NEW METHODS FOR DISCOVERY, FINGERPRINTING, AND ANALYSIS OF CANNABIS SATIVA NATURAL PRODUCTS

by

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Dedication

In 25 years, you end up with a long list of people you would like to thank for all your biggest accomplishments, and I could easily say that this is by far the biggest one I have ever achieved. It sounds cliché, and probably this is the reason why it is so true, but I could have never reached this point without the help of each single person that was behind the curtains pushing me to go forward and not give up. Or, in an even more cheesy way, telling me to fall down seven times and stand up eight.

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Success is the goal ahead and the team behind you.

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Abstract

NEW METHODS FOR DISCOVERY, FINGERPRINTING, AND ANALYSIS OF CANNABIS SATIVA NATURAL PRODUCTS

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Cannabis sativa is an herbaceous annual that has always been of great interest due to its uses in the manufacturing of textiles and cordage, as well as for its medicinal properties. The two main bioactive components of *C. sativa* are cannabinoids and terpenes; the former confer the medicinal and pharmaceutical benefits, as well as the psycho-activity, to the plant, while the latter are largely responsible for aroma and flavor. Because of the importance of both sets of analytes, it is crucial to have a comprehensive understanding and characterization of these compounds, and different methods have been developed to address these different objectives.

Two methods were developed for the analysis of cannabinoids using gas chromatography (GC), coupled either with mass spectrometry (MS), or with the vacuum ultraviolet detector (VUV). The first method uses tandem mass spectrometry (MS/MS) for the analysis of cannabinoids, which may exist as trace components in *C. sativa* products. That methodology may also be used as a tool for the discovery of new species, due the combination of high sensitivity and the availability of common fragmentation pathways. Multiple reaction monitoring (MRM) mode, which allows the selection of a precursor and a

product ion for each transition, was used to attain the highest specificity and sensitivity possible for the method. A greater sensitivity is achieved even if the signal of each peak is lower than in scan mode, because there is almost no background noise detected in MRM mode.

The second methodology developed featured the vacuum-ultraviolet detector (VUV) for the quantification of the eight most abundant cannabinoids, which can be found in different specimens. For both MS and VUV detection, relatively fast separations were achieved, but samples needed to be derivatized prior to analysis to avoid the degradation of Δ9-tetrahydrocannabinolic acid (Δ9-THCA) into Δ9-tetrahydrocannabinol (Δ9-THC); other cannabinoid acids would have a similar fate. These two instruments were also used to develop methods for the analysis of Δ9-THC metabolites in urine and plasma. Metabolite analysis is important because cannabinoids are broken down into other molecules inside our body, and such molecules can also have unique physiological effects.

Terpenes were also analyzed by GC-VUV, using a headspace extraction method featuring a hydrophilic room temperature ionic liquid (RTIL) co-solvent. This new technique was created and validated using hops, due to their similar matrix to *C. sativa,* and it has shown to be more efficient that normal solvent extraction. The main advantages are given by the elimination of matrix components of the plant, giving a cleaner and easier to understand chromatogram.

Finally, a comparison among the different fragmentation pathways that cannabinoids undergo in the MS was drawn between different ionization sources (electron ionization (EI), electrospray ionization (ESI), and atmospheric pressure chemical ionization (APCI)) and in subsequent tandem mass spectrometry fragmentation experiments. Similar or identical pathways were highlighted for different cannabinoids with different sources.

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Chapter 1

A REVIEW OF METHODS FOR CHEMICAL CHARACTERIZATION OF CANNABIS NATURAL PRODUCTS[1]

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1.1 Abstract

Cannabis has garnered a great deal of new attention in the past couple of years in the United States due to the increasing instances of its legalization for recreational use and indications for medicinal benefit. Despite a growing number of laboratories focused on cannabis analysis, the separation science literature pertaining to the determination of cannabis natural products is still in its infancy despite the plant having been utilized by humans for nearly 30,000 years and it being now the most widely used drug world-wide. This is largely attributable to the restrictions associated with cannabis as it is characterized as a Schedule 1 drug in the United States. Presented here are reviewed analytical methods for the determination of cannabinoids (primarily) and terpenes (secondarily), the primary natural products of interest in cannabis plants. Focus is placed foremost on analyses from plant extracts and the various instrumentation and techniques that are used, but some coverage is also given to analysis of cannabinoid metabolites found in biological fluids. The goal of this work is to provide a collection of relevant separation science information, upon which the field of cannabis analysis can continue to grow.

Abbreviations: **Δ9-THC**, Δ9-Tetrahydrocannabinol; **Δ9-THCA**, Δ9-Tetrahydrocannabinolic acid; **Δ9-THC-OH**, 1-hydroxy-Δ9-tetrahydrocannabinol; **11-nor-9-carboxy- Δ9-THC,** 11-nor-9-carboxy-Δ9-tetrahydrocannabinol; **AAS,** atomic absorption spectroscopy; **AES,** atomic emission spectroscopy; **AMD,** automated multiple development; **APCI,** atmospheric pressure chemical ionization; **BSTFA+1%TMCS**, *N*,*O*-Bis(trimethylsilyl)trifluoroacetamide; **CBC**,cannabichromene; **CBD**, cannabidiol; **CBDV**, cannabidivarin; **CBE**, cannabielsoin; **CBG**, cannabigerol; **CBL**, cannabicyclol; **CBN**, cannabinol; **CBT**, cannabitriol; **CE**, collision energy; **CO_2,** carbon dioxide; **CV**, coefficient of variation; **DEA**, drug enforment agency; **ECD,** electron capture detector; **EI**, electron

impact; **FID,** flame ionization detector; **FPD,** flame photometric detector; **FUSE,** focused ultrasound extraction; **HS,** headspace; **ICP-MS,** inductively-coupled plasma-mass spectroscopy; **IMS,** ion mobility spectroscopy, **LLE,** liquid-liquid extraction; **MeOH**, methanol; **MIC,** minimum inhibitory concentration, **MRM**, multiple reaction monitoring; **NPD,** nitrogen-phosphorus detector; **OPLC,** optimum performance layer chromatography; **Q-TOF,** quadrupole-time-of-flight; **QC,** quality control; **QQQ,** triple quadrupole; **QuEChERS,** quick easy cheap effective rugged and safe; **SFC,** supercritical fluid chromatography; **SFE,** supercritical fluid extraction; **SIM**, single ion monitoring; **THCV**, tetrahydrocannabivarin; **TMS**, trimethylsilyl; **TQ**, triple quadrupole; **VUV,** vacuum-ultraviolet detector; **XRF,** X-ray fluorescence.

Keywords: cannabinoids, cannabis sativa, cannabis indica, natural products, terpenes

1.2 Introduction

The probable origin of cannabis is the Altai mountains of southern Siberia, Russia, considering that this wild plant is common in that region [1]. *C. sativa* and *C. indica* have been widely used in the past both in the making of textiles and basketries, and in the medical/pharmacological field. The oldest evidence of the use of these plants dates back to the Gravettian settlements in Eastern Europe, 29000 years ago [2]. The first medical evidence dates back to 5000 years ago in China, with the emperor Chen Nung, who is believed to be the discoverer of medicinal plants [3]. In his Shen-nung pen ts'ao ching (Divine Husbandman's Materia Medica) he explains that the female plant of *C. sativa* could be used against menstrual fatigue, rheumatism, malaria, constipation, and other maladies [3]. This plant was also used as a medicine by the Assyrians (3000-2000 BC) [4], the

Egyptians (ca. 1500 BC) [5], the Indians [6], the Persians [7], the Greeks [8], and the Romans [9]. *C. sativa* was introduced to the modern Western society by French physician Jacques Joseph Moreau, who in the 1830s hypothesized the plant could be used to treat mentally ill patients [10]. However, a decline of the plant's usage started in the 1880s, with the publication of the so-called "Reefer Madness articles" [11]. In 1937, the Marijuana Tax Act was introduced, resulting in an extra $1/ounce tax on all cannabis medical products [12]. The plant and its medical use was definitively banned in 1970 [13]. Even so, it is currently reported to be the most widely used drug worldwide, with 3.6% of the population aged between 15 and 60 years old reported as users [14].

C. sativa and *C. indica* are herbaceous annuals that belong to the *Cannabaceae* family and can grow up to 8-12 feet tall. Their flowers, which contain the highest percentage of the biologically active components, blossom from late summer to mid-fall [15]. The female flowers are extremely leafy, unbranched, and live for 3-5 weeks after blossoming. The male plant flowers are branched, have fewer leaves, and die right after blossoming [15].

The aim of this review is to provide a comprehensive and detailed analysis of the different techniques used for the characterization of cannabis natural products. An overview of the primary classes of compounds of interest, cannabinoids and terpenes, is presented first. Next, a discussion of different means for analyzing cannabinoids is given. Gas chromatography (GC), liquid chromatography (LC), and mass spectrometry (MS) feature prominently in this discussion, but there are a variety of other techniques ranging from thin-layer chromatography (TLC) to new spectroscopic techniques, which add to the picture. The analysis of Δ9-THC metabolites from biological fluids is also covered. A shorter review of relevant analytical methods for determination of terpenes is also presented, but less coverage is given due to extensive prior published research on this

topic, as these aromatic and highly volatile molecules are also natural products of countless other plants. Some comment is also given to routine testing of cannabis plants for pesticides, metals, and microbes. This review serves as a collection of literature to date in a rapidly expanding and exciting field, which intersects forensic, medicinal, clinical, pharmaceutical, and agricultural chemistry. Highly specific and reliable analytical methods will be central to future development of cannabis science.

1.3 Natural Products of Primary Interest

1.3.1 Cannabinoids

To date, more than 500 different chemical components have been reportedly found in cannabis, 100 of which were identified as cannabinoids, the main bioactive principles of the plant. Gaoni and Mechoulam [17] were the first ones to define these terpenophenolic compounds "as the group of C_{21} compounds typical of and present in *Cannabis sativa,* their carboxylic acids, analogs, and transformation products". Currently, 10 different types of cannabinoids have been classified according to general structural variants [18]. Cannabigerol (CBG) was the first one to be isolated in 1964 by Gaoni and Mechoulam; it was shown to exhibit antibacterial activity against Gram-positive bacteria [17]. Currently, 7 types of CBG have been identified, varying based on the length of their carbon side chain [18]. Cannabichromene (CBC) was discovered almost simultaneously by Claussen et al. [19] and by Gaoni and Mechoulam [20], with 5 known different types. Cannabidiol (CBD) was discovered by Adams et al. [21], and it exists in 7 different types; this cannabinoid has the highest number of medical benefits, which vary between anti-epileptic [22], antipsychotic [23], antianxiolytic [24], and other biological activities. Δ9-tetrahydrocannabinol (Δ9-THC), the main psychoactive cannabinoid, was discovered by Gaoni and Mechoulam in 1971 [20], and 9 different types exist, including its acid precursor

(Δ9-tetrahydrocannabinolic acid, Δ9-THCA) [18]. Δ8-tetrahydrocannabinol (Δ8-THC) is a more stable isomer of Δ9-THC, but Δ8-THC is characterized as being 20% less active than Δ9-THC [18]. Cannabicyclol (CBL) was isolated in 1967 [17], and 3 different types exist, all having no reported bioactivity. Cannabielsoin (CBE) was isolated in 1983 [25], and its 5 different forms also lack known bioactivity to this point. Cannabinol (CBN) is the oxidation artifact of Δ9-THC, isolated by Wood in 1899 [26], and 7 different types have been identified. The propylated forms of THC and CBD (THCV and CBDV) are effective thermogenics and anti-convulsants, respectively. Cannabinodiol (CBDN) was discovered in 1972, and is the oxidation artifact of CBD [27]. Cannabitriol (CBT) was identified by Obata and Ishikawa in 1966 [28]. Figure 1.1 depicts representative structures of each of the different cannabinoid classes.

