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Executive Summary of the Thesis

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

Laurea Magistrale in Food Engineering - Ingegneria chimica

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## 1. Introduction

In the last few decades, the increase in world population and its needs highlighted the criticalities of the existing industrial system, therefore a process of rethinking production has begun in order to integrate the existing technologies and direct them toward greater long-term sustainability. A manufacturing system based on the extraction of raw materials and their utilization until the final disposal has become unacceptable in the modern scenario. For that reason, starting from the 1970s, the vision of a **circular economy** was proposed by many economists and researchers. 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 [1]. Nowadays, the 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 by observing three pillars:

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

A visual representation of the possible actions that can be implemented to achieve these objectives is presented in Figure 1.

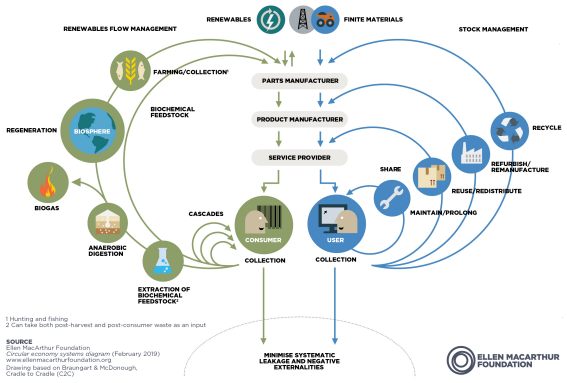


Figure 1: Butterfly iconography for circular economy as proposed by the Ellen MacArthur Foundation

One of the first feasible options to follow the circularity fundamentals for what concerns the biological product is the extraction of biochemical feedstocks; indeed, it is possible to obtain high-value compounds from biomass. Since it appears as a potential industrial opportunity, process plants such as biorefineries have been

developed, in which organic matter is fractionated and converted into a spectrum of valuable products, based on the petrochemical refinery concept. The biorefineries are in fact an example of the modern adaptation of already existing technologies for a new sustainable objective in accordance with all the circular economy pillars.
Similar ideas applied to the chemistry field and based on a triple-bottom-line sustainable approach are promoted by the twelve principles of **green chemistry**, but they are more oriented to the design of chemical products and processes to reduce the use and generation of hazardous substances. A difference is that circular economy is more widely applied to business models for its intrinsic economic orientation, while green chemistry implies multiple issues and is commonly seen as a final process certification.
However, strict adherence to these twelve guidelines guarantees increased process efficiency while lowering the environmental burden of chemical synthesis and the associated wastes.
In this situation, green chemistry principles have opened up a new world of possibilities and among these **biocatalysis** is gaining ground to be one of the most environmentally friendly technologies, which is predicted to revolutionize the chemical manufacturing system through higher synthetic efficiency in a low-emission, sustainable and more innovative economy. The use of enzymes and microorganisms as catalysts is in fact strongly related to green chemistry and so to circular economy [2]; it emerged rapidly and it offers many attractive features in the industrial context. A few examples are:

1. the substitution of wasteful chemical methodologies with more atom- and step-economical catalytic alternatives;
2. the reduction in the use of solvents, replaced by aqueous media;
3. the lower energy consumption since enzymatic reactions occur at mild temperature, and pressure;
4. the simplification of processes thanks to the high chemo-, regio-, and stereo-selectivities of enzymes which do not require group activation, protection/deprotection steps and reduce the downstream purification processes.

A solution to overcome biocatalyst disadvantages, such as activity inhibition or thermal instability, has been found in the immobilization process. That strategy allows to decrease enzyme costs while increasing their features: since immobilized enzymes are more resistant, can be recycled with the proper technology and further reutilized in processes.
In this project thesis, the sustainable ideas of circular economy, green chemistry and biocatalysis have been applied to the valorization of waste of the *Cannabis sativa* essential oil production. The agricultural residue derived from steam distillation process was mostly destined for waste-to-energy use or it was returned to fields as fertilizer when the nitrogen content allows it; otherwise, the final waste disposal represents a production cost. However, the cannabis plant is rich in high-value compounds (Figure 2), which could be extracted after distillation.

