Valorization of Hemp Biomass: Terpene Extraction at Different Stages of Cannabidiol Isolation Technology

Master thesis

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Kanepi biomassi väärindamine: terpeenide ekstraheerimine kannabidiooli isoleerimise tehnoloogia eri etappidel

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Declaration

Hereby I declare that I have compiled the paper independently and all works, important standpoints and data by other authors have been properly referenced and the same paper has not been previously presented for grading.

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The paper conforms to requirements in force.
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List of Abbreviations

CBCA	Cannabichromenic acid
CBD	Cannabidiol
CBDA	Cannabidiolic acid
CBGA	Cannabigerolic acid
CBNA	Cannabinolic acid
CBNDA	Cannabinodiolic acid
CB_1R	Cannabinoid receptors type 1
CB_2R	Cannabinoid receptors type 2
CRM	Certified reference material
EI	Electron ionization
FID	Flame ionization detector
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
HPLC	High-performance liquid chromatography
IPU	Isoprene unit
IDL	Instrumental detection limit
IQL	Instrumental quantification limit
IS	Internal standard
LOD	Limit of detection
LOQ	Limit of quantification
MS	Mass spectrometry
NIST	National Institute of Standards and Technology
RMSE	Root mean square error
RMSE%	Relative root mean square error
RSD	Relative standard deviation
R^2	Coefficient of determination
SD	Standard deviation
SFE	Supercritical fluid extraction
SIM	Selected ion monitoring
S/R	Signal to noise ratio
THC	Tetrahydrocannabinol
THCA	Tetrahydrocannabinolic acid

Introduction

Hemp is a highly productive crop, which contains a wide range of compounds that have been an interest of research for the past decades. These compounds include fatty acids, phenolic compounds, flavonoids, cannabinoids, and terpenes. Cannabinoids are a diverse group of terpenophenolic compounds, which interact with endocannabinoid receptors in the human body. The most researched cannabinoids are cannabidiol (CBD) and tetrahydrocannabinol (THC). CBD is associated with relaxing and sedative effects. Its potential therapeutic applications include treatment of various medical conditions like chronic pain, anxiety and depression, epilepsy, Alzheimer's and Parkinson's diseases, and sleep disorders. The anti-inflammatory effects of CBD can be utilized for reducing inflammation associated with conditions like asthma and acne. On the other hand, THC is a psychoactive cannabinoid. Some of the effects of THC include altered perception of time and space, impairing memory, concentration, decision-making abilities, and psychomotor function. In European Union, THC is illegal in most of the countries, including Estonia, and is considered a narcotic. The permitted concentration of THC in cultivated hemp biomass and derived products is <0.3% *w/w* [1]. The hemp biomass, which THC concentration is below this limit is classified as "industrial hemp". Terpenes are volatile organic compounds, that are derived from isoprene units. Terpenes contribute to the aroma of the hemp plant, and synergistically enhance the effects on the nervous system, working in conjunction with cannabinoids. Terpenes have potential applications in aromatherapy, flavoring, cosmetics, and pharmaceuticals, thus being a valuable component of hemp biomass.

Industrial hemp biomass typically contains CBD levels ranging from 5% to 15% or more, depending on the variety of hemp and cultivation and cultivation practices. The large content of CBD in industrial hemp makes it an excellent source for CBD extraction. The R&D project conducted by the TalTech Analytical Chemistry group (Department of Chemistry and Biotechnology) focused on isolating CBD from industrial hemp biomass. Since the isolating and concentrating CBD from industrial hemp also means doing the same to THC, the aim was to develop and optimize the isolation process to obtain purified CBD products with a THC content below the permitted level of 0.3% *w/w*. The CBD isolation process included several stages, during which terpenes were lost or discarded. Since terpenes are valuable compounds, another important aim of the project was to integrate the terpene extraction into the main CBD isolation process. This approach enhances the overall efficiency of hemp biomass valorization.

Figure 1 gives a schematic overview of the CBD isolation process hemp biomass, as well as the specific steps where terpenes isolation can be potentially performed. First, hemp biomass undergoes cold ethanol extraction at -40°C. The received hemp extract is further concentrated by evaporating the ethanol, yielding crude extract and recovered ethanol, which retains some amount of terpenes. The crude extract undergoes a decarboxylation process, by heating the extract under reduced pressure, aiming to minimize the acidic form of cannabinoids. During this process, volatile terpenes evaporate from the extract and can be collected. The decarboxylated crude extract then undergoes distillation, followed by crystallization or column chromatography, to receive a high

purity CBD final product. In the beginning of the process, prior to the cold extraction, raw hemp biomass can be subjected to steam distillation to collect the terpene-rich essential oil (EO). The steamed biomass can then undergo the previously described CBD isolation process.

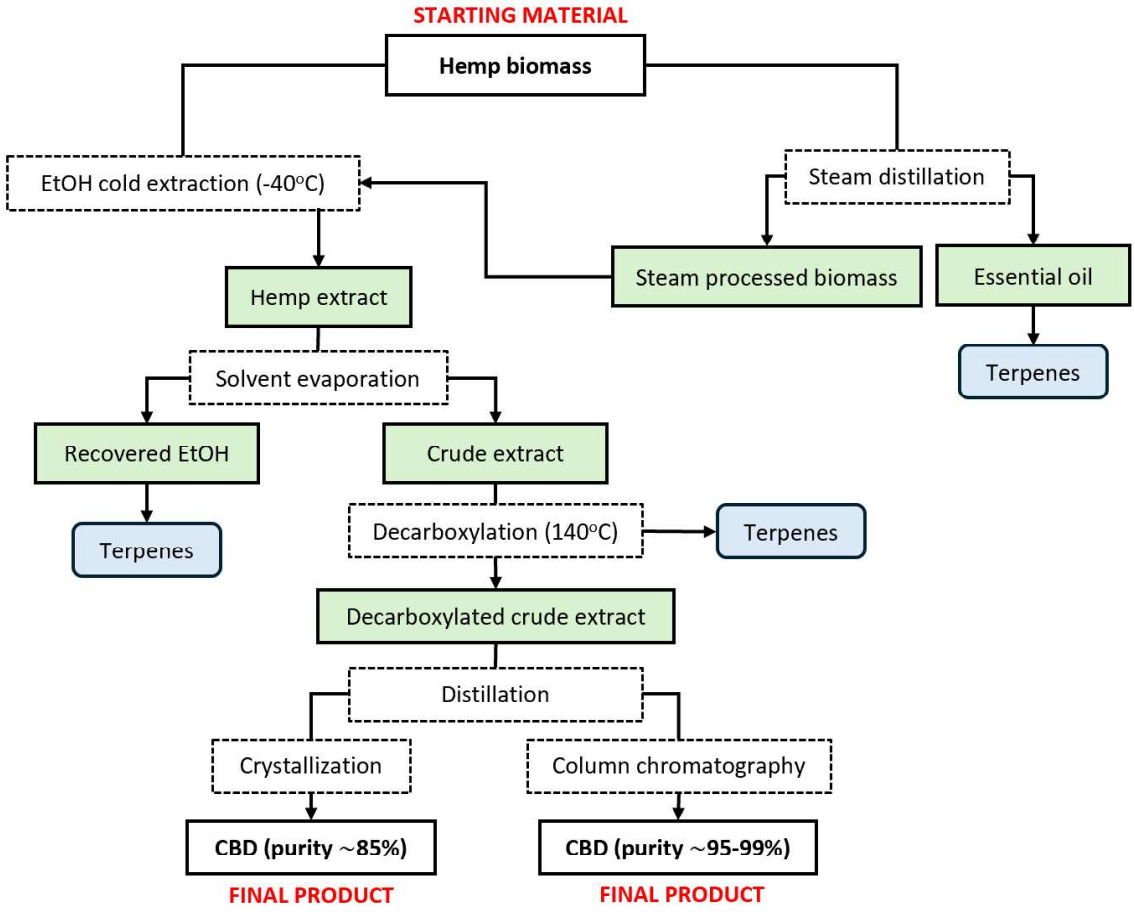

Figure 1. Schematic representation of high purity CBD isolation from hemp biomass.

The objective of this thesis was to investigate terpenes isolation at three different stages of the CBD isolation process, as well as develop and validate a gas chromatography-mass spectrometry (GC-MS) method to quantify these compounds in the resultant products. Through comparative analysis of the obtained data, this thesis aimed to identify the most efficient approach for terpene extraction, that is integrated into the CBD isolation process from hemp biomass. Therefore, the thesis contained the following tasks:

1. Development of a GC-MS method for quantitative analysis of terpenes in hemp products.
2. Isolation of terpenes via solvent extraction and their quantification.
3. Isolation and quantification of terpenes from recovered ethanol and evaluation of process efficiency.
4. Isolation and quantification of terpenes from crude extract during the decarboxylation process.
5. Isolation of hemp EO from raw plant material via steam distillation. Quantification of terpenes in hemp EO.

1 Literature Review

1.1 Hemp biomass

Hemp (*Cannabis sativa L., Cannabinaciae family*) is a herbaceous annual plant with a long cultivation history, primarily in Central Asia [2]. Hemp is a cropping productive crop, capable of yielding up to 22 tons of dry mass per hectare during a cropping season in favorable environments [3]. It stands out for its low input requirements, as it does not need pesticides and has minimal demand for nitrogen. Furthermore, hemp is known for its resilience to various environmental stresses, making it well-suited for growth in challenging conditions. [4]

The structure of the hemp plant is presented in **Figure 2**. At the top of the hemp plant are the flower clusters, also known as inflorescences or buds. These are the reproductive structures of the plant. Below the flower clusters are the leaves, which are typically palmate with serrated edges. Leaves are arranged alternately along the sturdy and fibrous stem, the main structural component of the hemp plant, which provides support for the plant and transporting nutrients and water between the roots and leaves. Hemp plant can have multiple branches that extend from the main stem. These branches may bear additional flower clusters and leaves. Nodes are the points along the stem where leaves, branches, or flower clusters emerge. Trichomes are small hair-like structures that are found on the surface of the hemp plant, particularly on the flowers, leaves, and stems. These structures are responsible for producing and storing bioactive compounds. Hemp is a dioecious[1], rarely monoecious[2] plant. Male and female hemp plants have distinct characteristics and play different roles in reproduction. Female hemp plants produce larger and more resinous flower clusters compared to male plants and produce pistils that catch pollen from male plants. The flowers of male plants are typically smaller and less densely packed. Additionally, female hemp plants have the potential to produce seeds and have longer flowering period compared to male plants. [1,4]

Hemp biomass composition highly depends on various factors, including the type of cultivar, growing conditions, cultivation practices, harvesting time, and processing methods. Similarly to other plant fibers, hemp is generally composed of cellulose (53-91%), hemicellulose (4-18%), pectin (1-17%), and lignin (1-21%) [6]. Hemp is a source of a wide range of bioactive compounds, which are produced and stored in glandular trichomes of the plant. These compounds include fatty acids, phenolic compounds, flavonoids, cannabinoids, and terpenes. The most abundant are cannabinoids, which content can reach 20% dry weight. Terpenes typically constitute less than 1% of the dry weight of hemp biomass. Other bioactive compounds are typically present in smaller quantities compared to cannabinoids and terpenes. [5]

There are two main categories of hemp plants, fiber-type and cannabinoid type. Fiber-type hemp plants are characterized by their tall stature (up to 5 meters [1, 4]). They are cultivated primarily for their fibers and seeds, and they have minimal cannabinoids content. In contrast, cannabinoid-type

[1] Dioecious plant – having either only male or only female organs in each plant. [57]
[2] Monoecious plant – having both male and female organs. [58]

hemp plants are shorter and possess a high concentration of cannabinoids (up to 20% [5]). Depending on the cannabinoid THC concentration, hemp can be further categorized into industrial hemp (THC <0.3% *w/w*) and marijuana (THC >0.3% *w/w*). [8]

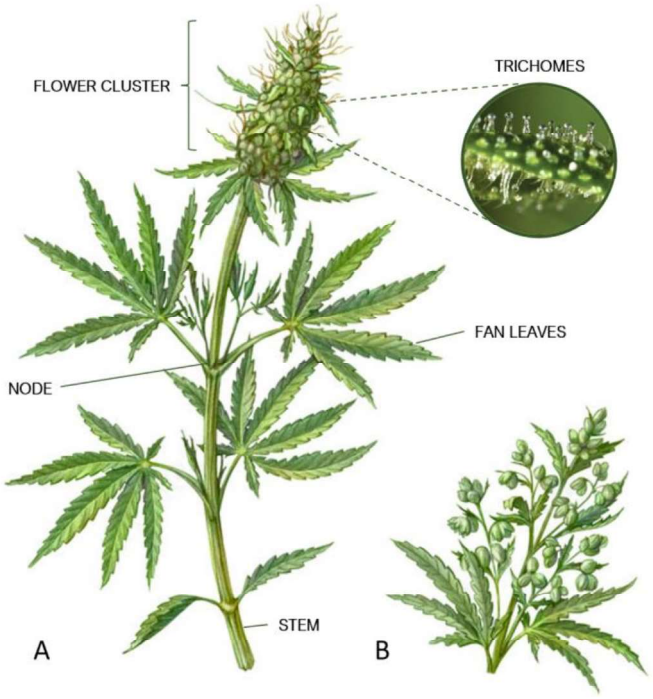

Figure 2. The structure of the (A) female and (B) male hemp plant. Figure based on Fordjour et al. (2023). [9]

Hemp has been valued for its medicinal properties and uses in textiles for centuries [5]. Today, hemp biomass finds application in a diverse range of industries due to its sustainability and versatility. One prominent application of hemp biomass is in the production of biofuels, with its cellulose and lignin content serving as valuable resources for biodiesel and ethanol production [10]. Hemp biomass is increasingly recognized in the construction industry for its role in sustainable building materials. One such material is hempcrete. This material is produced by mixing hemp hurds (the woody core of the hemp plant stem) with lime and water, resulting in a lightweight and breathable material that is resistant to mold, pests, and fire. [11] Furthermore, hemp biomass demonstrates potential in phytoremediation, aiding in soil remediation by absorbing heavy metals and toxins from contaminated sites [12]. The hemp fibers are also utilized in the textile industry for sustainable clothing and textiles, offering strength, durability, and breathability of the material [13].

