26 to 34 min. The concentration of Δ^8-Tetrahydrocannabinol was 10.61% at different retention times ranging from 34.33 to 35.404 min. The concentration of Δ-1(2)- tetrahydrocannabinol was 6.63% at a retention time of 34.871 min.

The order of elution was:

Δ^8-tetrahydrocannabinol<cannabichromene<cannabinol<cannabicoumaronone. This was in disagreement with an elution order discovered by (Santos et al., 2019), where the order of elution was found to be cannabidiol <cannabichromene < Δ^8-tetrahydrocannabinol by GC/MS. The retention times of all the cannabinoids detected in this study were detected between 26 to 36 min, which is longer than the retention time of cannabinoids detected in a retention time between 21 to 24 min (Richins et al., *2018)* but in agreement with the retention time of 26.5 min reported by (Hillig and Mahlberg, 2004).

The genetic characteristics such as climate, light, humidity, the elevation of the cultivated region influence and drying, heating, and storage influence the chemical composition of the cannabinoids in the Cannabis plant. During the drying, heating or storage, the plant undergoes decarboxylation and forms neutral delta-tetrahydrocannabinol and cannabidiol. The THCA was decarboxylated to form THC, and CBDA was decarboxylated to form CBD, as shown in Figure 28. Also, CBD is the most abundant compound in the cannabinoids, and in this study, the concentration of CBD was detected to be high, with a concentration of 35.17%.

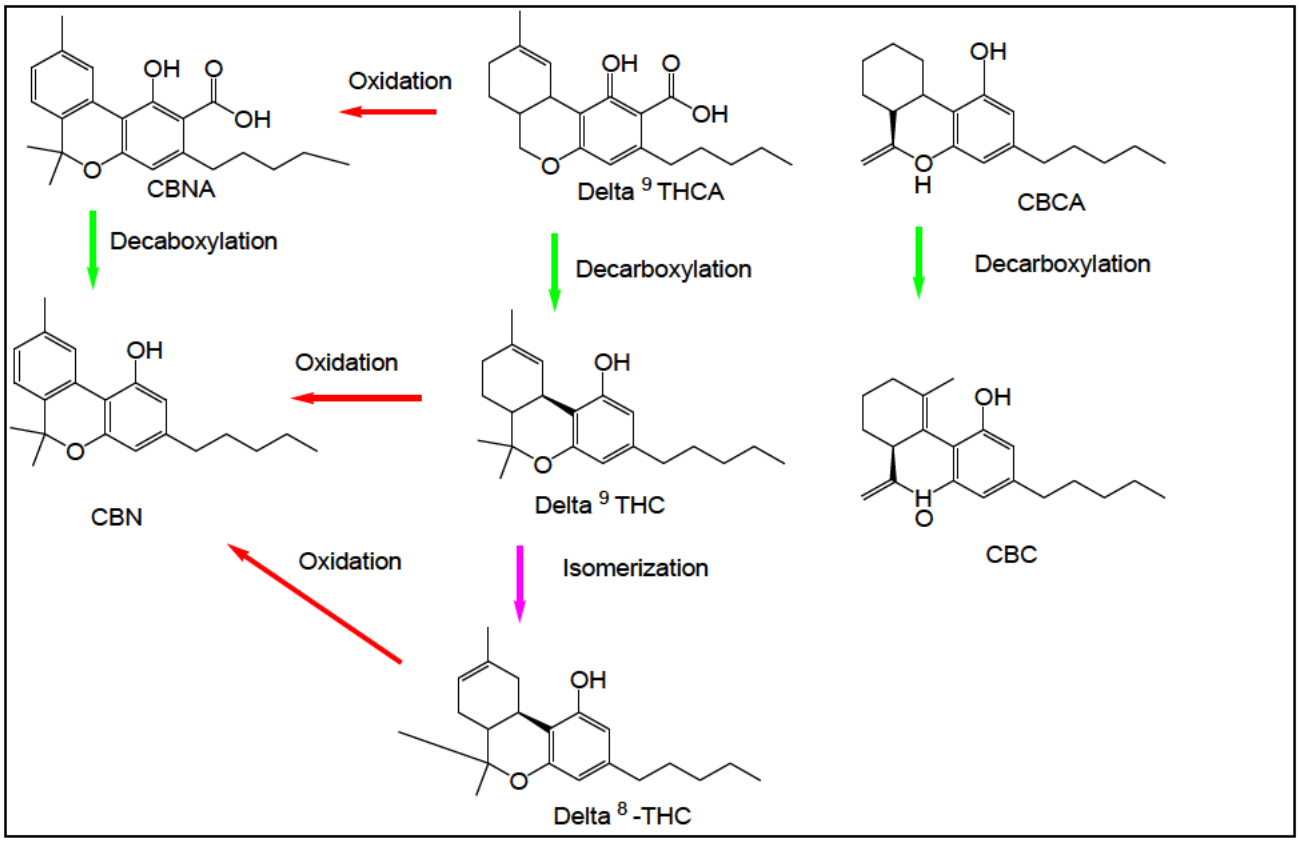

Figure 28: Chemical structures of main cannabinoids present in Cannabis *Sativa* L showing changes brought by heat. Abbreviation: Δ = heating; ox = oxidation; is = isomerization. Drawing inspired by (Pellati et al., 2018a)

5.9.3 Sterols

The sterols, steroids and resin acids were part of the compounds identified from the Cannabis *Sativa* L. extracts in Table 17. The stigmast-24(28)-en-3-one, (5. alpha.)- were the only steroids identified in the study. The stigmast-24(28)-en-3-one, (5. alpha.)- was eluted at 34.33 min with a concentration of 1.23%. Certain chemicals, such as sterols, have restricted solubility at the supercritical threshold, which explains their low abundance (Attard et al., 2018).

Table 17: Composition of lipophilic compounds from Cannabis *Sativa* L. by Py-GC/MS.

