METHOD DEVELOPMENT FOR QUALIFICATION AND QUANTIFICATION OF CANNABINOIDS AND TERPENES IN EXTRACTS BY GAS CHROMATOGRAPHY-MASS SPECTROMETRY

by
ALLEGRA LEGHISSA

THESIS
Submitted in partial fulfillment of the requirements for the degree of Master of Science in Chemistry at The University of Texas at Arlington August, 2016

Arlington, Texas

Supervising Committee:
Kevin A. Schug, Supervising Professor
Daniel W. Armstrong
Subhrangsu Mandal

ABSTRACT

METHOD DEVELOPMENT FOR QUALIFICATION AND QUANTIFICATION OF CANNABINOIDS AND TERPENES IN EXTRACTS BY GAS CHROMATOGRAPHY-MASS SPECTROMETRY

Allegra Leghissa, Ms.

The University of Texas at Arlington, 2016

Supervising Professor: Kevin A. Schug

Cannabinoids are a class of chemical compounds that act on cannabinoid receptors in cells. These receptors have different kinds of ligands, including endocannabinoids (produced in the body by humans and animals), phytocannabinoids (found mainly in *Cannabis sativa*), and synthetic cannabinoids. At least 85 different phytocannabinoids have been isolated; different cannabinoids are believed to have different effects and possible benefits in medical treatments, and it is therefore fundamental to classify cultivars prior to their medical use. The other class of compounds that characterize *C.sativa* are terpenes. They confer flavor and fragrance that are unique to each plant and further confer botanical differentiation of cultivars.

Methods were developed for the potential to evaluate terpene and cannabinoid content in extracts of *C. sativa*. Quantification was performed by creating a calibration curve with the standards of the examined cannabinoids (THCV, CBD, CBC, Δ8-THC, CBG, CBN, Δ9-THC, and Δ9-THCA). The quantification of Δ9-THCA was carried out with both the products from the two different silylation agents. Terpenes were also quantified; considering the wide broad of terpenes in the *C. sativa* extracts, only the six most abundant ones (linalool, exo-fenchol, caryophyllene, guaiol, and α-bisabolol) were considered.

AKNOWLEDGEMENT

This work would have never been possible without the help of Prof. Kevin A. Schug and of the people who work in his laboratory, who always helped me through and showed me that, even in the darkest moments of the research, you should never give up, because everything is going to turn around. A special thanks to Dr. Doug Carlton, Dr. Jonathan Smuts, and Jonathan Thacker, for always helping me with the instrument.

I would like to thank Dr. Zacariah L. Hildenbrand, Sean Jun, and Aaron Hicks from C4 laboratories LLC and Inform Environmental LLC, for their guidance in carrying out this research.

Finally, I would like to thank the member of my committee Dr. Daniel W. Armstrong and Dr. Subhrangsu Mandal, both for their time and their suggestions for improvement.

DEDICATION

Moving to another continent is a tough decision to make, impossible without the needed support. Therefore, I would like to thank all those people who helped me through this transition. I would like to thank my brother Stefano and his wife Jennifer, without whose example I would have never had the courage to leave my country. I would like to thank my friends, who even 8,000 miles away are still the first ones to reply whenever I ask for help. I would like to thank my boyfriend Austin LeRoy and his family, who has always treated me like one of them. I would like to thank my friends in Texas, who showed me that the transition from one country to the other is easier if you have someone by your side.

Finally, I would like to thank my parents. Without them, all of this would have not been possible. Not only I would like to thank them for giving me the opportunity to come to Texas, but also for passing down the passion for science and the desire of knowledge. They are my greatest fans and without their support I would have never accomplished all my goals. Thank you.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

THCV……..... Tetrahydrocannabivarin

CBD…….....Cannabidiol

CBC……... Cannabichromene

Δ8-THC……..Δ8-Tetrahydrocannabinol

CBG……...… Cannabigerol

CBN……...Cannabinol

Δ9-THC……..……Δ9-Tetrahydrocannabinol

Δ9-THCA……Δ9-Tetrahydrocannabinolic Acid

CBND……..... Cannabinodiol

CBL……..……... Cannabicyclol

CBE……...……... Cannabielsoin

CBT……. Cannabitriol

MeOH……..…… Methanol

BSTFA + 1%TMCS.. N,O-bis(trimethylsilyl) trifluoroacetamide +1% trimethylchlorosilane

MSTFA…… N-Methyl-N-(trimethylsilyl) trifluoroacetamide

EtOAc……...……. Ethyl Acetate

LOD……..…… Limit of Detection

Chapter 1. *Cannabis sativa*

1.1. The History of *Cannabis sativa*

It is more likely that the natural origin of *Cannabis sativa* is in the Altai mountains of southern Siberia, Russia, since this plant (also called hemp) is a common wild plant in this region [1]. Discoveries from Southern Moravia, Czech Republic, supplied evidence of the oldest use of hemp. Czech archeologist Klìma discovered that the fiber of this plant was used by inhabitants of famous eastern Gravettian settlements (29,000 to 22,000 years ago) for the manufacture of textiles, basketry, and cordage [2]. The first fragment of hemp clothes was found in 1972 in a grave from the late Chou dynasty (1,122-249 BC) in Shansi province, China. This piece of clothes represent the oldest preserved specimen of *C. sativa* [3].

To find the first evidence of medical use of *C. sativa*, it is needed to go back 5,000 years to the emperor Shen Nung, who is believed to be the discoverer of medicinal plants. He is believed to have tasted hundreds of herbs to test the medicinal value, and to be the author of Shen-nung pen ts'ao ching (Divine Husbandman's Materia Medica), that is the earliest existent Chinese pharmacopoeia. According to this manual, the flowers of the female *C. sativa* plant contain the greatest amount of yin energy, and therefore they were used to treat menstrual fatigue, rheumatism, malaria, beri-beri, constipation, and absentmindedness [4].

Another medical legacy, found on hundreds of clay tablets, was left by the Assyrians (3,000-2,000 BC), where it is clear that *C. sativa* was one of the major drugs, and the plant was named according to its use [5]. *C. sativa* was used as a medicine even by the ancient Egyptians. In the tomb at Thebes (ca. 1,534 BC), the Ebers Papyrus was found, that is the

oldest complete medical book in existence. It is a collection of approximately 900 prescriptions, among which two formulas mentioned the use of *C. sativa* [6].

In the Indian scripture of the Atharva Veda, that is the fourth book of the Vedas (ancient scriptures of the Brahman religion, 2,000-1,400 BC), bheng (identified as hemp) was one of the five sacred plants of India [7]. The Persian prophet Zarathustra (ca. 1,200 BC) [8], author of the Zend-Avesta, was a user of hemp, that occupies the first place in a list of 10,000 medical plants. Pliny the Elder (79 AD), Roman nobleman, historian, scientist and author of Naturalis Historia, gave evidences of the use of *C. sativa* by the Northern Mediterranean societies [9]. Even the Greeks utilized *C. sativa* for medical purposes, as it is described by the physician, pharmacologist, and botanist Pedanius Dioscorides in the Materia Medica (90 AD) [10]. Last, French physician Jacques Joseph Moreau hypothesized in the 1830s that *C. sativa* could be used as treatment of the mentally ill, and this explains the introduction of *C. sativa* into modern Western society [11].

1.2. Physical properties of *C. sativa*

1.2.1. Description of the plant

The scientific classification of *C. sativa* plant is:

Kingdom: Plantae

Phylum: Angiosperms

Order: Rosales

Family: Cannabaceae

Genus: *C. sativa*

Species: *C. sativa sativa*

Subspecies: *C. sativa* ssp. s*ativa*

C. sativa ssp. i*ndica*

C. sativa ssp. *ruderalis*

Between the subspecies, the most used ones are *sativa* and *indica,* whose products are physically combined to obtain desired psychotropic effects [12]. The plant is an herbaceous annual that can grow to a height of 8-12 feet. The plant has flowers that bloom from late summer to mid-fall, and these flowers can be male or female. Usually, *C. sativa* plants have just one type of flower, but some plants do have both. Male flowers grow in elongated clusters along the leaves, and they turn yellow and die after blossoming, while female flowers grow in spike-like clusters and they remain dark green for a month after blossoming. From the resin of the *C. sativa* flowers, it is possible to obtain Hashish, a more potent drug than Marijuana [13].

1.2.2. Descriptions of cannabinoids

1.2.2.1. Cannabinoids

C. sativa plants contain over 500 compounds, approximately 100 of which have been identified as cannabinoids, which are a group of C_{21} terpenophenolic compounds found until now uniquely in *C. sativa* L. These 66 compounds have been divided into 10 categories [14]:

1. Cannabigerol (CBG) type: It was the first identified, and it derives from cannabigerolic acid (CBGA), that the first biogenic cannabinoid formed in the plant (Figure 1-1, Table 1-1).

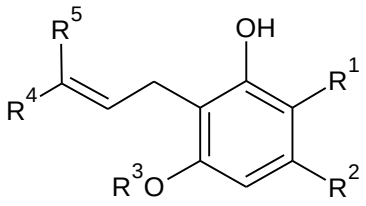


Figure 1.1: CBG-type Cannabinoids

Table 1-1: CBG-type Cannabinoids

Compound	R^1	R^2	R^3	R^4	R^5
Cannabigerolic acid A	COOH	*n*-C_5H_{11}	H	$(CH_2)_2CH=C(CH_3)_2$	Me
Cannabigerolic acid A monomethyl ether	COOH	*n*-C_5H_{11}	Me	$(CH_2)_2CH=C(CH_3)_2$	Me
Cannabigerol	H	*n*-C_5H_{11}	H	$(CH_2)_2CH=C(CH_3)_2$	Me
Cannabigerol monomethyl ether	H	*n*-C_5H_{11}	Me	$(CH_2)_2CH=C(CH_3)_2$	Me
Cannabigerovarinic acid A	COOH	*n*-C_3H_7	H	$(CH_2)_2CH=C(CH_3)_2$	Me
Cannabigerovarin	H	*n*-C_3H_7	H	$(CH_2)_2CH=C(CH_3)_2$	Me
Cannabinerolic acid A	COOH	*n*-C_5H_{11}	H	Me	$(CH_2)_2CH=C(CH_3)_2$

2. Cannabichromene (CBC) type: Just five CBC-type cannabinoids have been identified, and they are present as C_5 analogs (Figure 1-2, Table 1-2).

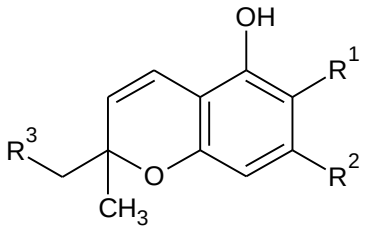


Figure1-2: CBC-type Cannabinoids.

Table 1-2: CBC-type Cannabinoids

Compound	R^1	R^2	R^3
Cannabichromenic acid	COOH	*n*-C_5H_{11}	$(CH_2)_2CH=C(CH_3)_2$
Cannabichromene	H	*n*-C_5H_{11}	$(CH_2)_2CH=C(CH_3)_2$
Cannabichromevarinic acid	COOH	*n*-C_3H_7	$(CH_2)_2CH=C(CH_3)_2$
Cannabivarichromene	H	*n*-C_3H_7	$(CH_2)_2CH=C(CH_3)_2$
Cannabichromevarin	H	*n*-C_3H_7	$(CH_2)_2CH=C(CH_3)_2$
2-Methyl-2-(4-methyl-2-pentenyl)-7-propyl-2H-1-benzopyran-5-ol	H	*n*-C_3H_7	$CH_2CH=C(CH_3)_2$

3. Cannabidiol (CBD) type: it was isolated in 1940, but only in 1963 Mechoulam and Shvo [15] were able to identify its correct structure. Seven CBD-type cannabinoids with C_1 to C_5 side chains have been described. The corresponding acid of CBD is CBDA, which was the first discovered cannabinoid acid, isolated in 1955 (Figure 1-3, Table 1-3).

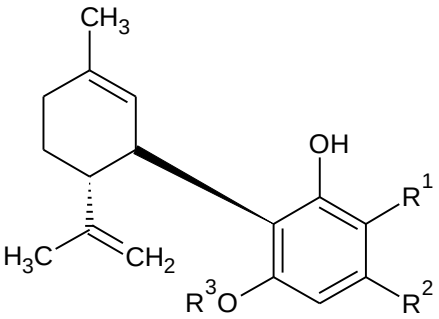


Figure1-3: CBD-type Cannabinoids.

Table 1-3: CBD-type Cannabinoids

Compound	R^1	R^2	R^3
Cannabidiolic acid	COOH	*n*-C_5H_{11}	H
Cannabidiol	H	*n*-C_5H_{11}	H
Cannabidiol monomethyl ether	H	*n*-C_5H_{11}	Me
Cannabidiol-C_4	H	*n*-C_4H_9	H

Cannabidivarinic acid	COOH	*n*-C_3H_7	H
(-)-Cannabidivarin	H	*n*-C_3H_7	H
Cannabidiorcol	H	CH_3	H

4. Δ9-Tetrahydrocannabinol (THC) type: Nine THC-type cannabinoids with C_1 to C_5 side chains have been identified. These mainly derive from THC acid A, even though also THC acid B is present as well. THC is the main psychotropic principle, and it was isolated in 1942, but the correct structure (6a,10a-trans-6a,7,8,10a-tetrahydro-6,6,9trimethyl-3-pentyl-6H-dibenzo[b,d]pyran-1-ol) was first identified by Gaoni and Mechoulam in 1964 [16] (Figure 1-4, Table 1-4).

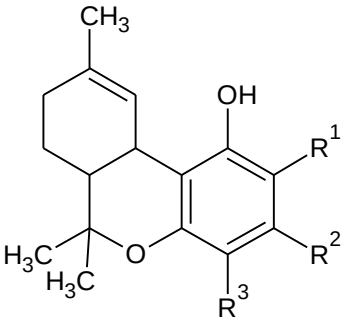


Figure1-4: Δ9-THC-type Cannabinoids.

Table 1-4: Δ9-THC-type Cannabinoids.

Compound	R^1	R^2	R^3
Tetrahydrocannabinolic acid A	COOH	*n*-C_5H_{11}	H
Tetrahydrocannabinolic acid B	H	*n*-C_5H_{11}	COOH
Tetrahydrocannabinol	H	*n*-C_5H_{11}	H
Tetrahydrocannabinolic acid-C_4	COOH or H	*n*-C_4H_9	H or COOH
Tetrahydrocannabinol-C_4	H	*n*-C_4H_9	H
Tetrahydrocannabivarinic acid A	COOH	*n*-C_3H_7	H

Tetrahydrocannabivarin	H	*n*-C_3H_7	H
Tetrahydrocannabiorcolic acid	COOH or H	CH_3	H or COOH
Tetrahydrocannabiorcol	H	CH_3	H

5. Δ8-THC type: It is considered, along with its acid precursor as THC and THC acid artifacts, respectively. The 8,9 double-bond position is thermodynamically more stable than the one found in THC in 9,10 position. Nevertheless, '8-THC is 20% less active than THC (Figure 1-5, Table 1-5).

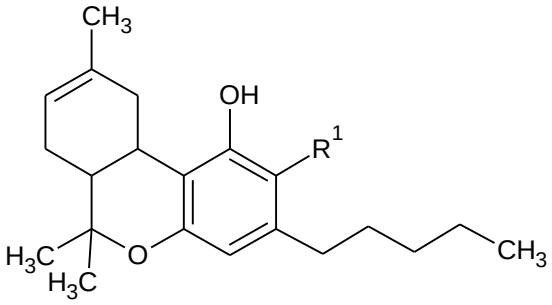


Figure1-5: Δ8-THC-type Cannabinoids

Table 1-5: Δ8-THC.type Cannabinoids.

Compound	R^1
(-)-Δ8-*trans*-(6aR,10aR)-Tetrahydrocannabinolic acid A	COOH
(-)-Δ8-*trans*-(6aR,10aR)-Tetrahydrocannabinol	H

6. Cannabicyclol (CBL) type: It is characterized by a five-atom bridge and C_1-bridge instead of the typical ring A, but just three of these cannabinoids are known (Figure 1-6, Table 1-6).

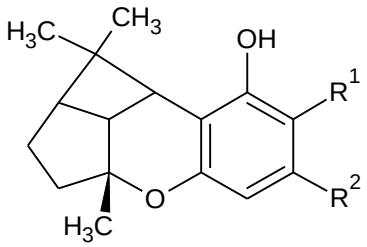


Figure 1-6: CBL-type Cannabinoids.

Table 1-6: CBL-type Cannabinoids.

Compound	R^1	R^2
(±)-(1aS,3aR,8bR,8cR)-Cannabicyclolic acid	COOH	*n*-C_5H_{11}
(±)-(1aS,3aR,8bR,8cR)-Cannabicyclol	H	*n*-C_5H_{11}
(±)-(1aS,3aR,8bR,8cR)-Cannabicyclovarin	H	*n*-C_3H_7

7. Cannabielsoin (CBE) type: It is formed from CBD, and only five CBE-types are known (Figure 1-7, Table 1-7).

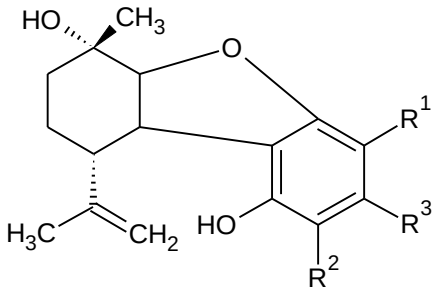


Figure 1-7: CBE-type Cannabinoids.