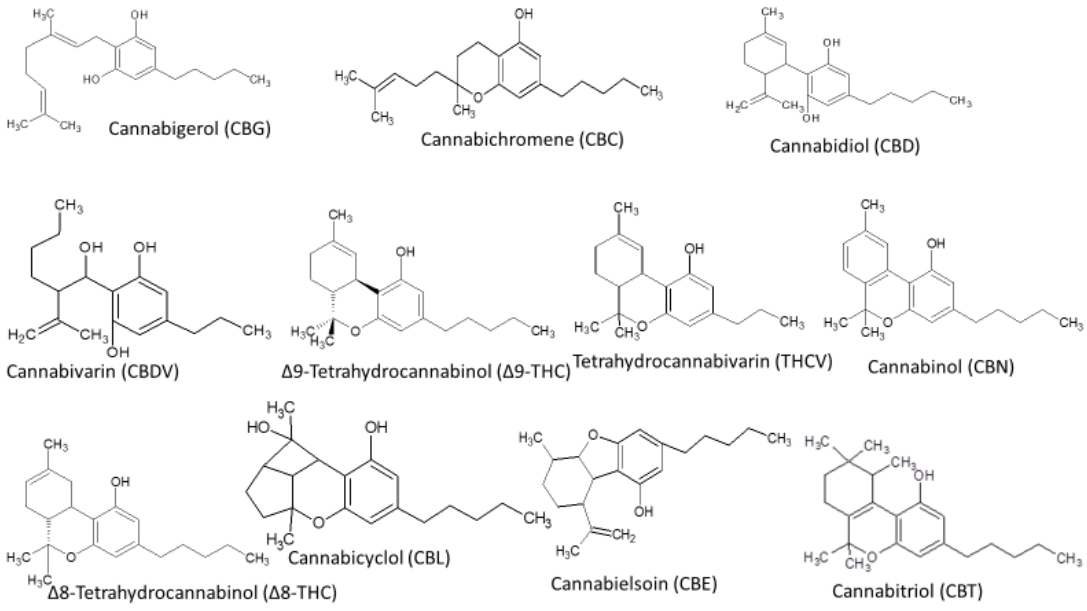

Figure 3.1 Structures of the 10 most prevalent cannabinoids in cannabis.

Less than 1% of Δ9-THC is eliminated from the human body in its original form. Once this cannabinoid reaches the liver, the lungs or the intestine, it is transformed primarily into its hydroxylated metabolite 11-hydroxy-Δ9-tetrahydrocannabinol (Δ9-THC-OH) by cytochrome P450 [29]. This is considered to be the most active metabolite of Δ9-THC. Δ9-THC-OH is further transformed in the body into its corresponding carboxylic acid 11-nor-9-carboxy-Δ9-tetrahydrocannabinol (11-nor-9-carboxy- Δ9-THC), which is biologically inactive and has been detected in plasma, urine, and faeces [30, 31]. Similar metabolic processing has been recorded for Δ8-THC and CBD, but their metabolites are not as well studied, presumably because of the diminished interest in the limited psychotropic activity compared to Δ9-THC. The major part of cannabinoids (65%) is excreted in faeces, while only 13% is excreted in urine. 80-90% of the total cannabinoids introduced into the body are excreted in the first 5 days after consumption [31]. Another way to detect the use of cannabis is through the analysis of hair. Because it is only limited by the length of the hair itself, older usages can be tracked with longer hair. This type of analysis leads to a detection range between a week and several months, but it can lead to false negatives, due to limited sensitivities of assays, as well as to false positives, due to passive exposure [32, 33].

1.3.2 Terpenes

Another important class of cannabis constituents is terpenes; components that derive from varied combinations of C_5 isoprene subunits [34]. These volatile and semi-volatile variants can be divided into two different classes based on the number of carbon atoms in their structure, specifically monoterpenes (C_{10}) and sequiterpenes (C_{15}). Larger terpenes exist as waxes and resins, as well as oxygenated terpenoids. Here we use the term “terpenes” as a collective primarily to indicate the volatile and semi-volatile terpenes

and terpenoids of greatest interest. Terpenes are the main aromatic principles of the plant and they reflect the immediate environment, such as the growing conditions of the plant and the surrounding environment. Terpenes are also known to exhibit various medicinal and pharmacological properties [35]. As of now, there does not exist a complete list of terpenes found in cannabis, but it is reported that a given cultivar can have as many as 100 different terpenes and terpenoids [35]. Among them, the most prevalent ones are: α-pinene and β-pinene, which are characterized by a pine fragrance and antiseptic effect; myrcene, which has a musky fragrance and can exhibit anti-oxidant and anti-carcinogenic properties; limonene, which has a citrus fragrance and is an antifungal and anti-carcinogenic compound; caryophyllene, characterized by a pepper fragrance and exhibits gastroprotective and anti-inflammatory effects; and linalool, which can help with anxiety and convulsions, and displays a floral fragrance [36-38]. Structures of come major terpenes found in cannabis are shown in Figure 1.2.

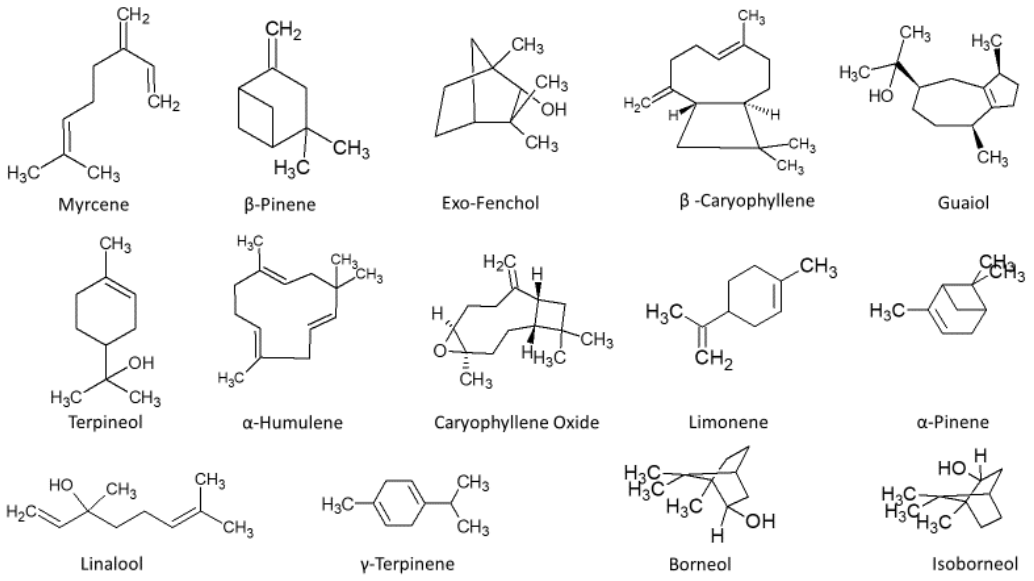

Figure 1.2 Structures of a selection of common terpenes and terpenoids found in C. sativa.

Worthy to note, while some pure standards and well characterized mixtures of terpenes can be purchased, a general lack of pure standards across the extremely large range of terpenes that exist naturally, ultimately hampers more extensive quantitative analysis of this class using available analytical methods.

1.4 Products, Legalization, and Testing in the United States

There are different types of products that can be produced from cannabis plant material, the most famous being herbal cannabis (consisting of the 'buds' or flowers of the cannabis plant that contain the majority of the psychoactive cannabinoids), cannabis resin (used to produce hashish), liquid cannabis (also known as hashish oil), and cannabis seeds (used to produce cannabis oil or to be eaten raw) [39]. Around the world, cannabis is controlled by different laws and regulations. In Uruguay, it has been legal since 2013. In the Netherlands, it is legal as well. It is legal in Spain as well, albeit in designated places, and in Portugal it is legal to carry up to 25 g of cannabis, but it is illegal to sell and buy it. In Czech Republic, it is legal to possess up to 15 g. In India, its use is allowed for some festivities. Cannabis use for the treatment of medical illnesses is also permitted in Canada, with recreation use being legalized in July of 2018. Currently, a legalization process is taking place in the United States, with 29 states plus the District of Columbia allowing the medical use of the plant, among which eight plus the District of Columbia allowing for recreational use, as well [40] (Figure 1.3).

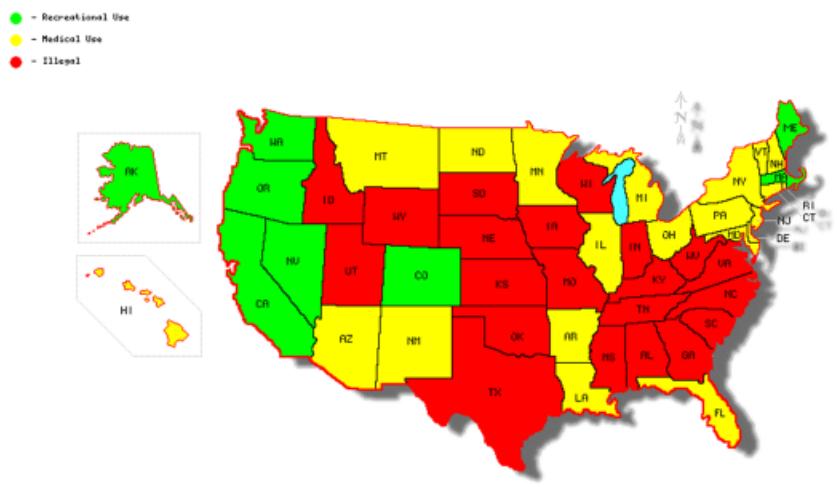

Figure 1.3. Map of the current legalization process in the United States.

Each state has its own rules regarding the use of cannabis, and this comes with different required testing for state compliance and product safety as well. Table 1.1 indicates some of the testing requirements in some of the most prolific “cannabis states” [41-44].