Compound name	Retention time min	Area%	Compound name	Retention time min	Area%
Cannabinoids			**Sterols**		
Δ8-Tetrahydrocannabinol	34,424	2,33	Stigmast-24(28)-ene, (5.alpha.)-	44,129	3,24
Cannabichromene	36,843	2,53	**Other Compounds**		
Cannabichromene	38,729	1	Benzene, methyl-	4,956	0,3
Cannabidiol	39,189	1,31	Cyclohexane, 1,2,3-trimethyl-, (1.alpha.,2.beta.,3.alpha.)-	5,428	0,34
Cannabidiol	39,485	0,47	Hexanal	5,587	0,49
Cannabidiol	40,398	0,75	Benzene, 1,3-dimethyl-	7,397	0,58
Cannabidiol	44,576	3,4	2,6-DIMETHYL-1,5-HEPTADIENE	7,777	0,37
Cannabidiol	46,618	29,24	2-Heptanone	7,936	0,57
Cannabicoumaronone	47,938	0,8	Heptanal	8,242	3,82
Terpenes			1,5-Heptadiene, 3,3,6-trimethyl-	8,369	0,56
α-Ocimene	12,138	0,38	Glycerin	10,324	1,16
α-Pinene	12,7	0,64	5-Hepten-2-one, 6-methyl-	10,797	0,97
L-Linalool	14,287	0,88	2-Heptene, 2,6-dimethyl-	10,935	0,65
Caryophyllene	23,497	1,34	1,6-Octadiene, 2,7-dimethyl-	11,006	1,5
Caryophyllene	25,094	0,85	Bicyclo[3.1.1]heptan-3-one, 2,6,6-trimethyl-	11,259	0,55
Caryophyllene	25,405	0,47	Pyrimidine-4,6-dione, hexahydro-4-(3-phenyl-2-propenyl)-2-thioxo-	14,006	0,33
Isocaryophillene	25,986	0,56	Benzene, (ethenyloxy)-	17,816	0,59
Isocaryophillene	26,395	2,61	Benzene, (ethenyloxy)-	17,816	0,32
Globulol	29,729	4,5	Ethanol, 2-butoxy-, acetate	18,664	0,38
citronellyl 2-methylpropanoate	33,945	0,77	1-Undecene	19,837	0,24
Geranyl linalool isomer b	34,934	0,52	Geranyl angelate	20,482	0,45
Citronellol	41,895	0,77	1-Propene, 2-nitro-3-(1-cyclooctenyl)	21,261	0,66

Fatty Acids			Cyclopentanone, 3-[3,5-decadienyl]-, (E,E)-	22,118	0,31
Pentanoic acid,	14,776	0,66	3-Tetradecene, (Goldberg and Rokem)-	22,522	0,35
Formic acid	26,866	0,73	cis-.beta.-Farnesene	24,225	1,8
Pentadecanoic acid	35,046	0,3	Octadecane, 1-chloro-	27,64	0,91
Octadecanoic acid	37,095	0,57	2-Naphthalenemethanol, 1,2,3,4,4a,5,6,7-octahydro-.alpha.,.alpha.,4a,8-tetramethyl-, (2R-cis)-	28,64	0,74
Terpenoids			2-Naphthalenemethanol, decahydro-.alpha.,.alpha.,4a-trimethyl-8-methylene-, [2R-(2.alpha.,4a.alpha.,8a.beta.)]-	29,111	1,93
Isoborneol	16,367	0,49	Acetic acid, 3,7,11,15-tetramethyl-hexadecyl ester	30,59	0,34
Terpineol	17,073	0,43	Cyclohexanecarboxylic acid, 4-pentyl-, 4-methoxyphenyl ester, trans-	31,216	3,32
Guaiol	27,826	2,4	Octadecane	32,059	0,39
Guaiol	28,426	2,06	2-Pentadecanone, 6,10,14-trimethyl-	33,081	1,02
Globulol	29,729	4,5	3-Isobutyl-4,5-dimethyl-3H-isobenzofuran-1-one	34,569	0,59
Phenol, 3-methoxy-2-methyl-	29,892	0,49	Oxalic acid, dodecyl neopentyl ester	35,704	0,76
Phenol, 4-(2-aminoethyl)methoxy-	32,324	1,53	Benzoic acid, 4-(4-pentylcyclohexyl)-, 4'-cyano[1,1'-biphenyl]-4-yl ester	36,205	1,95
			Borinic acid, diethyl-, 3,3,5-trimethyl-1-cyclohexen-1-yl ester	41,265	0,65
			1,3-Benzenediol, 2-(3,7-dimethyl-2,6-octadienyl)-5-pentyl-	42,639	0,35
			Acetic acid, 8a,10a-dimethyl-7-oxohexadecahydrodicyclopenta[a,f]naphthalen-1-yl ester	43,467	0,73

5.10 Pyrolysis-gas chromatography/mass spectrometry in the presence of TMAH analysis of lipophilic compounds from Cannabis *Sativa* L extracted by SFE.

The identified lipophilic compound in the presence of TMAH and their peak areas are listed in Table 18 and their pyrograms is shown in the appendix below as Figure F. The total lipophilic compounds concentrations are defined in Table 18 below, excluding solvents.

5.10.1 Cannabinoids with TMAH

Cannabinol, Δ-1(2)-THC, Δ^8-THC, and dronabinol were the only cannabinol compounds determined in the presence of TMAH. They are obtained through decarboxylation of their acid forms and THC acid decarboxylate to form Δ^9-THC, an isomer to Δ^8-THC. The oxidation of Δ^9-THC results in CBN formation during the storage of THC. Further, decarboxylation of cannabinoids occurs naturally at room temperature and over time, and these factors can influence the composition of cannabinoids in the plant before extraction (Moreno et al., 2020). The cannabinols had 0.51% detected between 23,03 to 25,17 min, Δ-1(2)-THC was the predominant cannabinoid with a concentration of 59.09% detected at the retention of 30.529 to 40.564 min. Δ^8-THC had a concentration of 0.22% eluted at 33.398 and 33.603 min. The amount of cannabinol decreased compared to the cannabidiol detected in the absence of TMAH; the retention time was shorter for cannabinoids detected with the derivative. The cannabichromene and cannabicoumaronone were not detected when TMAH was added as a derivative, while Δ-1(2)-THC and dronabinol were the new cannabinoids detected with the presence of TMAH. A significant change in the concentration of Δ^8-THC was noted with the addition of TMAH, where a decrease of concentration from 2.3% to 0.22% was noted. However, the peak was more defined with the addition of TMAH, while the retention time was observed to decrease with the addition of TMAH. Dronabinol had a concentration of 15.99% eluted at different retention times starting from 36.848 to 38 min. The 8,9 double-bond position is more thermodynamically stable than the 9,10 locations seen in THC. '8-THC, on the other hand, is 20% less active than THC.