1.1.1 Cannabinoids

Cannabinoids are the main bioactive constituents of the hemp biomass. In terms of their chemical composition, cannabinoids are a class of molecules comprising terpenoids and acyclic units. Presently, researchers have identified over 120 different cannabinoids in hemp biomass [7,3]. They play a crucial role in defense mechanisms and interactions with herbivores and pests [7]. Cannabinoids in hemp plant are mostly present in acidic form. The most abundant of them are

cannabidiolic acid (CBDA), tetrahydrocannabinolic acid (THCA) and cannabinolic acid (CBNA). Less abundant cannabinoids are cannabigerolic acid (CBGA), cannabichromenic acid (CBCA), and cannabinodiolic acid (CBNDA). [5] The project focuses on two most researched cannabinoids THC and CBD, the neutral forms of THCA and CBDA.

Cannabinoids cause various physiological effects by interacting with cannabinoid receptors. Cannabinoid receptors are specialized proteins found in cells throughout the human body that are part of the endocannabinoid system. These receptors play a crucial role in various physiological processes, including mood regulation, pain sensation, appetite, memory, and immune function. There are two main types of cannabinoid receptors. Cannabinoid receptors type 1 (CB_1R) are primarily found in the central nervous system, including the brain and spinal cord, as well as in peripheral tissues such as the gastrointestinal tract. Cannabinoid receptors type 2 (CB_2R) are mainly located in peripheral tissues, particularly immune cells. Cannabinoids, including those produced by the human body (endocannabinoids) and those derived from plants (phytocannabinoids), trigger a series of signaling events within the cell when binding to a cannabinoid receptor. This signaling is leading to various physiological responses. THC is a partial agonist[3] of both CB_1R and CB_2R, having a higher affinity for the CB_1R, which appears to mediate its psychoactive effects. [14,15] CBD exhibits low binding affinity for both CB_1R and CB_2R, but it can act as an antagonist in their presence of THC. CBD functions as a non-competitive negative allosteric modulator[4] of the CB_1R, reducing the efficacy and potency of THC. [17,18] THC demonstrates anti-inflammatory, anti-cancer, analgesic, muscle relaxant, neuro-antioxidative, and anti-spasmodic activities. However, it is also associated with side effects such as anxiety, cholinergic deficits, and immunosuppression. Short-term use of THC can lead to memory and cognitive deficits, impaired motor coordination, and psychosis. Long-term use of THC has been linked to a higher risk of addiction, cognitive impairment, altered brain development, and an increased likelihood of developing chronic disorders such as schizophrenia. On the other hand, CBD exhibits a wide range of therapeutic properties, including anti-anxiety, anti-nausea, anti-arthritic, anti-psychotic, anti-inflammatory, and immunomodulatory effects. It shows a promise in the treatment of epilepsy, neurodegenerative diseases (Alzheimer's and Parkinson's diseases), schizophrenia, multiple sclerosis, affective disorders, and modulation of feeding behavior. Furthermore, CBD possesses potent anti-fungal and anti-bacterial properties. [5]

The cannabinoids are synthesized and released by glandular trichomes located on the surface of the flowers, leaves, and stems (**Figure 2**) [14]. The fundamental process in cannabinoid synthesis (**Figure 3**) involves the production of olivetolic acid from hexanoyl coenzyme A, catalyzed by olivetolic acid synthase. Follows the alkylation of the phenolic part of olivetolic acid with the terpenoid segment of geranyldiphosphate, resulting in the formation of CBGA. CBGA serves as the precursor for all major cannabinoids in hemp and can then undergo enzymatic catalytic reactions mediated by THCA-synthase and CBDA-synthase, leading to the formation of cannabinoids such as THCA and CBDA.

[3] Partial agonist – a ligand that attaches to a cell receptor and induces a response similar to but weaker than the body's naturally produced full agonist. [59]

[4] Competitive negative allosteric modulator – a ligand that indirectly affects the binding of an agonist by interacting with a secondary site on the receptor, thereby reducing the ability of the agonist to bind to the primary site. [18]

[19] The acidic forms of cannabinoids lack of pharmacological activity as they do not interact with cannabinoid receptors in nervous system in the same manner as their neutral counterparts. The conversion of THCA to THCA and CBDA to CBD occurs through a process known as decarboxylation. Decarboxylation occurs as a result of heating, for example smoking or cooking. The decarboxylation process involves the removal of a carboxyl group from the cannabinoid molecule, resulting in the formation of neutral form from acidic (**Figure 3**). [20]

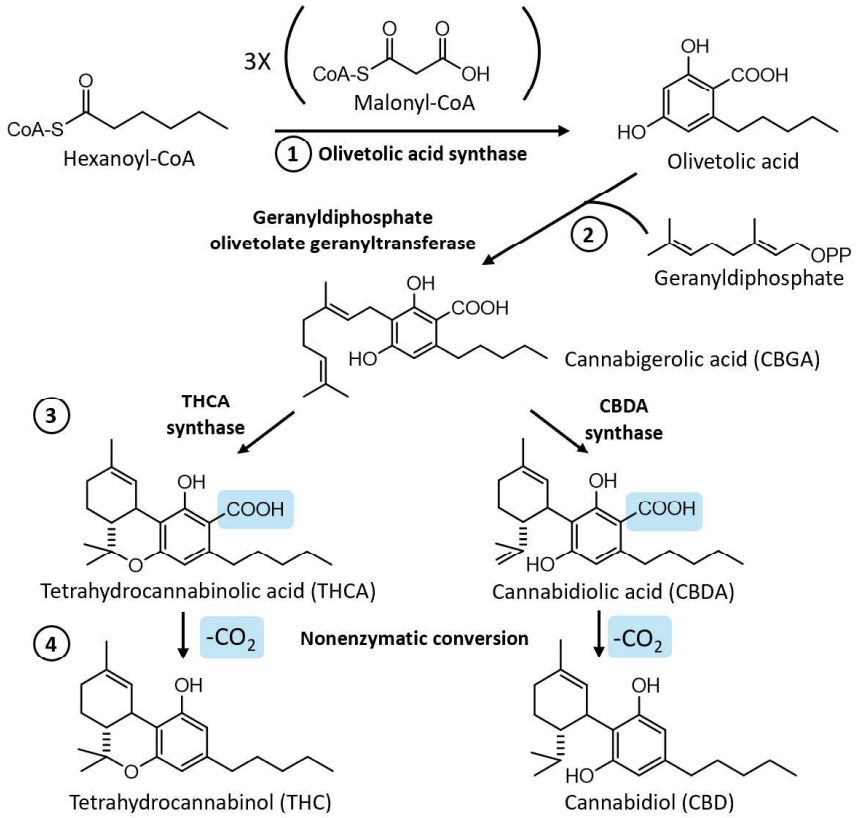

Figure 3. Cannabinoid synthesis pathway in the trichomes of hemp, **(1)** olivetolic acid synthesis by olivetolic acid synthase, **(2)** CBGA synthesis by olivetolate geranyltransferase, **(3)** THCA and CBDA synthesis by THCA synthase and CBDA synthase, **(4)** non-enzymatic conversion of compounds to neutral form by with heating (decarboxylation process). Adapted from Vergara et al. (2019). [19]

1.1.2 Terpenes

Terpenes and terpenoids represent another class of most abundant and chemically diverse bioactive compounds present in hemp [21]. These compounds play a crucial role in the distinct aroma of the hemp plant. They serve functions such as repelling and defending against herbivores, attracting pollinators, and inhibiting microbial growth. Terpenes and terpenoids are synthesized as a defense mechanism, and their production is influenced by environmental factors. Currently, more than 200 terpenes and terpenoids have been identified in hemp, constituting up to 10% of the trichome contents and up to 1% of the dry mass of hemp [5,22].

Terpenes are commonly used in cosmetics, perfumes, and aromatherapy. They have a wide range of therapeutic properties, including anti-inflammatory, antioxidant, and neuroprotective effects. Terpenes exhibit antimicrobial and antifungal activity, which can be utilized in natural pest control and disease treatment. [23] Additionally, terpenes are Food and Drug Administration approved additives, allowing their incorporation into food and beverages for flavor or nutritional purposes [8].

Terpenes are hydrocarbons that consist of small 5-carbon building blocks called isoprene units (IPU), which are linked together in a head-tail manner to form chains (**Figure 4**). These chains can undergo rearrangements to create cyclic structures. Terpenes that contain oxygen in their structure are called terpenoids. Terpenes are categorized into nine groups, based on the number of isoprene units in the molecule: hemiterpenes (1 IPU), monoterpenes (2 IPU), sesquiterpenes (3 IPU), diterpenes (4 IPU), sesterterpenes (5 IPU), triterpenes (6 IPU), sesquaterpenes (7 IPU), tetraterpenes (8 IPU), and polyterpenes (more than 8 IPU). [24]

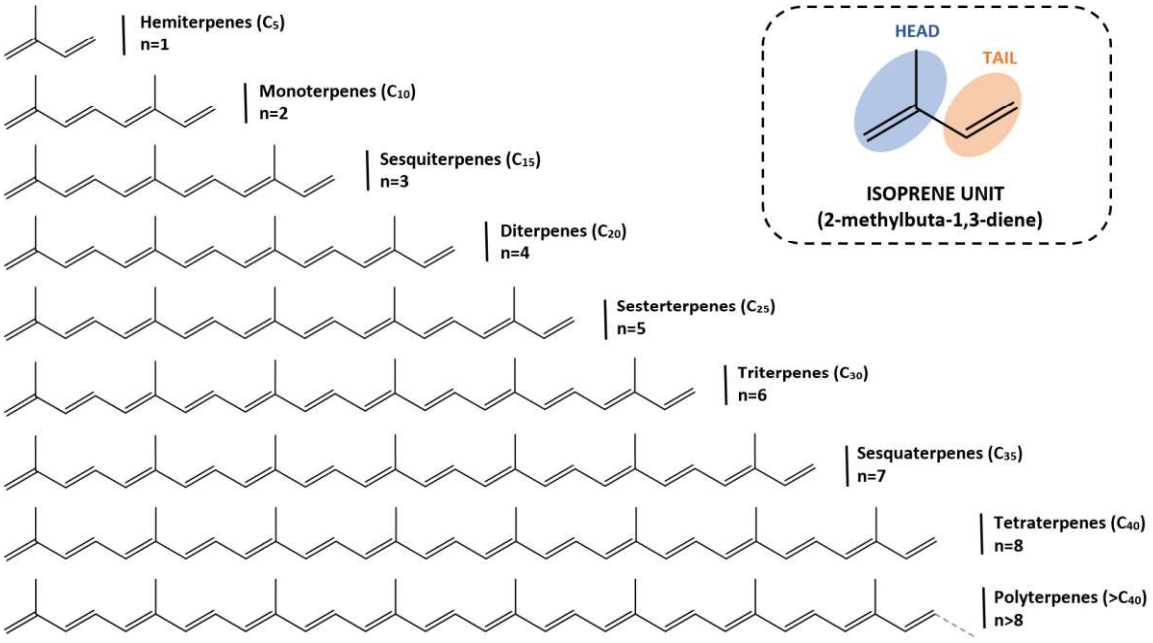

Figure 4. Classification of terpenes based on the linked isoprene units, which can rearrange to make cyclic structures. Adapted from Mosquera et al. (2021) and Dogra et al. (2022). [24,25]

Monoterpenes, monoterpenoids, sesquiterpenes, and sesquiterpenoids are the primary terpene types found in hemp. Monoterpenes and -terpenoids, composed of two IPU, are present in acyclic monocyclic and bicyclic forms. Sesquiterpenes and -terpenoids, composed of three IPU, represent the most diverse category of terpenes and can occur in various structural forms, including acyclic, monocyclic, bicyclic, and tricyclic forms. The less abundant terpenes in hemp include diterpenes and -terpenoids, and triterpenes and -terpenoids. Diterpenes, composed of four IPU, are mostly present in acyclic and monocyclic forms. Triterpenes, composed of six IPU, can occur in acyclic, tetracyclic, and pentacyclic forms. [24] The abundance of different terpenes in hemp can vary

depending on factors such as strain of hemp, growing conditions, etc. Some common terpenes found in hemp biomass are myrcene (acyclic monoterpene), limonene and terpinolene (monocyclic monoterpenes), α- and β-pinene (bicyclic monoterpenes), linalool (acyclic monoterpenoid), humulene (monocyclic sesquiterpene), and caryophyllene (bicyclic sesquiterpene). [26]

The physiochemical properties of terpenes depend on several factors, like molecular structure and molecular weight, the presence of oxygen-containing functional groups, and environmental conditions. The boiling points of hemp terpenes fall in the range of 100-320 °C at the atmospheric pressure. The boiling point increases with the molecular weight of terpenes, typically in the following order: monoterpenes, monoterpenoids, sesquiterpenes, and sesquiterpenoids. The vapor pressure of terpenes ranges from high to very low. In general, the vapor pressure of terpenes is decreasing in the order: monoterpenes, monoterpenoids, sesquiterpenes, sesquiterpenoids. At 20°C and atmospheric pressure, the vapor pressure of monoterpenes is significant and falls in the range of 0.8-4 mmHg. Monoterpenoids and sesquiterpenes have moderate vapor pressure in the range of 0.01-0.2 mmHg. Finally, sesquiterpenoids show extremely low vapor pressure (<0.001 mmHg), which makes these terpenes non-volatile. Terpenoids tend to be slightly more polar due to the oxygen-containing groups presence, which results in their greater solubility in aqueous solutions and lower volatility compared to terpenes. It is essential to consider the volatility of hemp terpenes, when choosing the biomass processing techniques and sample preparation procedure. The heating should be avoided, or the closed systems should be used, to prevent the loss of terpenes. [27]