Table 1.1 Testing requirements for Cannabis plants in a few selected states

State	Test Required
Oregon	Microbial Contaminants, pesticides, residual solvents. [41]
Colorado	Pesticides, Microbial Contaminants, residual solvents, Heavy metals. [42]
Washington	Moisture content, potency analysis, foreign matter inspection, microbiological screening, residual solvent. [43]

Maryland	Mercury, lead, cadmium, or arsenic, Foreign material such as hair, insects, or any similar or related adulterant, any microbiological impurity, pesticide residue. [44]

The analysis of pesticides in cannabis is important because they can be linked to adverse human health and environmental effects. As of now, there are over 700 pesticides that are used in the food industry, and the most common way to analyze their residue is through well-defined extraction procedures (such as that indicated by the well-known Quick Easy Cheap Effective Rugged and Safe (QuEChERs) protocol [45]) followed by gas chromatography – mass spectrometry (GC-MS) or liquid chromatography – mass spectrometry (LC-MS) [46, 47]. Other approaches for pesticides analysis include: Thin-layer chromatography (TLC) [48], which is easy and fast, but rarely used; GC with electron capture detection (ECD) [49], which is highly sensitive and selective to compounds containing electronegative functionality, such as halogen atoms; GC with a nitrogen-phosphorus detector (NPD) [50]; GC with a flame photometric detector (FPD) [51], especially for the analysis of sulfur-containing analytes; and high performance liquid chromatography with ultraviolet detection (HPLC-UV) [52]. Recently, the use of GC equipped with a vacuum ultraviolet absorption detector has been demonstrated as a highly effective method for pesticide analysis [53]. The chemical nature and physicochemical properties (e.g., volatility, thermal stability, etc.) of different pesticides generally dictate whether HPLC or GC approaches can be taken. Further consideration must be given to the complexity of the sample matrix, and this brings with it different possibilities also for sample preparation. Recently, a resurgence of supercritical fluid chromatography (SFC) has provided an alternative means to allow simultaneous analysis of both volatile and non-volatile pesticide compounds, but this technique has not yet been applied for cannabis product pesticide analysis to our knowledge [54-56].

Microbial life forms are another contaminant that can cause damage to the plant and the consumers, and should be controlled. Different states have different requirements. The term microbes collectively refers to fungi, bacteria, viruses, and protozoa. As of now there are no accepted standards for safe levels in *C. sativa.* They are usually analyzed through enumeration on Sabouraud Dextrose Agar media, and their identity is usually determined by polymerase chain reaction (PCR), while the mycotoxins produced by fungi can be analyzed through enzyme-linked immunosorbent assay (ELISA) [52]. The former is more sensitive than traditional staining and culture techniques, and the latter is easier to perform than other laboratory techniques. Another technique for the analysis of microbes is matrix-assisted laser desorption/ionization – mass spectrometry (MALDI-MS), which requires an initial burden of high instrumental costs, but then provides for rapid and low cost identification of microbial species [57, 58].

The presence of certain heavy metals must also be controlled as they can be toxic to humans. Metals are generally resistant to metabolism and many can bioaccumulate in the body. The metals that are usually mandatory to test for in cannabis are cadmium, lead, mercury, and arsenic. Among the different ways of analyzing heavy metals, there is atomic emission spectroscopy (AES), atomic absorbance spectroscopy (AAS), inductively-coupled plasma-mass spectroscopy (ICP-MS), and X-ray fluorescence (XRF) [59]. The XRF is simple, fast, and also non-destructive, whereas ICP-MS allows simultaneous analysis with extremely high sensitivity; however, it is expensive. AAS is accurate and provides good sensitivity for targeted analysis of many metals, but it is a poor technique for speciation of a wide range of metals in a sample.

1.5 Chemical Analysis of Cannabis Natural Products

Of course, potency testing, primarily for Δ9-THC, is an important part of the cannabis industry. In general, the broader range of cannabinoids can be analyzed using various techniques and instrumentation, among which the most common are gas chromatography (GC) and liquid chromatography (LC), coupled with mass spectrometry (MS). Both techniques have benefits and drawbacks; GC usually uses electron ionization (EI), which fragments the analytes in a consistent fashion through the application of a common ionization energy (70 eV). This enables the use of compound libraries for identification. The ionization sources used in LC (most commonly, electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI)) only generate typically a protonated molecule with little to no diagnostic fragmentation. Such diagnostic information could only be obtained through tandem mass spectrometry, and different instruments induce different fragmentation ions and intensities, rendering the generation of libraries only of value on given instruments. Considering that cannabinoids, which bear primarily phenolic and carboxylic acid functional units, are not highly efficiently ionized by ESI or APCI, the sensitivity obtained through LC-MS may not be as high as through GC-MS. On the other hand, considering that carboxylic acids will degrade in the hot injection port of a GC-MS, it is necessary to derivatize (mainly through silylation or methylation/esterification) the acidic cannabinoids to preserve their structure prior to analysis. Silylating the cannabinoids also makes them more volatile, which will improve peak shape during gas chromatographic analysis. Derivatization is not required when using LC-MS. A list of published works focused on cannabis analysis can be found in Table 1.2.

The other main components, terpenes, are solely analyzed through GC, coupled with different detectors. The most widely used is flame ionization detector (FID), due to its low cost and simplicity, followed by MS (mainly SIM or scan mode). Headspace sampling is also commonly used in order to streamline sample preparation in combination with GC-

FID or GC-MS analysis. Headspace (HS)-GC-FID is as an efficient way to simultaneously analyze terpenes and residual solvents, which can remain in concentrated cannabis products, resulting from extraction processes using alcohols and hydrocarbons. Such an approach removes the need to perform extractions of the plant prior to analysis. Lately, Qiu et al. also developed a new technique for the analysis of terpenes using GC-VUV [60]. Difficulties associated with terpene analysis include the sheer numbers of isomeric variants that exist and the wide range of abundance of different compounds that can be observed in different varietals. VUV detection may have some advantages over MS detection with respect to differentiating isomeric terpenes.

1.5.1 Cannabinoids

1.5.1.1 Gas chromatography

In general, GC analyses of cannabinoids feature use of low polarity phase stationary phases, mainly 5% diphenyl 95% dimethyl polysiloxane, from which these compounds elute at temperatures lower than 300 °C in a short analysis time (usually less than 20 min). The most widely used detectors are FID and MS. Both detectors are listed in the recommended methods for the identification and analysis of cannabis and cannabis products, issued in 2009 by the United Nations [39]. In that work, focus was placed on the identification of Δ9-THC, CBD, and CBN, using CBD as an internal standard. Considering the large number of people using *C. sativa* for medicinal purposes, Romano and Hazekamp [61] compared 5 different homemade methods for preparation of cannabis oil (using naphtha, petroleum ether, ethanol, and 2 types of olive oil). They performed the analysis of both terpenes and cannabinoids using GC-FID. They concluded that the best preparation method for medical cannabis oil was with olive oil, since it is cheap, non-toxic, and non-flammable. The analysis of silylated cannabinoids (Δ9-THC-TMS, CBD-2TMS,

CBN-TMS, and CBC-TMS) was performed on extracts from different matrices by Madea and coworkers [62, 63]. They developed a fully automated method for the analysis of cannabinoids in hair samples and hemp food, using headspace solid-phase dynamic extraction coupled with GC-MS (Figure 1.4).

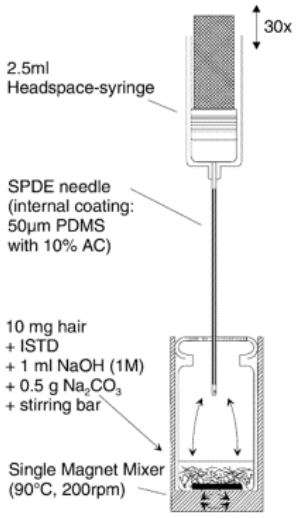

Figure 1.4 A process for automated headspace solid-phase dynamic extraction for the preparation of cannabinoids prior to GC analysis. Reprinted with permission from reference [63] (Copyright 2003, Elsevier).

In other work, Omar et al. [64] compared two different types of extraction, supercritical fluid extraction (SFE) and focused ultrasound extraction (FUSE), targeting their comparison on three cannabinoids (Δ9-THC, CBD, and CBC) and three terpenes (α-pinene, β-pinene, and limonene) from 13 different cannabis cultivars (AK-47, Amnesia, Somango, and Critical). They concluded that FUSE was more efficient after the first extraction, since 80% of terpenes and cannabinoids were recovered. Yet, overall SFE was concluded to be more advantageous, because it was possible to differentiate and isolate

the targeted compounds to be extracted. Some results of these experiments are shown in Figure 1.5.

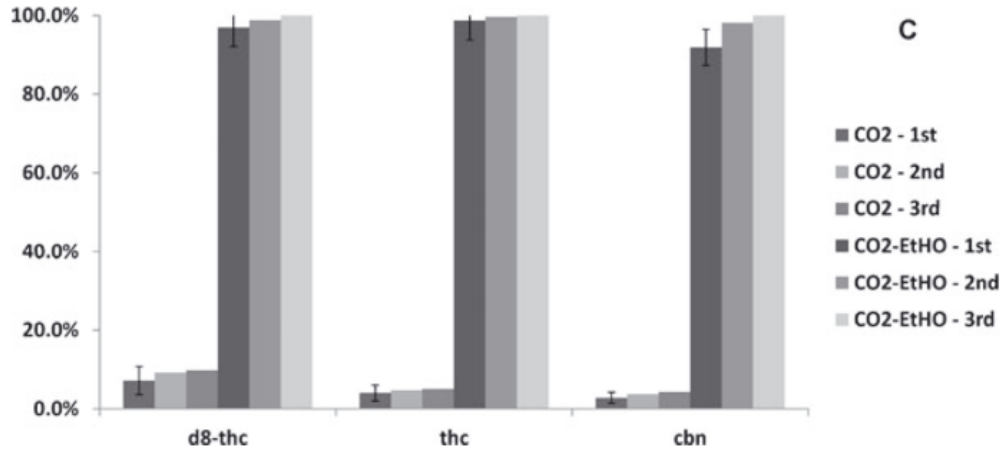

Figure 1.5 Differential extraction of cannabinoids using supercritical fluid extraction and carbon dioxide with or without the addition of ethanol. Reprinted with permission from reference [64] (Copyright 2013, Wiley-VCH).

Supercritical CO_2 is sufficient alone for the extraction of terpenes, while for cannabinoids, the best results were obtained when ethanol was added a co-solvent.

Another comparison was performed by Ross et al. [65], who used a GC-MS method to quantify the amount of Δ9-THC in cannabis and hemp seeds. They considered cannabis seeds as those with a higher amount of Δ9-THC (35.6 - 124 µg/g), while the hemp seeds were nearly devoid of Δ9-THC (0 - 12 µg/g). The samples were extracted with a mixture of chloroform and methanol (99:1), homogenized, and centrifuged. They tested the concentration of Δ9-THC, the effects of washing the seed with chloroform, and the location of the cannabinoid on the seed. They concluded that the highest amount of the cannabinoid

is found on the seed's surface, and this was attributed to the physical interaction between the seeds and the leaves.