Table 1-7: CBE-type Cannabonoids.

Compound	R^1	R^2	R^3
(5aS,6S,9R,9aR)-Cannabielsoic acid A	COOH	H	*n*-C_5H_{11}
(5aS,6S,9R,9aR)-Cannabielsoic acid B	H	COOH	*n*-C_5H_{11}
(5aS,6S,9R,9aR)-C_3-Cannabielsoic acid B	H	COOH	*n*-C_3H_7
(5aS,6S,9R,9aR)-Cannabielsoin	H	H	*n*-C_5H_{11}
(5aS,6S,9R,9aR)- C_3-Cannabielsoin	H	H	*n*-C_3H_7

8. Cannabinol (CBN) type: It is the oxidation artifact of THC. Six CBN-type cannabinoids are known. CBN was first named in 1896 by Wood et al. [17], and its structure discovered in 1940 (Figure 1-8, Table 1-8).

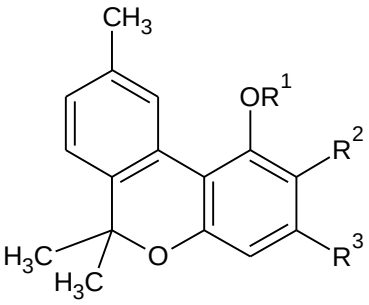


Figure1-8: CBN-type Cannabinoids.

Table 1-8: CBN-type Cannabinoids.

Compound	R^1	R^2	R^3
Cannabinolic acid A	H	COOH	*n*-C_5H_{11}
Cannabinol	H	H	*n*-C_5H_{11}
Cannabinol methyl ether	CH_3	H	*n*-C_5H_{11}
Cannabinol-C_4	H	H	*n*-C_4H_9
Cannabivarin	H	H	*n*-C_3H_7
Cannabinol-C_2	H	H	C_2H_5
Cannabiorcol-C_1	H	H	CH_3

9. Cannabitriol (CBT) type: It is characterized by an additional OH substitution. Nine CBT-type cannabinoids have been identified. CBT exists in the form of both isomers and the racemate (Figure 1-9, Table 1-9).

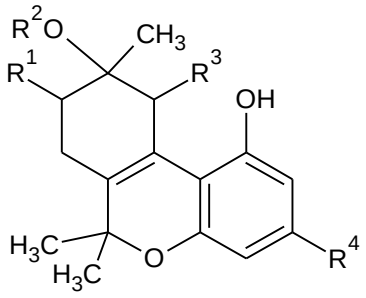


Figure1-9:CBT-type Cannabinoids.

Table 1-9: CBT-type Cannabinoids.

Compound	R^1	R^2	R^3	R^4
(-)-*trans*-Cannabitriol	H	H	OH	*n*-C_5H_{11}
(+)-*trans*-Cannabitriol	H	H	OH	*n*-C_5H_{11}
(±)-*cis*-Cannabitriol	CH_3	H	OH	*n*-C_5H_{11}
(±)-*trans*-Cannabitriol	H	H	OH	*n*-C_3H_7
CBT-C_3-homologue	H	H	OH	C_3H_7
(-)-*trans*-10-ethoxy-9-hydroxy-$^{\Delta 6a(19a)}$-tetrahydrocannabinol	H	H	OEt	*n*-C_5H_{11}
trans-10-ethoxy-9-hydroxy-$^{\Delta 6a(19a)}$-tetrahydrocannabivarin-C_3	H	H	OEt	*n*-C_3H_7
8,9-Dihydroxy-$^{\Delta 6a(19a)}$-tetrahydrocannabinol	OH	H	H	*n*-C_5H_{11}
Cannabidiolic acid tetrahydrocannabitriol ester	H	CBDA-C_5 ester	OH	*n*-C_5H_{11}

10. Miscellaneous types: Eleven cannabinoids of various unusual structure [with a furano ring (dehydrocannabifuran, cannabifuran), carbonyl function (cannabichromanon, 10oxo-G-6a-tetrahydrocannabinol), or tetrahydroxy substitution (cannabiripsol)] are known.

Among Δ9-tetrahydrocannabinols, there exists also a particular cannabinoid, tetrahydrocannabivarins (THCV), which instead of the pentyil side chain typical of Δ9-THC, has a propyl one. A similar propyl side chain is found in cannabidivarins (CBDV), found amongst cannabidiols. These two cannabinoids, due to the propyl side chain, do

not derive from cannabigerolic acid, but from cannabigerovarin acid (CBGVA), which undergoes enzyme binding, prenylation, and cyclization, and it is transformed to the acid precursors of these two cannabinoids (THCVA and CBDVA).

1.2.2.2. Identification Steps

The first important steps in the identification of cannabinoids was performed by Wood et al. [17], who were able to isolate cannabinol (CBN, $C_{21}H_{26}O_2$) from the resin of Indian hemp. After that, Cahn managed to identify the structure of CBN, leaving uncertain just the positions of a pentyl and a hydroxyl group [18].

The first natural cannabinoid, CBN, was determined independently both by Adam et al [19]. and Todd et al [20]. Subsequently, cannabidiol (CBD), that is the second *C. sativa* constituent, was identified, but its structure was not completely elucidated. Krejc ˇı ´ and S ˇantavy ´ [21] were able to isolate cannabidiolic acid (CBDA) from an extract that was effective against *Staphylococcus aureus* and other Gram-positive microorganisms. In 1963, Mechoulam et al. [15] were able to determine the correct structure and stereochemistry of CBD, and a year later they isolated and obtained a crystalline derivate of tetrahydrocannabinol (Δ 9-THC). They also managed to achieve a partial synthesis of D9-THC from CBD. Several years later, even Δ8-THC was isolated. At the same time, some non-psychotropic cannabinoids were isolated, such as cannabigerol (CBG), cannabichromene (CBC), and cannabicyclol (CBL) [22].

To understand the biogenesis of these cannabinoids, it was essential to identify cannabinoid acids. Firstly, cannabinolic (CBNA) and cannabigerolic (CBGA) acids were

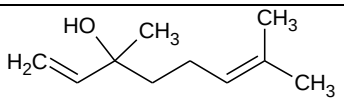
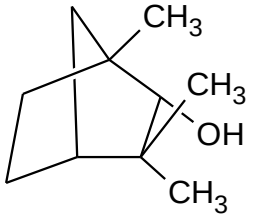
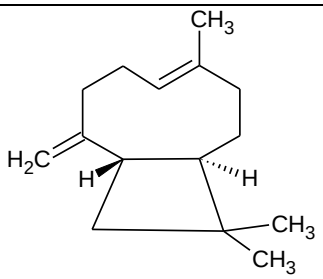
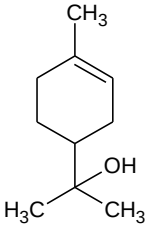
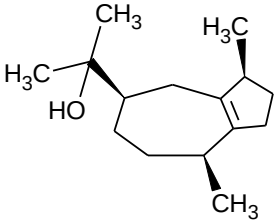
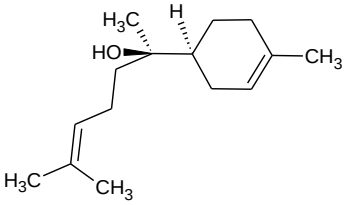
identified, followed by Δ9-THC acid, Δ8-THC acid, and cannabielsoic acid (CBEA) [23].

1.2.3. Description of Terpenes

It has been shown that terpenes play an important role in the classification of *C. sativa* [24]; in fact, although the cannabinoids are odorless and indicate the genetic relationship, terpenes are the primary aromatic principles found in the essential oil of the plant, and they reflect the immediate environment [25].

Terpenes comprise a large group of compounds derived from C_{10} isoprene subunits. In *C. sativa*, the most frequent classes of terpenes are monoterpenes (C_{10}) and sesquiterpenes (C_{15}). In each plant, no more than 40 known terpenes have been identified, even though a total of 100 named terpenes are present in *C. sativa* [26]; unfortunately, as of now, there is not a complete list of *C. sativa* terpenes; however, the six most recurrent ones are known (Table 1-10).

Table 1-10. Main classes of terpenes

Name	Structure	Properties
Linalool		It has a citrus, floral, and woody odor, with blueberry aromas. It works as an anti-anxiety, a sedative [27], and as an anti-convulsant [28].
Exo-Fenchol		It has a lemon, woody, and camphor odor.
Caryophyllene		It has a dry spicy odor, but with sweat notes. It is a gastric cell protectant and anti-inflammatory [29], and anti-malarial [30].
Terpineol		It is odorless.
Guaiol		It has a rose tea, and mild woody odor.
α-bisabolol		It has a mild floral odor, with a peppery note.

1.3. Receptors

Two cannabinoid receptors have been identified, and they belong to the superfamily of G-protein-coupled membrane receptors [31]. The first one was identified in the rat brain in 1988 [32]. This receptor was named CB1, and it is expressed on peripheral neurons and other cell types. It is negatively coupled with adenylate cyclase, and it can be either negatively or positively associated with selective ion channels [31]. CB1 is found in the central nervous system and some peripheral neurons, especially in the basal ganglia, hippocampus, cerebellum, and cerebral cortex, and this accounts for the effects of cannabinoids on short-term-memory processing, but also in the dorsal primary afferent spinal-cord areas, that are important in pain pathways [31]. Therefore, activation of CB1 receptors leads to retrograde inhibition of the neuronal release of acetylcholine, dopamine, GABA, histamine, serotonin, glutamate, cholecystokinin, D-aspartate, glycine, and noradrenaline. The second cannabinoid receptor, CB2, was identified in macrophages in the marginal zone of the spleen [33]. This receptor is mainly found in leucocytes, and it is not linked to ion channels. It has no known neurological activity [31]. Cannabinoids may also influence other receptors pathways.

1.4. Uses and Effects

1.4.1. Products and Uses

1.4.1.1. C. sativa and its products

The plant of *C. sativa* has many products that can be used, and among these, the

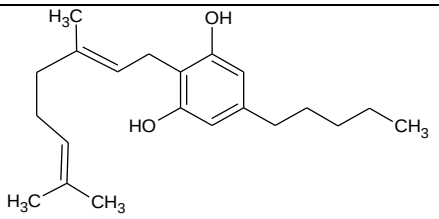
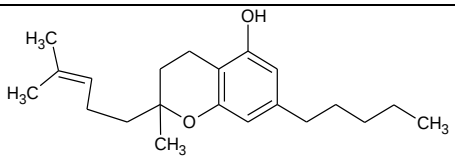
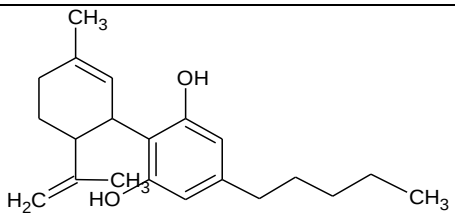
most common one is herbal *C. sativa*, important because it is believed that only flowers and leaves confer psychotropic effects. *C. sativa* resin is used to produce hashish, that is a more potent drug than marijuana. Finally, liquid *C. sativa*, that also known as hashish oil, and *C. sativa* seeds [13].

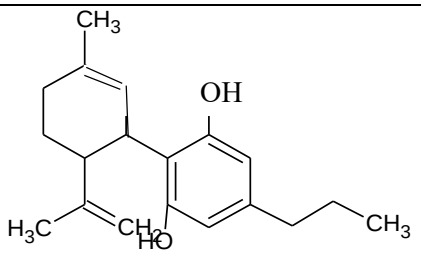
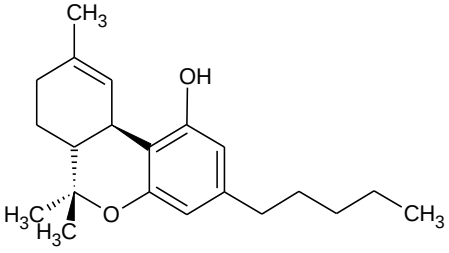
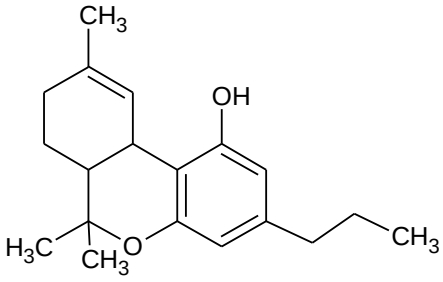
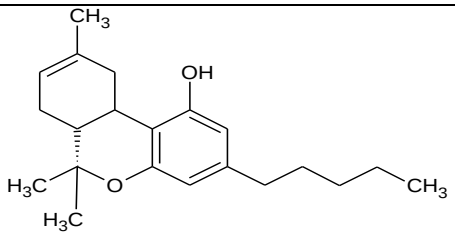
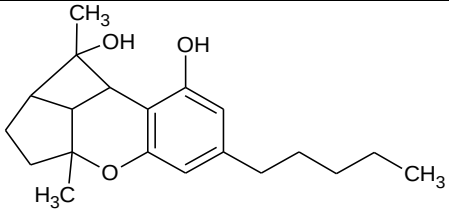
1.4.1.2. C. sativa and its effects

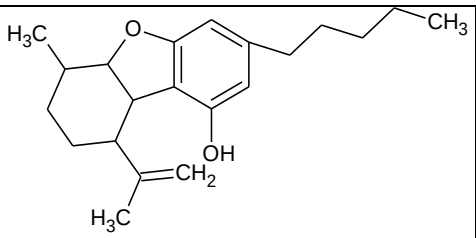
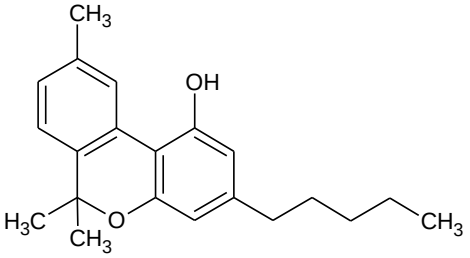
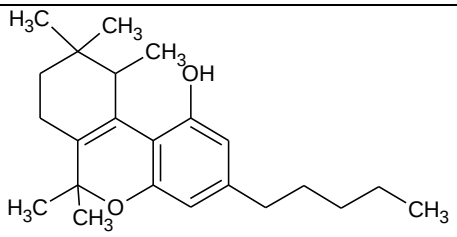
Considering the large number of products that are obtainable from *C. sativa,* it is important to remember that, besides the recreational uses, this plant has also many therapeutic uses (Table 1-11) [34]:

1. Obesity, anorexia, emesis: CB1 plays a very important role in central appetite control, peripheral metabolism, and body weight regulation.
2. Pain: THC, CBD and CBD-dimethyl heptyl (DMH) block the release of seratonin from platelets.
3. Multiple sclerosis, neuroprotection, inflammation.
4. Parkinson's disease, Huntington's disease, Tourette's syndrome, Alzheimer's disease, Epilepsy.
5. Bipolar disorder, schizophrenia, post-traumatic stress disorder (PTSD), depression, anxiety, insomnia.
6. Asthma, cardiovascular disorders, glaucoma.
7. Cancer.

Table 1-11. Main classes of cannabinoids

Name	Structure	Medical benefits
Cannabigerol (CBG)		It aids sleep. It inhibits cancer cell growth, and it slows bacterial growth, but it promotes bone growth [26].
Cannabichromene (CBC)		It inhibits cancer cell growth, but it produces bone growth. It reduces inflammation and it relieves pain [26]. It is an anti-bacterial and anti-fungal [27].
Cannabidiol (CBD)		It is an antibacterial, it inhibits cancer cell growth, it is neuro protective, it reduces seizure and convulsions, it reduces both blood sugar levels, inflammation, risk of artery blockage, vomiting, and nausea. It relieves pain and anxiety, and it suppresses muscle spasms. It is a tranquillizer, and a vasorelaxant [26]. Furthermore, it modulates THC-induced psychoactivity.

Cannabidivarin (CBDV)		It helps against hyperexcitability, it reduces epilepsy, and it reduces nausea [26].
Δ9-Tetrahydrocannabinol (Δ9-THC)		It reduces vomiting and nausea, it relieves pain, it stimulates appetite, and it suppresses muscle spasms [26]
Tetrahydrocannabivarin (THCV)		It reduces convulsions and seizures, and it promotes bone growth [26]. It stimulates energy metabolism, it improves glucose intolerance [27], and it is an anti-inflammatory and anti-convulsant [31].
Δ8-Tetrahydrocannabinol (Δ8-THC)		It relieves pain [26].
Cannabicyclol (CBL)		No reported bioactivity.

Cannabielsoin (CBE)		No reported bioactivity.
Cannabinol (CBN)		It aids sleep, it suppresses muscle spasms, and it relieves pain [26]. Furthermore, it is an appetite—stimulant and it is anti-tumorigenic against some types of lung cancer [31].
Cannabitriol (CBT)		No reported bioactivity.

In the liver, once Δ9-THC is digested, it is transformed into 11-Hydroxy-Δ9-THC. This conversion occurs at high rates if *C. sativa* is consumed in the edible form, and it is a reason why the edible consumption is more dangerous. 11-Hydroxy-Δ9-THC is in fact considered more potent than Δ9-THC, because it crosses the barrier between blood and brain easier. Afterwards, 11-Hydroxy-Δ9-THC is metabolized into 11-nor-9-carboxy-THC, which does not have any psychoactive effects, but still carries the anti-inflammatory and pain relief roles of *C. sativa.*

1.4.2. Laws and regulations

C. sativa also remains the most widely used drug worldwide, with 4% of the global population aged 15-64 currently using it. Moreover, every country in the world is affected by *C. sativa* trafficking; its products, in fact, are the most widely trafficked drugs worldwide, accounting for 65% of all global seizure cases (1.65 million cases) in 2006. North and South America produced 55 per cent of the global manufacturing in 2006, followed by the 22 percent by Africa [13].