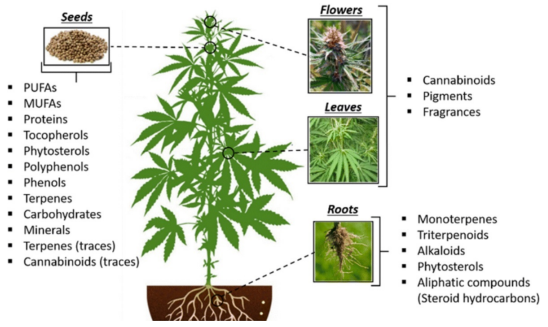


Figure 2: Valuable compound in cannabis plant

Cannabinoids are the most valuable compounds with proven pharmaceutical properties and they are still contained in the cannabis plant even after the distillation process due to their low water solubility and low volatility with respect to essential oils. Therefore, adhering to the circular economy principles, the valorization of industrial waste was investigated.
Cannabidiol (CBD) is the most abundant cannabinoid contained in hemp and is also the most medicinally studied one, in fact it has proven beneficial effects on human health [3]. Moreover, hemp seeds are utilized as a supplement in hemp-based food due to the nutritional properties related to the content of fatty acids, proteins and vitamins, and the anti-inflammatory advantages that CBD content and other compounds bring to the final consumer. As consequence, the hemp-based global food market had a significant growing trend and is predicted to achieve 7.08 billion of USD in 2027.

Due to the high interest from the pharmaceutical and food sectors, according to the first and second pillars of the circular economy, this project thesis utilized the hemp agricultural waste derived from essential oil production to perform the CBD extraction. Then, following the green chemistry and biocatalysis principles, was investigated the CBD functionalization involving the commercial lipase Novozyme 435.
As an alternative method to the traditional monovariate experimental approach, the Design of Experiments (DoE) was also applied. It consists of a statistical method that can be applied both in a preliminary screening phase and in a more advanced optimization phase; it aims to obtain the majority of reaction insights by setting the minimum number of experiments. It was utilized in order to save resources and time. The selected DoE with Full Factorial Design helped to define a chemical space in which are known the impact of the chosen variables in the studied range of value. The result was the knowledge of the reaction trend in the studied conditions, otherwise difficult to derive involving traditional methods.

## 2. Purpose of research

The purpose of this thesis project is to optimize the extraction methods on the residue of cannabis essential oil 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

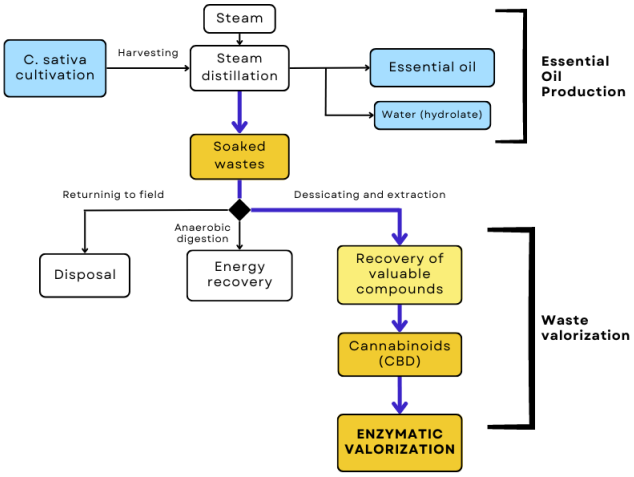


Figure 3: From essential oil to waste enzymatic valorization. Black lines=current method, Blue lines=this project

Figure 3 highlights the current practice and the new processes described in this work. The initial step of this work was CBD isolation and purification. Starting from the soaked waste of *C. sativa* essential oil production, the recovery of the most valuable component (CBD) has been performed through ethanol extraction. Then, maintaining the CBD scaffold, structural changes of aromatic ring substituents or terpene group have been studied in order to upgrade the initial molecule and to obtain a final substance with increased pharmaceutical activity or other beneficial properties.
The starting point for CBD functionalization was a detailed analysis of the numerous chemical cannabidiol derivatives reported in literature. In Figure 4 are reported some examples of the possible feasible reactions, and the demonstrated bioactivity of the products.