1.2 Isolation and characterization of terpenes from plant material

The isolation of terpenes from plant material involves extraction methods to obtain these compounds from the plant matrix. Commonly used extraction techniques are solvent extraction, supercritical fluid extraction (SFE), and steam distillation. Solvent extraction is the less complex method, which involves the use of organic solvents to dissolve the terpenes from the plant material. The choice of the extraction solvent, especially its polarity, can significantly influence the final composition and the yield of extracted terpenes. Polar organic solvents are better suited for extracting terpenoids, which contain oxygenated functional groups and thus have higher polarity (e.g. linalool, geraniol, nerolidol, and bisabolol). Non-polar solvents are more effective for extracting non-polar terpenes (e.g. pinene, limonene, myrcene, humulene). Various organic solvents with different polarity have been reported for terpene extraction from hemp biomass, including ether, ethanol, methanol, hexane, ethyl acetate, and chloroform, as well as mixtures of these solvents in different ratios. [28] The solvent extraction can be assisted by ultrasound or microwave to enhance the process efficiency [29]. Despite its simplicity, solvent extraction of terpenes from plant material is not favorable. This method produces a large amount of organic solvent waste, that is often not environmentally friendly and needs to be utilized or recovered. Another disadvantage is that terpenes are extracted along with other compounds (e.g. cannabinoids) and require additional concentration and purification procedures. SFE utilizes supercritical fluid (typically carbon dioxide) to extract terpenes under high pressure and temperature conditions. This method allows more selective extraction of terpenes by adjusting the pressure, temperature, and composition of the

supercritical fluid. SFE achieves high extraction yields in a shorter time compared to steam distillation. SFE typically uses carbon dioxide as the fluid, which is non-toxic, non-flammable, and can be recovered and reused within the same extraction process. However, SFE systems require specialized and expensive equipment, and scaling up the process from laboratory to industrial scale can be challenging. [28]

After the extraction of terpenes from hemp biomass, they can be analyzed qualitatively and quantitatively using different analytical techniques. Chromatographic techniques are commonly reported for terpene analysis, specifically high-performance liquid chromatography (HPLC) [30,31] and gas chromatography (GC). For terpene determination HPLC systems are mostly equipped with ultraviolet detection. GC systems are mostly coupled with either flame ionization (FID) or mass spectrometric (MS) detectors. GC coupled with electron ionization mass spectrometry (EI-MS) is often preferred, since it allows precise terpene identification using compound libraries. [31-33] The internal standard (IS) method is widely used for terpene quantification, to minimize the instrumental signal instability. Hydrocarbons are commonly used as IS, due to their chemically inert and non-polar nature, that is similar to terpenes [34-36].

1.2.1 Steam distillation

Steam distillation is a technique used to separate and purify volatile compounds from plant material or other substances. It involves passing steam through the mixture, which causes the volatile compounds to vaporize. The vapor is then condensed back to liquid and collected. Steam distillation is particularly useful for extraction of EOs. EOs are highly concentrated hydrophobic liquids extracted from plants. The EO of hemp biomass is a homogeneous mixture mainly consisting of terpenes. [37]

Steam distillation methods for isolation of EOs from hemp biomass can be classified into three types based on type of contact of solvent with the matrix (typically water): hydrodistillation, dry steam distillation, and direct steam distillation (**Figure 5**). In hydrodistillation (**Figure 5, 1**) the plant matrix is in direct contact with water, and extraction occurs through solid-liquid interaction. The disadvantage of hydrodistillation method is that some EO components may undergo hydrolysis, due to the higher extraction temperature. In dry steam distillation (**Figure 5, 2**), the steam generated in the first container is passing through the plant material in the second separate container, causing the volatiles to vaporize and carry over into the condensation apparatus, where they are collected. This method is particularly suited for plants containing compounds that are easily hydrolyzed when exposed to high temperatures. Direct steam distillation (**Figure 5, 3**) is the method that was used for terpene extraction in this thesis. In this method, the plant material is placed above the steam generator, supported by a metal mesh or perforated screen. Direct steam distillation is employed for plant material that requires higher temperatures to release EOs effectively. [34,35]

Steam distillation is the predominant extraction technique used for EO extraction due to its minimal investment needs and operational expenses in comparison to alternative methods. This method is

considered a clean method in the context of environmental safety, since the solvent used is water. However, steam distillation is marked by uncertainties in both yield and process. . [34,35]

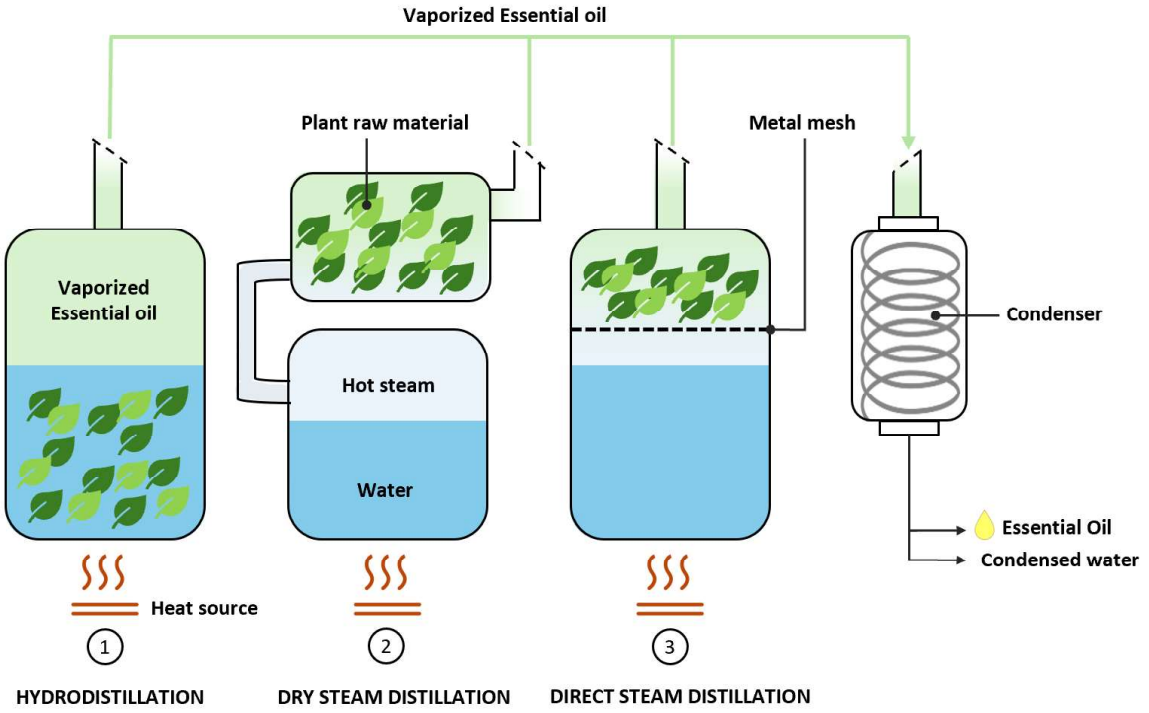

Figure 5. Schematic representation of **(1)** – hydrodistillation, **(2)** – dry steam distillation, and **(3)** – direct steam distillation process. Figure based on Shrivastava (2023). [38]

1.3 Gas chromatography-mass spectrometry

GC-MS is an analytical technique, which is widely used for the precise separation and identification of volatile compounds. GC relies on a gaseous mobile phase to transport sample components through a thin capillary column, where separation occurs based on interactions with a stationary phase. Meanwhile, MS detects and analyzes ionized molecules based on their mass-to-charge ratio. The GC-MS allows effective separation and enables identification of the compounds. This technique offers notable advantages, including rapid analysis, high efficiency, and sensitive detection at low concentrations (parts per billion). [35,36]

A typical GC-MS instrument comprises an inert carrier gas supply, an injector for sample introduction, a column oven for precise temperature control, and an MS detector (**Figure 6**). As the injected sample vaporizes and enters the GC column, variations in chemical properties result in their separation along the column's length. The column's efficacy depends on factors such as capillary dimensions and phase properties, ultimately leading to molecules' retention and subsequent release at distinct retention times. [35,36]

Upon entering the mass spectrometer through the transfer line, molecules undergo ionization through various methods, with electron ionization being common. Here, molecules encounter free

electrons emitted from a filament, inducing characteristic fragmentation patterns. The electron energy is typically set at 70 electron Volts (eV), enabling comparison of generated spectra with library spectra, such as those provided by the National Institute of Standards and Technology (NIST). [35,36]

Following fragmentation, the fragments enter the mass analyzer, typically a quadrupole, which allows only ions of a certain mass-to-charge ratio (m/z) to pass through to reach the detector. Mass spectrometers operate in full scan or selected ion monitoring (SIM) modes. While full scan mode is advantageous for identifying unknown compounds by allowing fragments within a set range of m/z to pass through, SIM mode enhances sensitivity and selectivity by only allowing fragments with specific m/z ratios to reach the detector. This mode reduces background noise and interference from co-eluting analytes, ensuring precise analysis. Finally, in the detection unit, the ionized mass fragments' signals are multiplied, usually by electron multiplier diode, and converted into an electrical signal for analysis. [35,36]

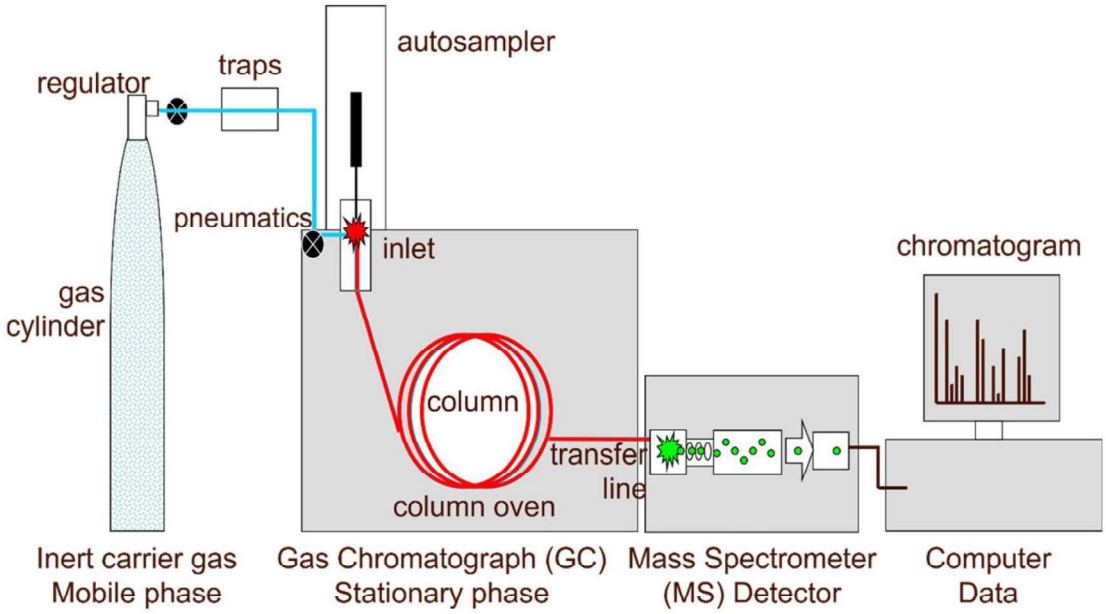

Figure 6. Schematic representation of GC-MS instrumentation. [39]

2 Experimental

2.1 Materials

2.1.1 Hemp biomass

In this study, organic certified hemp biomass from the Finola variety was used (**Figure 7**). Specifically, the upper segment of the plant (approx. 30-40 cm), including leaves, flowers, and seeds, was selected. The biomass was harvested in Karksi Nuia, Estonia, in the year 2023. The fresh biomass was freezed right after it was transferred to the laboratory.

Figure 7. Hemp plant material.

2.1.2 Chemicals

The reagents used for the experiments were the following: chloroform (Fisher Chemical, USA), purity >99.8%; ethyl acetate (Fisher Chemical, USA), HPLC grade; methanol (Honeywell, USA), HPLC grade; n-dodecane (Sigma-Aldrich, USA), purity >99%; n-hexane (Fisher Chemical, USA), purity 95%.

2.1.3 Terpene reference material

The certified reference material (CRM) (19 terpenes in the mix) used for terpenes analysis was obtained from LGC Standards Ltd (USA). The CRM had a concentration of 2500 µg/mL in hexane for each terpene. The compounds present in the CRM, along with their molecular structures and classifications, are illustrated in **Figure 8**. The details of the compounds present in the CRM, including their respective concentrations (in µg/mL), expanded uncertainties (U, in µg/mL), Chemical Abstracts Service (CAS) numbers, and the purity percentages of the compounds are presented in **Appendix 1**.