Since 1970s, drug producers in North America and Europe have tried to create a more potent *C. sativa*, the indoor-produced Sinsemilla, but in Europe it is imported *C. sativa* that dominates the market. Sinsemilla has a greater potency because it is cultivated using high THC breeds and intensive hydroponic techniques. The THC concentrations, in fact, have been raised up to 15-20 percent in certain herbal materials [13].

In the USA, dronabinol (Marinol) (a synthesized drug with the active components of *C. sativa*, mainly Δ9-THC) has been licensed since 1985 for the treatment of nausea and vomiting caused by cytostatic therapy, and since 1992 for loss of appetite in HIV/AIDS-related cachexia. The medicinal use of *C. sativa* is an open topic, and in the USA many states are now evaluating the possibility of amplifying the legal treatment.

In Germany, only one *C. sativa* extract is approved for use. It contains THC and CBD in a 1:1 ratio and it was licensed in 2011. This *C. sativa* extract, named nabiximols (Sativex), has been approved by regulatory bodies in Germany and elsewhere for use as a sublingual spray. In Great Britain, nabilone (Cesamet), a synthetic cannabinoid that mimics the THC action, is used for treatment of the side effects of chemotherapy in cancer patients [35].

1.5. Structure-Affinity Relationships of Cannabinoids in Men

The introduction of Δ9- tetrahydrocannabinoil (Δ9-THC) into the body by smoking is 2.6 to 3 times more potent than the Δ9- THC ingested orally, and the effects by smoking appear within seconds or minutes, while with orally assumed doses the symptoms are delayed from thirty minutes to two hours. Based on the clinical evaluation of various cannabinoids, the following structure-affinity relationships (SAR) in man are suggested [36]:

1. A benzopyran ring is mandatory for activity, since the ring-opened compound cannabidiol (CBD) is inactive. However, it was noticed that, since cannabichromene is inactive, the benzopyran by itself does not confer activity. Moreover, the oxygen in the benzopyran ring can be substituted by a nitrogen without loss of activity.
2. The aromatic hydroxyl group has to be free or esterified, and blocking of the hydroxyl group leads to inactivation of the molecule.
3. Variations of the length of the aromatic side change do not cause the loss of activity, but the minimal is three-carbon chain. Moreover, branching of the chain increases potency, and the attachment of this side chain can be via oxygen atom, without loss of activity.
4. Monohydroxylation on the 7-position of the benzopyran ring increases the activity, while dihydroxylation causes loss of activity. Monohydroxylation on other position of the benzopyran ring also usually leads to active derivatives.
5. Hydroxylation on the C-1" of the side chain causes inactivity, while hydroxylation

on the C-3" increases it.

6. Alkylation on the C-4' aromatic position of the benzopyran ring retains activity, while alkylation on the C-6' position of the same ring eliminates it. Anyways, adding elecronegative groups such as carbonyl or carboxyl, at either C-4' or C-6' of the benzopyran ring, eliminates activity.
7. The double bond isomers of THC have different activities in human: Δ1-THC> Δ6-THC> Δ3-THC.
8. The 7-methyl group is not an absolute requirement for activity.
9. The benzopyran ring may be exchanged by some heterocyclic systems.

Therefore, changing the position of the double bond from Δ9- to Δ8- or Δ6- reduces the potency, decreasing the length of the side chain by two carbons reduces potency by 75%, and substitution of a hydroxyl group in the 11- position maintains the potency, while in the 8-position reduces the potency by 80 per cent.

1.5.1. Endocannabinoids

The first endocannabinoid to be discovered (1992) was arachidonoylethanolamide (anandamide), followed by 2-arachidonoylglycerol (2-AG). These two compounds have a cannabinoid receptor binding activity, but their physiological roles are still uncertain. It is known that they are both produced on demand from precursors by pathways that involve phospholipases D and C, and they are able to bind and stimulate the CB receptors [31].

In general, the main function of the endocannabinoid system is to regulate synaptic neurotransmission; CB1 regulates the one of excitatory and inhibitory circuits. In fact, in

response to depolarization and Ca^{2+} fluxes, endocannabinoids are released to inhibit more stimulation of CB1 receptor. Furthermore, this system is believed to influence different functions; there is in fact evidence that cannabinoids affect the activity of most neurotransmitters [31].

Chapter 2: Gas Chromatography-Mass Spectrometry

All forms of chromatography involve the distribution of a compound between two different phases, one stationary and the other mobile. In a mixture, this partitioning is based on the solubility of the compounds in each phase. The different solubility causes the compounds to be physically divided, with the ones having greater solubility in the stationary phase eluting later than those with less solubility [40].

In gas chromatography, the mobile phase is an inert carrier gas, and the stationary phase is a high molecular weight liquid attached to the walls of a capillary column. A GC instrument usually uses helium, nitrogen or hydrogen as the carrier gas, and its flow into the sample injection device is controlled by pressure regulators and valves. The requirements for the carrier gas are <0.1 ppm oxygen, moisture, or trace hydrocarbons [41]. A column is attached to the injection port, and samples are introduced through the carrier gas, after having been vaporized at sufficient temperature; the sample is introduced via a microliter syringe which is forced into the injection port. The column is inserted in an oven, where the temperature control to ±0.1°C is the main aim. The oven can work in different methods; isothermal, single-gradient, step gradient, and multicycle [41]. A detector is directly attached to the end of the column, and it must be insensitive to the carrier gas, while it must be able to detect compounds as they elute; its response is recorded with time, creating a chromatogram (Figure 2-1). The qualitative information are found in the retention time of the analyte, while the quantitative ones in the peak area [41].

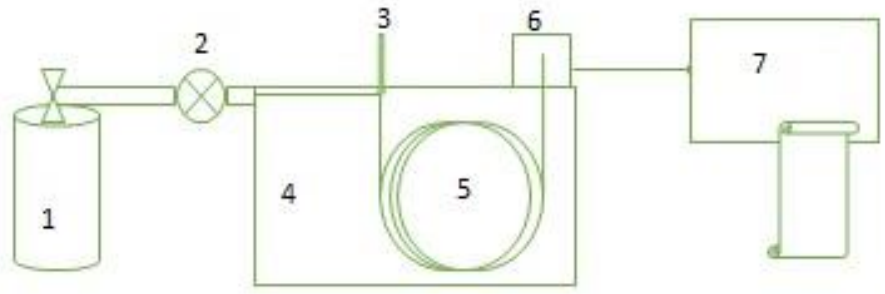


Figure 2-1:.Scheme of a GC instrument. 1, Gas Tank; 2, flow rate regulator; 3, injection port; 4, column; 5, oven; 6, detector; 7, recorder.

2.1. Types of injection

2.1.1. Split and Splitless injection

There are two different modes of injection, based on the concentration of the compounds; split and splitless. For high concentration analytes the split mode is used. To prevent overloading of the column the set ratio of flow between the column and the split vent splits the heated sample in two inequivalent portions; the larger portion is vented from the system, while the smaller one is injected into the column. The advantages are that this technique is simple, and it provides high column efficiencies, but on the other hand it discriminates toward higher boiling compounds.

The splitless injection is used for samples that contain traces of compounds, and that would not reach the limit of detection if vented out of the system. In this mode, the sample undergoes flash vaporization and it is reconcentrated at the head of the column; without reconcentration, the efficiency of the separation would be reduced and the band widths

would be increased. The main advantage of the splitless mode is a greater sensitivity, but the transfer in the column is slow, causing broad initial bands [42,43].

2.1.2. Direct Injection

The hardware is similar to the one for the splitless injection, but without the split outlet. This method is used with packed columns, and it is use for trace analysis, as well as permanent gas analysis [41].

2.1.3. On-column injection

The methods above described always require flash vaporization, that could lead to the decomposition of thermally labile compounds. This problem can be overcome by using a cool injection port directly onto the column, requiring a concentration of the sample at the head of the column. This method has the highest reproducibility and the lowest discrimination and decomposition of any inlet; the analytical sensitivity is very high and detection limits are as good as with a splitless injection, if not better. The problems that on-column injections show are solvent overload, peak splitting, premature degradation of the stationary phase, and contamination from non-volatile sample components [42,43].

2.2. Temperature program

There are three ways of changing the column temperature during a run; discontinuously with or without interruption of the gas flow, continuously in an uncontrolled

manner, and continuously with a linear relation between time and temperature increment. The first two programs are barely used, and the third technique modifies not only the retention times, but also the selectivity of the separation. This method is used with compounds that have different boiling points [44].

2.3. Types of columns

There are generally two different types of gas chromatography columns, packed and capillary. The first ones have a higher sample capacity, and they are preferable for the analysis of gas samples. For all the other analysis, capillaries provide narrow peaks that lead to better efficiency and greater peak separation. The material used in columns have to be as inert as possible, especially for trace analysis work; for capillaries, fused silica is usually chosen. There are two basic types of fused silica capillary columns: the wall coated open tubular (WCOT) columns and the porous layer open tubular (PLOT) columns. In the first type, the stationary phase is a liquid film coated to the wall of the column, while in the PLOT columns it is a solid substance coated to the column wall. Packed columns are usually made of stainless steel or glass. Metal, in fact, is durable and suitable for nonpolar materials; if the sample contains many polar components, then glass is preferred [45].

2.4. Mass Spectrometer

When a molecule is ionized, a molecular ion can be formed, and if there is sufficient additional internal energy, fragment ions may also form. A mass spectrometer is used to separate and measure the masses of ions based on their mass-to-charge ratios (*m/z*). For most

ionization techniques, an ion with a single positive charge ($z = 1$) is formed, so that the ion's *m/z* ratio is equal to its mass, but sometimes we can produce two, three, or more charges, leading to lower *m/z* ratios [46].

The mass spectrometer is composed of the inlet system, the ion source, the analyzer, and the detecting and recording device (Figure 2-2).

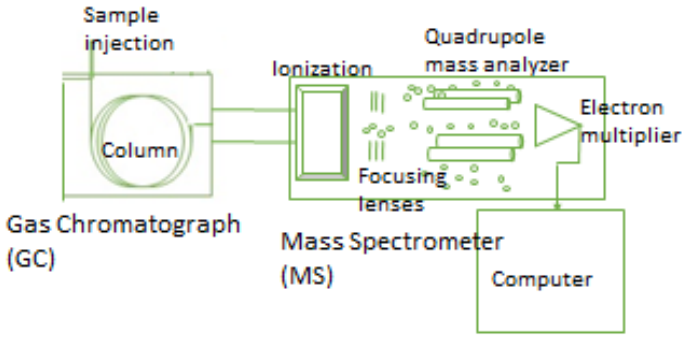


Figure 2-2: Scheme of a generic GCMS instrument

2.4.1 Ion Sources for GC-MS

2.4.1.1. Electron Ionization (EI)

The electrons are obtained by heating a filament in vacuum, and after this they are accelerated through some voltage and they pass through an ion chamber. Usually, the voltage used is 70 eV, to ensure efficient and reproducible fragmentation profiles for the wide range of molecules that might be subjected to electron ionization (EI) [41].

2.4.1.2. Chemical Ionization (CI)

The collision between the ion and the molecule can cause reactions that create new charged species; for these reactions to be effective, the source pressure has to be about 0.1 Torr greater than for other ion sources. The most used reagent gases are ammonia, isobutene, and methane. One can even produce negative ions, because the electron capture is efficient at high pressures [41].

2.4.2 Mass Analyzers for GC-MS

2.4.2.1. Quadrupole Mass Analyzer

Four parallel rods are used, and between each pair of opposite and electrically connected rods we apply a dc voltage and a superimposed radio-frequency potential. Because of this combination of fields, the ions move in complex trajectories, but always causing a separation according to mass. At a certain point of the scan, all ions but the selected one are excluded from the system (Figure 2-3). This system is usually paired with chromatography because it can scan the entire mass range in a few milliseconds [47].

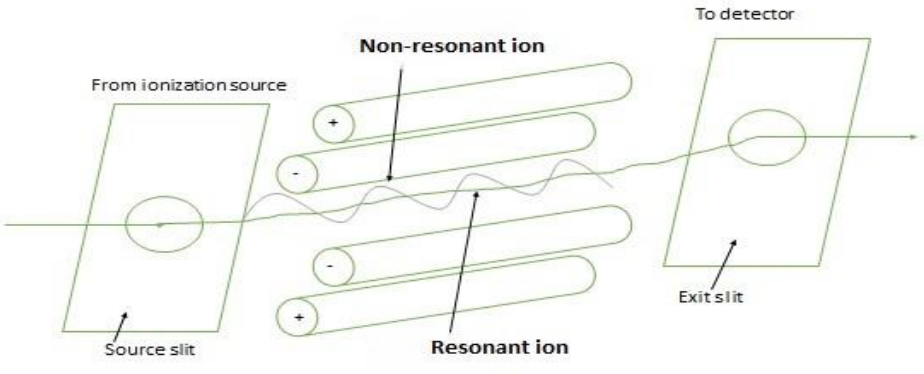


Figure 2-3: Scheme of a quadrupole analyzer.

2.4.2.2 Quadrupole Ion Trap Mass Analyzers

This ion trap foresees the use of dynamic electric fields for trapping molecules that are charged; these electric fields change in time, making it possible to have a confining force, and the directions are switched at a rate that is faster than the escaping speed of the particle itself. The quadrupole is the easiest geometry to obtain this trap, and metal electrodes are used for producing the fields [41].

2.4.2.3. Time-of-Flight Mass Analyzers (TOF)

This analyzer separates ions by accelerating them into a field-free drift tube with constant kinetic energy. The ion velocity is mass-dependent, so the ions will reach the detector at different time intervals. The time intervals are very short (nanosecond to microsecond), but with reflectron time-of-flight technology, much higher resolution mass spectra can be collected, compared to quadrupole and ion trap systems [41].

2.5. Other types of detectors

2.5.1. Flame Ionization Detector (FID)

2.5.1.1. Principle of Operation

The combustion of organic compounds in a small hydrogen air diffusion flame burning at a capillary jet produces a few ions per million molecules. The reaction is CH^{0}+O $\rightarrow$ CHO^{+} + e^{-}, and the ions are collected through an electric field, after which the current is measured. The background current is usually 10^{-14}, 10^{-13} A, and the signal is amplified by

an electrometer. Since each C atom produces the same signal, the overall response is given by the sum of the effective C atoms; the contribution to the response of various C atoms depends in fact on the nature of their substituents (Aliphatic=1, Olefinic=0.95, Acetylene=1.30, Carbonyl=0, and Nitro=0.3) [41].

2.5.1.2. Instrument

The carrier gas from the GC is premixed with H_2 and burned in a chamber with excessive air. The electrode (either cylindrical or hemicylindrical) is few millimeters above the flame, and the ion current is measured through a potential between the jet tip and the collector electrode.

The parameters that affect the response are the temperature and the size of the flame, hydrogen and air flow rates, carrier gas flow rate, and polarization voltage of the collecting electrode [43]. The average LOD is 10^{-14} g, therefore this detector is used mainly for sub-ppb determination. It has a linearity: 10^6-10^7, and it is used to detect all organic compounds, especially hydrocarbons, fatty acid methyl esters, and sterols. It is not an universal detector, and the process is destructive.

2.5.2. Thermal Conductivity Detector (Catharometer)

2.5.2.1. Principle of Operation

The thermal conductivity of a mixture of gases varies with its composition [41].

2.5.2.2. Instrument

The carrier gas flows through a heated thermostated cavity, where the resistor, that is the sensing element, is electrically heated. The heat loss is a function of the temperature difference between the sensor and the cavity and the thermal conductivity of the carrier gas, therefore when something changes in the carrier gas, there is a consequent change in the thermal conductivity. Resistors are usually connected to form a Wheatstone bridge, where one diagonal is swept by pure carrier gas, and the other cells are swept by the column eluent. These resistors may be operated in different modes, such as constant-current, constant-voltage, or constant-temperature compensation circuit.

Parameters that affect the results are the nature and the flow rate of the carrier gas, the nature of sensors, the intensity of the current, and the geometry of the cavity [48].
The LOD is 10^{-4} g, it is an universal detector, but it is especially suggested for permanent gases or light hydrocarbons. The dynamic range is about 10^{4}, and the process is not destructive.

2.5.3. Electron Capture Detector (ECD)

2.5.3.1. Principle of Operation

It is based on the difference between the recombination rates of positive and negative ions, and electrons and positive ions. Beta electrons are emitted by a radioactive source, and they collide with the carrier gas forming a plasma of positive ions, radicals, and electrons. The thermal electrons are collected by an electrode that produces the background current,

therefore a decrease in the current is linked to the number of captured electrons. The reaction is e^-+AB → AB^-, that can lead to A^0, B^- [41,42].

2.5.3.2. Instrument

The source is ^{63}N, and three techniques may be employed; constant-voltage, pulsed-voltage, and constant-current. With this type of detector, we measure the frequency. The parameters that may affect the response are the nature and the flow of the carrier gas, the temperature, and the polarization voltage of the electrodes. In this detector, the LOD depends on the nature of the solute, but it has a low dynamic range, 10^2-10^4. It is selective to halogenated compounds, and sensitive to pesticides, polychlorinated molecules, and herbicides. It is a destructive detector [41].