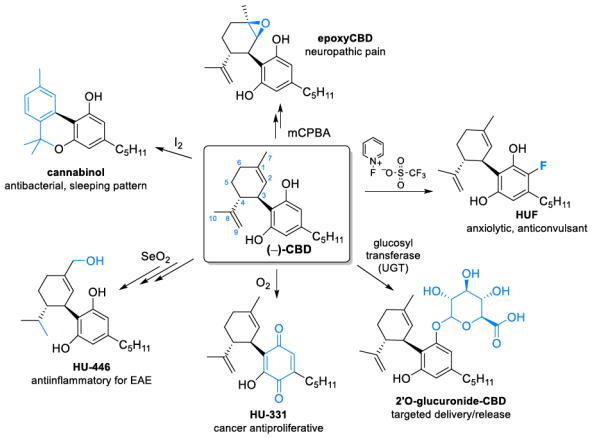


Figure 4: CBD chemical derivatives and their therapeutic effect

Of these options, it was decided to focus on the epoxy-CBD (CBO) synthesis considering its reported effect in the treatment of neuropathic pain [4]. Moreover, the epoxide functional group is highly versatile for additional chemical functionalization (e.g. diols, aminoalcohols, etc.).
Due to the high relevance for synthetic chemists, pharmacologists, and biologists, it was decided to investigate the CBO synthesis involving a biocatalyst aiming to obtain an environmentally-friendly alternative to chemical synthesis. Indeed, in literature CBO has been prepared only through the involvement of toxic or harmful reagents in extreme conditions, while this project studied enzymatic pathways in mild conditions.

## 3. Results and Discussion

This project is the continuation of previous work about extraction optimization on the same hemp waste lot and valorization of the most abundant isolated molecule (CBD). Therefore, the inherited information was our starting point.
Regarding CBD extraction, the previous optimization process evaluated different solvents as possible extraction solutions. The best result obtained through >2 hours of dynamic maceration of 2 g of fresh sample in 50 mL of various solvents, are reported in Table 1.
As visible, ethanol (EtOH) was the most effective solvent and it was utilized in this work also since it is an organic solvent with a low environmental footprint. The use of hardware-shore-grade derived bioethanol instead of reagent-grade ethanol did not show a significant difference.

| MeOH | **EtOH** | $CHCl_3$ | n-heptane |
|---|---|---|---|
| 10.8 | **12.4** | 10.7 | 9.8 |

Table 1: Highest yield of cannabinoid extracted, as mg of CBD for g of sample [mg/g]

In this project, following the optimized protocol for CBD extraction, after overnight maceration in bioethanol, the extraction solution was filtered and evaporated under reduced pressure in a rotary evaporator. Then GC-MS analysis was utilized to determine the extracted compound; in parallel, TLC was utilized to identify also the compounds undetectable by GC-MS, such as waxes. Then, automated chromatographic separation was performed on silica gel to ensure the repeatability of operations and the saving of solvents. To identify the correct fraction output of the purification silica gel column containing CBD were both used GC-MS and TLC with FastBlue staining. As final verification of product purity, $^1$H-NMR analysis was performed confirming the identity of CBD in >95% purity.
The agricultural matter utilized could be categorized into two groups:

1. Fresh hemp inflorescences;
2. Fresh fibrous post-distillation waste.

The maceration of 20g of fresh inflorescences was performed in bio-EtOH and it provided a dried crude extract of 740mg. After purification were obtained 550mg of product, that GC-MS and $^1$H-NMR analysis confirmed to be cannabidiol. Compared to the result reported in Table 1, was achieved a great yield equal to 27.5 mg,CBD/g,sample. Further extraction resulted in no additional recovery of the CBD molecule since most of the residue was composed of stems and seeds, which are the lowest CBD-rich part of the plant. Therefore, was necessary to change the extraction source. Starting from 20g of fresh fibrous residue, were obtained 814mg as crude extract and 39mg of purified product. The final outcome analyzed in GC-MS still contained many impurities and their separation was very problematic because CBD and impurities had similar polarity. Even if there were enough quantities of distillation residue to continue in the extraction processes, it was decided to don't keep it on because was not profitable with respect to the solvent consumption and the relative costs.
Since the climate conditions of the 2022 summer season had compromised the annual hemp harvesting for the essential oil price fluctuation, these phenomena affected the hemp replenishment. In order to have enough CBD quantities to investigate the molecule functionalization, was necessary to find an alternative CBD source. For that reason, was decided to purchase a commercial oil with a high CBD content and utilize it as the model source from which to obtain the target molecule. The claimed CBD content in the oil was 6000mg in 30mL, which was also confirmed by GC-MS analysis using acetophenone as the internal standard. 2 mL of crude oil was purified by automated chromatography affording 411 mg of isolated CBD, which was fully characterized by GC-MS, $^1$H-NMR, $^{13}$C-NMR study as well as bidimensional COSY and HSQC NMR analysis.
Then, other separation techniques were also tested to obtain CBD from the model source involving:

- Kugelrohr apparatus for horizontal distillation;
- winterization process at 4°C for approximately 10 weeks with a stationary position of the sample.