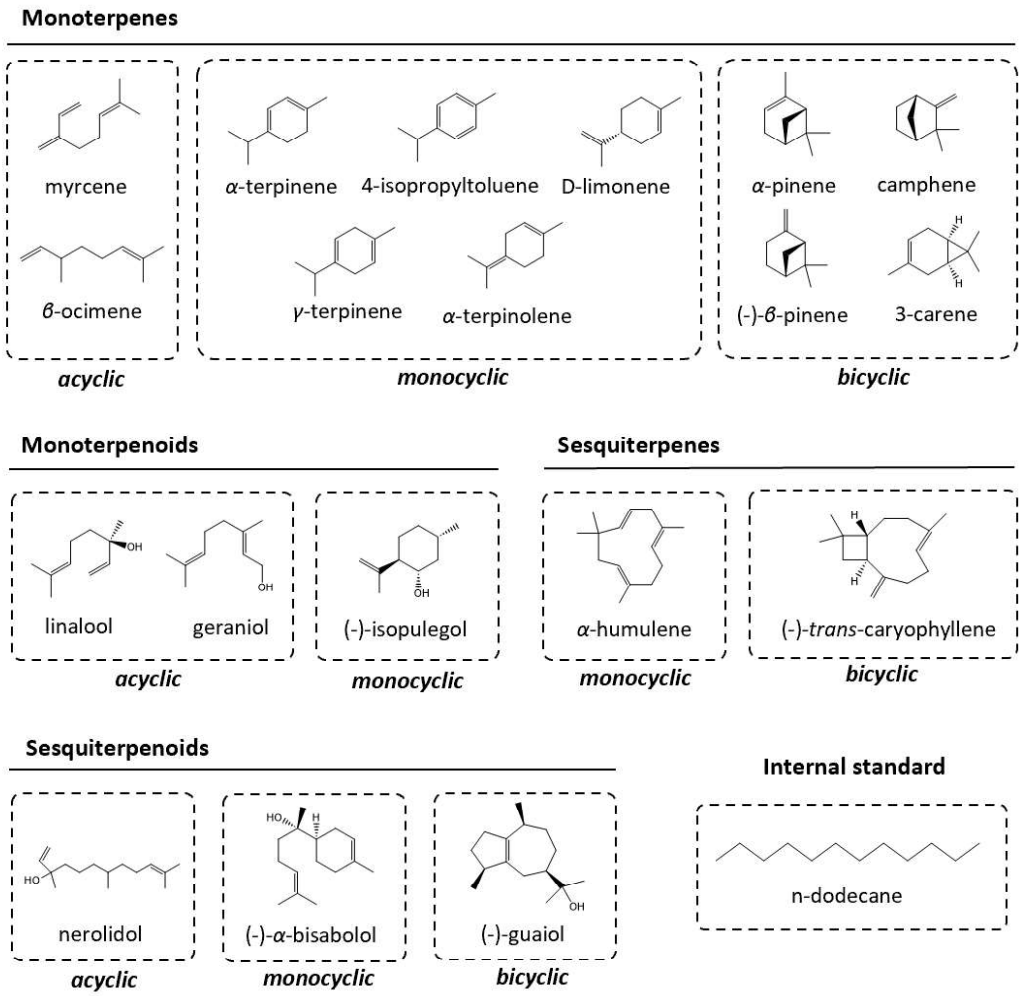

Figure 8. Molecular structures and classification of analyzed terpenes and the IS.

2.2 Methods

2.2.1 Gas chromatography-mass spectrometry

The GC analysis was conducted using Agilent Technologies 7890A gas chromatograph equipped with Phenomenex ZB-5plus column (30.0 m x 250 µm). The oven temperature program commenced at 50 °C, increased at a rate of 12 °C/min to 300°C, and was held at 300°C for 3 minutes. The total run time was 24 min. The injection temperature was set at 280°C, with a 1.5 µL injection volume. A 10:1 split was used for sample introduction. Helium (purity 6.0, Linde Gas AS) was employed as the carrier gas, maintained at a flow rate of 1 mL/min and a pressure of 14.4 psi. The gas chromatograph was coupled with mass spectrometry equipment.

The mass spectrometry analysis was performed using an Agilent Technologies 5975C VL MSD mass spectrometer with electron ionization at 70 eV. The transfer line temperature was set at 280°C, while the source and quadrupole temperatures were maintained at 230°C and 150°C, respectively. Mass spectra was acquired over a scan range from m/z 10 to 230, with a solvent delay of 4.0

minutes. GC-MS data was processed using Agilent MassHunter Qualitative Analysis program. Compound identification was based on NIST17 mass spectral library and retention time matching.

2.2.2 Analytical method validation

Calibration linearity range

Calibration solutions of 0.5, 0.7, 1, 2, 5, 7, 10, 20, 50, 70, and 100 µg/g were prepared by dissolving CRM of terpenes in methanol. The IS in the final concentration of 100 µg/g was added to all calibration solutions. The structure of the IS is provided in **Figure 8**. Calibration curves were constructed by plotting the concentration of each terpene against respective peak area and IS area ratio. Nerolidol is expressed as total concentration of two isomers (*cis* and *trans* mixture). The linearity of each calibration curve was evaluated to ensure a linear relationship between terpene concentration and peak area. To rate the linearity and statistical significance of the calibration curves obtained, the coefficient of determination (R^2), root mean square error (RMSE) in absolute and relative units, and *p*-value were calculated.

R^2 assesses how well the regression line fits observed data points. It indicates the proportion of the variance in the dependent variable (y) that is predictable from the independent variable (x) in the model. R^2 values range from 0 to 1, with higher values indicating a better fit. R^2 values were calculated by Eq. 1.

$$R^2 = 1 - \frac{SS_{res}}{SS_{tot}} \quad \text{(Eq. 1)}$$

SS_{res} – the sum of squares of the residuals (the differences between observed and predicted values);
SS_{tot} – the total sum of squares (the sum of squares of the differences between observed values and the mean of the observed values).

RMSE is a measure of the differences between values predicted by a model or estimator and the actual observed values. It quantifies the average difference between the predicted and observed values and provides a measure of the models' predictive accuracy. Lower RMSE values indicate better model performance. RMSE values were calculated by Eq. 2.

$$RMSE = \sqrt{\frac{\sum_{i=1}^{n}(y_i - \hat{y}_i)^2}{n}} \quad \text{(Eq. 2)}$$

n – the number of observations;
y_i – the observed value;
$\hat{y}_i$ – the predicted value.

Relative root mean square error (RMSE%) is the RMSE expressed as a percentage of the average observed values. It provides a relative measure of the error compared to the scale of the observed

values, allowing for better interpretation of the error magnitude. Like RMSE, lower RMSE% values indicate better model performance. RMSE% values were calculated by Eq. 3.

$$RMSE\% = \left(\frac{RMSE}{\bar{y}}\right) \times 100\% \quad \text{(Eq. 3)}$$

$\bar{y}$ – the average of the observed values.

In the context of a calibration curve, the p-value is a statistical measure that helps assess the significance of the relationship between the independent variable (concentration) and the dependent variable (terpene: internal standard peak ratio). The significance level was set to α=0.05 and one-sample t-test was performed using the Eq. 4.

$$t = \frac{\bar{x}-\mu}{s/\sqrt{n}} \quad \text{(Eq. 4)}$$

$\bar{x}$ – the sample mean;
μ – the expected value (the true concentration);
s – the sample standard deviation;
n – the sample size.

Limit of detection and limit of quantification

Instrumental detection limit (IDL) and instrumental quantification limit (IQL) were determined for each terpene to establish the lowest concentrations at which the compounds could be reliably detected and quantified. IDL and IQL values were calculated based on the signal to noise ratio (S/R) (Eq. 5 and Eq. 6). For IDL and IQL the SNR limits were set to 3:1 and 5:1, respectively. The noise was determined from the same chromatogram within an area with no peaks and SNR was calculated automatically using Agilent MassHunter program.

$$IDL = 3 \times \frac{S}{N} \quad \text{(Eq. 5)}$$

$$IQL = 5 \times \frac{S}{N} \quad \text{(Eq. 6)}$$

S – signal intensity;
N – noise intensity.

According to calculated IDL and IQL values, the Limit of detection (LOD) and Limit of quantification (LOQ) were determined, to establish the lowest concentrations at which the compounds can be detected and quantified from the hemp biomass. The LOD and LOQ were calculated using Eq. 7 and Eq. 8.

$$LOD\ [\mu g/g] = \frac{IDL \times V}{m} \quad \text{(Eq. 7)}$$

$$LOQ\ [\mu g/g] = \frac{IQL \times V}{m} \quad \text{(Eq. 8)}$$

V – added solvent [mL];
m – mass of biomass [g].

Accuracy of the method

Measurement accuracy refers to the degree of proximity between a single measurement and a reference value. Method validation aims to evaluate the accuracy of results by examining both systematic and random effects on individual measurements. Accuracy is thus assessed through two key components, which are precision and trueness. [40]

Precision, also known as measurement precision, gauges the degree of proximity between individual results. It accounts for the random error inherent in measurement outcomes. Precision is often quantified using statistical parameters that describe the dispersion of results, such as the standard deviation (SD) or relative standard deviation (RSD). These parameters are calculated based on replicate measurements conducted on a suitable material under specified conditions. Measurement repeatability and reproducibility represent two extremes of precision. Repeatability refers to the variation observed when the same operator measures the same sample multiple times under identical conditions, reflecting the precision of measurements within a single laboratory. Reproducibility, on the other hand, evaluates the variation in measurements obtained using the same method over an extended period, often several months, and under different conditions. Lower SD and RSD values indicate higher precision of the method. [40]

For repeatability assessment, six injections of six terpene standard concentrations (1, 5, 10, 20, 50 and 100 µg/g) were performed on the same day using the GC-MS system. To evaluate reproducibility, six injections of 50 µg/g terpene standard were conducted on different days (in the period 01.10.2023-01.02.2024) using the same GC-MS system, with a fresh standard prepared each time. The peak areas obtained for each injection were recorded, and ratios of terpene to internal standard area ratios were calculated. The RSD was calculated for both repeatability and reproducibility results.

Measurement 'trueness' reflects the degree of agreement between the mean of parallel measurements obtained from a method and a reference value. Trueness is typically evaluated quantitatively using parameters such as 'bias' or 'recovery rate'. [40]

A practical determination of bias involved comparing the mean results obtained from the GC-MS method with a suitable reference value. In this study, recovery experiments were conducted using spiked samples of hemp biomass organic solvent extracts. First, hemp biomass extract was prepared and analyzed for terpene concentration. Then, terpene standard solutions were spiked into the extracts to achieve concentrations of 5 µg/g, 10 µg/g, and 20 µg/g of spiked terpenes. Subsequently, these spiked samples were analyzed using the optimized GC-MS method. Bias was calculated using Eq. 9 and Eq 10.

$$R'(\%) = \frac{\bar{x}' - \bar{x}}{x_{spike}} \times 100\% \quad \text{(Eq. 9)}$$

$$bias(\%) = 100 - R(\%)$$ (Eq. 10)

$\bar{x}'$ – the mean value of the spiked sample (µg/g);
$\bar{x}$ – the mean value of the non-spiked sample (µg/g);
x_{spike} – the added (spiked) concentration (µg/g).

2.2.3 Isolation of terpenes via solvent extraction

To assess the abundance of terpenes in hemp biomass and compare these results with the composition of hemp essential oil, terpenes were extracted using an organic solvent. These extracts were further used for the GC-MS method recovery experiments. To investigate the extraction of terpenes from hemp biomass, two commonly used solvents were selected based on the previously reported data [36]: ethyl acetate and methanol. Hexane and chloroform were avoided due to their undesirability in the context of green chemistry [41]. Using mixtures of solvents was not recommended due to the need for further separation for recovery purposes.

To extract terpenes, 0.40 ± 0.01 g of hemp biomass (dried at 30°C for 24 h) was weighted into a 15 mL centrifuge tube and 10 mL of extraction solvent was added. The sample tube was sealed and placed into an ultrasound bath at room temperature for 60 min. Following the ultrasound assisted extraction, the sample was centrifuged at 8000 rpm for 5 min. The supernatant was transferred into the amber glass vials, and the IS in the final concentration of 100 µg/g was added. Finally, the sample was injected into the GC-MS system.

2.2.4 Isolation of terpenes from recovered ethanol

During the process of ethanol removal from the CBD extract, a certain amount of terpenes are separated together with the ethanol as well as remaining in crude extract. The distillation of recovered ethanol could be used to isolate terpenes. Distillation is the process of separating the components of a liquid mixture through selective evaporation and condensation. The basis of separation is the difference in the volatilities of the ethanol and terpenes. The boiling points and vapor pressures of terpenes studied, as well as ethanol, are presented in **Table 1**.

Table 1. Boiling temperatures and vapor pressures of analyzed terpenes, compared to ethanol; the values are given for atmospheric pressure and 20° C.

Compound	Category	Boiling point, °C	Vapor pressure, mmHg	Reference
Ethanol	organic solvent	78	44.00	[42]
α-pinene	monoterpene	155	3.57	[27]
Camphene	monoterpene	159	3.00	[43]
(-)-β-pinene	monoterpene	166	2.18	[27]
Myrcene	monoterpene	168	1.69	[27]
3-carene	monoterpene	170	3.72	[44]
α-terpinene	monoterpene	174	1.67	[45]

4-isopropyltoluene	monoterpene	177	1.46	[46]
***D*-limonene**	monoterpene	176	1.13	[27]
***β*-ocimene**	monoterpene	175	1.60	[47]
***γ*-terpinene**	monoterpene	182	1.09	[48]
***α*-terpinolene**	monoterpene	185	7.99×10^{-1}	[27]
Linalool	monoterpenoid	198	1.15×10^{-1}	[27]
(-)-isopulegol	monoterpenoid	217	2.00×10^{-2}	[49]
Geraniol	monoterpenoid	230	3.00×10^{-2}	[50]
(-)-*trans*-caryophyllene	sesquiterpene	263	2.12×10^{-2}	[27]
***α*-humulene**	sesquiterpene	276	1.00×10^{-2}	[27]
Nerolidol	sesquiterpenoid	276	6.21×10^{-4}	[51]
(-)-guaiol	sesquiterpenoid	290	4.98×10^{-5}	[27]
(-)-*α*-bisabolol	sesquiterpenoid	314	2.24×10^{-5}	[27]

Ethanol evaporation (distillation) was conducted using vacuum evaporator Laborota 4000 (Heidolph), equipped with vacuum pump EV-5 (Labfirst Scientific Instruments). The process was carried out under reduced pressure (35 mbar) at different temperatures (10°C, 15°C, and 20°C). Terpene concentrations were monitored throughout the distillation process. The distillation process was halted at different volume ratios between the distillation flask (residue – R) and receiving flask (distillate – D) (**Table 2**). The terpene content in bulk ethanol containers, as well as in residue and distillate fractions was analyzed using the developed GC-MS procedure.

