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Titre: Title: Development of Bioactive Cannabis Extracts Through Optimization of Green Supercritical Fluid Process

Auteur: Author: Hinane Boumghar

Date: 2023

Type: Mémoire ou thèse / Dissertation or Thesis

Référence: Citation: Boumghar, H. (2023). Development of Bioactive Cannabis Extracts Through Optimization of Green Supercritical Fluid Process [Mémoire de maîtrise, Polytechnique Montréal]. PolyPublie. https://publications.polymtl.ca/10816/

URL de PolyPublie: PolyPublie URL: https://publications.polymtl.ca/10816/

Directeurs de recherche: Advisors: Daria Camilla Boffito, Gregory Scott Patience, Boumghar Yacine, & Xavier Banquy

Programme: Program: Génie biomédical

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POLYTECHNIQUE MONTRÉAL

Affiliée à l'Université de Montréal

Development of bioactive cannabis extracts through optimization of green supercritical fluid process

HINANE BOUMGHAR

Institut de génie biomédical

Mémoire présenté en vue de l'obtention du diplôme de *Maîtrise ès sciences appliquées*

Génie biomédical

Janvier 2023

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Ce mémoire intitulé :

Development of bioactive cannabis extracts through optimization of green supercritical fluid process

présenté par **Hinane BOUMGHAR**

en vue de l'obtention du diplôme de *Maîtrise ès sciences appliquées*

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ACKNOWLEDGEMENTS

To Yacine, my co-supervisor and cousin, your expertise and refined knowledge in the area gave me a boost to start working with the industry, and the mental support you provided me made me a stronger woman than I was at the beginning, thank you is not enough. I am also very grateful for the opportunity you offered me to work in CEPROCQ's laboratories, surrounded by smart and talented scientists like Mathieu, Assia, Annie-Claude, Serge, Naima, Mounia, Svetlana, Sanaz and Smail, who inspired me at different levels. Nathalie, I would have burnt out without your help! I can say I have changed since I am here with you all. I learnt, grew and moved forward towards my dream life thanks to you.

Daria and Gregory, my supervisors, I wouldn't be here today without you, you believed in me, gave me a chance and supported me, I won't forget that I once thought to quit when times were difficult, and Professor Gregory reminded me that this is part of the process and I should never quit if I want to reach my goals. Thank you for making a place for me in your both research groups, EPIC and PACE, where I met very friendly people like Nooshin, Mina, Dalma, Paula, Christopher, Tugce, Nicolas, Fellipy, Song, Marie-Thérèse, Mahdi, Moha and Pierre who helped me when I asked for, and with who we enjoyed good time.

Leonardo Stella, my industrial and business mentor, thank you for supporting my project, you have always pushed me to give meaning to my work and to have a positive impact on society, you also taught me how business and industry work. I also learnt with you that serving and bringing value is at the heart of all success in life, I am blessed to have you by my side, and I wish to build greater projects with you.

This work was supported by the Natural Sciences and Engineering Research Council of Canada (stipend allocated to me via the NSERC-CREATE PREMIUM program). This research was undertaken, in part, thanks to funding from the Canada Research Chair program. Also, NSERC College and Community Innovation program - Innovation Enhancement grants (CÉPROCQ) the hosting laboratory, and Zollaris Laboratories Corporation the industrial partner, are both gratefully acknowledged.

To the amazing people around me, Nesrine, Djamel, uncle Tahar, Faouzia, Yacine, Mima, Sourour, Lamis, Nouha, Yesmine, Batoul, Hiba, Hadia, Ash, Badis, sharing love with you is one of the most beautiful parts of my life, I wouldn't survive without you during the hard times, thank you for believing in me and motivating me to pursue my dreams. Mom and Dad, when I see you proud of me, I find no words to describe how fulfilling it feels, Yanis, Yasmine, Yanis and my little nephew Adam, thank you for bringing joy to my life, this helps me to stay energetic and give it my all every day.

RÉSUMÉ

En raison du potentiel médicinal des substances chimiques actives pour traiter les patients atteints de maladies telles que la maladie d'Alzheimer, les maladies auto-immunes et le cancer, la recherche sur l'huile de cannabis s'est orientée vers le marché médical. Le traitement avec des cannabinoïdes comme le tétrahydrocannabinol, le cannabigérol et le cannabinol pour réduire la douleur neuropathique et activer le système immunitaire est le moyen le plus efficace de traiter ces maladies mortelles. L'absence de traitement industriel standard de ces composés bioactifs est à l'origine de l'échec de l'introduction des produits à base de cannabis sur le marché pharmaceutique. La contribution de cette étude vise à pousser plus loin l'optimisation des conditions d'extraction de ces cannabinoïdes. Les cannabinoïdes étant des composés non polaires, ils ont une grande affinité avec le CO_2 comme solvant, mais pas avec les autres composés polaires non nécessaires (polyphénols, terpènes).

Nous suggérons l'extraction au CO_2 supercritique comme traitement industriel afin de se rapprocher de la qualité pharmaceutique. Nous utilisons un plan d'expérience Box-Behnken à trois niveaux, et nous testons 27 expériences. Le débit de CO_2, la pression, la température et le temps sont les principaux facteurs examinés. Pour le THC, le CBG et le CBN, les conditions optimales sont 15 g/min, 235 bar, 55 °C et 2 h, mais 4 h pour le CBN. Une comparaison rapide avec l'extraction à l'éthanol montre que les extraits au CO_2 supercritique contiennent 24 % de cannabinoïdes de plus que l'extraction à l'éthanol.

ABSTRACT

Due to the medicinal potential of active chemicals to treat patients with diseases including Alzheimer's, auto-immune illnesses, and cancer, research on cannabis oil has shifted toward the medical market. Treatment with cannabinoids such tetrahydrocannabinol, cannabigerol, and cannabinol to reduce neuropathic pain and activate the immune system is the most effective way to treat these fatal diseases. The lack of standard industrial processing of these bioactive compounds is at the origin of the failure to get cannabis-based products into the pharmaceutical market. The contribution of this study aims to push further the optimization of these cannabinoids' extraction conditions. As CO_2 is non polar, it has a great affinity to cannabinoids, but not for other polar compounds such as polyphenols, terpenes.

We suggest supercritical CO_2 extraction as an industrial processing in order to get closer to the pharmaceutical grade quality. We use a three-level Box-Behnken design of experiment, and we test 27 experiments. The CO_2 flowrate, pressure, temperature, and time are the main factors examined. For THC, CBG, and CBN, the optimal conditions are 15 g/min, 235 bar, 55 °C, and 2 h but 4h for CBN. A quick comparison with ethanol extraction shows that the supercritical CO_2 extracts contains 24 % more cannabinoids than conventional ethanol extraction.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS III

RÉSUMÉ IV

ABSTRACT V

TABLE OF CONTENTS VI

LIST OF TABLES X

LIST OF FIGURES XI

LIST OF ABBREVIATIONS XII

CHAPTER 1 INTRODUCTION 1

1.1. Context 1

1.1.1 Decarboxylation 1

1.1.2 Supercritical CO_2 extraction 1

1.1.3 Purification 1

1.1. Problem statement 2

1.2. Research objectives 3

1.3. Thesis outlines 3

CHAPTER 2 CANNABIS PLANT 4

2.1 Geography of cannabis plant 4

2.2 Cultivars 5

2.3 Biology of cannabis plant 6

2.4 Cannabis compounds 10

2.4.1 Carotenoids 11

2.4.2 Terpenes 11

2.4.3 Flavonoids 11

2.4.4 Cannabinoids 12

2.5 Cannabinoid bioactivities 12

2.5.1 Cannabigerol 13

2.5.2 Δ^9-tetrahydrocannabinol 15

2.5.3 Cannabinol (CBN) 18

2.6 The relationship between cannabis use and processing technology 20

CHAPTER 3 LITERATURE REVIEW 22

3.1 Introduction 22

3.2 Definition and class of supercritical fluids 23

3.3 Extraction 24

3.4 Supercritical fluid chromatography 27

3.5 Supercritical particle design 28

3.5.1 Supercritical carbon dioxide as solvent 29

3.5.2 Supercritical carbon dioxide as antisolvent 30

3.5.3 Supercritical CO_2 as co-solute 31

CHAPTER 4 METHODOLOGY 33

4.1 Cannabinoid's extraction 33

4.1.1 Supercritical carbon dioxide extraction 33

4.1.2 Ethanol extraction 35

4.1.3 Microwave extraction 36

4.1.4 Ultrasound extraction 36

4.2 Design of experiments (BOX-BEHNKEN) 37

4.2.1 Introduction 37

4.2.2 Undertake an experimental project 37

4.2.3 Choice of the experimental plan 38

4.2.4 Types of experimental plans 39

4.2.5 Analysis of the experimental plan 40

4.3 Cannabinoid purification 42

4.3.1 Introduction 42

4.3.2 Solid-phase extraction (SPE) 43

4.3.3 Hydrophilic Interaction Liquid Chromatography (HILIC) 44

4.3.4 Supercritical Fluid Chromatography (SFC) 44

4.3.5 Fast Centrifugal Partition Chromatography (FCPC) 45

CHAPTER 5 ARTICLE1: OPTIMIZATION OF SUPERCRITICAL CARBON DIOXIDE FLUID EXTRACTION OF MEDICINAL CANNABINOIDS BY A BOX-BEHNKEN DESIGN OF EXPERIMENTS 47

5.1 Introduction 48

5.2 Material and methods 53

5.2.1 Plant material....53

5.2.2 Chemicals 53

5.2.3 Decarboxylation 54

5.3 Supercritical fluid extraction (SFE) protocol....54

5.4 Ethanol extraction 56

5.5 HPLC analysis 57

5.6 Δ⁹-THC concentration by fast centrifugal partition chromatography (FCPC)..57

5.7 Results and discussion 58

5.7.1 Decarboxylation 58

5.7.2 Supercritical extraction 60

5.7.3 Ethanol extraction 70

5.7.4 Recovery 70

5.7.5 Δ⁹-THC concentration by fast centrifugal partition chromatography (FCPC)..71

5.7.6 Optimization of the biomass-solvent contact. 72

5.8 Conclusions....73

5.9 References 75

CHAPTER 6 GENERAL DISCUSSION....80

CHAPTER 7 CONCLUSION AND PERSPECTIVES 81

REFERENCES....83

LIST OF TABLES

Table 1. Botanical classification of *Cannabis sativa* L. [26] 8

Table 2. Physicochemical properties of supercritical fluids 23

Table 3. Operating conditions SC-CO_2 mode 29

Table 4. Solubility of cannabinoids and CO_2 solvent 33

Table 5. Review on cannabis oil extraction using SC-CO_2 34

Table 6. Physicochemical properties of cannabinoids. 51

Table 7. Review on SCF cannabis oil extraction [20]. 52

Table 8. Range and variables for the experimental design. 56

Table 9. Raw material cannabinoid analyses 59

Table 10. Cannabinoids yield per extract and per raw material 60

Table 11. Effect estimates for the total yield 62

Table 12. ANOVA for cannabinoids. (a) Δ^9-THC; (b) CBN; (c) CBG. 62

Table 13. Error percentage for each cannabinoid 62

Table 14. Influence of designed factor on each response 64

Table 15. Ethanol cannabis extraction 70

Table 16. Recovery for ethanol and SC-CO_2 extractions 70

LIST OF FIGURES

Figure 1. Cannabis vernaculat taxonomy [19] 6
Figure 2. *Cannabis sativa* in flower, pistillate (female) plants at left, staminate (male) plants at right [24] 7
Figure 3. Cannabis plant phenology [30] 9
Figure 4. Chemical composition of different parts of *Cannabis sativa L.* [32] 10
Figure 5. Molecular structure of cannabigerol 14
Figure 6. Molecular structure of Δ^9-tetrahydrocannabinol 16
Figure 7. Molecular structure of cannabinol 19
Figure 8. Phase diagram of carbon dioxide 23
Figure 9. Schematic supercritical fluid extraction process 25
Figure 10. General view of supercritical fractionation flow diagram 27
Figure 11. RESS process 30
Figure 12. Schematic view of SC-CO_2 as an antisolvent (SAS process) 31
Figure 13. Schematic view of micronizing process (PGSS-drying process) 32
Figure 14. Classification of high-performance liquid chromatography. 43
Figure 15. FCPC principle 45
Figure 16. Supercritical carbon dioxide extraction set-up. 55
Figure 17. FCPC set-up 58
Figure 18. Simplified cannabinoid synthetic pathway: decarboxylation, biosynthesis 60
Figure 19. Surface response (a) and Pareto chart (b) for Δ^9-THC 65
Figure 20. Surface response (a) and Pareto chart (b) for CBG 67
Figure 21. Surface response (a) and Pareto chart (b) for CBN 68
Figure 22. Cannabis extract sample from SC-CO_2 (left) and ethanol (right) 71
Figure 23. Chromatogram of Δ^9-THC fraction 72

LIST OF ABBREVIATIONS

BPRV	Back-Pressure Relief Valve
CB	Cannabinoids (including THC, THCA, CBD, CBDA and CBN)
CB1-2	Cannabinoids receptors 1-2
CBD	Cannabidiol
CBDA	Cannabidiolic acid
CBN	Cannabinol
CO_2	Carbon dioxide
DOE	Design of experiment
EC	Endocannabinoids
GPR	G-protein-coupled receptor
GC	Gaz chromatography
LC	Liquid chromatography
HPLC	High performance liquid chromatography
IL	Interleukin
iNOS	Inducible nitric oxide synthase
MAE	Microwave assisted extraction
SFE	Supercritical fluid extraction
SOD-1	Super oxide dismutase
THC	Δ^9-tetrahydrocannabinol
THCA	Δ^9-tetrahydrocannabinolic acid
TNF	Tumor necrosis factor

CHAPTER 1 INTRODUCTION

1.1. Context

Medicinal cannabis is widely used around the world, for different applications such as pain management, psychiatric diseases, autoimmunity, and cancer. This date to before Christian era, where Indians had cannabis-based medicines for several infections and diseases. Israeli scientists were the first to study cannabis pharmacokinetics, and later in the 19th century [1], the western medicine started, showing interest in the plant's therapeutic effects. As such, for the pharmaceutical industry, having a standard extract approved by Health Canada is a more interesting starting point. This work aims to contribute to standardize the process to obtain a pharmaceutical grade cannabis extract. Main steps to extract cannabinoids from cannabis plant are:

1.1.1 Decarboxylation

Heating the cannabis in an oven between 100 °C and 140 °C, since cannabinoids are carboxylic acids in the cannabis plant, and their pharmacological bioactivity is lower, decarboxylation turns them into the neutral forms, and activates their therapeutic effects.

1.1.2 Supercritical CO_2 extraction

Cannabis biomass is transferred in a quarter-liter reactor. Supercritical CO_2 passes through the vessel. A separator allows depressurization, where we collect cannabinoids extract, and recycle gaseous CO_2. Supercritical fluid extraction (SFE) is one of the most used techniques in the cannabis industry.

1.1.3 Purification

Isolation of cannabinoids concentrates for its end use employs a Fast Centrifugal Partition Chromatography (FCPC). The goal is to separate a single cannabinoid, to meet the International Council for Harmonization threshold: 0.5 % and 0.03 % for ethanol and hexane, respectively.

1.1. Problem statement

COVID-19 negatively impacted various industries from 2020 to 2021, but the nutraceutical and pharmaceutical market grew exponentially during the first 6 months of the pandemic, since they help manage pain, but also improve immunity and reduce fatigue. Pain costs society billions of dollars in lower productivity and affects millions of people [2].

Cannabis sativa L. medicinal oil has therapeutic effects that helps to manage diseases like autoimmunity, cancer, and psychiatric disorders. The lack of standardization of final extract with fixed characteristics doesn’t fasten the formulation of pharmaceutical grade products to get into the market and help the medical area. The cannabinoids extract variability delays the understanding of dose-response pharmacological activities and the establishment of drug delivery systems for each targeted disease [3].

The scale and standardization of cannabis-based nutraceutical product is still a challenge. There is an urgent need to establish the efficacy of medical cannabis and standardize their properties for adjunctive therapy as an alternative to opiates [4].

Clinical trials have not substantiated the scientific literature of clinical data providing evidence of the benefit of cannabinoids. Of the 171 preclinical trials reported in the meta-analysis of Finn et al. [5], only 11 clinical trials were conducted. For example, in one trial, pain was higher after a third molar extraction in clinical trials than expected from preclinical studies. Pharmacokinetics investigations are inconclusive as they, among other things, consider different routes of administration. Thus, the industry is facing a real problem with product standardization and identifying specific molecules for each therapeutic effect.

For the cannabis industry, it is not enough to know the raw material (cannabinoids, terpenes, and polyphenols) to choose a related processing technology because the target molecules are sensitive to the end use (recreational vs pharmaceutical, for example).

First problem to solve in order to bring cannabis to pharmaceutic industry is to determine the extraction process conditions.

1.2. Research objectives

This project aims at determining and optimizing the process parameters of supercritical CO_2 cannabinoids (THC, CBG, CBN) extraction from *Cannabis sativa* L. using a Box-Behnken design of experiment, as well as comparing results to data from cannabis kinetics studies published in the open literature and establishing a purification step for THC cannabinoid using Fast Centrifugal Partition Chromatography.

1.3. Thesis outlines

Chapter 1 introduces this study and the motivations behinds it. Chapter 2 presents *Cannabis sativa* L. characteristics, including its morphology, and pharmaceutical properties. Chapter 3 provides a literature review of the supercritical carbon dioxide technology and its application in the cannabis industry, including purification techniques. Chapter 4 details the methodology used to design experiment protocol for this project. Chapter 5 is an article describing the key findings for the optimization of the cannabinoids extraction conditions, with a preliminary purification step using fast centrifugal partition chromatography. Chapter 6 points out the most important results as a general discussion, while Chapter 7 is the conclusion and statement of the limitations of the project and future perspectives.

CHAPTER 2 CANNABIS PLANT

Eight genera that were originally in the Celtidaceae, including Celtis, Pteroceltis, Aphananthe, Chaetachme, Gironniera, Lozanella, Trema, and Parasponia, make up the family Cannabaceae today, along with Cannabis and Humulus. However, only Parasponia plants generate nitrogen-fixing nodules in conjunction with rhizobial bacteria. Some botanists confuse Parasponia and Trema. Only legumes share this characteristic. On the other hand, cannabis and other Cannabaceae collaborate with arbuscular mycorrhizal fungus [6].

2.1 Geography of cannabis plant

Weedy hemp is common throughout Eurasia, with southeast and central Asia and many European nations being notably affected. Less frequently, *C. sativa* L. is discovered in South America, Australia, and Africa outside of cultivation [7]. *C. sativa* L. rarely gets naturalized as a result of escapes from cultivated hemp in subtropical and tropical locations, according to Haney and Kutscheid [8], possibly a sign that the species is naturally adapted to a north-temperate climate. While North American wild plants have only existed for a short period of time, those in the Old World have evolved to fit varied ecosystems over thousands of years. Unsurprisingly, the species grow wild in a considerably wider range of climates and altitudes in Eurasia than they do in North America. In Eurasia, Vavilov [9] noted extensive stands of wild hemp. *C. sativa* L. grows to elevations of thousands of meters in the Himalayas, although in North America, uncultivated plants are rare at locations higher than a few hundred meters.

C. sativa L. has been harvested naturally in North America from the 48 contiguous states and Washington, DC, as well as from British Columbia to New Brunswick in Canada [10-12]. However, many plants that appear to be growing untamed are illegally grown or recent ("spontaneous") escapes from cultivation (in either case, the seeds reveal that they are domesticated races). In North America, the Midwest and Northeast, as well as southern Ontario and southern Quebec (especially along the St. Lawrence and lower Great Lakes) are the regions where weedy populations—plants with characteristics of those adapted to wild existence—are best established. These are all regions where hemp cultivation was historically concentrated. During the rise of cultivation in both Canada and the United States during World War II, several

wild populations in North America might have sprung from escapes. Naturalized hemp is extremely rare in Mexico, rare in the United States south of 37°N latitude, and uncommon in the western United States [8].

2.2 Cultivars

The large variety of end use of cannabis plant diversifies the number of cultivars in today's agronomy. Despite the 45 industrial hemp cultivars that Health Canada has approved, there is currently very little knowledge about how well these cultivars perform and adapt in eastern Canada. The three cultivars with the largest and most steady biomass yields were Anka, Ferimon, and Jutta, according to Aubin M-P et al's evaluation of eleven cultivars (Alyssa Anka CanMa CFX-1 CFX-2 CRS-1 Delores Ferimon Finola Jutta and Yvonne). Additionally, several cultivars, including as Anka, Ferimon, and Jutta, possessed these qualities, exhibiting high yields and consistent performance in a variety of settings [13].