The distillation process obtained pure CBD in a few hours. On the other hand, the winterization process resulted in pure CBD crystallization

but required industrially impracticable time. In both cases, $^1$H-NMR analysis confirmed that the CBD structure was not affected by the utilized purification method and even the source from which it was obtained had any influence.
Since the CBD from agricultural waste and from the alternative model source were identically recognized at $^1$H-NMR analysis, was decided to utilize them without any distinction for the study of CBD functionalization.

Therefore, with CBD available in sufficient amounts started the functionalization study. Based on the literature information on the selected CBD epoxidation, CBO synthesis was reported involving hydroxyl group protection and deprotection in rather extreme experimental conditions [5]. The synthesis was also performed with harmful reagents and low atom efficiency due to the use of mCPBA for the incorporation of one oxygen atom only.
The sustainable alternative applied to carry out the CBO synthesis was investigated by employing $H_2O_2$ as oxygen source in ethyl acetate solution, with the crucial involvement of the commercial immobilized lipase B Novozyme 435, derived from *Candida antartica*, in mild condition. A deeper analysis of the perhydrolysis reaction mechanism mediated by lipase reported in Figure 5 [6], suggested that the peroxyacetic acid build-up could lead to side reactions, which decreases the selectivity of the target process.

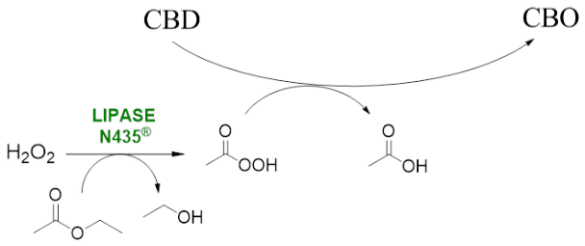


Figure 5: Lipase perhydrolysis mechanism in the studied reaction conditions

The experimental test was performed following the literature method for lipase perhydrolysis as a sustainable alternative for chemical synthesis. The pilot test performed on 75 mg of purified CBD at 30°C for 18 hours afforded complete conversion after the quenching to neutralize the residual $H_2O_2$ and peroxyacids content. Then the organic phase was separated, dried and concentrated through evaporation under reduced pressure. The GC-MS analysis revealed the generation of the target product (MM=330 g/mol), which resulted from the addition of an atom of oxygen to CBD. However, other compounds were detected in small amounts. The purification by chromatographic separation of 70 mg of the crude reaction product from 75 mg of CBD substrate afforded 21 mg of the target product, isolated and analyzed at $^1$H-NMR. This first experiment revealed that the CBD scaffold was maintained, but the target alkenes signal was absent; comparing it to the GC-MS output, was deduced that CBO was obtained. However, the reaction was not optimized and further experiments were set to achieve better results.
Initially, was tested if hydrogen peroxide could degrade CBD in the reaction solution. So, a test with only CBD, $H_2O_2$, and ethyl acetate was performed. After 5 hours under magnetic stirring agitation, the GC-MS analysis confirmed that no reaction occurred; therefore, could be claimed that CBD did not degrade in the reaction mixture without enzyme activity. As consequence, the subsequent reactions were set in presence of the enzyme; the other experimental parameters were changed and were observed the effects. By monitoring the reaction with periodical withdrawals, the GC-MS analysis revealed the occurrence of the reaction, but with some selectivity problems probably due to the build-up of peroxy acid. It was therefore decided to saturate EtOAc with a phosphate buffer pH 7.5, due to the optimal pH range in which the enzyme works and to protect at the same time the CBD derivatives dissolved in the reaction solution. It seemed to bring a beneficial effect on the reaction yield but was not the crucial aspect thanks to which reaction yield optimization was achieved. Then, the enzyme quantity was decreased from 2 mg to 0.8 mg: it resulted in a very low reaction rate with a positive effect on selectivity, but after 22 hours the complete CBD conversion led anyway to several side products, as revealed by $^1$H-NMR analysis. Further parallel tests investigated the optimal reaction temperature comparing one reaction at 45°C and one at 4°C. They revealed an unexpected result: Novozyme 435 has a high activity also at very low temperatures resulting in higher selectivity even at complete CBD conversion. From this preliminary test, the product was isolated and

