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Maximizing industrial hemp waste value by recovery and enzymatic functionalization of cannabidiol

Tesi di Laurea Magistrale in
Food Engineering - Ingegneria chimica

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Abstract: This thesis project will investigate the extraction and functionalization of cannabidiol, which is the most abundant and pharmaceutically interesting component in the organic waste related to the production of *C. sativa* essential oil by steam distillation. Among the cannabinoids present in the hemp plant, cannabidiol already possesses biological benefits and operates in synergy with some medical drugs. Basing on literature, also the product of cannabidiol epoxidation reaction could enhance or develop new attractive bio-activities. The objective of this work was to validate a suitable cannabidiol extraction method from spent hemp distillation waste and to optimize the oxidation reaction with an enzymatic pathway, involving the lipase Novozyme 435 as a biocatalyst. The end purpose was to purify, isolate, and completely characterize the products of the oxidation process with particular attention to sustainable experimental practices while maximizing the reaction yield for the more interesting product. In order to achieve these results and simultaneously save time and resources, was applied the statistical tool known as Design of Experiments (DoE), which is an alternative method with respect to the traditional monovariate techniques used in organic chemistry, to better understand the reaction trend. In accordance with the circular economy principles, the implementation of the recovery of the most valuable compound from agricultural waste and the later biocatalytic valorization is essential for the sustainable development of the chemical industry and resulted in a high-added-value product with superior characteristics and potential high medical importance.

Key-words: cannabidiol, biocatalysis, Novozyme435, agri-food waste, circular economy, DoE

1. Introduction

1.1. Circular Economy and Green Chemistry

In the last few decades, the increase in world population and its needs highlighted the criticalities of the existing production system, therefore has begun the rethinking of the production system in order to integrate the current technologies and direct them toward greater long-term sustainability. The industrial system used to be based on a linear flow, which starts with the extraction of raw materials and their utilization until the final disposal. Unfortunately, this way of acting has rapidly become unsuitable for the modern scenario where there

are higher demands and limited resources. In 1976, the architect Walter Stahel and the economist Genevieve Reday submitted a report [1] to the European Commission outlining the vision of a circular economy and its impact on job creation, resource savings and waste reduction. Their study claimed that an economy focused on servicing recycled and remanufactured products was less energy-intensive and required less manpower than the creation of new goods [2]. It was the first time that the idea of a circular system was proposed as the solution to the manufacturing problems and during the 1990s publications related to sustainable systems for goods and services multiplied. Since them, many organizations emerged developing the principles of the circular economy to reduce waste by closing the natural cycle of the products, but at the same time, the fundamentals were also proposed to be a solution for harmonizing goals of economic growth and environmental protection [3]. Among these, nowadays a reference for the green transition is the Ellen MacArthur Foundation, which was formed in 2010 to inspire a generation to rethink, redesign and build a positive future [4]. It defines the three pillars of circular economy as:

1. Eliminate waste and pollution;
2. Circulate products and materials (at their highest value);
3. Regenerate nature.

In order to satisfy these pillars, the entire manufacturing system needs to be partially redesigned and integrated with the engineering improvements proposed by researchers. Since circular economy principles could be extended to many industrial fields, an ideal final scenario in which each actor observes these sustainable fundamentals is called "integrated system". It consists in an industrial reality in which individual components are connected to achieve in synergy the final common goal for the benefit of the whole system [5]. Process integration, oriented toward the minimization of resource and energy consumption, has ancient roots but emerged as a powerful engineering-oriented methodology at the end of the last century and has led to numerous modern design methods; today it still represents one of the objectives for the development of future industrial applications because it can provide the necessary connecting concepts for enabling the industrial implementation of circular economy [5].

A visual representation of the possible actions implementable to achieve these objectives has been presented by Ellen MacArthur Foundation in a butterfly diagram (Figure1). This representation provides numerous strategies to avoid extracting new raw materials and prevent their final disposal. Indeed, is possible to reach the regeneration of nature following two different flow cycles: the biological cycle, which regards degradable products such as food and wood, and the technical cycle concerning anything that does not degrade like metals.

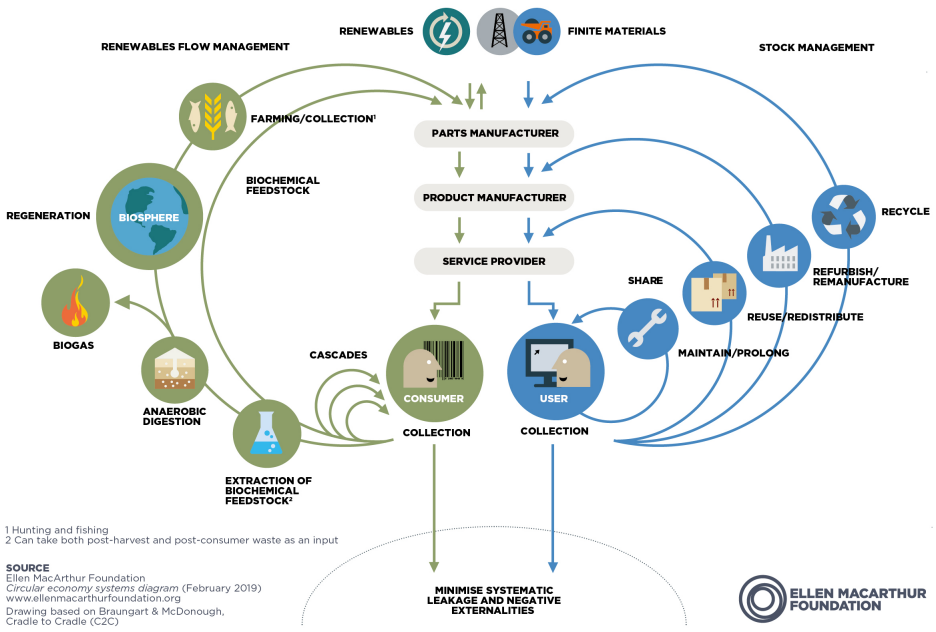

Figure 1: Ellen MacArthur "butterfly" iconography for circular economy

The focus of this thesis is agricultural waste as starting material for the experiments, so the application of circular economy principles is mainly adherent to the biological life cycle. Given that bio-products are the main users of environmental resources in industrialized and developing countries, it is really important to define an efficient strategy in order to align with the modern circular economy ideas [6]. From these needs, the "cradle to cradle" approach was developed aiming to close the loop (pillars 2 and 3 of circular economy), so that the efficiency of resources in use is enhanced.

To measure and determine the whole impact of a product during its entire life cycle in a practical and objective manner, the life cycle assessment (LCA) can be used, which is an innovative technique thanks to its intrinsically complete effect evaluation. It is a quantitative tool able to support preliminary process evaluation, eventually allowing to redesign of the critical process steps when the expected results are not satisfactory, but it is also applicable as a final control test for sustainability. Through software dedicated, the impact analysis can be evaluated on different indicators such as water resource depletion, human toxicity, mineral and fossil resource depletion or climate change, which consider emission of greenhouse gases in kg of CO_2 equivalent. In this way, it is possible to acquire a global vision of the problems derived from the processes. There are many instruments to establish the sustainability of the operations, and hence, when these instruments are correctly used and implemented, the result is a business model designed for sustainable development in harmony with nature [3].
One of the first feasible actions to consider in order to apply the principles of circularity for a biological product is the extraction of biochemical feedstocks and their valorization, therefore it is industrially appealing. Based on these principles, biorefinery plants have been developed. The term "biorefinery" is defined by The United States Department of Energy, in its Energy, Environmental, and Economics Handbook (U.S. Department of Energy 1997) as an overall concept of a processing plant where biomass feedstocks are converted and extracted into a spectrum of valuable products, on the model of the petrochemical refinery. In fact, as already mentioned, sustainable technologies like biorefineries are the modern adaptation of old concepts to new objectives. In the first step of biological refineries, the precursor-containing biomass is separated using physical methods. The main products and by-products are then subjected to microbiological or chemical transformations. The final products obtained can be further converted or enter into a conventional refinery. Therefore, the concept of biorefinery becomes relevant on two levels: on the one hand, because of the biological genesis of the corresponding raw material and on the other hand since biological methods increased the added value of outputs [7]. For example, biofuels are profitable biorefinery outcomes because they are obtained from biomasses using chemo-enzymatic reactions. In order to not compete with food production, the substrate for biofuels production has been shifted from wheat, soy and corn to plants that grow on non-cultivable soils such as forest residues, or agri-food waste achieving a win-win situation in accordance with first and second circular economy principles. In general, in bio-industries these sustainable ideas have driven industrial production toward a valorization of waste in order to exploit more than once the chemical complexity of the biomass before incineration or disposal. It happened mainly because when wastes become resources, it is advantageous in both economical and environmental terms. Food waste and crop residues represent some valuable potential bio-resources because, after the fractionation of raw materials, the single components extracted can be also functionalized giving a wide range of output products. As consequence, this operation implies a shift from a low value of the starting material to high added value for the final products in according with circular economy principles [8].
Another possibility to take into account during operations is the exploitation of unavoidable side streams for sustainable value creation. Side streams and waste streams are reinvented as an opportunity to be seized: instead of being only undesirable and unavoidable flows that lead to losses, they might serve as new inputs for value generation. To recycle and up-cycle the sides streams, new and innovative technologies need to be developed. Therefore, the valorization of waste requires a higher level of collaboration between industries and academic researchers from various fields. As already mentioned about the integrated systems, collaboration is a core aspect in achieving results of circularity and sustainability.

The circular economy rules can be observed and applied in the field of chemical production; in fact, sustainability in the chemical industry is encouraged by Green Chemistry, which is defined as the “design of chemical products and processes to reduce or eliminate the use and generation of hazardous substances” [9]. The principles derived are founded on these basic concepts and ideas, moreover it can be deduced that green chemistry aims to eliminate the risk at the source rather than solve it afterward. The twelve green chemistry fundamental principles introduced by Anastas P. et. al. should be followed to drive the circularity transition [9].
Principles of green chemistry can be listed as follows:

1. Waste prevention instead of remediation;
2. Atom efficiency;
3. Less hazardous materials;
4. Safer products by design;
5. Innocuous solvents and auxiliaries;
6. Energy efficient by design;
7. Preferably renewable raw materials;
8. Shorter synthesis (avoid deviation);
9. Catalytic rather than stoichiometric reagents;
10. Design products for degradation;
11. Analytical methodologies for pollution prevention;
12. Inherently safer processes.

Examining the twelve fundamentals carefully, it is possible to notice that circular economy and green chemistry

share several common ideas, in particular for what concerns waste prevention and elimination. It happens because both views can be referred to as a triple-bottom-line sustainable approach trying therefore to achieve a trade-off between economic, environmental and social sustainability. But as a difference, these work philosophies are used in a slightly different way: circular economy is widely applied in industrial business models as a priority, because is more oriented to economical profit in business reality; instead, the twelve green chemistry principles are more commonly seen as a final certification tool to prove that an already implemented method is also green. These different applications of similar sustainable codes are due to the numerous issues that green chemistry application implies during the development of a new process.
Despite this, green chemistry principles have opened up a new world of possibilities for the creation of new technologies to enhance chemical processes. The implementation of these twelve guidelines guarantees increased process efficiency while lowering the environmental burden during chemical synthesis. In this situation, biocatalysis is gaining ground to be one of the most environmentally friendly technologies and is predicted to revolutionize chemical manufacturing through higher synthetic efficiency in a low-emission, sustainable and more innovative economy [10].

1.2. Biocatalysis

Based on the principles of green chemistry and sustainable development, biocatalysis is intrinsically a green and sustainable technology [11], which adheres in particular to the circularity principles number 2, 9 and 12 listed before. These aspects contribute to the recent increasing use of biocatalysis in industrial synthetic chemistry and its application as an attractive technology in green manufacturing. In the modern era, one of the major causes of waste generation in the production of fine chemicals and pharmaceuticals is the use of stoichiometric inorganic reagents in organic synthesis. The resulting situation is additional waste generation during reaction or in downstream processes, due to the presence of side products formed in multi-step synthesis. The solution to these problems could be the substitution of these wasteful chemical methodologies with some more atom- and step-economical catalytic alternatives, such as the use of various catalysis strategies [11]. Among all the possible catalysis approaches, biocatalysis emerges and is particularly valuable because of the use of enzymes and microorganisms as catalysts in a bioprocess has many attractive features in the sustainable industrial context. Enzymes are proteins with high molecular weight and have the function of biological catalysts. It means that enzymes are able to decrease the activation energy of a specific reaction by changing the synthetic pathway into a faster one, without consuming itself. The catalytically active part of the enzyme, so where reactions take place, is called the active site. Thanks to the complicated protein folds, this region is typically highly specific to its exact substrate. However, enzyme selectivity can be categorized on the basis of what is recognized: a single substrate, a group of similar substrates, or a characteristic functional group. Due to the different specificities presented, the old lock-and-key model for substrate-active site interaction has been updated with the modern induced-fit model, which considers reciprocal adaptation and flexibility for bond actors. Although various substrate specificities, the active site stereoselectivity and stereospecificity imply that only one specific reaction is catalyzed and the product has one specific enantiomer configuration.
From an industrial point of view, enzymes can revolutionize the design of a chemical synthesis. Some of the main positive consequences of their application are here listed [11]:

- reducing the use of solvents, because water is used as an environmentally compatible solvent;
- eco-efficient catalytic cascade processes are easier to implement with respect to other catalytic solutions because biocatalytic reactions are conducted under similar conditions;
- enzymes operate mainly at mild temperature and atmospheric pressure pressure ranges;
- high chemo-, regio-, and stereo-selectivities generally do not require functional group activation, protection, and deprotection steps present in traditional organic syntheses; these simplifications make the process more environmentally and economically attractive than the conventional route;
- reducing of downstream process and increasing purity of final products are a direct result of the high selectivity and mild reaction conditions.

In addition, operating in mild conditions of temperature, pressure and pH contributes to reducing energy consumption. This is not always true and further case-by-case evaluation should be conducted to assert it, but in many industrial applications, the energy saving of agitated biocatalytic pathway is significant comparing the same process conducted with high-temperature chemical synthesis. It happens mainly because, unlike the chemical business, the biotechnology sector is primarily oriented to the pharmaceutical industry and so does not have huge volume sales [12]. Biocatalysts are also appreciated for their ability to ensure the high quality of final products by maintaining bioactive functional attributes. For these reasons, it finds profitable implementation in the food and beverage industry: enzymes can modify the rheological, nutritional and physicochemical characteristics of food without side effects on human health. An example of how biocatalysis has brought clear improvements over the chemical route is margarine production. Interesterification is a crucial step to arrive at the desired margarine features: performing chemical reactions requires more processing steps, uses higher

temperature and so more energy; while the enzymatic process is simpler as takes place in one single step in a fixed bed reactor at mild condition [13]. Furthermore, the risk of contamination in the final product is completely avoided due to the absence of chemical reagents, whose residues represent a risk to human health. Thus, changes in production paradigms have not only brought industrial benefits, but in addition were reflected on the final consumer, which has a higher quality product also suitable for vegan dietary or as a butter alternative for allergic needs for what specifically concerns the margarine.
For all these reasons, biocatalysis is aligned and in complete accordance with circular economy fundamentals so much that it is possible to make a parallelism between the two ideas. In Table 1 [11], a parallel comparison between green chemistry principles and the corresponding features of biocatalysis is reported.

Green Chemistry Principles	Biocatalysis
Waste prevention	Enables more sustainable routes with significantly reduced waste
Atom economy	Enables more atom and step economic routes
Less hazardous syntheses	Generally low toxicity
Design for safer products	Not relevant
Safer solvents and auxiliaries	Usually performed in water or Class 3 solvents
Energy efficient	Mild conditions are conducive with energy efficiency
Renewable feedstocks	Enzymes are renewable
Reduce derivatization	Biocatalysis obviates the need for protection/deprotection
Catalysis	Enzymes are catalysts
Design for degradation	Not really relevant but enzymes themselves are biodegradable
Real-time analysis for pollution prevention	Can be applicable to biocatalytic processes
Inherently safer processes	Performed under mild and safe conditions

Table 1: Parallelism between green chemistry and biocatalysis principles

In essence, biocatalysis integrates very well with at leat ten out of twelve green chemistry principles validating itself as a truly green and sustainable technology [11]. Consequently, supported by the trend of the last decades, biocatalysis has established itself as a vital technology for meeting the growing demand for green and sustainable chemical processes.
On the other hand, enzyme catalysis has some disadvantages too. Considering that they are biomolecules that work under optimal cellular conditions, biocatalysts might have significant thermal instability, could suffer from activity inhibition and be prone to protein degradation. The loss of enzymes in the purification phases results in a considerable rise in running costs, because enzymes also play a relevant role in cost consideration in biological processes when they are difficult to recycle. [14]. In fact, since both biocatalysts and products are in the dissolved state, the separation process of a diluted aqueous solution becomes harder, consequently more costly [15].
A solution to some of these drawbacks is enzyme immobilization. This technique refers to the use of physical support onto which the biomolecules is covalently or non-covalently linked so that they can be used repeatedly and continuously, reducing the necessity to frequently produce or purchase new enzyme and avoiding dead time typical of discontinuous processes. From a different point of view, immobilization is also the key to improving the performance of biocatalysts in industrial processes [16], because it improves enzyme properties by increasing the stabilization of the 3D structure and reducing inhibitions. Thanks to the industrial interest in immobilization, nowadays it is one of the most effective and well-studied systems to carry out production on an industrial scale with higher productivity [15]. In fact, immobilization enables enzyme reuse: it decreases industrial investments and increases revenue by making the entire process economically feasible. Are present even some disadvantages in biocatalyst immobilization, such as the high cost of this new technique, the risk of reduction of enzyme activity due to the high density of immobilization material and the need for a specific design for their use [14]. But despite this, immobilization remains an important and widely used technique.
For this thesis, the enzyme used is a commercially available immobilized lipase B enzyme from the fungus *Candida antartica* called Novozyme 435 (N435). It is immobilized on a resin, Lewatit VP OC 1600, which is a macroporous support formed by poly(methyl methacrylate) crosslinked with divinylbenzene [17]. Even if it is one of the most widely used commercial enzymes in both academia and industry due to its excellent activity and versatility in performing trans-esterification, kinetic resolution of chiral esters and epoxidation reactions, N435 still presents some problems. Despite the immobilization disadvantages reported in the literature, during the experiments conducted for this thesis N435 turned out to be a good choice thanks to the simple work-up and for the chemical and physical robustness of its immobilized form, but has also shown some critical aspects that will be better investigated in the experimental discussion.

1.3. Essential oil production

In this thesis project, circular economy, sustainability and green chemistry principles find their application in the valorization of waste of essential oil production.
Essential oils can be generally defined by the Oxford Languages Dictionary as "a natural oil typically obtained by distillation and having the characteristic odor of the plant or other source from which it is extracted". They are a complex mixture of volatile compounds, which are generated and present in all parts of aromatic and medicinal plants, looking like a colorless or yellowish liquid with a strong distinctive odor at room temperature. Containing an average of over 300 different volatile compounds [18], among these terpenes are the principal components. They are formed from combinations of several 5-carbon-base (C5) units called isoprene; for instance, monoterpenes are made from the coupling of two isoprene units (C10), while diterpenes are formed from the assembly of four isoprene units (C20). Monoterpenes are the most representative molecules constituting 90% of essential oils and possess a great variety of structures such as terpenoids, which are terpenes that contains oxygen-based functional groups. Terpenes can be functionalized by many functional groups, but they remain highly volatile molecules intrinsically hydrophobic. Due to these features, they are easily extracted by steam distillation and then separated from the condensed aqueous phase. In addition, is possible to mention alcohols, ethers or oxides, amines, amides, aldehydes, ketones, esters, phenols and heterocycles contained in essential oils. Among them, especially aldehydes and ketones could give a wide variety of aromatic notes, since most are partially in a vapor state at atmospheric pressure and room temperature due to their low molecular weight, so the volatility characteristic is enhanced. Essential oil composition is highly variable both in qualitative and quantitative terms and is possible to distinguish two different category factors affecting that: intrinsic factors, connected to the plant, soil, and climate type, but also maturity of the plant and even harvest time, and extrinsic factors related to extraction method and process steps.
In recent years, the attraction to aromatic and medicinal plants is continuously growing because the final consumers are more informed about wellness and health benefits derived from the pharmaceutical applications of these natural products. Indeed, the chemical composition of essential oil gives them numerous biological activities interesting for cosmetic and food industries as well as in the area of human health. The most common and well-demonstrated biological activities of essential oil are listed:

- Antimicrobial activity: due to their hydrophobic property, essential oils are enabled to partially enter into bacterial membrane, separate the phospholipid structure and disrupt it by making it more permeable. It results fatal to cells that can not withstand the leakage of ions and other cellular molecules [19];
- Antioxidant activity: since most essential oils are rich of compounds containing conjugated double bonds, they often possess anti-oxidative properties. Moreover, the ability of essential oils to eliminate free radicals may be crucial in the prevention of several diseases, including brain dysfunction, cancer, heart disease, and immune system decline [20];
- Anti-inflammatory activity: antioxidant activities combined with their signaling interaction involving cytokines and regulatory transcription factors of the human immune system provide a new strategy for inflammatory disease treatment;
- Cancer chemoprotective activity: due to their complex formulation, when essential oils are administered, their metabolic transformation in the liver produces some pharmacologically effective metabolites called "prodrugs" even if the original substance was an inactive drug; this process has good effects on prevention of cancer, as a correct dietary education can do[21].

Essential oils are soluble in organic solvents and insoluble in water. This is a crucial aspect of the most common extraction process, which is based on the physical and chemical properties of the final product. Avoiding the use of hazardous or toxic solvent, is possible to obtain essential oil by steam distillation when the desired final product profile allows it. This method is most frequently applied o fresh plant materials such as flowers, leaves, and stems, but some chemical components contained in plants are too delicate and are easily degraded by the high heat used in the distillation process.
For what concern the essential oil production from Cannabis sativa by steam distillation, fresh plants are collected in a truck container immediately after the harvesting. Water is heated in a boiler and the resulting steam is fed into the truck container from a sparger in the bottom, coming into contact with the raw plant material and vaporizing the volatile compounds. When steam flow is recondensed to liquid in a separate apparatus with respect to the boiler, natural separation of essential oil easily occurs on the liquid surface at atmospheric pressure. It is an efficient procedure, with low operating cost and a high-value product obtained making the entire process economically sustainable. The substances extracted from cannabis are particularly interesting since they are bioactive valuable compounds [22].
Other methods extract essential oils from plant raw materials and it is possible to group them into classical or advanced methods . The investment in these techniques and the innovations of the last decades, were oriented to increase extraction efficiency and final product quality while decreasing time and energy consumption [23].
Hydro-distillation is the oldest extraction method. It is based on azeotropic distillation: occurring in the

alembic, water boils with raw material immersed in it, then gases are condensed in a condenser and lastly, water is separated from essential oil by decantation. It is a simple and selective method that is still used because has the advantage to allow the recycling of condensates, but the long extraction time needed could lead to chemical alteration of heat-sensitive molecules as well as overheating, causing bad energy management. Steam distillation exploits the same principle of hydrodistillation, but steam is generated outside the distillation alembic. Injection of water steam from bottom to top through the plant matter is the core extraction step for essential oils and volatile compounds. The advantages of this method are the same of hydro-distillation, but this technique eliminates several artifacts. However, steam distillation requires a longer extraction time with respect to hydro-distillation [22]. A more delicate treatment of plant matter to avoid chemical alteration of the natural products is organic solvent extraction. In this case, the raw plant material is macerated into an organic solvent and then the crude extract is concentrated by solvent evaporation by lowering pressure. Even if the temperature remains low, some contaminants can be extracted by the solvent limiting the human consumption for nutritional o pharmaceutical applications. An extraction method that combines the steam distillation process and the organic solvent technique, utilizing low boiling temperature liquids, could guarantee satisfying results avoiding drawbacks, as happened for the extraction of *Cuminum cyminum* essential oil [24].

The residue of the essential oils extraction process is a solid bio-waste to be treated. In order to gain some advantages it can be utilized for energy generation in incinerators, which are also called waste-to-energy plants to highlight their purpose, or can be partially composted and utilized as fertilizers. Unfortunately, there are several factors that do not always allow composting; for instance, a low nitrogen content prevents fast decomposition of the organic residue but has a detrimental effect on soil microorganisms due to antimicrobial properties [25]. Currently, even landfilling is a waste treatment option, but it does not contribute to any waste valorization and is just a disposal cost. To maintain a circular economy approach through the entire industrial process, organic wastes should be used as input in a valorization operation to increase the final product value and gain profit from a potentially valuable biological source. An economical and environmentally sustainable management of the residual wet plant matter is feasible in biorefineries, where the agricultural waste is fractionated in its single components. Among the compounds present in plants, lignin is a complex organic polymer that could be utilized as a fermentation substrate due to its ability to be digested by fungi and bacteria [26].