Soler et al. [14] described the genetic makeup of 154 people from 20 cultivars of *Cannabis sativa* subsp. *indica* and 2 cultivars of *C. sativa* subsp. *sativa*. He was the only person to be receptive to the potential of employing molecular tools for breeding purposes, including the generation of new types, despite the fact that a great research works has been done to improve breeding [15].

The therapeutic effects of active cannabis compounds are strongly sensitive to the type of cultivars. These compounds are generally obtained from plants harvested at maturity but not all cannabinoids are present or abundant during the last stage of hemp development. Hammami et al. [16] tested, in 2021, five hemp genotype dual-purpose cultivars in Atlantic Canada, to evaluate their impact on the cannabinoid compositions, and results show that with the exception of CBGA, grain cultivars (such as Katani, CFX-2, Grandi, and CRS-1) typically had high cannabinoid content, although certain dual-purpose cultivars (such as Anka, Silesia) and two grain cultivars (such as Ferimon, USO 31) showed the opposite pattern.

2.3 Biology of cannabis plant

According to morphological, geographic, ecotypic, or chemotypic differences, the cannabis genus may consist of one extremely variable species (*Cannabis sativa* L.), two species, or three species [17].

Two significant subdivisions typically arise. One of them makes the distinction between cannabis used as a drug and cannabis used to create fiber, which is particularly significant for legal reasons. The Sativa and Indica subspecies of *Cannabis sativa* L. are distinguished in the other category, which is based on botanical considerations. The bulk of cannabis plants that are sold today are hybrids of Sativa and Indica [18].

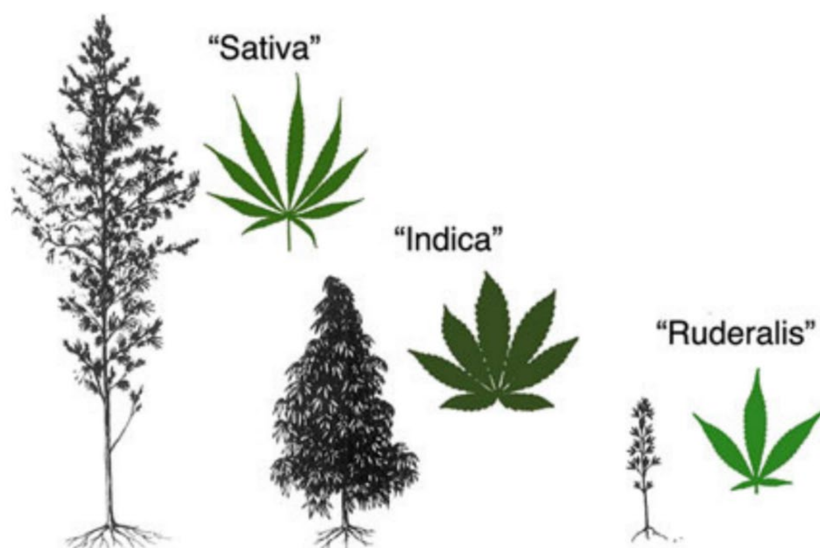


Figure 1. Cannabis vernaculat taxonomy [19]

Anderson depicted a Schultes-consistent plant, not a Janischevsky-consistent one (Figure 1). In one of the earliest seed bank catalogues, "Ruderalis" plants were depicted as growing close to the Hungary–Ukraine border. About 75 Ruderalis plants are depicted in photographs as having little branching and a strong apical dominance. These characteristics differ from those proposed by Vavilov and Janischevsky [19] and are consistent with a spontaneous escape of farmed hemp.

In today's taxonomy, the term "Ruderalis" is used to describe plants that have one to three of the

following traits: CBD-THC, wild-type morphology, or early blooming (also known as "autoflowering," which is day-neutral, light-independent flowering). Some authors have made an effort to harmonize "Sativa" and "Indica" with the official *Cannabis sativa* and *C. indica*. [19].
Indica plants often mature more quickly than Sativa kinds under similar conditions, and the two types tend to smell differently, due probably to distinct terpene profiles.
Cannabinoids are cannabis-specific terpenophenolic compounds. Most the plant's aerial surfaces are covered in glandular trichomes, which are responsible for producing them [20, 21].
Unisexual blooms become bored in the early stages of terminal inflorescences and the latter stages of terminal or lateral inflorescences (Figure 2) [22].

Figure 2. Cannabis sativa in flower, pistillate (female) plants at left, staminate (male) plants at right [23]

Cannabis plant derives from the Cannabaceae family, it is well known for the diversification history in two regions, the family contains 10 genera and about 117 species [24]. Table 1 provides the botany of the plant to give information's that makes it easy to understand the various properties of the plant and its different compounds linked to biological effects.

Table 1. Botanical classification of Cannabis sativa L. *[25]*

Category	**Botanical nomenclature**
Kingdom	Plantae- plants
Subkingdom	Tracheobionta – Vascular plants
Superdivision	Spermatophyta – Seed plant
Division	Magnoliophyta – Flowering plants
Class	Magnoliopsida – Dicotyledons
Subclass	Hamamelididae
Order	Urticales
Family	Cannabaceae
Genus	Cannabis
Species	*Cannabis sativa* L.

Most plants are dioecious, meaning that pistillate plants only produce female flowers and staminate plants only produce male flowers (Figure 2). Male (staminate) plants die after anthesis, whereas female (pistillate) plants endure the first cold snap. Even if they steadily deteriorate in strength over time, female plants kept in greenhouses or in climates without severe winters can thrive for years. The plants have been characterized to as "annual or perennial depending on climate" even though the species is normally an annual.

In a century of research, Indian hemp resin has produced nearly 80 phenolic compounds derived from benzopyran, which are not nitrogenous (alkaloids), forming the distinctive cannabinoid family [26]. Ninety-five percent of angiosperms (flowering plants) have flowers with both male and female reproductive organs, while the remaining five percent have flowers with only one reproductive organ. A plant is said to be monoecious if it produces both male and female flowers on the same individual, and dioecious if it produces male and female flowers on different individuals.

Since the genus Cannabis is dioecious, male plants have shorter life cycles and taller, thinner shoots than female plants. However, there are well-known varieties that additionally produce hermaphrodite or monoecious blooms, which have distinct male and female flowers on the same

plant. Male plants produce massive volumes of pollen, which can cover vast distances to pollinate female flowers on plants growing far from pollen-bearing blossoms. Cannabis plants are widely grown, and there are no barriers that would stop or limit interbreeding. Therefore, many fertile hybrids with durable features are produced [27]. Male plants are often taller and leaner than female plants, and their life cycles are frequently shorter.

Cannabis sativa, which is most known for generating marijuana, is undoubtedly the plant that is the most well-known, notorious, and divisive in the entire globe [23]. *Cannabis Sativa* L. is an annual plant in the Cannabis genus that originates from China and is a part of the Cannabaceae family (Figure 3) [28].

Figure 3. Cannabis plant phenology [29]

There is infrequently recognized a unique subspecies of ruderalis that resembles cannabis. It is a smaller, more weed-like plant that is indigenous to Central Russia.

The main element of marijuana that contributes to its narcotic potential is called "bracts." A

modified or specialized leaf, especially one connected to flowers, is referred to in botany as a "bract." In *C. sativa*, the bract-like structures are very minute and resemble tiny unifoliolate leaves (i.e., leaves with only one leaflet). They are in fact connected to the flowers. A "perigonal bract" surrounds in a cup-like form each female flower, and enlarges significantly, becoming densely covered with tiny secretory glands that create the bulk of the THC that the plant produces [30].

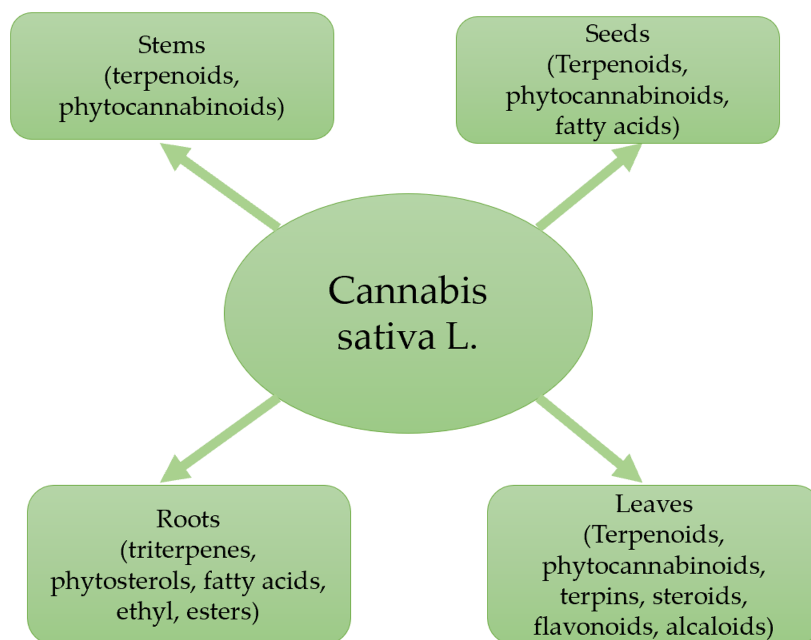


Figure 4. Chemical composition of different parts of cannabis sativa L. [31]

The bracts of sinsemilla marijuana, which is made by preventing pollination on the female flowers, remain small and are quite thickly covered with secretory glands. On the other hand, fertilized flowers turn into "seeds" (achenes), the perigonal bract expands, and the density of secretory glands significantly decreases [30].

2.4 Cannabis compounds

Over the past few decades, a greater variety of compounds originating from cannabis have been discovered. Up to now, 750 different bioactive substances, including cannabinoids, flavonoids, terpenoids, stilbenoids, alkaloids, steroids, polysaccharides, benzoquinone, phenanthrenes, spiroindans, lignans, fatty acids, sugars, hydrocarbons, amino acids, and protein, have been found in *Cannabis sativa* L. [32]. To illustrate the diversity of the cannabis plant components and to show

why it is one of the richest and most complex species, the most known groups of compounds are as follows.

2.4.1 Carotenoids

Carotenoids are auxiliary pigments for photosynthetic processes with absorbance spectra in the 400–550 nm range [33]. By thermally dissipating the excess energy of the single excited chlorophyll (1Chl*) and possibly a triplet excited chlorophyll (3Chl*) within light reaction centers and scavenging any evolved singlet-oxygen, carotenoids protect other cell components from photo-oxidative damage brought on by the photosynthetic light harvesting apparatus [34, 35].

2.4.2 Terpenes

The majority of terpenes in cannabis plants, such as monoterpenes and sesquiterpenes, are found in the glandular trichomes and have a variety of functional properties despite being present in considerably lesser amounts than cannabinoids [36, 37]. Terpenes are volatile aromatics that affect or contribute to how plants taste and smell, protect plants from biotic stressors, and act as plant hormones to control growth [38]. Furthermore, certain terpenes aid plants in coping with light and drought stress [39]. Both Schnarrenberger and Mohr [40], and Tanaka et al. [41] noted that phytochrome, a red-light photoreceptor, controls the formation of carotenoid and monoterpene compounds.

2.4.3 Flavonoids

Because flavonoids are sensitive to the type of light they receive, plants grown under ultraviolet, blue, and Far Red light treatment have higher concentrations. Flavonoids are a physically and functionally diverse group due to their two-ring, 15-carbon basic structure. The many different kinds of flavonoids (flavonols, flavones, flavanones, anthocyanins, and isoflavonoids) are distinguished by different accessory groups connected to the primary 15-carbon skeleton. This enables their crucial functions as oviposition stimulants, feeding deterrents, pollinator and feeding attractants, as well as in plant disease resistance and light stress management. The right light spectrum must be used in lighting systems for cannabis growth and manufacturing in order to produce flavonoids. Benefits from ultraviolet, blue, and Far Red wavelengths should be more carefully considered [42].

2.4.4 Cannabinoids

Before senescence, unfertilized female flowers have an abundance of glandular trichomes, which contain secretory cells that produce cannabinoids. According to Shoyama et al., cannabis leaves exude cannabinoids into the leaf tissue through glandular trichomes, which causes cell death [43]. Classification of cannabinoids is still an ongoing task for the scientific and industrial diaspora. Until now, there are 11 defined groups of cannabinoids with a total of 104 compounds, including, 17 from the CBG type, 18 from Δ^9-THC, 10 from CBN, 9 from CBT, 8 from each CBC and CBD, and between 2 to 5 types from Δ^8-THC, CBL, CBE and CBND [25].

Their important bio-interaction with G-protein-coupled membrane receptors, particularly cannabinoid receptors (CB1 and CB2), to which different members of the group have extremely varying affinities, is thought to be how they exert their effects on the human body, similarly to endocannabinoids. In addition, several cannabinoids have recently been found to have molecular targets outside of the endocannabinoid system. Nuclear receptors, ligand-gated ion channels, transient receptor potentials, opioid or serotonin receptors, as well as other G protein-coupled receptors (GPR55 or GPR18 receptors), have all been demonstrated to interact with plant cannabinoids [30].

2.5 Cannabinoid bioactivities

Cannabinoids are active compounds that we find in the cannabis plant material, and they cause many of their biological effects via the endocannabinoid system and through cannabinoid (CB) receptors. This system oversees controlling hunger, anxiety, memory and learning, processing rewards, growth, and development. The synaptic connection is also impacted. Neuronal excitability has been linked to endocannabinoids (EC), a term for the endogenous ligands of the CB receptors. CB1 receptors are found in most peripheral organs, including the brain. Despite the fact that CB2 receptors can be expanded in many organs, hematological and immune cells largely contain them. THC binds to CB1 and CB2 receptors to exert its effects. Conversely, several non-cannabinoid receptors pathways enable CBD to have some of its most notable neuronal effects [44]. Cannabis extracts and inflorescence inhibit inflammatory responses in vitro and in pre-clinical and clinical trials. The endocannabinoid system (ECS) is a modulator of immune system activity, and dysregulation of this system is involved in various chronic inflammations. This

system includes cannabinoid receptor types 1 and 2 (CB1 and CB2), arachidonic acid-derived endocannabinoids, and enzymes involved in endocannabinoid metabolism. Cannabis produces a large number of phytocannabinoids and numerous other biomolecules such as terpenes and flavonoids. In multiple experimental models, both in vitro and in vivo, several phytocannabinoids, including Δ^9-tetrahydrocannabinol (THC), cannabidiol (CBD) and cannabigerol (CBG), exhibit activity against inflammation. These phytocannabinoids may bind to ECS and/or other receptors and ameliorate various inflammatory-related diseases by activating several signaling pathways [45].

Alternative therapeutic options are required for patients who do not respond well to opioid analgesics or who experience severe side effects when taking conventional analgesics. Anecdotal data suggests that medical cannabis may be able to manage pain in this patient population in an effective manner.

2.5.1 Cannabigerol

In the scientific literature as well as the media, medical marijuana, and specific cannabinoids like cannabidiol (CBD) and Δ^9-tetrahydrocannabinol (Δ^9-THC) are gaining popularity. Cannabigerol (CBG) is the precursor molecule for the majority of phytocannabinoids, which are produced by the cannabis plant and number over 100 distinct cannabinoids.

CBG is a compound with a structure made up of 21 carbon atoms (22 in the acidic form) and a molar mass of 316.48 g/mol. Its chemical formula is $C_{21}H_{32}O_2$ (Figure 5).

The systematic name of cannabigerol, according to IUPAC nomenclature, is 2-[(2E)-3,7-dimethylocta-2,6-dienyl]. -5-pentylbenzene-1,3-diol. In 1964, the substance was initially separated from a hashish extract in hexane. Chemical synthesis was used to establish its structure and stereochemistry [30].

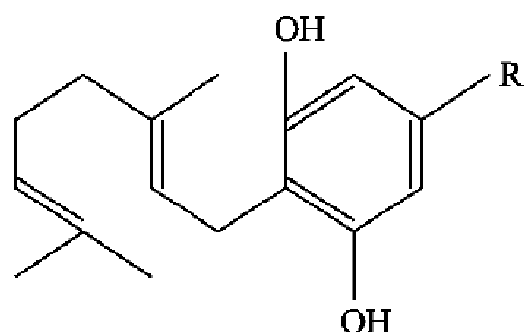


Figure 5. Molecular structure of cannabigerol

Cannabigerol (CBG) was the first chemical to be isolated in its purest state. It has been shown to block the 5-HT1A receptor and is a phytocannabinoid that is non-psychoactive and has a low affinity for the cannabinoid CB1 and CB2 receptors. CBG inhibited the CBD-induced reduction of vomiting in shrews and rats, but only at modest dosages on its own. A strong TRPA1 agonist, a strong TRPM8 antagonist, a strong TRPA1 agonist, and a modest TRPV1 and TRPV2 agonist are additional roles that CBG performs [45].

CBG has been shown to have anti-inflammatory and neuroprotective characteristics in models of neurodegenerative illnesses, suggesting that it may be used as a treatment for neuroinflammation and oxidative stress. Additionally, it has been shown that CBG increases rats' food intake and enhances their ability to respond to the sweetener saccharin. These results suggest that CBG may be helpful in the treatment of cancer patients, possibly reducing inflammation, enhancing appetite, and alleviating nausea.

By influencing the production of the superoxide dismutase SOD-1, whose activity is boosted by pro-inflammatory factors, cannabigerol has been demonstrated to regulate redox equilibrium by lowering the activity of one of the primary pro-oxidant factors, iNOS (inducible nitric oxide synthase) which activates the membrane receptor PARP-γ (e.g., lipopolysaccharide, LPS). As a result, CBG helps prevent cell death by tipping the redox equilibrium in favor of the antioxidant. The nuclear factor NFB, which is in charge of transcription of pro-inflammatory cytokines, is

greatly reduced in transcriptional activity by the phytocannabinoid, which modifies the inflammatory processes. As a result, levels of cytokines such as TNF and IL-1 are decreased [31]. Interleukin-6 (IL-6) and lactate dehydrogenase (LDH) levels in astrocytes were reduced by CBG. In astrocytes, CBG lowered DNA damage proteins, including p53. The reductions in LDH caused by CBG (3 lM) in astrocytes were not affected by antagonists for CB1, CB2, PPAR-c, PPAR-a, 5-HT1A, or TRPV1 [46].

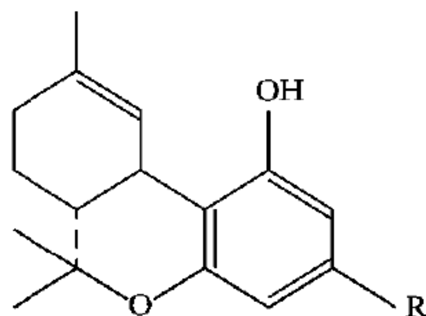
Three separate BBB-forming cells were protected from OG-mediated damage by CBG, which modulated several symptoms of ischemic stroke pathogenesis. These findings call for more research into the preventive properties of CBG in ischemic stroke and improve our understanding of its effects. Future research should determine further potential CBG neuroprotective properties and its associated mechanisms of action [47].

CBG shares similar affinity and activity characteristics with both THC and CBD (cannabidiol) substances at cannabinoid receptors (5-HT1A). According to studies, CBG has antimicrobial activity, therapeutic potential for treating inflammatory bowel disease, multiple sclerosis, and neurologic illnesses like Huntington's disease and Parkinson's disease [48].

2.5.2 Δ^9-tetrahydrocannabinol

After being detected by Gaoni and Mechoulam [49-51], the cannabinoid activity of Δ^9-THC was subsequently examined in rhesus monkeys, dogs, gerbils, mice, and rats, and only Δ^9-THC was shown to elicit the usual psychoactive effects of cannabis.

THC is a compound with a structure made up of 21 carbon atoms (22 in the acidic form with 2 more oxygen atoms) and a molar mass of 314.5 g/mol (Figure 6). Its chemical formula is $C_{21}H_{30}O_2$. The systematic name of Δ^9-THC, according to IUPAC nomenclature, is 6,6,9-trimethyl-3-pentyl-6a,7,8,10a-tetrahydrobenzo[c]chromen-1-ol.



R = C_3H_7 Δ⁹-Tetrahydrocannabivarin (THCV)
R = C_5H_{11} Δ⁹-Tetrahydrocannabinol (THC)

Figure 6. Molecular structure of Δ⁹-tetrahydrocannabinol

Roger Pertwee [52] discovered that Δ^9-THC was truly the cause of catalepsy after testing it in the ring test, a quantitative *in vivo* evaluation for catalepsy (muscular rigidity and stationary posture). Billy Martin's team then adapted the mouse tetrad assay to incorporate the ring test in addition to catalepsy, hypokinesia (inactivity), hypothermia (lower body temperature), and antinociception [53]. Usually, the mouse tetrad test is used to check for psychotropic cannabinoids. The CB1 and CB2 receptors only partially bind to Δ^9-THC.