purified, in order to perform a full characterization by NMR.
For the first time, it was observed that the NMR spectra of the product obtained could not match the structure of CBO. Despite the GC-MS analysis indicating that the final product has the correct molecular weight (330 g/mol), a more accurate $^1$H-NMR analysis proved that a different cannabinoid structure had been obtained. Indeed, the spectral data matched with those of cannabielsoin (CBE), a cannabinoid with a 5-membered ring structure. It was confirmed by a literature report [6], which also claimed that the biological study regarding CBO biological activity [4] was instead referred to CBE due to the same misleading structural assignment met by Nally Y. *et. al.* researchers. In fact, the high epoxide reactivity combined with the nonprotected CBD phenolic groups could lead to the generation of the regioisomer CBE (Figure 6), as also shown in a recent publication [7] about CBD biocatalytic functionalization.

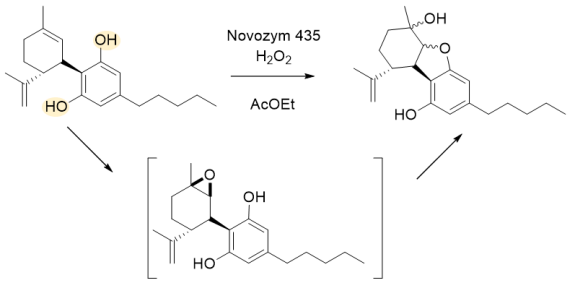


Figure 6: Hypothesized CBD epoxidation pathway

With this approach, however, the obtained product appeared as a mixture of two diastereoisomers of CBE. Thus, based on the assumed reaction trend derived from the previous experiments, were tested two parallel reactions in identical conditions except for the enzyme loading. The result was that the reactions occurred at different rates, but with the same reaction products. The main issue was that the reaction selectivity appeared strictly coupled to conversion even before a high CBD consumption, compromising the final yield. Therefore, an alternative strategy was needed to obtain the highest amount of CBE from CBD selective oxidation in experimental conditions able to afford high selectivity of the occurred reaction.

## 4. Design of Experiment (DoE)

Since a clear reaction trend was not derived, it was decided to use the design of experiment (DoE) to optimize the reaction. The innovation introduced by DoE was strongly related to the multivariate approach. Indeed, 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. With respect to the traditional monovariate approach, its application results in the definition of an experimental region in which the considered variables in the considered range have a well-established relevance. The DoE was defined with the objective to establish the optimal conditions of the enzymatic reaction.
To achieve that goal, based on the preliminary experimental data, four variables of interest were defined (Temperature (T), Enzyme quantity (E), Hydrogen peroxide volume ($H_2O_2$), time (t)) and their relative extreme values, as reported in Table 2.

| Extremes | T [°C] | E [mg] | $H_2O_2$ [eq] | t [h] |
|---|---|---|---|---|
| Min (-1) | 4 | 4 | 1.1 | 2 |
| Max (+1) | 24 | 8 | 4 | 6 |

Table 2: Extreme value of the selected variables investigated in DoE

Applying a full factorial design, the set of $2^4$=16 experiments in multivariate conditions was defined. It was the chosen design since considering only four variables was a problem simplification, but the resulting number of experiments was feasible and from them complete information could be obtained. Moreover, assuming that the withdrawals of 40$\mu$L from 5 mL of reaction solution did not affect the reaction trend, the number of experiments was halved avoiding to perform two identical experiments and stop them at different times. Therefore, 8 experimental conditions were set up: each one was subjected to sampling at 2 and 6 hours and then was stopped, saving time and resources; also 4-hour-withdrawals were performed to obtain additional data for model validation. As system responses, it was decided to utilize the GC-MS peak area of the detected compounds. $^1$H-NMR analysis was also

performed for each experimental crude product at the end of the considered reaction time (6 hours). The experiments were set up with the identical procedure, on different days to avoid the "block effect" and some stock solutions for CBD and the work-up solution were prepared in order to decrease the experimental variability. The CBD stock solution contained n-decane as an internal standard utilized to compensate potential slight dilution errors.
The elaboration of the GC-MS data was performed by normalizing the peak areas with respect to the internal standard the conversion and selectivity as follows:

- 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 sample at time t and the reference internal standard area at t in the same 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 the target product and the relative internal standard area at t in the same sample, while $A_0$ and $A_t$ were calculated with the same formula utilized for the conversion:

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

  In this way, selectivity quantifies how much target product (CBE) was generated with respect to the consumed reagent (CBD).

Preliminary data management was performed on Microsoft Excel and it was immediately noticed that only one reaction achieved complete conversion in six hours, then data was imported and elaborated through Chemometric Agile Tool (CAT) affording Lenth's plots which show the impact of variables on the selected outcomes (Figure 7).

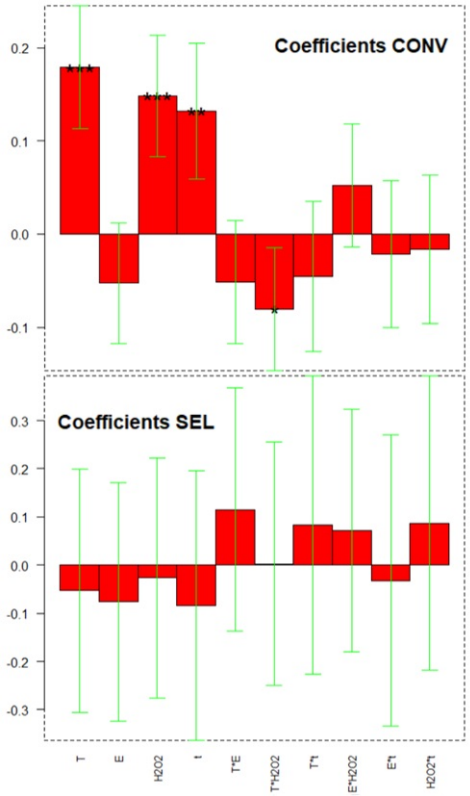


Figure 7: Lenth's plot of calculated coefficient of variable affecting conversion (top plot) and selectivity (bottom plot)

From these plots, it is evident that the conversion elaboration was the most reliable since it has narrower confidence intervals, while the selectivity response factor had rather low significativity since all the selected variables seemed irrelevant. Focusing on the graphical representation of the variables which affect the conversion (Figure 7), it was clear that the conversion is positively influenced by time as expected and the other relevant parameters for conversion were T and $H_2O_2$, both with positive effects on the response factor. Instead, the amount of enzyme involved was not relevant in the range investigated in this study; therefore, in scale up that value could be minimized resulting in lower costs. These observations are confirmed by the unique reaction that achieved complete conversion, which was set with high T, low E, and high $H_2O_2$ values.
Considering the interaction between variables, T-$H_2O_2$ was the most important relationship due to the high relevance of T and $H_2O_2$ even when considered individually. To evaluate the trend of that variable interaction the response surface T-$H_2O_2$ was analyzed with respect to the

conversion (Figure 8).

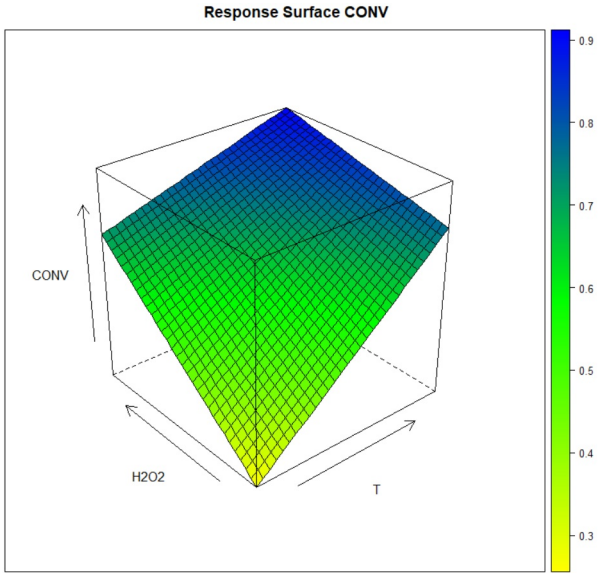


Figure 8: Response surface of temperature-hydrogen peroxide affecting CBD conversion