1.4. *Cannabis sativa*, cannabinoids and CBD

For essential oils production and related wastes, the natural raw material considered in this work is Cannabis sativa hemp. Among the residual valuable organic molecules present in the waste of essential oil production from *Cannabis s.*, cannabinoids are certainly one of the most relevant molecules because of their bio-active properties. With further extraction, cannabinoids can be recovered and used as a springboard for further cascade valorizing procedures. In relation to green chemistry and biocatalysis, an enzymatical valorization of bio-waste is the best option to functionalize the initial molecule and obtain a more complex product with added value. *Cannabis sativa* is probably one of the world's most versatile crops. It has been employed for millennia primarily as a source of a stem fiber derived from the plant which is termed "hemp", then for the resinous psychoactive intoxicant contained in the plant popularly known as "marijuana" and destined to drug preparation [27] as well as for illegal recreational consumption.
The hemp plant *Cannabis sativa* is a crop cultivated in temperate climate areas, exists in mainly three varieties (*sativa, indica,* and *rudelaris*) [28] and is appreciated for its numerous useful properties. For centuries, hemp has been employed as raw material for papers, ropes, clothes and building materials, but also as the source of fibers and proteins for food purposes as well as a medicine in the oriental tradition. The plant seeds can be used for producing flour or seed oil for human consumption due to their nutritional properties, while the essential oil extracted from hemp has cosmetic applications. Currently, interest in hemp is growing because it is seen as a low-cost, sustainable and versatile material suitable as a fossil-based materials replacement, and acoustic and thermal insulator. Its cultivation has proved environmental benefits and, due to its high harvest yield and sustainability, is an attractive biomass for chemical and energetic valorization [29]. Nowadays, hemp is cultivated in at least 47 countries and global leaders in hemp production are in particular Canada, China, Chile and France [30]. The global hemp industry has the potential to expand significantly since consumer demand for eco-friendly and organic products rises. Especially in the food sector, in the last decades hemp-based food market has steadily grown in relation to the increasing cases of nutritional disorder, vegan population and nutritional consciousness among consumers [31]. As shown in Figure 2, the global hemp-based food market share is predicted to increase from 3.92 billion USD in 2020 to almost double within 2027.

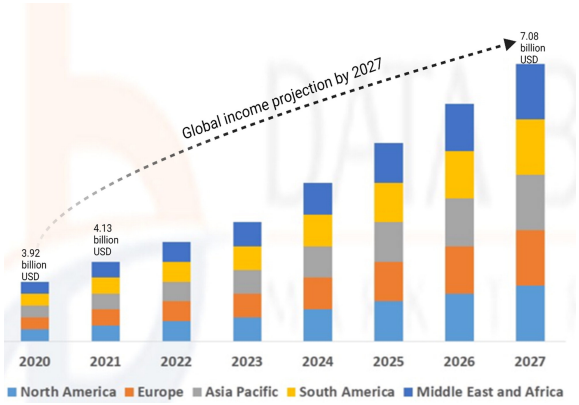

Figure 2: Hemp-based global food market projection [31]

The hemp-based food market most commonly is composed of food products based mainly on seed or seed oil, which are added to foods as a supplement. For instance, hemp seeds are considered an excellent source of ω-fatty acids and protein to add to other meals such as breakfast cereal and smoothies, while hemp seed oils have the same nutritional properties and are used in salad dressing or baked goods. Other hemp-based food products are energy bars, veggie burgers, granola, hemp milk, hemp flour, hemp tea and other infused drinks. As result, the increasing trend of the hemp market and the numerous realizable hemp valorizations are able to attract new stakeholders and investments.

Focusing on the chemical composition, hemp is made by a complex mixture of constituents: so far >560 components have been reported, including at least 120 plant-synthesized phytocannabinoids in Cannabis sativa extract [32]. Their content changes varying the herbal extracts or botanical factors such as genetics, the growing conditions, and the harvesting methods of the plant. Each part of the cannabis plant contains valuable compounds; in Figure 3 is reported an overview of the beneficial substances found in the cannabis plant along with where they are located in the various plant components [22].

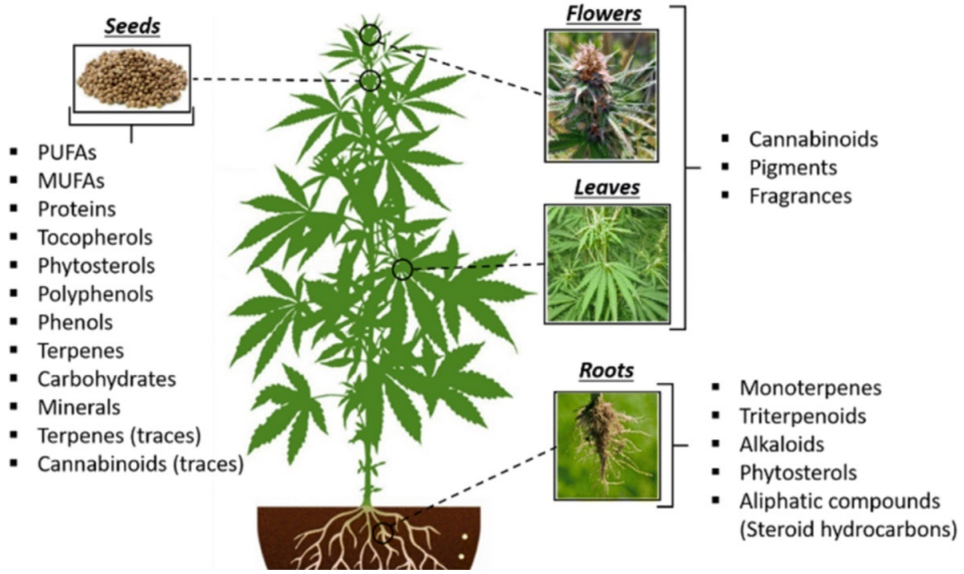

Figure 3: Most important compounds contained in the cannabis plant (PUFA: polyunsaturated fatty acids; MUFA: monosaturated fatty acid)

As particularly interesting chemical classes of secondary plant metabolites, have to be mentioned terpenes, cannabinoids and phenolic compounds such as flavonoids, stilbenes and lignans. Terpenes have shown in numerous studies anti-inflammatory, analgesic, anxiolytic, anti-bacterial, and anti-fungal activities; while phenolic compounds have numerous beneficial effects in the prevention of various diseases such as atherosclerosis and coronary heart disease thanks to their antioxidant activity [33]. It is also relevant to mention research about the "entourage effect", which results in synergy between cannabis metabolites: some terpenes and flavonoids are presumed to interact with cannabinoids and reciprocally modify their properties achieving a new wide range of pharmacological effects [33]. In the group of cannabinoids, the most present in the carboxylic

acids form are cannabigerolic acid (CBGA), cannabidiolic acid (CBDA), cannabichromene acid (CBCA) and Δ^9-tetrahydrocannabinolic acid (Δ^9-THCA); followed by their decarboxylated forms cannabigerol (CBG), cannabidiol (CBD), cannabichromene (CBC), Δ^9-tetrahydrocannabinol (Δ^9-THC) and the Δ^9-THC-derivative cannabinol (CBN) [34] [35], which are shown in Figure 4. Among cannabinoids, the cannabidiol (CBD or 2-[(6R)-6-isopropenyl-3-methyl-2-cyclohexen-1-yl]-5-pentyl-1,3-benzene-diol) is the most well-known compound since has numerous pharmacological properties such as analgesic, anti-inflammatory, and anti-cancer activities. Since the compounds present in the cannabis plant are of great interest to certain industries fields, such as pharmaceuticals and food sectors, the implementation of strategies to recover these molecules seems relevant. For instance, the valorization of wastes derived from cannabis essential oil production could find interesting applications because these valuable compounds are still present in the final organic residue, in particular CBD. Recovering these molecules is relevant for implementing a sustainable business model, which starts from industrial waste and explores their possible derivatives achieving new products with higher value.
For this research, CBD is the focus and the most important as well as abundant chemical compound in raw material, so is interesting to analyze its origin and structure. The precursor of CBD is CBGA, whose biosynthetic pathway starts with geranyl diphosphate (Geranyl-PP) and olivetolic acid. Olivetolate geranyl transferase (GOT) afford cannabigerolic acid (CBGA) as result of a condensation reaction, as shown in Figure 4.

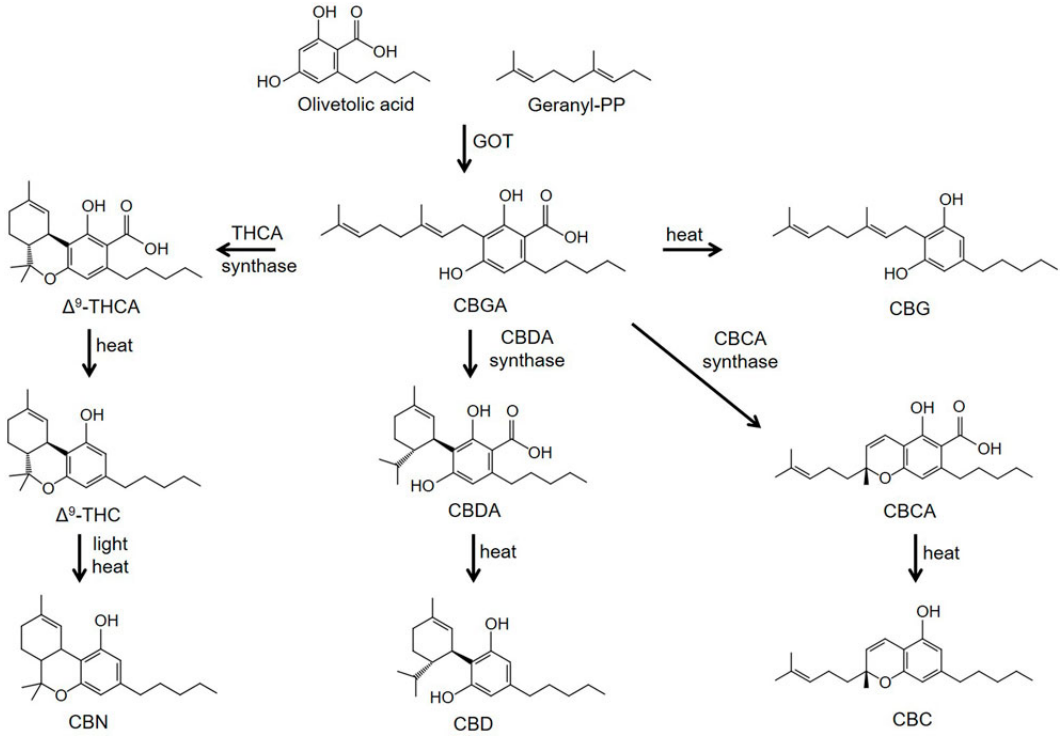

Figure 4: Biosynthesis of the main cannabinoids present in Cannabis species

CBGA is then transformed by giving different acids such as cannabidiolic acid (CBDA) by CBDA synthase, and also Δ^9-tetrahydrocannabinolic acid (Δ^9-THCA) by THCA synthase if the relative synthase enzymes are present in the plant species. [36]. Commonly, these acids content is very low because of spontaneous non-enzymatic decarboxylation, which converts precursor acid in case of light exposure, heating or aging, resulting in CBD and Δ^9-THC.
Focusing on CBD structure, which was first isolated from Cannabis by Adams *et al.* in 1940 [37], it can be described as a resorcinol ring linked to a molecule of limonene to the carbon atom between the two hydroxyl groups, and to a pentyl chain on the opposite side. Although the CBD and THC molecules represented in Figure 4 seem different only in a ring closure, it is also important to consider the spacial representation of these molecules: THC is essentially planar, while CBD has a bent conformation in which aromatic and terpene ring are rotated at almost right angles to each other. The difference in CBD and THC structure accounts for the very different biological effects. The first clear difference in bioactivity is that THC has a psychotropic effect, while CBD does not have psychotropic effects [38]. THC acts as a partial agonist ligand on both cannabinoids CB1 and CB2 receptors, which are in the central nervous system and in the immune system. Its pharmacological action is carried out through the binding of CB1 receptor: inhibition of the uptake of various neurotransmitters and the stimulation of areas that inhibit the ascending pathways of pain are two direct effects of THC. On the other hand, negative effects of THC highlighted in the literature are euphoria, alteration of spatiotemporal perceptions, confusional state, drowsiness, cardiovascular effects such as tachycardia and changes in blood

pressure, neuronal degeneration, and increased risk of cancer and fibrosis in patients with chronic hepatitis [39]. Instead, CBD has a low affinity for CB1 and CB2 receptors, but acting as an indirect antagonist it potentiates THC by increasing the density of CB1 and extending its effects.
For what concerns CBD, it is currently used in consumer products and for its potential therapeutic effects; it is known to have anti-inflammatory, pain-relieving, antioxidant, immunomodulatory, antidepressant, antiepileptic, anticonvulsant and antineoplastic effects, with only partial data on the effects on oral neoplasms [40]. Recent researches focus on its pharmaceutical investigation because it is not psychoactive and many possible derivatives have been synthetized and tested for bioactivity. Since CBD has benefits on human health and in general cannabinoids find pharmaceutical applications, the interest in studying its derivatives has been considerably growing and an increased or new therapeutic activity of CBD derivatives is expected.

1.5. Design of Experiments (DoE)

Traditionally, planning an experiment provides a piece of information about the studied process and based on the results further tests are decided. The classical approach is carried out by studying one variable at a time, but the fundamental assumption of this technique is that the parameters involved do not influence each other [41]. In reality, in many industrial and academic cases, this statement is known to be not correct. In fact, following that strategy the information obtained is valid only in these experimental conditions, no interaction between factors is considered, the results could be misunderstood and the chance of finding the global optimum is very low [41]. For these reasons, it was decided in this thesis project to further investigate the reaction behavior with an innovative multivariate statistical approach known as Design of Experiments (DoE). The DoE is a statistical method that was invented in 1920 by the English statistician Ronald A. Fisher in order to maximize the information derived from experimental data. The Japanese engineer Genichi Taguchi is instead responsible for the global diffusion of this approach [42] because he focused on the industrial application to reduce cost while increasing revenue. Regarding the field of chemistry, Rolf Carlson was the first to apply and spread the use of DoE as a mathematical and computational tool for solving organic synthesis problems [43]. Indeed, in synthetic chemistry it is crucial understand interferences deduced from experimental observations in order to develop new methods (or to improve already existing methods) able to reach the desired target molecule. Therefore, especially in that scenario, the experiments performed need to be designed in the best possible manner. As a statistical analysis-based approach to design and set experiments, DoE aims to define the associations between a group of input variables (ex. mixture components) and one or more output variables (e.g. reaction yield). Thanks to its application, some important advantages such as a reduction of experimental trials, more efficient use of resources and increased reliability of the process can be achieved. It also promises to be able to overcome traditional problems derived from a monovarietal strategy obtaining more complete data at a lower expense in terms of money and time, detecting simultaneously the optimum setting of every variable by systematically varying them and using statistical procedures to analyze the results. Nowadays, DoE can be applied to a wide range of industrial fields as a substitute for the screening of variables in an initial phase which aims to identify the key variables, then this statistical instrument can lead to optimizing processes, reducing cost and increasing product quality. For instance, manufacturing systems [44], pharmaceutical formulations and quality control [45], engineering [46], and organic synthesis [43] could apply this powerful tool for achieving the desired outcomes.
In particular for what concern organic chemistry, in literature are reported evidence about how the DoE approach explores more efficiently the reaction space [47] observing at the same time as many dimension as there are variables. Following this, is covered a larger response space volume and can be revealed reciprocal parameters interactions. In this perspective, differently from what happens when one variable per time (OVAT) is changed, not only the best but even the worst experiments are useful to define the operational process space. Once the correct procedure is performed, with no additional experimental expense and thanks to the flexibility of method, many responses may be modeled concurrently such as yield, residual starting material and contaminants produced.
The DoE implementation follows some mandatory steps:

1. Problem definition and identification of variables;
2. Choice of factors and levels;
3. Choice of the experimental plan;
4. Experiments;
5. Result analysis and interpretation.

A factor is defined as an input manipulable variable, while the level defines its value. The number of factors and the levels decided by the experimental designer establishes the type of design, which can be full factorial design or fractional factorial design. In the first case are collected as much information as possible and the entire range of combinations are considered, with higher cost and time to perform the study. Therefore, the DoE output in full factorial configuration completely characterizes and describes a chemical space as a result of several properly-planned experiments. A visual representation of the comparison between the insights obtained

by OVAT method with respect to a full factorial study through DoE is reported in Figure 5.

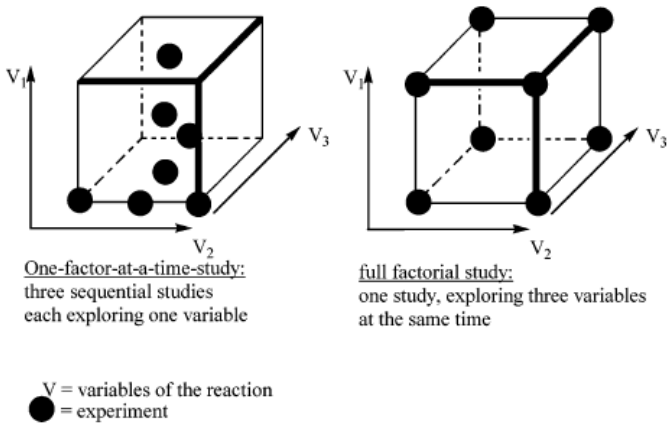

Figure 5: Visual representation of a traditional OVAT study vs. a full factorial DoE study [47]

On the other hand, a fractional factorial design of experiments monitors only a few variable interactions and does not consider several combinations. As result, the data derived are partial, but this model has the advantage of requiring lower cost and time. For that reason, when there are a lot of parameters to consider in a preliminary analysis, a fractional factorial design is preferable since also allows to ignore the degree of interaction over the third order, which is usually negligible.
For the above-mentioned reasons, the DoE is a precious resource when there is the necessity to obtain informations, in a short time, with few resources available and can be applied either to the investigation of a new process or to the optimization of an already-known process.

2. Purpose of the research

The purpose of this thesis project is to optimize the extraction methods on the residue of *Cannabis s.* essential oils production by steam distillation and study the reactivity of the principal component extracted (CBD) in an oxidation reaction using a biocatalyst. The flow diagram in Figure 6 shows the current process and highlights the innovative path developed for the treated product: starting with the agricultural waste of essential oil production, the recovery of the valuable compound is applied as a sustainable operation to extract cannabinoids and mainly cannabidiol, then is performed the enzymatic valorization to synthesize a high-value product.

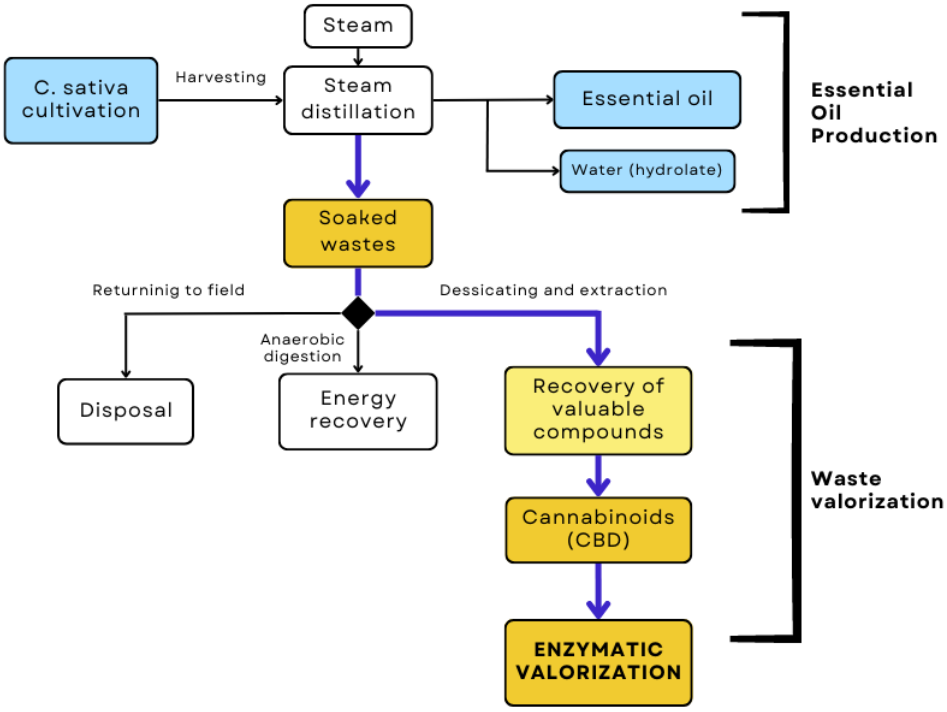

Figure 6: From essential oils process to waste valorization. Black lines=current process, blue lines=this project

Indeed, following the circular economy practices and principles, industrial wastes of plant treatments need to be recovered and valorized instead of being considered as material to be disposed of, with relative costs. Among the numerous compounds not removed by the distillation process and hence recoverable through extraction, cannabinoids are the most relevant components. They are still present in the soaked wastes due to their low solubility in water and their high molecular weight which makes them slightly volatile.
Cannabidiol (CBD) is the most valuable cannabinoid: once it is extracted, purified and isolated, can follow further molecule upgrading to obtain some derivatives with promising biological activities as all cannabinoids have proven to have. Indeed, in literature several interesting studies are already reported about the endocannabinoid system interaction mechanism stimulated by cannabinoids and in particular by CBD [48] While maintaining the CBD scaffold, structural changes of the aromatic ring or terpene group can give rise to new compounds with enhanced therapeutic activity or other beneficial properties. An example of some already studied chemical synthesis starting from CBD to obtain cannabinoids with improved pharmaceutical effects is reported in Figure 7.

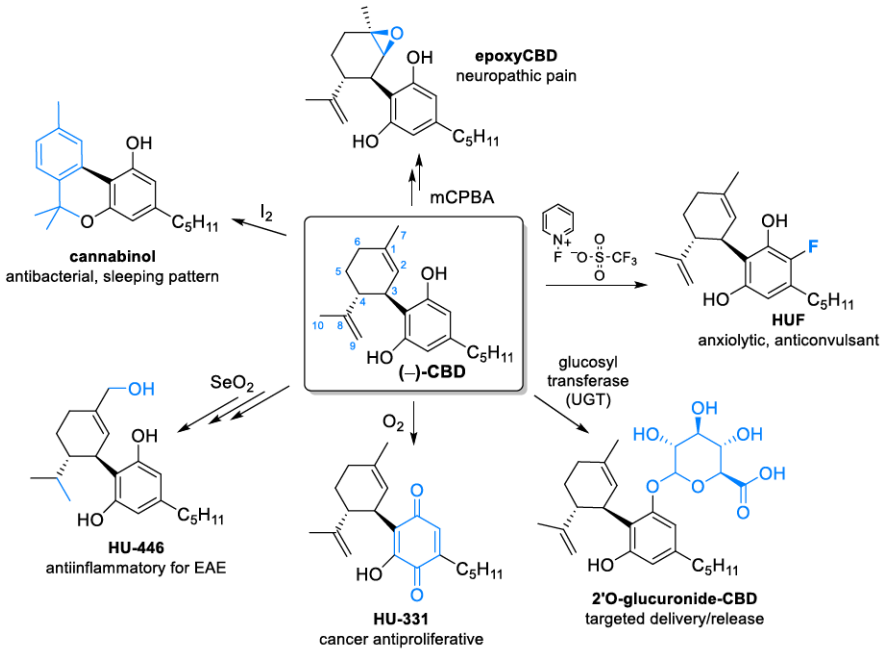

Figure 7: Already studied chemical synthesis from CBD to cannabinoids with therapeutic activity

The modifications of the terpene group can be carried out by selective epoxidation (Epoxy-CBD) [49], by iodine-promoted aromatization (cannabinol) [50], or by hydrogenation and subsequent hydroxylation (HU-466) [51]. On the other hand, structural changes in the benzene ring can be made to obtain fluorine-halogenated CBD derivatives (HUF), quinone derivatives by oxidation (HU-311), and other CBD analogs by biocatalytic monoglycosidation (2'-O-glucuronide) [51].
Among the range of possibilities, epoxy CBD (CBO) captured our attention because of its potent inhibitory activity against Wnt/β-catenin signaling pathway, concerning pain sensation [49] and its potential bioactivity with antitumorigenic properties. In fact, in literature, some documentations are present about the beneficial role of epoxides in preventing cancer due to their anti-inflammatory properties, which are able to inhibit tumoral formation and proliferation [52].
CBO is an attractive molecule not only because have proven to be biologically active, but also because it carries the epoxide functional group that is highly functionalable, as is shown in Figure 8.