Recently, a selected-ion monitoring (SIM) analysis on GC-MS was reported by Leghissa [66] for the fast detection of 9 different cannabinoids (THCV, CBD, CBC, Δ8-THC, Δ9-THC, Δ9-THC-TMS, CBG, CBN, and Δ9-THCA-2TMS). Two different derivatization agents, N,O-bis(trimethylsilyl)trifluoroacetamide + 1% chlorotrimethylsilane (BSTFA + 1% TMCS), and N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) were compared, and it was concluded that BSTFA + 1% TMCS is a better derivatization agent for the preparation and analysis of cannabinoids.

Leghissa et al. [67] were also the first to develop a GC-MS-based multiple reaction monitoring (MRM) database for normal and silylated cannabinoids using a triple quadrupole MS. Tandem mass spectrometry fragmentation patterns of primary radical cations for each cannabinoid in their derivatized and underivatized forms were elucidated in order to provide high specificity MRM transitions. The fragmentation pattern elucidated for Δ9-THC is shown in Figure 1.6, as an example.

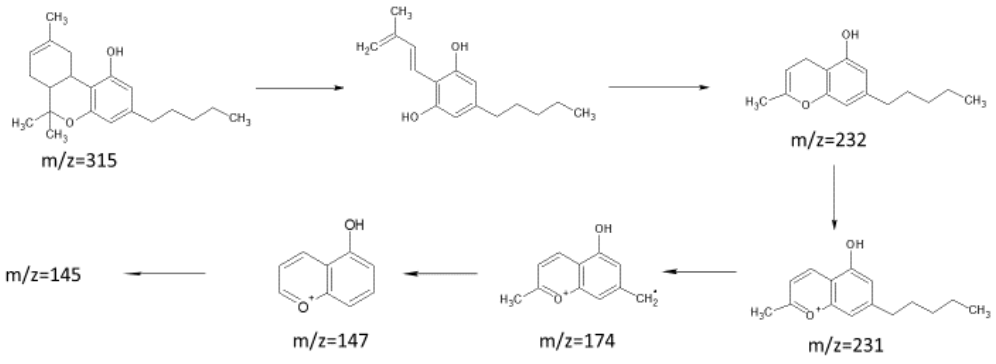

Figure 1.6 Tandem mass spectrometry fragmentation pathway for Δ9-THC following electron ionization in a GC-triple quadrupole-mass spectrometry. Adapted from reference [67].

The method was validated for cannabinoids spiked onto hops, as a surrogate matrix. Hops used in brewing beer are relatively similar to cannabis as they are both part of the *cannabaceae* family. Hops are also more widely available to researchers compared to federally-regulated (Schedule 1) cannabis plant material, and they contain similar types of waxes and other compounds, which allows for an effective emulation of a cannabis matrix. The analysis provided both good linearities and recoveries for the cannabinoid analytes, exhibiting low LODs (low pg on-column), due to the sensitive and specific MRM mode. Both techniques have advantages and disadvantages; underivatized cannabinoids showed slightly lower LODs compared to their silylated versions. However, acidic cannabinoids are decarboxylated in the hot injection port of the GC, and such a process confounds accurate speciation of all cannabinoid types of interest. Derivatization requires extra sample preparation, but it improved chromatographic separation, both due to increased volatility of the products and the potential for some cannabinoids to be better differentiated depending on whether they supported addition of only one or multiple silyl units. While it was not specifically investigated in that work, the use of GC-triple quadrupole-MS in MRM mode could be a good approach for profiling cannabinoids in other cannabis products, such as edibles and textiles, which will exhibit a wide range of interferences. The MRM mode provides enhanced specificity due to the monitoring of optimized and unique precursor – product ion transitions.

Even though FID and MS are the most widely used detectors coupled with GC for the analysis of cannabinoids, other alternatives have been used. Hillig and Mahlberg [68] used the GC technology and starch gel electrophoresis to perform a chemotaxonomic analysis of 157 cultivars of cannabis. Their starch gel electrophoresis analysis was based on the differences between the B_D and B_T allele frequencies, which are the locations where

the CBD-acid synthase and the THC-acid synthase are encoded. The frequencies of these alleles were used to determine the Δ9-THC/CBD ratio. They concluded that there are 2 different types of the plant, with *C. sativa* having the Δ9-THC/CBD ratio <25%, and *C. indica* >25%.

As another alternative, a vacuum ultraviolet detector coupled with GC was used by Leghissa et al. [69] to speciate different cannabinoids in their underivatized and silylated forms. The GC-VUV instrument, which records full range absorption spectra from 120 – 240 nm, enables the development of faster separation methods due to the possibility to deconvolve co-eluting peaks based on unique absorbance spectra for each analyte [70, 71]. It is also particularly adept at distinguishing isomers, which might exhibit similar mass spectra and fragmentation patterns during MS analysis [72, 73]. GC-VUV analysis was also utilized for the analysis of silylated versions of Δ9-THC metabolites. Even so, this technical note did not report a specific determination of cannabinoids from a sample matrix, and was presented simply to show that the VUV detector could be used as a viable tool in future work for enhanced differentiation of cannabinoids.

1.5.1.2 Liquid chromatography

Cannabis testing laboratories generally prefer the use of LC for determination of cannabinoids, because additional sample preparation steps associated with derivatization for GC analysis and the decarboxylation of pertinent precursory molecules (i.e., Δ9-THCA and CBDA), can both be avoided. Non-polar columns are usually used, mainly C18 or biphenyl variants, using 0.1% formic acid in water and 0.1% formic acid in methanol as mobile phases. Among the different detectors coupled with LC, the most widely reported used is MS [39]. In 2014, Aizpurua-Olaizola et al. [74] used LC-MS to analyze 30 different strains of *C. sativa*, in order to determine 6 cannabinoids (CBD, THCV, CBG, CBN, Δ9-

THC, and Δ9-THCA). They were further able to identify 7 more cannabinoids through the use of LC coupled with a quadrupole-time-of-flight (Q-TOF) detector (Figure 1.7).

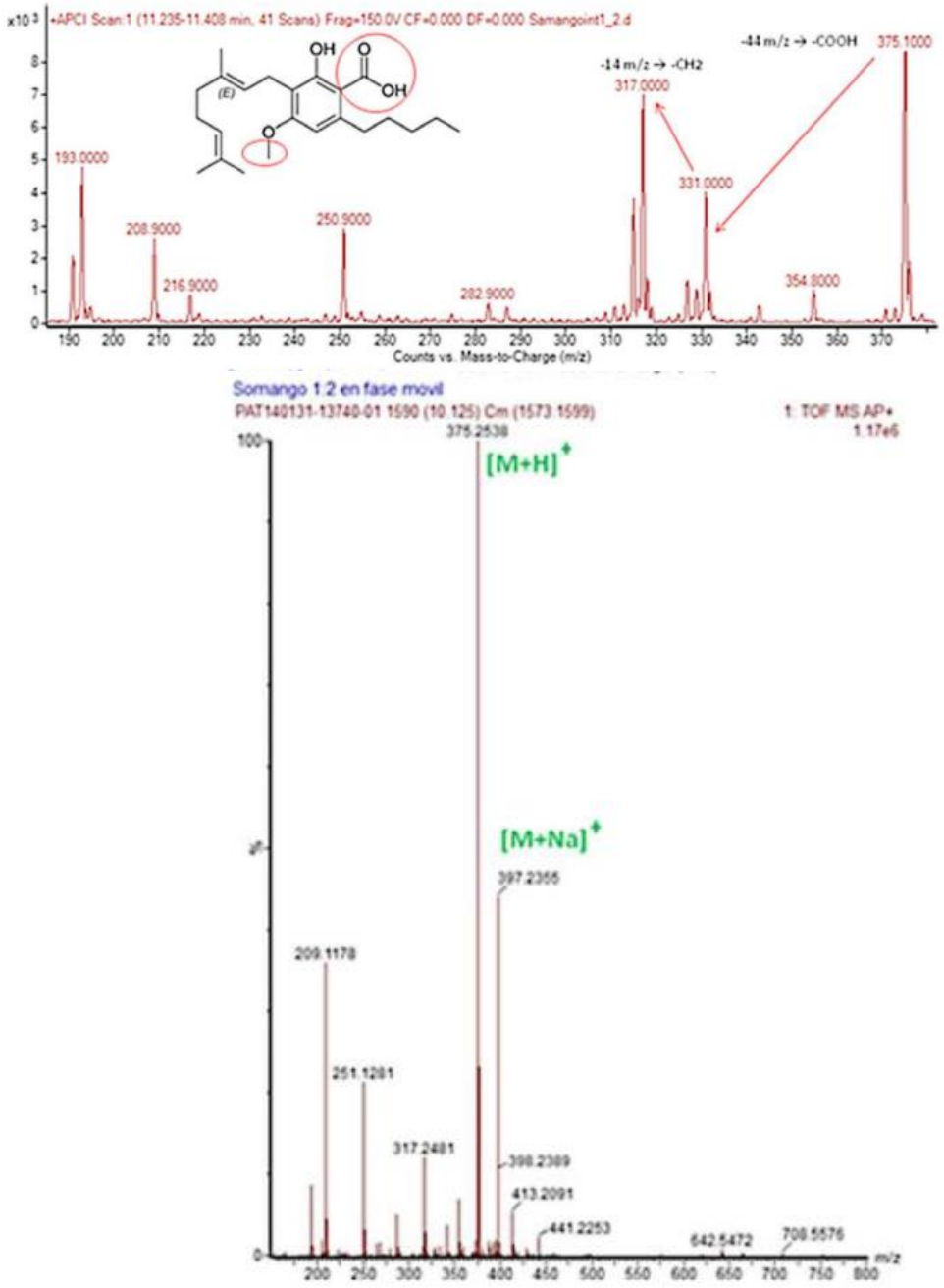

Figure 1.7 Use of HPLC-MS/MS (upper spectrum) and LC-Q-TOF-MS (lower spectrum) to identify a cannabis plant product, cannabigerolic acid monomethylether. Reproduced with permission from Reference [74] (Copyright 2014, Springer).

In the different samples, they were able to differentiate between plants grown indoors and those grown outdoors, with the former having higher concentrations of CBD, CBN, and Δ9-THC. The Q-TOF detector is attractive because it allows for tandem mass spectrometry, as in a triple quadrupole MS, but further provides higher mass accuracy for detected ions, due primarily to the higher resolving power of the TOF component.

A similar approach, using both LC-MS-MS and LC-QTOF-MS was reported by Jung et al. [75] for the identification of Δ9-THC, Δ9-THCA, and their metabolites in rat brain. As presented previously for GC-MS applications, the use of tandem mass spectrometry provides higher specificity for cannabinoid determination, when the analytes are extracted from a complex matrix. In this case, solid-phase extraction was used to extract the analytes from the rate brain prior to analysis.