In fact, it has been shown that Δ^9-THC can reduce neuropathic pain and both acute and chronic pain. Studies by Carter et al. [54], Cichewicz et al. [55], Lynch and Clark [56], and others suggest that Δ^9-THC possesses opioid-sparing qualities because it interacts synergistically with the majority of opioid medicines.

In addition, Δ^9-THC improves sleep quality and prevents nightmares brought on by upsetting situations, notably in combat veterans with post-traumatic stress disorder. Nelson et al. [57] discovered that Δ^9-THC stimulated appetites in advanced cancer patients and decreased nausea and vomiting in chemotherapy patients. Tourette's syndrome has also been shown to benefit with Δ^9-THC.

Studies on animals show that Δ^9-THC reduced inflammation and problems with *in vitro* motility in rat colitis. According to studies on mice, Δ^9-THC has a biphasic effect on behaviors associated to anxiety, with low dosages lowering anxiety and high dosages creating anxiogenic effects. Δ^9-THC has been demonstrated to have antidepressant, and antiemetic effects in animal models.

Δ^9-tetrahydrocannabinol also delays motor deterioration and lengthens survival in a mouse model of amyotrophic lateral sclerosis. It also enhances activity and hand-eye coordination in animal models of Parkinson's disease. In animal models of neurodegeneration, it also reduces neurological impairments [58].

These findings point to a novel, albeit sex-specific, physiological explanation for the symptoms of dry eyes associated with cannabis use: tear production is decreased by the lacrimal gland's neuronal CB1 receptors.

THC or the cannabinoid agonist CP55940 can diminish ripping in male mice by activating CB1 receptors. THC has little effect on female mice, whereas CP55940 causes more tearing. In CB1 knockout mice, the impact of CP55940 is absent in both sexes.

Males have roughly a four-to five-fold higher level of CB1 mRNA and protein than females. Δ^9-THC promotes tearing in male knockouts, indicating that it also affects several receptors [59].

We present a selection of representative clinical studies that assessed the effectiveness of cannabinoid-based therapies containing tetrahydrocannabinol (THC) and cannabidiol (CBD) for reducing cancer-associated pain, ranging from small pilot studies carried out in 1975 to double-blind placebo-controlled trials carried out in 2014. Five clinical trials that examined the impact of THC on reducing cancer pain were found in a survey of literature published on Medline between 1975 and 2017. There is some indications that THC oil capsules, THC: CBD oromucosal spray (nabiximols), or THC or mucosal sprays help reduce cancer pain, according to five studies that looked at these treatments. Different dosages of 0 to 40 mg/day of CBD and 2.7 to 43.2 mg/day of THC were given. In some trials, higher THC doses were linked to greater pain alleviation. According to one study, considerable pain alleviation can be obtained at doses as low as 2.7-10.8 mg THC combined with 2.5-10.0 mg CBD. There is data that indicates people with advanced cancer experience less chronic or neuropathic pain while using medical cannabis [60].

THC was successfully formulated as a dry powder, to opens up new routes of administration, a formula that chemically labile lipophilic Δ^9-tetrahydrocannabinol (THC), and dissolves quickly in water to boost bioavailability. By lyophilizing a THC and inulin solution in a solution of water and tertiary butyl alcohol, these solid dispersions were created (TBA). A glassy inulin matrix could contain 4 or 8 weight percent of THC.

THC and inulin dissolved at the same rate, according to dissolution tests with tablets made from inulin glass dispersion material. A combination of polyvinylpolypyrrolidone (PVPP), mannitol, and a solid dispersion of THC containing 2 mg per 125 mg tablet was used to make the tablets. These pills showed promise for sublingual administration in dissolution studies, with 80% of the THC dissolving from them in less than 3 minutes. It was determined that adding THC to an inulin matrix can strongly stabilize it. The high rate of water dissolution may have improved bioavailability [61].

2.5.3 Cannabinol (CBN)

Among the remaining substances, which have traditionally been referred to as "minor cannabinoids," cannabinol (CBN) stands out for its enormous effects and repercussions on the global scientific community [62].

The crude material's separation from the other closely related compounds used crystalline cannabinol acetate as an intermediary. The ester was hydrolyzed to produce pure cannabinol, an amber liquid or resin with a boiling point of 263-264 °C [63].

CBN was the first cannabinoid to be isolated from hashish in the late 19th century, but its structure wasn't fully figured out until 1940, due to some issues related to both nomenclature misunderstandings and the type of plant material used for the extraction. As a result of its unfortunate and confusing discovery, CBN was likely regarded as a "minor" cannabinoid.

CBN is a compound with a molecular structure made up of 21 carbon atoms (22 in the acidic form with 2 more oxygen atoms) and a molar mass of 310.4 g/mol. Its chemical formula is $C_{21}H_{26}O_2$ (Figure 7). The systematic name of cannabinol, according to IUPAC nomenclature, is 6,6,9-trimethyl-3-pentylbenzo[c]chromen-1-ol. CBN is isolated from the plant cannabis that is a metabolite of tetrahydrocannabinol (THC).

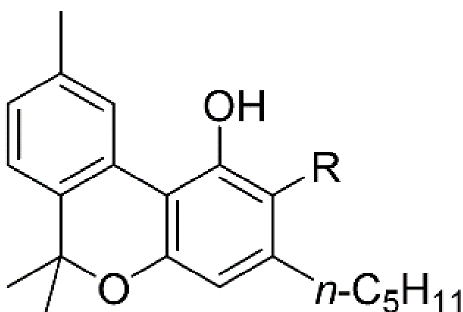


Figure 7. Molecular structure of cannabinol

The biological and pharmacological characterization of the substance was severely hampered by the confusing situation around it's nomenclature and corresponding plant biomass, as well as its minor composition in the plant and the discovery of more intriguing bioactive cannabinoids such as CBC, CBA, etc.

By highlighting its well-known synthetic routes and the urgent need to understand its biological comprehension, especially it's mechanism of action and cell communication pathways, the aim is to raise attention to this underappreciated and underutilized cannabinoid [60].

Because it regulates a few physiological and mental processes necessary for maintaining the body's equilibrium, the endocannabinoid system is an important biological system and the main target of cannabinoids.

Cannabis ligands are thought to significantly activate pathways linked to cyclic AMP/receptor activation, while the precise method by which the ECS regulates metabolism is still unclear.

A preclinical trial showed that cannabinol enhanced appetite by lengthening and sizing meals, and increased consummatory behavior by lowering the latency to eat through the CB1 receptor [64].

CBN functions also as both an analgesic and an anti-inflammatory. When provided in a 1:1 mixture with CBD, it lessens the mechanical sensitivity brought on by intramuscular injections of nerve growth factor in the masseter muscles of rats, reducing the symptoms of myofascial pain

syndromes such fibromyalgia and temporomandibular disorders. CBN has been classified as a potential treatment for allergic airway diseases since it may lower the production of interleukins 2, 4, 5, and 13 and decrease allergen mucus formation in OVA-sensitized and challenged A/J mice. The use of CBN as a glaucoma treatment has also been suggested because it lessens inflammation, which increases intraocular pressure. Early studies have also demonstrated that CBN has antioxidant properties and reduces cell oxidative stress in a cell culture model of Huntington disease, in addition to promising treatment for staph infections since it works well against several bacteria that are resistant to antibiotics, including methicillin-resistant *Staphylococcus aureus* (MRSA).

CBN is also effective in treating epidermolysis bullosa, a group of rare medical conditions characterized by blisters that grow easily on the skin and mucosal membranes. Phase 2 investigations are now being carried out by InMed Pharmaceuticals as a follow-up to the phase 1 research, which showed that the CBN-based preparation in development was safe and well-tolerated on induced open epidermal wounds and caused no systemic or significant side effects [60].

2.6 The relationship between cannabis use and processing technology

Many researchers already reported that cannabis holds more than 100 identified cannabinoids, and each one of them has a distinct therapeutic effect. Also, the therapeutic effect of any pharmaceutical formulation is dose dependent. And the starting point of a formulation is a cannabis oil extract.

It is well established that oil extract composition is a function of the processing conditions, starting from decarboxylation to extraction and purification techniques [64]. The chemical structures of cannabinoid molecules are sensitive to conditions like temperature, pressure, nature of the solvent, processing time and others. For example, high temperature breaks quickly chemical bonds, long processing time results in higher quantity of products of degradation. Especially, biodegradation reactions between several cannabinoids occur simultaneously and depend also on the process parameters [65].

For food-infused goods, this begins in the "raw" condition of the cannabinoid. However, it's possible that the conversions that take place when cannabinoids are subjected to "heat" or are

affected by "age" are more significant. As a result, changing the decarboxylation temperatures for example from 100 °C to 140 °C, can also cause Δ^9-THC to transform into Δ^8-THC and/or CBN as the cannabis-infused food product ages [65].

CHAPTER 3 LITERATURE REVIEW

3.1 Introduction

Supercritical fluid extraction (SFE) is a growing and green extraction method for different applications such as natural products, pharmaceutical products and so on. Particularly for natural products, SFE has attracted industrial interest to replace toxic and petrochemical solvents such as hexane. This was due to safety and health problems encountered with this type of solvents, the increasing costs due to environmental constraints.

Due to its properties such as low critical temperature, the ability, without degradation, to recover thermolabile molecules, non-toxic, nonflammable and widely available at relatively low cost, CO_2 appears as a promising alternative. Consequently, the products are totally free from residues and obtained in a single step just by adjusting pressure or temperature, the solvent power and selectivity can be easily adjusted from gas-like to liquid-like by changing the operating temperature and pressure, allowing fractionation of the extract.

Although, SFE is viewed as a technology having higher costs than solvent extraction, some considerations will surely make SFE economically viable during coming years:

- Regulations issues: most petrochemical solvents are either banned for food and cosmeceutical products or authorized with extremely low residual concentrations. The growing environmental constraints will ease the development of SFE in relation with some issues such as work ambiance control, volatile compounds release control and residual concentration in the final product either for consumer or environment;
- Quality considerations: SFE is used to develop high values products as nutraceuticals and cosmeceuticals, for which the "natural" character of the extracting mode has a high marketing value;
- Innovative products: new food or cosmeceutical products that are not at all comparable with those obtained from conventional organic extraction can be manufactured. The pharmaceutical industry is paying great attention to Drug Delivery Systems that are opening new therapeutic routes for many major drugs related to different diseases (diabetes, cancer, …).

3.2 Definition and class of supercritical fluids

The vaporization curve (liquid-gas) has a triple point with a specific temperature and pressure where all three phases are in equilibrium; this curve ends at a critical point (see figure 8). Beyond this critical point (pressure (P) > critical pressure (P_c) and temperature (T) > critical temperature (T_c)), only one state exists: the fluid is said to be supercritical and has very specific properties on which the implementation processes will be based. Indeed, supercritical fluids and "subcritical" liquids (pressure (P) > critical pressure (P_c) and temperature (T) < critical temperature (T_c)) are generally used because they have a density close to that of liquids, a viscosity slightly higher than that of gases, a diffusivity between that of liquids and that of gases (see Table 2).

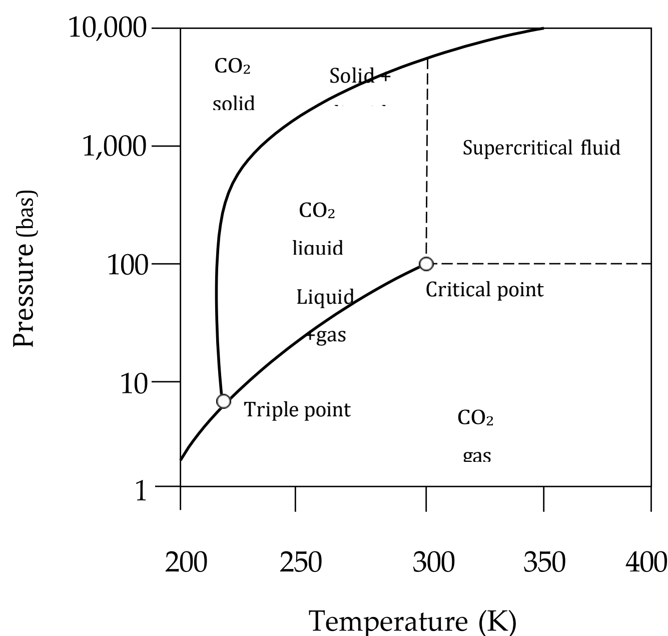


Figure 8. Phase diagram of carbon dioxide

Table 2. Physicochemical properties of supercritical fluids

Phase	Density (g/mL)	Viscosity (poise)	Diffusion coefficient (cm^2/s)
Gas	0.001-0.004	0.5-3.5 (x10^{-4})	001-1.00
Supercritical Fluid	0.2-0.9	0.2-1.0 (x10^{-3})	0.1-3.3 (x10^{-4})
Liquid	0.8-1.0	0.3-2.4 (x10^{-2})	0.5-2.0 (x10^{-5})

The advantages of supercritical fluids relate to the fact that they can be used in different areas of

chemical processes and can be also used at different stages of the manufacturing processes such as:

- Extraction / purification;
- Isolation and separation of a molecule of interest from a mixture using supercritical chromatography;
- Production of micrometric and nanometric particles. Depending on the nature of the raw material, the technology will be adapted. If the material is solid, we will talk about extraction and purification and/or fractionation when the material is in liquid phase.

3.3 Extraction

The extraction of the solids is performed in batch or semi-batch mode with a continuous supply of supercritical fluid (e.g., CO_2). A high-pressure pump sends the CO_2, maintained in a liquid state by means of a condenser, to the extraction reactor or autoclave previously loaded with the biomass and preheated to the working temperature. The extract loaded with supercritical fluid is sent to the separator by means of a pressure reduction valve. At reduced temperatures and pressures, the extract precipitates in the separator while the pure CO_2 gas is purified and reintroduced into the reactor, as shown in Figure 9.

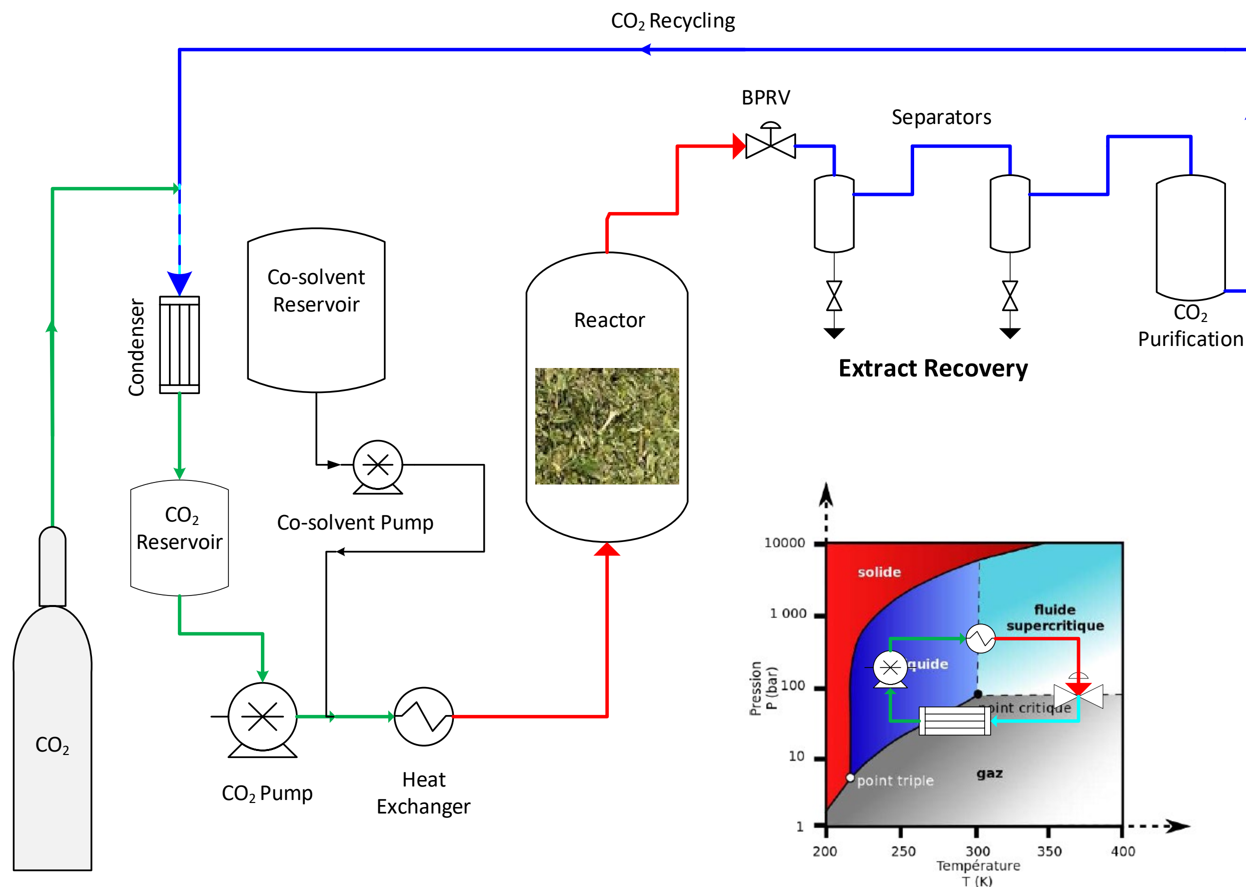


Figure 9. Schematic supercritical fluid extraction process.

There are four primary involved steps: extraction, expansion, separation, and solvent conditioning. The four corresponding critical components needed are a high-pressure extractor, a pressure reduction valve, a low-pressure separator, and a pump for intensifying the pressure of the recycled solvent. Other ancillary equipment includes heat exchanger, condenser, storage vessels, fluid make-up source, etc.

The feed, generally ground solid, is charged into the extractor. CO_2 is fed to the extractor through a high-pressure pump. The extract-laden is sent to the separator via a back pressure relief valve (BPRV). At a reduced temperature and pressure conditions, the extract precipitates in the separator, while CO_2 free of any extract, is recycled to the extractor. SFE for solid feed is a semi batch process in which carbon dioxide flows in a continuous mode, whereas the solid feed is charged in the extractor basket in batches. For making the process

semicontinuous at the commercial scale, multiple extraction vessels are sequentially used, such that when one vessel is on loading or unloading, the other vessels are kept in an uninterrupted extraction mode. Separation is carried out in stages by maintaining different conditions in two or three separators for fractionation of the extract, depending on the solubilities of the components and the desired specifications of the products. Similarly, by varying the pressure, it is possible to alter the solvent power of the extractant, the effect of which is equivalent to changing the polarity of an extraction solvent. Thus, a production plant can have flexible operating conditions for multiple natural products, and it is also possible to obtain different product profiles from a single botanical material by merely using a single solvent, namely, supercritical CO_2.

CO_2 as a solvent diffuses (mostly temperature-dependent effect) inside the substance like gas, while the density (mostly a pressure-dependent effect), which behaves like a liquid and dissolve the desired material, thus increases the yield of desirable bioactive molecules. This phenomenon derives itself from a combination of Charles's and Boyle's Laws where, for example, a lower temperature and higher pressure increases the molar volume/density and decreases diffusivity (important for less volatile compounds) [66].

Carbon dioxide is chosen because it is cheap, recognized as safe (GRAS) and presents critical point under mild operating conditions (31 °C and 73.8 bar) which favor the extraction of thermolabile compounds [67]. Supercritical carbon dioxide (SC-CO_2) is a lipophilic solvent, but its low polarity is overcome, employing polar modifiers (alcohols, water, acids, etc.) as co-solvents, expanding, in consequence, the extraction range to include more polar components [68, 69].

When the biomass is liquid (e.g. oils, liquid by-products, etc.), we refer to a fractionation or counter-current extraction, which has the advantage of being a continuous operation unlike a batch extraction while allowing a unitary operation with several trays or stages, thus promoting better separation. There are two types of fractionations: counter-current extraction and supercritical fractionation.

The fractionation or counter-current extraction requires a vertical column, filled with a packing in which, due to differences in density, the liquid phase flows downwards along the column

while the supercritical fluid flows upwards. The supercritical fluid emerging from the top of the column is a solution containing the extract and the liquid leaving the bottom of the column is raffinate. The difference between supercritical counter-current extraction and supercritical fractionation is that the former is conducted at a constant temperature while for the latter, a temperature gradient is imposed at the top of the column. Furthermore, in the case of extraction, the liquid feed to be extracted is injected at the top of the column while, in the case of fractionation, the feed is located somewhere between the top and the bottom of the column with the presence of a reflux. Figure 10 below illustrates the flow diagram of a typical supercritical fractionation system. The feed is from the center or top of the column and the supercritical fluid enters from the bottom. The wash section is at low temperature while the enrichment section is at high temperature.