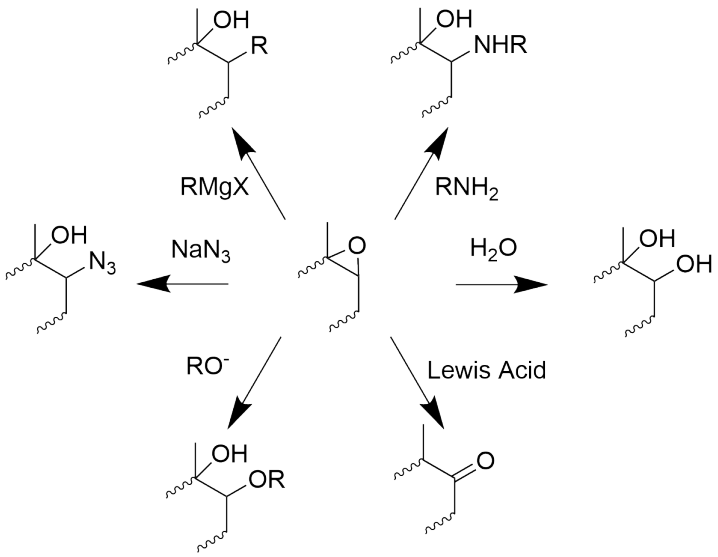

Figure 8: Possible epoxide functionalization with different reagents

In fact, epoxides are a very reactive class of compounds that is often a reaction intermediate in more complex reaction pathways. Therefore, the epoxide isolation becomes relevant also for its probable use as starting reagent from which obtain various final products such as alcohols, diols, ethers and carboxylic acids by ring opening or

to even perform complicated S_N2 mechanism with amines (R-NH2) and Grignard reagents (R'MgX) (Figure 8).
Given that the reagent of the considered epoxidation reaction (CBD) has already proved beneficial bioactivities and in scientific literature cyclohexane epoxides are frequently displayed having a wide range of interesting biological activities [53], these substances are very interesting to synthetic chemists, pharmacologists, and biologists.
For these pharmaceutical and chemical reasons, there is a significant interest in deeply investigating the synthesis of CBO. The chemical synthesis of CBO is already known, but occurs with and not environmentally friendly reagents and solvents, which are necessary for an initial step of hydroxyl group protection and a final deprotection step. In addition, rather extreme experimental conditions are necessary, therefore the process is challenging to industrially scale the process. For these reasons, the objective of this work is the CBD functionalization by enzymatic pathway, adhering to green chemistry and biocatalysis guidelines, to transform the wastes related to the industrial production of *Cannabis s.* essential oil into a high-value product. In particular, the CBD epoxidation reaction using a commercial lipase (Novozyme 435) is the focus of this work and the final aim is to purify and isolate CBO maximizing the reaction yield. Medical studies on the possible applications of the final isolated product and its effects on human health are left as tasks for future researchers.

3. Result and discussion

This project thesis was the continuation of previous research on the valorization of industrial hemp waste related to essential oil production. The aim was to extract the most valuable compound (CBD) contained in the industrial residue and functionalize it involving a lipase enzyme (N435). To introduce the preliminary information known at the beginning of this work, chapter 3.1 resumes the results obtained in earlier hemp extractions, then are summarized the literature information concerning the possible CBD functionalization, focusing on the CBD epoxidation and the chemical mechanism of the lipase utilized to perform it. In light of the preliminary data, the practical study of the processes of interest conducted in this thesis will be investigated and discussed starting from chapter 3.2.

3.1. Preliminary data

ABOUT CBD EXTRACTION
Starting from the results reported in literature [54], the extraction of cannabinoids from hemp was investigated with the objective to find the optimal technique to extract the majority of the valuable compounds contained in the plant while keeping as low as possible the solvent used. The optimization of the extraction procedure is more relevant on the industrial scale than in the laboratory because brings economic advantages limiting the environmental impact of the process; however, the laboratory has the possibility to test different conditions and then the one with the highest yield is easily scalable for industrial application. In a previous project thesis, knowing the polar nature of cannabinoids, dynamic maceration was studied on different feasible solvents such as MeOH, EtOH, $CHCl_3$, MeOH-chloroform 1:1 (v/v), and n-heptane. In Table 2 are reported the best results of cannabidiol extraction obtained from 2 g of fresh sample macerated under magnetic stirring for >2 hours in 50 mL of different solvents.

Solvents	MeOH	**EtOH**	$CHCl_3$	MeOH-$CHCl_3$	n-eptane
Yield [mg,cannabidiol/g,sample]	10.8	**12.4**	10.7	11.4	9.8

Table 2: Best extraction results on fresh sample

As observable, the highest yield was achieved using EtOH due to its appropriate polarity and for that motivation it was the solvents employed in this work. The waste batch on which these extractions were performed was the same batch used in this project thesis, but these results were obtained almost one year earlier with respect to when the first extraction was performed again for this work, therefore the natural lot matrix was significantly changed in content during time.

ABOUT CBD FUNCTIONALIZATION
Once the recovered cannabidiol is extracted and isolated, can follow further molecule upgrading to obtain some derivatives with promising biological activities, as cannabinoids have proven to have. While maintaining the CBD scaffold, structural changes of the aromatic ring or terpene group can give rise to a new compound with enhanced therapeutic activity or other beneficial properties. Among the possible functionalization of the CBD

scaffold, it was decided to focus on the epoxidation of the chemically favored alkene bond present in the terpene ring.

ABOUT EPOXIDATION

CBD epoxidation reaction has been already analyzed in literature and there are documentations that epoxy-CBD is obtainable by chemical synthesis. An example of an epoxidation procedure utilizing mCPBA [55] to achieve the target molecule in a two-step reaction scheme is reported in Figure 9.

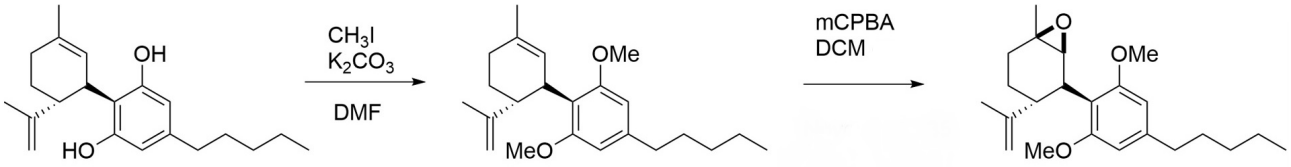

Figure 9: Chemical synthesis scheme of CBO obtained from CBD utilizing CH_3I, K_2CO_3 in DMF and then 3-chloroperbenzoic acid in CH_2Cl_2 [55]

In fact, the hydroxyl groups on the aromatic ring of CBD are very reactive to many compounds, so it is necessary to have a reaction intermediate with protected functional groups. Involving iodomethane in the first methylation step, the intermediate molecule is dimethoxy CBD (diMe-CBD), which is able to protect the sensible hydroxyl groups in order to direct oxidation reaction on the favored double bond in the non-aromatic ring of CBD structure. Once diMe-CBD has reduced the group reactivity, m-chloroperbenzoic acid (mCPBA) is able to perform the epoxidation reaction following the well know mechanism of the Prilezhaev reaction and obtain epoxy-diMe-CBD. In order to have the isolated target molecule, a second step is mandatory to remove the protective groups; it is possible utilizing and intermediate Grignard reagent in inert condition under N_2 at 210°C for more than one hour. This method has reached the target molecule with a final yield equal to 65% and the protective groups have a crucial role in multistep organic synthesis But it presents some unavoidable disadvantages such as the solvent waste generated, the final reaction step in rather extreme conditions and a very low atom economy. In fact, the use of mCPBA (MM=172,57 g/mol) is finalized to add only an oxygen atom (MM=15,99 g/mol) while the remaining part of the molecule is unutilized and is part of chlorinated reaction wastes.

An alternative procedure to synthesize CBO without involving protective groups is also reported [56]. In that case, atom efficiency is solved by involving hydrogen peroxide as an oxygen source to perform epoxidation. The other reagents utilized are potassium bicarbonate ($KHCO_3$) and benzonitrile (PhCN) in a methanol solution to keep agitated for 40 hours at room temperature. Comparing this procedure with the previous one, the main drawbacks are related to the long reaction time and to the involvement of PhCN, which is harmful to human health and to the environment. However, the possibility to obtain the target molecule in a one-pot-reaction without protection and deprotection steps, combined with the employment of a well-known reagent such as hydrogen peroxide and mild reaction conditions are good reasons to prefer this synthesis pathway. The final yield of CBD epoxidation to obtain the target molecule in the last cited method is 43%.

Starting to these considerations and following the green chemistry and biocatalysis principles, in this project thesis it was tried to find an enzymatic alternative method to obtain CBO. The recent technological trend has focused on enzyme application since they represent a sustainable method to achieve the final result. Biocatalysts are safe, renewable and biodegradable as green chemistry wants; in addition, enzymatic reactions occur at mild temperature and pressure conditions, in aqueous media, at physiological pH and not require protection and deprotection reaction steps because of enzyme selectivity. One of the most relevant disadvantages of a biocatalytic reaction is the enzyme cost, but it can be overcome through enzyme immobilization. That strategy allows the recycling and reuse of a biological catalyst or its application in continuous flow reactions, but specific process developments are needed to make it feasible. For these motivations and to increase the sustainable character of this work, the biotransformation of CBD into CBO was deeply investigated.

Basing on the literature model where octanoic acid was utilized to produce the peroxy acid, which works in a water/toluene biphasic mixture as solvent to perform epoxidation of alkenes [57], the starting experimental conditions were decided. Ethyl acetate was identified as the solvent with appropriate chemical and environmentally sustainable features for our purpose, hydrogen peroxide as oxygen source is easy to manage and satisfies the atom efficiency biocatalytic requirement, and Novozyme 435, a commercial immobilized lipase B from *Candida antartica*, is a suitable biocatalyst. The objective was to arrive with an enzymatic pathway to CBO starting from CBD and preliminary tests have confirmed its feasibility.

A deeper analysis of the lipase mechanism highlighted some possible problems. As shown in Figure 10, in our reaction ethyl acetate and hydrogen peroxide are transformed by perhydrolysis lipase mediated reaction resulting respectively in peroxyacetic acid and ethanol. Then, the acid presents in the reaction environment is

able to release one oxygen atom that is inserted into the alkene double bond following the Prilezhaev reaction mechanism. As result, EtOAc is regenerated inside the solution while epoxide is generated on the target molecule.

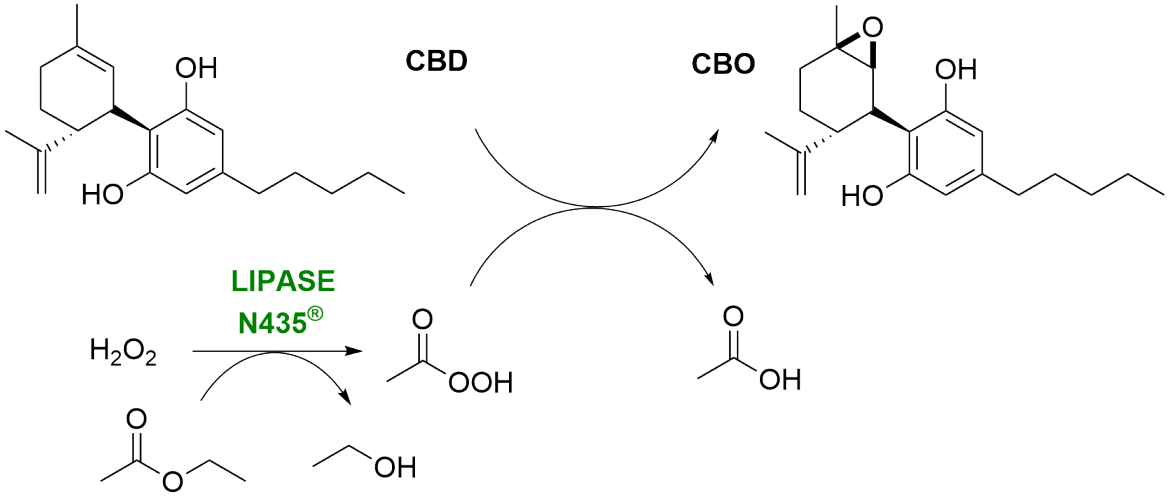

Figure 10: Lipase perhydrolysis mechanism in CBD epoxidation reaction

The peroxyacetic acid build-up could lead to many side reactions and decrease the selectivity of the main process lowering the final yield and reaction performances. Indeed, focusing on the target molecule CBO is important to mention its high reactivity [56]: due to the high stability of a five-ring structural conformation with respect to an epoxide, it has shown a strong tendency to react further in the generation of cannabielsoin (CBE) (Figure 11), also known by the IUPAC name (5aS,6S,9R,9aR)-6-methyl-3-pentyl-9-prop-1-en-2-yl-7,8,9,9a-tetrahydro-5aH-dibenzofuran-1,6-diol. CBE is a cannabinoid with a 5-membered ring structure and it could be chemically favored with respect to CBO.

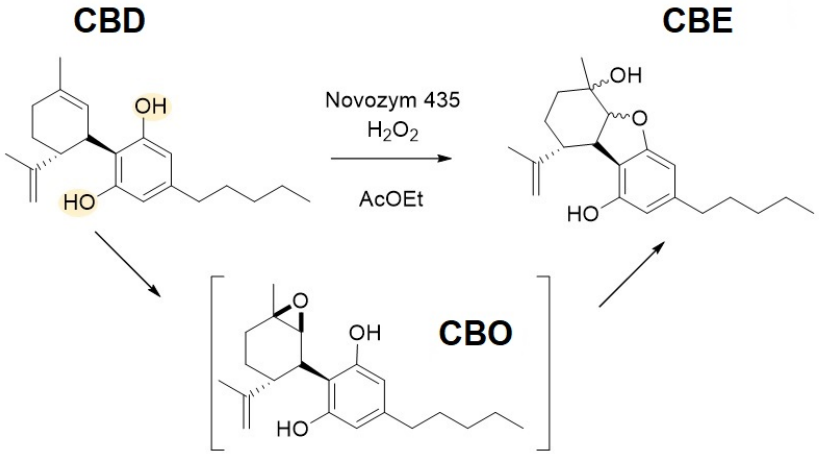

Figure 11: CBO as possible intermediate in CBE synthesis starting from CBD

In addition to theoretical chemical consideration about CBO stability, further proofs that in reality precisely CBE could be the result of our work are motivated by a recent experimental study [58]. Maybelle Kho Go *et. al.* had in fact obtained CBE starting from CBGA (the biological precursor of CBD) involving oxidation reaction with enzymes as biocatalysts. The reaction mechanism observed in these experiments to have CBE from CBGA is reported in Figure 12. Their result confirms the high probability to arrive at CBE as previously hypothesized describing some possible side reactions for CBO, even if are involved different enzymes with respect to Novozyme 435.

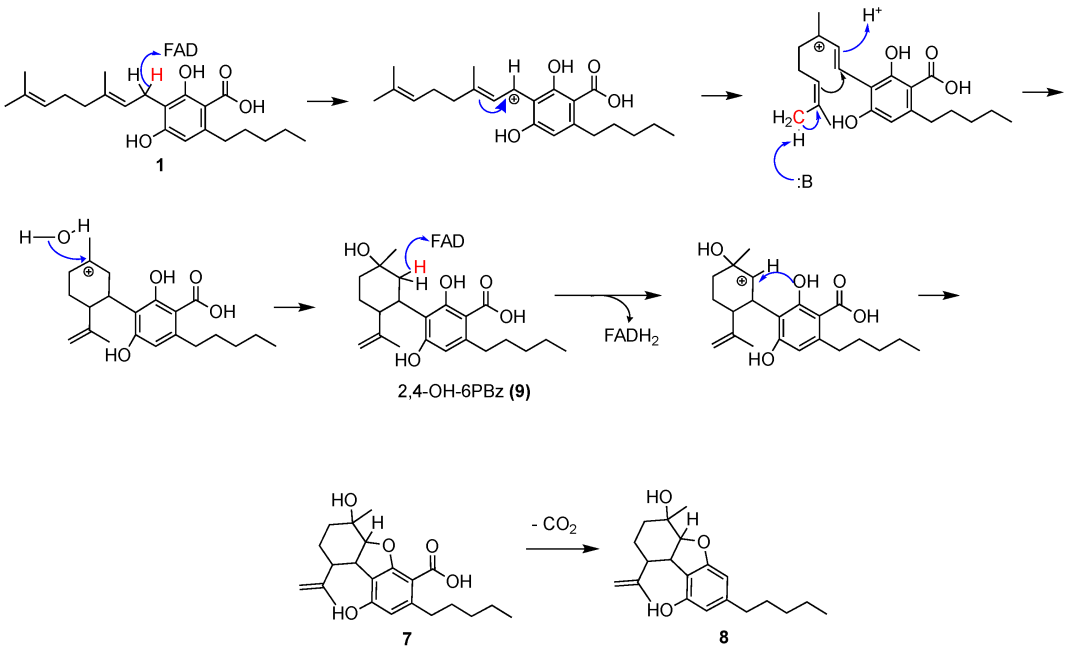

Figure 12: CBE mechanism formation from the CBD biological precursor (CBGA) [58]

Cannabielsoin was discovered for the first time in 1973 [59], then in 1983 at Ohio State University researchers reported the first biocatalytic synthesis of CBE and better clarifications about its process formation were carried out in Japan in 1988. It is curious to remember that CBE is the only eponymic cannabinoid: it was called "cannabielsoin" in honor of the technician Elsa Boyanova, who isolated it before passing away soon after. CBE is a metabolite of cannabidiol since it can be obtained by enzymatic transformation of CBD, so can not be directly obtained from cannabis or hemp. It is believed that has non-psychoactive activity because is a cannabinoid formed from CBD, so is highly probable that interacts at the same way with CB1 and CB2 receptors of the endocannabinoid system. Even if there are no available data regarding its medical benefits, researchers are thinking that CBE can contribute to the "entourage effect". As result, when mixed with other cannabinoids it can contribute to providing health benefits like anti-psychotic and anti-tumor effects or could be applied in the treatment of substance use disorder.
As a final consideration, the possibility to observe a natural rearrangement of CBO to a similar cannabinoid with the same molecular weight but a different structure is not negligible. Even if CBO is the target molecule for its bioactivity and the possibility to have further functionalization, also the isolation and purification of CBE are relevant and the molecule still maintains the CBD scaffold, so the related cannabinoids features are not loose. Moreover, CBE biological activity has not been studied yet, as can be deduced by the trend of CBE patents [56] reported in Figure 13. Indeed, before 2016 patents applications were very low, but they had an increasing trend in the last years.

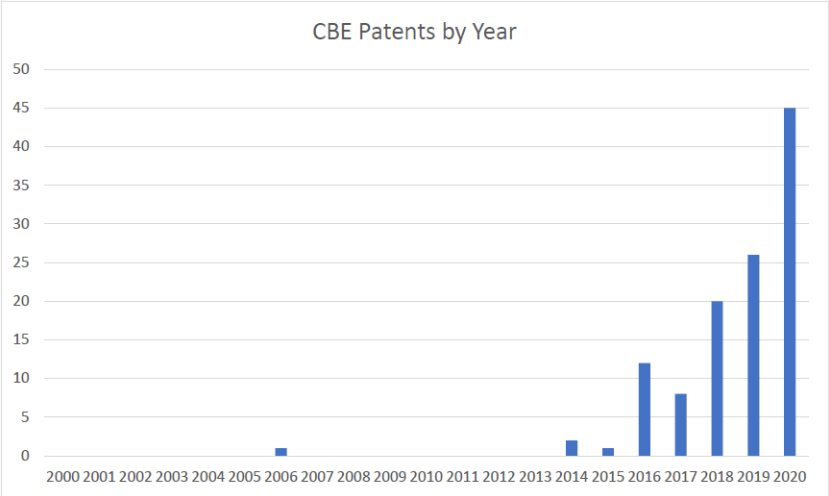

Figure 13: CBE Patents trend in USA by year

As visible in Figure 14 and 15, is evident that starting from the 70s the number of articles and patents on CBD and CBE has grown with an exponential trend. Comparing the CBD and CBE document tendencies, whose data were obtained from SciFinder, in both cases there was a significant increase at the beginning of the 2000s. The CBD-related studies were some order of magnitude more numerous than the ones on CBE, achieving more than 2000 CBD-related documents with respect to the almost 100 CBE-related publications. This fact means that CBD is a highly studied molecule and its properties are well known, while CBE investigation represents an opportunity to be seized in order to discover new syntheses and applications for that cannabinoid.

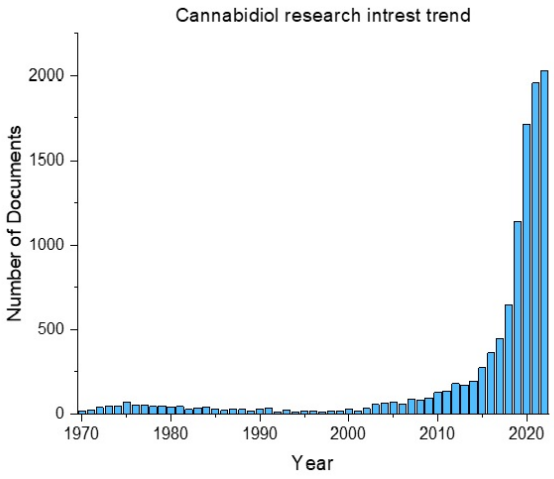

Figure 14: CBD interest research trend

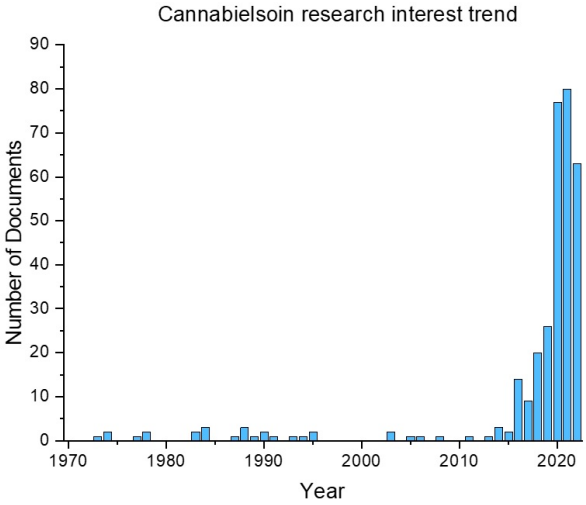

Figure 15: CBE interest research trend

In this section were reported the preliminary insights basing on which this project thesis was designed. Starting from the next chapter, the experimental work begins and is reported the discussion of the obtained results.

3.2. Extraction of cannabidiol from agricultural waste

The extraction of cannabinoids from agricultural waste is the first step to consider in order to obtain a sufficient amount of cannabidiol (CBD), which will be used for further functionalization. Therefore, the optimized solvent extraction method previously studied on the same agricultural waste lot was applied. The protocol was initially set with ethanol, but the use of hardware-shore-grade derived bioethanol instead of reagent-grade ethanol did not show a significant difference in the efficiency; it instead increased the sustainability and the greenness of the process. The procedure consisted of the maceration of hemp in bioethanol solution under agitation overnight. After that, the extraction solution was filtered and a secondary maceration occurred on the filter cake with half of the previously utilized solvent for 2 hours. The amount of organic solvent utilized in the first maceration operation was 25 mL per gram of hemp waste. The optimization of the extraction process aimed to find the most suitable solvent, but also to reduce its quantity as much as possible. Indeed, as final step was necessary to evaporate the organic solvent under reduced pressure in a rotary evaporator, so it would become a longer operation if there was an abundant solvent amount to remove. Once the crude extract was obtained, it was necessary the purification because the solvent extracted cannabinoids but also chlorophyll, waxes, and others impurities. Especially waxes were difficult to separate from our target product (CBD) since they had similar polarity. Based on that information and aiming to reduce solvent consumption, the automated chromatographic separation was performed on a silica gel column to ensure the repeatability of the procedure. To identify the correct fraction output of the purification column containing CBD were both used GC-MS and TLC. In TLC, the utilization of Fast Blue saltB reagent made easier CBD identification because the cannabinoid colors were orange-red after staining. As final confirmation of an efficient chromatographic separation and isolation of CBD ^{1}H-NMR was performed.

Due to the high variability of the natural matrix, enhanced by the fact that it was an agricultural waste, it was necessary to categorize the experiments into three main groups:

1. fresh hemp: after harvesting, the drying process in the oven at 60°C was the only pre-treatment for the organic product and it was performed with the objective to extend the sample stability and improve its storability in a hermetic plastic bucket, kept at room temperature and far from heat sources;
2. fibrous post-distillation residue: after harvesting, hemp was subjected to steam distillation and then was left drying in the sun for some days, finally was stored as fresh hemp in a hermetic plastic bucket;
3. frozen fibrous post-distillation residue: after harvesting, hemp was subjected to steam distillation, then

it was frozen in a plastic bag to extend its stability; before utilization, it should defrost overnight in an oven at 60°C.