5.10.2 Fatty Acids with TMAH

Fatty acids extracted by SFE are dependent on the solubility and affinity of the solvent used. In contrast, solubility is dependent on the temperature and pressure, resulting in a wide range of concentration ranges. A variety of fatty acids with various concentrations were detected in the presence of TMAH. 9,12-Octadecadienoic acid (2.83%) was the most abundant fatty acid eluting at different retention times from 42.52 to 45,889 min. Followed by 11-Octadecenoic acid, methyl ester with a concentration of 1.58% eluted at a retention of 41,297 and 41.54 min. Hexadecanoic acid methyl ester had a concentration of 1.37% eluted at the retention of 35.07 min. Propanoic acid, 2-hydroxy-, 2-methylpropyl ester, lactic acid, geranic acid propanoic acid, 2-hydroxy-, ethyl ester, Propanoic acid, 2-hydroxy-, ethyl ester hexanoic acid methyl ester, Hexanoic acid, 5-methyl-, methyl ester, 2-pentenoic acid, 2-methoxy-4-methyl-, methyl ester, 3-nonenoic acid, methyl ester, octanoic acid, 8-hydroxy-, methyl ester, and 2-octenoic acid, methyl ester, were eluted before 20 min of running the GC-MS. Their concentrations were very low, ranging from 0.03 to 0.23%, the highest concentration belonging to Propanoic acid, 2-hydroxy-, ethyl ester. Compounds eluted between 20 to 30 min were 9-octadecenoic acid, methyl ester, 10-octadecenoic acid, methyl ester, glutaric acid, 3-methyl-, dimethyl ester, nonanedioic acid, dimethyl ester, adipic acid, 2-methyloct-5-yn-4-yl pentyl ester with low concentrations ranging from 0.03 to 0.45%. Tetradecanoic acid, 9-hexadecenoic acid, methyl ester, tridecanoic acid, 12-methyl-, methyl ester, 2-propenoic acid, and 3-(2-oxo-2H-naphtho[1,2-b] pyran-3-yl)-, ethyl ester were other fatty acids detected in the Cannabis extract in the presence of TMAH. An increase in the number of fatty acids detected was observed with the addition of TMAH; however, the concentrations were deficient.

5.10.3 Amino acids with TMAH

Glycine, N,N-dimethyl-, methyl ester Glycine, N-benzoyl-, L-Proline, 1-methyl-5-oxo-, methyl ester, d-Proline, N-methoxycarbonyl-, methyl ester, and L-2-Aminobutyric acid, N-methyl-, methyl ester were detected in the presence of TMAH. Glycine, N,N-dimethyl-, methyl ester (0.74%) was the most abundant amino acid eluted at 6.403 min, followed by L-proline, 1-methyl-5-oxo-, methyl ester (0.38%) eluted at the retention of 21.207 min. L-2-aminobutyric acid, N-methyl-, methyl ester is a common amino acid found in the Cannabis plant; the concentration was detected to be 0.355% at the retention of 7.795 min. d-Proline, N-methoxycarbonyl-, methyl ester and Glycine,

N-benzoyl- had the lowest concentration of 0.05%, the d-Proline, N-methoxycarbonyl-, methyl ester was eluted last at the retention of 22.44 min. Most of the amino acids identified in the extracts with the derivative have been identified in a study where Cannabis *Sativa* L. leaves, stems and seeds were investigated for their potent feed materials, allowing them to be used in aquaculture to augment fish nutrition. It was found that the C. Sativa leaf contained a high number of amino acids, which were rich in methionine and lysine (Audu et al., 2014). However, in this study, both these compounds were not identified.

5.10.4 Sterols and steroids with TMAH

(-)-Spathulenol, spathulanol, stigmast-24(28)-ene, (5.alpha.)-, and stigmasta-4,24(28)-dien-3-one were detected in the presence of TMAH, stigmasta-4,24(28)-dien-3-one detected as the most abundant with a concentration of 0.46% at the retention of 48.21 min. The order of elution was (-)-Spathulenol < spathulanol < stigmast-24(28)-ene, (5.alpha.) < stigmasta-4,24(28)-dien-3-one. The compound that was eluted first had the lowest concentration of 0.04%, while the one that was eluted last had had the highest concentration of 0.46%.

5.10.5 Terpenes with TMAH

The order of elution of terpenes detected in this study was L-linalool< caryophyllene < Guaiol< Pyrazine, 2-methoxy-3-(1-methylpropyl)- < citronellol. Guaiol was the most abundant terpene with a concentration of 0.75% at the retention of 27.816 min. The low concentration of terpenes may also be associated with its growth conditions. The concentration of terpenes in Cannabis increases with exposure to light but decreases soil fertility. As well as the pre-treatment of the sample before extraction such as the drying method and grinding. Rotary vapor has also been a contributing factor in monoterpenes loss (Micalizzi et al., 2021). Other compounds detected in this study are displayed in Table 18 below

Table 18: Py-GC/MS analysis of Cannabis *Sativa* L. lipophilic compounds in the presence of TMAH, Py(TMAH)-GC/MS.