Table 2. Ethanol distillation experiments residue and distillate ratios; R – residue, D – distillate.

Exp. Nr	Sample ID	Residue, mL	Sample ID	Distillate, mL	Residue/distillate ratio
1	**R1**	250	**D1**	50	5/1
2	**R2**	200	**D2**	100	1/1
3	**R3**	150	**D3**	150	2/1
4	**R4**	50	**D4**	250	1/5
5	**R5**	25	**D5**	275	1/11

The quantitative distribution of each terpene between residue and distillate was evaluated according to the Eq. 11 and Eq. 12, and results were presented in %:

$$m_{init}[mg] = C[mg/L] \times V[L] \quad \text{(Eq. 11)}$$

$$m[\%] = \frac{m_x[mg]}{m_{init}[mg]} \times 100\% \quad \text{(Eq. 12)}$$

m_{init} – amount of terpene in the volume of ethanol used for experiment (V=300mL), [mg].
m_x – the amount of terpene in fraction (residue or distillate), [mg].
m – the relative amount of terpene in fraction, [%].

2.2.5 Separation of terpenes from crude extract during decarboxylation process

During the ethanol removal process from the CBD extract, a portion of the terpenes is carried away with the ethanol, while some remain in the crude extract. Prior to distilling the CBD crude extract, it is decarboxylated through heating under reduced pressure. The heating process causes volatile terpenes to evaporate and be lost. The objective of the experiment was to assess the terpene loss during decarboxylation. To achieve this, a vacuum oven was connected to a cold trap filled with dry ice, allowing volatile terpenes to condense and be collected (**Figure 9, A**).

3 kg of air-dried hemp biomass were extracted using 30 liters of cold ethanol (T≤-40°C). The obtained extract was concentrated through ethanol evaporation. Subsequently, the extract was winterized for 24 hours at -60°C, followed by filtration, and the remaining ethanol was evaporated. Crude extracts from 20 extractions were mixed and decarboxylated. Decarboxylated extract was analyzed by GC-MS procedure.

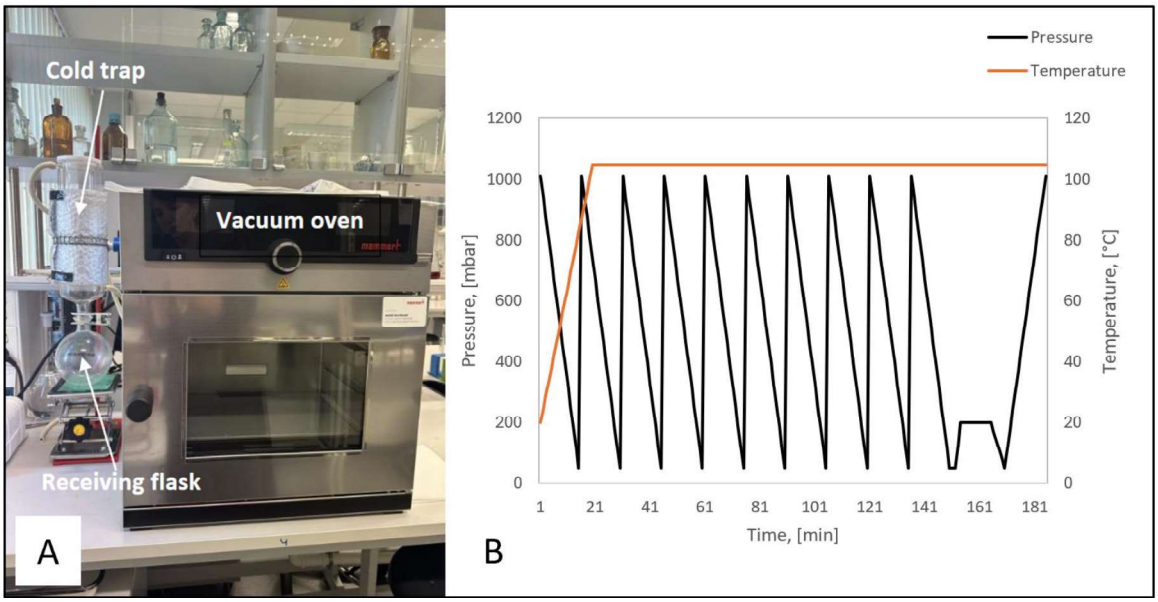

Figure 9. Decarboxylation and condensation equipment **(A)**, temperature and pressure program **(B)**.

A vacuum oven operates by removing air and creating a vacuum environment within the chamber. Once the desired vacuum level is reached, the vacuum pump stops removing air, and the oven maintains the vacuum by sealing the chamber and preventing air from entering. A cyclic ramping pressure program was employed to facilitate the release of vapors from the oven (**Figure 9, B**), allowing them to condense in the cold trap and subsequently be collected in the receiving flask. For decarboxylation crude extract was held for 3h in vacuum oven. The temperature program ramped from 20°C to 105°C in 20 minutes and was held constant at 105°C for 2 hours 45 minutes. Pressure ramped from atmospheric pressure to 50 mbar in 15 minutes and repeated for 8 cycles; then it was held constant at 50 mbar for 2 minutes, ramped from 50 mbar to 200 mbar in 2 minutes, was held

constant for 10 minutes at 200 mbar, ramped back to 50 mbar in 5 minutes, and finally ramped to atmospheric pressure in 15 minutes.

2.2.6 Isolation of terpenes from raw plant material by steam distillation

Steam distillation is a separation process which consists of distilling water together with other volatile and non-volatile components. The water is heated up in the spherical vessel separately and the steam from the boiling water goes into the reactor where it reacts with the masses of trees and plants and further carries the vapor of the volatiles to a condenser, where both are cooled and returned to the liquid or solid state, while the non-volatile residues remain behind in the reactor.

1L of water was added to a 2L flask for steam generation. Boiling aids (cork pieces) were added to the flask to reduce bumping during boiling. A strainer was installed between the steam generation flask and the extraction flask with biomass (**Figure 10**). To avoid evaporation of terpenes, freshly -60°C freezed biomass (approx. 300 g) was used for experiments. The biomass was unfreezed, cut in pieces and placed into the extraction flask. Recirculation glassware with a condenser and a burette were installed on top of the extraction flask. The whole system was wrapped with heat isolation material. The distillation was run for 45 minutes from the point when the steam entered the condenser. Collected EOs were drained from the separation funnel, placed into a centrifuge tube and centrifuged at 8000 rpm for 10min. Four experiments were carried out, and EO yields were recorded. Concentrated terpene EO samples, obtained after steam distillation, were diluted in methanol prior to GC-MS analysis. Several dilutions were prepared to ensure that the concentrations of all terpenes fell within their respective calibration ranges.

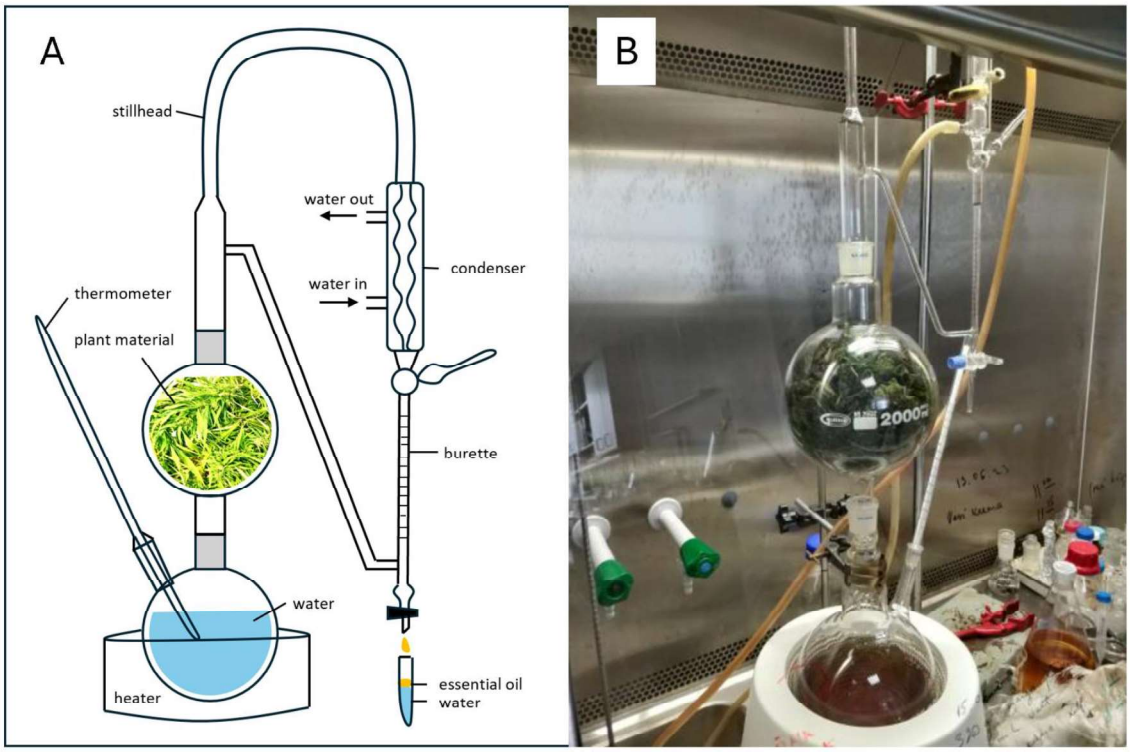

Figure 10. Steam distillation equipment schematic representation **(A)**, and the real setup **(B)**.

2.2.7 Data processing

Microsoft Excel 2021 was employed for all calculations and graph generations. Microsoft PowerPoint 2021, along with ChemDraw 22.2.0, aided in the creation of figures and molecular structure illustrations.

3 Results and Discussion

3.1 Gas chromatography-mass spectrometry method optimization

Following the optimization of GC-MS parameters, efficient peak separation was achieved within a relatively short timeframe, less than 15 minutes. The resulting GC-MS chromatogram is depicted in **Figure 11**.

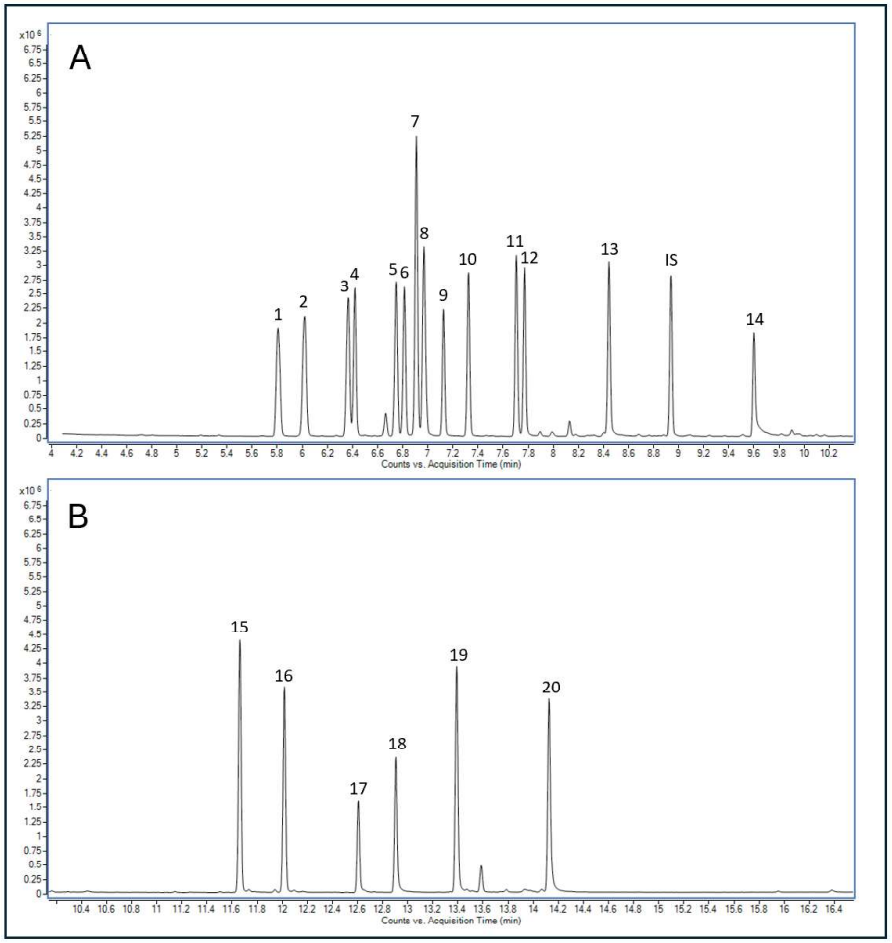

Figure 11. Total ion chromatogram of 100 µg/mL terpene CRM, **(A)** – monoterpene and monoterpenoid region, **(B)** – sesquiterpene and sesquiterpenoid region; **1** – α-pinene; **2** – camphene; **3** – (-)-β-pinene; **4** – myrcene; **5** – 3-carene; **6** – α-terpinene; **7** – 4-isopropyltoluene; **8** – D-limonene; **9** – β-ocimene; **10** – γ-terpinene; **11** – α-terpinolene; **12** – linalool; **13** – (-)-isopulegol; **IS** – internal standard (n-dodecane); **14** – geraniol; **15** – (-)-trans-caryophyllene; **16** – α-humulene; **17-18** – nerolidol (isomers); **19** – (-)-guaiol; **20** – (-)-α-bisabolol.

Peak groups are discerned within two distinct regions: the monoterpene and -terpenoid region (**Figure 11, A**), and the sesquiterpene and -terpenoid region (**Figure 11, B**). The first eluted compounds comprised monoterpenes such as α-pinene (5.81 min), camphene (6.03 min), (-)-β-pinene (6.34 min), myrcene (6.43 min), 3-carene (6.75 min), α-terpinene (6.82 min), 4-isopropyltoluene (6.92 min), D-limonene (6.98 min), β-ocimene (7.13 min), γ-terpinene (7.33 min),

and α-terpinolene (7.71 min), followed by monoterpenoids like linalool (7.78 min), (-)-isopulegol (8.45 min), and geraniol (9.61 min). Subsequently, sesquiterpenes (-)-trans-caryophyllene (11.66 min) and α-humulene (12.02 min) were eluted, followed by sesquiterpenoids nerolidol (presenting as two isomeric peaks at 12.62 and 12.92 min), (-)-guaiol (13.39 min), and (-)-α-bisabolol (14.14 min). The internal standard was detected at 8.94 min. Additionally, several peaks were observed that were not associated with the compounds of interest, likely originating from solvent or impurities within the CRM.