2.5.4. Thermoionic Detector (Alkaline Flame Ionization Detector)

2.5.4.1. Principle of Operation

The addition of an alkali metal salt to a flame causes the response of compounds containing S,P,B, or N_2. There are three types of reaction that may occur: solid-phase reactions, photoevaporation, and gas-phase reactions. The latter involves free alkali metal atoms that are ionized in the flame with the carrier gas: A + M → A^+ + e^- + M, and the free radicals resulting from the pyrolysis of organic compounds with P or N react with alkali metal atoms: A + R^0 → A^+ + R^-, Rb + R^0 → Rb^+ + R^-[41].

2.5.4.2. Instrument

A ceramic or glass matrix is doped with an alkali metal salt that can be electrically heated. The used salt is most of the times rubidium silicate, because it yields to a higher response. The parameters that affect the response are the hydrogen and the air flow rates, and the carrier gas flow rate. The LOD changes with the molecule detected; 10^{-3} g for N_2, and 5 x 10^{-14} g for P. The dynamic range is around 10^4, and the response is greater for N and P derivatives, and halogens. The process is destructive [41].

2.5.5. Flame Photometric Detector

2.5.5.1. Principle of Operation

When atoms are heated in a flame, some of them shift to an excited state and then return to the ground state, accompanied by the emission of light at a characteristic wavelength.

2.5.5.2. Instrument

Carrier gas and air are mixed, and the combustion occurs in a hydrogen-rich atmosphere. The light is collected by a photomultiplier, and a monochromator separates the signals. The parameters that affect the response are the photomultiplier voltage, and the flame stability. The LOD is around 10^{-10} g, and it is very selective towards S and P. it is not linear, and the calibration is required for all compounds. The process is destructive [41].

2.5.6. Helium Ionization Detector

2.5.6.1. Principle Of Operation

The ionization is caused by collisions between He atoms and β particles generated by radioisotopes. Metastable He atoms are formed during secondary collisions, and they have an energy of 19.8 eV, much higher than any other atom.

2.5.6.2. Instrument

A hollow electrode is polarized to a voltage between 500 and 2000 eV; the higher the voltage, the greater the response. The parameters that affect the response are the purity of He, He flow rate, electric field, temperature, column bleed, and equipment. It is an universal detector, and the process is destructive [41].

2.5.7. Chemiluminescence Detector

2.5.7.1. Principle of Operation

A chemical reaction produces electronically excited species that undergo emission of photons. There are different types of this detector: TEA (thermal energy analyzer), where a catalytic pyrolysis breaks the N-NO bond forming nitrosyl radical, that can react with ozone to create electronically excited nitrogen dioxide. SCD (sulfur chemiluminescence detector), on the other hand, burns sulfur-containing solutes in a hydrogen-rich flame to form sulfur monoxide, that reacts with ozone to form SO_2* (it emits in the 260-480 nm range) [41].

2.5.7.2. Instrument

It includes a reaction chamber, a light sensor, and a system to generate the reagent gas.

2.5.8. Hall Electrolytic Conductivity Detector (HECD)

2.5.8.1. Principle of Operation

The solutes from the column are pyrolysed, and electroactive species are formed. The products are swept from the furnace into a gas-liquid contactor, where they are mixed with a solvent. The liquid phase is then sent to a conductivity cell.

2.5.8.2. Instrument

The detector uses one cell for pure solvent, and one cell for the solvent plus the reaction products. The parameters that affect the response are the pH of the conductivity solvent, the contamination of the transfer line, the catalyst, and the flow rate of the solvent. The LOD varies from 10^{-12} g for N, to $5x10^{-13}$ g for Cl, and 10^{-2} g for S; it is sensitive to Cl, S, and N, and it is a destructive process [49].

2.6. Silylation

The silylation reaction involves the substitution of a hydrogen atom that is bound to a hetero atom, such as –OH, =NH, and –SH, by a silyl group, without any further alteration

of the molecule. In analytical chemistry, this reaction has been used in GC-MS to derivate a wide variety of functional groups; in fact, it allows the study of compounds otherwise non-volatile or unstable for this technique [50].

The trimethyl silyl group is the most popular silyl group for these purposes, and a variety of trimethyl silylating agents has been developed. The stability of a compound R^1 R^2 R^3 SiX (Figure 2-4) towards the breaking of the Si-X bond depends on both the nature of R^1, R^2, R^3, and of X.

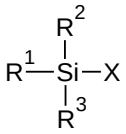


Figure 2-4: R^1 R^2 R^3 SiX.

The influence of X on the solvolytic stability of $R^1R^2R^3SiX$ depends on the bond energies and the polarization of the SiX bond, and on the steric bulk of X. Following these rules, we can state that the stability of R^3SiX decreases normally in the order R^3Si0- > $R^3SiN=$ > R^3SiS-, therefore decreasing along with the bond energies, that since silicon is generally more electropositive than X, the nucleophilic attack occurs at silicon and electrophilic attack at X, and that the steric bulk of X enhances the stability to both acidic and basic hydrolysis. Similar rules apply for the nature of R^1, R^2, and R^3: the more bulky, the higher the stability of the silyl group [50].

2.6.1. N,O-Bis(trimethylsilyl)trifluoroacetamide, BSTFA

BSTFA, introduced by D. L. Stalling et al., is one of the most commonly used silylation agents. Its analytic applications have two main advantages over other agents in

gas chromatography: BSTFA and its by-products mono(trimethylsilyl)trifluorocetamide and trifluoracetamide are extremely volatile and so they cause less interference in chromatograms. Because of its polar nature, BSTFA can act as its own solvent. The silylating power of BSTFA can also be increased by the addition of a catalyst, mainly trimethylsilyl chloride (TMCS) between 1–50% (Figure 2-5) [50].

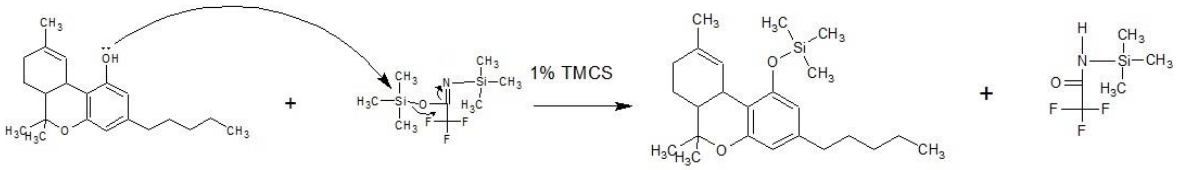


Figure 2-5: Mechanism of the reaction between Δ9-THC and BSTFA+1%TMCS.

2.6.2. N-Methyl-N-trimethylsilyltrifluoroacetamide, MSTFA

MSTFA, introduced by M. Donike, has similar reaction properties to BSTFA as tetramethylsilane (TMS). Its particular advantage is that the reagent itself and its by-product, N-methyltrifluoroacetamide, are even more volatile than BSTFA and its by-products. It can be used without solvent, and due to its polarity it can dissolve even highly polar substances (Figure 2-6). Such as for BSTFA, the silylating power can be increased by addition of a catalyst, such as TMCS, TMSIM, TMBS, and TMIS [50].

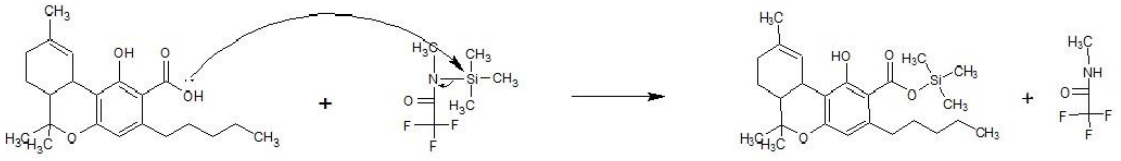


Figure 2-6: Mechanism of the reaction between Δ9-THCA and MSTFA.

Chapter 3: Analytical Methods in the Literature

3.1. Sampling and preparation methods

For each *C. sativa* field, 30 fruiting or flowering tops (one per plant) are randomly chosen and cut to a length of 20 cm, and then stored in a bag. The sample should be dried before sending to the laboratory, when possible. If it has to be stored, the room must be dark and cool. Once dried, the degradation of the cannabinoids stops, but THC is still sensitive to oxygen and UV light, which can oxidize THC to CBN [13].

Wet plant material is either dried at 70°C until the leaves become brittle, or dried at room temperature for several days. At this point, the percentage of water in the plant is usually 8-13 per cent. The dried sample is then selected, since only flowers and leaves are used, pulverized and sieved (mesh size 1 mm) [13]. If the analyzed sample is *C. sativa* resin, it must be reduced to small pieces by a grater, or cooled down with liquid nitrogen and pulverized [13], while *C. sativa* oil can be directly used for analysis [13].

3.2. Gas Chromatography Analysis

3.2.1. Chemotaxonomic analysis of cannabinoid variations in *C. sativa*

According to Hillig et Al. [51], 50 g of the sample material must be placed in a test tube with 1 mL of chloroform. The plant material was pulverized with a glass rod and sonicated; at this point, the sample was left at room temperature for at least one hour, then sonicated again. Finally, the solvent was evaporated and the residue was redissolved in acetone containing 0.25 mg/mL of n-eicosane (the internal standard) [51].

The instrument used was a Hewlett-Packard 5710A Gas-Liquid Chromatograph fitted with a 30mX0.53mm DB-5ms column (J & W Scientific, Rancho Cordova, California, USA), that had a film thickness of 1.5 mm.

Table 3-1: Results of chemotaxonomic analysis.

Retention Time (min)	Compound
13.71	*N*-Eicosane (Internal standard)
19.02	Cannabidivarin (CBDV)
21.23	Δ9-Tetrahydrocannabidivarin (THCV)
23.44	Cannabidiol (CBD)
23.59	Cannabichromene (CBC)
24.54	Cannabigerol monomethylether (CBGM)
25.46	Δ9-Tetrahydrocannabinol (THC)
26.5	Cannabigerol (CBG)

These conditions allowed a good separation of the cannabinoids.

3.2.2. Gas Chromatography-Flame Ionization detection (GC-FID)

According to the United Nations office on drugs and crime, each 200 mg of dry herbal *C. sativa* must be extracted with 20 mL of internal standard solution, Tribenzylamine (TBA) in ethanol (0.5mg/ml), for 15 minutes in an ultrasonic bath [13].

A capillary column technique was used, with dimensions of 15mX0.25mm, and a film thickness of 0.25 µl. The final temperature suggested is 240°C, and the detector used

was FID [13]. This study states that, since THC degrades rapidly, its quantification can be performed with CBN reference material. The calibration with CBN instead of THC is known and widely accepted. In theory the correlation factor is 1.00 [13].

3.2.3. Gas Chromatography-Mass Spectrometry (GC-MS)

Another gas chromatography technique suggested by the United Nations office on drugs and crime states that it is necessary to add 1 ml of medium-polar or non-polar solvents such as methanol, ethanol, acetonitrile, ethyl acetate, acetone or isooctane to a small portion of sample (e.g. 100 mg of plant material or 1-2 mg of solid material) [52].

The column used was 30mX0.25 mm, with a film thickness of 0.25 µl. Using the mass spectrometer detector, the final temperature reached was 320°C, and the identification was accomplished by comparing the retention time and mass spectrum of the analyte with that of a reference standard [52].

3.3. Liquid chromatography analysis

3.3.1. LC-MC-MS

Lebel et Al. [53] studied 10 samples in two different forms: tablets and herbal products. They were ground, and then aliquotes of 5-10 mg of the powder were transferred to flasks and dissolved in 70:20:10 methanol:water:acetonitrile containing 1% formic acid. The solution was vortexed and the supernatant was filtered through a 0.45 µm pore polytetrafluoroethylene (PTFE) syringe filter [53].

The column used was Accucore aQ column coupled to a 4mmX2.0mm Phenomenex

C18 guard column, but since many of the analyzed compounds have similar structures, therefore the chromatographic separation is hard [53].

3.3.2. HPLC

According to the United Nations office on drugs and crime, dry and homogenized herbal *C. sativa* samples were extracted with 9:1 v/v methanol:chloroform [13].

The column used was 250mmX4mm RP-8, with a film thickness of 5 μm, coupled with a 4mmX4mm precolumn, with the same film thickness. The detection method suggested was photodiode array (220 nm and 240 nm) [13].

Table 3-2: Results of HPLC analysis

Retention Time (min)	Substance
4.9	Cannabidiol (CBD)
6	Cannabinol (CBN)
7.1	Δ9-Tetrahydrocannabinol (THC)
7.4	Δ9-Tetrahydrocannabinol acid (THCA)

3.3.3. Identification by HPLC-MS-MS of cannabinoids in plants

Aizpurua-Olaizola et Al. prepared the samples by pulverizing the plant samples with liquid Nitrogen at 660 rpm during 4 min and stored frozen (–20 °C) until their analysis [45].

The column was a 150mmX3mm Kinetex C18, with a film thickness of 2.6 μm, coupled with a guard column of 0.5 μm depth filter×0.1 mm. They were able to identify six different cannabinoids using pure standards in the *Somango C. sativa* plant. A part from the

known compounds, seven unknown ones were identified with the mass spectra collected in the scan mode and based on the literature [45].

3.4. Other types of analysis

3.4.1. Color test

This method was suggested by the United Nations office on drugs and crime, and this test provides indication of the presence of *C. sativa*-containing material. It is therefore mandatory to confirm the results with a more discriminative technique [13].

3.4.1.1. Fast Corinth V salt test

It is performed on a filter paper. Three reagents must be used; petroleum ether, Fast Corinth V salt (1% v/v in anhydrous sodium sulphate), and sodium bicarbonate (1% v/v aqueous solution). If a purple red stain appears where the sample was placed, *C.sativa* is contained [13].

3.4.1.2. Fast Blue salt test

It is performed on a filter paper. Three reagents must be used; petroleum ether, Fast Blue salt (1% v/v in anhydrous sodium sulphate), and sodium bicarbonate (1% v/v aqueous solution). If a purple red stain appears where the sample was placed, *C.sativa* is contained, in particular THC if the stain is red, CBN if the stain is purple, and CBD if the stain is orange [13].

3.4.1.3. Rapid Duquenois test

It is performed in a test tube. Three reagents must be used; the first one is either Acetaldehyde or Vanillin (A2), then Concentrated Hydrochloric acid, and finally chloroform. If the lower layer (chloroform) becomes purple, than *C. sativa* is contained [13].

3.4.2. Ion Mobility Spectrometry (IMS)

It is a fast technique used to detect trace of cannabinoids under atmospheric pressure conditions. The herbal mixture has to be transferred with a wooden rod onto a Teflon filter, and IMS can operate both in positive and negative ion modes [54].

The United nations office on drugs and crime report the use of a ^{63}Ni beta-emitting source or x-ray tube as the ionization source. Classical cannabinoids give sharp signals in positive ion mode within a characteristic detection window at high drift times. On the other hand, non-classical cannabinoids can be detected with lower sensitivity in negative ion mode within a characteristic detection window [54].

3.4.3. Automated Multiple Development (AMD)

Automated multiple development is a high-performance thin-layer chromatography technique that foresees the performance of multiple runs with decreasing solvent strength and increasing developing distances. Galand et Al. suggest the extraction of hemp samples was extracted with hexane. After filtration, the extract must beevaporated in vacuum and the residue dissolved in toluene [55].

The different Cannabinoids were identified by their hRf and the color of the spots: purple for Δ9-THC, orange red for CBD and violet for CBN; this method confers a better

resolution than TLC, and a higher reproducibility due to its automatism [56].

3.4.4. Thin-Layer Chromatography (TLC)

The United nations office on drugs and crime suggests four different visualizing methods for the detection of cannabinoids [52]:

1. UV light at 354 nm: It is possible to see dark spots against a green background.
2. Freshly prepared Fast Blue RR reagent: Dissolve 0.10 g of Fast Blue RR in 10 ml of distilled water and add 4 ml of 20 % w/v sodium hydroxide solution. Cannabinoids appear as orange-reddish spots.
3. Iodine: Put the dried plate in a TLC chamber with solid iodine crystals. Synthetic cannabinoids appear as yellow to brown spots.
4. Iodoplatinate: Dissolve 5 g of chloroplatinic acid hexahydrate and 35 g of potassium iodide in 1650 ml of distilled water, then add 49.5 ml of concentrated hydrochloric acid. Synthetic cannabinoids appear as green, white or purple spots.

3.4.5. Supercritical Fluid Extraction (SFE)

This technique is used because of the preparative separations issues. The extraction of cannabis is usually done by organic solvent or SFE; firstly, the extract is decarboxylated to convert all cannabinoid acids into their neutral form [57]. SFE foresees the use of supercritical fluids to remove analytes from the matrices. The definition supercritical fluid stands for a substance with temperature and pressure above the thermodynamic critical point, causing the elimination of the interface between the liquid and the vapor phase. This

technique has higher separation efficiency and selectivity, even at low concentrations. On the other hand, this method is expensive and complicated [57].

The extraction is performed at ca. 10°C and 870 psi with a CO_2 mass flow of 1250 kg/h for 8-10 hours. Organic modifiers with a 0-20% v/w concentration have to be used, eg. ethanol, methanol, 2-propanol, diethyl ether, ethyl acetate, chloroform, dichloromethane, carbon tetrachloride, acetonitrile, cyclohexane, acetone, acetic acid, nitromethane, dioxane, n-hexane, n-pentane and pyridine [57].