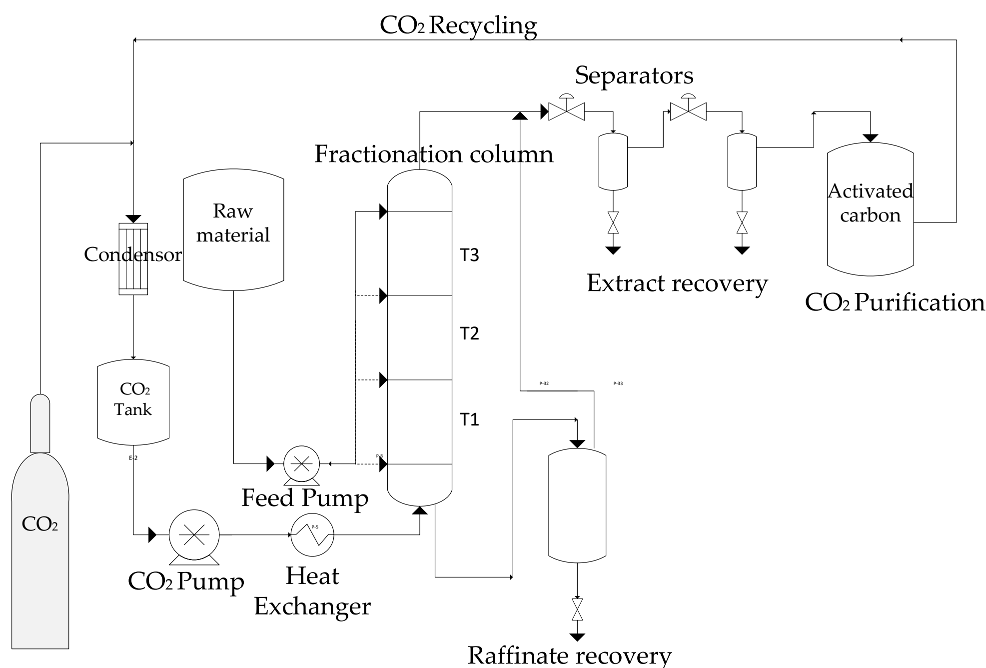


Figure 10. General view of supercritical fractionation flow diagram

3.4 Supercritical fluid chromatography

Once the extract is obtained, the next step is to separate and isolate the molecule(s) of interest. Among the existing separation technologies, supercritical chromatography is identified as an emerging one [70].

It is a recent separation method that can be considered as intermediate between gas

chromatography (GC) and liquid chromatography (HPLC). Supercritical chromatography has the advantages related to the use of supercritical fluids (solvent recycling, no organic solvents, product purity, etc.) and its ability to use all HPLC and gas chromatography (GC) detectors, which makes it attractive as an ultimate purification step in the pharmaceutical and fine chemical sectors.

In this mode of chromatography, like conventional chromatography (HPLC), the mobile phase is a supercritical fluid. The low viscosity of the fluid enables providing high flow rates, which do not affect the efficiency of the separations. This is performed in a few minutes (typically in the order of a few tens). Comparative studies of separation by supercritical fluid chromatography (SFC) and liquid chromatography (LC) have shown that liquid chromatography (LC) requires 4.2 more time and uses 12 times more solvent than supercritical fluid chromatography (SFC) for the isolation of an active principle in the pharmaceutical field.

The principle is like that of liquid chromatography since the mobile phase, which is the supercritical fluid, pushes the sample through a column packed with a stationary phase. At the exit of the column, the compounds, having different retention times, are detected.

Supercritical chromatography is suitable for non-volatile molecules (not analyzable by gas chromatography) and those that do not have adequate UV chromophores to be analyzed by HPLC. Most of its applications are in the pharmaceutical field to separate small quantities of active ingredients during the development phases of a drug. This separation technique has experienced a rapid development over the past years and many companies are now offering preparative chromatography systems that can separate up to a few tens of grams per day.

The applications relate to the separation of natural products and fatty acid esters such as omega-3 when pure CO_2 is used. The field of application extends to the separation of chiral molecules and active ingredients when CO_2 is spiked with a polar cosolvent such as ethanol, methanol, etc.

3.5 Supercritical particle design

For nutraceuticals, pharmaceuticals and microelectronics, the size distribution and the shape of dry product have to be well controlled as the needed particle size is often in the order of a micron-meter [71]. Thus, the production of micro- and nanoparticles, when supercritical fluids are used, is the subject of increasing interest by mainly pharmaceutical companies that have to deal with

the following major challenges [72, 73]:

- Increasing the bioavailability of molecules with low solubility;
- Designing and developing controlled-release powdery formulations;
- Effecting a less-intrusive release than conventional methods (e.g., orally, pulmonary, transdermal patch).

There are four main categories of SCF-assisted micronization (Table 3), depending on the role played by the supercritical fluid which can be used as a solvent, anti-solvent, solute, or a co-solute. Each of them has unique characteristics that will guide the selection depending on the targeted product. Table 3 shows the conditions to be met depending on the type of the process.

Table 3. Operating conditions SC-CO_2 mode

Role of Supercritical CO_2	Conditions
Solvent	The substance to be processed should be soluble in SC-CO_2.
Antisolvent	The substance to be processed must be practically insoluble in SC-CO_2, but the base solvent should have a high affinity for SC-CO_2.
Solute	SC-CO_2 should be soluble in the substance to be processed.
Co-solute	The substance to be processed must be practically insoluble in SC-CO_2, which should have a good solubility in the solvent.

3.5.1 Supercritical carbon dioxide as solvent

Rapid expansion supercritical solutions (known as RESS process) involve dissolving the solute of interest in the supercritical fluid under high pressure before directing this solution to a depressurizing chamber in which the supercritical mixture is expanded rapidly via a nozzle or a capillary (Figure 11). This causes a precipitation due to the expansion of the solution, which greatly modifies the solubility of dry product. The nozzle orifice is one of the decisive components of the RESS process because it is subject to clogging.

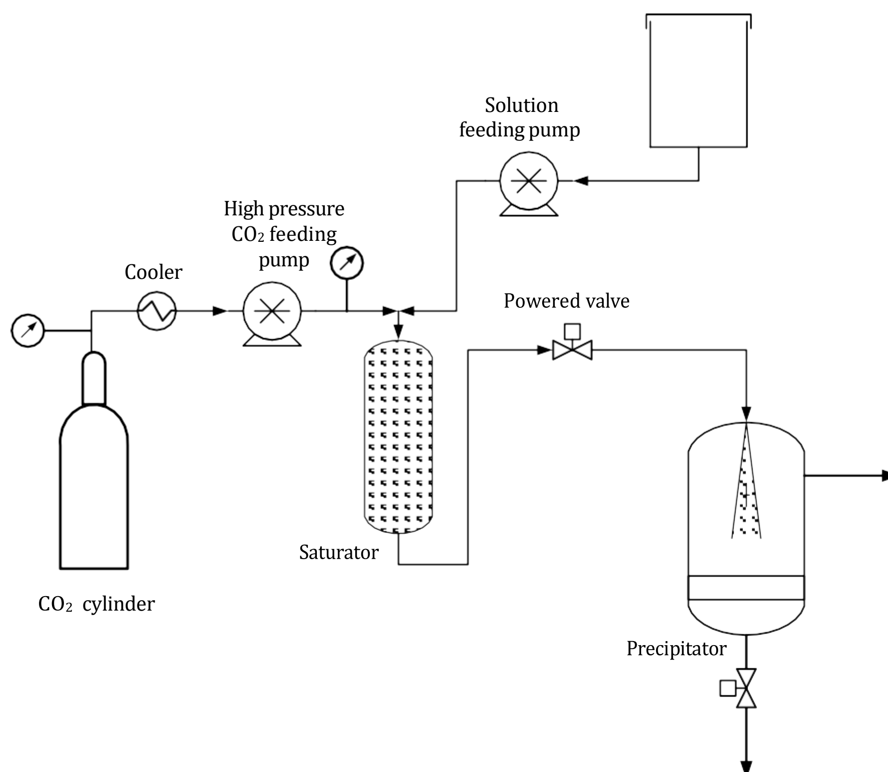


Figure 11. RESS process

3.5.2 Supercritical carbon dioxide as antisolvent

The solute of interest is first dissolved in a classic solvent before the addition of the SC-CO_2, which triggers the precipitation since it acts as an anti-solvent. In anti-solvent process, in addition to the substances to be micronized and SC-CO_2, a third compound is used. It is an organic solvent in which the product of interest solvates straight forwardly and must also have a good compatibility with supercritical fluid as an anti-solvent or be perfectly miscible.

When the solvent evaporates in the supercritical phase, the solute will be concentrated and brought to supersaturation level. When the SC-CO_2 dissolves in the organic phase, it decreases the density and, therefore, its solvent power. The solute begins to precipitate, that is, the microparticles are produced (Figure 12).

There are two crucial conditions to make successful use of the antisolvent process:

- The solute (i.e., the substance to be powdered) must have low solubility in the supercritical fluid;
- The solvent (used to dissolve the solute) should have maximum solubility in the supercritical fluid.

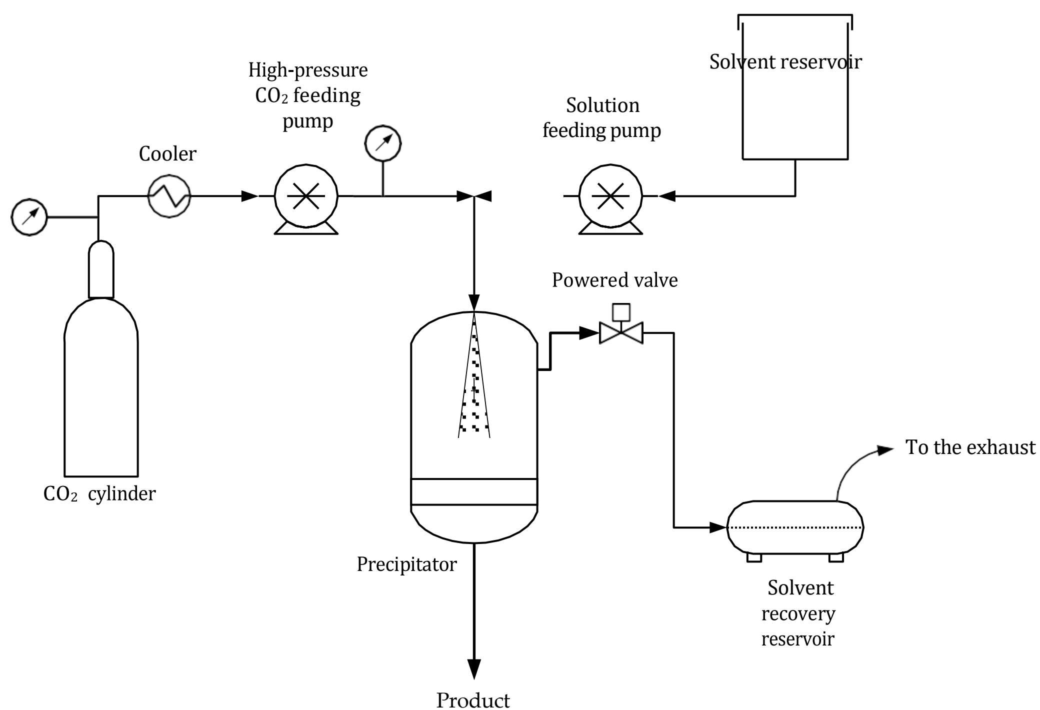


Figure 12. Schematic view of SC-CO_2 as an antisolvent (SAS process)

3.5.3 Supercritical CO_2 as co-solute

In this type of process, SC-CO_2 is dissolved in the liquefied compound. The gas-saturated mixture is then decompressed through a nozzle, inducing the precipitation and the formation of particles within the atomization region. This process is commonly called PGSS, an acronym for particles from gas- saturated solution (Figure 13).

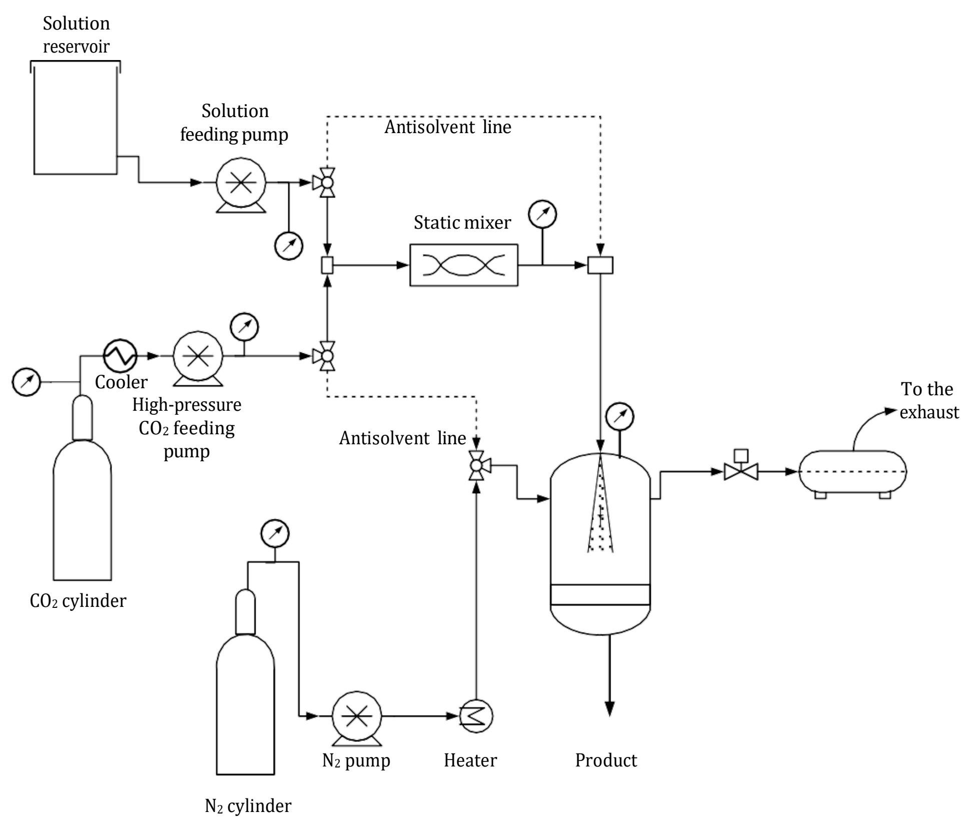


Figure 13. Schematic view of micronizing process (PGSS-drying process)

CHAPTER 4 METHODOLOGY

4.1 Cannabinoid's extraction

4.1.1 Supercritical carbon dioxide extraction

The ability of cannabinoids to dissolve in extraction and processing solvents as well as in product formulation is crucial to processing and product formulation. In order to do that, the solubility parameter theory (SPT) describes the solvency of cannabinoids in processing solvents including hydrocarbon fluids, sub-critical, super-critical CO_2, and ethanol.

Table 4. Solubility of cannabinoids and CO_2 solvent

Chemical compound	Solubility ($MPa^{1/2}$)	References
Δ^9-THC	21.6	[64]
CBG	21.1	[74]
CBN	17.8	[75]
SC-CO_2	0-21.8	[64]

The essential of SPT is that such cannabinoids will optimally dissolve and mix when they fall within or equal the solubility parameters of the solvent media [76]. The diffusivity of the CO_2 as a supercritical solvent is higher than the other usual solvents, and this makes the mass transfer easier and the extraction process faster. Low viscosity decreases the surface tension and allows it to penetrate the botanical material from which the active ingredient is to be extracted.

Extraction with supercritical CO_2 depends on the occurrence of the individual functional groups in the biomass, their molecular weight and polarity.

Here, we present a review of previous studies on cannabis oils extractions using the SC-CO_2 (Table 5) technology in order to show what are the most studied parameters, point out that the choice of the parameters of our project, and the added value we bring to the field.

Table 5. Review on cannabis oil extraction using SC-CO_2.

P (bar); *T* (°C)	Solvent	CO_2 flow rate (g/min)	Ref
100-1300; 50	SC-CO_2 (0-5 % ethanol)	/	[76] Baldino et al.
250-450; 45	SC-CO_2	116.6	[77] Vagi et al.
170-340; 55	SC-CO_2 (25 % ethanol)	200	[78] Rovetto et al.
80-400; 35-65	SC-CO_2	35	[79] Attard et al.
150-330; 40-80	SC-CO_2 (0-5 % ethanol)	9.15	[80] Gallo-Molina et al.
75-500; 31-80	SC-CO_2	/	[81] Mueller et al.
150-320; 60	SC-CO_2	40-150	[82] Rochfort et al.
180-320; 40-80	SC-CO_2 (0-16.7% ethanol)	/	[83] Monton et al.
150-250; 35-45	SC-CO_2	/	[84] Qamar et al.
150 & 330; 40 & 60	SC-CO_2 (0-2% ethanol)	/	[85] Kargili et Aylac

Since the review published by Baldino et al. [76], some researches were dedicated to optimize the supercritical extractions of cannabinoids. It will be difficult to draw a consensual conclusion, knowing that cultivar of cannabis is not always the same and also, the raw material was not systematically decarboxylated.

Moreno et al. have concluded that neutral cannabinoids are more efficiently extracted with pure CO_2 at 50 °C, 200 bar. Trying to optimize the extraction, Vagi et al. have observed that increasing pressure from 250 to 450 bar, while working at 45 °C, the extraction yields increased (up to 6.59% on dry basis), but not for cannabinoids yields. Rovetto et al. recommended to work at 55 °C, 340 bar to shorten the extraction time. As they stated, higher is the pressure, larger is the solvent power and the smaller is the extraction selectivity. For a better extraction of waxes, Attard et al. suggested 350 bar and 50 °C. To extract THC from *Cannabis sativa* L., Gallo-Molina et al. suggested to operate at 60 °C, 330 bar and 2% of ethanol as cosolvent. Rochfort et al. have concluded that the maximum yield of decarboxylated cannabis was 7.1% at 60 °C, 320 bar, 150 g/min of CO_2 and ten hours as extraction time.

Qamar et al. have concluded that the maximum yield of CBD dominant cultivar was 14.8% at 35 °C, 250 bar and 3 h as extraction time.

Monton et al. have optimized the extraction of non carboxylated cannabis using pressures from 180 to 320 bar, temperatures between 40 and 80 °C and ethanol as cosolvent (0-16.7%). They obtained a maximum yield of 7.23% at 60 °C, 320 bar and 16.7% EtOH.

Finally, Kargili et Aytac have extracted four different cultivars of decarboxylated cannabis at two temperatures (40 and 60 °C), two pressures (150 and 330 bar), two ethanol percentages as cosolvent (0-2%). They found that the maximum yield of 9.7% was obtained at 40 °C, 330 bar and 2% of cosolvent.

Mechanical extraction or solvent extraction are frequently used in commercial large-scale seed oil extraction. Oil from seeds is frequently extracted mechanically, which depends on pretreating the raw seed material. However, only around 70% of the oil is recovered via mechanical pressing procedures [86].

Commercial-scale solvent extraction uses organic solvents like hexane [87]. Due to the potential for the extraction of some compounds that are more soluble in organic cosolvents than carbon dioxide (CO_2), SFE employs a variety of organic cosolvents including petroleum ether, chloroform, acetone, etc.

The proportions of non-polar and polar lipids recovered from the seeds depend on the solvent media used. A common technique for preparing analysis samples in the lab is the Soxhlet extraction method, which is utilized on an analytical scale. However, there are significant disadvantages to Soxhlet extraction, such as lengthy extraction durations [88]. There has been a growing trend in recent decades to use alternative extraction procedures for the extraction of seed oils to eliminate these disadvantages [89].

Devi and Khanam investigated supercritical fluid extraction, Soxhlet, percolation, coupled with ultrasonication and pyrolysis, as well as pretreatment processes to assess viable extraction techniques for hemp seed oil for the oleochemical industry [90]. The parameters for the supercritical fluid extraction and ultrasonication procedures were optimized using a central composite design. R2 values (> 0.93) for the constructed quadratic models were satisfactory. Using ultrasonication and Soxhlet extraction, a maximum yield of 37.30% was achieved.

4.1.2 Ethanol extraction

Ethanol is originally a substitute for the hexane extraction technique, since it is considered a safer

solvent, less hazardous, for both the environment and the health [91]. It is widely used in the cannabis industry, mainly because the investment is very low, the equipment's are affordable and easy to handle. Also, large quantities of biomass are processed simultaneously (30 to 250 kg per batch), as easy as just putting the solvent and the biomass together in a batch reactor for 5-10 hours, depending on the desired extracted oil and the volume/size of the reactor and the biomass fed.