Since the hemp lot was already employed to study the optimal extraction method for CBD, the remaining part was the material available for the current project.

About category 1, the maceration of 20g of fresh inflorescences was performed in bioethanol and it provided a dried crude extract of 740mg. After purification were obtained 550mg of product, that GC-MS and ^{1}H-NMR confirmed to be cannabidiol. Comparing to the result reported in Table 2, a great yield equal to 27.5 mg,CBD/g,sample was achieved. Further extraction resulted in no additional recovery of the CBD molecule. Unfortunately, the fresh inflorescence was a minor part of the available batch with respect to the abundance of stems and seeds. In subsequent extraction, stems and seeds have proven to contain few quantities of CBD and have shown to be difficult to manage because they made physical obstruction at mechanical agitation, preventing its proper functioning. For these aspects, CBD-rich plant parts were scarce.

After a few extractions, since the climate conditions of the 2022 summer season had compromised the annual hemp harvesting, we were forced to change the raw material category and the fibrous distillation residue was the first choice. The extractions were performed adhering to the same procedure utilized for the hemp category 2, but with worse results. Starting from 20g of sample, 814mg of crude extract was obtained, and 39mg of purified CBD was afforded at the end. The final outcome analyzed in GC-MS still contained many impurities and their separation was very problematic because they have similar polarity, so chromatographic separation was not efficient. Even if there were enough quantities of distillation residue to continue in the extraction processes, it was decided to don't keep it on since was not profitable with respect to the solvent consumption and the relative costs in terms of money and time.

The last opportunity to have furthermore CBD was represented by the hemp category 3. Since it was frozen, it was hypothesized that half of the sample weight was ice, therefore the usual procedure was seized on the double sample in weight. 40 mg of frozen fibrous post-distillation residue macerated in 500mL of bioethanol because was considered 20g of biomass, then the extraction solution was evaporated and analyzed in GC-MS. From the initial sample were obtained 154mg of crude extract, but its dark colors visibly suggested that it was rich in impurities. After purification in the silica gel column were obtained 24 mg, but even a qualitative TLC confirmed the presence of many compounds, therefore CBD was not isolated from that agricultural product and no NMR analysis was performed. Moreover, after defrosting overnight in the oven at 60°C, were noticed mold and fungi (probably *Aspergillus* species) on the biomass. Their presence was presumably due to the lack of an appropriate pre-treatment of biomass before the storage: the low temperature slowed down mold proliferation but did not stop it and the entire lot was compromised after one year in the freezer. The useful information derived from this hemp category was about the effects of inappropriate storage, but no pure CBD was extracted; as consequence, it was decided to don't keep on extractions.

It can be derived that the optimized extraction methods lead to significant amounts of CBD with an average yield of 10-15 mg of CBD per gram of waste. Unfortunately, the hemp replenishment was negatively affected by the unprofitable harvesting season, therefore the others extractions performed resulted in poor extraction yields. At the end of this phase, a total of 252mg of clean CBD have been reached by extractions and purifications.

3.3. Alternative CBD recovery from a model source

Given the need to do as many as possible experimental tests to study the CBD epoxidation reaction, it was necessary to have the higher amount of reagent possible. Unfortunately, as evidenced by the extraction results, the available batch of fresh hemp contained many stems and few flowers, so there was a lack of CBD-rich plant parts. Even the fibrous distillation residue was not suitable for deriving sufficient CBD quantities since cannabidiol was less present in that raw material. Therefore the obtained results confirmed the high variability of the natural matrix and in this case it was more evident since the hemp batch was an agricultural waste. Moreover, the waste derived from the distillation process had an increased component fluctuation due to the storage condition. In our case, also the annual fluctuation of agricultural yield and the essential oil price volatility affected the hemp replenishment. In fact, when was needed a supplementary quantity of *C. sativa* to perform further CBD extraction, the suppliers decided that was not profitable to harvest and produce essential oil. Therefore, experimental research needed an alternative source from which to extract high amounts of CBD from.

In order to be able to investigate the epoxidation reaction with a sufficient CBD quantity, it was decided to use commercial cannabis oil as a model source. The commercial oil was sold to reconcile sleep thanks to its high cannabidiol content, but it was selected as the new CBD source because the oil was certified with a high content of the interesting compound. The decision to use a commercial oil as a source model was also motivated by the necessity to anyway purify the ethanol solution when CBD extraction was performed in organic solvent from the natural waste hemp. Hence, purifying the extraction solution of an agricultural raw material or purifying a commercial oil were both mandatory separation operations and the final objective in both cases was

to obtain the isolated cannabinoid, which was hypothesized to be the same molecule regardless of the source from which it would be obtained. Obviously, the chromatographic separation in the silica gel column changes its parameters depending on the required operation, so a new separation method had to be studied and developed for CBD commercial oil purification. In conclusion, the results obtained were still valid and legitimate even if the originally conceived biomass was not utilized.

When purchased, crude commercial oil was analyzed by GC-MS and by ^{1}H-NMR to better comprehend the composition of the new material available, to verify the declared features, and to quantify the true obtainable CBD amount. The claimed CBD content was 6000mg in 30mL, in accordance with the concentration calculated with GC-MS investigation using acetophenone as the internal standard. TLC in hexane-ethyl acetate (9:1) was performed with the aim of preliminarily understanding if the compound separation could be feasible. It was observed that TLC performed on an already-opened commercial oil sample contained more substances with respect to a new and never opened sample of CBD oil. Therefore, commercial CBD oil had a strong tendency to oxidate itself with air during utilization, varying its composition with time. The pilot test to analyze the CBD stability in formulation confirmed the possibility to use the chromatographic column to perform separation, therefore 2 mL of crude oil was purified. The operation was performed using 100% hexane for the initial phase, then the eluent changed with a gradient concentration profile for ten column volume achieving the ratio 96:4 of hexane-ethyl acetate. The separation process obtained 411 mg of isolated CBD. Then the product was subjected to GC-MS, ^{1}H-NMR, and ^{13}C-NMR analysis as well as bidimensional COSY, HSQC and HMBC NMR analysis to have a complete cannabidiol characterization. The results were compared with the CBD characterization reported in literature [60], confirming the efficiency of the chromatographic separation on the commercial oil.
An initial winterization test was also carried out, but with no appreciable effect in one day, so the separation by chromatography was chosen as the technique to apply to isolate CBD.
By coincidence, the winterization separation method was discovered to be successful in a long time when the experimental tests were not performed for more than two months and the commercial oil was stationarily stored in the fridge at 4°C without ever being used. When restarted the research, was observed some crystals in the commercial oil sample. Further analysis proved that happened the selective crystallization of CBD at 4°C. That phenomenon was not studied more in-depth but it was really relevant to cite it as a useful, precise and low-cost separation method becase it did not require any solvent involvement. The main drawback of that technique was the long time needed, which made it industrially unfeasible.
Another attempt to obtain isolated CBD from the commercial oil without solvent consumption was performed involving the Kugelrohr apparatus for horizontal distillation. The oil was disposed in the rotating boiler at a high temperature, and based on the boiling point of the target compound (170°C), CBD was collected. ^{1}H-NMR analysis confirmed the hypothesized scenario: commercial oil distillation was performed correctly and efficiently achieved the cannabidiol isolation. With the same results obtained, the distillation process seemed to be the most sustainable purification method even if operated for some hours, which is more time respect than what chromatographic separation perform in dozen minutes, but it is less than crystallization required. As drawbacks, the energy consumption to keep high temperatures for distillation was not a negligible aspect compared with the low energy consumption required by an automated chromatographic column, which separates the components based on the affinity with solvents and the different polarity of substances, but involving high cost for silica column and solvents.
It can be concluded that CBD structure was not affected by the origin of the material from which was obtained or by the extraction method utilized, as proved by the ^{1}H-NMR analysis reported in the Appendix with identical CBD spectra despite the different sources and the different extraction methods involved.
Therefore, after the appropriate preliminary analysis and the purification processes, it was decided to use commercial oil as a source model from which deriving CBD and to use the cannabidiol extracted to study the epoxidation reaction. Indeed, when the CBD available was in sufficient quantity, the experimental study of CBD functionalization started.

3.4. Preliminary tests on epoxidation reaction

Once that CBD was isolated as a pure substance, a wide range of functionalizations were possible. Among these, it was decided to use Novozyme 435 to perform an epoxidation reaction to synthesize epoxy CBD (CBO). The valorization of the most abundant cannabinoid extracted from agricultural waste gives the possibility to arrive at a final product with an increased added value, in accordance with the circular economy principle. Because of that, in the previous project thesis on cannabis hemp, the reaction was already tried. The results lead to the conclusion that was a feasible operation, therefore we inherited the basilar information to perform it and our purpose was to optimize the reaction parameters to increase the final yield. The following conditions were initially used to validate the reaction feasibility: 75 mg of isolated CBD was dissolved in 12 mL of EtOAc, then were added in solution 1.5 equivalents (35 μL) of H_2O_2 as a 35% w/w aqueous solution and 2 mg of Novozyme 435. After 18 hours at 30 degrees Celsius in an agitated thermoshaker, the reaction was worked up and quenched

with the addition of Na_2SO_3, $NaHCO_3$ sat. solution. Then the organic phase was extracted in EtOAc, dried with Na_2SO_4 and concentrated through evaporation under reduced pressure and 40°C. Thanks to GC-MS analysis was observable the reaction product (MM=330 g/mol), but also other compounds were detected. As consequence, the purification process was necessary by involving a chromatographic silica gel column: loading 70,7 mg of the concentrated reaction product obtained from 75 mg of pure CBD, 21 mg of CBO was finally isolated. A more accurate ^{1}H-NMR analysis demonstrated the success of the reaction because the target alkene signals of CBD were absent in the NMR spectra, but there was room for improvement of reaction conditions in order to increase selectivity and yield. Indeed, for sure CBD was converted to another cannabinoid and the target carbon-carbon double bond was involved in the reaction, but was not characterized the final compounds since they had superimposed signals in NMR analysis. This first experiment was the reference point in our reaction study.

Since CBD epoxidation could be the starting point for further functionalization and the part of the molecule interested in that reaction have the same conformation of limonene oxide, had been tried to obtain the opening of the ring epoxide of limonene achieving the diol conformation; it was initially tested on a substrate similar to CBO in order to not compromise an important molecule in case of failure. The preliminary attempt on 254 mg of limonene oxide was performed by dissolving it in 50 mL of buffer solution of sodium acetate pH 4 as reported in literature [61]. The reaction occurred for 3 hours under magnetic stirring agitation providing the desired limonene diol as detected by GC-MS and ^{1}H-NMR. Therefore, were tested the same condition on the target substrate (CBO). The experiment on CBO failed, but the criticism was that the final GC-MS analysis did not detect either the reagent or the final product and the entire CD scaffold seemed degrading. The suspicion that something did not work as expected in the previous CBD epoxidation made us focus on that reaction.

Concentrating on the CBD epoxidation reaction, the key parameters seemed to be substantially three: the quantity of lipase enzyme, the volume of hydrogen peroxide and the temperature. Considering also other variables, it was deduced that the agitation was not a negotiable parameter because the lipase operates in biphasic conditions, so a stationary situation would have led to not exploiting all the reagents inside the reaction environment but only the one at the interface surface of organic and aqueous phases. Also the variables time, CBD concentration and impurities presence could have affected the reaction selectivity, but in that case, it could be possible to study the reaction kinetics by monitoring the reaction trend with sampling or by comparing the same reactions performed in different conditions.

In order to better understand the reaction behavior and optimize it, furthermore experiments were carried out. First of all, was tested the CBD stability in the reaction solution without the presence of enzyme: in 10 mL of EtOAc 50 mg of purified CBD was dissolved, then 2 equivalents (31 uL) of H_2O_2 as a 35% w/w aqueous solution was added. The reaction was kept under magnetic stirring agitation at 24°C for 5 hours. Since there were no lipase, H_2O_2 and EtOAc perihydrolysis had not the key component thanks to which the cannabidiol substrate was oxidized. Periodic sampling of 200 μL from the reaction was treated and analyzed in GC-MS. The results made us conclude that CBD was not degraded due to the sole reaction solution, but it would be transformed only by the enzyme activity. It was an important starting point that allowed asserting with certainty the crucial role of lipase to made the CBD substrate react. As consequence, all the subsequent experiments involved the lipase enzyme selected for this project (N435). The same procedure utilized for the last experiment was repeated, but adding also 2,3 mg of Novozyme 435. Due to the regular sampling, it was observed that epoxidation reaction occurred, but with selectivity problems. Were indeed identified several side products, which were also detected by the final ^{1}H-NMR analysis, but not by the periodic sampling subjected to GC-MS analysis. The latter, detected just that CBD (MM=314 g/mol) quantity decreased but the reaction product (MM=330 g/mol) did not increase by the same quantity. The build-up of peroxy-acid derived by enzyme activity was probably responsible for that, favoring CBD side reactions or transforming the target product into other non-detectable products such as polymers, decreasing the reaction selectivity and the final yield. Was therefore decided to saturate EtOAc with a phosphate buffer pH 7.5, in order to have the optimal pH range in which the enzyme works. This operation also aimed to facilitate phase separation, which is a mandatory step in the workup of reaction. Moreover, the buffer should protect both CBD and CBD derivatives dissolved in EtOAc for hours, disadvantaging the generation of side products. The experiment with EtOAc saturated with buffer solution was set up with 50 mg of CBD, 2 equivalents of H_2O_2 at 24°C as before, but with a minor quantity of Novozyme 435 (0.8 mg instead of 2.3 mg). The reaction occurred for 5 hours (as before) with periodic monitoring sampling, which showed a slow transformation; therefore it was decided to achieve complete conversion of CBD letting continue overnight for 22 hours. In the end, 46 mg of concentrated products were obtained and then purified. The objective to increase reaction selectivity and have less side product failed as can be concluded by ^{1}H-NMR spectra of the final separated products. Indeed, achieving complete CBD conversion, the reaction yield was strongly affected because it was observable that overnight (between the 5 hours-sampling and the 22 hours-sampling) the reaction product value increased from 9% to 19% with respect to the internal standard utilized, while CBD passed from 52% to 2%. As observable, the generation of CBO was significantly lower than CBD consumption.

The reaction behavior was not clear, moreover the cannabidiol extracted from the organic waste was really

limited. Since there was the necessity to continue with the experimental test, commercial cannabis oil was purchased. Then, initially, it was directly utilized in the epoxidation reaction. The commercial oil was voluntarily not pretreated because it would have involved an initial purification process from which to obtain the isolated CBD and further final separation of the reaction products. Aiming to save one purification step in case the enzyme was not affected by impurities, it was decided to utilize crude cannabis oil as the epoxidation reagent. Two parallel tests were performed both at 24°C, with a low quantity of enzyme (0.5 mg) and 1.1 equivalents (17 μL) of H_2O_2, but they occurred with different reaction time and CBD sources: the first test had purified CBD as reagent and occurred for 5 hours, while the second experiment had crude cannabis oil as reagent and 22 hours of reaction time. The final GC-MS analysis has shown the relevance of impurities in crude oil and long reaction time (at 24°C): despite a complete CBD conversion, these parameters reduced selectivity, resulting in a high presence of side products and a low yield of the target product (CBO).
The main problem experienced until this point was related to the reaction selectivity, which seemed to be highly related to the CBD conversion. In order to decouple these reaction patterns, the changing of other reaction parameters was evaluated .
Since the temperature range was not investigated until now and was still not clear how limitate the generation of parallel reactions, which led to many undesirable products and decreased the reaction yield, from the central value of 24°C different reaction temperatures were studied. Oxidation reaction on purified CBD (50 mg) was carried out at 45°C, with a low quantity of enzyme (0.5 mg) and 1.1 equivalents of H_2O_2 for 22 hours and it was compared with the same quantity of CBD in the epoxidation reaction at 4°C, with a high quantity of enzyme (5 mg) and 1.1 equivalents of H_2O_2 for 48 hours. In the end, both experiments were analyzed in GC-MS and the result was unexpected. At 45°C complete CBD conversion was achieved in 22 hours despite the very low amount of Novozyme 435 involved, but the selectivity of chemical transformation in the target product was very low, decreasing consequently the final yield. On the other hand, at 4°C the reaction occurred very slowly (also due to the low amount of H_2O_2, which was probably consumed after two days), therefore the conversion of CBD was lower, but the reaction selectivity was significantly higher. This means that the enzyme activity was improved at low temperatures, which is an unusual experimental consideration but focal in our study. The fact that Novozyme 435 has a better activity at 4°C than at 45°C was also important since the enzyme optimal temperature is indicated by the manufacturing company between 30°C and 60°C. The product obtained from the experiment at 4°C, so the one with higher selectivity, ^{1}H-NMR analysis had been performed after the purification process. The resulting spectra reported the presence of a couple of diastereoisomers, but undoubtedly the separation was efficient since only one single molecule was detected, which was for sure a CBD derivative.
For the first time, with well-performed NMR analysis, was understood that the product obtained by the CBD oxidation reaction and detected in GC-MS with a molecular weight equal to 330 g/mol, as expected due to the addition of an oxygen atom (MM=16 g/mol) on CBD (MM=314 g/mol), was not the target molecule CBO but was instead its conformational isomer cannabielsoin. The CBE identity was confirmed by its complete characterization reported in literature [56]. Moreover, A. Monroe *et. al.* claimed that the biological activity reported by Nally Y. *et. al.* for the CBO molecule was instead referred to CBE. It happened because Nally Y. *et. al.* studied the bioactivity of a molecule that was not correctly characterized due to the same detection problems that were encountered by us in the initial phase of this project. The fact that CBD epoxidation enzymatically catalyzed afforded CBE as the final product was partially confirmed also by a recent study [58] on the same reaction pathway, even if in that case different biocatalysts were involved.
However, low temperatures value seemed to be a crucial parameter to guarantee higher selectivity in the CBE synthesis. For that reason, the subsequent experiments involved lower reaction temperatures with respect to the initial reference experiments at 24°C. Therefore, an additional parallel test was performed always on CBD oxidation with Novozyme 435 as biocatalyst keeping approximately 13°C. The reactions involved both 31.4 mg of CBD dissolved in ethyl acetate previously saturated with the buffer solution, 2 equivalents of H_2O_2 as a 35% w/w aqueous solution, but had a different enzyme quantity: one contained 2 mg of enzyme, while the other had slightly more than 5 mg of Novozye 435. In both cases, every two hours have been performed withdrawals for GC-MS analysis until the final workup at 6 hours. The trend observed in these last parallel experiments was exactly the same, with an extremely high precision in possible overlapping for the sampling at 4 hours of the reaction which had more enzyme with the 6 hours sampling analysis of the reaction with lower enzyme quantity. That meant that increasing only the enzyme amount reflected in higher kinetics, but seemed not possible to decouple the target epoxidation reaction with respect to the other phenomena and changing the amount of enzyme appeared to influence only the time at which a certain situation was observed without changing the reaction trend.
Until this point, the useful pieces of information obtained were:

- the CBD needs to be utilized as a pure substance instead of as crude commercial oil, because impurities significantly decrease the reaction selectivity;
- the ethyl acetate needs to be saturated with phosphate buffer pH 7.5, because it is a favorable pH for the enzyme and sensible molecules dissolved in EtOAc are protected from H_2O_2;

- the H_2O_2 as a 35% w/w aqueous solution is the most suitable oxygen source and its volume does not degrade CBD;
- the enzyme quantity increases the kinetics, but decoupling the epoxidation reaction and side reactions seems not possible;
- the optimal reaction time could be related to the amount of enzyme involved;
- at mild temperature: in the initial phase the CBD conversion was low, but the selectivity of the epoxidation reaction was appreciable, while at higher reaction time the CBD conversion was completed but the reaction selectivity was strongly affected to side reactions;
- at lower temperature: the reaction occurred slower than at mild temperature, therefore the CBD conversion was not high, but it had positive effects on the reaction selectivity;
- as the last but most important aspect, was clarified that CBD epoxidation afforded CBE instead of CBO in the investigated reaction conditions.

Since the situation was not clear and all the variables seemed correlated to each other resulting in low CBE yield, changing a few parameters at time has not led to the desired reaction optimization. The solution to overcome a such difficult situation has been tried to be found in the application of the Design of Experiment (DOE), which is a mathematical method based on the use of statistical analysis and it was applied to maximize the information derived from the minimum number of experiments.

3.5. Design of Experiment (DoE)

The experiments performed until this point of the research were useful to understand the complexity of the reaction and the large number of variables that could impact the final yield, but how to achieve the optimal condition able to oxidate cannabidiol with an appreciable reaction yield was not clear. Since the CBD conversion proceeded at a different rate with respect to the reaction selectivity, it was difficult to quantify the yield due to the detection problem of the GC-MS analysis, which reported a progressive decrease of reagent (CBD) without a proportional increase of the product (CBE), but did not even show the generation of other compounds. One possibility to consider was that the reaction product was generated and then transformed into not detectable products, hence the target product was consumed. In order to change the approach to the problem, the design of experiments (DoE) was applied as an innovative and alternative strategy. It is a statistical method able to establish a multivariate group of experiments which allows to test a set of variables minimizing the experiments and maximizing the derived hints. However, it is not an automated operation, since the DoE is a mathematical instrument that needs to be integrated with specific knowledge of the problem in the field in which it is applied. Therefore, in this case, is mandatory to understand organic chemistry and it is necessary to have some prior information about the reaction.
The result of the DoE application is the definition of an experimental region in which the considered variables in the considered range have a well-established relevance. The statistical method known as DoE can be utilized both in a preliminary screening phase on many variables to understand which are more important and as an optimization tool in a more advanced study phase to define the optimal value of these variables.
Since the oxidation reaction had been already studied with previous experiments, in this project DoE was applied to find the optimal conditions in which the yield of CBD oxidation affording CBE was maximized. The results obtained were strongly related to the considered variables and their relative range, so the initial phase in which the variables and their values were decided was a crucial step in the design of experiments. As consequence, in fact, the derived model will be accurate just in the studied range.

The first step in the design of experiments was the definition of the objective. For our purpose, the DoE was used to determine the optimal condition of the enzymatic reaction, therefore the reaction conditions to achieve the highest reaction yield were the target of this innovative method application.
In the second phase, to achieve the claimed objective, the variables of interest must be specified. In our case, have been chosen the investigated variables on the basis of the previous experimental result: Temperature (T), Enzyme quantity (E), Hydrogen peroxide volume (H_2O_2) and time (t) were the selected parameters. Since there were only four factors, it was convenient defining a full factorial design that resulted in a set of 2^4=16 experiments. It was the defined design because full factorial design generates the experimental set through which are derived the most informations. Indeed, in full factorial design are taken in consideration all the variable interaction levels and the resulting number of experiments was not prohibitive. Moreover, the selected design was in accordance with the available experimental resources, both in terms of time and materials needed. The experimental set would need some central point to validate the model and some repetition of random experiments to decrease the response coefficient variances. Given that before the DoE many experiments were carried out, it was established to assume two of them as explorative experiments and they were selected as the central points of the developed model due to the improvable compromise between the conversion and selectivity obtained. It was decided to reutilize already performed tests in order to save time and not waste already gained useful information. The selected explorative tests were both performed on 31.4 mg of CBD dissolved in 5 mL

of ethyl acetate at 13°C, with 5 mg of enzyme and differed for the H_2O_2 volume involved (2 or 4 equivalents). In both cases the reaction solution was subjected to sampling at 2, 4 and 6 hours, then occurred the work-up. The GC-MS analysis was utilized for the sampling study and a final ^{1}H-NMR was performed after 6 hours of running reaction. The same procedure will be exactly repeated for the DoE's experiments.
Based on the explorative experiments, their experimental conditions were considered as the central points, therefore the extreme values of the selected variables were established and they are reported in Table 3, defining the considered experimental range.

Values	T [°C]	E [mg]	H_2O_2 [eq]	t [hours]
Minimum	4	4	1.1	2
Maximum	24	8	4	6

Table 3: Extreme values of DoE's variables

The resulting experimental coded matrix generated by a full factorial design with four variables is reported below (Figure 16), highlighting that eight experimental conditions are identically repeated at two different times. As shown, was considered 20 experiments: 8 experimental conditions at the minimum time value (-1), the same 8 conditions at the maximum time value (+1), and 4 identical tests with central value of T,E and H_2O_2 but both at the maximum (-1) and minimum (+1) time of reaction.