Compound name	Retention time min	Area%	Compound name	Retention time min	Area%
Cannabinoids			**Fatty Acids**		
Cannabinol	23,03	0,14	Propanoic acid, 2-hydroxy-, 2-methylpropyl ester	4,071	0,2
Cannabinol	24,86	0,28	Propanoic acid, 2-hydroxy-, ethyl ester	5,394	0,05
Cannabinol	25,174	0,09	Propanoic acid, 2-hydroxy-, ethyl ester	7,409	0,23
Δ-1(2)-tetrahydrocannabinol	30,529	0,32	hexanoic acid methyl ester	8,897	0,16
Δ-1(2)-tetrahydrocannabinol	30,824	0,13	Hexanoic acid, 5-methyl-, methyl ester	11,922	0,04
Δ-1(2)-tetrahydrocannabinol	31,031	0,13	2-Pentenoic acid, 2-methoxy-4-methyl-, methyl ester	10,77	0,04
Δ8-Tetrahydrocannabinol	33,398	0,17	2-Propenoic acid, 2-methyl-, 2-(dimethylamino)ethyl ester	14,797	0,05
Δ8-Tetrahydrocannabinol	33,603	0,05	2-propenoic acid, 3-(2-oxo-2H-naphtho[1,2-b] pyran-3-yl)-, ethyl ester	36,482	0,06
Dronabinol	36,848	0,12	3-Nonenoic acid, methyl ester	14,676	0,05
Dronabinol	37,233	0,62	Octanoic acid, 8-hydroxy-, methyl ester	14,994	0,03
Dronabinol	37,615	3,23	2-Octenoic acid, methyl ester, (Goldberg and Rokem)-	16,35	0,03
Dronabinol	38,2	6,71	9-Octadecenoic acid, methyl ester	23,148	0,1
Dronabinol	38,486	5,31	10-Octadecenoic acid, methyl ester	23,74	0,04
Δ-1(2)-tetrahydrocannabinol	38,896	6,44	11-Octadecenoic acid, methyl ester	41,297	1,06
Δ-1(2)-tetrahydrocannabinol	39,318	12,47	11-Octadecenoic acid, methyl ester	41,54	0,52
Δ-1(2)-tetrahydrocannabinol	40,086	24,33	9-Octadecenoic acid, 12-(acetyloxy)-, methyl ester, [R-(Zhen et al.)]-	42,532	0,54
Δ -1(2)-tetrahydrocannabinol	40,564	15,27	9,12-Octadecadienoic acid, methyl ester, (E,E)-	43,462	1,26
Sterols and steroids			9,12-Octadecadienoic acid (Z,Z)-, methyl ester	43,861	0,63

(-)-Spathulenol	31,21	0,04
Spathulanol	32,34	0,17
Stigmast-24(28)-ene, (5.alpha.)-	47,372	0,11
Stigmasta-4,24(28)-dien-3-one	48,211	0,46
Other compounds		
Propane, 1,2-dimethoxy-	4,509	0,03
Propane, 1,2,3-trimethoxy-	8,137	0,1
Heptanal	8,219	0,05
1,4-Butanediol, 2,3-dimethoxy-	8,336	0,33
Benzoic acid, 4-amino-2-phenylaminooxazol-5-yl ester	24,357	0,04
1,3-2H-Isobenzofuranone, 3,3,7-trimethyl-	24,61	0,07
1,4-Benzenedicarboxylic acid, dimethyl ester	25,34	0,1
Phenol, 4-ethyl-5-methoxy-2-(4-phenyl-5-isoxazolyl)the concet-	26,528	0,15
1,3-Benzenediol, 2-(3,7-dimethyl-2,6-octadienyl)-5-pentyl-	27,545	0,22
9,12-Octadecadienoic acid (Z,Z)-, methyl ester	44,929	0,49
9,12-Octadecadienoic acid, methyl ester, (E,E)-	45,889	0,45
Glutaric acid, 3-methyl-, dimethyl ester	23,26	0,03
Nonanedioic acid, dimethyl ester	26,403	0,18
Tetradecanoic acid, (3,3a,4,6a,7,8,9,10,10a,10b-decahydro-3a,10a-dihydroxy-2,10-dimethyl-3,8-dioxobenz[e]azulen-5-yl) methyl est	32,544	0,36
Tetradecanoic acid, (3,3a,4,6a,7,8,9,10,10a,10b-decahydro-3a,10a-dihydroxy-2,10-dimethyl-3,8-dioxobenz[e]azulen-5-yl) methyl est	32,87	0,33
9-Hexadecenoic acid, methyl ester, (Zhen et al.)-	34,507	0,13
Hexadecanoic acid, methyl ester	35,06	1,37
Linoelaidic acid	41,006	0,41
Tridecanoic acid, 12-methyl-, methyl ester	30,457	0,16
Adipic acid, 2-methyloct-5-yn-4-yl pentyl ester	26,863	0,06
Lactic acid	4,808	0,21
Geranic acid	10,998	0,04
Amino acids		

1,3-Benzenediol, 2-(3,7-dimethyl-2,6-octadienyl)-5-pentyl-	27,985	0,78	Glycine, N,N-dimethyl-, methyl ester	6,408	0,74
1,3-Benzenediol, 2-(3,7-dimethyl-2,6-octadienyl)-5-pentyl-	28,333	1,01	Glycine, N-benzoyl-	14,152	0,05
1,3-Benzenediol, 2-(3,7-dimethyl-2,6-octadienyl)-5-pentyl-	28,7	1,14	L-Proline, 1-methyl-5-oxo-, methyl ester	21,207	0,38
1,3-Benzenediol, 2-(3,7-dimethyl-2,6-octadienyl)-5-pentyl-	28,959	0,4	d-Proline, N-methoxycarbonyl-, methyl ester	22,44	0,05
1,3-Benzenediol, 2-(3,7-dimethyl-2,6-octadienyl)-5-pentyl-	29,194	2,54	L-2-Aminobutyric acid, N-methyl-, methyl ester	7,795	0,35
2-Pentadecanone, 6,10,14-trimethyl-	33,072	0,17	**Terpenes**		
Lactic Anhydride	49,352	4,52	Guaiol	27,816	0,12
			Citronellol	41,9	0,09
			Caryophyllene	23,486	0,06
			Pyrazine, 2-methoxy-3-(1-methylpropyl)-	29,886	0,07
			L-linalool	14,273	0,05

5.11 Comparison of chemical compounds identified by Py-CG/MS and Py(TMAH)-GC/MS of Cannabis extract

The total concentration of lipophilic compounds is summarized in Figure 29 below. The cannabinoids percentage increased upon TMAH, and the concentration increased by 30% more than without the derivative. The fatty acid concentration increased upon the addition of TMAH, and the concentration increased from 2.26 to 10.28%. Terpenes concentration was high in the absence of TMAH, 14.29% it decreased to 0.39%. Sterol/steroids decreased from 3.24 to 0.78% upon addition of TMAH. Amino acids of 1.57% were detected with the presence of TMAH. While the percentage of other compounds was determined to decrease upon TMAH. As stated in section 4.5.5, adding the TMAH derivative eliminates peak tailing compounds from getting stuck in the column or the systems analyzer. It also attaches to readily volatile compounds and helps with their detection.