It represents a reaction trend that could be predictable, but here is mathematically calculated and experimentally proved. It was in fact shown that the highest conversion value was obtained with high T and high $H_2O_2$, while keeping only one variable at its highest value and the other at its minimum value gives elevated conversion values, but they are far from the optimal situation. That absolute optimum was obtained and proved due to DoE properties; on the other hand, a monovariate approach could just suspect it and further experiments would be necessary to confirm it.
In conclusion, the GC-MS derived data and their elaboration allowed us to state that in the considered range, 1) the variable E did not affect both conversion and selectivity, 2) the other parameters (t, T, $H_2O_2$) positively influenced the conversion, but made the selectivity response not significant, therefore was needed to find a compromise.
Analyzing the final $^1$H-NMR spectra of the crude reaction product obtained in six hours, a mathematical elaboration was difficult. The final mixture contained CBD, CBE, and other cannabinoids whose peaks were superimposed, therefore precise compound quantification was not possible. For sure, it was established that the studied enzymatic reaction afforded CBE instead of the initial target product CBO (as evidenced in the diagnostic region between 3.25 and 3.80 ppm). Due to the nature of the data, only a qualitative analysis was possible. From that, it could be concluded that hydrogen peroxide was probably the main variable responsible for impurities generation, while temperature and enzyme high values seemed to generate fewer impurities probably due to the increased selectivity of the enzyme.

In conclusion to this work, the comparison between the most selective DoE reaction and the reference chemical synthesis of CBE [6] was performed (Figure 9). Both reactions started from isolated CBD and occurred for 40 hours.

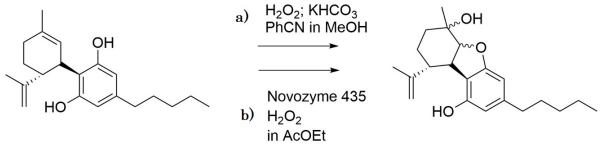


Figure 9: Comparison of the chemical (a) and the chemo-enzymatic (b) synthesis of CBE from CBD

Independently of chemical or enzymatic methods, the studied CBD reaction resulted in CBE. The advantage of the enzymatic pathway is that it involved a biocatalyst, in mild conditions and required an easy work-up, that is also industrially scalable inexpensively. On the other hand, the chemical synthesis needed the utilization of benzonitrile, which is harmful to human health and to the environment, and the necessity to set the experiment for 40 hours under argon made it less industrially attractive despite its higher yield (58%) compared to the enzymatic method yield (28%).

## 5. Conclusions and future developments

In this project, firstly the extraction of CBD from industrial organic waste was validated and optimized using a simple extraction method with an environmentally benign and sustainable solvent (bioethanol). An average yield of 10-15 mg of CBD per gram of waste could be achieved. When replenishment problems occurred due to seasonal fluctuation, it was also confirmed that the CBD extracted from an alternative source was not affected by the origin of the material or the extraction method (chromatography,

distillation, crystallization). Then, a chemoenzymatic selective oxidation of CBD was studied and developed. After a traditional monovariate experimental investigation, DoE was applied to optimize the reaction condition to synthesize CBE, a naturally occurring cannabinoid derivative with interesting bioactivity. The DoE-derived reaction trend opened new research options depending on the selected reaction configuration (batch or flow) and on the final aim (complete conversion or high selectivity). Indeed, in future experiments, the reaction configuration could be changed from batch to continuous flow reactors: keeping high temperature and low residence time, selectivity could be improved further. However, if the CBD conversion could not be complete, it would be necessary to develop a method to separate the unreacted CBD in the final reaction mixture to recirculate it in feedback. Despite CBE was not the initial target molecule, its isolation and characterization represent an opportunity to seize since it is a not deeply studied cannabinoid and recent publication [6] claimed that the therapeutical properties referred to CBO [4] are instead proper of CBE, enhancing the pharmaceutical interest for the obtained molecule. Further optimization of CBE synthesis and its medicinal applications are left to future researchers.

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