#	T [°C]	E [mg]	H_2O_2 [eq]	t [h]
1	-1	-1	-1	-1
2	1	-1	-1	-1
3	-1	1	-1	-1
4	1	1	-1	-1
5	-1	-1	1	-1
6	1	-1	1	-1
7	-1	1	1	-1
8	1	1	1	-1
9	-1	-1	-1	1
10	1	-1	-1	1
11	-1	1	-1	1
12	1	1	-1	1
13	-1	-1	1	1
14	1	-1	1	1
15	-1	1	1	1
16	1	1	1	1
17	0	0	0	1
18	0	0	0	1
19	0	0	0	-1
20	0	0	0	-1

Figure 16: Coded experimental DoE matrix (+1=max value, -1=min value, 0=central value)

The same procedure applied to the explorative experiments (the already performed two central points of the DoE model) was replicated for the DoE experiments and it was reported in the following part. In order to facilitate the experimental set up and to decrease the experimental variability, it was decided to prepare a stock solution both for starting ("CBD stock") and for working up ("quench. sol.") the reaction. The "CBD stock" was prepared by dissolving cannabidiol in ethyl acetate and an internal standard; in this way to start the experiments was just necessary to uptake 5 mL of stock solution and then add the determined quantity of hydrogen peroxide and enzyme. The decided internal standard was n-decane ($C_{10}H_{22}$), MM=142,29 g/mol) and it was present in the reaction mixture in an almost identical concentration as CBD in order to correct slight dilution error and quantify in GC-MS analysis the conversion and the selectivity with respect to the standard. The calculation of selectivity and conversion of CBD oxidation was in fact a necessary step for data elaboration to obtain the reaction yield. On the other hand, the internal standard had a crucial role and it was

intentionally in equal concentration to the initial CBD quantity since otherwise the reaction yield calculation would not be possible in case the CBD was consumed and the resulting compounds were not totally detected by the laboratory equipment. The other utilized stock solution was the "quench. sol.", which was composed of distilled water (9.05 g) and sodium sulfite anhydrous (1.002 g).
To be able to control how the reactions proceeded, it was decided to withdraw 40 μL from the reaction solution of 5 mL and analyze the samples in GC-MS after diluting them with 250 μL of ethyl acetate and treating them with the quenching solution. It was possible to perform withdrawals since it was assumed from the experimental experience that removing 40 μL from 5 mL of reaction solution did not affect the reaction trend. Sampling was carried out at 2 and 6 hours, but also intermediate sampling at 4 hours was performed in order to have more data for a more reliable elaboration in case the 2-6 hours data had no clear outcomes. Therefore, in addition to the experiments reported in Figure 16 could be integrated the experimental matrix in Figure 17, with the same reaction conditions at the intermediate time (4h).

#	T [°C]	E [mg]	H_2O_2 [eq]	t [h]
1	-1	-1	-1	0
2	1	-1	-1	0
3	-1	1	-1	0
4	1	1	-1	0
5	-1	-1	1	0
6	1	-1	1	0
7	-1	1	1	0
8	1	1	1	0

Figure 17: Experimental data additional to Figure 16 if were considerate also intermediate withdrawals at the central time (central time value=4h)

Due to sampling at different times on the same reaction, from the elaboration point of view, was as if we carried more times the same experiment and stopped it with the withdrawals. The result was that one experiment with three withdrawals at 2, 4 and 6 hours was equivalent to three experiments performed in the same conditions and stopped respectively at 2, 4 or 6 hours. Therefore, the sampling procedure was applied to save time and resources by avoiding experimental repetition. With that strategy, the number of experiments decreased from 30 (consideing three different reaction times) to 10, but two of them are the already performed central points. As result, the simplification had brought the experiments to practically set up to 8; they are reported in Figure 18 and 19 respectively in the coded and uncoded version. Since the selected design of experiments was limited, to generate the experimental matrix was not needed any software, but it was manually accomplished.

#	T [°C]	E [mg]	H_2O_2 [eq]
1	-1	-1	-1
2	1	-1	-1
3	-1	1	-1
4	1	1	-1
5	-1	-1	1
6	1	-1	1
7	-1	1	1
8	1	1	1
9	0	0	0
10	0	0	0

Figure 18: Coded matrix of the practically set experiments

Name	T [°C]	E [mg]	H_2O_2 [eq]
MV028 H	4	4	1,1
MV028 G	24	4	1,1
MV028 D	4	8	1,1
MV028 F	24	8	1,1
MV028 E	4	4	4
MV028 B	24	4	4
MV028 C	4	8	4
MV028 A	24	8	4
MV027	13	5	4
MV026	13	5	2

Figure 19: Uncoded matrix corresponding to Figure 18 with named experiments

The experiments were performed on two different days to avoid the "block effect". Indeed, it was essential to obtain reliable data: executing the entire experimental set in one day could seem the best option because allow

to saves time, but on the other hand, splitting data on some different days permits to decrease significantly the impact of random variables, resulting in more reproducible procedures and reliable outcomes.

Once the standard experimental setting was established, the GC-MS and ^{1}H-NMR analysis were chosen as response factors of the experimental matrix, therefore the data to elaborate will be taken from these analyses. The set of experiments was randomized and split into two days to prevent external factors or prejudices interference with data collection.
Onetime that the introductory DoE features were decided, all experiments were performed, and they were analyzed by GC-MS. Then, from that laboratory equipment the integrated peaks area of the detected compounds (CBD and target product) were collected in an Excel sheet (Figure 20) to facilitate a preliminary data elaboration.

				2h			4h			6h		
	T [°C]	E [mg]	H_2O_2 [eq]	S.I.	CBD	PROD	S..I.	CBD	PROD	S.I.	CBD	PROD
MV028 A	24	8	4	1,33E+08	3,15E+07	1,98E+07	1,23E+08	2,84E+07	2,45E+07	1,32E+08	1,72E+07	1,71E+07
MV028 C	4	8	4	1,28E+08	4,06E+07	2,31E+07	1,30E+08	4,44E+07	3,02E+07	1,34E+08	8,95E+06	2,59E+07
MV028 B	24	4	4	1,31E+08	1,63E+07	2,79E+07	1,26E+08	4,15E+06	2,83E+07	1,28E+08	0,00E+00	9,93E+06
MV028 E	4	4	4	1,75E+08	8,48E+07	9,08E+06	1,52E+08	3,77E+07	2,29E+07	1,66E+08	2,35E+07	3,36E+07
MV028 F	24	8	1,1	1,65E+08	5,84E+07	1,58E+07	1,53E+08	4,04E+07	2,29E+07	1,50E+08	2,76E+07	1,88E+07
MV028 D	4	8	1,1	1,31E+08	8,40E+07	1,09E+07	1,23E+08	9,25E+07	1,29E+07	1,31E+08	5,48E+07	1,88E+07
MV028 G	24	4	1,1	1,66E+08	5,80E+07	2,04E+07	1,55E+08	2,86E+07	3,20E+07	1,60E+08	1,38E+07	3,07E+07
MV028 H	4	4	1,1	1,74E+08	1,16E+08	5,53E+06	1,69E+08	9,34E+07	1,73E+07	1,77E+08	7,02E+07	2,09E+07
MV027	13	5	4	1,07E+08	2,43E+07	7,80E+06	1,21E+08	9,77E+06	1,49E+07	1,10E+08	3,93E+06	1,18E+07
MV026	13	5	2	1,18E+08	5,27E+07	6,53E+06	1,19E+08	2,90E+07	1,42E+07	1,17E+08	1,58E+07	1,67E+07

Figure 20: Integrated peaks of CBD and reaction product detected by GC-MS

The first comment was derived just by the collection of data in Excel (Figure 20). Indeed, can be observed that most of the reactions did not reach complete conversion since CBD was still present at the final time (6 hours). Reaction B, which occurred with high temperature and hydrogen peroxide values but a low enzyme quantity, was the only one to consume all the available CBD reagent in six hours. It was also noticed that reaction B had the lowest value for the reaction product; it was an interesting result that confirmed the huge relevance of chemical phenomena able to transform the reagent but consuming contemporaneously the target product.
Based on these crude data, followed a computational adaptation in order to be able to elaborate the DoE responses through Chemometric Agile Tool (CAT). The following assumptions and introductory evaluations have been made for data management:

- To have reliable numerical values, the integrated areas of compound peaks were considered with respect to the integrated peak area of internal standard. Indeed, the internal standard function was to correct slight dilution errors during withdrawal operations, which could affect the absolute value of peaks of the detected compounds;
- Since the experiments were carried out on different days, even if was used the "CBD stock" to ensure to have the same initial quantity of CBD in all reactions, a daily sampling was taken from the stock solution at t_0 (t_0=0 hours) to verify that CBD concentration in ethyl acetate solution did not change over time;
- CBD and CBE were assumed to interact and respond in the same way at GC-MS measurements since are chemically similar molecules;
- Given the previous assumptions, the compound peak areas compared to the internal standard area were utilized to elaborate more physically interpretable parameters such as the CBD conversion and the reaction selectivity;
- The CBD conversion was calculated with the following equation (1), in which A_0 was the ratio between the area of CBD in t_0 sampling and the internal standard area of the same t_0 sample, while A_t was the ratio between the area of CBD in the withdraw at time t and the internal standard area at t in the corresponding withdraw:

$$\boldsymbol{CONV} = \frac{A_0 - A_t}{A_0} \tag{1}$$

  In this way, conversion corresponded to the ratio between the reacted reagent and its initial quantity. It was a useful parameter to quantify how much CBD was consumed in the reaction;
- The reaction selectivity was calculated with the following equation (2), in which B_t was the ratio between the area of CBE and the relative internal standard area at t in the corresponding withdraw:

$$\boldsymbol{SEL} = \frac{B_t}{A_0 - A_t} \tag{2}$$

After these assumptions, it was possible to elaborate on data. for the sake of simplicity, in the first phase of elaboration only the data collected at 2 hours and 6 hours was considered. The result of data elaboration was the experimental matrix (Figure 21) with the uncoded experiment conditions and the calculated system response; each experiment was also associated with a serial number and an alphabetic letter.

	T [°C]	E [mg]	H_2O_2 [eq]	t [h]	CONV [%]	SEL [%]
MV028 D	4	8	1,1	2	8,3%	143,9%
MV028 D	4	8	1,1	6	39,8%	51,9%
MV028 C	4	8	4	2	70,9%	23,3%
MV028 C	4	8	4	6	93,8%	19,0%
MV028 F	24	8	1,1	2	49,4%	27,7%
MV028 F	24	8	1,1	6	73,7%	24,3%
MV028 E	4	4	4	2	30,7%	24,1%
MV028 E	4	4	4	6	79,7%	36,4%
MV028 B	24	4	4	2	88,5%	22,2%
MV028 B	24	4	4	6	100,0%	7,2%
MV028 A	24	8	4	2	78,1%	17,6%
MV028 A	24	8	4	6	88,0%	13,5%
MV028 G	24	4	1,1	2	67,8%	16,8%
MV028 G	24	4	1,1	6	92,0%	19,2%
MV028 H	4	4	1,1	2	4,0%	113,1%
MV028 H	4	4	1,1	6	43,1%	39,3%
MV027	13	5	4	2	64,1%	17,9%
MV027	13	5	4	6	94,4%	18,0%
MV026	13	5	2	2	64,1%	17,9%
MV026	13	5	2	6	94,4%	18,0%

Figure 21: Elaborated experimental matrix imported to CAT

The experimental matrix reported in Figure 21 was then imported and furthermore elaborated in CAT. Thanks to that software, Lenth's plots showing the impact of variables on the selected outcomes were obtained. When the red bars were below the central line, the considered variable had a negative impact on the observed response, otherwise had a positive effect. The yellow candles indicate the confidence interval of the calculated value. In addition, in some cases are also present some stars. They were derived from the p-values, which indicate the precision of the calculated value and under certain thresholds the p-values were indicated with graphical symbols (*): lower was the calculated p-value and higher was the number of stars. Here are reported the plots of the calculated coefficient of variables with respect to the conversion and selectivity response factors.

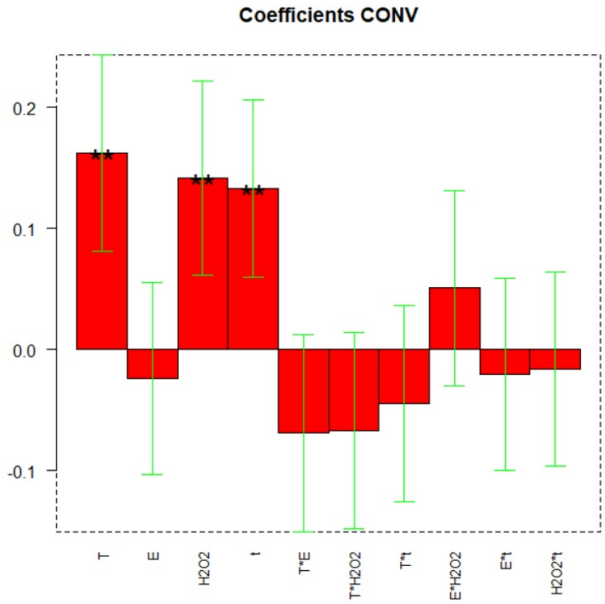

Figure 22: Plot of variable coefficient affecting conversion

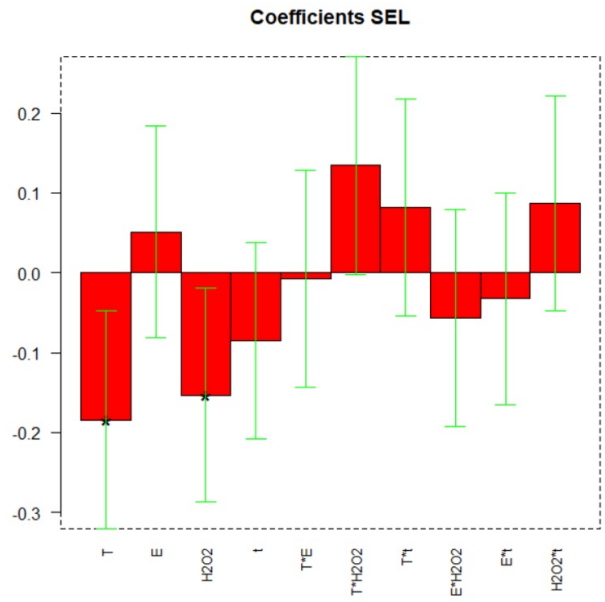

Figure 23: Plot of variable coefficient affecting selectivity

From initial observation, the conversion calculated with respect to CBD and the selectivity calculated involving the target reaction product (CBE) had almost specular aspects. The fact that the two graphs were almost

mirrored images was a preliminary indication that the analysis was correct, since were referred to reagents and products of the same reaction so the variable which had a positive impact on one response factor must have also a negative impact on the other response factor. Moreover, can be also claimed that no mass loss occurred in the reaction, otherwise the two box plot would not be specular. It was also noticed that the confidence intervals were narrower in the conversion plot. This phenomenon was related to the method of calculation of these parameters: conversion was strongly related to the CBD detection in GC-MS, while selectivity was connected to the target reaction product. Therefore, since in GC-MS the CBD was more abundant than CBE, its measurement was more accurate, while the target product was less present and its values ware closer to the detection limit of the involved machinery, so was less precisely measured.
For these reasons, the plot obtained for selectivity was considered not reliable and the data was elaborated again in a second phase considering also the data collected at the intermediate time (4h). Therefore, this step considered also the experimental additional data (Figure 17), which needed to be integrated into CAT elaboration together with the data collected at 2 and 6 hours. The obtained outputs considering an initial, an intermediate and the final data time were considered to generate elaborations closer to reality due to the addition of data. As result, the plot for conversion was slightly changed, while the selectivity graph was significantly modified and the results obtained are shown in Figure 25.

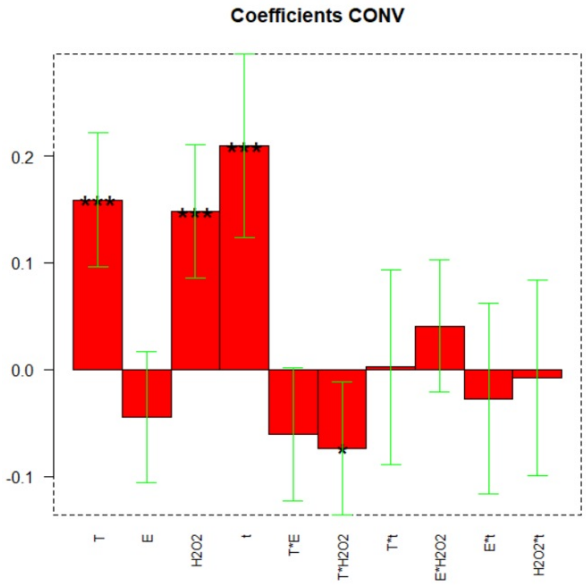

Figure 24: New plot of variable coefficient affecting conversion, obtained considering also the collected data at central value of time (4h)

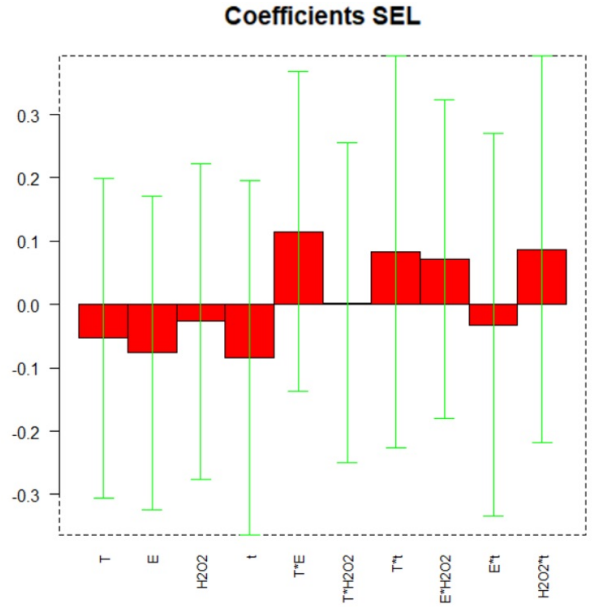

Figure 25: New plot of variable coefficient affecting selectivity, obtained considering also the collected data at central value of time (4h)

Focusing on one response factor per time, can be discussed the effects of the considered variables within the studied range. For what concern the conversion, an initial examination of the first-grade parameters was applied. Can be easily asserted that the enzyme quantity in the considered range did not influence the CBD conversion. The red bar of the enzyme variable was the lowest and the closest to the central line, so its impact was not significant. Focusing on the time variable, it was instead derived that it was the most important parameter that positively affects the conversion. Therefore, more time occurred and more the CBD conversion increased, as expected. This conclusion seemed obvious, but it confirmed the correctness of the analysis and data elaboration. Secondly, temperature and hydrogen peroxide quantity were relevant variables and positively influenced the final conversion for what concerned the investigated chemical space. All these evaluations were made for the single variables, then were also considered the interaction of second order between two variables. From what can be understood by watching the conversion graph, the sole important interaction was between temperature and hydrogen peroxide; the others binary interactions were negligible since had low values and large confidence intervals. The interaction between T-H_20_2 was so important because it considered the relationship between two variables which were very relevant even if individually considered. However, the response surface methodology was applied for that relationship and the resulting output is shown in Figure 26.

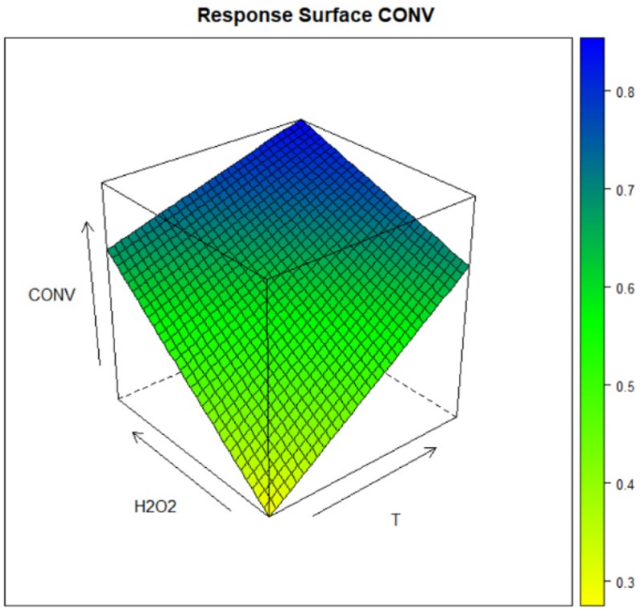

Figure 26: Response surface of temperature-hydrogen peroxide affecting CBD conversion

The observed trend described that the highest conversion value was reached at high values of both T and H_20_2. That was mathematically calculated and proved by DoE, while a monovariate approach could obtain a high conversion value at low T and high H_20_2 (or vice-versa), considering them the optimal situation. In reality, the absolute optimum could be in a different condition an DoE can claim it without further investigation experiments, differently from a monovariate investigation.
Regarding the conversion response was affirmed in conclusion that to achieve high reagent consumption was suggested to save the enzyme and reduce its quantity with respect to the minimum value investigated, to keep a long reaction time in order to allow complete conversion and to manage the hydrogen peroxide quantity and temperature close to higher values than the ones studied. The DoE was revealed to be a powerful tool to understand these trends, which were hypothesized but were never proven until now.
Then, analyzing the selectivity calculated with respect to CBE, the obtained plots was the one shown in Figure 25. As already mentioned, the confidence intervals for this response factor were very larger, therefore the binary interaction between variables and their response surfaces was negligible because were not significant. Since the derived model had background noise, the analysis was focused on evaluating how the single variables affected the selectivity because their values had higher weight. However, even the individual variables had rather low significativity due to the large confidence interval calculated for the low coefficient of the variables which influenced selectivity. Therefore, the selectivity analysis was fairly not significant. It could be just affirmed that the parameters which positively affect the conversion had instead a negative impact on selectivity, but it was deduced more by analyzing the raw data than the elaboration. Time (t) seemed to be the most relevant variable which affected the selectivity, so probably due to side rection the resulting data elaboration was not efficient. It is a common situation reported in literature when conversion and selectivity responses are considered [43], but there was no other strategy to achieve a reaction yield quantification. Therefore, a trade-off has to be found in order to find optimal variables which achieve a high value of yield and proper response factors.
In conclusion, the GC-MS-derived data and their elaboration allowed us to claim the following consideration. In the considered range, the variable E did not affect both conversion and selectivity, therefore it can be minimized with a consequent significant saving of funds. The other parameters (t, T, H_20_2) were contrasting if were considered the conversion or the selectivity response, therefore were needed to find a compromise. The CBD conversion indicated the rate at which the reagent was consumed, while the selectivity gave insight on the target reaction rate. Depending on which response is the priority, especially in the economy of scale, different evaluations of operative variables and the related costs could be possible. For instance, high energy costs are related to high reaction temperatures which consume the whole reagent utilized with low selectivity and resulted in mandatory downstream operation for separation and purification of the reaction mixture. In our case, longer reaction time contributed to increasing the CBD conversion while decreasing in the reaction selectivity, even with a lower effect on the later response factor. On the other hand, T and H_20_2 were relevant and with completely opposite effects on the two responses considered.
In a future investigation, will be necessary to design a second DoE based on the already gained insights to find the optimal value of the considered variable by investigating them in a different range. As an alternative, can be changed the reaction configuration and it could be developed in flow reactors. Keeping the high temperature and low residence time, will be reached high selectivity since the side reactions were still not relevant in the

initial phase of CBD selective oxidation by an enzymatic pathway. However, the CBD conversion would be not completed, therefore would be necessary to develop and study a method to separate the final reaction mixture to recirculate the unreached CBD in feedback.

Since all the DoE experiments performed were also subjected to a final ^{1}H-NMR analysis of the crude reaction product obtained in six hours, the NMR spectra reported in Figure 27 were an additional analysis to utilize as a possible test response.