Stolker et al. [76], on the other hand, used LC-ion trap-MS for the detection of 5 different cannabinoids using a matrix of cannabinoids spiked hops. Ion trap MS has been less utilized for cannabis analysis according to the scientific literature; however, ion traps can be powerful for qualitative analysis based on the potential for using multi-stage fragmentation to more comprehensively elucidate atom connectivity. While ion traps may be useful for the discovery and identification of new cannabinoids, they may be less well suited for quantitative determinations because of their limited dynamic range compared to triple quadrupole systems.

In other work, Grauwiler et al. [77] used an APCI method on a LC-QQQ-MS to detect CBD, CBN, Δ9-THC, Δ9-THC-OH, and 11-nor-9-carboxy-Δ9-THC in human plasma (EDTA-treated) and urine. The analysis featured a 25 min run time and a limit of quantitation of 0.2 ng/mL in urine. This LOQ is a reasonable level of analysis, considering that regular users of cannabis are reported to have a Δ9-nor-9-carboxy-THC concentration higher than 75 ng/mL in the first 8 days after consumption. Occasional users generally

exhibit concentrations of this metabolite below 5 ng/mL. Even though this method requires longer analysis time, the LOQs are such that reliable quantitation for both major cannabinoids and two Δ9-THC metabolites can be achieved.

Different approaches were taken by Citti et al. [78], who analyzed medicinal cannabis using HPLC-UV. This approach was meant to serve as a simple assay, which could be used by pharmacists; where necessary, follow-up analysis to confirm identity and purity using LC-MS could be used. HPLC-UV is a very straightforward approach. The instrument is simpler and less expensive, relative to MS-based systems. However, UV detection lacks specificity compared to MS. With UV detection, cannabinoid analytes are primarily assigned based on reproducible retention times, determined through the analysis of standards. Absorption profiles for cannabinoids in solution will be similar, but are not highly featured and well suited to differentiate various cannabinoids and make qualitative assignments. HPLC-UV methodology was also used by Gambaro et al. [79], in combination with GC-FID, for the quantification of the 3 primary constituents, Δ9-THC, CBD, and CBN, from cannabis plants.

As mentioned previously for the determination of pesticides, SFC is enjoying a resurgence in the marketplace and in research labs. SFC analysis of cannabinoids was reported by Backstrom et al. [80] and by Wang et al. [81]. The former used 2% MeOH in CO_2 as modifier, reaching 7% in 15 minutes at a column temperature of 70 °C on a cyanopropyl silica-based column. Detection was based on APCI-MS. Growth in the application of SFC for cannabis analysis is expected to increase, because it is considered greener than HPLC. The amounts of solvents used are greatly reduced, and as such, the amount of waste that needs to be disposed are minimal. Obtainable efficiency and specificity by SFC are comparable to that of HPLC [82- 84].

1.5.1.3 Other techniques

A wide variety of other techniques have been used for the quantification and qualification of cannabinoids. In 2009, the United Nations suggested that fast and easy qualification of cannabis products could be achieved using a variety of color tests (e.g., the Fast Corinth V salt test, the Fast Blue salt test, and the Rapid Duquenois test) [39]. However, these tests only confirm the potential presence of some cannabinoids, without providing further information. Color tests used for drug identification can, in general, be prone to having poor specificity and generating false positives because they cannot specifically identify the substance. These tests are usually referred to as “presumptive,” and confirmation of suspected positive hits need to be followed by more specific laboratory tests.

Ion mobility spectrometry (IMS) [39] is a fast technique that can be used to detect traces of organic substances. This technique has been used for the analysis of trace level drugs, but not for bulk samples due to the possibility of saturating the detector. IMS alone is also likely not suitable for analysis of highly complex mixtures. That said, new LC-IMS-MS systems are now commercially available. This arrangement adds another dimension for separation in the gas phase, providing increased separation capacity relative to LC-MS. To our knowledge, LC-IMS-MS has yet to have been utilized for the analysis of cannabis natural products.

Galand et al. [85] suggested that two newer techniques, automated multiple development (AMD) and optimum performance (or over-pressure) layer chromatography (OPLC), would be considerable improvements to the use of classic thin-layer chromatography (TLC) for cannabis analysis. These alternatives provide faster separations and decreased band broadening relative to TLC. AMD allows for the use of a gradient, even though air or compressed nitrogen are required, while OPLC features the

development of the plate with two different solvents, creating a two-dimensional chromatogram. The separation of the cannabinoids of interest was achieved using AMD and a 20-step step-gradient with acetone, diisopropylether, and hexane, while OPLC was developed with hexane and diethylether (80:20).

Lastly, Beasley et al. [86] used matrix-assisted laser desorption/ionization-MS (MALDI-MS) to detect and image cannabinoids in hair samples. MALDI-MS is another technique capable of detecting low-level cannabinoids, though requiringderivatization. The hair was limited to 5 cm due to the target plate size, and it was spiked by soaking it in 300 µL of a 0.5 µg/mL cannabinoids solution. Further extraction was not required; hairs were directly placed on the target plate with matrix and imaged. Figure 1.8 exemplifies this analysis and shows how MALDI-MS imaging can be used to determine the spatial distribution of cannabinoids in a hair sample.

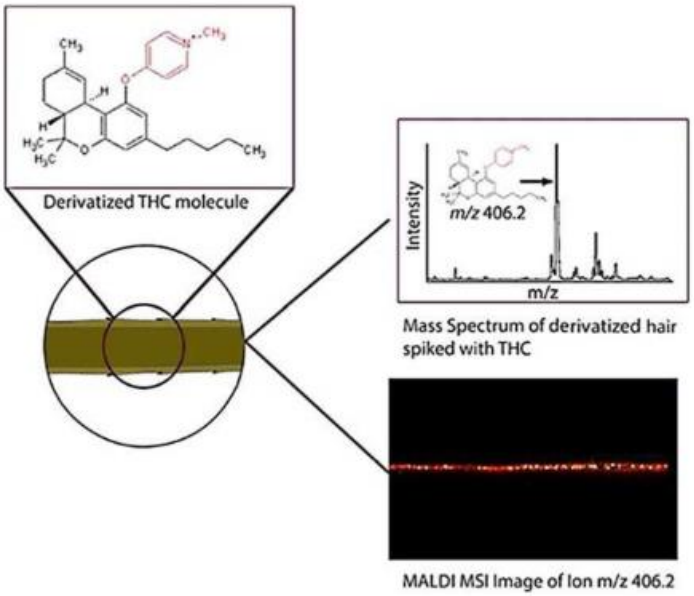

Figure 1.8 MALDI approach on a hair sample. Reproduced with permission (open access and reproduction granted) from reference [86] (Copyright 2016, American Chemical Society).

While the technique provides the ability to detect Δ9-THC and its metabolites in a single analysis, MALDI-MS is not generally considered to be a high performance quantitative technique. This is because the reproducibility of matrix deposition can vary across the sample, and from sample to sample. Additionally, while MALDI-MS may be attractive for this and other more forensic-oriented applications [87], such instrumentation are not yet commonplace in modern forensic laboratories.

1.5.2 Metabolites

Even though all cannabinoids are subjected to degradation in the body through various metabolic pathways, the focus of scientists has been primarily directed to the

analysis of Δ9-THC metabolites (11-nor-9-carboxy-Δ9-THC and Δ9-THC-OH), due to their high potency and psychoactive effects. Both GC-MS and LC-MS analysis have been reported. Selected ion monitoring on a GC-MS instrument equipped with electron ionization can be used, such as that reported by Kemp et al. [88] and Steimeyer et al. [89]. In both works, metabolites were extracted from biological samples (urine and blood). The analytes were derivatized by methylation. As stated previously, the use of GC-EI-MS can be attractive given that fragmentation patterns are consistent and analyte assignments can be confirmed by matching measured mass spectra with those in a database. As an alternative, negative chemical ionization (GC-NCI-MS) in SIM mode was used by Kala et al. [90]. This ionization mode was used because of the good responsiveness of the target analytes; however, less qualitative information can be obtained using NCI-MS compared to EI-MS.

On the other hand, Moore et al. [91], Karschner et al. [92], and Lowe et al. [93] have suggested the use of comprehensive two-dimensional GC-MS (GCxGC-MS), as an alternative to traditional one-dimensional GC. The use of this technique, which takes the effluent from one column and injects it directly on a second column for additional separation, provides better resolution for substances in a complex mixture. While GCxGC-MS is more expensive, requires additional hardware and software, and is a bit more complicated to optimize compared to traditional GC-MS, many new commercial systems have been introduced on the market, and applications of this technology will continue to grow.

Recently, Leghissa et al. [94] developed a GC-triple quadrupole-MS MRM database for the analysis of Δ9-THC-OH and 11-nor-9-carboxy-Δ9-THC, followed by general method validation in spiked urine and plasma samples. This was the first example of a cannabinoid metabolite analysis using a triple quadrupole system. Such an approach

offers superior sensitivity and specificity for the analysis of cannabinoid metabolites from complex biological matrices. Leghissa et al. [69] also reported the analysis of these compounds using the GC-VUV detector. The LODs are higher than those obtained using GC-MS/MS, but the additional verification and differentiation provided by the gas phase absorption spectra may be another tool to aid forensic laboratories in their investigations. As an example, Figure 1.9 shows the gas phase VUV absorbance spectra for Δ9-THC and its two primary metabolites.

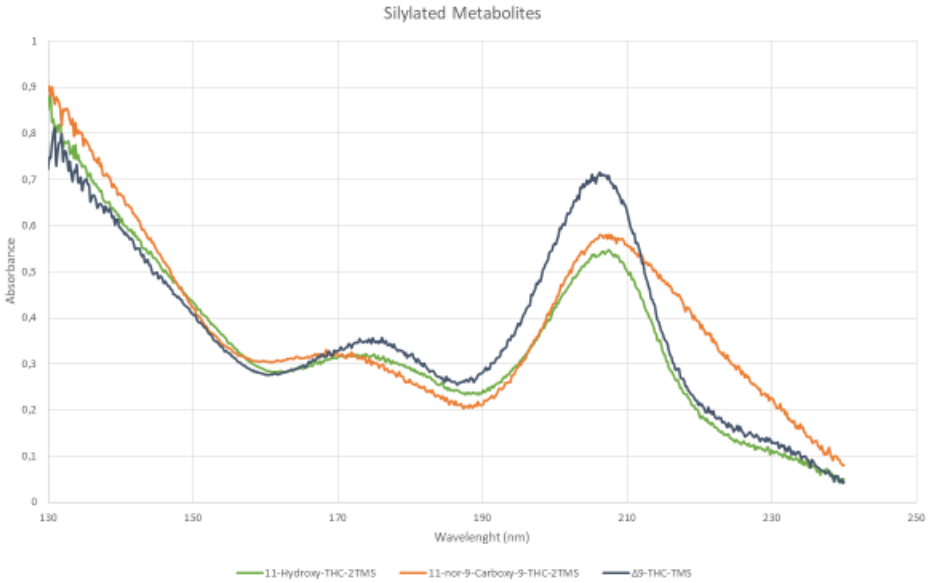

Figure 1.9 Gas phase vacuum ultraviolet absorptions spectra for Δ9-THC and its primary metabolites.