Ethanol as a solvent dissolve, at high degrees, non-polar compounds like cannabinoids, but also polar molecules like terpenes and polyphenols that are undesired for the pharmaceutical formulation. Further purifications are needed before getting a pharmaceutical grade extract. This is the main disadvantage of the ethanol extraction, in addition to the high amount of solvent consumption and the time-consuming process.

4.1.3 Microwave extraction

Microwave assisted extraction is one of the most important intensified processes since companies became concerned with environmental issues. This technology requires moderate investment to implement it at industrial scale. Its main advantages are rapidity, easy to handle and moderate solvent consumption. Average extraction time is between 3-30 min, sample is immersed in solvent and submitted to microwave energy inside the reactor. There are two major drawbacks for the technology: first, the solvent must absorb microwave energy, and second, a filtration step is required to separate the extract from the solvent [92].

4.1.4 Ultrasound extraction

A Chinese study found that ultrasound-assisted (US) extraction has unique ultraviolet-absorbing properties and better 2,2-diphenyl-l-picryhydrazyl radical-scavenging activity during hempseed oil extraction process. It has also been proved that its extracts are of pharmaceutical grade and that it improves efficiency compared to the conventional techniques [93]. It is easy to manipulate, the sample is immersed in solvent and submitted to ultrasound using a US probe or US bath, processing time varies between 10–60 min, the investment is considered low, but same as the last two technologies, a filtration step is required since the biomass is immersed in the solvent, and the amount of the extracted oil depend on the quantity of the processed biomass and solvent, and this consumes large amounts of solvent [92].

4.2 Design of experiments (BOX-BEHNKEN)

4.2.1 Introduction

While optimizing a chemical extraction process, experiments are conducted. The evaluation of experimental data is based on responses or features of any product such as yield, purity, or process related properties. According to the goals of the experiments, the responses should be properly assessed. The factors are those that have an impact on the experiments.

The following are typical objectives for experiments:

- Measuring or assessing character values without statistically evaluating them;
- Determining through experiment which elements are important to the responses and how much of an impact they have. Statistics are used to examine experiment findings;
- Identifying of the significant factors using statistics;
- Calculating the values of important factors, the optimal conditions are established.

The allocation and strategy for the experiments that will best achieve the goals are decided by the Design of Experiments (DOE). Analysis of the experimental results yields several factors. DOE has been used, as was already noted, to conduct effective experiments and analyze the data.

The DOE characteristic value and DOE factors correspond, respectively, to the design's objective function and design variables. The experiments have a lot of errors. The functions are typically assessed numerically when the DOE approach is used in the process design [94].

4.2.2 Undertake an experimental project

In order to conduct an experimental project, it is important to briefly describe the process (product or manufacturing process) which will be the subject of the experimental project. Defining the purpose of the experiment and the associated objectives are a key step to a successful project, and then it becomes easy to identify the response variable(s) (process output variables). Also, the more data we collect about both the product, and the process

the best it is to identify all the factors that may affect the variable's answer. For example, make an Ishikawa diagram (cause and effect) will help to screen the inputs and their relationship to the response. Before starting the experiments identify the factors that will be held constant during testing, and the factors (primary variables) that will be varied.

It is important to specify whether or not there are any known adverse secondary factors (variables) that will be checked: i) primary factors: those who are at the origin of the project; ii) harmful secondary factors: those that vary during experimentation, and which cannot be kept constant.

If we can control them, we can build a plan in blocks, which makes it possible to neutralize their real effects or not on the response. Also, we can identify the variables (factors) that are not controlled but that can be measured (they are considered as covariates during the analysis of the data).

Once the different factors are identified, specify the minimum and the maximum value (range of variation) of each quantitative primary factor that will be varied during the tests. It is important to explore as much space as possible but avoid problematic regions. Finally, specify the list of modalities for each qualitative primary factor.

If possible, anticipate the relationship (increase/decrease) of the response with each factor and how it will be measured. Afterward, depending on the state of knowledge on the process, propose one or more plans, mainly, sifting plan to separate the important factors from those that are not, or clear separation of main effects and interaction effects plan for optimizing responses.

After checking the project finances and feasibility, determine the number of repetitions (n) of each trial, consider adding trials in the center of the experimental space. It could save time to check if there are any known mathematical relationships between the answer and the factors, also if there's any restrictions on full randomization of the tests. Once the plan set up, specify the experimental protocol for the execution of the tests, and plan tests to validate the solution(s) resulting from the project [95].

4.2.3 Choice of the experimental plan

The experimental method chosen should facilitate the interpretation of the results. It

should also minimize the number of trials without sacrificing quality. The design of experiments theory ensures the conditions for which we obtain the best possible accuracy with the minimum of trials. We therefore have the maximum efficiency with the minimum of experiments and therefore the minimum cost.

However, two main categories of type of statistical plan stand out. It all starts with analyzing the level of knowledge on the factors involved in the process, and whether they exceed 5 factors or not. If few factors influence the process or few factors are known (> 5), or/and we have little information on their impacts and on the process in general, it is a question of an exploratory analysis. We go through a sieving, using all the factors, and the corresponding designs are in general the fractional factorial design, definitive Jones & Nachtcheim sieving designs with 3 modalities.

In the case where the number of factors is equal to or greater than 5, and that we have prior information on the factors, their influences, and the process, we proceed to a confirmatory analysis, by carrying out a modeling or an optimization, with a selection of only critical factors, and in this case the most appropriate designs are the full factorial design, the central composite or Box-Behnken design [94, 96].

4.2.4 Types of experimental plans

There are a lot of types of experimental plans. We describe some of the most used in the industry and scientific research, like complete/reduces plans, Taguchi plans, then we point put the type of Box-Behnken, the one that served us for this project.

4.2.4.1 The complete plans

This strategy consists of testing all possible combinations of selected settings. In this case, the number of trials can quickly become too large, which means that this type of plan is rarely used. On the other hand, it makes it possible to take into account all the interactions between the factors.

4.2.4.2 Reduced plans (fractional)

This strategy consists of testing a fraction of the possible combinations of selected settings. This achieves the effects of several factors with minimal testing. The difficulty remains in the selection of the trials since this does not make it possible to obtain all the

interactions between factors. The objective is to identify and isolate the most influential parameters with part of the complete plan for saving analysis time [96, 97].

4.2.4.3 The Taguchi Plans

This strategy consists in optimizing the reduced plans. This simplifies setting implementation of experimental plans by proposing standard tables (the Taguchi tables).

4.2.4.4 Box-Behnken

The Box-Behnken experiment design is frequently used to assess the impact of processing parameters using fewer materials and experiments. It is a second-order multivariate technique based on three-level incomplete factorial designs that has found widespread use for determining the maximum or minimum of response function, which is a critical experimental condition. N = 2k (k 1) + C0, where (k) is the factor number and (C0) is the replication number of the central point, is the total number of tests (N) required to construct the Box-Behnken matrix [96, 97].

4.2.5 Analysis of the experimental plan

In order to analyze the influence of the process factors, the statistical analysis is based on a model function, f, to represent a relation between input X and output Y. Once established, we generally estimate the model parameters, evaluate the variability decomposition (ANOVA), then we test the hypothesis. Sometimes, if necessary, we proceed to iteration steps (1 to 5). Depending on the choice of the plan, we optimize the response, and graphically represent the results [96, 97].

4.2.5.1 Effect estimates

In general, we use multiple linear regression to model the process, we develop a matrix, according to the following formula.

$X_{N \times p} = [\ x_{ij}\ ]$ p =1+m YN x 1: vector N x 1 4.1

βp x 1 = (β0 , β1 , β2 , β3 , ……. , βk)': vector p x 1 4.2

Subsequently, we need to estimate the values of the coefficients of each factor, and for that, we use the least squares method. Many softwares such as Statistica, JMP Pro or Design Expert facilitate the calculation of least squares to directly estimate the coefficients for the factors introduced [96, 97].

4.2.5.2 ANOVA analysis

Analysis of variance is a term that precisely captures what is actually done to examine sample data obtained to address process issues of both exploratory and optimization nature. It is used to test differences between two or more means, examining variance allows one to draw conclusions about means. ANOVA is used to assess general differences between means rather than specific ones. It tests the initial hypothesis, for example for a p-value less than 5 %, the impact of factors or interactions between factors is significant, whether for p-value higher than 5 %, it is insignificant and then neglected, unless further analysis and tests proves it's wrong.

4.2.5.3 Response Surface Methodology

Response Surface Methodolgy (RSM) is a set of mathematical and statistical techniques in which a response of interest is influenced by multiple variables. It reproduces the variations of a given phenomenon in a three-dimensional space. The horizontal plane of this space materializes the domain of variation of 2 factors while the vertical axis materializes the variation of the response from the empirical model. If there are more than 2 factors, it is necessary, in this case, to fix, at a constant level, the other factors which do not appear in the horizontal plane.

This method applies within a defined framework. If the plan is conducted for exploration purposes, the study should be finalized. The plan used (2k – p), including quantitative or qualitative factors, with a first-order model (with/without Xi Xj interaction) and the optimization is in the form of examining a finite list of possibilities.

Then, conduct a characterization/optimization, with the central-composite plan (Box-Wilson) or Box-Behnken. The only factors included are quantitative, and the model is second order (quadratic), and the optimization becomes equation solving. The RSM aims first to cover the optimal region of the process, but also to test the adequacy of the established model and the possibility of blocking the tests. It helps to accurately estimate the model coefficients allowing a uniform accuracy of the response in all directions. Also, since the goal of statistical plans is to save time and money, it is important to reduce the number of trials as well as the number of modalities for each factor.

The experimental approach of exploring the space of the process, empirical modelling allowing to develop a relationship appropriate approximation between yield and process variables, as well as methods optimization tool to find the values of the process variables that produce desirable values of the response are all included in the methodology for response surfaces.

Equation 4.3 can be used to represent the second-order polynomial model.

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum \sum_{i<j}^{k} \beta_{ij} x_i x_j + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \epsilon \quad (4.3)$$

Where Y is the expected response, β_0 represents the model constant coefficient, βi represent the input parameter xi's, βii the linear coefficient, ii represents its quadratic coefficient, xi, βij represents the interaction between xi and xj, and ε is the model error. The vector of the model's estimated coefficients will be denoted by the symbol β.

According to the following formula, the predicted coefficients are obtained by the multiple linear regression already described.

$$\hat{\beta} = (X'X)^{-1} X'y \quad (4.4)$$

Where X' is the matrix's transposition, X is the model's matrices, which depend on the experimental locations selected to carry out the model's plan, and y is the vector of responses.

Reviewing a model like (Y) serves the following three goals:

- First, build a relationship between y and $x_1, x_2, \ldots, x_k$ that can be used to forecast response values for given values of the variables. This relationship need not be exact;
- Second, use hypothesis testing to ascertain the importance of the variables;
- Third, ascertain the ideal $x_1, x_2, \ldots, x_k$ values that will result in the response's maximum (or minimum) over the area of interest [97].

4.3 Cannabinoid purification

4.3.1 Introduction

As shown in Figure 14, based on packed and non-packed columns chromatography, there are

two main classes of liquid chromatography.

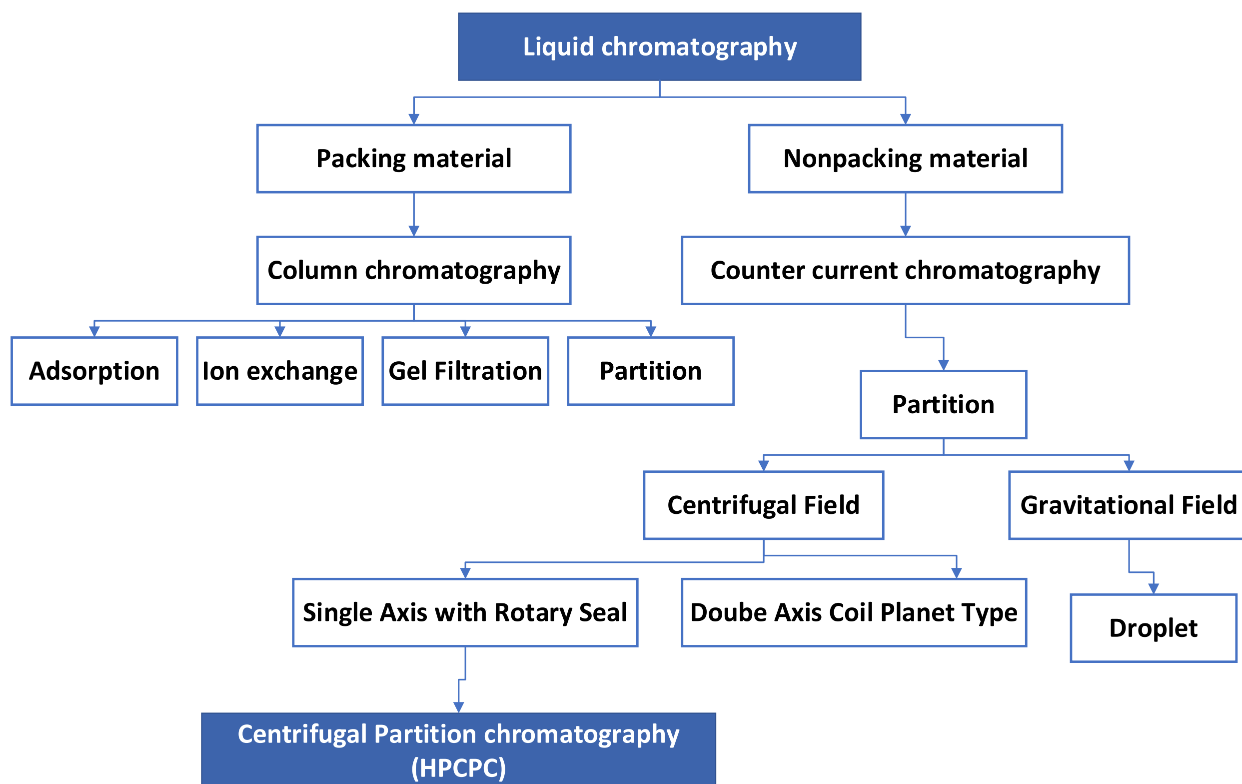


Figure 14. Classification of high-performance liquid chromatography [98].

In the process of creating bioactive chemicals, separation and purification are crucial phases. The phytochemicals in *C. sativa* that are the subject of the following discussion have been separated and purified using the techniques of solid-phase extraction (SPE), hydrophilic interaction liquid chromatography (HILIC), supercritical chromatography, and fast centrifugal partition chromatography (FCPC).

4.3.2 Solid-phase extraction (SPE)

SPE is a separation method that divides liquid substances based on their physicochemical properties. The main variables determining the separation and purification process, as well as the final quality of the bioactive chemicals in *C. sativa*, are the types and qualities of adsorbents and the elution parameters in SPE.

In the work published by Di Palo et al. [99], N-trans-caffeoyltyramine cannabisin B was purified

from the ethanol extract of hempseed hull using HPD-600 macroporous resin adsorption and LH-20 gel chromatography. In a different investigation, THC was extracted and purified from a SPE extract of cannabis inflorescences with a high purity (36.18%).

Gallo-Molina et al. reported that they obtained THC content of 37.85% by supercritical fluid extraction at 60 °C, 33 MPa and 2% ethanol as cosolvent. Using SPE, they claim that the purity of THC was 90.1% [80].

SPE was also used to quantify trace presence of cannabinoids in wastewater and blood. Klu et al. have developed a method using SPE and LC-MS/MS to quantify THC in blood and they concluded that, at 99.7% confidence level, the expanded uncertainty was 0.393 mg/L for a THC concentration of 2 mg/L [100].

A method was developed and applied practically to detect six naturally occurring cannabinoids (CBG, CBD, CBDV, CBN, THC, THCV), two cannabinoids in acidic form (CBDA, THCA-A), and the major cannabis-related human metabolite (THC-COOH) in wastewater [101]. After SPE offline enrichment, the authors used UPLC-ESI-MS/MS system.

4.3.3 Hydrophilic Interaction Liquid Chromatography (HILIC)

HILIC is a promising alternative separation method for polar compounds. It combines stationary phases used in the normal phase liquid chromatography (NPLC) method, and mobile phases used in the reversed phase liquid chromatography (RPLC) method.

Coupled with the chemical profile from high resolution mass spectrometry (HRMS) detection, a novel hydrophilic liquid chromatography (HILIC) CBD quantification method was developed, specifically for CBD blended sample hemp seed oil materials, which can overcome the matrix buildup in reverse phase columns [102].

4.3.4 Supercritical Fluid Chromatography (SFC)

Supercritical fluid chromatography (SFC) has attracted great interest from scientific community, particularly in the pharmaceutical sector. This attention was due to several advantages of SFC: i) versatility; ii) shorter analysis than conventional or classical chromatography; iii) and lower amounts of organic solvents. Although few papers were published for dosing CBD by SFC [103, 104], some application notes developed by industrial manufacturers are available.

A work was conducted recently to develop a SFC method to study the applicability of SFC for

the analysis of cannabinoids and to compare it with standard UHPLC. Using nine cannabinoids (CBC, CBN, CBD, CBG, Δ^8-THC, Δ^9-THC, CBCA, CBGA, Δ^9-THCA), the authors conclude that SFC analysis method is a viable method with a shorter analysis time (6 min vs 18 min for UPHLC) [105].

4.3.5 Fast Centrifugal Partition Chromatography (FCPC)

A FCPC device is made to carry out chromatography by maintaining the stationary phase in a liquid-liquid biphasic system without the use of solid support. A FCPC instrument is essentially a collection of channels connected in cascade by ducts and arranged in a circle around a rotor using cartridges or discs; when the rotor is in motion, this assembly is subjected to a constant centrifugal field (Figure 15).

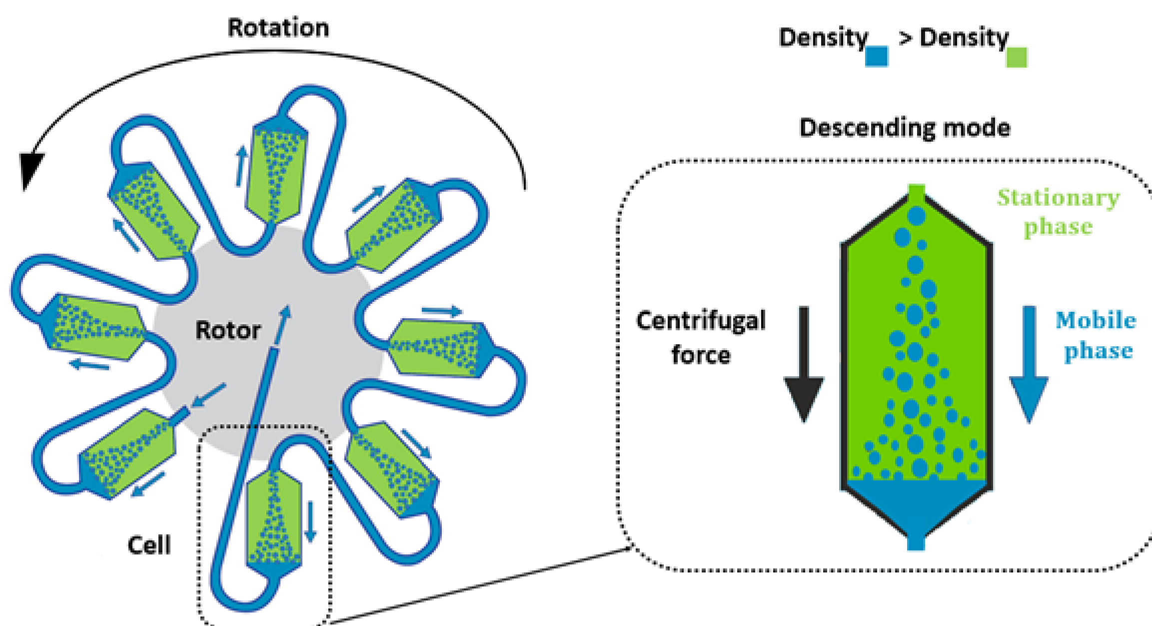


Figure 15. FCPC principle

Unlike traditional chromatography, there is no solid stationary phase in this method. Instead, a liquid is retained inside the device by centrifugal forces as the mobile phase, which is also a liquid, flows through. As a result, there is no possibility of sample loss due to irreversible attachment to

the stationary phase.

Two-phase solvent system is used as a separation medium in FCPC. One serves as the stationary phase, the other serves as the mobile phase. The solvents may be selected from an infinite variety of possible combinations. There are countless solvent mixtures that form immiscible phases.