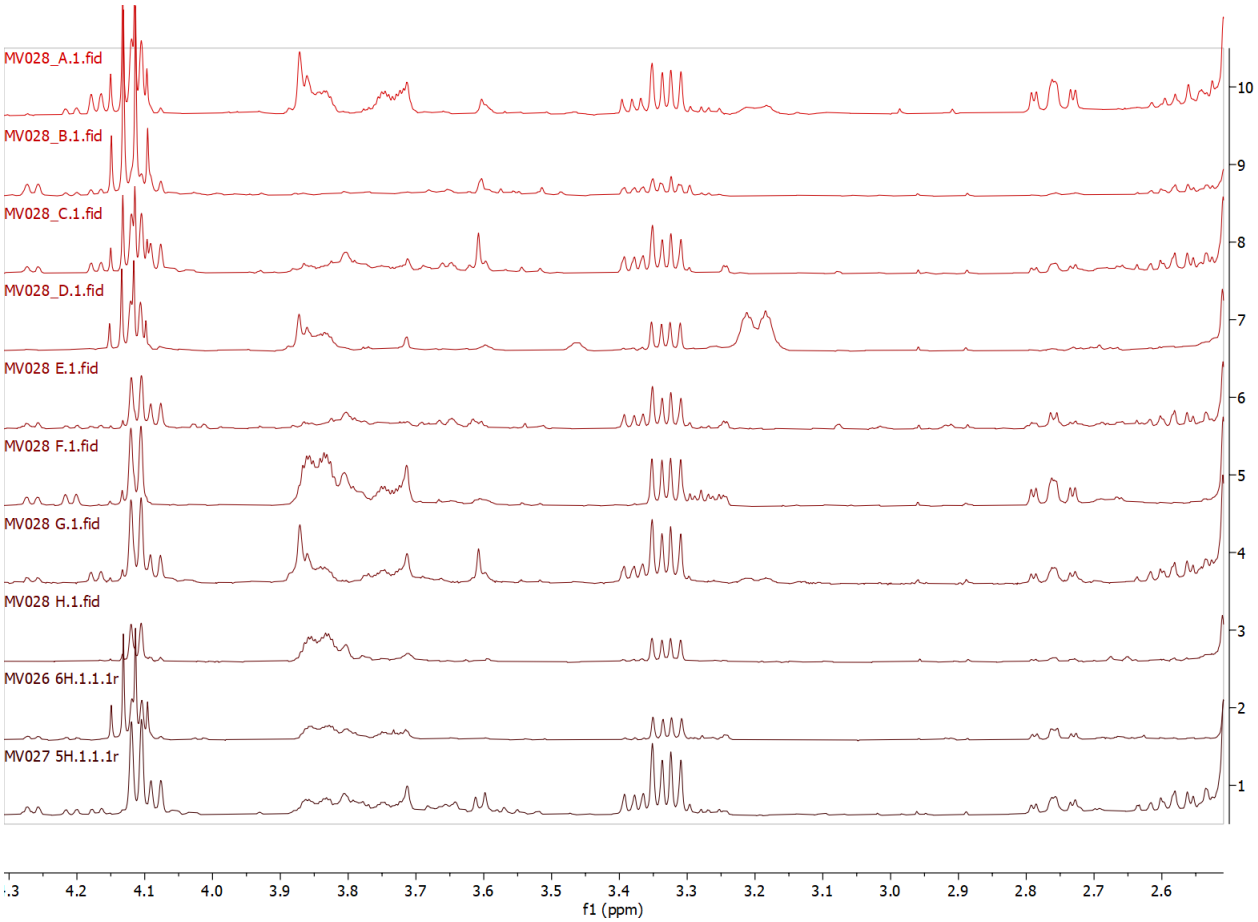

Figure 27: ^{1}H-NMR spectra of DoE experimental matrix (MV028) and the explorative earlier experiment (MV026,MV027) utilized as DoE central points

They have been processed by using 3 order baseline correction with a 9 Hz filter, manually phased, and referenced at 7.26 ppm with a residual peak of $CDCl_3$. The diagnostic region of this reaction is between 4 and 3 ppm as shown by Table 4 and Figure 28.

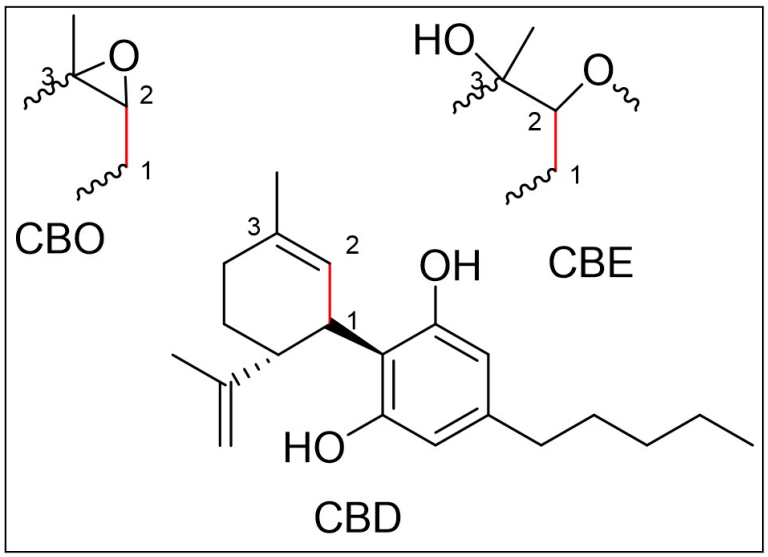

Figure 28: Carbons characterization of CBD, CBE and CBO molecules

Molecule	1C	2C
CBO	3.25	3.82
CBE	4.11	3.33
CBD	8.16	-

Table 4: Characteristic ppm of H shift to check in ^{1}H NMR spectra

From these ten NMR spectra (Figure 27), in the first place was described that the CBD oxidation with lipase afforded CBE. That result was difficult to achieve due to the identification and detection problems that occurred during this project thesis, but was in accordance with what was reported in literature [56]. As reported in section 3.4, the enzymatic reaction led to two CBE diastereoisomers, but they resulted in two partial superimposed doublet of doublet signals at 3.33 ppm and were isolable by chromatographic purification.
Since the DoE-derived spectra had the peaks of two or more different molecules superimposed between 3.90 and 3.70 ppm, it was impossible to elaborate the integrated value of NMR spectra without introducing an error. It was also tried to give some marks from 100 (the best) to 0 (the worst) to the spectra, but no significant DoE elaboration was reached. However, by observing NMR spectra, some qualitative data were observed; as reported in Table 4 the identified compounds in the final reaction mixture are CBD, CBO, CBE, and another cannabinoid to investigate.
A qualitative comment on spectra could be made keeping in mind the experimental data elaborated from GC-MS analysis. Indeed, can be observed that experiment B, which reached a conversion of 0.06% by GC-MS measurement, had also the lowest NMR peaks. That was an experimental confirmation that the final target reaction product was in a very limited quantity. However, seemed that also reaction B generated CBE, as had carried out 7 of the 8 experiments. The only test condition which obtained a compound that was not CBE, was the D experiment. Indeed, D spectra had unique signals between 3.15 and 3.25 ppm. In accordance with the highest decrease in selectivity measured by the GC-MS analysis for D experiments, probably that reaction condition led to a different cannabinoid synthesis. Focusing on the other reactions, the most interesting region to observe in ^{1}H-NMR spectra was the one between 3.25 and 3.40 ppm, because is where were visible the CBE diastereoisomer signals. The test condition H, so keeping the low value for all the considered variables, provided the cleanest signals within the diagnostic diastereoisomer region; in fact, were not revealed the presence of extra peaks which are characteristic of a molecule that is not the target one. Observing the diagnostic region, the reactions A, C, E, and G had a relevant quadruplet on the left of the diastereoisomer signals, but no common conditions can be defined to extract a specific reaction trend. A final qualitative comment can be derived from the central point spectra considering that they were carried out in equal conditions, except for the hydrogen peroxide volume involved. The central point with the highest H_20_2 value was also detected in ^{1}H-NMR spectra with a higher number of peaks with respect to its parallel experiments. Therefore, seemed that the reaction selectivity decreased with the increasing of H_20_2 value, but would be necessary further studies to affirm it for sure.
Since NMR-derived pieces of information were not mathematically obtained, it was impossible to define a

reaction trend depending on experimental conditions. Therefore, the elaboration of data from GC-MS produced more reliable outputs and was mainly considered those. In the future, the reaction could be monitored with GC-MS until complete conversion and then perform ^{1}H-NMR analysis, in order to have a more precise response factor on that instrument.

3.6. Final comparison between chemical and biocatalytic reaction

As the final step of this project, a comparison was carried out between the chemical synthesis of CBE reported in literature [56] and the best reaction condition achieved in the design of the experiment and resulting in CBE. The reaction considered as the best of DoE was H, so the one performed on 31.4 mg of CBD at 4°C with 4 mg of enzyme and 1.1 equivalents of hydrogen peroxide. It has been chosen because it had the highest selectivity and yield of the reaction product. Moreover, the chemical synthesis required 40 hours, therefore was not relevant that H did not reach complete CBD conversion in 6 hours during DoE optimization, since chemical and enzymatic synthesis were both performed with equal reaction time (40 hours) to achieve high conversion value in both cases. The procedure utilized to set the enzymatic reaction was the same utilized in the DoE, without withdrawals for monitoring the already known trend. It was decided to conduct three biologically catalyzed reactions with the same method, except for changing the pH of the phosphate buffer solution utilized to saturate ethyl acetate. Therefore, three parallel experiments were saturated with the buffer solution at pH 4.5, pH 7 or pH 8.5 and occurred for 40 hours in order to have the same reaction time of the chemical synthesis and comparable results. Following the procedure executed by Monroe *et. al.* [56], the selective CBD oxidation involved potassium bicarbonate added in a methanol solution under argon with benzonitrile and hydrogen peroxide. After this time, the suspension was filtered, concentrated and then purified by silica gel chromatography. In both chemical and biocatalytic synthesis, the final isolated product was analyzed with GC-MS and ^{1}H-NMR technique.
From the ^{1}H-NMR spectra was immediately clear that the two methods led to the same substance. As correctly reported in the literature, it was confirmed by our experiments that the compound obtained by chemical synthesis from CBD was CBE. It was demonstrated feasible (yield=58%) by involving chemical reagents without the implication of protective groups for the CBD hydroxyl functional groups. The main drawback remains the non-environmentally friendly nature of the utilized reagents, in particular benzonitrile. On the other hand, the chemo-enzymatic method afforded mostly pure CBE at neutral conditions (yield=28%), while minor amounts of different impurities were obtained in acidic and basic conditions. The generation of other cannabinoids was due to the high/low pH, which contributes to favoring side reactions that involve also the target product, transforming it. The fact that CBE was the final product of the enzymatic process at neutral pH was in accordance with the results observed in the previous DoE. The comparison between the two performed synthesis techniques is reported in Figure 29. The advantages of chemo-selective CBE synthesis with respect to chemical synthesis were the milder reaction conditions, which make the reaction industrially attractive, and the use of a biocatalyst (N435) instead of toxic and harmful chemical reagents (PhCN) even if the final yield achieved by enzymatic technique (28%) was lower than that of the chemical method (58%).

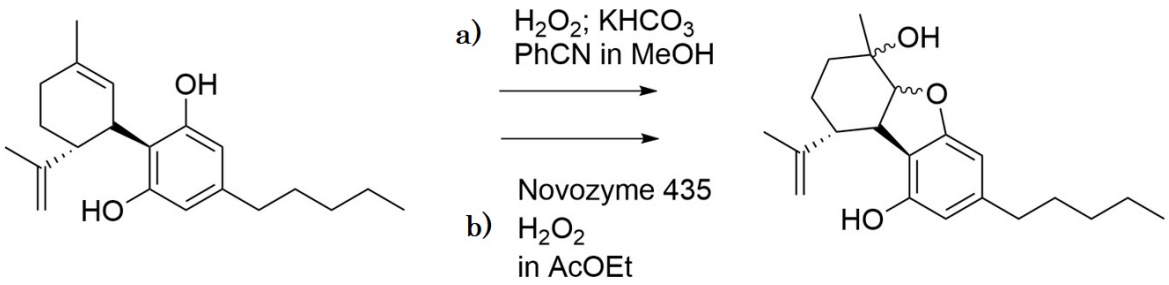

Figure 29: Comparison of chemical (a) and chemo-enzymatic (b) CBE synthesis performed from CBD

As a final consideration, it can be stated that the synthesis and the characterization problem met with the enzymatic pathway, which afforded CBE instead of the initial target molecule (CBO), was not due to the involvement of biocatalysis, but seemed intrinsically linked to the high reactivity of epoxide against the phenolic groups on the benzene ring in aqueous conditions. In literature [56] was reported a report of the chemical process which utilizes Oxone in CBD epoxidation [49] in which the final results obtained by Monroe *et. al.* were different from the one claimed by Nalli *et. al.*. The inconsistencies led to the conclusion that the reaction of CBD with Oxone resulted in CBE, even if was published an article declaring that the final product was CBO. The misleading structural assignment was discovered by further investigation and experimental repetitions, then

was concluded by an accurate ^{1}H-NMR analysis. It was an issue not related to the application of biocatalysis, indeed it was demonstrated that the same situation occurred when a chemical synthesis of CBE was performed. However, both CBE and CBO are cannabidiol derivatives and they maintain the CBD scaffold, so the biological activities are still relevant and a new synthetical pathway has been discovered to synthesize CBE, which is a valuable compound not deeply studied but with an added value due to its the enzymatic synthesis and potential medical application.

4. Experiments

The waste material has been collected from Sacmar (Milan, Italy). All chemicals and solvents were purchased from Zentek s.r.l. (Milan, Italy) and Merck (Merck Life Science s.r.l., Milan, Italy), employed without further purification. All purifications were carried out on a PuriFlash XS-420+ (Interchim) using Purezza-Daily Standard Flash cartridges (Sepachrom, Italy). TLC analyses were performed on Merck Kieselgel 60F254 plates purchased from Merck. Novozyme 435 (Novozymes) was purchased from Strem Chemicals Inc.(Bischheim, France). ^{1}H-NMR and ^{13}C-NMR spectra were recorded on a 400 MHz spectrometer in $CDCl_3$ solution at room temperature. Analyses were executed using gas chromatography with a mass detector (GC-MS). Detected substances were identified using GC-MS by comparing the mass spectra and relative retention index based on homologous series of n-alkanes with the literature, the National Institute of Standards and Technology (NIST) database and the available standards. GC-MS analyses were performed using a HP-5MS column (30 m $\times$ 0.25 mm $\times$ 0.25 μm), Agilent Technologies Italia S.p.A. (Cernusco sul Naviglio, Italy). The following temperature program was employed: 60°C (1 min)/6°C min−1 /150°C (1 min)/12°C min−1 /280 °C (5 min); this temperature profile is called FUGMS. FUGMS method takes almost 40 minutes to run, considering the sample analysis time and the dead time between two analytical tests. For that reason, was also used FSCAN method when necessary to obtain information more quickly, in order to take decisions on the current reaction without time delay. Indeed, FSCAN method takes half the time of FUGMS following the temperature program as: 60°C (1 min)/12°C min−1 /150°C (1 min)/24°C min−1 /280°C (5 min). Both methods are able to generate distinguished peaks for the reagent and products of interest: with FUGMS, cannabidiol is released at 28.75 min and the product of oxidation reaction is at 29.8 min, while with FSCAN, cannabidiol is released at 17.7 min and the product of oxidation reaction is at 18.4 min. Therefore, using both methods is possible to quantify the presence of chemical compounds without peaks overlapping and consequentially is possible a resolution.

Solvent extraction: was performed on three different sample of the same waste batch (fresh inflorescence, fresh and frozen fibrous distillation residue) taking 20g of raw material and 500mL of bio-EtOH. First maceration occurred overnight in a 0.75L flask at room temperature (around 25°C) under mechanical rod agitator or in a thermoshaker. Then the solution was filtered and the solid residue was subjected to a secondary maceration for 2 hours with the same procedure, except for using half of the previously utilized bio-EtOH volume. The first and secondary extraction solutions were finally combined together in the round bottom flask and they were subjected to evaporation at 40°C using a rotary evaporator with decreasing pressure.

Identification by TLC: eluents mostly used were hexane-ethyl acetate (9:1) or (98:2), depending on the impurities present in the sample. To run on a TLC of 10cm length, separation took 10-15 minutes. CBD (MM=314.047 g/mol) was identified under UV light thanks to the proper reference substance or its known retention factor. CBD was also directly visible by spraying Fast Blue saltB TLC reagent (H_2O+MeOH solution 0.2% v/v), which colored the interesting cannabinoids orange-red. Even the product of CBD epoxidation (MM=330 g/mol) was identified with the same procedure; its retention factor was slighly lower and reacted with Fast Blue saltB giving a less intensive color with respect to CBD.

Cannabidiol (CBD): the structure was characterized by ^{1}H-NMR, ^{13}C-NMR, COSY, HSQC, HMBC spectroscopy and GC-MS analysis; the spectra were compared with those reported in literature [62] and are reported in appendix (Figure 34 35, 36, 37, 38, 39).
[65,66] MS m/z: 314 (M+), 246 (13), 231 (100), 193 (9), 174 (9), 121 (10). ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 6.22 (2H, br s, 3'+5'), 5.96 (1H, br s, 2'-OH), 5.57 (1H, s, 2), 4.67 (1H, br s, 6'-OH), 4.67–4.65 (1H, m, 9-trans), 4.57–4.55 (1H, m, 9-cis), 3.89-3.81 (1H, dm, J = 10.2 Hz, 1), 2.44 (2H, t, J = 7.6 Hz, 1"), 2.39 (1H, dt, J = 10.6, 3.7 Hz, 6), 2.29-2.17 (1H, m, 4a) , 2.13-2.05 (1H, m, 4b), 1.87-1.74 (2H+3H, m, 5ab+7), 1.65 (3H, s, 10), 1.56 (2H, quint, 2", J = 7.2 Hz), 1.36-1.24 (2H+2H, m, 3"+4"), 0.88 (3H, t, 5", J = 7.1 Hz). ^{13}C-NMR ($CDCl_3$, 101 MHz): 155.1 (br d, J = 199.1 Hz), 149.3, 143.1, 140.0, 124.3, 113.9, 111.0, 108.9 (br d, J = 163.6 Hz), 46.3, 37.3, 35.6, 31.6, 30.7, 30.5, 28.5, 23.8, 22.6, 20.5, 14.1. MS m/z: 314 (M+), 246 (13), 231 (100), 193 (9), 174 (9), 121 (10).

CBD purification from commercial oil: the crude commercial oil (2 mL) was loaded in cartridge (120 g) by syringe. After a preliminary TLC using as eluents hexane-ethyl acetate (98:2), the retention factors were

calculated and the separation profile was set. The sample was eluted in 100% hexane for 1 column volume (CV), then hexane-ethyl acetate (96:4) were reached with concentration gradient over 20 CV and finally concentration remained isocratic for 1 CV. TLC and GC-MS were used to identify the correct column fractions containing CBD. ^{1}H-NMR analysis was used to characterize the isolated molecule.

Chemo-enzymatic epoxidation of CBD: Novozyme 435 enzyme
Purified cannabidiol (CBD) (75 mg) was dissolved in EtOAc (10 mL), previously saturated with phosphate buffer solution pH 7.5. Novozyme 435 (5 mg) and then H_2O_2 (3,5% w/w aqueous solution) were added (1.1 equivalents, 23 μL) to the solution, which was kept in agitation thanks to magnetic stirring. The reaction was carried out for 22 h at 24°C; the temperature was kept constant using a heating plate and a water bath. The reaction trend was monitored with sampling every 2 hours (5 μL from reaction solution diluted 50 times in EtOAc). Samples were quenched with the prepared solution (Na_2SO_3 1% w/w, $NaHCO_3$ 1% w/w, in H_2O), centrifugation (1000 rpm for 1 minute) was utilized for separating aqueous and organic phase, Na_2SO_4 was added for removing residual H_2O from organic phase and then samples were analyzed in GC-MS. The reaction was stopped following the same procedure on the entire reaction solution, in more enzyme was left behind by filtration on cotton when Na_2SO_4 was added and organic phase was transferred from a falcon lab to a round bottom flask. The organic phase was finally concentrated under reduced pressure using a rotary evaporator. The residue was then chromatographed on a silica gel column, which concentration gradient profile was defined thanks to preliminary TLC hexane-ethyl acetate (98:2). The obtained product was analyzed by GC-MS and by ^{1}H-NMR. For a complete characterization were used the results reported in literature [56] and were compared with the obtained spectra.

Purification of CBD oxidation reaction products The concentrated products (70 mg) of epoxidation reaction on CBD were dissolved in hexane and loaded in the cartridge (4 g) by syringe. After a preliminary TLC using as eluents hexane-ethyl acetate (98:2), the retention factors were calculated and the column profile was set. The sample was eluted in hexane-ethyl acetate (99.6:0.4) for 3 column volume (CV), then hexane-ethyl acetate (96:4) was reached with a concentration gradient over 18 CV. When the chromatogram has shown a stationary situation where purification seemed blocked (25 CV), therefore eluents polarity was raised achieving hexane-ethyl acetate (92:8) and occurred separation. After 37 CV, polarity was increased again up to hexane-ethyl acetate (86:14) in order to ensure to obtain the target product. TLC and GC-MS analysis were used to identify the correct column fractions containing the reaction product. ^{1}H-NMR was used to characterize the isolated molecule.

Cannabielsoin (CBE): the structure was characterized by ^{1}H-NMR, ^{13}C-NMR spectroscopy and GC-MS analysis; the spectra were compared with those reported in literature [56]; the spectra are reported in appendix (Figuere 40, 41, 42).

Limonene oxide ring-opening to obtain diaxial limonene diol Limonene oxide (250 mg) *cis/trans* isomers mixture was added to a buffer solution of NaOAc (50 mL, 100mM, pH 4). Magnetic stirring agitation at constant volume was continued until total consumption of the limonene epoxide (18 hours), as detected by GC-MS analysis. The mixture was neutralized with aq sat. $NaHCO_3$, then the organic phase was extracted with EtOAc (25 mL x 3 times), dried (Na_2SO_4) and concentrated under reduced pressure using a rotary evaporator to have the diaxial limonene diol, as detected by GC-MS. The procedure followed the instructions reported in literature [61].

DoE explorative experiments A set of 2 experiments was set up and worked up involving the stock solutions. A stock solution of CBD (630 mg), EtOAc (100 mL) previously saturated with phosphate buffer solution pH 7.4, and n-decane (975 μL) was prepared to set up experiments. Quenching solution (Na_2SO_3 1% w/w, $NaHCO_3$ 1% w/w, in H_2O) was prepared to work up the experiments. Experiments were both prepared with 5 mL of CBD stock solution (31.4mg of CBD per reaction), then was added the enzyme (Novozyme 435, 5mg). The added volume of hydrogen peroxide was 2 equivalents in one test and 4 equivalents in the other. Reactions occurred under magnetic stirring agitation for 6 hours. Every 2 hours, 40 μL of the reaction solution was taken as sample, diluted in 250 μL of EtOAc, quenched with quenching solution (2 μL), dried with Na_2SO_4 and analyzed in GC-MS. After 6 hours the whole reaction was worked up with the quenching solution (400 μL) and centrifuged to ensure phase contact. The organic phase was separated to the aqueous solution, dried (Na_2SO_4), concentrated under reduced pressure using a rotary evaporator and analyzed by ^{1}H-NMR.

DoE on CBD oxidation A set of 8 experiments was set following a full factorial design of experiments (DoE). A stock solution of CBD (630 mg), EtOAc (100 mL) previously saturated with phosphate buffer solution pH 7.4, and decane (975 μL) was prepared. Quenching solution (Na_2SO_3 1% w/w, $NaHCO_3$ 1% w/w, in H_2O) was prepared too. The variables temperature (T, [°C]), enzyme (E, [mg]), hydrogen peroxide (H_2O_2, 35% w/w aqueous solution, [μL]) and time (t, [hours]) were selected; their respective extreme values were 4°-24°C, 4-8 mg, 1.1-4 equivalents and 2-6 hours. Experiments were prepared with 5 mL of stock solution (31.4 mg of CBD per reaction), then was added a certain quantity of enzyme (Novozyme 435) and hydrogen peroxide in different

reaction temperatures, as previously decided and set by the experimental method. Reactions were performed in two different days (4 reactions per day). Reactions occurred under magnetic stirring agitation for 6 hours. Every 2 hours, 40 μL of the reaction solution was taken as sample, diluted in 250 μL of EtOAc, quenched with quenching solution (2 μL), dried with Na_2SO_4 and analyzed in GC-MS. After 6 hours the whole reaction was worked up with a quenching solution (400 μL) and centrifuged. The organic phase was separated to the aqueous solution, dried (Na_2SO_4), concentrated under reduced pressure using a rotary evaporator, and the crude reaction product was analyzed by ^{1}H-NMR.