3.2 Gas chromatography-mass spectrometry method validation

Calibration curves linearity

A wide linearity range was achieved for most of the terpenes analyzed (**Table 3**). Among them, 4-isopropyltoluene exhibited the widest linearity range of all terpenes, ranging from 0.7 to 100 µg/g. The linearity range for *α*-pinene, camphene, *(-)-β*-pinene, 3-carene, *D*-limonene, and *γ*-terpinene fell within the range of 1 to 100 µg/g. Myrcene, *α*-terpinene, *β*-ocimene, *α*-terpinolene, linalool, *(-)*-isopulegol, *(-)-trans*-caryophyllene, and *α*-humulene had a linearity range of 2 to 100 µg/g. *(-)*-Guaiol exhibited a linearity range of 5 to 100 µg/g, while geraniol and *(-)-α*-bisabolol fell within the range of 7 to 100 µg/g. The narrowest linearity range was observed for nerolidol, ranging from 10 to 100 µg/g.

The obtained R^2 values for the calibration curves of terpenes ranged between 0.995 and 0.998 (**Table 3**). These R^2 values signify a strong linear relationship between the concentrations of terpenes and their corresponding peak and internal standard area ratios.

The RMSE values obtained for the calibration curves of terpenes exhibited a narrow range, falling between 0.017 and 0.043 (**Table 3**). These low values indicate that the deviations between the observed and predicted values of terpene concentrations are minimal, highlighting the accuracy of the calibration models.

The RMSE% values obtained for the calibration curves of terpenes demonstrated a range spanning from 4.73% to 9.63% (**Table 3**). With RMSE% values in this range, the average percentage deviation between the predicted and observed concentrations of terpenes remains relatively low, indicating a high level of precision in the analytical measurements. Despite minor fluctuations across the different calibration curves, the consistency in RMSE% values suggest a consistent performance of the analytical method in quantifying terpenes across various concentrations.

The obtained *p*-values for the calibration curves of terpenes consistently fell below the significance threshold of 0.05 (**Table 3**), indicating strong evidence against the null hypothesis of no relationship between the concentration and instrumental response. This signifies that the linear regression models developed for the calibration curves are statistically significant and provide a reliable means of predicting terpene concentrations based on instrumental responses.

Detection and quantification limits

Detection and quantification limits results are presented in **Table 3**. For most terpenes, the calculated IDL fell below 1 µg/g, ranging from 0.3 to 0.9 µg/g. However, certain terpenes (geraniol, α-humulene, nerolidol, *(-)*-guiaol, and *(-)*-α-bisabolol) exhibited higher detection limits, ranging from 1.2 to 4.5 µg/g. Similarly, the IQL values were generally low for most terpenes, spanning from 0.6 to 1.6 µg/g. Geraniol, α-humulene, nerolidol, *(-)*-guiaol, and *(-)*-α-bisabolol resulted in higher IQL values ranging from 2.0 to 6.6 µg/g. The LOD and LOQ values varied from 9 to 112 µg/g, and from 14 to 187 µg/g, respectively, with elevated values observed for geraniol, α-humulene, nerolidol, *(-)*-guiaol, and *(-)*-α-bisabolol. Overall, the achieved detection and quantification limits are satisfactory, enabling the detection and quantification of most terpenes of interest at relatively low concentration levels.

Repeatability and reproducibility

Repeatability experiments revealed consistent and precise results, with the highest of 1.0% for α-pinene at the concentration of 50 µg/g (**Table 3**). This level of repeatability ensures that the measurements are close to each other when repeated under the same conditions. Repeatability experiments were conducted across various concentrations. **Figure 12** illustrates the exponential dependence between the relative repeatability and analyte concentration level. The lower the measured concentration, the higher is the variability of the results observed. According to the guidelines for standard method performance requirements, repeatability under 11% is predicted for concentration of 1 µg/g [52]. Therefore, it is essential to consider the lower repeatability of the signal, when analyzing terpenes close to their IDL/IQL levels. Reproducibility experiments demonstrated RSD values lower than 5.0% for all terpenes at the concentration of 50 µg/g (**Table 3**). The results demonstrate a satisfactory level of precision, ensuring that the method is robust and reliable across the board.

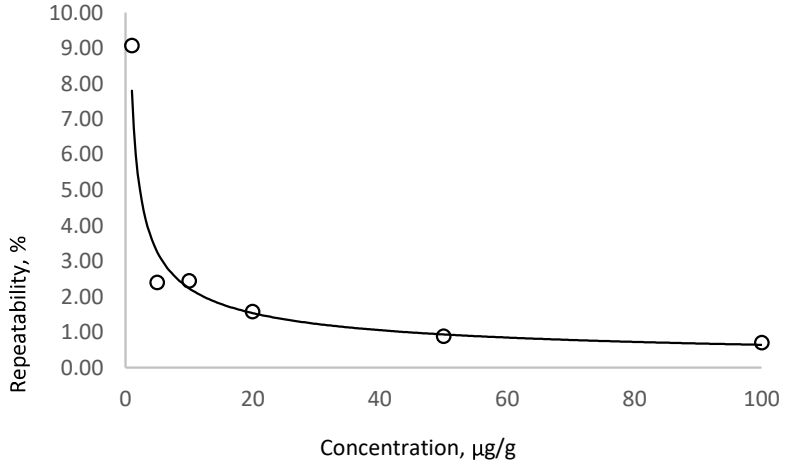

Figure 12. Dependence between relative repeatability and terpene concentration.

Table 3. Calibration linearity parameters.

Terpene	R^2	RMSE	RMSE%	p-value	Linearity range, µg/g	IDL, µg/g	IQL, µg/g	LOD, µg/g	LOQ, µg/g	Repeatability (at 50 µg/g), %	Reproducibility (at 50 µg/g), %
α-pinene	0.997	0.020	7.6	1.54×10^{-11}	1 - 100	0.5	0.8	12	20	1.0	4.1
Camphene	0.997	0.022	7.8	7.57×10^{-13}	1 - 100	0.4	0.8	11	20	0.8	3.4
(-)-β-pinene	0.997	0.022	7.7	6.62×10^{-13}	1 - 100	0.4	0.7	10	19	0.7	2.1
Myrcene	0.997	0.022	8.9	2.28×10^{-12}	2 - 100	0.9	1.6	24	39	0.9	1.3
3-carene	0.997	0.023	8.4	1.44×10^{-12}	1 - 100	0.5	0.9	12	22	0.9	2.4
α-terpinene	0.996	0.023	9.6	4.64×10^{-12}	2 - 100	0.6	1.1	15	27	0.6	1.9
4-isopropyltoluene	0.996	0.044	9.1	2.84×10^{-12}	0.7 - 100	0.3	0.6	9	14	0.8	1.4
***D*-limonene**	0.996	0.033	9.2	3.48×10^{-12}	1 - 100	0.5	0.9	12	22	0.6	3.2
β-ocimene	0.997	0.017	8.8	1.99×10^{-12}	2 - 100	0.5	1.2	12	31	0.7	0.9
γ-terpinene	0.996	0.024	9.3	3.56×10^{-12}	1 - 100	0.6	1.0	15	26	0.6	2.3
α-terpinolene	0.997	0.023	7.9	8.70×10^{-13}	2 - 100	0.6	1.1	16	29	0.8	2.2
Linalool	0.996	0.026	9.2	7.12×10^{-11}	2 - 100	0.8	1.4	20	34	0.5	1.3
(-)-isopulegol	0.997	0.026	7.4	4.13×10^{-10}	2 - 100	0.9	1.4	22	36	0.6	1.8
Geraniol	0.997	0.019	4.7	5.54×10^{-5}	7 - 100	3.7	6.1	92	153	0.8	7.2
(-)-*trans*-caryophyllene	0.997	0.034	8.3	1.09×10^{-12}	2 - 100	0.8	1.4	20	34	0.5	1.1
α-humulene	0.996	0.030	8.9	2.65×10^{-12}	2 - 100	1.2	2.0	30	50	0.8	1.3
Nerolidol	0.997	0.037	7.6	1.33×10^{-8}	10 - 100	4.5	7.5	112	187	0.5	4.4
(-)-guaiol	0.998	0.027	6.7	5.31×10^{-12}	5 - 100	1.8	3.0	46	76	0.7	2.7
(-)-α-bisabolol	0.995	0.043	9.0	4.62×10^{-8}	7 - 100	4.0	6.6	99	165	0.6	3.8

Recovery rate and trueness

The results of the average recovery rate fell between 82.1 – 102.2% (**Table 4**). The range of 80-110% is considered acceptable for the terpene concentration range observed in hemp extract (up to 20 µg/g) [51,52]. This range indicates that the method is capable of accurately recovering the terpenes from the sample without significant bias. The obtained recovery rate for three different spiked concentrations remained relatively consistent, which suggests that the method is not significantly affected by the concentration of the analyte in the sample. The bias (trueness) values, respectively, fell in the range of 1.9 – 17.9%.

Table 4. Recovery rate and trueness of detected terpenes in hemp biomass extract.

Terpene	Recovery rate, (%)			Average recovery rate (%) ± SD	Trueness (bias), %
	Spiked concentration:				
	5 µg/g	10 µg/g	20 µg/g		
α-pinene	82.0	81.7	82.5	82.1 ± 0.4	17.9
Camphene	101.9	94.5	91.3	95.9 ± 5.4	5.4
(-)-β-pinene	88.6	88.3	87.9	88.3 ± 0.4	11.7
Myrcene	87.1	86.7	87.3	87.0 ± 0.3	13.0
3-carene	99.5	95.4	91.8	95.6 ± 3.9	4.4
α-terpinene	98.8	94.7	90.5	94.7 ± 4.2	5.3
4-isopropyltoluene	100.6	95.9	92.3	96.2 ± 4.2	4.1
***D*-limonene**	95.4	94.0	92.7	94.0 ± 1.4	6.0
β-ocimene	94.7	92.2	91.6	92.9 ± 1.7	7.1
γ-terpinene	100.2	94.4	92.1	95.6 ± 4.2	4.6
α-terpinolene	86.8	87.6	86.5	87.0 ± 0.6	13.0
Linalool	108.5	101.0	97.1	102.2 ± 5.8	4.1
(-)-isopulegol	107.5	99.1	95.0	100.5 ± 6.4	4.5
Geraniol	101.8	101.5	102.3	101.9 ± 0.4	1.9
(-)-*trans*-caryophyllene	85.0	84.4	85.4	84.9 ± 0.5	15.1
α-humulene	88.6	88.7	89.0	88.8 ± 0.2	11.2
Nerolidol	102.9	98.2	97.9	99.7 ± 2.8	2.3
(-)-guaiol	97.7	95.1	95.0	95.9 ± 1.6	4.1
(-)-α-bisabolol	99.6	96.8	97.3	97.9 ± 1.5	2.1

3.3 Terpene isolation via solvent extraction

Terpene extraction using MeOH and ethyl acetate as an extraction solvent revealed no significant difference in the amount of extracted terpenes (**Table 5**). With both solvents the total concentration of terpenes in hemp biomass was 0.19g/100g dry weight. Therefore, the methanol was used for further experiments that involved terpene extraction from hemp biomass, due to its lower price and availability.

The most abundant extracted terpenes were α-pinene, *(-)-trans*-caryophyllene, and myrcene (**Figure 13**). Followed by α-humulene, α-terpinolene, *(-)*-β-pinene, nerolidol, β-ocimene, *D*-limonene, and *(-)*-α-bisabolol. The less abundant terpenes were α-terpinene, 3-carene, camphene, γ-terpinene, and 4-isopropyltoluene. Linalool, *(-)*-isopulegol, geraniol, and *(-)*-guaiol concentrations were below the LOD.

Table 5. Results of terpene solvent extraction from hemp biomass.