Chapter 4: Method Development for Qualification and Quantification of Cannabinoids and Terpenes in Plant Extracts using Gas Chromatography-Mass Spectrometry

4.1. Abstract

The two main classes of compounds found in *C. sativa* cultivars are cannabinoids and terpenes; eight types of cannabinoids (THCV, CBD, CBC, Δ8-THC, Δ9-THC, CBG, CBN, and Δ9-THCA), and six types of terpenes (linalool, exo-fenchol, caryophyllene, guaiol, and α-bisabolol) were analysed for the development of this method.

The analysis were performed using a Shimadzu GCMS 8030 (triple quadrupole mass spectrometer) with a Restek Rxi-5ms column (20 m L x 0.18 mm i.d. x 0.18 μm d_f). Silylation was performed using two different silylation agents (BSTFA+1% TMCS and MSTFA), due to the decarboxylation of Δ9-THCA at the high temperatures of the GC-MS. Analyses were performed with two different injection modes: splitless for the low concentration cannabinoids and split (80:1) for the high concentration cannabinoids. Terpenes were studied with a splitless injection method.

4.2. Aim of investigation

Every *C. sativa* plant has different characteristics; the relative amount of each cannabinoid and terpene varies a lot from plant to plant, causing each extract to be more indicate for the treatment of some disease. This approach represents a guideline to the use

of medical *C. sativa*, and a novel, rapid method for the analysis of cannabinoids and terpenes by GC-MS.

4.3. Materials and methods

4.3.1. Standards and reagents

Cannabinoids standards (CBC, THCV, CBD, Δ8-THC, CBG, CBN, Δ9-THC, and Δ9-THCA) were purchased from Cerilliant Corporation, Round Rock, TX, while terpene standards (linalool, exo-fenchol, caryophyllene, terpineol, guaiol, and α-bisabolol) were purchased from Restek Corporation, Bellafonte, PA. To study Δ9-THCA and Δ9-THC, two silylation agents were purchased; BSTFA+1%TMCS (Restek), and MSTFA (Restek). All samples analyzed in this work were laboratory-prepared samples, based on standards dissolved in methanol, to mimic actual natural product extracts.

4.3.2. Instrument parameters

For the analysis of the components of *C. sativa*, a GC-MS 8030 (Shimadzu Scientific Instruments, Inc., Columbia, MD) gas chromatograph - triple quadrupole mass spectrometer was used. It was equipped with a Rxi-5ms column (20 m L x 0.18 mm i.d. x 0.18 μm d_f) (Restek). Different analytical methods were used, including different injection methods for components with different concentration ranges, and different temperature programs between terpenes and cannabinoids.

In the study of cannabinoids, the temperature program started at 40.0 °C for 1 min, was raised to 200.0 °C at 20.0 °C/min, and then raised again to 300 °C at 3 °C/min, where

it was held for 3 min. Two different methods were used, based on the concentration range expected in real samples. A splitless injection method (Figure 4-1a), where 1 µl of sample was injected with a 1 min hold time, was designed for analysis of low concentration cannabinoids (THCV, CBD, CBC, Δ8-THC, CBG, and CBN), while a split injection method 80:1 (Figure 4-1b), with an injection volume of 1µl, was designed detection of high concentration cannabinoids (Δ9-THC, Δ9-THCA, and in some cases, CBD). Both methods were performed with a constant linear velocity of 53.40 cm/sec of helium as carrier gas.

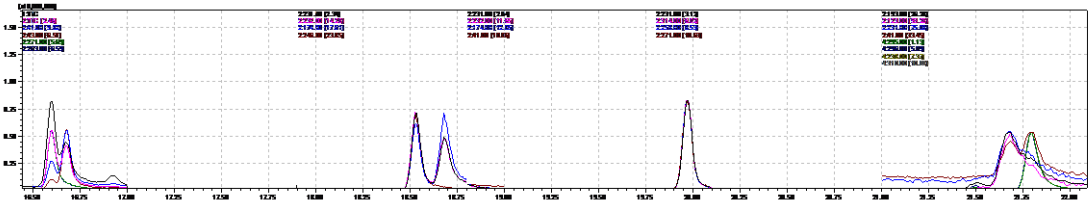


Figure 4-1a:Splitless chromatogram

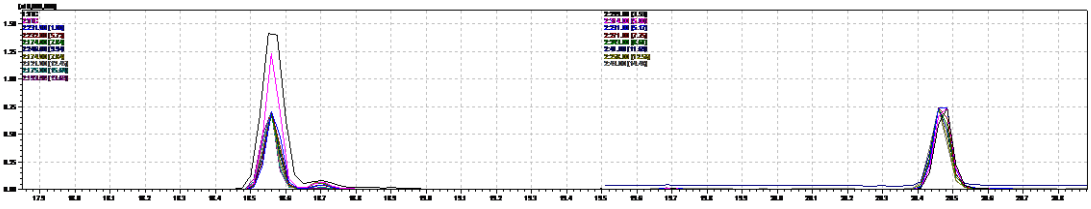


Figure 4-1b: Split chromatogram

For the analysis of terpenes, a splitless injection method was used, with a linear velocity of 54.00 cm/s starting at 35 °C and raising the temperature to 200 °C at 3 °C/min, where it was held for 5 min.

The MS was operated in electron ionization mode with an ion source temperature of 230 ˚C, and an interface temperature of 250 ˚C. It was operated through a SIM mode, and the monitored ions are displayed in Table 4-1:

Table 4-1: Monitored ions.

CANNABINOIDS		TERPENES	
COMPOUND	m/z	COMPOUND	m/z
THCV	41, 43, 271, 203	Linalool	71, 93, 55, 43
CBD	231, 232, 174, 246	Exo-fenchol	81,43, 80, 41
CBC	231, 232, 174, 41	Caryophyllene	93, 133, 91, 41
Δ8-THC	231, 314, 258, 271	Terpineol	59, 93, 121, 136
CBG	193, 123, 231, 41	Guaiol	161, 59, 107, 105
CBN	295, 296, 238, 310	α-bisabolol	93, 109, 41, 43
Δ9-THC	299, 314, 231, 271	-	-
Δ9-THCA-2TMS	487, 73, 365, 147	-	-

4.3.3. Sample preparation

Since the decarboxylation of Δ9-THCA occurs at the high temperatures of the GC-MS, a silylation reaction was needed to stabilize this cannabinoid, and two different silylation agents were used, to compare the efficiency of the reactions. In the first method 100 µL of sample was dried at 50 ˚C with gentle N_2. After the addition of 50 µL of ethyl acetate and 50 µL of BSTFA+1%TMCS, the sample was incubated at 70 ˚C for 30 min, and cooled down before GC-MS analysis. The second method consisted in just drying 50 µL of sample with gentle N_2, and adding 50 µL of MSTFA.

4.3.4. Data analysis

The quantification was performed by creating a calibration curve with the standards of the examined cannabinoids (THCV, CBD, CBC, Δ8-THC, CBG, CBN, Δ9-THC, and Δ9-THCA). Terpenes were also quantified; considering the wide broad of terpenes in the plant extracts, the most abundant terpenes found in *C. sativa* (linalool, exo-fenchol, caryophyllene, α-bisabolene, α-terpineol, and α-bisabolol) were considered.

4.3.5. Method validation

The calibration curves (Table 4-2, and Table 4-3) were built using triplicate analyses of cannabinoid and terpene standards at different concentrations.

Table 4-2: Method validation results for cannabinoids.

	Linearity (R^2)	**LOD (ppm)**	**Prepared Concentration (ppm)**	**Measured Concentration (ppm)***	**Precision (CV %)**	**Percent error in Accuracy (%)**
THCV	0.9965	0.02	0.5	0.68± 0.01	1.47	36.00
			1	0.96± 0.02	2.08	4.00
			2	1.9± 0.1	5.7	3.0
			5	4.9 ± 0.2	4.1	1.2
			8	7.6± 0.3	3.9	4.5
			10	10.3 ± 0.1	1.0	3.2
CBD	0.9926	0.01	0.5	0.56 ± 0.01	1.79	12.00
			1	0.82 ± 0.05	6.10	18.00
			2	1.80 ± 0.03	1.67	10.00
			5	5.4 ± 0.1	2.1	7.2
			8	8.4 ± 0.0	0.0	5.0
			10	9.54 ± 0.02	0.21	5.00
CBC	0.9921	0.01	0.5	0.39 ± 0.02	5.13	22.00
			1	0.89 ± 0.02	2.25	11.00
			2	2.02 ± 0.02	0.99	1.00
			5	5.14 ± 0.07	1.36	8.00
			8	8.56 ± 0.04	0.47	7.00

			10	9.49 ± 0.05	0.53	5.00
Δ8-THC	0.9946	0.02	0.5	0.50± 0.02	4.00	0.00
			1	0.83 ± 0.03	3.6	17.00
			2	1.9 ± 0.2	8.1	8.0
			5	5.4 ± 0.2	3.7	9.0
			8	8.24 ± 0.05	0.61	3.00
			10	9.64 ± 0.03	0.30	4.00
CBG	0.9912	0.009	1	1.770 ± 0.007	0.400	77.000
			2	2.00 ± 0.02	0.80	0.50
			5	5.02 ± 0.06	1.20	4.00
			8	6.9 ± 0.1	1.8	14.0
			10	9.9 ± 0.1	1.4	1.0
			20	20.46 ± 0.09	0.44	2.30
CBN	0.9903	0.01	0.5	0.71 ± 0.02	2.82	42.00
			1	1.20 ± 0.05	4.17	20.00
			2	2.18 ± 0.08	3.67	9.00
			5	5.02 ± 0.04	0.80	4.00
			8	8.910 ± 0.007	0.080	11.000
			10	9.76 ± 0.04	0.36	2.40
CBD SPLIT	0.9989	1.3	1.25	1.5 ± 0.4	23.3	20.0
			2.50	2.9 ± 0.8	26.6	16.0
			5.00	4.9 ± 0.5	9.1	1.4
			10.00	9.8 ± 0.2	2.2	3.0
			20.00	19.2 ± 0.4	1.8	4.0
			40.00	40.4 ± 0.9	2.3	1.0
Δ9-THC	0.995	1.3	1.25	1.9 ± 0.2	10.8	49.0
			2.50	2.8 ± 0.2	7.3	10.0
			5.00	5.5 ± 0.6	10.6	9.0
			10.00	9.2 ± 1.0	11.3	8.0
			20.00	18.1 ± 1.2	1.1	9.7
			40.00	41.0 ± 2.1	5.0	2.0
Δ9-THC-TMS (BSTFA +1% TMCS)	0.9934	0.85	5	15.38±0.04	0.26	208.00
			10	16.93±0.07	0.41	69.00
			20	21.5±0.2	0.8	8.0
			40	33.7±0.1	0.4	16.0
			80	72.0±0.2	0.3	10.0
			160	142.3±0.6	0.4	11.0
			250	259.0±1.2	0.7	4.0
			320	324.1±2.2	0.7	1.0
Δ9-THC-TMS (MSTFA)	0.9916	1.21	5	13.33±0.05	0.38	167.00
			10	14.8±0.2	1.4	48.0
			20	17.68±0.05	0.28	12.00
			40	34.7±0.5	1.6	13.0

			80	77.9±0.8	1.0	3.0
			160	157.1±0.6	0.4	2.0
			250	267.1±0.8	0.3	7.0
			320	318±1	0	1
Δ9-THCA-2TMS (BSTFA +1% TMCS)	0.9912	3.8	10	23.1±0.1	0.5	131.0
			20	29.8±0.1	0.4	49.0
			40	40.3±0.6	1.4	0.8
			80	73.3±0.2	0.3	8.0
			160	141.6±0.1	0.0	12.0
			250	240±1	0	44
			320	310±5	1	3
			400	422±5	1	6
Δ9-THCA-2TMS (MSTFA)	0.9926	6.0	10	21.7±0.2	1.1	117.0
			20	29±1	4	47
			40	36.3±0.1	0.5	9.0
			80	64.1±0.4	0.5	20.0
			160	150.4±0.5	0.4	6.0
			250	250±3	1	0
			320	330±1	0	3
*Mean±SD, n=3						

Table 4-3: Method validation results for terpenes.

	Linearity (R^2)	**LOD (ppm)**	**Prepared Concentration (ppm)**	**Measured Concentration (ppm)***	**Precision (CV %)**	**Percent error in accuracy (%)**
LINALOOL	0.9953	0.010	0.5	0.730 ± 0.007	0.960	46.000
			1	0.770 ± 0.007	0.910	23.000
			2	2.0 ± 0.1	5.4	15.0
			5	5.09 ± 0.06	1.18	18.00
			8	7.60 ± 0.02	0.26	5.00
			10	10.28 ± 0.04	0.39	3.00
EXO-FENCHOL	0.9908	0.27	0.5	0.77 ± 0.02	7.79	54.00
			1	1.6 ± 0.4	25.6	64.0
			2	2.8 ± 0.5	19.2	38.0
			5	5.4 ± 0.5	9.0	8.6
			8	9.2 ± 0.5	5.7	14.5
			10	10.2 ± 0.2	1.8	2.0
CARYOPHYLLENE	0.9964	0.010	0.5	0.780 ± 0.007	0.900	56.000
			1	0.880 ± 0.007	0.800	12.000
			2	2.04 ± 0.04	1.96	20.00
			5	4.70 ± 0.04	0.85	6.00

			8	7.84 ± 0.08	1.02	2.00
			10	10.2 ± 0.1	1.2	8.0
TERPINEOL	0.9925	0.010	0.5	0.800 ± 0.007	0.880	60.000
			1	1.04 ± 0.01	1.15	4.00
			2	1.98 ± 0.05	2.53	1.00
			5	4.48 ± 0.08	1.79	10.00
			8	7.79 ± 0.05	0.06	3.00
			10	10.42 ± 0.03	0.29	4.00
GUAIOL	0.9921	0.009	0.5	0.870 ± 0.007	0.800	74.000
			1	1.00 ± 0.01	1.00	0.00
			2	1.41 ± 0.07	4.96	30.00
			5	5.160 ± 0.007	0.140	3.000
			8	8.25 ± 0.01	0.12	3.00
			10	9.8 ± 0.2	1.9	2.0
α-BISABOLOL	0.9925	0.010	0.5	0.800 ± 0.007	0.880	60.000
			1	1.04 ± 0.01	1.15	4.00
			2	1.98 ± 0.05	2.53	1.00
			5	4.48 ± 0.08	1.79	10.00
			8	7.79 ± 0.05	0.06	3.00
			10	10.42 ± 0.03	0.29	4.00
*Mean±SD, *n*=3						

4.4. Results and discussion

The calibration curves for each cannabinoid and terpene showed a good linearity (R^2> 0.99) and reproducibility, meaning that this method and these conditions are appropriate for the future analysis of *C.sativa* cultivars.

The silylation of Δ9-THCA showed different yield of reaction, as proved by the Δ9-THC residual; if not sylilated, in fact, the carboxyl group of Δ9-THCA wass eliminated, forming Δ9-THC, causing a difference in the amount of Δ9-THC and silylated Δ9-THC. It has to be considered, in fact, that part of the Δ9-THC detected and quantified without silylation may be the sum of the actual cannabinoid and the decarboxylated Δ9-THCA,

therefore a GC-MS analysis of Δ9-THC must always be integrated with the analysis of silylated Δ9-THC and Δ9-THCA.

In most of the standards, the silylation via MSTFA showed a higher concentration for both Δ9-THCA-2TMS, and Δ9-THC-TMS, meaning that MSTFA is a more reactive agent, but BSTFA is more stable. In fact, MSTFA has a higher yield of reaction in the standard, where the cannabinoids were without impurities and other compounds that could degrade the silylation agent.

4.5. Conclusion

This study involved the development of methods to determine the types and different amounts of cannabinoids and terpenes in plant extracts. Characterization of such content would help to establish a guide that can help fully satisfy the needs of patients for different medical indications. This novel method would allow for a rapid ranking and a rapid quantification of the compounds of interest, to characterize extracts of individual strains of *C.sativa,* a critical step when targeting a specific or a series of desired effects.

Chapter 5: Development of a MRM database for Cannabinoids

5.1. Abstract

With the growing importance of *C.sativa* in the medical field, and with the differentiation of cannabinoids due to their therapeutic action, there is a strong need for the development of a methods that enable easy and fast quantification of these compounds in a large number of cultivars. Analyses using a triple quadrupole GC-MS allows the one to use a multiple reaction monitoring (MRM) mode of operation which can increase the sensitivity and specificity for analysis of compounds from complex sample matrices. This mode enhances both sensitivity and selectivity; it works in fact as a double mass filter, resulting in a reduced noise level. Furthermore, the detected signal-to-noise (S/N) is higher, allowing lower limits of quantitation (LOQ). Additionally, it allows a more accurate quantification and a higher reproducibility.

This is the first report about the development of a MRM database for cannabinoids for GC-MS, and it could be the starting point for more sensitive and specific analysis of *C. sativa* cultivars.