CBE synthesis by chemical pathway for final comparison Adhering to the literature procedure [56], a glass vial with flat bottom was charged with (31.4 mg) of purified cannabidiol (CBD). Potassium bicarbonate (10 mg) and methanol (0.5 mL) were added, forming a suspension solution that was kept under argon and agitated by magnetic stirring bar. Then, benzonitrile (15 μL) and hydrogen peroxide 35% w/w aqueous solution (15 μL) were added in this order. At this point, reaction was carried out for 40 hours under argon and agitation. After this time, the reaction was filtered through methanol wash and the filtrate was concentrated to dryness by decreasing pressure. Once obtained the final mixture, it was purified by chromatographic separation by applying solvent ramping from 100% hexane to 80:20 hexane-ethyl acetate over 15 CV. Final GC-MS and ^{1}H-NMR analysis were performed at the end on the purified reaction product

CBE synthesis by chemo-enzymatic pathway for final comparison A set of three parallel experiments was carried out following the DoE procedure. Each reaction was set up with CBD (31.4 mg), which was dissolved in EtOAc (5 mL) previously saturated with phosphate buffer (0.1 M) at a different pH value (8.5, 7, and 4.5). Then were added N435 (4 mg) and H_2O_2 50% w/w aqueous solution (10 μL) diluted with the respective buffer solution (10 μL), achieving approximately a H_2O_2 concentration of 25% w/w aqueous solution. The reaction was kept agitated at 4°C for 40 hours. After this time, the reaction was worked up with the same quenching solution of DoE (Na_2SO_3 1% w/w, $NaHCO_3$ 1% w/w, in H_2O) and centrifuged to obtain the organic phase, then it was dried (Na_2SO_4) and concentrated under reduced pressure using a rotary evaporator. Chromatographic separation occurred by loading the three reaction product together in a 4 mg caritage. The eluent profile maintained 100% hexane for 4 CV, then occurred a gradient ramping for 10 CV achieving hexane-ethyl acetate (9:1). TLC hexane-ethyl acetate 9:1 was utilized involving Fast Blue saltB staining to identify the CBE containing fractions. Final GC-MS and ^{1}H-NMR analysis were performed at the end on the purified reaction product.

5. Conclusions and future development

The aim of this project was to validate and optimize the cannabinoid extraction from the residual material derived from the industrial production of *Cannabis s.* essential oil and to study the functionalization of the most abundant extracted compound (cannabidiol, CBD). The extraction of CBD was performed involving a simple extraction method with an environmentally benign and sustainable solvent (bioethanol); also an alternative source model was utilized for the CBD extraction and were not observed changes in the obtained molecule due to the origin of the material or the extraction method (chromatography, distillation, crystallization). Then, several possible valorizations of CBD molecule were investigated focusing on the epoxidation reaction utilizing a commercial biocatalyst (Novozyme 435). Numerous experiments were carried out in order to achieve the reaction optimization following the traditional monovariete method and then was applied an innovative strategy known as the Design of Experiments (DoE). It proved to be a precious tool capable of extracting many essential insights and reaction trends from ten specific experiments. The multivariate approach of DoE helped to clarify the reaction behavior and was useful to understand phenomena that would need greater effort to be observed with a monovariate approach. As reported also in literature [56], structural assignment problems of the reaction product occurred, but thanks to our experiments was confirmed that CBD oxidation provided the cannabielsoin (CBE) instead of the epoxy CBD (CBO). These cannabidiol derivatives are isomers identically detected by GC-MS and with very similar NMR spectra, but the latter revealed the true nature of the final isolated compound. Concerning the studied chemo-enzymatic selective oxidation of CBD, the DoE results could be applied in the future for further optimization processes by changing the range of the investigated variables, for instance by decreasing the enzyme quantity with respect to the considered value. As an alternative, the reaction configuration could be modified from batch to continuous flow reactors: keeping high temperature and low residence time, the reaction selectivity could be improved due to the minor relevance of side reactions in the initial phase of CBD oxidation. However, if the CBD conversion could not be completed, it will be necessary to develop a method to separate the unreacted CBD from the final reaction mixture in order to recirculate it in feedback. It would result in a two-step production with the optimal compromise between the operative variables, achieving a high CBE yield.
Cannabielsoin is a functionalized molecule not naturally synthesized by plants and it still maintains the CBD scaffold, therefore is a cannabinoid with an intrinsic added value. Since CBE is not deeply studied, it represents

an opportunity to seize and its bioactivity on human health could be investigated for possible pharmaceutical applications. Further optimization of CBE synthesis and its medicinal applications are left to future researchers.

6. Bibliography and citations

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A. Appendix

Bioethanol: ^{1}H-NMR analysis of the solvent utilized in cannabidiol (CBD) extraction.

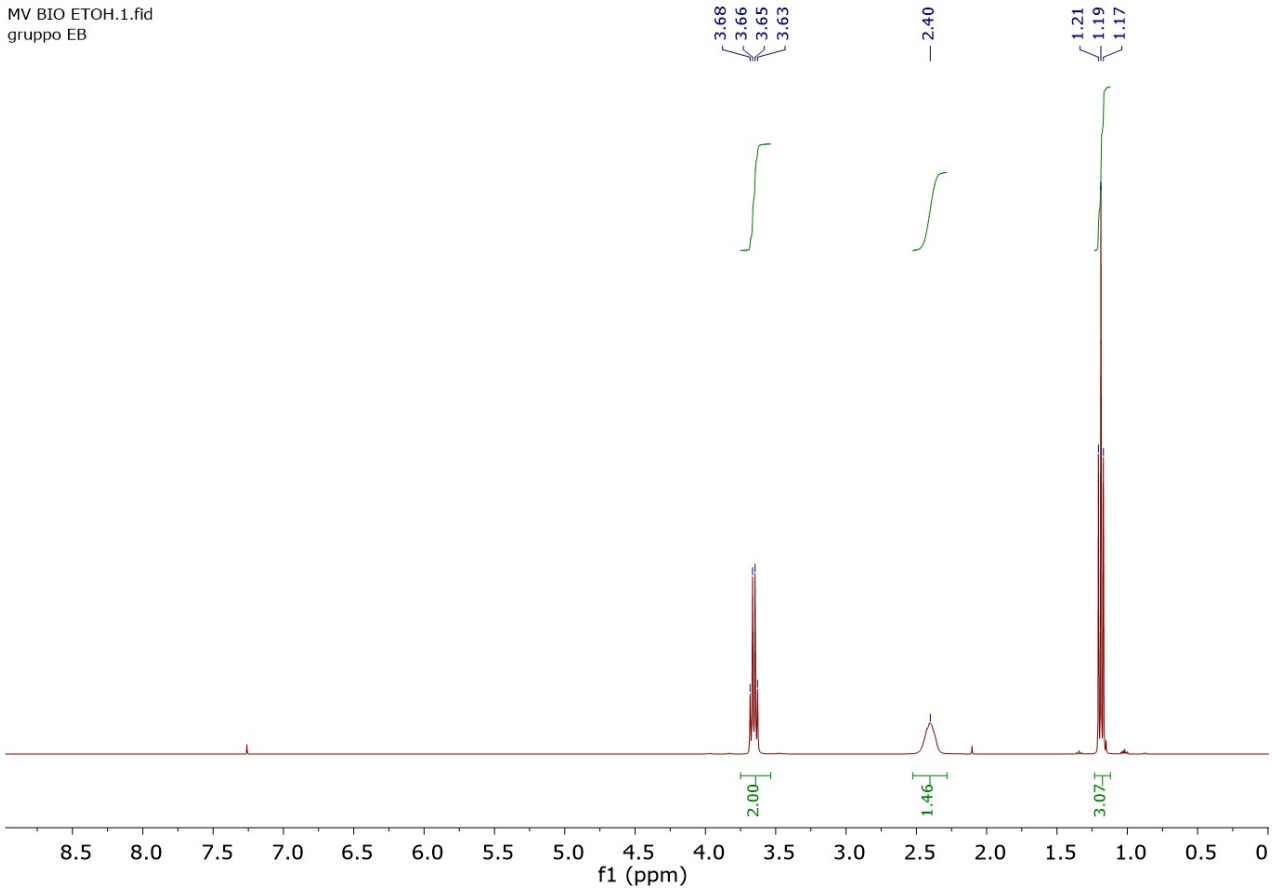

Figure 30: Bioethanol, ^{1}H-NMR in $CDCl_3$, 400 MHz

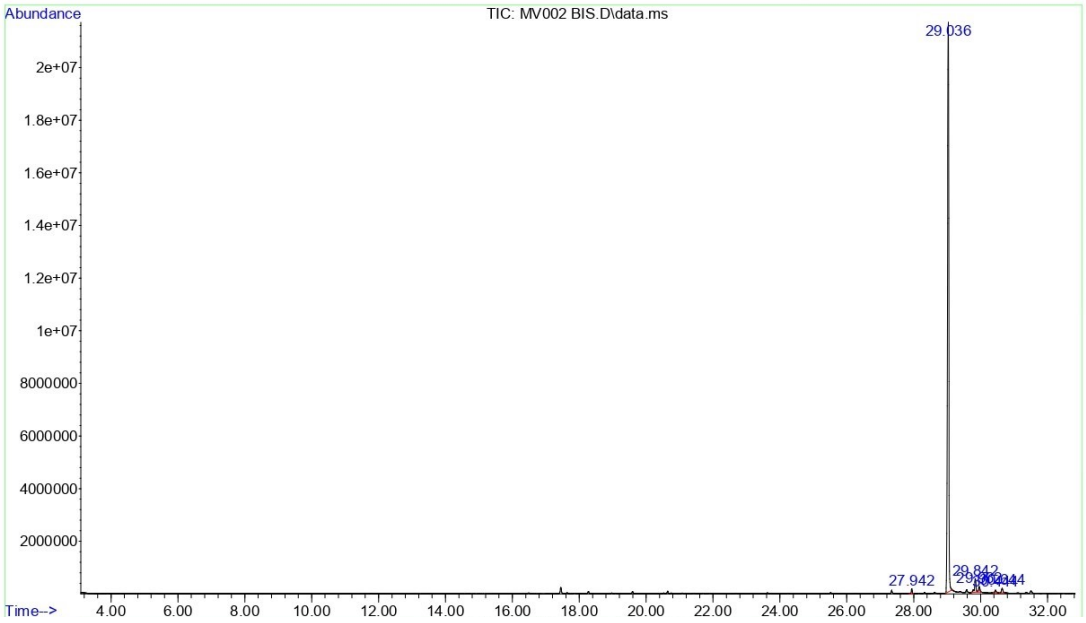

Figure 31: GC-MS of CBD obtained from fresh hemp

Cannabidiol (CBD)

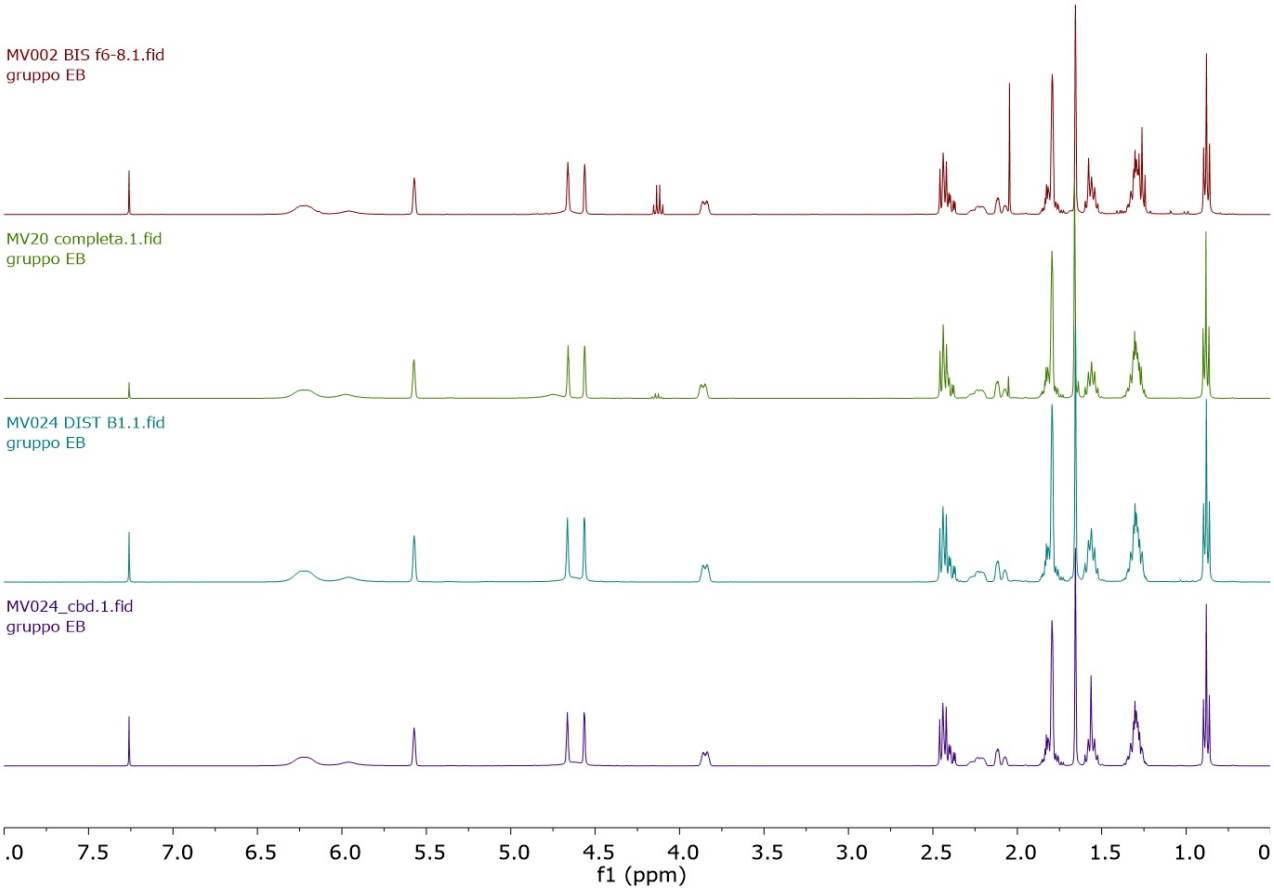

Figure 32: Comparison between cannabidiol (CBD) obtained from different sources in different methods ("MV002 BIS f6-8" is obtained from the fresh hemp and purified by chromatographic separation, "MV020 completa" is obtained from the model source and purified by chromatographic separation, "MV024 DIST B1" is obtained from the model source and purified by distillation, "MV024 cbd" is obtained from the model source and purified by winterization), ^{1}H-NMR in $CDCl_3$, 400MHz

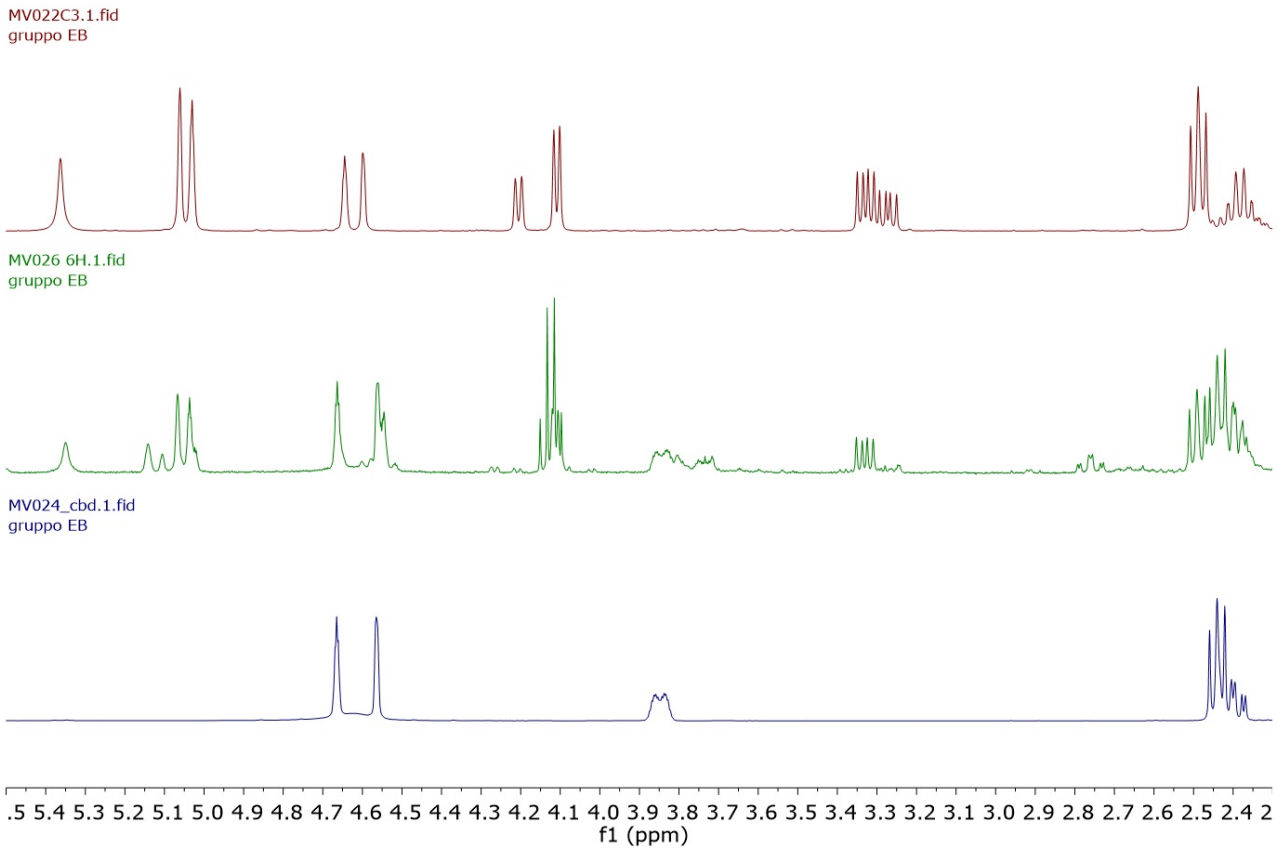

Figure 33: Comparison between purified cannabielsoin (CBE) with two diastereoisomers (MV022C3), crude CBE with only one diastereoisomer (MV026 6H) and purified CBD (MV024 cbd), ^{1}H-NMR in $CDCl_3$, 400 MHz