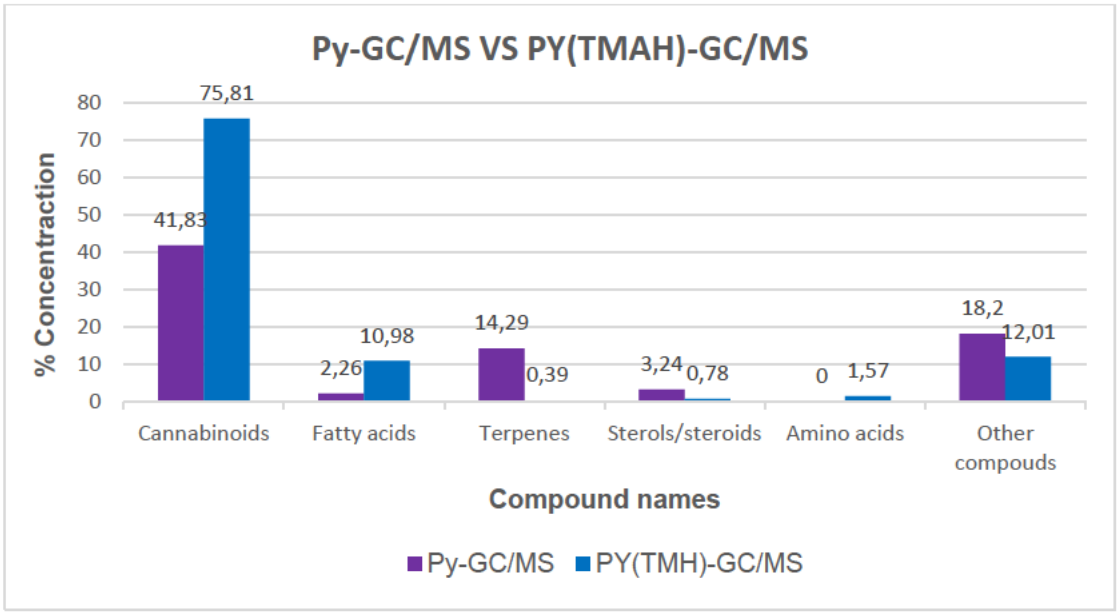

Figure 29: Summary of lipophilic compounds detected in the Cannabis *Sativa* L. by Py-GC/MS and Py(TMAH)-GC/MS.

6. CONCLUSION

Pinus patula sawdust and Cannabis *Sativa* L. may consist of high-value compounds. Finding safe and innovative ways of extracting these high-value compounds from these biomasses can transform the economy and reduce the environmental impacts of the medical and forestry industry. Traditional extracting methods produce a significant amount of waste material during extraction, which requires disposal, which can be costly and environmentally unfriendly. For example, in South Africa, there is a strong drive to divert all organic waste from landfills. Green extraction technologies such as SFE and ASE can beneficiate these waste materials by extracting high-value products like lipophilic compounds. This type of application could make it possible to reduce the amount of waste produced during extractions and provide environmentally-friendly extractions.

Overall, the quadratic polynomial models developed in this study strongly supported the optimization of lipophilic compounds from pinewood sawdust by ASE and SFE. The maximum yield of lipophilic compounds were 4.2% for ASE and 2.5% for SFE. These maximum yields were achieved under optimal conditions: 160°C, 12.5 mins and the static cycle of 1. For SFE, the optimum conditions were 50 °C, 300 bar, a CO_2 flow rate of 3.2 ml/min, and a 2 ml/min co-solvent flow rate. The yield obtained by ASE was greatly influenced by temperature and the number of static cycles.

In contrast, SFE was influenced by pressure, temperature and flow rate of extraction fluid and cosolvent. ASE was observed to have a high extraction efficiency compared to SFE. FTIR was used to identify functional groups present in the lipophilic extracts, and TGA/DSC was used to study the thermal stability of the extract.

Py-GC/MS and Py(TMAH)-GC/MS was used to identify lipophilic compounds extracted by ASE and SFE. Fatty acids were the major compounds identified in both extraction methods, with the ASE yielding a higher concentration than the SFE. An increase in the concentration of fatty acids was noted with the addition of TMAH, and the ASE increased from 49.98 to 69.38%. SFE increased from 17.72 to 43.58%. The increase in the concentration may be associated with less tailing and trapping of compounds in the column due to the addition of TMAH. A significant difference in the compounds was observed, as sterol compounds were only identified in the SFE extract. A slight change in terpenoids was observed as both methods displayed the

same terpenoid in the presence of TMAH, which was terpineol, which contrasted with the analysis of the SFE extract by Py-GC/MS, where no terpenoid compounds were detected. A high concentration of resin acids was detected upon the addition of TMAH for both methods, with methyl abietate as the main constituent. These findings show that in the presence of TMAH, the concentration of methyl abietate was higher than without the derivative, 7,81% and 7.39% for ASE and SFE, respectively. The SFE was more selective for resin acids than ASE without the TMAH. Aromatic compounds, monoterpenes, sterols, and long-chain fatty acids were not detected upon adding TMAH in the SFE extract. However, a higher amount of monoterpenes was detected in the absence of TMAH than ASE. Sterols were only detected in the SFE extract before the addition of TMAH. It can be concluded that compounds extracted are dependent on the extraction method employed. Using both ASE and SFE methods can extract a wide range of lipophilic compounds identified by Py-GC/MS and in the presence of TMAH. The SFE showed high selectivity for lipophilic compounds, and the concentration of unwanted compounds classified as other compounds was less than that identified in the ASE. The SFE is a better method for extracting lipophilic compounds from pinewood sawdust. It extracted a high amount of an important industrial monoterpene, α-pinene, compared to the ASE. Monoterpene adds value to pharmaceuticals, insecticides, repellents, solvents, and antimicrobials.