With a resolution of 0.5 nm, these spectra have high dissimilarity and could be easily distinguished and identified, even if they co-eluted with each other or other interferences.

LC-MS has also been reported for metabolite analysis, using ESI [95, 96] and APCI [97], with the main attention being on urine, oral fluids, and hair. Park et al. prepared hair samples using liquid-liquid extraction (LLE), and used 3 columns (precolumn, trap column, and analytical column) to carry out the separation, using a column switching system. The LOQ observed for 11-nor-9-carboxy-Δ9-THC was 0.1 pg/mg. The human samples were found to vary in their content of this metabolite between 0.13 and 15.75 pg/mg, making this a reliable method for the quantification of these analytes [95]. Mercolini et al. developed a method for the analysis of Δ9-THC, 11-nor-9-carboxy-Δ9-THC, and Δ9-THC-OH from human dried blood spots. The goal was to create a reliable method for roadside testing [96], specifically to avoid the problems of transportation and storage that are usually associated with police road tests. Weinmann et al. [97] used APCI to detect 11-nor-9-carboxy-Δ9-THC in urine samples, preceded by automated SPE. Even if this analysis was focused on only one metabolite, it showed a low LOD (2.0 ng/mL) and performance appropriate for quantifying 11-nor-9-carboxy-Δ9-THC in biological samples.

Table 1.2 Applications for chemical analysis of cannabinoids and their metabolites.

Analytes	Technique	Matrix	Other	Reference
Cannabinoids	GC-MS	Plant	-	[66], [65]
Cannabinoids	GC-MS	Plant	SFE	[64]
Cannabinoids	GC-MS	Plant	Headspace-solid phase microextraction	[98]
Cannabinoids	GC-MS	Plant	MRM database	[67]
Cannabinoids	GC-MS	Plant	2D-GC-MS	[99]

Cannabinoids	GC-MS	Hemp food	-	[100]
Cannabinoids	GC-MS	Hemp food	Headspace-solid phase microextraction	[63]
Cannabinoids	GC-MS	Hair	-	[101], [102]
Cannabinoids	GC-MS	Hair	Headspace	[62], [103], [104]
Cannabinoids	GC-MS	Air	In-injector thermal desorption	[105]
Cannabinoids	GC-VUV	Plant	-	[69]
Cannabinoids	GC-FID	Plant	-	[79], [106], [39]
Cannabinoids	GC	Plant	-	[68]
Cannabinoids	LC-IT-MS	*C. sativa* products	-	[76]
Cannabinoids	LC-MS	Oral fluids, plasma, urine	-	[107], [108] [109]
Cannabinoids	LC-MS-MS	Oral fluids, plasma, urine	-	[[110], [111], [112], [77], [113], [75], [109]

Cannabinoids	LC-chemiluminescence detector	hemp	2D-LC-MS	[114]
Cannabinoids	LC-QTOF	Urine	-	[75]
Cannabinoids	HPLC	Plant	-	[74], [39], [115]
Cannabinoids	HPLC	Plasma	-	
Cannabinoids	HPLC-UV	Plant	-	[78], [116], [79], [117]
Cannabinoids	HPLC- densitometry	Cannabis resin	-	[118]
Cannabinoids	CPC	Plant	-	[119]
Cannabinoids	EI-FT-ICR-MS	Plant	-	[120]
Cannabinoids	Capillary electrophoresis-MS	Urine	-	[121]
Cannabinoids	Overpressured-layer chromatography	Hemp	-	[122]
Cannabinoids	Color Test, IMS	Plant	-	[39]
Cannabinoids	MALDI	Hair	-	[86]
Cannabinoids	AMD, TLC	Plant	-	[85]
Cannabinoids	Laser Desorption Ionization	Plant	Silica plate extraction	[123]
Cannabinoids	ENose	Skin	-	[124]

Cannabinoids	SFC-MS	Plant	-	[80], [81]
Δ9-THC metabolites	GC-MS	Urine, Plasma	MRM	[94]
Δ9-THC metabolites	GC-MS	Urine	-	[125], [126], [90], [88]
Δ9-THC metabolites	GC-MS	Blood Serum	-	[89], [127], [90], [128], [129],
Δ9-THC metabolites	GC-MS	Plasma	-	[88], [130]
Δ9-THC metabolites	GC-MS	Hair	-	[101], [131]
Δ9-THC metabolites	GC-MS	Oral Fluids	-	[129], [128]
Δ9-THC metabolites	GC-MS	Oral fluids, plasma	GCxGC-MS	[132], [92], [133], [91]
Δ9-THC metabolites	GC-VUV	Standards	-	[69]
Δ9-THC metabolites	LC-MS-MS	Urine	-	[75], [77], [134], [97], [109], [135], [136]
Δ9-THC metabolites	LC-MS-MS	Plasma	-	[77], [137]

Δ9-THC metabolites	LC-MS-MS	Oral fluids	-	[112], [110], [129], [112], [138], [128]
Δ9-THC metabolites	LC-MS-MS	Oral fluids	Microflow	[108]
Δ9-THC metabolites	LC-MS-MS	Oral fluids	Orbitrap	[139]
Δ9-THC metabolites	LC-MS-MS	Hair	-	[140], [95], [141]
Δ9-THC metabolites	LC-MS-MS	Blood	-	[96], [142], [143]

1.5.3 Terpenes

To date, there are very few studies in the literature focused specifically on terpene analysis in cannabis. However, it is clear that a great many terpene measurements are routinely performed in commercial cannabis testing facilities. Even though cannabinoids and their metabolites can be analyzed both with GC and LC, terpenes are mainly analyzed trough GC, due to their high volatility. They are readily ionized by electron ionization, but the most common methods of analysis do not take in consideration the isomeric nature of the major portion of terpenes and their different aromatic properties, leading to a less than comprehensive characterization. To overcome this shortcoming, chiral GC columns (with a cyclodextrin-based stationary phase) should be used, even if highly increasing the analysis time due to the slow temperature ramps required for the differentiation of enantiomers. Another issue in the analysis of terpenes is the structural similarities of these

components, which lead to similar and identical fragments on EI, causing problems in their qualification.

Giese et al. [116], as well as Casano et al. [144] reported the use GC-FID to detect and analyze terpenes. They noted that a primary choice of this technology was based upon the low cost of instrumentation. On the other hand, Ferioli et al. [145] and Leghissa [66] used a GC-MS instrument to carry out the analysis. Nissen at al. [146] decided to focus their attention on the antimicrobial activity of cannabis oils, using a minimum inhibitory concentration (MIC) assay. Finally, Qiu et al. were able to develop a method for the identification of these components with the use of the VUV detector, creating a gas phase absorbance spectral library, which can be used for the confirmation of the different species [60]. In that work, a standard commercial mixture of cannabis terpenes was primarily evaluated, even if the main application focus of the study was on commercial turpentine samples.

Considering the growing importance of the plant, it is predictable that more exploratory studies will be carried out in the future. Of course, different terpenes may have different flavors, fragrances, and therapeutic benefit, but it is also worth noting that terpene profiles could be useful to help track and understand the origin and the growing conditions of a specific cannabis cultivar. It is possible that terpene profiles, and specifically characterization of the enantiomer ratio of chiral terpenes, may be valuable for future forensic analysis to connect confiscated cannabis from its region of origin.

1.6 Conclusions

The importance of cannabis is rapidly growing due to the legalization process that is taking place in the United States. Therefore, it is necessary to develop methods that satisfy all the requirements for different endogenous and exogenous analytes of interest.

For the latter, the lists of dictated compounds, metals, and microbes may vary from state to state. With respect to endogenous compounds, numerous studies are being carried out to prove and discover new medicinal benefits of cannabinoids, including also cancer research. Realistically, the complex interplay and synergism of multiple constituents, whether they be cannabinoids or terpenes, which contribute to apparent or potential therapeutic benefits, will be a rich area of research for many years to come. Progress in this area will only be slowed by governmental regulations, which will limit traditional funding opportunities for research and access to obtain an appropriate variety of samples for exploration.

Considering the wide variety of analytes that can be found in *C. sativa* cultivars, and the high volume of testing that is currently performed, a real challenge is to create a universal method for their comprehensive detection. This effort will ultimately be limited by the variable nature of the compounds present and their relative abundances. Further, appropriate methods for field testing or forensic analyses are needed. These needs are more immediate for routine analysis, but other methodologies related to discovery and testing of therapeutic effect will also grow; however, it is not clear whether the majority of such work will make it into the scientific literature or if it will be held as proprietary information.

It was stated before that the analysis through GC-MS has a higher sensitivity for the analysis of cannabinoids due to their fragmentation on EI, as opposed to their ionization through APCI or ESI, but it requires a prior derivatization for the protection of carboxyl groups. To overcome this problem it is necessary to develop a method that would reduce lab preparation before the analysis, or a preparation/analysis online. As cannabis is incorporated into different products, significant additional analytical challenges will result from dealing with the complexities of different matrices. Again, the wide range of

compounds of interest makes this a rich research area for future study, if appropriate support and materials can be garnered. The path for a comprehensive methodology for the analysis of cannabis is still long, but it is in the interest of scientists, care-givers, and consumers to fully understand its composition and its full medical/pharmaceutical benefits.

1.7 Acknowledgements

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1.8 Conflict of interest

The authors declare no conflict of interest related to this work.

1.9 References

[1] Hanus L.O., Pharmacological and Therapeutic Secrets of Plant and Brain (Endo) Cannabinoids. *Med. Res. Rev.*, 2009, *29*, 213-271.

[2] Pringle, H., Ice Age Communities may be earliest known net hunters. *Science*, 1977, *277,* 1203-1204.

[3] Unschuld P.U., Medicine in China: a history of pharmaceutics. University of California Press, Berkeley 1986.

[4] Thompson R.C., Dictionary of Assyrian botany. British Academy, London 1949.

[5] Ebbell B., The papyrus Ebers. The greatest egyptian medical document. Oxford University Press, Oxford 1937.

[6] Bloomfield M., Hymns of the Atharva-Veda, Together with Extracts from the Ritual Books and the Commentaries. Claredon Press, Oxford 1897.

[7] Darmesteter J., Heyworth Mills L., The Zend-Avesta: The Sîrôzahs, Yasts, and Nyâyis, translated by James Darmesteter. Claredon Press, Oxford 1897.

[8] Elder P.t., Natural History: Books XX-XXIII. Harvard University Press, Cambridge 1999.

[9] Dioscorides P., De Materia Medica. Knobloch, 1829.

[10] Moreau J.J., Hashish and mental illness. Raven Press, 1973.

[11] Sloman L., Reefer madness: a history of marijuana. Macmillan, London 1988.