	Concentration (µg/g, *d/w*) ± SD (n=3)	
Terpene	**MeOH**	**Ethyl acetate**
α-pinene	447.0 ± 26.8	452.4 ± 15.4
Camphene	**13.7 ± 2.0*	**10.4 ± 0.5*
(-)-β-pinene	126.4 ± 8.6	132.0 ± 2.8
Myrcene	229.1 ± 15.5	248.1 ± 4.7
3-carene	**18.7 ± 2.2*	**18.4 ± 0.7*
α-terpinene	**20.9 ± 0.2*	**16.4 ± 1.3*
4-isopropyltoluene	**9.6 ± 0.4*	**9.2 ± 0.6*
***D*-limonene**	71.5 ± 2.1	75.7 ± 1.1
β-ocimene	73.5 ± 4.4	79.7 ± 0.5
γ-terpinene	**13.5 ± 2.1*	**13.1 ± 1.5*
α-terpinolene	143.8 ± 6.2	127.7 ± 5.3
Linalool	***ND*	***ND*
(-)-isopulegol	***ND*	***ND*
Geraniol	***ND*	***ND*
(-)-*trans*-caryophyllene	424.4 ± 29.8	458.1 ± 8.5
α-humulene	147.0 ± 10.2	152.5 ± 2.6
Nerolidol	**81.5 ± 4.2*	**64.9 ± 0.9*
(-)-guaiol	***ND*	***ND*
(-)-α-bisabolol	**63.2 ± 4.3*	**41.8 ± 1.8*

** Values below LOQ; **ND – not detected, values below LOD.*

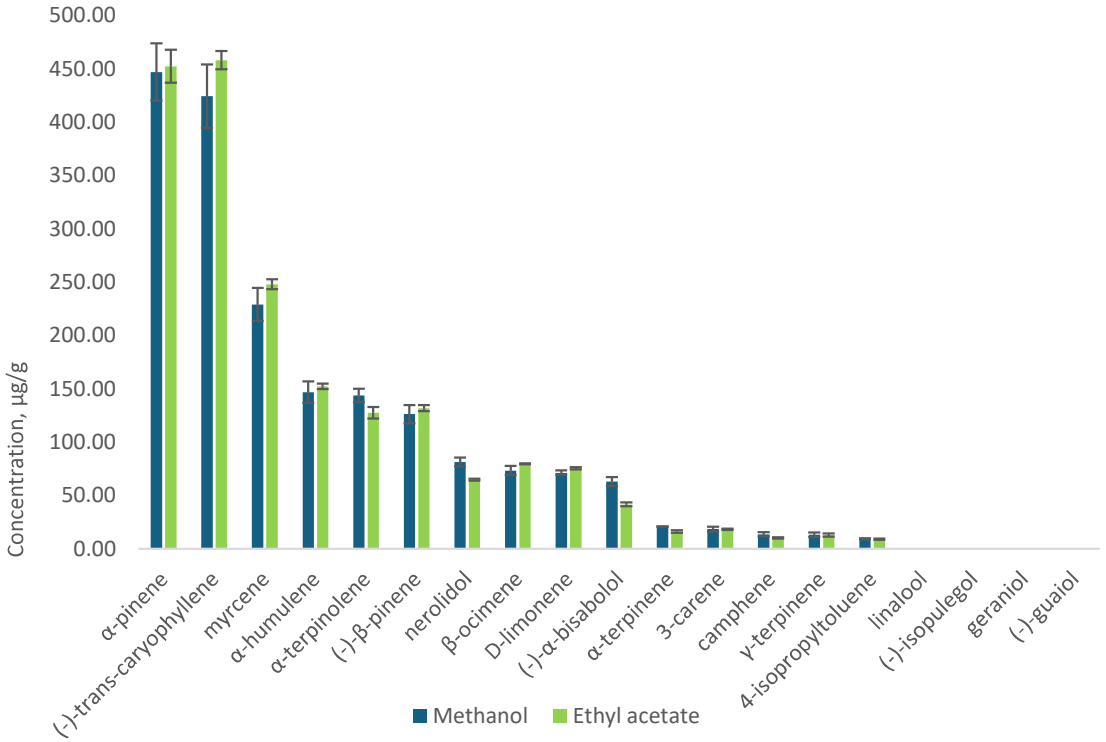

Figure 13. Terpene concentrations obtained from hemp biomass using methanol and ethyl acetate as an extraction solvent.

3.4 Terpene isolation from recovered ethanol

The recovered ethanol distillation experiments aimed to explore the possibility of separating terpenes from ethanol, using the difference in volatility of ethanol and terpenes. The terpene concentrations were assessed using a validated GC-MS method applied to samples obtained from two bulk recovered ethanol containers. Analysis revealed the presence and of six distinct terpenes in both containers: *α*-pinene, *(-)-β*-pinene, myrcene, *D*-limonene (monoterpenes), as well as *(-)-trans*-caryophyllene and *α*-humulene (sesquiterpenes). **Table 6** provides the numerical values (in µg/g) representing the concentrations of these terpenes in each container, as well as the total concentration of terpenes in each container. Subsequent distillation experiments were conducted using ethanol from container 1 under conditions of 10°C and 15°C, while ethanol from container 2 was subjected to distillation at 20°C.

Figure 14 represents the distribution of terpenes between residue flask (orange) and distillation flask (green). Throughout the distillation process, the distillation was stopped at different residue/distillate ratios (x-axis, numbers 1 to 5) (**Table 2**). Therefore, number 1 stands for the beginning of the distillation process, and number 5 for the end. The Y-axis represents the total terpene content measured in ethanol. The results indicate that monoterpenes (such as α-pinene, (-)-β-pinene, myrcene, and D-limonene), which have higher vapor pressure compared to sesquiterpenes, distribute between the residue and distillate during the distillation experiment. This implies that it is not possible to separate ethanol from the monoterpenes fraction and concentrate them. Additionally, no clear dependence between the distribution of terpenes and distillation

temperature was observed. Meanwhile, sesquiterpenes such as (-)-trans-caryophyllene and α-humulene are observed to remain in the residue flask, which could be attributed to their reduced volatility. Consequently, the residue becomes enriched with these terpenes. This phenomenon could further be utilized for the isolation of sesquiterpenes from ethanol recovered after hemp cold extraction.

Table 6. Content of terpenes in recovered ethanol.

Terpene	Type	Concentration, µg/g	
		Bulk container 1	Bulk container 2
α-pinene	bicyclic monoterpene	13.0	9.5
(-)-β-pinene	bicyclic monoterpene	3.2	2.7
myrcene	acyclic monoterpene	2.1	2.2
***D*-limonene**	monocyclic monoterpene	0.6	0.6
(-)-*trans*-caryophyllene	bicyclic sesquiterpene	6.4	5.0
α-humulene	monocyclic sesquiterpene	3.0	1.8
Total terpene concentration		**28.3**	**21.8**

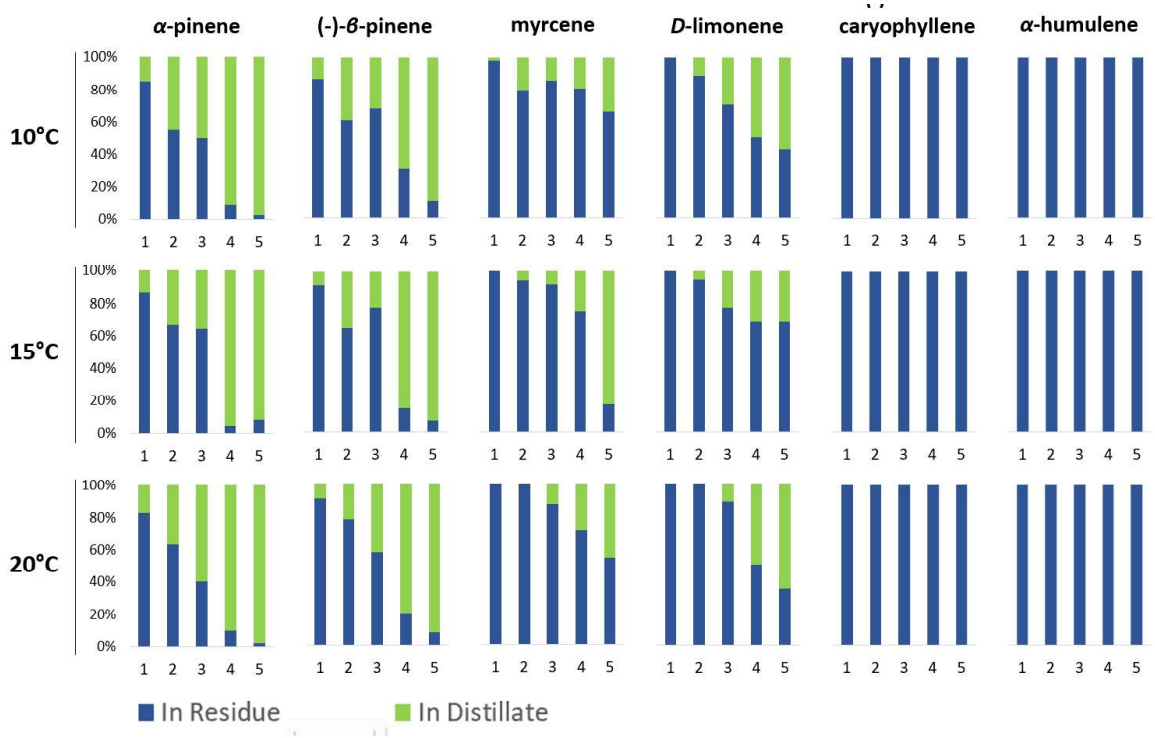

Figure 14. Terpene distribution between the residue (orange) and distillate (green); x-axis: distillation fraction (1-5), y-axis: percent of total terpene content in ethanol.

3.5 Terpene isolation from crude CBD extract during decarboxylation process

The present experiment aimed to evaluate the loss of terpenes during the decarboxylation of the crude hemp extract. Based on the results of the decarboxylation experiment, the amount of collected condensate comprised the mixture of water, ethanol and terpenes. The GC-MS analysis of oily part (0.04g/100g crude extract) revealed the main constituents to be sesquiterpenes (-)-trans-caryophyllene (58.0% by mass) and α-humulene (19.0% by mass). After decarboxylation, crude extract contained in total 72.6% of terpenes initially contained in the hemp biomass. It means that only a small amount of terpenes, 0.2% of initial amount, was vaporized and caught by the cold trap, while the main part remained inside the crude extract.

3.6 Terpene isolation from raw biomass by steam distillation

Steam distillation was assessed as a potential method for terpene isolation from hemp biomass before the CBD extraction process. The obtained terpene content was taken as a total terpene concentration in hemp biomass. The average essential oil yield obtained by steam distillation was 0.37 ± 0.06% *d/w* (**Table 7**). The amount of terpene essential oil (0.37 ± 0.06% d/w) is in coincidence with previously reported data for essential oil content of 0.1 – 0.5% [22,54-56] in hemp biomass. The quantitative GC-MS analysis revealed the predominance of myrcene above all the other chemical constituents (21.08g/100g) (**Figure 15**). Secondly, *β*-ocimene was a significant compound, which was present at a concentration of 19.70g/100g, followed by (-)-*trans*-caryophyllene (16.94g/100g) and *α*-terpinolene (13.10g/100g). Other compounds, detected in lower amounts, were *α*-pinene (6.34g/100g), (-)-*β*-pinene (2.69g/100g), and *D*-limonene (3.56g/100g) among monoterpenes, and *α*-humulene (7.64g/100g) among sesquiterpenes. The components of the obtained hemp essential oil are consistent with those presented in other research papers [22,54-56]. In total, the identified terpenes constitute 94.21g/100g of the essential oil composition (**Table 8**).

Table 7. Steam distillation results.

Exp. nr	Biomass wet weight, g	Moisture content, %	Dry matter, g	Obtained EO weight, g	EO content, %*dw*	EO sample ID
1	296	67.4	96.5	0.36	0.38	TRP1
2	652*	67.4	212.6	0.95	0.45	TRP2
3	337	67.4	109.9	0.39	0.36	TRP3
4	309	63.6	112.5	0.32	0.29	TRP4
5	314	63.6	114.3	0.42	0.37	TRP5

**Two batches pooled*

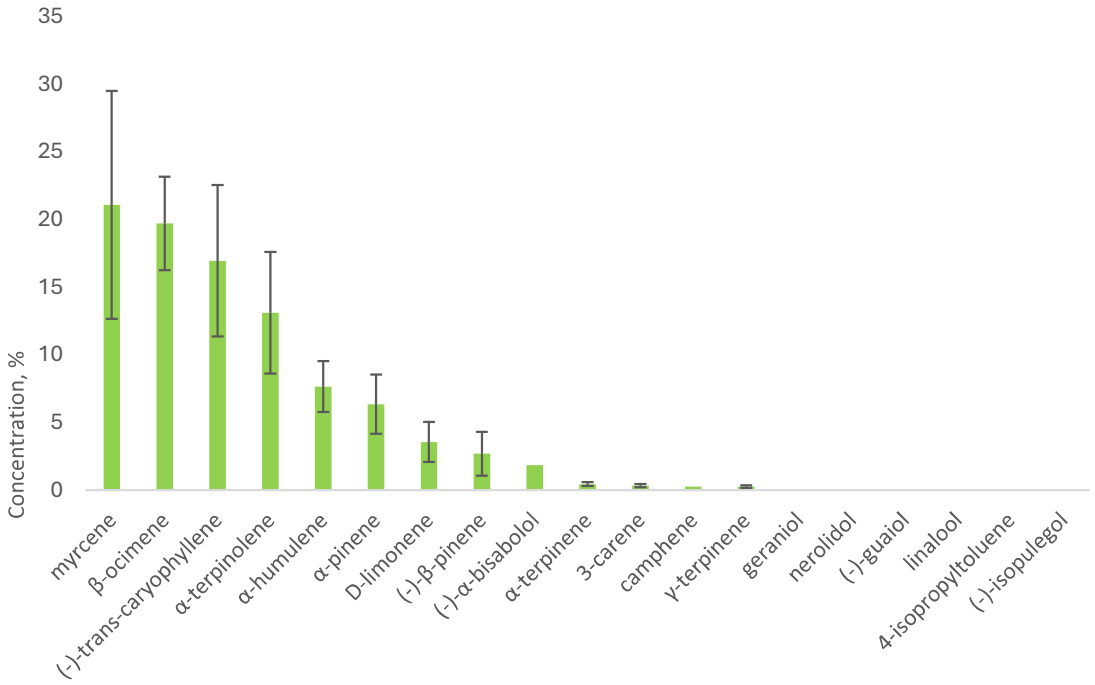

Figure 15. Terpene concentrations in hemp essential oil.

Table 8. Terpene content in essential oil samples.