5.2. Introduction

The triple quadrupole is perhaps the most common tandem-in-space MS instrument (Figure 5-1). This instrument is composed of three sets of quadrupoles in series, where the first and third quadrupoles (Q1, Q3) are used as mass analyzers, and the second one (Q2) as

a collision cell. The ions of wanted *m/z* (precursor ions) that can therefore pass through Q1, are fragmented in Q2 through the presence of an inert gas (usually N_2 or Ar, called collision-induced dissociation gas (CID gas)) and an acceleration of the ions by applying a "collision energy" voltage. The collisions of accelerated ions with the inert gas creates fragmentations that are specific to the compound; the fragments are finally analyzed in the Q3. In the newer instruments, the second quadrupole is replaced with a more effective collision cell, which can be a hexapole, an octapole, or a transverse wave guide [58].

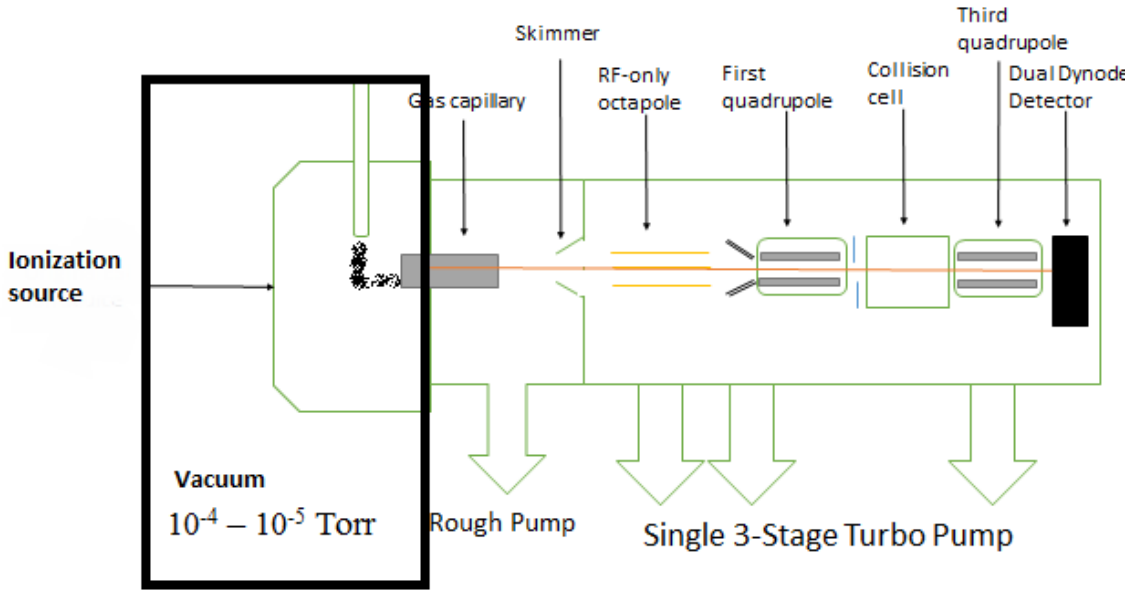


Figure 5-1: Scheme of a Triple Quadrupole Mass Spectrometer.

There are different types of triple quadrupole operation modes, and each one of them has different sensitivity and applications (Table 5-1) [59].

Table 5-1: Types of Mass Spectrometer Modes.

MODE	APPLICATIONS
Full Scan Q1 &Q3	Qualitative
Single Ion Monitoring (SIM)	Selective Quantitative
Full Scan Product Ion	Structural
Precursor Ion	Screening
Neutral Loss	Screening
Selected Reaction Monitoring (SRM), and Multiple Reaction Monitoring (MRM)	Quantitative Target Analysis

The Full Scan MS Q1 & Q3 is used for the qualitative identification of substances. The plot, also called Total Ion Current Plot (TIC), is a plot of the total ion current plotted as an intensity point; as the compound elutes from the column, the relative intensity grows creating a peak plotted against time. Finding the compound of interest in a mixture can be hard, since many peaks are created, and the intact mass is not a unique identifier [60].

The Single Monitoring Scan (SIM) is used for the quantification of a compound, since the molecular ion is monitored in a narrow amu window (typically one mass unit), but all the other analytes can be monitored in scans. SIM gives a better selectivity and a higher sensitivity than full scan modes [57,58].

The product ion scans are divided in two different categories; Full Scan Product Ion, used for obtaining structural information (qualitative), and selected reaction monitoring product ion, used for quantitative target analysis. These scans are also called daughter ion

scans, because Q1 allows only the *m/z*, and the "parent" ion collides with the CID gas (Ar) in Q2 to create the product ions, that are finally scanned through Q3 (Figure 5-2) [58].

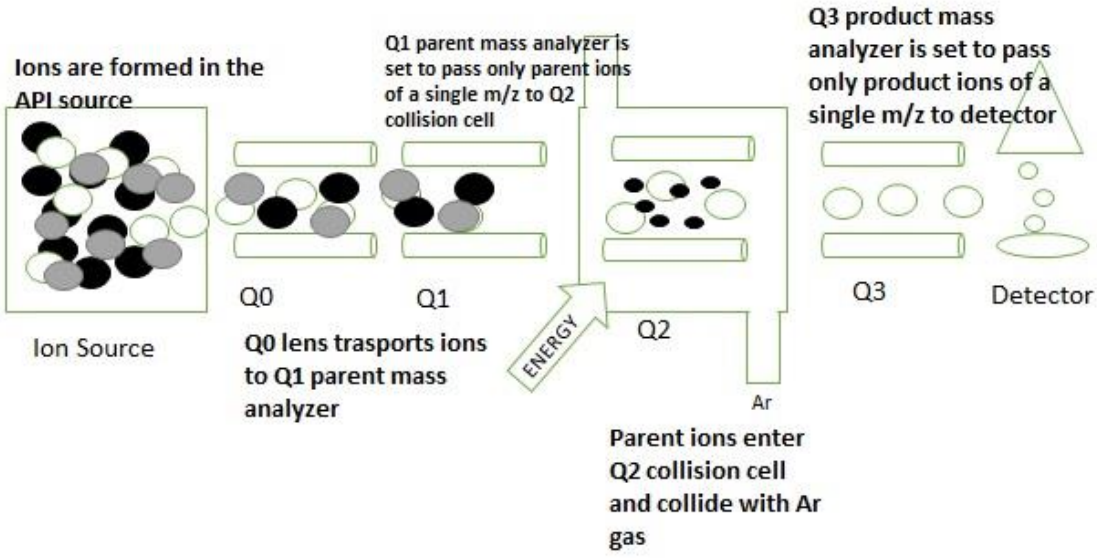


Figure 5-2: Scheme of a Selected Reaction Monitoring

The precursor ion scans, also known as "parent" ion scans, foresee the passage of only a fragment ion of one *m/z*, and Q1 is scanned. The precursor collides with CID gas in Q2, creating the fragment ions, but only the compounds who provide that specific fragment ions are detected. This mode is used when a group of compounds give the same fragment.

The neutral loss scans utilize both Q1 and Q3 scanned together, but Q3 is offset by the neutral loss that is being studied. The precursor collides with CID gas in Q2 creating the fragment ions, and only the compounds that undergo that specific loss are detected. These scans are used for screening experiments to identify classes of compounds that yield the same neutral loss upon fragmentation [58].

Finally, multiple reaction monitoring (MRM) involves two stages of mass filtering; in the first stage, the precursor ion is preselected in Q1 and induced to fragment in Q2 by the CID gas. In the second stage, only a small number of sequence-specific fragment ions that are formed are analyzed in Q3, allowing a lower detection limit, and a rapid and continuous monitoring of the ions of interest [58]. MRM plots are simplified, since they ideally just contain one peak for each targeted analyte [59]. This experiment is known as "transition", and it can be written as "parent mass"> "fragment mass". The main strength points of this technique are that it results is almost a complete removal of the background noise [60], and that it reduces the risk of false positives and false negatives [59] (Figure 5-3).

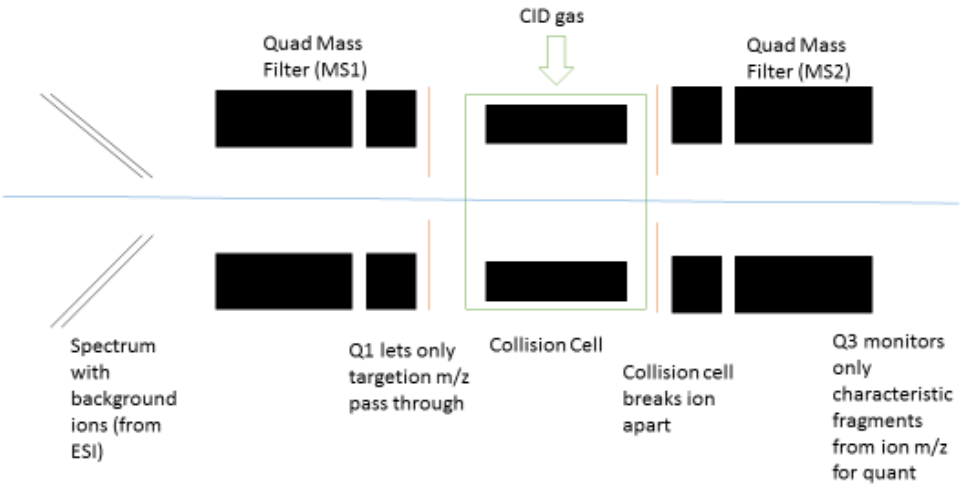


Figure 5-3: Scheme of a Multiple Reaction Monitoring Scan.

The creation of a MRM method requires different points; the first run is important for the retention time and the identification of the most intense ions as the precursor ones,

then the number of transitions per compound has to be defined, both for quantitation and for ion ratio confirmation. The second run is a product ion spectrum for each precursor ion, using the CID gas, and noting the most intense product ion for quantification and ion ratio confirmation. Finally, the collision energies have to be optimized, by starting at low V and increasing it, so that the collision energy of the highest product ion intensity can be used for the method [60].

5.3. Materials and Methods

5.3.1. Standards and reagents

Cannabinoid standards (CBC, CBD, THCV, CBG, CBN, Δ8-THC, Δ9-THC, and Δ9-THCA) were purchased at Cerilliant Corporation, and the derivatization of Δ9-THCA and Δ9-THC, was performed with MSTFA (Restek).

5.3.2. Instrument parameters

For the creation of the MRM database, the GCMS-8030 (Shimadzu Scientific Instruments) gas chromatograph – triple quadrupole mass spectrometer was used, in conjunction with a Rxi-5ms column (20 m L x 0.18 mm i.d. x 0.18 μm d_f) (Restek Corporation).

The method involved a splitless injection of 1 μL, and a temperature program started at 40.0 °C for 1 min, raised to 200.0 °C at 20.0 °C/min, and again to 300 °C at 3 °C/min, where it was held 3 min. The chromatography was performed with a linear velocity of 53.40 cm/sec of helium carrier gas.

5.3.3. Sample preparation

All the standards were prepared and analyzed at a concentration of 5 ppm, to avoid the saturation of the detector. Since the analysis of Δ9-THCA by GCMS has to be performed after the protection of the carboxyl group, to avoid its decarboxylation and therefore its transformation to Δ9-THC, both Δ9-THC and Δ9-THCA were silylated by drying 50 µL of extract with gentle N_2, and adding 50µL of MSTFA.

5.3.4. Data analysis

The procedure used for the creation of the MRM database was supplied by Shimadzu Scientific Instrument technical service experts. The MRM Smart Database Tool was used to create a Product Ion Scan mode batch at different CE, starting from a Scan mode batch. The compound table used for the identification of the ions is represented in Table 5-2.

Table 5.2: Compound Table

Compound	Retention Time	Ions
THCV	16.590	203, 271, 243, 286
CBD	18.520	231, 232, 174, 121
CBC	18.665	231, 174, 232, 175
Δ8-THC	19.965	231, 258, 201, 314
Δ9-THC	20.440	299, 231, 243, 314
CBG	21.705	193, 231, 123, 107
CBN	21.780	295, 296, 238, 223
Δ9-THC-TMS	17.905	371, 315, 386, 73
Δ9-THCA-2TMS	24.000	487, 488, 73, 489

5.3.5. Results

Two cannabinoids databases were created, one for the normal compounds, the other one for the silylated ones. The MRM Optimization tool offered by Shimadzu represents the results on the forms of both graphs (Figure 5-4, Figure 5-5, Figure 5-6, Figure 5-7, Figure 5-8, Figure 5-9, Figure 5-10, Figure 5-11, and Figure 5-12) and tables (Table 5-3, and Table 5-4).