RSM and BBD was used to investigate and optimize the extraction of the lipophilic compounds from Cannabis by SFE. The effect of pressure, cosolvent flow rate and CO_2 flow rate were modelled and optimized. The coefficients of determination (R^2) of 0.98 and 0.91 were obtained for the SFE. The optimum extraction conditions were 300 bar, 1ml/min co-solvent, and 5 ml/min CO_2 flow rates, which gave a maximum yield of 55%. The compounds were identified using Py-GC/MS and Py(TMAH)-GC/MS; an increase in cannabinoids, fatty acids and amino acids was observed in the presence of TMAH. The addition of the TMAH derivative improved the detection of highly volatile compounds by forming a methylated version that can be detected in the MS without being stuck in the column, increasing the amount of cannabinoids, fatty and amino acids. A decrease in terpenes and sterols was observed with the addition of TMAH, which contradicted the literature finding.

This study showed that pinewood sawdust and Cannabis *Sativa* L. might be used as feedstocks to recover valuable materials. The efficiency of lipophilic compounds

depends not only on the type of feed material used, but the effect of the extraction method and parameters also has a significant impact on the compounds. This study would benefit small farmers of Cannabis, the forestry industry, and the local Green Economy in South Africa by providing insight into employing safer methods for attaining lipophilic compounds.

6.1 RECOMMENDATIONS FOR FURTHER RESEARCH

As a way forward, the study's focus will be on the calibration of industrial important monoterpenes such as α-pinene extracted from pinewood sawdust using Py-GC/MS and interpreting the MS spectra.

Quantification and comparison of cannabinoids from Cannabis *Sativa* L. flowers, stems and leaves extracted by SFE and ASE using Py-GC/MS

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Appendix

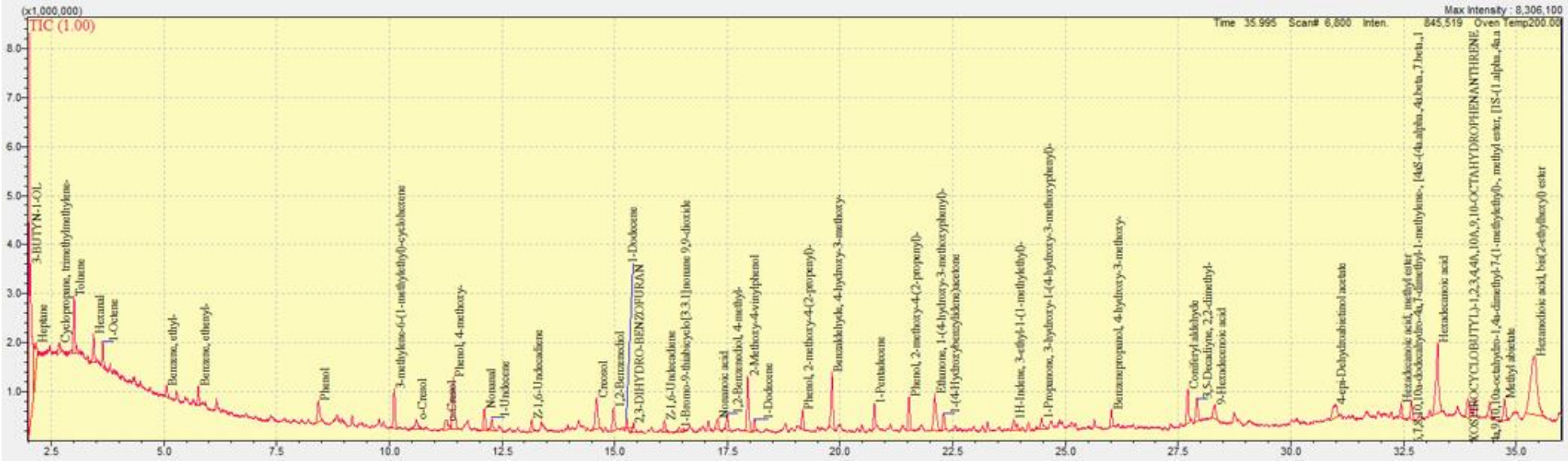

Figure A: Pyrogram showing the lipophilic compounds of pinewood sawdust their extracted by ASE.