Compound	Terpene content, %					Average, %
	TRP1	TRP2	TRP3	TRP4	TRP5	± SD (n=5)
α-pinene	4.7	6.7	8.5	3.6	8.2	6.3 ± 2.2
Camphene	<0.2	<0.2	<0.2	<0.2	0.3	0.3
(-)-β-pinene	0.8	2.7	3.4	1.5	5.0	2.7 ± 1.6
Myrcene	22.4	21.6	33.1	9.6	18.7	21.1 ± 8.4
3-carene	0.2	0.3	0.4	0.3	0.5	0.3 ± 0.1
α-terpinene	0.3	0.3	0.6	0.5	0.6	0.4 ± 0.1
4-isopropyltoluene	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2
D-limonene	1.4	4.3	3.0	3.8	5.3	3.6 ± 1.5
β-ocimene	21.0	20.1	24.5	17.7	15.3	19.7 ± 3.5
γ-terpinene	0.2	0.2	0.3	0.3	0.3	0.3 ± 0.1
α-terpinolene	13.5	10.8	20.8	10.4	10.0	13.1 ± 4.5
Linalool	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3
(-)-isopulegol	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2
Geraniol	<3.1	<3.1	<3.1	<3.1	<3.1	<3.1
(-)-*trans*-caryophyllene	11.2	11.3	20.0	24.0	18.1	16.9 ± 5.6
α-humulene	6.8	5.1	7.3	9.5	9.5	7.6 ± 1.9
Nerolidol	<2.7	<2.7	<2.7	<2.7	<2.7	<2.7
(-)-guaiol	<0.4	<0.4	<0.4	<0.4	<0.4	<0.4
(-)-α-bisabolol	<1.7	<1.7	<1.7	1.9	<1.7	1.9

3.7 Mass balance of terpenes

Figure 16 presents the mass balance of the terpenes, within parts studied in this thesis, expressed in grams and percent of the initial amount. The initial amount of terpenes inside 3kg of hemp biomass was determined by steam distillation (yield of essential oil). Received terpene amounts in all processes were recalculated on 3kg of hemp biomass starting material. Process started with 10.5g of terpenes (100%) contained in raw hemp biomass. Ethanol that was recovered after cold extraction retained only 0.6g (5.7%) of terpenes. 0.02g (0.2%) of terpenes were collected in the decarboxylation process, while 7.62g (72.4%) remained in the crude extract. Calculating the total amount of determined terpenes, it can be concluded, that some amount of them was lost before the parts studied in this thesis. Most likely, 2.38g (22.9%) of terpenes were lost in the solvent evaporation process, after the cold extraction.

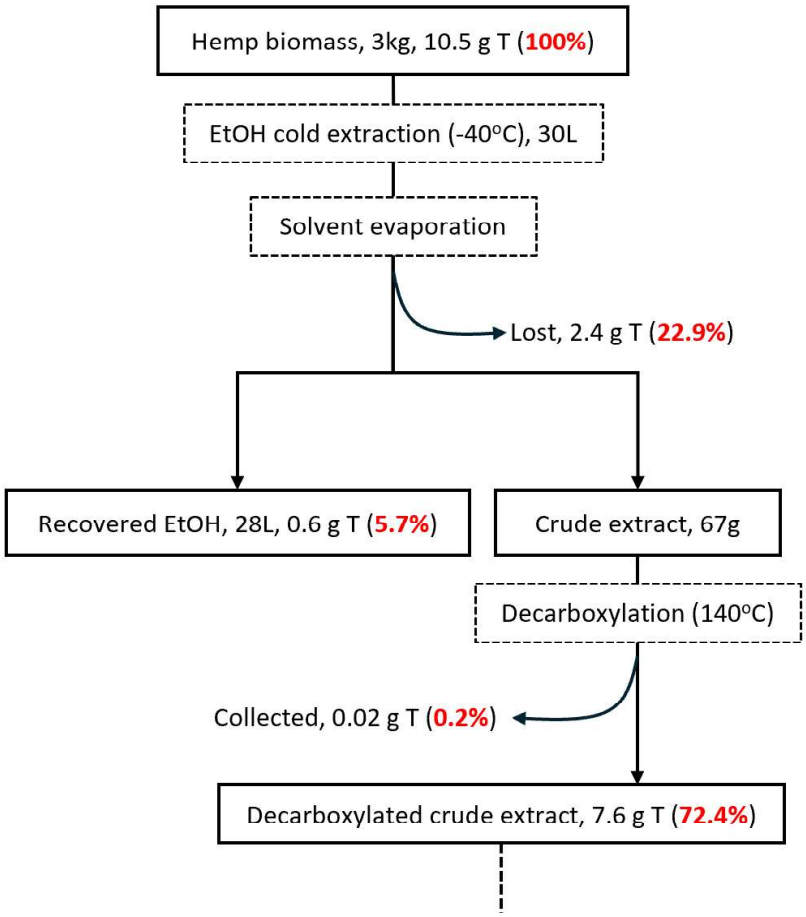

Figure 16. Mass balance of terpenes; T – terpenes.

4 Conclusions

The objective of this thesis was to investigate terpene isolation at three different stages of the CBD isolation process and to identify the most efficient approach for terpene extraction that could be integrated into the CBD isolation process, providing more effective valorization of hemp biomass. Through comparative analysis of the obtained data, the following conclusions were made:

1) A reliable GC-MS method for terpene quantification was optimized and validated for the 19 most abundant terpenes in hemp biomass. The method was successfully applied for the quantitative characterization of raw hemp biomass, essential oils, hemp ethanol extracts, and recovered ethanol.
2) The most effective method for quantitatively isolating terpenes from hemp biomass was found to be steam distillation. The product received is almost a pure mix of terpenes that does not need additional purification and isolation steps. It could be incorporated at the beginning of the CBD isolation process, before cold ethanol extraction. The steam distillation process is widely used in industrial-scale processes and can be used for mass production of hemp terpenes.
3) Results showed that recovered ethanol contained a negligible amount of terpenes (approx. 6% by mass of the total terpene content), and only sesquiterpenes could be isolated by distillation. Monoterpenes, having higher vapor pressure, are distilled together with ethanol, and their pre-concentration was not successful.
4) During the crude extract decarboxylation process, terpenes mainly remain in the extract, and only a negligible part of terpenes volatilizes (approx. 0.2% by mass of the total terpene content). Thus, terpene isolation from recovered ethanol and capturing during decarboxylation are not cost-effective processes to integrate them into the CBD isolation technology.
5) A significant loss of terpenes occurs at the ethanol evaporation stage (approx. 23% by mass of the total terpene content), and a better solution for cold trapping of terpenes should be used.

The majority of terpenes (approx. 72% by mass of the total terpene content) remain in the decarboxylated crude extract, and further developments should be made to capture them during the crude extract distillation process.

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Abstract

Hemp is a highly productive crop with a rich history of cultivation. Hemp consists of various compounds, including fatty acids, phenolic compounds, flavonoids, cannabinoids, and terpenes. Cannabinoids are terpenophenolic compounds, which interact with specific receptors in the human body, causing a variety of physiological and psychoactive effects. Cannabidiol (CBD) is known for its sedative and relaxant effects and has large potential in medical treatment. Tetrahydrocannabinol (THC) possesses psychoactive properties and is subject to strict regulatory constraints across the European Union. Terpenes are organic volatile compounds derived from isoprene units, which contribute to the aroma and therapeutic properties of hemp. Terpenes have various applications in different fields, mostly in food and beverage production, medicine, and cosmetics.

According to regulations set by the European Union, hemp biomass containing less than 0.3% *w/w* THC is permitted for cultivation and is classified as “industrial hemp”. The same limit is set for the derived hemp products, including extracts. The R&D project conducted by the Analytical Chemistry group (Department of Chemistry and Biotechnology, TalTech) focused on isolating CBD from industrial hemp biomass. The aim was to obtain purified CBD products with a THC content below <0.3% *w/w*. The CBD isolation process involved several stages, during which terpenes were lost or discarded due to their volatile properties. Therefore, another important aim of the project was to combine the CBD isolation process with terpene extraction, thereby enhancing the overall efficiency of hemp biomass valorization. To achieve this goal, the methods for terpene extraction were investigated at different stages of the CBD isolation process. Terpene essential oil was isolated from raw hemp biomass via steam distillation. Additionally, terpenes were isolated via distillation of recovered ethanol used in cold extraction of hemp biomass; and during the decarboxylation of the crude extract using the cold trap. For the quantification of terpenes in received samples, a gas chromatography-mass spectrometry (GC-MS) method was developed and validated for 19 terpenes most abundant in hemp biomass.

The main objective of this thesis has been achieved by identifying the most effective method for extracting terpenes from hemp biomass, which can be integrated into CBD isolation technology, thus increasing the hemp biomass valorization efficiency. The results revealed steam distillation as the optimal approach for terpene extraction from hemp biomass, yielding a nearly pure terpene mixture. Steam distillation is suitable for incorporation before the CBD isolation process. Recovered ethanol retained only a small amount of terpenes, and distillation was only able to isolate sesquiterpenes. During the decarboxylation process, the main amount of terpenes remained within the crude extract. Consequently, attempts to isolate terpenes from recovered ethanol or capture them during decarboxylation were deemed impractical. Further investigations are required to develop methods for capturing the terpenes lost in the subsequent processes following decarboxylation of the crude extract.

Annotatsioon

Kanep (ing. k. *hemp*) on pikka kultiveerimis ajalooga ja kõrge produktiivsusega põllukultuur. Kanep koosneb erinevatest ühenditest, sh rasvhapetest, fenoolühenditest, flavonoididest, kannabinoididest ja terpeenidest. Kannabinoidid on terpenofenoolsed ühendid, mis interakteeruvad spetsiifiliste retseptoritega inimese organismis, põhjustades erinevaid füsioloogilisi ja psühhoaktiivseid toimeid. Kannabidiool (CBD) on tuntud oma rahustava ja lõõgastava toime poolest ning sellel on suur potentsiaal meditsiinilises ravis. Tetrahüdrokannabinoolil (THC) on psühhoaktiivne mõju ja selle suhtes kehtivad kogu Euroopa Liidus ranged regulatiivsed piirangud. Terpeenid on isopreeni ühikutest koosnevad orgaanilised lenduvad ühendid, mis panustavad kanepi lõhna ja terapeutilistesse omadustesse. Terpeenidel on mitmesuguseid rakendusi mitmetes valdkondades, peamiselt toiduainete ja jookide tootmises, meditsiinis ja kosmeetikatoodetes.

Vastavalt Euroopa Liidu kehtestatud määrusele, on kanepi kasvatamine lubatud, kui see sisaldab alla 0,3% THC-d (massi järgi). Selline kanep on klassifitseeritud kui „tööstuslik kanep“. Sama piirmäär on kehtestatud kanepist saadud toodete suhtes, sh ekstraktide. Analüütilise keemia rühma (TalTech Keemia ja biotehnoloogia instituut) läbiviidud teadus- ja arendusprojekt keskendus CBD eraldamisele tööstuslikust kanepist. Eesmärk oli saada puhastatud CBD tooteid, mille THC sisaldus on alla 0.3%. CBD isoleerimisprotsess hõlmas mitut etappi, mille käigus terpeenid nende lenduvate omaduste tõttu kadusid. Seetõttu oli projekti teiseks oluliseks eesmärgiks integreerida terpeenide ekstraheerimist CBD isoleerimisprotsessi, tõstes kanepi biomassi väärindamise efektiivsust. Eesmärgi saavutamiseks uuriti terpeenide ekstraheerimise meetodeid CBD isoleerimisprotsessi erinevatel etappidel. Terpeenide eeterlik õli eraldati toorkanepist aurdestillatsiooni meetodil. Lisaks eraldati terpeenid destilleerimise teel kanepi biomassi külmekstraheerimisel kasutatud taastatud etanoolist, ja paralleelselt kanepi toorekstrakti dekabroksüleerimisprotsessiga kasutades külmlõksu. Terpeenide kvantifitseerimiseks saadud proovidest optimeeriti ja valideeriti gaaskromatograafia mass-spektromeetria (GC-MS) meetod 19 kanepi biomassis kõige levinuma terpeeni analüüsiks.

Antud lõputöö põhieesmärk oli saavutatud, tuvastades kõige tõhusama meetodi kanepi biomassist terpeenide estraheerimiseks, mida saab integreerida CBD isoleerimistehnoloogiasse ning seekaudu tõsta kanepi biomassi väärindamise efektiivsust. Tulemused näitasid, et aurdestillatsioon on optimaalne lähenemisviis kanepi biomassist terpeenide ekstraheerimiseks, saavutades peaaegu puhta terpeenide segu. Aurdestillatsiooni meetod sobib biomassi töötlemiseks enne CBD isoleerimisprotsessi. Taastatud etanool sisaldas madala koguse terpeene ja destilleerimisel õnnestus isoleerida ainult seskviterpeene. Dekarboksüleerimisprotsessi käigus jäi suurem osa terpeenidest toorekstrakti. Sellest tulenevalt, peeti katseid eraldada terpeene taastatud etanoolist ja paralleelselt dekarboksüleerimisprotsessiga ebapraktiliseks. Edasised uuringud on vajalikud, et töötada välja meetodid toorekstrakti dekarboksüleerimisele järgnevates protsessides terpeenide isoleerimiseks.

Appendix

Appendix 1. Certified reference material (LGC Standards Ltd (USA)) compound information table.

Compound Name	Concentration (ug/mL)	Expanded uncertainty U (ug/mL)	CAS number	Purity (%)
α-pinene	2482	120	80-56-8	98.2
Camphene	2459	120	79-92-5	99.8
(-)-β-pinene	2489	120	18172-67-3	99.8
myrcene	2457	120	123-35-3	93.6
3-carene	2451	120	13466-78-9	92.9
α-terpinene	2539	130	99-86-5	90.7
4-isopropyltoluene	2547	130	99-87-6	99.9
D-limonene	2503	130	5989-27-5	99.4
β-ocimene	2503	130	13877-91-3	94.5
γ-terpinene	2467	120	99-85-4	97.7
α-terpinolene	2467	120	568-62-9	97.2
linalool	2481	120	78-70-6	98.9
(-)-isopulegol	2460	120	89-79-2	100
geraniol	2513	130	106-24-1	99.2
(-)-*trans*-caryophyllene	2486	120	87-44-5	98.8
α-humulene	2461	160	6753-98-6	95
nerolidol (cis & trans mix)	2512	130	7212-44-4	99.3
(-)-guaiol	2460	120	489-86-1	100
(-)-α-bisabolol	2480	120	23089-26-1	95

Annex
to Rector's directive No 1-8/17 of 7 April 2020

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