The mobile phase percolates through the stationary phase with the help of a pump and the centrifugal field using two immiscible liquids that are created by combining two or more solvents or solutions. Upper and lower phases of the biphasic system can be chosen as mobile or stationary due to their liquid nature; in the ascending mode, the lighter phase percolates through the heavier one in the direction of the centrifugal field (from the outside to the center of the rotor in each channel), and in the descending mode, the heavier phase percolates through the lighter one in the opposite direction (from center to periphery of the rotor in each channel).

Compared to other chromatography techniques, FCPC benefits from a number of advantages: i) no irreversible adsorption; ii) total recovery of injected sample; iii) tailing is minimized; iv) low risk of sample denaturation; v) low solvent consumption; vi) and most importantly, easily scalable and the possibility to purify from milligrams to kilograms.

To ensure large yields of bioactive chemicals in *C. sativa* and industrial-grade preparative separation and purification, FCPC with a high flow rate is very selective. Cannabidiolic acid (CBDA) and cannabivarinic acid (CBDVA) were extracted from hemp using the pH-zone-refining technologies by FCPC in a study [106].

In a different investigation, FCPC and HPLC were set up for the large-scale separation and purification of CBD in crude *C. sativa* extract, in which CBD had a purity of greater than 95% [32]**.**

CHAPTER 5 ARTICLE1: OPTIMIZATION OF SUPERCRITICAL CARBON DIOXIDE FLUID EXTRACTION OF MEDICINAL CANNABINOIDS BY A BOX-BEHNKEN DESIGN OF EXPERIMENTS

Article submitted to The Journal of Supercritical Fluids on November 30th 2022

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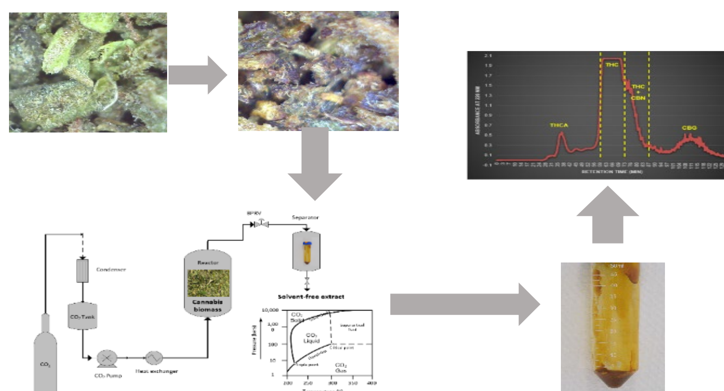
Graphical abstract



Keywords: Cannabinoids, Box-Behnken, Optimization, Supercritical carbon dioxide, Fast Centrifugal Partition Chromatography

Abstract

Research on cannabis oil has evolved to encompass the pharmaceutical industry for the therapeutic potential of the active compounds for pathologies such as Alzheimer, auto-immune disorders, and cancer. These debilitating diseases are best treated with cannabinoids such as tetrahydrocannabinol, cannabigerol, and cannabinol that relieve neuropathic pain and stimulate the immune system. The extraction by supercritical CO_2 produces extracts with 24 % more cannabinoids than traditional ethanol extraction. We optimized the yield of Δ^9-THC, CBG, and CBN in a batch laboratory scale vessel based on a 3 level Box-Behnken design of experiments comprising CO_2 flowrate, pressure, temperature, and time. The yield of Δ^9-THC and CBG after 2 h and 15 g/min of CO_2, 235 bar, 55 °C, was 14.6 and 0.54 g/100 g of raw material, respectively and after another 2 h the yield of CBN was 1.1 g/100 g.

5.1 Introduction

The cannabis plant was originally used in central Asia during the Neolithic period. Western medicine adopted medicinal cannabis in the 1800s, when the Irish physician William O'Shaughnessy and French psychiatrist Jacques-Joseph Moreau attempted to treat tetanus, rabies, and mental disorders [1]. For example, dronabinol is a synthetic tetrahydro-cannabinoid (Δ^9-THC) to treat anorexia, nausea, and vomiting, initially approved by the FDA on May 31, 1985 [2]. The department of psychiatry and mental health of the University of Cape Town, South Africa reported that dronabinol and nabilone (also a synthetic Δ^9-THC) are available in Canada, USA, UK, Spain to treat nausea after chemotherapy, pain, and spasticity. This raised the interest in the use of psychoactive ingredients of the cannabis plant in other medical contexts, like weight gain in HIV-positive patients [3].

Chronic pain affects 30 % of the adult population, with prognostic factors of age, baseline pain, mental health complaints, and genetic factors. The study shows that patients

experience the same pain at multiple sites [4]. Ointments and tinctures were the first forms of medical cannabis applied to relieve soldier's pain during the American civil war [1] but they also come as oils and resins [5]. Besides these medicinal forms, people consume cannabis for recreational purposes: they smoke and vape dried herb and hash or kief, apply it as a wax (shatter and budder), drink it in soda, teas and coffees, and eat it [6].

However, cannabis use degenerated during the subsequent decades and was perceived as dangerous for young people. It was first banned in Egypt during Napoleon's occupation, followed by British Burma in 1891. In 1909, an international meeting on opium was initiated by the United States. The meeting was followed by the Shanghai Opium Commission, and consequently the Hague Opium Convention of 1912, to discuss the use of cannabis. Cannabis was regulated under the 1925 International Opium Convention, on the request of Egypt and support from South Africa, Italy, Turkey, and others [7]. Consequently, many countries criminalized it, including Canada who had already banned the use of opium and psychedelic drugs, including cannabis, in 1923 [1]. Meanwhile, patients continued to use cannabis illegally, until mid 2010 where support for cannabis legalization gained attraction, especially since scientific research provided evidence for its potential in medicine.

In July 2000, the Ontario Court concluded that Terrance Parker (a patient suffering from a severe form of epilepsy since childhood) was exempted from cannabis restrictions for personal medical use. This led Canada, in 2001, to be the first country to implement a system for regulating medical cannabis. In October 2018, cannabis became legal for recreational use [1]. In 2019, cannabis plant seeds and female flowers were recognized as pharmaceuticals and nutraceuticals by Health Canada [8]. Results from clinical trials published in 2022 confirmed the safety, tolerance, and pain reduction properties of medical cannabis for older adults [9].

Universities and research centers were attracted again to conduct research on cannabis,

after it's legalization, considering the lack of data for process conditions to extract each cannabinoid. Furthermore, each has its own therapeutic effect including efficacy and safe dosing limits [3].

Journals publish 10 times more articles on cannabidiol and delta 9-tetrahydrocannabinol (CBD and Δ^9-THC) than on other cannabinoids. These two phytocannabinoids are the focus of medical research. Cannabinoids and modulators of endogenous cannabinoids in the body are proven to have antinociceptive properties according to preclinical studies [10]. CB1 and CB2 receptor agonism is the main mediator of the analgesic effects of Δ^9-THC, whereas CBD targets receptors like 5-HT1A receptor agonism, negative allosteric modulation of CB1, GPR55 antagonism, TRPV1 activation, PPARγ activation, and reuptake inhibition (e.g., anandamide and adenosine) [10].

COVID-19 negatively impacted various industries from 2020 to 2021, but the nutraceutical market grew exponentially during the first 6 months of the pandemic, since they help manage pain, but also improve immunity and reduce fatigue. Pain costs society billions of dollars in lower productivity and affects millions of people [11]. However, the scale and standardization of cannabis-based nutraceutical product is still a challenge. There is an urgent need to establish the efficacy of medical cannabis and standardize their properties for adjunctive therapy as an alternative to opiates [12]. The European Pain Federation advise patients to use medical cannabis as an oil extract [13].

Clinical trials have not substantiated the scientific literature of clinical data providing evidence of the benefit of cannabinoids. Of the 171 preclinical trials reported in the meta-analysis of Finn et al. [14], only 11 clinical trials were conducted. For example, in one trial pain was higher after a third molar extraction in clinical trials than expectation from preclinical studies. Pharmacokinetics investigations are inconclusive as they, among other things, consider different routes of administration. Thus, the industry is facing a real problem with product standardization and identifying specific molecules for each therapeutic effect.

For the cannabis industry, it is not enough to know the raw material (cannabinoids, terpenes, and polyphenols) to choose a technology because the target molecules are sensitive to the end use (recreational vs pharmaceutical, for example). Cannabis research to extract cannabinoids include maceration, solvent extraction, microwave, supercritical fluid, and ultrasound. Some studies compare the total extraction yields from the different technologies – microwave vs ultrasound, for example.

The International Council for Harmonization threshold of solvent residues for nutraceuticals is 0.03 % for hexane and 0.5 for ethanol. The residue varies with extraction technology, and this is one of the motivations to choose supercritical fluid extraction (SFE) since it is a selective process. SFE separates, or dissolves, plant matrix components based on their solvating properties. Changing the temperature above the critical point maintains the solvating property of the component extraction [15].

SFE technical properties reduce the purification steps of the extracts to meet the demands of the pharmacological market [16, 17].

The knowledge of physicochemical properties of compounds of interest is relevant for defining the optimal conditions of any extraction technique (Table 6). Wong et al. determined the physicochemical properties of synthetic cannabinoids, and their data helps improve the natural cannabinoids extraction technologies [18].

Table 6. Physicochemical properties of cannabinoids.

Properties	Δ^9-THC	CBN	CBG	References
Molecular formula	$C_{21}H_{30}O_2$	$C_{21}H_{26}O_2$	$C_{21}H_{32}O_2$	/
Molecular weight(g/mol)	314.2	310.4	316.22	/
Density (g/L)	1.02	1.06	/	[18]
Dipole moment (μ)	0.88	1.43	1.55	[19]

Since the review published by Baldino et al. [20], some research were dedicated to

optimize the supercritical extractions of cannabinoids. It will be difficult to draw a consensual conclusion, knowing that cultivar of cannabis is not always the same and also, the raw material was not systematically decarboxylated (Table 7).

Moreno et al. have concluded that neutral cannabinoids are more efficiently extracted with pure CO_2 at 50 °C, 200 bar [21]. Trying to optimize the extraction, Vagi et al. have observed that increasing pressure from 250 to 450 bar while working at 45 °C, the extraction yields increased (up to 6.59% on dry basis), but not for cannabinoids yields [22]. Rovetto et al. recommended to work at 55 °C, 340 bar to shorten the extraction time. As they stated, higher is the pressure, larger is the solvent power and the smaller is the extraction selectivity [23].

Table 7. Review on SCF cannabis oil extraction.

P (bar); *T* (°C)	Solvent	CO_2 flowrate (g/min)	Ref
100-1300; 50	SC-CO_2 (0-5 % ethanol)	/	[20]
250-450; 45	SC-CO_2	116.6	[21]
170-340; 55	SC-CO_2 (25 % ethanol)	200	[22]
80-400; 35-65	SC-CO_2	35	[23]
150-330; 40-80	SC-CO_2 (0-5 % ethanol)	9.15	[24]
75-500; 31-80	SC-CO_2	/	[25]
150-320; 60	SC-CO_2	40-150	[5]
180-320; 40-80	SC-CO_2 (0-16.7% ethanol)	/	[26]
150-250; 35-45	SC-CO_2	/	[27]
150 &330; 40 &60	SC-CO_2 (0-2% ethanol)	100	[28]

For a better extraction of waxes, Attard et al. suggested 350 bar and 50 °C [24]. To extract THC from *Cannabis sativa* L., Gallo-Molina et al. suggested to operate at 60 °C, 330 bar and 2% of ethanol as cosolvent [25]. Rochfort et al. have concluded that the maximum

yield of decarboxylated cannabis was 7.1% at 60 °C, 320 bar, 150 g/min of CO_2 and ten hours as extraction time [5].

Qamar et al. have concluded that the maximum yield of CBD dominant cultivar was 14.8% at 35 °C, 250 bar and 3 h as extraction time [26].

Monton et al. have optimized the extraction of non carboxylated cannabis using pressures from 180 to 320 bar, temperatures between 40 and 80 °C and ethanol as cosolvent (0-16.7%). They obtained a maximum yield of 7.23% at 60 °C, 320 bar and 16.7% EtOH [27]. Finally, Kargili et Aytac have extracted four different cultivars of decarboxylated cannabis at two temperatures (40 and 60 °C), two pressures (150 and 330 bar), and two ethanol percentages as cosolvent (0-2%). They found that the maximum yield of 9.7% was obtained at 40 °C, 330 bar and 2% of cosolvent [28].

The present study contributes to the optimization of supercritical CO_2 extraction for THC, CBG and CBN, and to initiate standardization of a pharmaceutical grade extract based on a Box-Behnken design. This standardization step is important to compare effectively between different pre-clinical and clinical studies, and we choose to optimize for each cannabinoid separately since their therapeutic effects are different.

5.2 Material and methods

5.2.1 Plant material

We purchased 1 kg of dried and crushed cannabis flowers from QC Gold TECH, (Saint-André-Avellin, QC; Canada). The cannabis was stored in the dark, at room temperature, right after its reception and secured in a safe-store box.

5.2.2 Chemicals

CO_2 (99% purity) used for the supercritical extraction was from Oxymed (Montréal, Canada). HPLC solvents, methanol (MeOH) and formic acid (HCOOH), were of analytical grade from Techni Science (Oisterwijk, Netherland). The standards used for the HPLC quantification are Δ^9-THC, CBD CBG, and CBN, of 1.0 mg/mL in methanol,

bought from Sigma Aldrich (Oakville, Canada).

5.2.3 Decarboxylation

Based on data from literature [39], we choose to perform the decarboxylation at 120 °C, 90 min in a Thermo Electron Corp. oven (model 6520 series) that employs gravity convection as a method of heat transfer. 1 kg of biomass (A) was split between two trays (B and C). B and C were superimposed in the oven during the decarboxylation, and this induced a non-uniformly distributed heating, as the bin below received more heating than the one above. Afterward, the two biomass samples were mixed and homogenized (BC). Analyses are all in triplicate.

5.3 Supercritical fluid extraction (SFE) protocol

The reactor consisted in a 225 mL stainless steel cylinder from Thar Technologies which was equipped with a heating element. In each run, 15 g of dried decarboxylated cannabis was placed in the reactor (Figure 1). The system was filled with CO_2 from a 50 L cylinder, compressed to the desired extraction pressure by a model P50 CO_2 pump from Thar Technologies (Pittsburgh, Pennsylvania, USA). It passed through a heat exchanger which was working at 4 °C (to avoid formation of gaseous CO_2) with a mixture of 50% ethylene glycol, as cooling agent, CO_2 flowrate is controlled via a Thar keypad controller. A manual backpressure regulator maintained the pressure at prescribed settings (BPRV, model BP66-1A11QEQ151, 0-10000 psig, GO Regulator). A heat exchanger brought the CO_2 to the operating temperature before entering the reactor. The extract separated from the solvent when the pressure dropped to 50 bar. To minimize ice formation during depressurization, which would block the line, the backpressure valve is equipped with a heating element. The extracts were recovered from the separator and stored in the dark until the analysis by HPLC-UV.

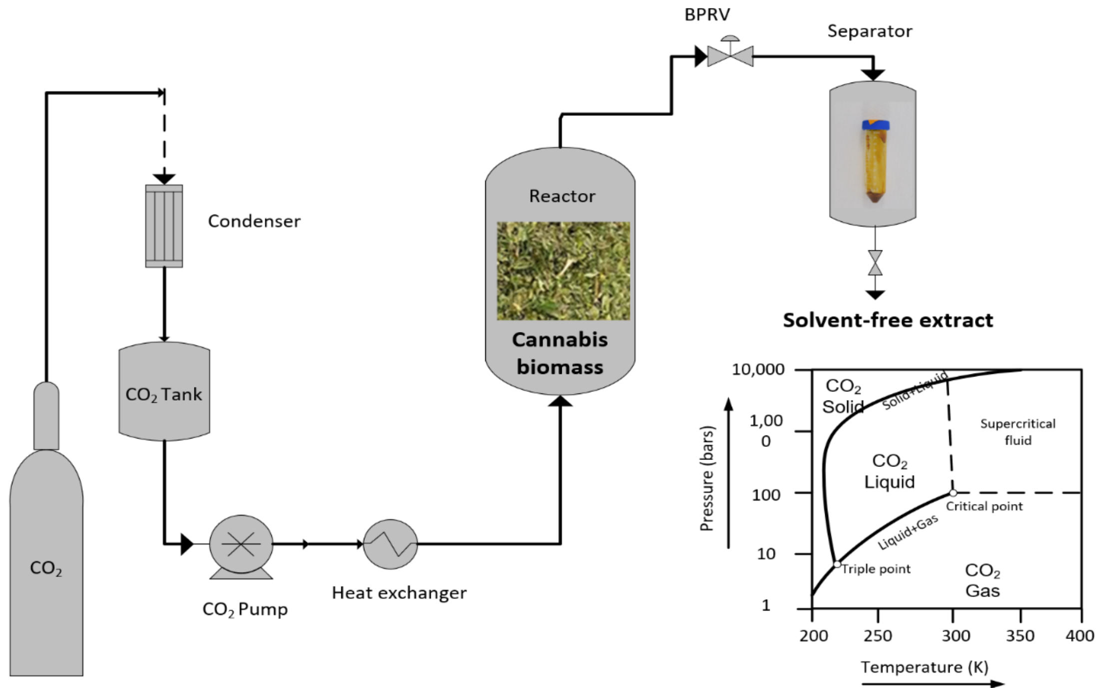


Figure 16. Supercritical carbon dioxide extraction set-up.

Total yield is the ratio of extract mass, M_e, to M_{rm}:

$$Y = \frac{M_e}{M_{rm}} * 100 \qquad 5.1$$

For each cannabinoid, M_i, we calculate the yield:

$$Y_{\text{Cannabinoid},i} = \frac{M_i}{M_{rm}} * 100 \qquad 5.2$$

$$M_i = \frac{X_i . M_e}{100} \frac{X_i . M_e}{100} \qquad 5.3$$

$$X_i = \frac{C_i}{C_s} . 100 \frac{C_i}{C_s} * 100 \qquad 5.4$$

$$C_s = \frac{M_a}{V_s} \frac{M_a}{V_s} \qquad 5.5$$

where:

$Y_{\text{Cannabinoid},i}$: extraction cannabinoid yield in g/100g of raw material,

M_i: mass of the cannabinoid i,

M_{rm}: mass of the raw material put into the reactor.

X_i: mass percentage of the cannabinoid i, in g/100g of extract,

M_e: mass of the extract,

C_i: concentration of cannabinoid i, measured by HPLC,

C_s: concentration of the analyzed solution of cannabinoid i,

M_a: mass of analyzed cannabinoid i,

V_s: volume of analyzed solution of cannabinoid i.

2.3.3. Box-Behnken experimental design

We applied a Box-Behnken experimental design with 4 factors at 3 levels (Table 3).

A randomization factor was added to the experimental plan to restrict variability of the response due to external factors. All responses are expressed in a second-order polynomial equation, as a function of independent variables, and Statistica® software estimated the coefficients, a_i, of the response surface equation, and to test the model with ANOVA analysis.

$$Z = a_0 + a_1X_1 + a_2X_2 + a_3X_3 + a_{11}X_1^2 + a_{22}X_2^2 + a_{33}X_3^2 + a_{12}X_1X_2 + a_{13}X_1X_3 + a_{23}X_2X_3 \quad 5.6$$

where Z is the response variable.

Table 8. Range and variables for the experimental design.

Parameters	Levels		
	Low	Average	High
Temperature (°C)	40	55	70
Pressure (bar)	150	235	320
CO_2 flowrate (g/min)	5	10	15
Time (min)	120	180	240

5.4 Ethanol extraction

2.8 grams of cannabis biomass were transferred into a filter paper extraction thimble and extracted with 250 mL ethanol for 5 h in a Soxhlet extractor. Ethanol was removed at 40 °C under reduced pressure, in a rotary evaporator (Rotavapor R210, Buchi, Flawil,

Switzerland). Subsequently, the flask was placed in a desiccator chamber for 1 h.

5.5 HPLC analysis

All chromatographic analysis were performed using an Agilent 1260 Infinity Quaternary HPLC, including a quaternary pump, a solvent degasser, autosampler, and column compartment. A 1260 Agilent photodiode-array detector (DAD) with a Phenomenex Kinetex® C18 100 Å column (50 x 2.1 mm ID and 2.6 μm particle size) measured the concentration of the extract at a wavelength of 220 nm. Data acquisition and integration were performed with MassHunter Quantitative Analysis Software.

The mobile phase A was a mixture of 5 % MeOH, 94.9 % H_2O, and 0.1 % HCOOH. Mobile phase B was 99.9 % MeOH and 0.1 % HCOOH. The column temperature was maintained at 40 °C with a mobile phase flowrate of 0.4 mL/min. We injected 7 μL and the total run time was 26 min. A variable gradient was used, starting with 48 % B, gradually increased to 88 % B over 18 min, then to 100 % B after 1 min, and decreased to 48 % B after 7 min. Standards at 100, 50, 25, 12.5, 6.25 and 3.125 mg/L were prepared for the calibration curve, for each cannabinoid (THCA, Δ^9-THC, CBDA, CBD, CBN and CBG).