[12] Musto D.F., The marijuana tax act of 1937. *Arch. Gen. Psychiatry*, 1972, *26,* 101-108.

[13] Press A., Marijuana Nation: the legalization of cannabis across the USA. Mango Media Inc., Miami 2015

[14] National Survey on Drug Use and Health, U.S., National Estimates of Marijuana Use and Related Indicators. *Surveillance Summarie*s, 2016, 1-25.

[15] Van der Werf H., Crop physiology of fibre hemp (Cannabis sativa L.)Crop physiology of fibre hemp (Cannabis sativa L.). Van der Werf, 1994.

[16] Emboden W.A., Cannabis- a polytypic genus. *Econ. Bot.*, 1974, *28,* 304-310.

[17] Mechoulam R., Gaoni Y., Recent advances in the chemistry of hashish*. Fortschr. Chem. Org. Naturst.*, 1967, *25,* 175-213.

[18] ElSohly M.A., Slade D., Chemical constituents of marijuana: The complex mixture of natural cannabinoids. *Life Sci*., 2005, *78*, 539-548.

[19] Claussen U., Von Spulak F., Korte F., Hashish. XIV. Information on the substance of hashish. *Tetrahedron*, 1968, *24*, 1021-3.

[20] Gaoni Y., Mechoulam R., The isolation and structure of delta-1-tetrahydrocannabinol and other neutral cannabinoids from hashish*. J. Am. Chem. Soc.*, 1971, *93*, 217-24.

[21] Adams R., Hunt M., Clark J.H.., Structure of cannabidiol, a product isolatedfrom the marihuana extract of Minnesota wild hemp*; J Am Chem Soc,* 1940,*62,* 196-200.

[22] Cunha J.M., Carlini E.A., Pereira A.E., Ramos O.L., Pimentel C., Gagliardi R., Sanvito W.L., Lander N., Mechoulam R., Chronic administration of cannabidiol to healthy volunteers and epileptic patients. *Pharmacology*, 1980, *21*, 175-185.

[23] Rohleder C., Müller J.K., Lange B., Leweke F.M., Cannabidiol as a Potential New Type of an Antipsychotic. A Critical Review of the Evidence. *Front. Pharmacol.*, 2016, *7,* 422.

[24] Fogaca M.V., Reis F.M.C.V., Campos A.C., Guimaraes F.S., Effects of intra-prelimbic prefrontal cortex injection of cannabidiol on anxiety-like behavior: involvement of 5HT 1A receptors and previous stressful experience. *Eur. Neuropsychopharmacol.*, 2014, *24*, 410-419.

[25] Hartsel S.C., Loh W.H., Robertson L.W., Biotransformation of Cannabidiol to Cannabielsoin by Suspension Cultures of Cannabis sativa and Saccharum officinarum. *Planta Med*., 1983, *48,* 17-9.

[26] Schultz O.E., Haffner G., On the question of the biosynthesis of cannabinol. Part III. *Arch. Pharm*., 1960, *293,* 1-8.

[27] Vree T.B., Breimer D.D., Ginneken C., Rossum J.M., Identification in hashish of tetrahydrocannabinol, cannabidiol and cannabinol analogues with a methyl side-chain. *J. Pharm. Pharmacol.*, 1972, *24*, 7-12.

[28] Obata Y., Ishikawa Y., Studies on the constituents of hemp plant (Cannabis sativa L.). *Agr. Biol. Chem.*, 1966, *30,* 619-620.

[29] Iribarne C., Berthou F., Baird S., Dréano Y., Picard D., Bail J.P., Beaune P., Ménez, J.F., Involvement of cytochrome P450 3A4 enzyme in the N-demethylation of methadone in human liver microsomes. *Chem. Res. Toxicol.*, 1966, *9*, 365-373.

[30] Lemberger L., Weiss J.L., Watanabe A.M., Galanter I.M., Wyatt R.J., Cardon, P.V., Delta-9-tetrahydrocannabinol: Temporal correlation of the psychologic effects and blood levels after various routes of administration. *N. Engl. J. Med.*, 1972, *286*, 685-688.

[31] Wall M.E., Perez-Reyes M., The metabolism of delta 9-tetrahydrocannabinol and related cannabinoids in man. *J. Clin. Pharmacol.*, 1981, *21*(Suppl): p. 178s-189s.

[32] Musshoff F., Madea B., Review of biologic matrices (urine, blood, hair) as indicators of recent or ongoing cannabis use*. Ther. drug monit.*, 2006, *28,* 155-163.

[33] Cary P., The marijuana detection window: Determining the length of time cannabinoids will remain detectable in urine following smoking*.* National Drug Court Institute, Alexandria 2006.

[34] Brenneisen, R., in: ElSohly, M.A. (Eds), Marijuana and the Cannabinoids. Humana Press, New York 2007, pp. 17-49.

[35] Clarke R.C., D.P. Watson, in: ElSohly, M.A. (Eds), Marijuana and the Cannabinoids. Humana Press, New York 2007, pp. 1-15.

[36] Gertsch J., Leonti M., Raduner S., Racz I., Chen J.Z., Xie X.Q., Altmann K.H., Karsak M., Zimmer A., Beta-caryophyllene is a dietary cannabinoid. *Proc. Natl. Acad. Sci. U.S.A.*, 2008, *105*, 9099-9104.

[37] Russo E.B., Taming THC: potential cannabis synergy and phytocannabinoid-terpenoid entourage effects. *Br. J. Pharmacol*., 2011, *163,* 1344-1364.

[38] Fine P.G., Rosenfeld M.J., The endocannabinoid system, cannabinoids, and pain. R*ambam Maimonides med.J.,* 2013, *4,* 4*.*

[39] Recommended Methods for the Identification and Analysis of Cannabis and Cannabis Products. Laboratory and Scientific Section United Nations Office on Drugs and Crime, New York 2009.

[40] http://www.ncsl.org/research/health/state-medical-marijuana-laws.aspx. (last time accessed: June 1, 2017)

[41] Farrer D.G., Technical report: Oregon health authority's process to determine which types of contaminants to test for in cannabis products, and levels for action. 2015

[42] Department of Revenue, Medical Marijuana Rules. Code of Colorado Regulations.

[43] Washington State Legislation*, Section 314-55-102*.

[44] Annotated Code of Maryland*, §§13-3301—13-3303*.

[42] Department of Revenue, Medical Marijuana Rules. Code of Colorado Regulations.

[43] Washington State Legislation*, Section 314-55-102*.

[44] Annotated Code of Maryland*, §§13-3301—13-3303*.

[45] Schenck F.J., Hobbs J.E., Evaluation of the Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) Approach to Pesticide Residue Analysis. *Bull. Environ. Contam. Toxicol.*, 2004, *73,* 24–30.

[46] Payá P., Anastassiades M., Mack D., Sigalova I., Tasdelen B., Oliva J., Barba A., Analysis of pesticide residues using the Quick Easy Cheap Effective Rugged and Safe (QuEChERS) pesticide multiresidue method in combination with gas and liquid chromatography and tandem mass spectrometric detection. *Anal. Bioanal Chem.*, 2007, *389,* 1697-714.

[47] Mastovska K., Dorweiler K.J., Lehotay S.J., Wegscheid J.S., Szpylka K.A., Pesticide multiresidue analysis in cereal grains using modified QuEChERS method combined with automated direct sample introduction GC-TOFMS and UPLC-MS/MS techniques. *J. Agric. Food Chem.,* 2009, *58,* 5959-72.

[48] Tuzimski T., Soczewinski E., Use of a database of plots of pesticide retention (RF) against mobile-phase composition. Part I. Correlation of pesticide retention data in normal- and reversed-phase systems and their use to separate a mixture of ten pesticides by 2D TLC. *Chromatographia,* 2002, *56,* 219-23.

[49] Correia M., Delerue-Matos C., Alves A., Development of a SPME-GC-ECD methodology for selected pesticides in must and wine samples. *Fresenius J. Anal. Chem.*, 2001, *369,* 647-51.

[50] Eisert R., Levsen K., Determination of organophosphorus, triazine and 2, 6-dinitroaniline pesticides in aqueous samples via solid-phase microextraction (SPME) and

gas chromatography with nitrogen-phosphorus detection. *Fresenius J. Anal. Chem.*, 1995, *351,* 555-62.

[51] Dugo G., Di Bella G., La Torre L., Saitta M., Rapid GC-FPD determination of organophosphorus pesticide residues in Sicilian and Apulian olive oil. *Food Control,* 2005, *16,* 435-8.

[52] Daley P., Lampach D., Sguerra S., Testing Cannabis for ContaminantsTesting Cannabis for Contaminants. Coresta, 2013.

[53] Fan H., Smuts J., Walsh P., Harrison D., Schug K.A., Gas chromatography–vacuum ultraviolet spectroscopy for multiclass pesticide identification. *J. Chrom. A.,* 2015, *1389,* 120-7.

[54] McNally M.E.P., Wheeler J.R., Supercritical fluid extraction coupled with supercritical fluid chromatography for the separation of sulfonylurea herbicides and their metabolites from complex matrices. *J. Chrom. A,* 1988, *435,* 63-71.

[55] Dost K., Jones D.C., Auerbach R., Davidson G., Determination of pesticides in soil samples by supercritical fluid chromatography–atmospheric pressure chemical ionisation mass spectrometric detection. *Analyst,* 2000, *125,* 1751-5.

[56] Ishibashi M., Ando T., Sakai M., Matsubara A., Uchikata T., Fukusaki E., Bamba T., High-throughput simultaneous analysis of pesticides by supercritical fluid chromatography/tandem mass spectrometry. *J. Chrom. A,* 2012, *1266,* 143-8.

[57] Havlicek V., Lemr K., Schug K.A., Current Trends in Microbial Diagnostics Based on Mass Spectrometry. *Anal. Chem*., 2013, *85,* 790-7.

[58] Santos I.C., Hildenbrand Z.L., Schug K.A., Applications of MALDI-TOF MS in environmental microbiology. *Analyst*, 2016, *141,* 2827-37.

[59] Leiterer M., Einax J.W., Löser C., Vetter A., Trace analysis of metals in plant samples with inductively coupled plasma–mass spectrometry. *Fresenius J. Anal. Chem.*, 1997, *359,* 423-6.

[60] Qiu C., Smuts J., Schug K.A., Analysis of terpenes and turpentines using gas chromatography with vacuum ultraviolet detection. *J. Sep. Sci.,* 2017, *40,* 869-877.

[61] Romano L.L., Hazekamp A., Cannabis Oil: chemical evaluation of an upcoming cannabis-based medicine. *Cannabinoids*, 2013, *1*, 1-11.

[62] Musshoff F., Lachenmeier D.W., Kroener L., Madea B., Automated headspace solid-phase dynamic extraction for the determination of cannabinoids in hair samples. *Forensic sci. int.*, 2003, *133*, 32-38.