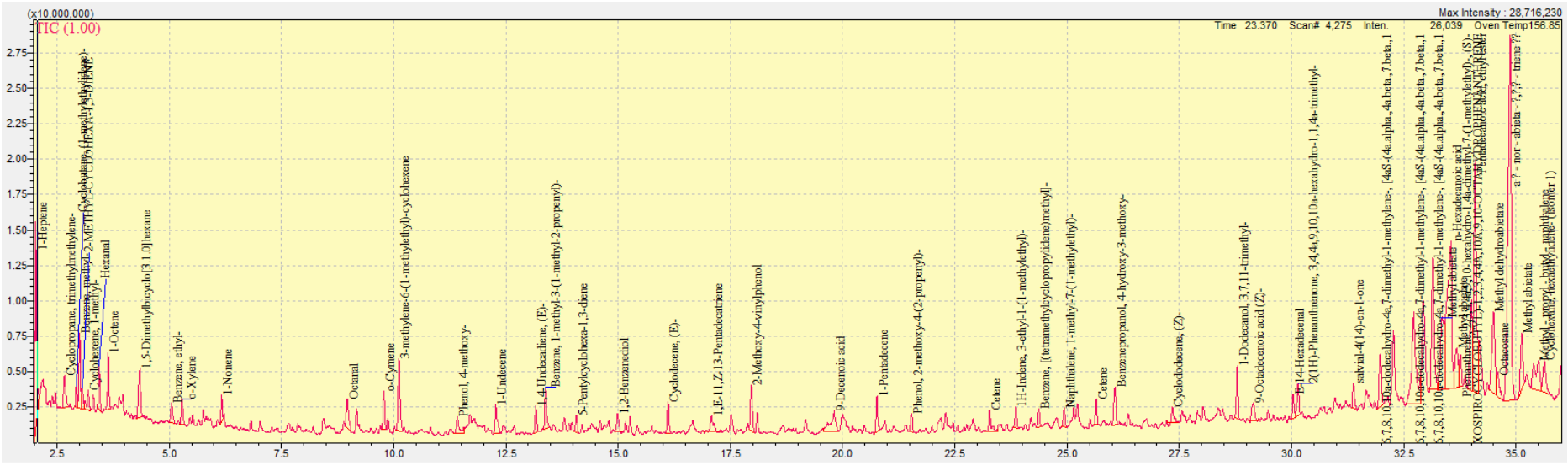

Figure B: Pyrogram showing lipophilic compounds of pinewood sawdust extracted by SFE.

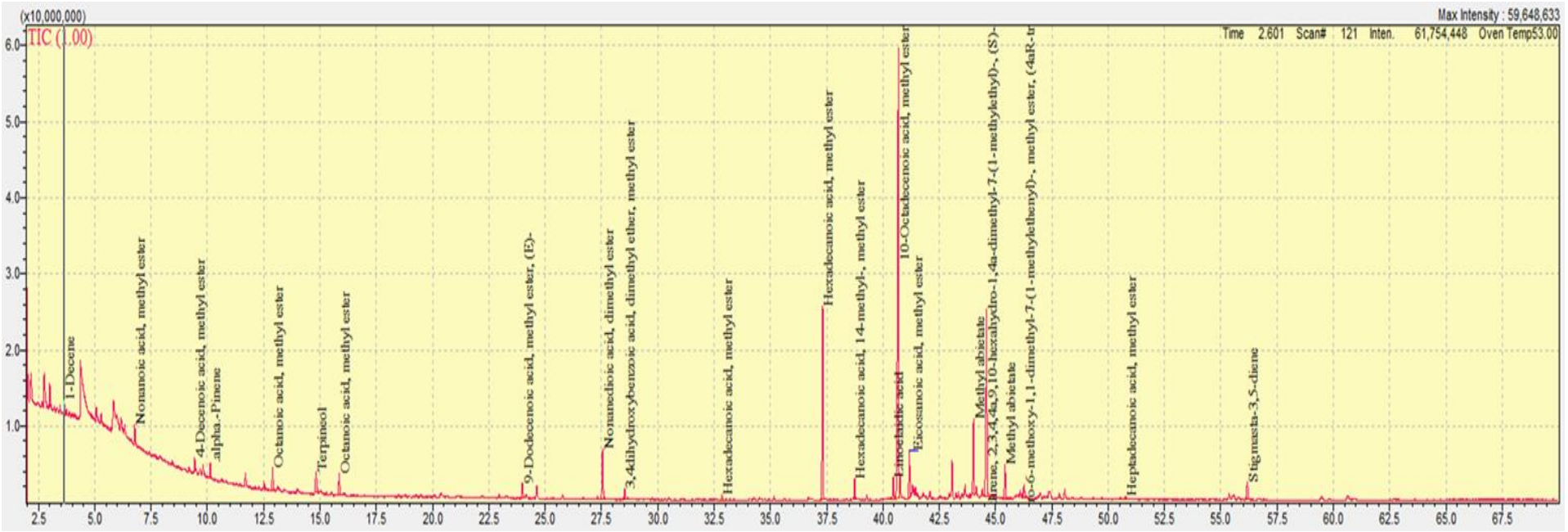

Figure C: Pyrogram showing lipophilic compounds of pinewood sawdust detected after the addition of TMAH for extracts by ASE.

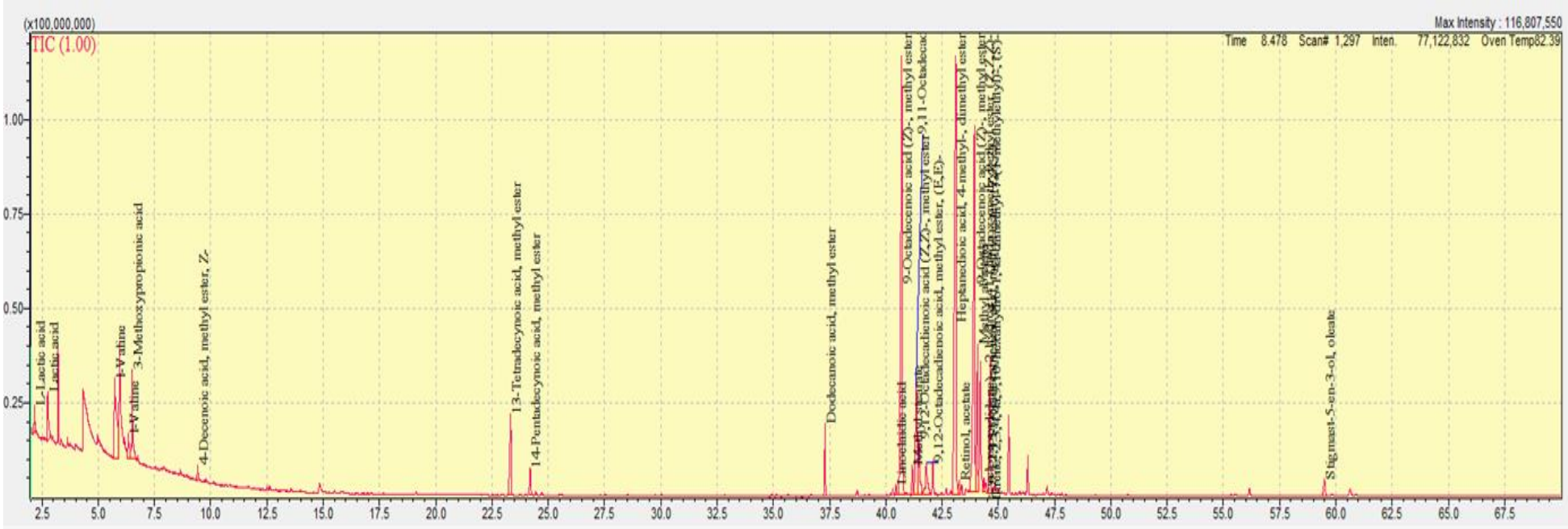

Figure D: Pyrogram showing lipophilic compounds of pinewood sawdust detected after the addition of TMAH for extracts by SFE.