The 7 μL sample was drawn from solution of 25 mg of extract charged to a 25 mL flask with 20 mL of methanol that was sonicated for 5 min. All samples were filtered and loaded to the sample vial, then injected into the HPLC. For Δ^9-THC analysis, we diluted the sample by a factor of 10 with methanol.

5.6 Δ^9-THC concentration by fast centrifugal partition chromatography (FCPC)

A fast centrifugal partition chromatograph (FCPC® A200, Kromaton Technologies Annonay, France), equipped with a 200 mL rotor measured Δ^9-THC concentration (Figure 17). It was connected to a Series 2500 pump (LabAlliance™, France), a manual injector with a 5 mL loop, and a microcomputer-controlled fraction collector.

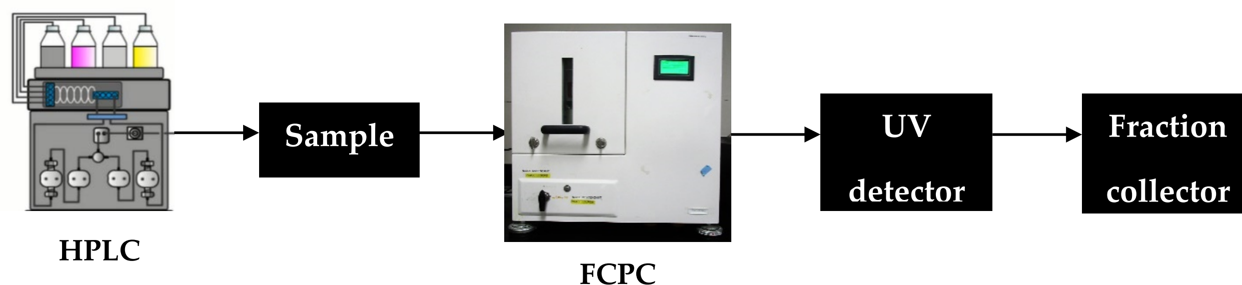


Figure 17. FCPC set-up

FCPC chromatography separates and concentrates compounds. A polar solvent is pumped into a rotor which, then, is centrifuged to form a stationary phase. A non-polar mobile phase immiscible with the stationary phase is then pumped into the system. The sample is injected into the mobile phase and separated on the stationary phase into different fractions that are collected in a fraction collector. FCPC fractionated 100 mg of the neutral extracts from both Soxhlet and SC-CO_2 with the two-phase system hexane/acetone/acetonitrile, 5:2:3 (v/v/v) [30]. The acetonitrile-rich lower phase served as the stationary phase and the hexane-rich upper phase as the mobile phase, so the FCPC was operated in ascending mode. The flowrate was set at 4 mL/min and a rotation speed of 1000 rpm. The samples from the Soxhlet and SC-CO_2 were dissolved in hexane. 8 mL aliquots were injected, and 60 fractions were collected before eluting the stationary phase. The selected fractions were further analyzed by HPLC. Fractions containing a high proportion of the desired compound were combined and subsequently evaporated under reduced pressure.

5.7 Results and discussion

5.7.1 Decarboxylation

Decarboxylation increased Δ^9-THC yield and reduced total Δ^9-THCA (Table 9). Also, the quantity of CBN increased due to the oxidation of Δ^9-THC in addition to the CBNA oxidation. CBG not only comes from the oxidation of CBG but also from Δ^9-THC degradation and CBN degradation.

Decarboxylation converts the acidic cannabinoids into their neutral forms, making them

more extractable because their low polarity [29]. Acidic cannabinoids are considered non active [5].

Table 9. Raw material cannabinoid analyses

Biomass	Name	CBN	Δ⁹-THCA	Δ⁹-THC	CBG
		mg/100g raw material (RM)			
None decarboxylated	A	0.31	22.4	1.11	/
	B	0.85	/	16.3	0.47
Decarboxylated	C	0.19	/	18.6	0.47
	BC	0.67	/	15.7	0.48

Moreno et al. summarized the 3 natural synthetic pathways for the most studied cannabinoids [31]. CBGA decarboxylates to CBG, biosynthesizes CBDA, CBCA, and Δ^9-THC A (Figure 18). CBCA decarboxylates to CBC, CBDA to CBD, Δ^9-THCA to Δ^9-THC but also oxidizes to CBNA. CBNA decarboxylates to CBN, and finally Δ^9-THC also oxidizes to CBN.

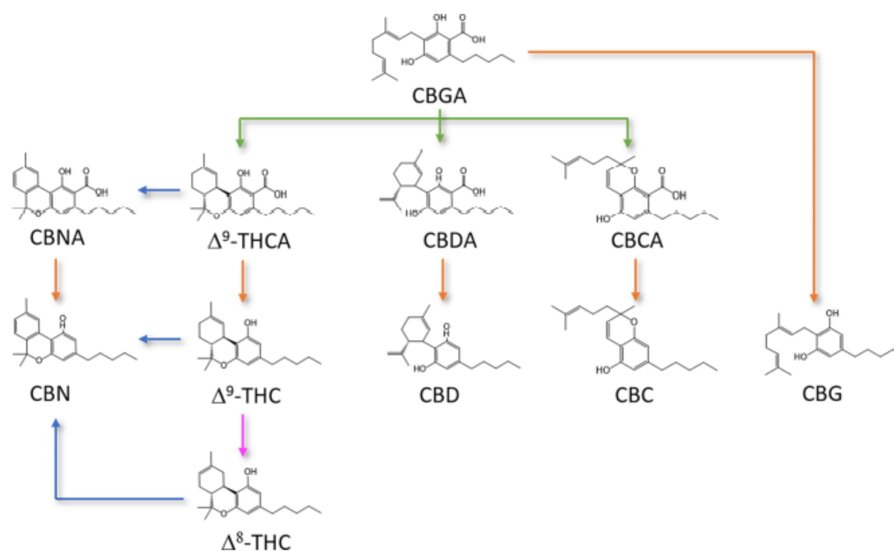


Figure 18. Simplified cannabinoid synthetic pathway: decarboxylation, biosynthesis

5.7.2 Supercritical extraction

5.7.2.1 Total yield and cannabinoids content

The cannabinoid yield from SC-CO_2 extraction was higher than for ethanol extraction, but the total mass of cannabinoids extracted with ethanol was higher as it extracts polyphenols and other compounds, i.e. it is less selective than SC-CO_2 (Table 10). The lowest total yield for the DOE was 2.4 % at 55 °C, 150 bar, 10 g/min CO_2, 120 min (run 23). Increasing the pressure to 320 bar in run 24, more than sextupled the yield 16 %.

The total yield was highest in runs 4, 6, 8 and 24 with a maximum of 26 %, which is reached in run 8 at 55 °C, 235 bar, 15 g/min and 4 hours. The pressure and CO_2 flowrate for these averaged 235 bar and 10 g/min with the highest at 320 bar and 15 g/min. The conditions for run 6 are the same as run 8, except that processing time was reduced to 2 hours, and this change dropped the total yield by more than 3 %.

Table 10. Cannabinoids yield per extract and per raw material.

Run	T	P	F	t	Total yield	Δ⁹-THC	CBN	CBG	Δ⁹-THC	CBN	CBG
	°C	bar	g/min	min	%	g/100g extract			g/100g RM		
1	40	150	10	180	11.1	62.0	2.1	3.7	6.9	0.4	0.2
2	70	150	10	180	18.2	51.1	1.7	4.1	9.3	0.7	0.3
3	40	320	10	180	11.6	48.2	1.8	2.6	5.6	0.3	0.2
4	70	320	10	180	22.9	50.3	1.8	4.6	11.5	1.1	0.4
5	55	235	5	120	7.4	62.6	2.2	3.9	4.6	0.3	0.2
6	55	235	15	120	22.7	64.3	2.4	4.6	14.6	1.0	0.5
7	55	235	5	240	16.0	59.1	2.2	4.0	9.5	0.7	0.4
8	55	235	15	240	25.9	52.3	2.0	4.3	13.6	1.1	0.5

9	55	235	10	180	11.3	45.0	1.8	3.1	5.1	0.4	0.2
10	40	235	10	120	15.2	55.7	1.5	5.8	8.5	0.9	0.2
11	70	320	10	120	18.0	51.9	1.9	3.8	9.4	0.7	0.3
12	40	235	10	240	21.1	48.9	1.8	4.1	10.3	0.9	0.4
13	70	235	10	240	21.7	55.0	1.9	4.4	11.6	0.9	0.4
14	55	150	5	180	12.0	62.0	2.0	3.8	7.5	0.5	0.2
15	55	320	5	180	11.0	60.7	2.2	4.6	6.7	0.5	0.3
16	55	150	15	180	6.7	62.6	2.1	4.0	4.2	0.3	0.1
17	55	320	15	180	19.0	49.8	1.5	3.9	9.5	0.8	0.3
18	55	235	10	180	9.3	53.9	2.0	6.0	5.0	0.6	0.2
19	40	235	5	180	12.1	52.8	1.9	4.1	6.4	0.5	0.2
20	70	235	5	180	8.5	57.8	2.2	5.1	5.0	0.6	0.2
21	40	325	15	180	19.5	61.1	1.8	3.7	11.9	0.7	0.4
22	70	235	15	180	12.7	49.7	1.8	4.5	6.3	0.6	0.2
23	55	150	10	120	2.4	50.2	1.6	4.0	1.2	0.1	0.0
24	55	320	10	120	16.2	50.2	1.8	3.8	8.4	0.6	0.3
25	55	150	10	240	11.0	54.9	2.1	4.8	6.0	0.5	0.2
26	55	320	10	240	24.9	51.6	1.8	3.2	12.9	0.8	0.5
27	55	235	10	180	12.2	59.7	2.1	3.6	9.1	0.6	0.3

Processing conditions of run 8 had the highest total yield while run 6 produced the most Δ^9-THC and CBG with half the solvent and energy. Lowering the pressure to 150 bar at CO_2 flowrate to 10 g/min drops the Δ^9-THC yield to a minimum of 1.18 g/100 g of raw material even if temperature and processing time are the same as the optimal conditions.

5.7.2.2 Effect estimates and ANOVA analysis

ANOVA analysis confirmed that the pressure and time have a significant impact on extraction yield, $p<0.05$ (Table 11). The fitted equation for Δ^9-THC content in the cannabis extract shows that only pressure positively affected the content, while temperature,

flowrate and time had no effect. However, a median temperature (55 °C) and pressure (235 bar) with high flowrate and low time were associated with high Δ⁹-THC and CBG yields. When time increases, Δ⁹-THC and CBG yields decrease, whereas CBN yield increases.

Table 11. Effect estimates for the total yield.

Factors	*Effect*	*P-value*	*Coefficient*
Mean interaction	16	0.0000	16
Temperature	0.4	0.89	0.2
Pressure	8	0.014	4
Flowrate	5.5	0.081	2.7
Time	-4.2	0.049	-2.1

Also, according to the three best extraction conditions for CBN yield, high temperature increases CBN yield, while, conversely, the Δ⁹-THC and CBG yield decreases (Table 12). The optimal SFE runs were selected using the ANOVA analysis and the RSM. Run 6 (235 bar, 55 °C, 15 g/min, 120 min) was determined to be optimal and was used for verification. Conditions in run 8 (235 bar, 55 °C, 15 g/min, 240 min) are the same as run 6, but with an extended processing time.

Table 12. ANOVA for cannabinoids. (a) CBN; (b) Δ⁹-THC; (c) CBG.

Factor	*SS*	*MS*	p-*value*
T	*0.01*	*0.01*	*0.04*
P	*0.04*	0.04	0.02
Q	*0.05*	0.05	0.02
t	*0.15*	0.15	0.01
T*(Quad) x* Q*(Lin)*	*0.16*	0.16	0.01
P*(Quad) x* Q*(Lin)*	*0.21*	0.21	0.01

(a)

Factors	*Sum of Squares*	*Means Squared*	*p-value*
T	0.001	0.001	0.77
P	*0.06*	*0.06*	*0.03*
Q	*0.01*	0.01	0.24
t	*0.02*	0.02	0.15

(b)

Factors	*Sum of Squares*	*Means Squared*	*p-value*
T	0.01	0.01	0.97
P	*39.8*	39.8	0.04
Q	*14.9*	14.9	0.19
t	*11.5*	11.5	0.25

(c)

5.7.2.3 Response surface methodology analysis: cannabinoids yield

A response surface plot of the equation expresses the effect of the design factor on each response within the experimental space (Table 14). Equations calculate optimized yields out and inside the experimental extraction points.

Table 13. Error percentage for each cannabinoid

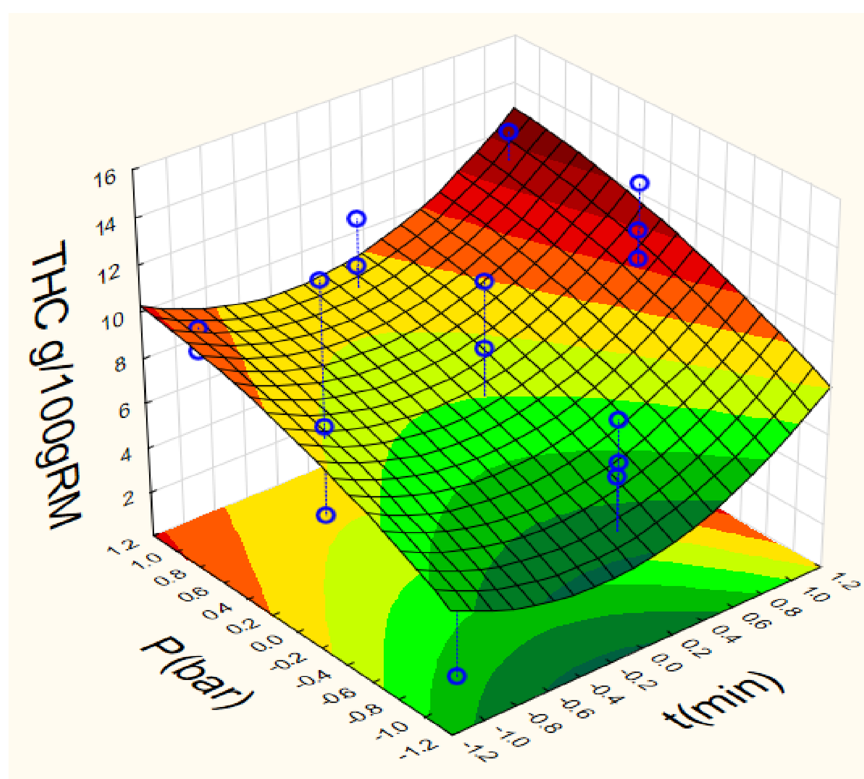
Responses	**Models**	**Equations**	p-value
Total yield	Linear	$Y=16 + 4.0\,P - 2.1\,t$	0.05
Δ^9-THC Y	Linear	$Y= 8.6 + 2.0P$	0.05
CBG Y	Linear	$Y= 0.296 + 0.075P$	0.05
CBN Y	Quadratic	$Y= 0.657 + 0.148T + 0.183T^2{*}Q + 0.199P^2Q$	0.009

Table 14. Influence of designed factor on each response

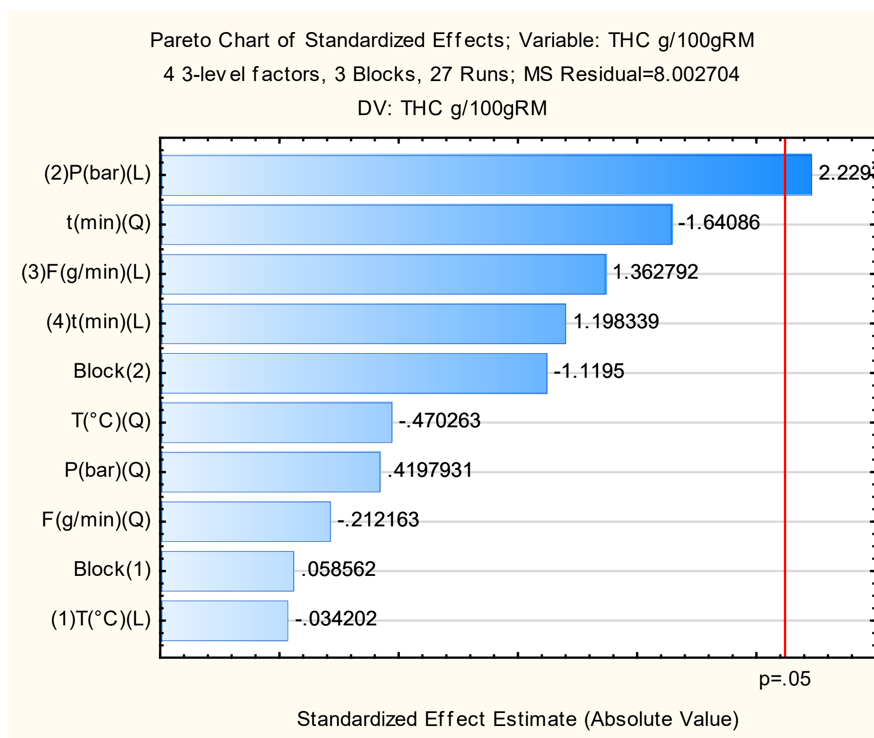
Yield	Models	Equations	p-value
Total yield	Linear	$Z = 11.864 + 4.017x - 0.3722x^2 + 2.8001y + 4.1935y^2$	0.05
Δ^9-THC	Linear	$Z = 7.037 + 2.135y - 0.276y^2 + 1.274x + 1.794x^2 + 0.881y + 0.779x + 1.972y - 0.005yx - 0.473x$	0.05
CBG	Linear	$Z = 0.258 + 0.073y - 0.026y^2 + 0.051x + 0.069x^2 + 0.026y - 0.008x + 0.046y + 0.002yx - 0.023x$	0.05
CBN	Quadratic	$Z = 0.499 + 0.201y - 0.049y^2 + 0.350x + 0.132x^2 + 0.155y - 0.047y^2 - 0.075y + 0.120y^2 - 0.099x - 0.341x^2 - 0.366x - 0.103x^2 + 0.166yx - 0.007yx^2 - 0.399y^2x - 0.170y - 0.013y^2$	0.009

Where y is the pressure and x is a time for CBG and Δ^9-THC, but CO_2 for CBN.

The fitted surface shows that the maximum Δ^9-THC yield varies from 8 to 17 g/100g RM in the range of 269-337 bar and 240 to 252 min. High pressure increases the density of CO_2: this explains the higher solubility of oil in CO_2. However, the maximum Δ^9-THC yield is restricted in time since it has the second largest impact, according to the Pareto chart (Figure 19b).