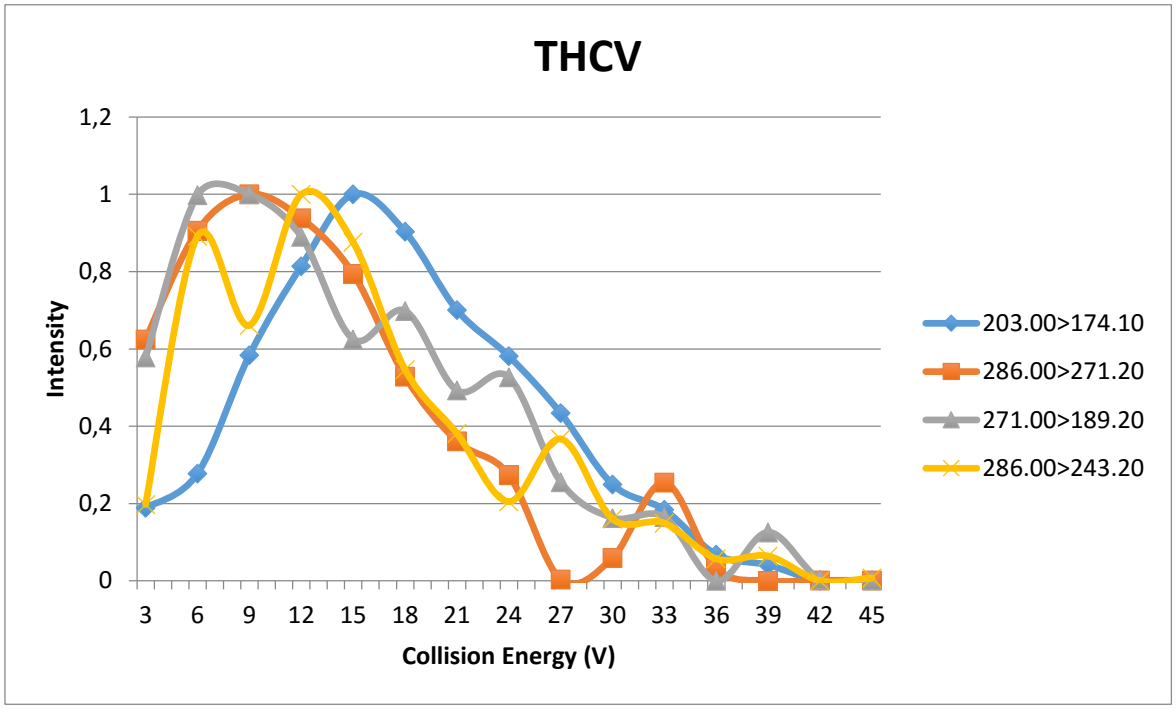


Figure 5-4: THCV optimization

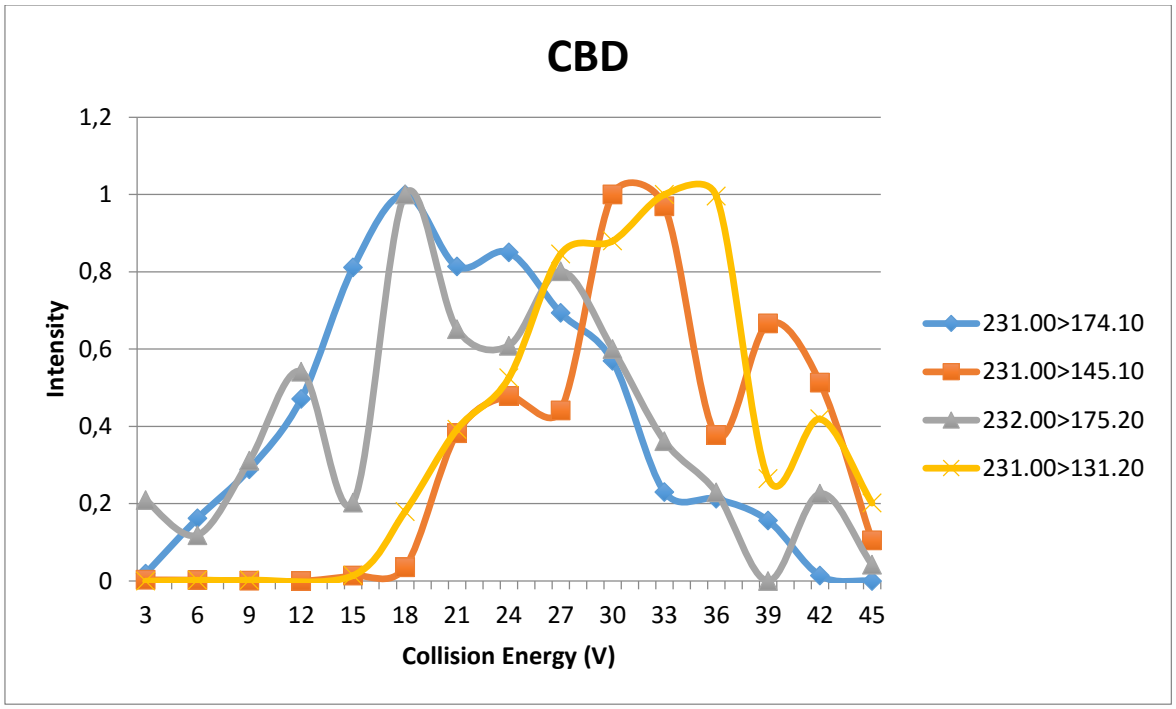


Figure 5-5:CBD Optimization

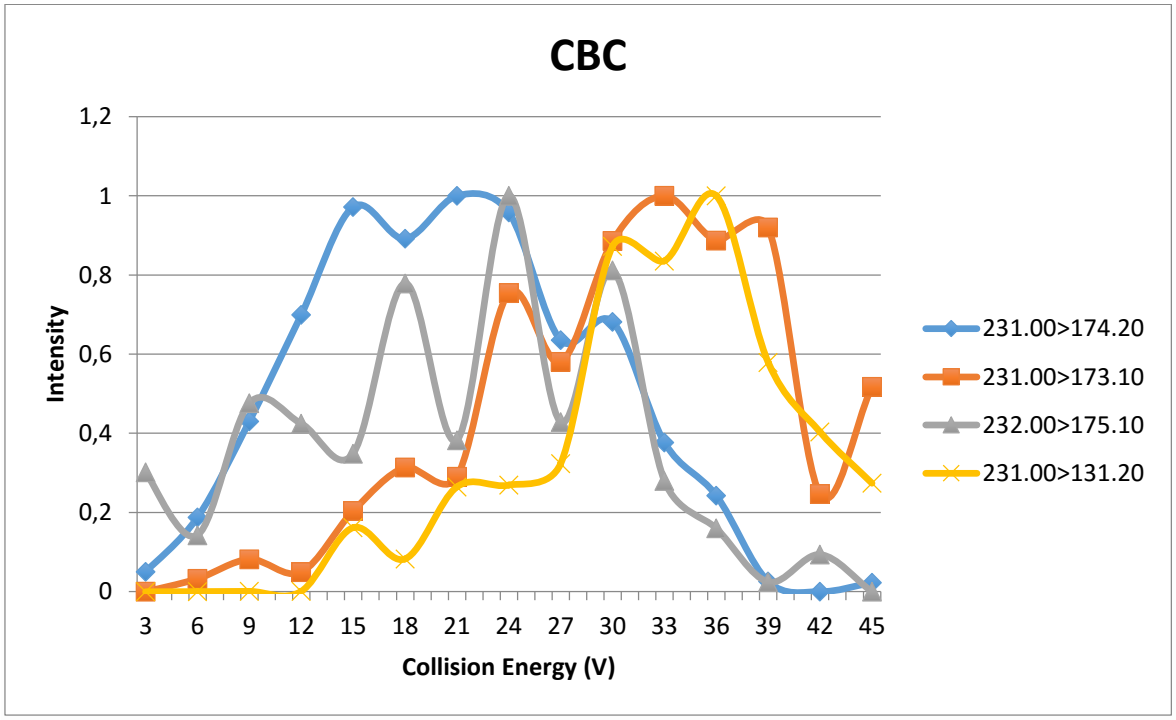


Figure 5-6: CBC Optimization

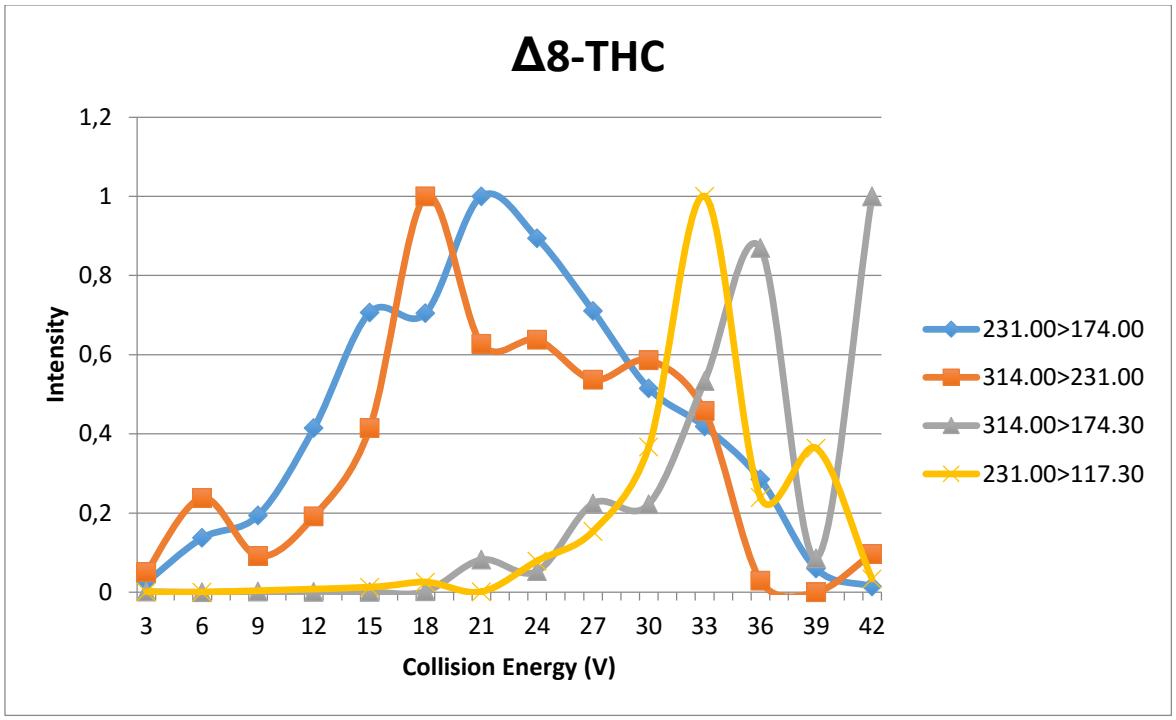


Figure 5-7: Δ8-THC Optimization

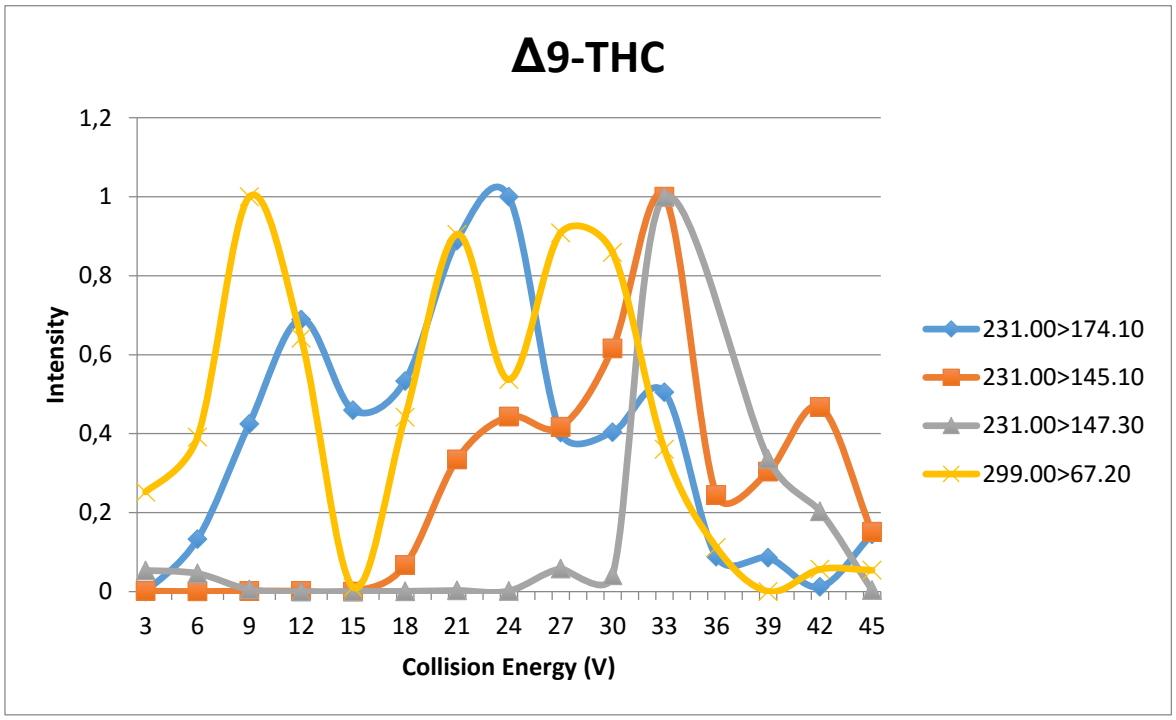


Figure 5-8:Δ9-THC Optimization

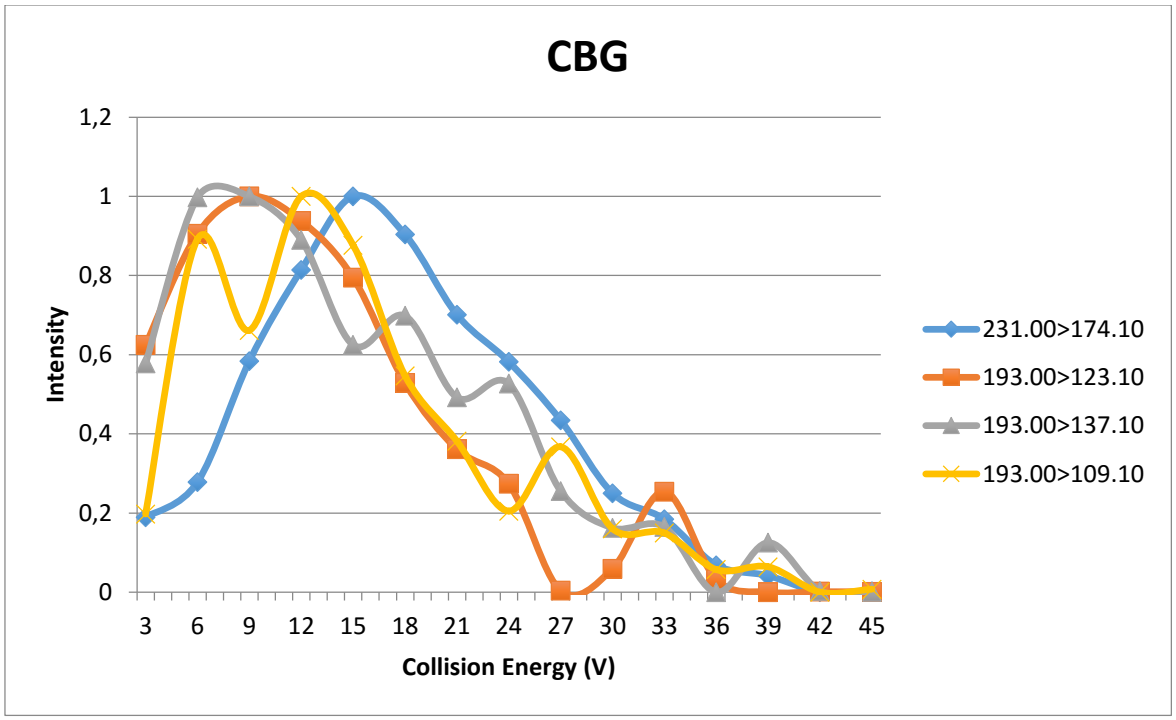


Figure 5-9: CBG Optimization

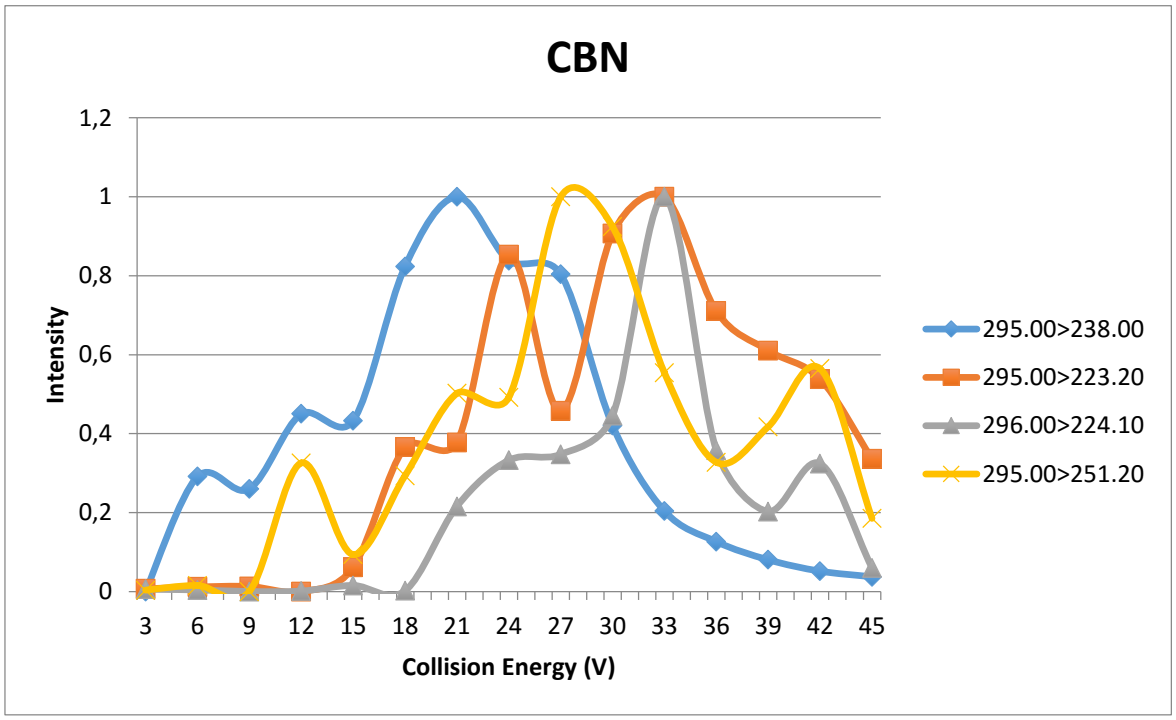


Figure 5-10: CBN Optimization

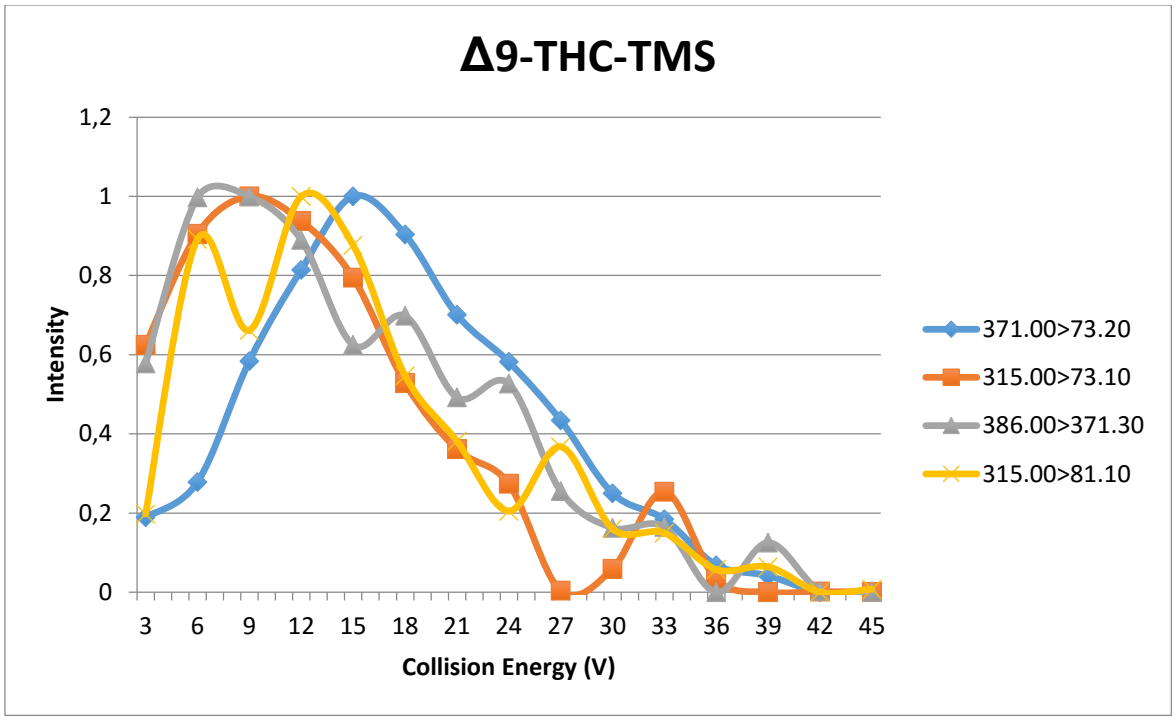


Figure 5-11: Δ9-THC-TMS Optimization

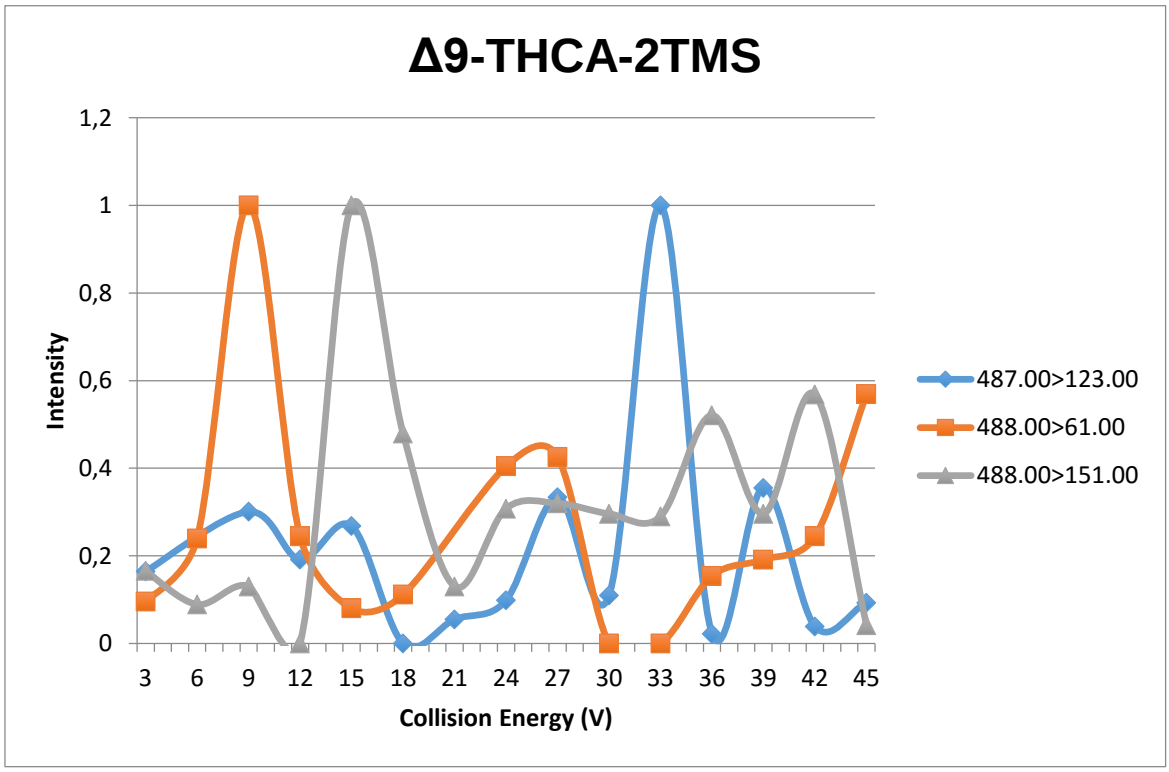


Figure 5-12: Δ9-THCA-2TMS Optimization

Table 5-3: MRM Database for Cannabinoids

Compound Name	Ret. Index	Ion1				Ion2				Ion3				Ion4			
		Type	m/z	CE	Ratio	Type	m/z	CE	Ratio	Type	m/z	CE	Ratio	Type	m/z	CE	Ratio
THCV	16.575	T	203.00> 174.10	15	100.00	Ref.1	286.00> 271.20	9	24.63	Ref.2	271.00> 189.20	9	17.85	Ref.3	286.00> 243.20	12	17.53
CBD	18.506	T	231.00> 174.10	18	100.00	Ref.1	231.00> 145.10	30	13.10	Ref.2	232.00> 175.20	18	11.72	Ref.3	231.00> 131.20	33	11.20
CBC	18.658	T	231.00> 174.20	21	100.00	Ref.1	231.00> 173.10	33	22.35	Ref.2	232.00> 175.10	24	16.07	Ref.3	231.00> 131.20	36	15.15
8THC	19.955	T	231.00> 174.00	21	100.00	Ref.1	314.00> 231.00	18	20.64	Ref.2	314.00> 174.30	42	18.56	Ref.3	231.00> 117.30	33	15.36
9THC	20.432	T	231.00> 174.10	24	100.00	Ref.1	231.00> 145.10	33	30.84	Ref.2	231.00> 147.30	33	28.63	Ref.3	299.00> 67.20	9	26.12
CBG	21.696	T	231.00> 174.10	15	100.00	Ref.1	193.00> 123.10	12	97.49	Ref.2	193.00> 137.10	12	48.33	Ref.3	193.00> 109.10	15	32.79
CBN	21.772	T	295.00> 238.00	21	100.00	Ref.1	295.00> 223.20	33	51.80	Ref.2	296.00> 224.10	33	16.64	Ref.3	295.00> 251.20	27	15.71

Figure 5-4: MRM Database for Silylated Cannabinoids

Compound Name	Ret. Index	Ion1				Ion2				Ion3				Ion4			
		Type	m/z	CE	Ratio	Type	m/z	CE	Ratio	Type	m/z	CE	Ratio	Type	m/z	CE	Ratio
9THC-TMS	17.883	T	371.00> 73.20	30	100.00	Ref.1	315.00> 73.10	27	84.18	Ref.2	386.00> 371.30	15	81.43	Ref.3	315.00> 81.10	15	75.89
9-THCA-2TMS	24	T	487.00> 123.00	33	100.00	Ref.1	488.00> 61.00	9	97.93	Ref.2	488.00> 151.00	15	90.67	Ref.3	487.00> 338.00	9	84.46

5.4. Conclusions

The Shimadzu, Inc. MRM optimization tool allowed a fast and simple creation of a cannabinoids MRM database. Both the CEs and the transitions for the Target Ions and the Reference Ions were optimized for these conditions on the GC-MS; this database is going to be useful for future characterization of cannabinoids on triple quadrupole GCMS, allowing a better sensitivity and a greater linearity.

The formation of the *m/z*=231 is caused by a Retro Diels-Alder reaction followed by the loss of a geminal methyl group, while the *m/z*= 314 is due to the elimination of an electron. The fragment *m/z*=295 and *m/z*=299 are caused by the loss of a germinal methyl group that is common in almost all cannabinoids, and the fragment *m/z*=271 in THCV is given due to a ring opening followed by a possible hydrogen transfer or ring reclosure [78]. The fragment *m/z*= 488.00 in Δ9-THCA-2TMS corresponds to the loss of a methyl in the $COOSi(CH_3)_3$ on the ring [61].

Chapter 6: Summary and Future Work

The study and characterization of a drug is a mandatory step for its approval on the market, and this is no different for *C. sativa*. With this study, we developed a method for the fast analysis of cannabinoids and terpenes, allowing an easy ranking and quantification of the compounds of interest. The need to run these experiments is given by the hypothesis that different cultivars of different strains of *C. sativa* have different concentrations of cannabinoids and terpenes. These differences would lead to more specific uses of *C. sativa* in the medical field, but all these hypothesis have to be tested and proved on actual samples.

The first step that has to be done is getting the Drug Enforcement Agency (DEA) approval for performing these analysis, in order to proceed to testing of the drug. After getting the approval, the cultivars should be run with the described conditions to perform an easy quantification and qualification.

Other parameters should be taken in consideration; are there two components that allow all the different cultivars to cluster? Finding these variables would simplify the characterization, because we would be able to carry out a Principle Component Analysis (PCA). This graph would make different strains group and show their cannabinoid ranges, creating an easy way to quantify the extract by just knowing its strain.

Furthermore, performing a test on the thermal stability would be critical for the approval of medical *C. sativa*. It is important to show that cannabinoids do not deteriorate,

because their degradation would lead to the loss of their medical benefits and therefore to the inactivation of the drug. It is in fact mandatory to prove that the cannabinoids do not degrade at room temperature over a long period of time, to allow a correct and preservative storage of the drug.

The last examination that should be done on these cultivars is the detection of pesticides; considering the medical application, it is ideal for the extracts not to have any pesticide, but it is mandatory to have a lower concentration of these compounds than the levels approved by the Law.

All of these studies are important points for the better understanding of *C. sativa* and its components, and luckily enough they will allow us to fully understand the nature of this plant, that has been employed from human beings since the early 29,000 BC.

References

[1]: Hanusˇ L. Personal observations, 2006

[2]: Klı´ma B, Dolnı´ Veˇstonice. Praha: Nakladatelstvı´ Cˇ eskoslovenske´ akademie veˇd; 1963.

[3]: Li H-L. 1974; An archeological and historical account of Cannabis in China. Econ Bot 1974;28:437– 448.

[4]: Unschuld PU. Medicine in China: A history of pharmaceutics. Berkeley: University of California Press; 1986. p. 367

[5]: Campbell Thompson R. A Dictionary of Assyrian Botany. London: The British Academy; 1949. p. 405

[6]: The papyrus Ebers. The greatest Egyptian Medical document. Translated by Bendix Ebbell. Copenhagen: Levin & Munksgaard; 1937, 135 pages.

[7]: Sacred Books of the East. Translated by Maurice Bloomfield. Volume 42, Hymns of the Atharva-Veda. Oxford: The Clarendon Press; 1897, 792 pages.

[8]: The Vendidad (The Zend-Avesta, part I, Fargard 15, II, 14(43)) (1880) translated by James Darmesteter, Sacred books of the East, volume 4., Oxford University Press; 1880.

[9]: Pliny the Elder. The natural history. London: Taylor and Francis; 1855.

[10]: Dioscorides Pedanius. De Materia Medica. Johannesburg: IBIDIS Press; 2000. p. 836.

[11]: Green J. Cannabis. New York: Thunder's Mouth Press; 2002. p. 256.

[12]. Optimisation and characterisation of marihuana extracts obtained by supercritical fluid extraction and focused ultrasound extraction and retention time locking GC-MS. Jone Omar,