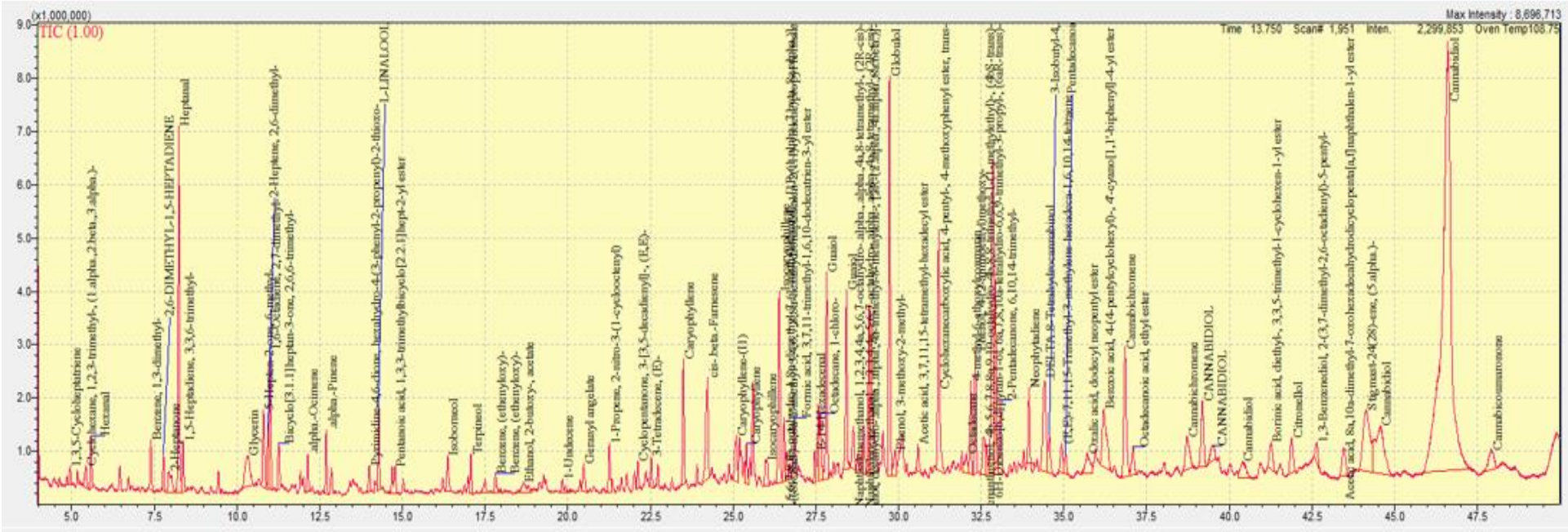

Figure E: Pyrogram showing lipophilic compounds of Cannabis *Sativa* L. extracted by SFE.

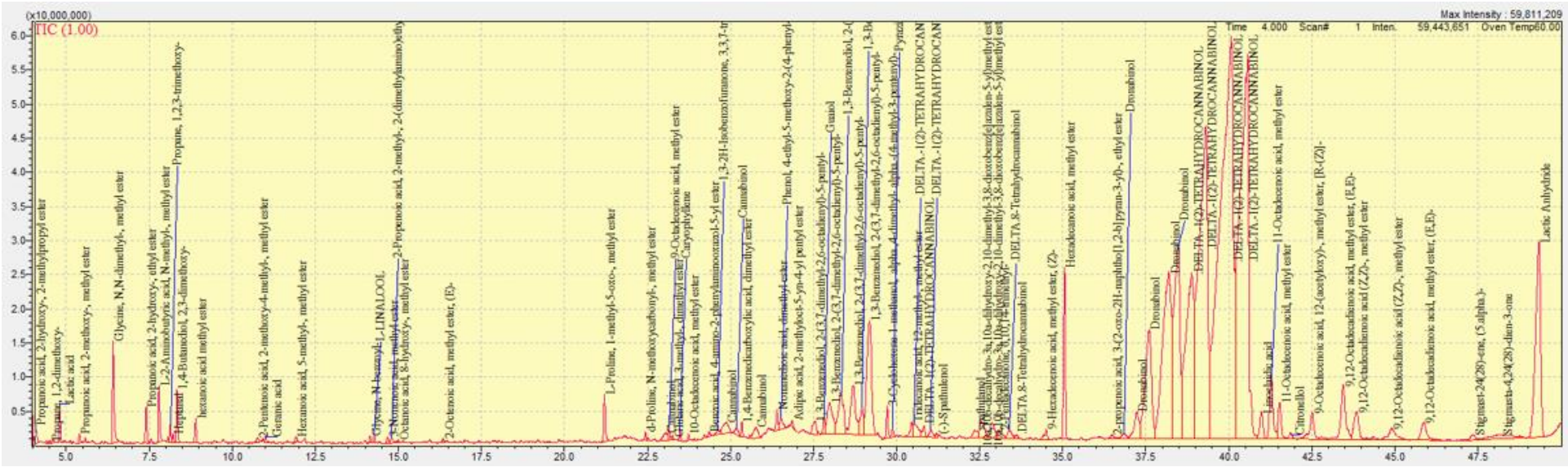

Figure F: Pyrogram showing lipophilic compounds of Cannabis *Sativa* L. detected after the addition of TMAH for extracts by SFE.