(a)

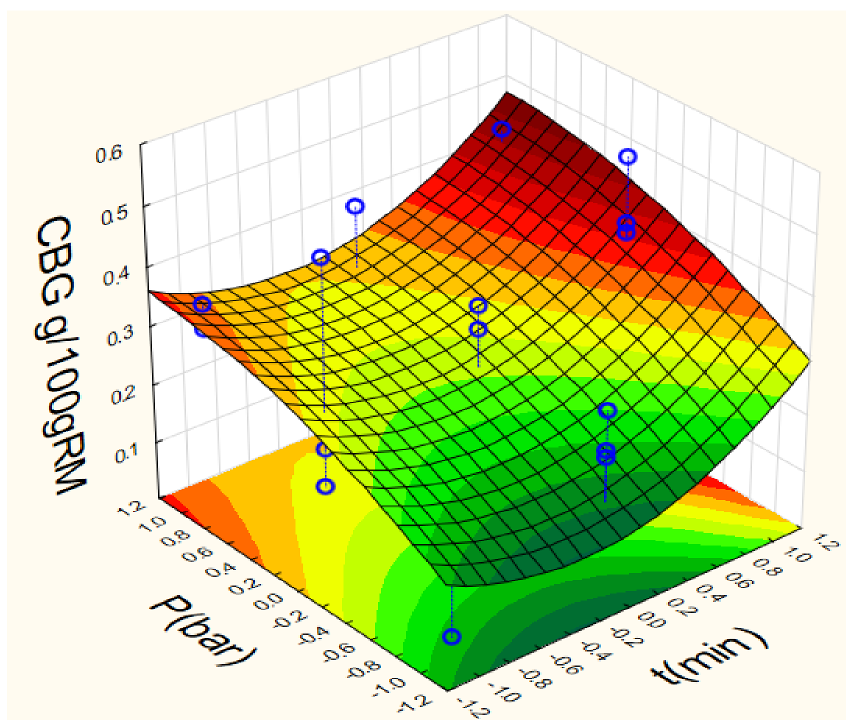


(b)

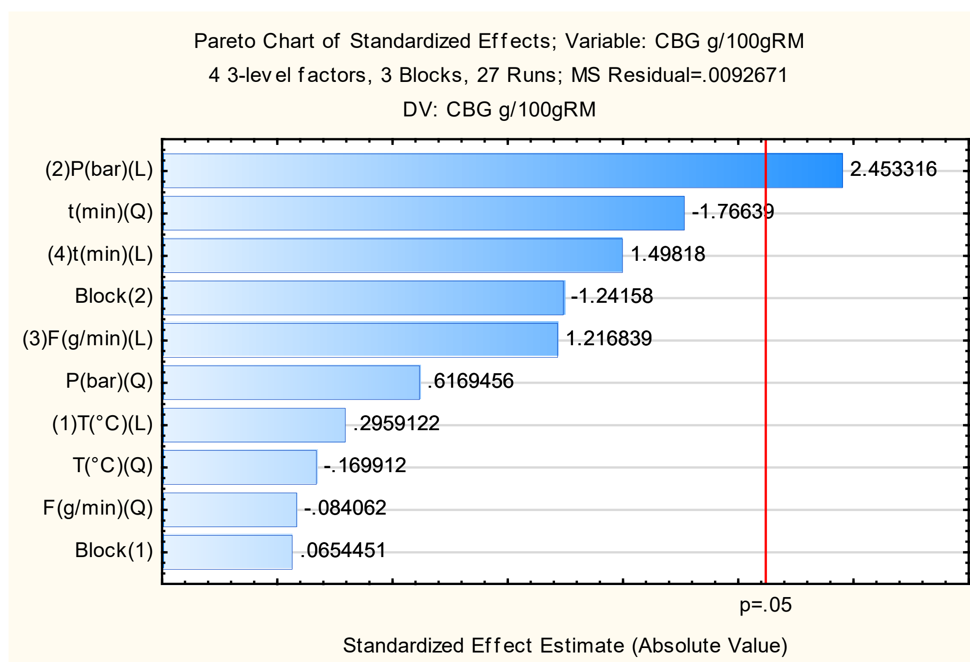
Figure 19. Surface response ***(a)*** *and Pareto chart* ***(b)*** *for Δ⁹-THC*

Moreno et al. confirms that increasing the pressure increases Δ⁹-THC extraction [31], since it has a low solubility in CO_2 (0.20-2.95 × 10^{-4}) [32], and King confirms that SC-CO_2's solubility is a function of both pressure and temperature [33].

The nature of the fitted surface shows a maximum CBG yield from 0.33-0.52 g/100g RM, obtained within a pressure range of 269-337 bar, and time from 240 to 252 min. The response surface and Pareto chart show that pressure and time have a comparable effect on CBG extraction yield and Δ⁹-THC extraction yield. This is due to the similar nature of the chemical structure of these compounds (Δ⁹-THC: $C_{21}H_{30}O_2$, CBG: $C_{21}H_{32}O_2$) (Figure 20b). Furthermore, they almost have the same molecular weight, 315 g for Δ⁹-THC and 317 g for CBG, and degree of polarity.



(a)



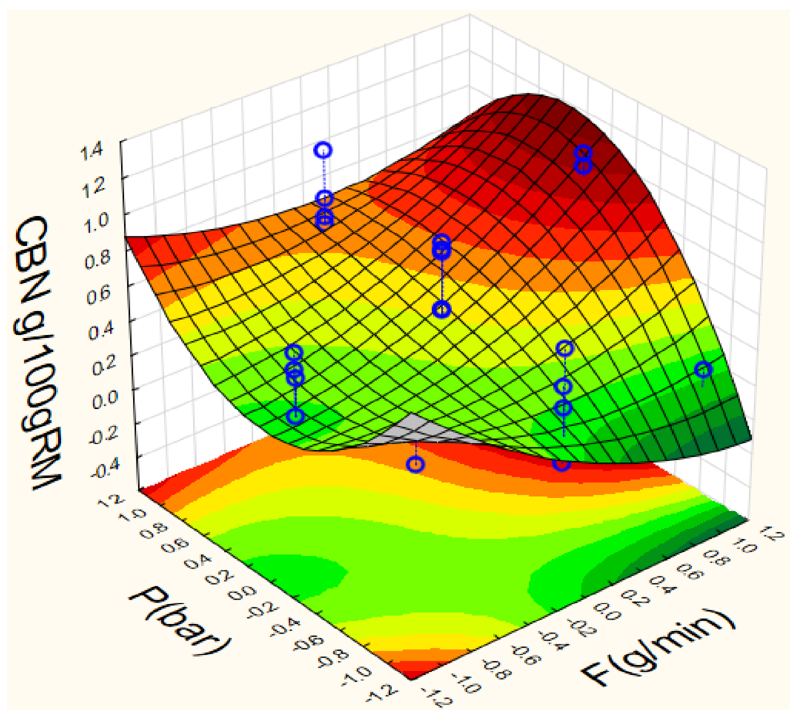
(b)

Figure 20. Surface response (a) and Pareto chart (b) for CBG

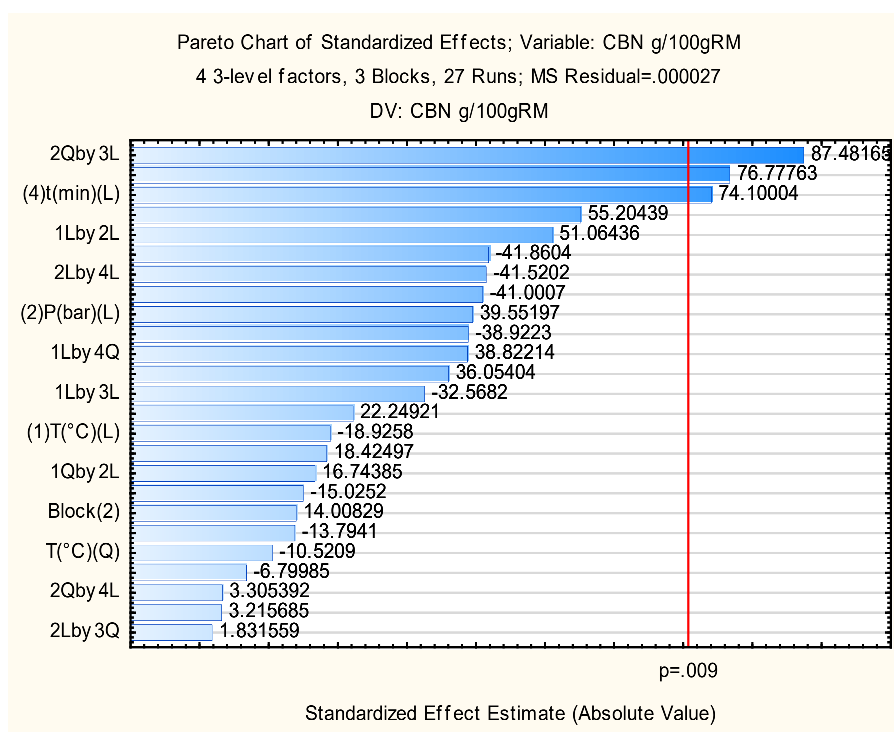
These physicochemical properties contribute to the extractive power of CO_2, which dilutes the biomass material membrane, dissolves the molecules, then carries the cannabinoids depending on their density and polarity levels.

The CBN content in the cannabis extract ranged from 0.094 (run 23) to 1.103 (run 8). The response surface is concave downwards with a maximum CBN yield ranging from 0.14 to 0.52, from 201 to 320 bar and CO_2 flowrate from 14 to 16 g/min. The corresponding Pareto chart (Figure 21b) identifies the interaction between pressure and CO_2 flowrate is the key factor contributing to the variance in the data (explains the most variability), followed by the interaction of temperature and flowrate, and finally time.

This suggests that processing at high pressure produces CBN right at the beginning and then the second peak at 2h30.



(a)



(b)

Figure 21. Surface response (a) and Pareto chart (b) for CBN

The cannabinoid yield is influenced by the operating conditions, which depend on their respective solubilities. Perrotin et al. determined the solubility of four cannabinoids in supercritical carbon dioxide [34]. Molar solubility for Δ^9-THC lies between 0.20 and 2.95×10^{-4}, from 42 to 72 °C and 130 to 250 bar. Molar solubility for CBN (1.26 to 4.16×10^{-4}), CBG (1.17 to 1.91×10^{-4}) and CBD (0.88 to 2.69×10^{-4}) was determined in the range of 41 to 61 °C and 110 to 200 bars.

The Δ^9-THC solubility at 53 °C, determined by Perrotin et al. based on the Peng-Robinson equation of state combined with Van der Waals mixing rules, was higher than CBG, which was higher than CBN, which agrees with the trends in our experimental data [35]. This order, Δ^9-THC $<$ CBG $<$ CBN, also corresponds to the molecular weight of the compounds mentioned earlier, but also confirms the significance of the cannabinoids yield by the optimization of the Box-Behnken experimental design.

5.7.2.4 Effect of time on Δ^9-THC and CBG degradation and CBN formation

The optimal extraction parameters (235 bar, 55 °C and 15 g/min) produce the most Δ^9-THC and CBG at 2 h processing, but at 4h, the CBN yield increases at the expense of Δ^9-THC and CBG (Figure 7). This trend agrees with kinetic models suggesting that both CBG and Δ^9-THC degrade to CBN. Therefore, Δ^9-THC and CBG formulations should not exceed 2h, while if CBN is the target molecule, longer times are better.

5.7.2.5 Effect of pressure on total extraction yield and cannabinoids content

Pressure plays an important role in both the overall extraction yield and the quantity of bioactive compounds extracted. Increasing the pressure from 150 to 320 bar increases the yield of cannabis extract. However, it increases total extract yield at the expense of Δ^9-THC, CBN, and CBG content. Higher pressures increase solvent strength and decrease extraction selectivity, making the optimization of multiple extraction factors an important tool to achieve high extraction yields and selectivity. Temperature affects extraction yield but not cannabinoids content, which is confirmed in the literature [36].

5.7.3 Ethanol extraction

Ethanol extraction results in a high extraction yield, but the extract contains more than 1 % solvent residue, which is the upper threshold for pharmaceutical applications. Also, a winterization step is required before FCPC separation.

Ethanol extraction yield is 8 % greater than SC-CO_2 yield. However, the cannabinoids content in the extract is much lower since ethanol extraction synthesizes also terpenoid and phenolic compounds (Table 15).

Table 15. Ethanol cannabis extraction

Volume	Flowrate	Time	Yield	Δ^9-THC	CBN	CBG	Δ^9-THC	CBN	CBG
(mL)	(cycles/h)	(h)	%	g/100g extract			g/100g RM		
250	6-10	5	3.66	32.6	1.70	0.70	10.97	1.21	0.44

5.7.4 Recovery

Concentrations of Δ^9-THC, CBN and CBG in supercritical CO_2 extracts at optimal conditions mentioned earlier, were 2, 1.4 and 6.5 times higher than those in ethanol extracts, respectively. Recovery for these cannabinoids is 93.1 %, 164.2 % (which includes CBN produced from the degradation of Δ^9-THC and CBG), and 164.2 %, respectively. This recovery data is comparable to data reported in the literature (Table 16) [29].

Table 16. Recovery for ethanol and SC-CO_2 extractions

Recovery	Δ^9-THC (%)	CBG (%)	CBN (%)
SC-CO_2	93.1	112.5	164.2
EtOH	69.9	91.6	180.5

5.7.5 Δ^9-THC concentration by fast centrifugal partition chromatography (FCPC)

CO_2 extract is very soluble in the FCPC mobile phase (hexane), contrary to ethanol extract. Moreover, some of the compounds in the ethanol extract are polar (polyphenols, terpenes, etc.) and precipitates in hexane, making purification more complicated.

The chromatogram (Figure 23) shows that the Δ^9-THC fraction elutes between 60 and 72 min, and we collected 6 aliquots of 8 mL during this time interval. After evaporation, 80 % of the extract was pure Δ^9-THC, while the remaining 20 % of the Δ^9-THC was with the CBN fraction at $t > 72$ min with 57 % Δ^9-THC and 6 % CBN. This promising result must be refined to concentrate Δ^9-THC but also to separate between the different other cannabinoids.

Figure 22. Cannabis extract sample from SC-CO_2 (left) and ethanol (right)

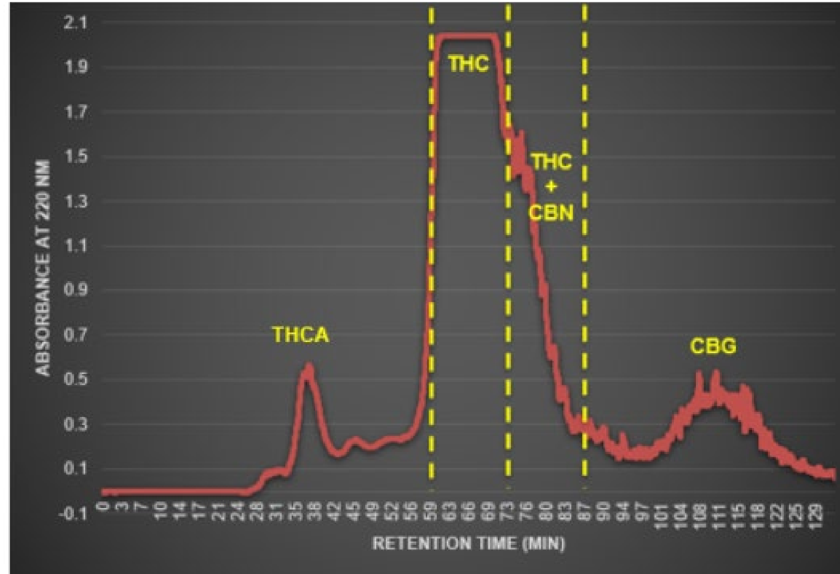


Figure 23. Chromatogram of Δ⁹-THC fraction

5.7.6 Optimization of the biomass-solvent contact.

A plug flow hydrodynamic regime optimizes the contact between the biomass and solvent. The pore Reynolds number (Re_p) is a guideline to estimate the expected flow patterns as a function of conditions [37]. The laminar flow regime, $Re_p < 6$, is more plug flow like than the transient regime [38, 39]:

$$Re_p = \frac{\text{Inertia forces}}{\text{Viscous forces}} = \frac{\rho_{CO_2} * Up_{CO_2} * d_g}{\mu_{CO_2}} \frac{\text{Inertia forces}}{\text{Viscous forces}} = \frac{\rho_{CO_2} * Up_{CO_2} * d_g}{\mu_{CO_2}} \quad 5.7$$

$$U_{CO_2} = \frac{\text{Mass CO2 flowrate}}{\rho_{CO_2} * \text{Autoclave surface area}} \frac{\text{Mass CO2 flowrate}}{\rho_{CO_2} * \text{Autoclave surface area}} \quad 5.8$$

$$U_{pCO_2} = (U_{CO_2}/\varepsilon) * \tau \quad 5.9$$

With:

a_g: cannabis grain surface = $6/d_g$.

ρ_{CO_2}: supercritical carbon dioxide density.

ε: bed void fraction.

τ: tortuosity (considered equal to $\pi/2$ for spherical particles).

μ_{CO_2}: supercritical carbon dioxide viscosity.

Axial dispersion is more prominent in the transient flow regime. Considering cannabis

biomass as spherical particles, the tortuosity (τ) is considered equal to $\pi/2$. For the optimal extraction conditions in this case (55 °C, 235 bar), the CO_2 flow corresponds to a laminar regime, since Re_p is 3.8, and so we consider axial dispersion was negligible.

The bed void fraction of cannabis particles ε was estimated by immersing 30 g of biomass in a funnel for filtering solvents with 200 mL ethanol. The mass of introduced ethanol led to the value of bed void fraction of $\varepsilon = 0.36$ for 2 mm cannabis particles. The CO_2 density at optimal conditions is 785 kg/m^3. This low void fraction is surprising as we would have expected it to be greater than 0.41 so we hypothesize that the plant matter may have absorbed some of the solvent.

5.8 Conclusions

SC-CO_2 is a selective extraction technique for cannabinoids, while ethanol extracts more polar compounds like polyphenols and terpenes (69 % Δ^9-THC in the ethanol extract, 93% in the SC-CO_2 extract). The CBN concentration increases with time as Δ^9-THC and CBG degrade, which agrees with literature kinetic data. The optimal conditions to extract Δ^9-THC and CBG were 55°C, 235 bar, 15 g/min CO_2 and 2h. The optimal conditions were the same for CBN but at 4h rather than 2h. Pressure is the first factor affecting Δ^9-THC yield but considering Δ^9-THC degradation into CBN time is a limiting factor. It is also identified as a significant factor to increase total yield. Results from our experimental work, based on the Box-Behnken DOE, agree with literature data: maximum extract total yields of 20 % and Δ^9-THC content of 1.54% can be achieved by SC-CO_2. Optimal experimental conditions were validated by statistical optimization, since they're all included in the ranges of the conditions corresponding to maximum yields from the Box-Behnken DOE. FCPC is a viable technique to purify Δ^9-THC from the SC-CO_2 extract (80 % Δ^9-THC) whereas, it is inapplicable for the ethanol process as the polar compounds precipitate in the hexane phase. This result introduces an opportunity to develop and isolate different cannabinoids for either nutraceutical or pharmaceutical applications.

$SC\text{-}CO_2$ is a sustainable and economic alternative [40], because it minimizes the use of chemicals. The main challenge is the costs of equipment, which motivates a future work idea of technical-economic study on a scaled-up process for commercialization.

ACKNOWLEDGMENTS

This work was supported by the Natural Sciences and Engineering Research Council of Canada (stipend allocated to Hinane BOUMGHAR via the NSERC-CREATE PrEEmium program). This research was undertaken, in part, thanks to funding from the Canada Research Chair program.

Also, NSERC College and Community Innovation program - Innovation Enhancement grants (CÉPROCQ) the hosting laboratory, and Zollaris Laboratories Corporation the industrial partner, are both gratefully acknowledged. The authors have declared no conflict of interest.

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CHAPTER 6 GENERAL DISCUSSION

This work brings chemical engineering to the service of biomedical engineering, usually in the long journey of pharmaceutical product development. There are a lot of research gaps, that doesn't get enough attention from doctors and pharmacists, and as a result not enough financial support and investment. This covers, the statistical plans before starting the development to ensure reducing the number of experiments and targeting the most important ones, but also, the industrial and process development parts, that has a big influence on the products profile. Our successful work is one of the few studies that enables pharmaceutical industry filling research gaps of the cannabis extract knowledge, as well as provides insights on the selectivity of SC-CO_2 extraction method for three different cannabinoids.

CHAPTER 7 CONCLUSION AND PERSPECTIVES

This work is an optimization of cannabis active ingredients extraction, where THC was the main focus, since it is the molecule that is getting the most attention. We purified it using a new technique, FCPC, to get up to 80 % Δ^9-THC. We exclude ethanol extract for this purification technique, due to the polar compounds that it contains, and it precipitates in hexane phase.

We also analyzed and compared concentrations of the cannabinoids before and after decarboxylation and extraction. We confirmed previous kinetic data about the chemical reactions leading CBN to increase with time as Δ^9-THC and CBG degrade.

It is important to point out that there is no best extraction technique for all industries, there are only suitable technologies depending on the end use and desired properties of the final products. For pharmaceuticals, and concerning therapeutic effects of Δ^9-THC, CBG and CBN, we suggest SC-CO_2, with the optimal conditions to extract the first 2 (THC, CBG) at 55°C, 235 bar, 15 g/min CO_2 and 2h, then same conditions for CBN but at 4h rather than 2h.

The initial factor impacting Δ^9-THC yield is pressure. However, time is also a limiting factor when Δ^9-THC degrades into CBN. It is also noted as a key element in raising total yield. Results from our experimental work, based on the Box-Behnken DOE, are consistent with data from the literature: SC-CO_2 can produce maximum extract yields of 12% and Δ^9-THC contents of 1.54%. Statistical optimization was used to confirm the best experimental settings because they all fell within the parameters that produced the highest yields in the Box-Behnken DOE.

Next step to this work, can go in two ways: first, a biomedical focus, by pushing further purification of Δ^9-THC, test FCPC techniques for CBG and CBN, then to target a therapeutic effect, and start developing a drug delivery system which leads to new

formulations that can help improve the human health and the economics of the pharmaceutical industry. A second important focus of the work is the technical-economic study of the process and a scale-up. The ideal scenario is to have both projects working simultaneously in order to optimize realistically each one according to the other.

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