Cannabidiol (CBD): complete characterization by NMR and GC-MS

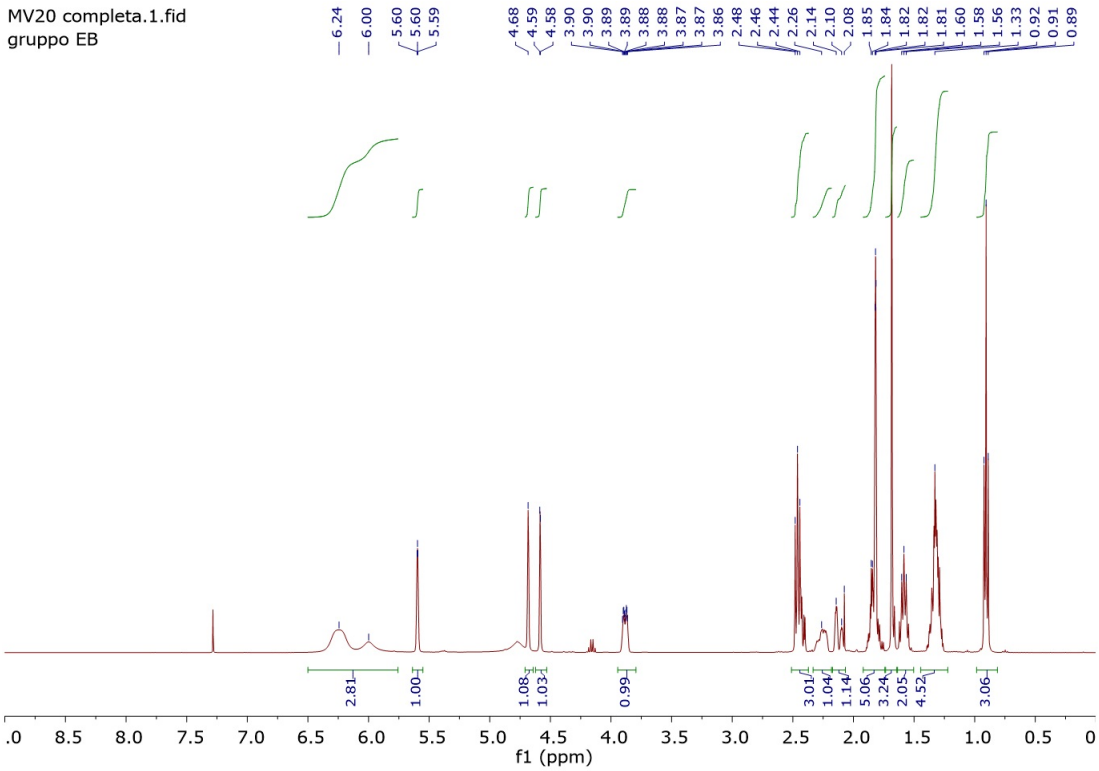

Figure 34: Cannabidiol (CBD), ^{1}H-NMR in $CDCl_3$, 400 MHz

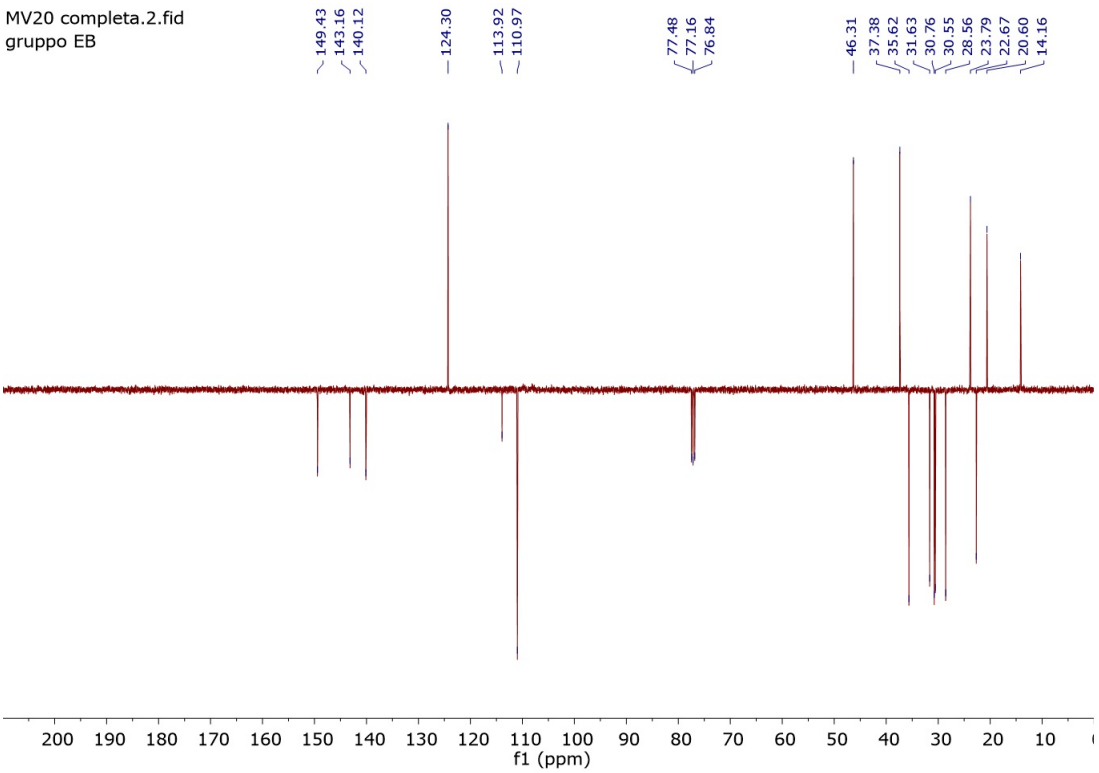

Figure 35: Cannabidiol (CBD), ^{13}C-NMR in $CDCl_3$, 101 MHz

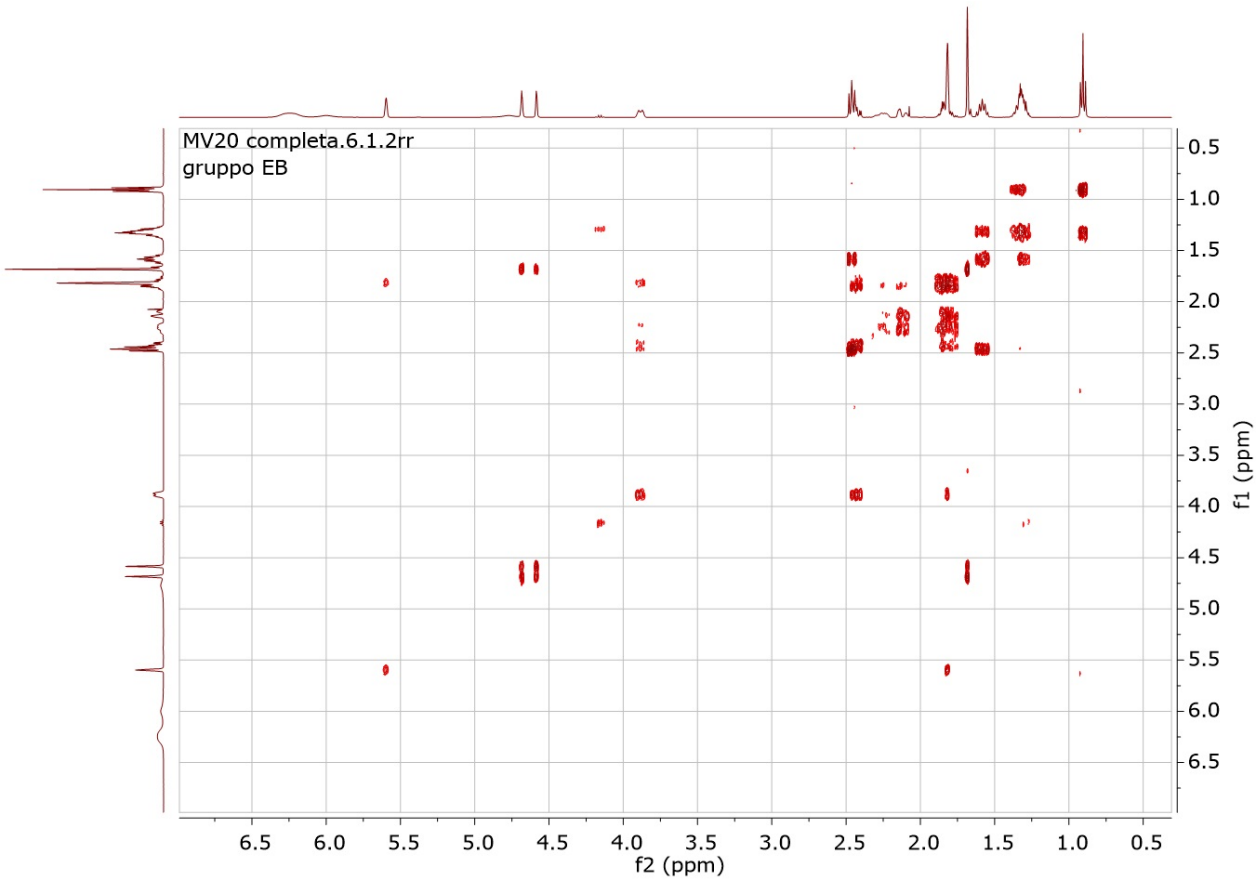

Figure 36: Cannabidiol (CBD), ^{1}H-^{1}H COSY in $CDCl_3$

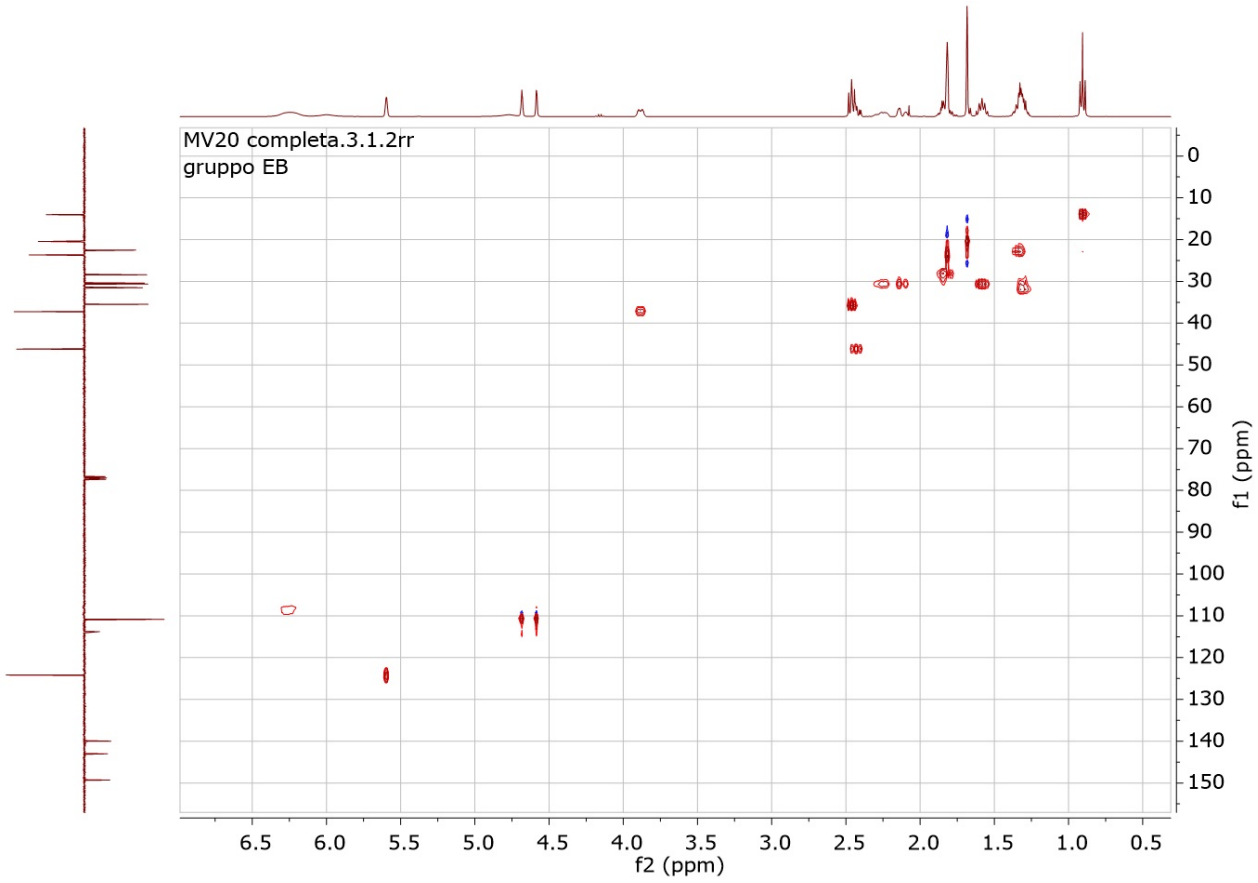

Figure 37: Cannabidiol (CBD), ^{1}H-^{13}C HSQC in $CDCl_3$

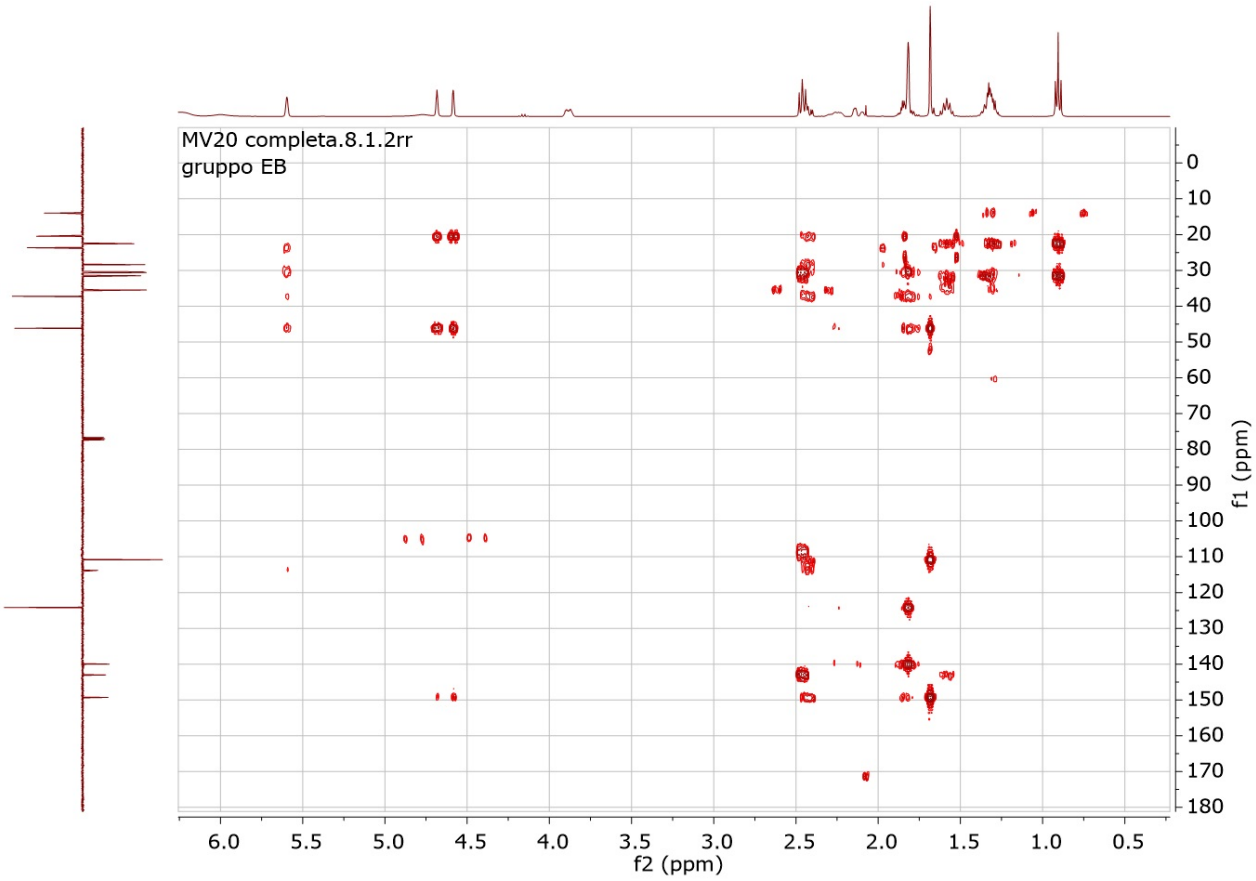

Figure 38: Cannabidiol (CBD), ^{1}H-^{13}C HMBC in $CDCl_3$

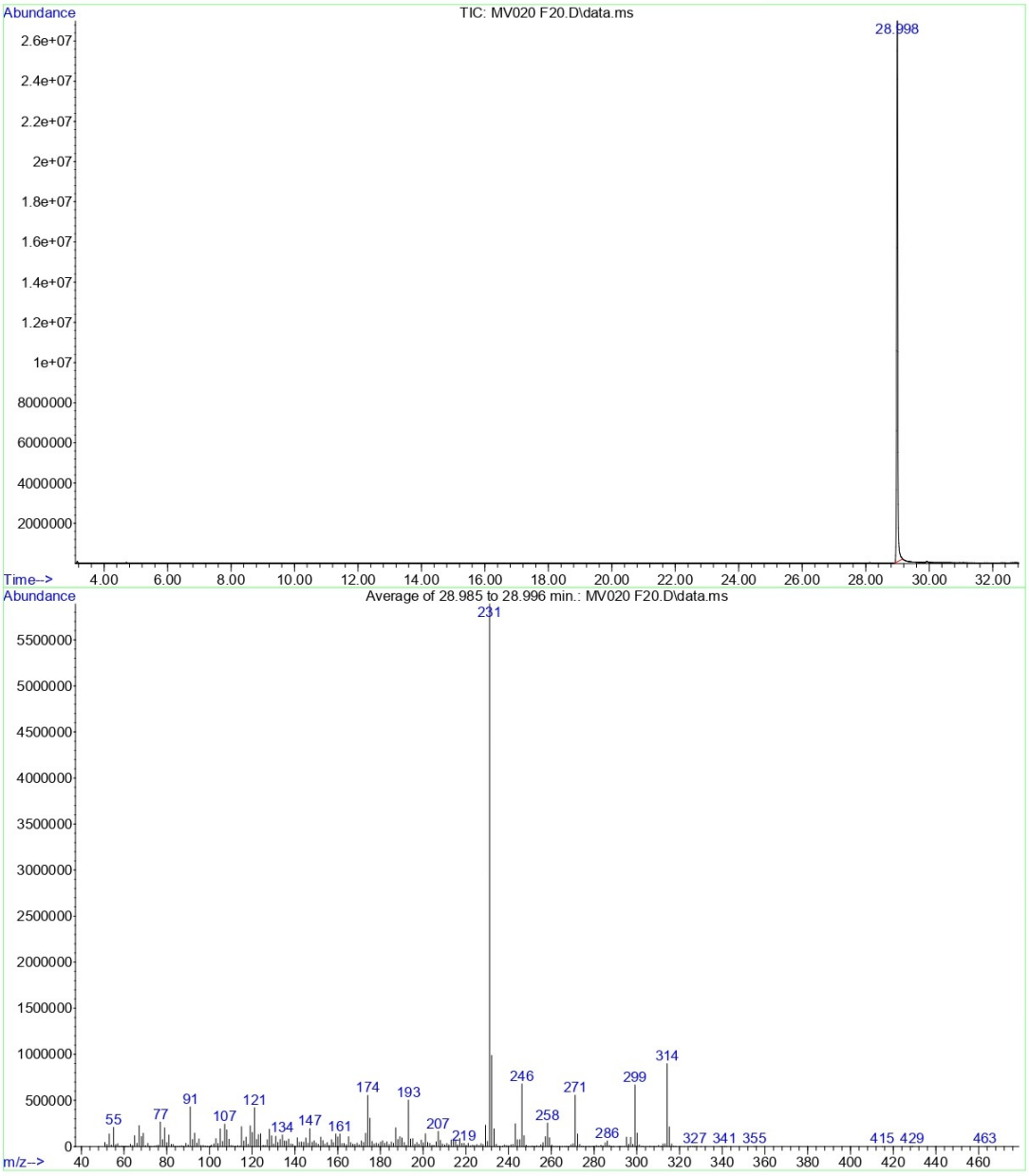

Figure 39: (Top) GC-MS of CBD purified, (bottom) MS of the peak of CBD at 28.99 min with FUGMS method

Cannabielsoin (CBE): ^{1}H-NMR, ^{13}C-NMR and GC-MS characterization

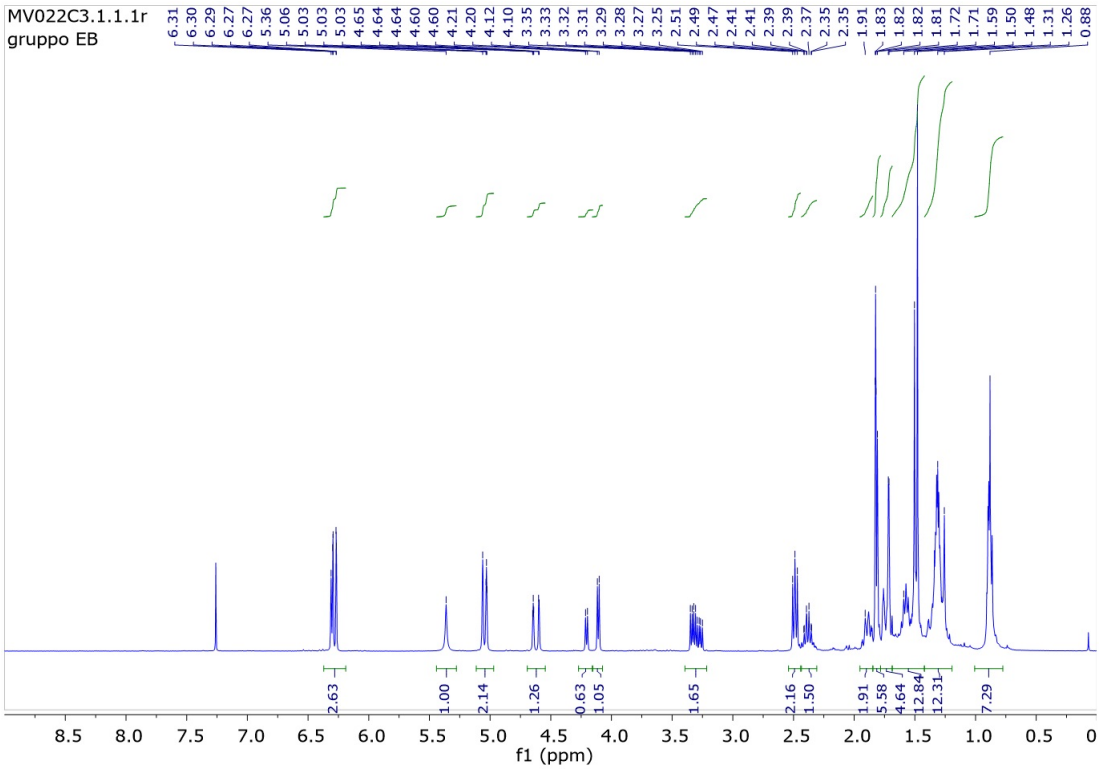

Figure 40: Cannabielsoin (CBE) purified with two diastereoisomers, ^{1}H-NMR in $CDCl_3$, 400 MHz

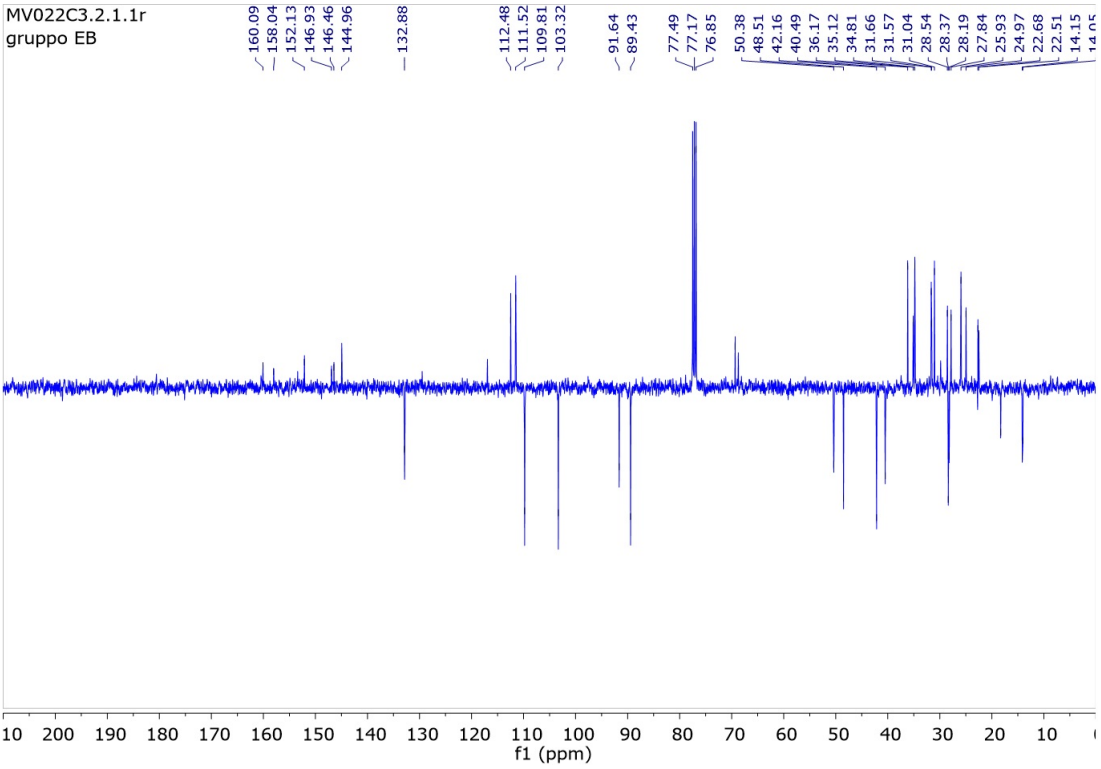

Figure 41: Cannabielsoin (CBE) purified with two diastereoisomers, ^{13}C-NMR in $CDCl_3$, 400 MHz

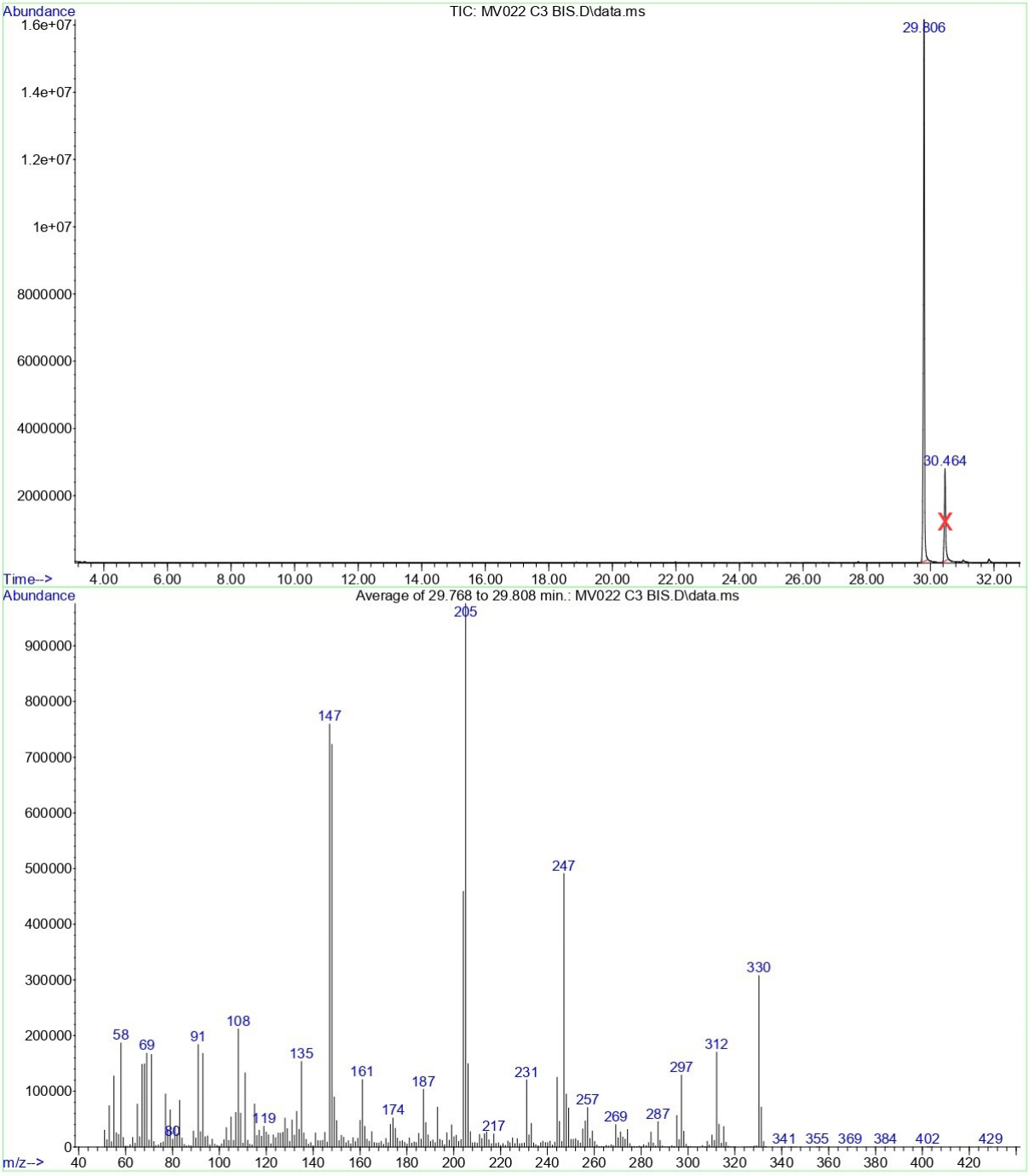

Figure 42: (Top) GC-MS of CBE purified, (bottom) MS of the peak of CBD at 29.8 min with FUGMS method

Abstract in lingua italiana

Questo progetto di tesi esaminerà l'estrazione e la funzionalizzazione del cannabidiolo (CBD), che è il componente più abbondante e di maggiore interesse farmaceutico nei rifiuti organici legati alla produzione di olio essenziale di *C. sativa* mediante distillazione a vapore. Tra i cannabinoidi presenti nella pianta di canapa, il cannabidiolo possiede proprietà biologiche benefiche e opera in sinergia con alcuni farmaci. Basandosi sulla letteratura, anche il prodotto della reazione di epossidazione di cannabidiolo potrebbe migliorare o sviluppare nuove interessanti bioattività. L'obiettivo di questo lavoro era quello di validare il metodo di estrazione sostenibile del cannabidiolo dai rifiuti organici derivanti dalla distillazione della canapa e di ottimizzare la reazione di ossidazione con una via enzimatica, coinvolgendo la lipasi Novozyme 435 come biocatalizzatore. Lo scopo finale era quello di purificare, isolare e caratterizzare completamente i prodotti del processo di ossidazione con particolare attenzione alle pratiche sperimentali sostenibili massimizzando la resa di reazione per il prodotto più interessante. Per ottenere questi risultati e contemporaneamente risparmiare tempo e risorse, è stato applicato lo strumento statistico noto come Design of Experiments (DoE), che è un metodo alternativo rispetto alle tradizionali tecniche sperimentali monovariate utilizzate in chimica organica, per comprendere meglio l'andamento della reazione. In conformità con i principi dell'economia circolare, il recupero del composto più prezioso (CBD) dai rifiuti agricoli e la sua successiva valorizzazione biocatalitica è essenziale per lo sviluppo sostenibile dell'industria chimica e ha portato a ottenre un prodotto con un altro valore aggiunto, con caratteristiche superiori e una potenziale elevata importanza medica.

Parole chiave: cannabidiolo, biocatalisi , Novozyme435, rifiuti agroalimentari, economia circolare, DoE