Maitane Olivares, Mikel Alzaga, Nestor Etxebarria. J. Sep. Sci. 2013, 36, 1397–1404 1397

[13]. Recommended Methods for the Identification and Analysis of *C. sativa* and *C. sativa* Products. Laboratory and Scientific Section United Nations office on drugs and crime. New York 2009

[14]. Chapter 2: Chemistry and Analysis of Phytocannabinoids and Other *C. sativa* Constituents. Rudolf Brenneisen

[15]: Mechoulam, R., Gaoni, Y., 1967a. Recent advances in the chemistry of hashish. Fortschritte der Chemie Organischer Naturstoffe 25, 175– 213.

[16]: Mechoulam, R., Gaoni, Y., 1965. Hashish. IV. Isolation and structure of cannabinolic, cannabidiolic, and cannabigerolic acids. Tetrahedron 21 (5), 1223–1229.

[17]: Wood, T.B., Spivey, W.T., Easterfield, T.H., 1896. XL-Charas. The resin of Indian hemp. Journal of the Chemical Society 69, 539.

[18]: Cahn RS. Cannabis indica resin, Part III. The constitution of Cannabinol. J Chem Soc 1932; 1342–1353

[19]: Adams R, Pease DC, Cain CK, Clark JH. Structure of cannabinol. VI. Isomerization of cannabidiol to tetrahydrocannabinol, a physiologically active product. Conversion of cannabidiol to cannabinol. J Am Chem Soc 1940;62:2402–2405.

[20]: Simonsen JL, Todd AR. Cannabis indica. X. The essential oil from Egyptian hashish. J Chem Soc 1942; 188–191.

[21]: Krejcˇ´ı Z, Sˇantavy´ F. Isolace dalsˇ´ıch la´tek z listı´ indicke´ho konopı´ Cannabis sativa L. Acta Univ Palacki Olomuc 1955;6:59–66.

[22]: Gaoni Y, Mechoulam R. The structure and synthesis of cannabigerol, a new hashish constituent. Proc Chem Soc, March 1964; 82. 62.

[23]: Shani A, Mechoulam R. Cannabielsoic acids. Isolation and synthesis by a novel oxidative cyclization. Tetrahedron 1974;30:2437–2446

[24]. The Volatile Oil Composition of Fresh and Air-Dried Buds of *C. sativa* sativa. Samir A. Rossi and Mahmoud A. El Sohly. J. Nut. Prod. 1996, 59, 49-51 49.

[25]. Chemistry and Analysis of Phytocannabinoids and Other *C. sativa* Constituents. Rudolf Brenneisen. Forensic Science and Medicine: Marijuana and the Cannabinoid.

[26]. *C. sativa* and Natural *C. sativa* Medicines. Robert C. Clarke and David P. Watson. Forensic Science and Medicine: Marijuana and the Cannabinoid.

[27]: Fragrance compounds and essential oils with sedative effects upon inhalation. G. Buchbauer et al. Journal of Pharmacological Sciences 82, 1993.

[28]: Effects of linalool on glutamatergic system in the rat cerebral cortex. E. Elisabetsky et al. Neurochemical Research 20, 1995.

[29]: Gastric cytoprotection of the non-steroidal anti-inflammatory sesquiterpene, beta-caryophyllene . Y. Tambe et al. Planta Med 62, 1996.

[30]: Composition and anti-malarial activity in vitro of essential oil of Tetradenia riparia. W.E. Campbell et al. Planta Med 63, 1997

[31]. The Therapeutic Potential of Cannabis. Baker, Pryce, Giovannoni, J.Thompson. Lancet Neurology 2:291.298. 2013

[32]: Devane WA, Dysarz FA III, Johnson MR, Melvin LS, Howlett AC. Determination and characterization of a cannabinoid receptor in rat brain. Mol Pharmacol 1988;34:605–613.

[33]: Shire D, Calandra B, Rinaldi-Carmona M, Oustric D, Pessegue B, Bonnin-Cabanne O, Le Fur G, Caput D, Ferrara P. Molecular cloning, expression and function of the murine CB2 peripheral cannabinoid receptor. Biochim Biophys Acta 1996;1307:132–136

[34]. Cannabinoids in health and disease. Natalya M. Kogan, and Raphael Mechoulam. Dialogues Clin Neurosci. 2007 Dec; 9(4): 413–430.

[35]: Synthetic and plant-derived cannabinoid receptor antagonists show hypophagic properties in fasted and non-fasted mice. G. Riedel et al. British Journal of Pharmacology 156, 2009.

[36]: Cannabis Pharmacy. M. Backes, 2014.

[37]: Cannabinoids and cancer: pros and cons of an antitumor strategy. M. BiFulco et al. British Journal of Pharmacology 148, 2006.

[38]: The Therapeutic Potential of *C. sativa* and Cannabinoids. Franjo Grotenhermen, Kirsten Müller-Vahl, Deutsches Ärzteblatt. Dtsch Arztebl Int 2012; 109(29–30): 495–501.

[39]: Structure-Activity Relationships of the Cannabinoids. Rao S. Rapaka, Alexandros Makriyannis.

[40]: Basic Gas Chromatography-Mass Spectrometry: Principles and Techniques. Di F.W. Karasek,R.E. Clement.

[41]: Instruments and Techniques. A.M.Siouffi and C.Delaurent. Handbook of food analysis, 1996, 1907-1987.

[42]: Choosing the right column, Agilent.

[43]. A.E. Messner, D. M. Rose, and P.A. Argabright. Anal. Chem, 31:230. 1959.

[44]: D.R. Deans. Chromatographia, 1:8. 1968.

[45]: Identification and quantification of cannabinoids in *C. sativa sativa* L. plants by high performance liquid chromatography-mass spectrometry. Oier Aizpurua-Olaizola, Jone Omar, Patricia Navarro, Maitane Olivares, Nestor Etxebarria, Aresatz Usobiaga. Anal Bioanal Chem.

[46]: Separation and identification of *C. sativa* components by different planar chromatography techniques (TLC, AMD, OPLC). N. Galand, D.Ernouf, F. Montigny, J. Dollet and J. Pothier.

[47]: The Electron-capture detector and green Politics. J.E. Lovelock. LC-GC, 8:11.

[48]: Applications of Stable, Nonradioactive Isotope Tracers in In vivo Human Metabolic Research. Kim, Suh, Lee, Wolf. Experimental and Molecular Medicine, 48(1).2016

[49]: An introduction to mass spectrometry based proteomics—Detection and characterization of gonadotropins and related molecules. Kickman, Parkin, Iles. Molecular and Cellular Endocrinology, 260-262:212-27. 2007

[50]: Silylating Agents Derivatization Reagents Protecting-Group Reagents Organosilicon Compounds Analytical Applications Synthetic Applications. Dr. Gert van Look. 1988.

[51]: A chemotaxonomic analysis of cannabinoid variation in *C. sativa (Cannabaceae).* Karl W. Hillig, and Paul G. Mahlberg. *American J*ournal of Botany 91(6): 966–975. 2004.

[52]: Recommended methods for the Identification and Analysis of Synthetic Cannabinoid Receptor Agonists in Seized Materials. Laboratory and Scientific Section United nations office on drugs and crime. New York, 2008.

[53]: Rapid Determination of 24 Synthetic and Natural Cannabinoids for LC–MS-MS Screening in Natural Products and Drug Inspection Applications. Philippe Lebel, Karen C. Waldron, Alexandra Furtos. LCGC, North America Issue 1, Mar 2015.

[54]: Naturally Occurring and Related Synthetic Cannabinoids and their Potential Therapeutic Applications. Ahmed M. Galal, Desmond Slade, Waseem Gul, Abir T. El-Alfy, Daneel Ferreira and Mahmoud A. Elsohly. Recent Patents on CNS Drug Discovery, 2009, 4, 112-136, © 2009 Bentham Science Publishers Ltd.

[55]. Mass Spectrometry of Natural Compounds. MASONACO.

[56]. Introduction to MS quantitation ad Modes of LC/MS Monitoring. Mass Spectrometry Quantitation.

[57]. Mass Spectrometer Scan Types. ThermoFinnigan.

[58]. MRM (Multiple Reaction Monitoring). Broad Institute of MIT and Harvard.

[59]. Introduction to GC-MS Coupling. Stéphane Bouchonnet. 2013.

[60]. Handbook of GC-MS: Fundamentals and Applications. Hans-Joachim Hübschmann. 2015.

[61]: Mass Spectrometry of Cannabinoids. T.B. Vree. Journal of pharmaceutical science. 1977.