[63] Lachenmeier D.W., Kroener L., Musshoff F., Madea B., Determination of cannabinoids in hemp food products by use of headspace solid-phase microextraction and gas chromatography–mass spectrometry. *Anal. Bioanal. Chem.*, 2004, *378*, 183-189.

[64] Omar J., Olivares M., Alzaga M., Etxebarria N., Optimisation and characterisation of marihuana extracts obtained by supercritical fluid extraction and focused ultrasound extraction and retention time locking GC-MS. *J. Sep. Sci.*, 2013, *36*, 1397-1404.

[65] Ross S.A., Mehmedic Z., Murphy T.P., ElSohly M.A., GC-MS analysis of the total δ9-thc content of both drug-and fiber-type cannabis seeds. *J. Anal. toxicol.*, 2000, *24*, 715-717.

[66] Leghissa A., Method development for qualification and quantification of cannabinoids and terpenes in extracts by Gas chromatography-mass spectrometry. The University of Texas at Arlington, Arlington 2016.

[67] Leghissa A., Hildenbrand Z.L., Foss Jr. F.W., Schug K.A., Determination of cannabinoids from a surrogate hops matrix using multiple reaction monitoring gas chromatography – triple quadrupole – mass spectrometry. Submitted.

[68] Hillig K.W., Mahlberg P.G., A chemotaxonomic analysis of cannabinoid variation in Cannabis (Cannabaceae)*. Am. J. Bot.*, 2004, *91*, 966-75.

[69] Leghissa A., Smuts J., Qiu C., Hildenbrand Z.L., Schug K.A., Detection of cannabinoids and cannabinoid metabolites using gas chromatography-vacuum ultraviolet spectroscopy. Submitted.

[70] Santos, I.C., Schug. K.A., Recent advances and applications of gas chromatography vacuum ultraviolet spectroscopy. *J. Sep. Sci,* 2016, *1,* 138-151.

[71] Schug K.A., Sawicki I., Carlton Jr D.D., Fan H., McNair H.M., Nimmo J.P., Kroll P., Smuts J., Walsh P., Harrison D. Vacuum ultraviolet detector for gas chromatography. *Anal. Chem.*, 2014, *86*, 8329-8335.

[72] Schenk J., Mao X., Smuts J., Walsh P., Kroll P., Schug K.A., Analysis and Deconvolution of Dimethylnaphthalene Isomers Using Gas Chromatography Vacuum Ultraviolet Spectroscopy and Theoretical Computations. *Anal. Chim. Acta* , 2016, *945,* 1-8.

[73] Skultety L., Frycak P., Qiu C., Smuts J., Shear-Laude L., Lemr K., Mao J.X., Kroll P., Schug K.A., Szewezak A., Vaught C., Resolution of isomeric new designer stimulants using gas chromatography – vacuum ultraviolet spectroscopy and theoretical computations. *Anal. Chim. Acta,* 2017, *971,* 55-67.

[74] Aizpurua-Olaizola O., Omar J., Navarro P., Olivares M., Etxebarria N., Usobiaga A., Identification and quantification of cannabinoids in Cannabis sativa L. plants by high performance liquid chromatography-mass spectrometry. *Anal. Bioanal. Chem.*, 2014, *406*, 7549-7560.

[75] Jung J., Meyer M.R., Maurer H.H., Neusüß C., Weinmann W., Auwärter V., Studies on the metabolism of the Δ9-tetrahydrocannabinol precursor Δ9-tetrahydrocannabinolic

acid A (Δ9-THCA-A) in rat using LC-MS/MS, LC-QTOF MS and GC-MS techniques. *J. mass spectrom.*, 2009, *44*, 1423-1433.

[76] Stolker A.A.M., Van Schoonhoven J., De Vries A.J., Bobeldijk-Pastorova I., Vaes W.H.J., Van Den Berg R., Determination of cannabinoids in cannabis products using liquid chromatography–ion trap mass spectrometry. *J. Chrom. A.,* 2004, *1058*, 143-151.

[77] Grauwiler S.B., Scholer A., Drewe J., Development of a LC/MS/MS method for the analysis of cannabinoids in human EDTA-plasma and urine after small doses of Cannabis sativa extracts*. J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci.*, 2007, *850,* 515-522.

[78] Citti C., Ciccarella G., Braghiroli D., Parenti C., Vandelli M.A., Cannazza G., Medicinal cannabis: Principal cannabinoids concentration and their stability evaluated by a high performance liquid chromatography coupled to diode array and quadrupole time of flight mass spectrometry method. *J. Pharm. Biomed. Anal.*, 2016, *128*, 201-209.

[79] Gambaro V., Dell'Acqua L., Farè F., Froldi R., Saligari E., Tassoni G., Determination of primary active constituents in cannabis preparations by high-resolution gas chromatography/flame ionization detection and high-performance liquid chromatography/UV detection. *Anal. Chim. Acta*, 2002, *468*, 245-254.

[80] Bäckström B., Cole M.D., Carrott M.J., Jones D.C., Davidson G., Coleman K., A preliminary study of the analysis of Cannabis by supercritical fluid chromatography with atmospheric pressure chemical ionisation mass spectroscopic detection. *Sci. Justice*, 1997, *37*, 91-97.

[81] Wang M., Wang Y.H., Avula B., Radwan M.M., Wanas A.S., Mehmedic Z., ElSohly M.A., Khan I.A., Quantitative Determination of Cannabinoids in Cannabis and Cannabis Products Using Ultra-High-Performance Supercritical Fluid Chromatography and Diode Array/Mass Spectrometric Detection. *J. forensic Sci.*, 2017, *62*, 602-611.

[82] Geryk R., Švidrnoch M., Přibylka A., Kalíková K., Maier V., Tesařová E., A supercritical fluid chromatography method for the systematic toxicology analysis of cannabinoids and their metabolites. *Anal. Methods*, 2015, *7*, 6056-6059.

[83] Radcliffe C., Maguire K., Lockwood B., Applications of supercritical fluid extraction and chromatography in forensic science. *J. Biochem. Bioph. Meth.*, 2000, *43,* 261-272.

[84] Cole M.D., in: Williams J.R., Clifford A.A. (Eds), Supercritical Fluid Methods and Protocols. Humana Press, New York 2000. pp. 145-148.

[85] Galand N., Ernouf D., Montigny F., Dollet J., Pothier J., Separation and identification of cannabis components by different planar chromatography techniques (TLC, AMD, OPLC). *J. Chromatogr. Sci.*, 2004, *42*, 130-134.

[86] Beasley E., Francese S., Bassindale T., Detection and Mapping of Cannabinoids in Single Hair Samples through Rapid Derivatization and Matrix-Assisted Laser Desorption Ionization Mass Spectrometry. *Anal Chem*, 2016, *88,* 10328-10334.

[87] Spencer S.E., Kim S.Y., Kim S.B., Schug K.A., Matrix-Assisted Laser Desorption/Ionization - Time of Flight Mass Spectrometry Profiling of Trace Constituents of Condom Lubricants in the Presence of Biological Fluids. *Forensic Sci. Int.,* 2011, *207,* 19-26.

[88] Kemp P.M., Abukhalaf I.K., Manno J.E., Manno B.R., Alford D.D., Abusada G.A., Cannabinoids in humans. I. Analysis of Δ9-tetrahydrocannabinol and six metabolites in plasma and urine using GC-MS. *J. Anal. toxicol.*, 1995, *19,* 285-291.

[89] Steinmeyer S., Bregel D., Warth S., Kraemer T., Moeller M.R., Improved and validated method for the determination of Δ 9-tetrahydrocannabinol (THC), 11-hydroxy-THC and 11-nor-9-carboxy-THC in serum, and in human liver microsomal preparations using gas chromatography–mass spectrometry. *J. Chrom. B*, 2002, *772*, 239-248.

[90] Kala M., Kochanowski M., Determination of Delta(9)-tetrahydrocannabinol (9THC) and 11-nor-9-carboxy-Delta(9)-tetrahydrocannabinol (THCCOOH) in blood and urine using gas chromatography negative ion chemical ionization mass spectrometry (GC-MS-NCI). *Chem. Anal. Warsaw*, 2006, *51,* 65-78.

[91] Moore C., Coulter C., Rana S., Vincent M., Snares J., Analytical procedure for the determination of the marijuana metabolite 11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid in oral fluid specimens. *J. Anal. Toxicol.,* 2006, *30,* 409-412.

[92] Schwilke E.W., Schwope D.M., Karschner E.L., Lowe R.H., Darwin W.D., Kelly D.L., Goodwin R.S., Gorelick D.A., Huestis M.A., Δ9-tetrahydrocannabinol (THC), 11-hydroxy-THC, and 11-nor-9-carboxy-THC plasma pharmacokinetics during and after continuous high-dose oral THC. *Clin. Chem.,* 2009, *55,* 2180-2189.

[93] Lowe R.H., Karschner E.L., Schwilke E.W., Barnes A.J., Huestis M.A., Simultaneous quantification of Δ 9-tetrahydrocannabinol, 11-hydroxy-Δ 9-tetrahydrocannabinol, and 11-nor-Δ 9-tetrahydrocannabinol-9-carboxylic acid in human plasma using two-dimensional gas chromatography, cryofocusing, and electron impact-mass spectrometry. *J. Chrom. A*, 2007, *1163*, 318-327.

[94] Leghissa A., Hildenbrand Z., Foss Jr. F.W., Schug K.A., Determination of the metabolites of Δ 9-Tetrahydrocannabinol using multiple reaction monitoring gas chromatography – triple quadrupole – mass spectrometry. In preparation.

[95] Park M., Kim J., Park Y., In S., Kim E., Park Y., Quantitative determination of 11-nor-9-carboxy-tetrahydrocannabinol in hair by column switching LC–ESI-MS 3. *J. Chrom. B*, 2014, *947*, 179-185.

[96] Mercolini L., Mandrioli R., Sorella V., Somaini L., Giocondi D., Serpelloni G., Raggi M.A., Dried blood spots: Liquid chromatography–mass spectrometry analysis of Δ 9-tetrahydrocannabinol and its main metabolites. *J. Chrom. A*, 2013, *1271*, 33-40.

[97] Weinmann W., Goerner M., Vogt S., Goerke R., Pollak S., Fast confirmation of 11-nor-9-carboxy-Δ 9-tetrahydrocannabinol (THC COOH) in urine by LC/MS/MS using negative atmospheric-pressure chemical ionisation (APCI). *Forensic Sci. Int.*, 2001, *121*, 103-107.

[98] Ilias Y., Rudaz S., Mathieu P., Christen P., Veuthey J.L., Extraction and analysis of different Cannabis samples by headspace solid-phase microextraction combined with gas chromatography-mass spectrometry. *J. Sep. Sci.*, 2005, *28*, 2293-2300.

[99] Omar J., Olivares M., Amigo J.M., Etxebarria N., Resolution of co-eluting compounds of Cannabis sativa in comprehensive two-dimensional gas chromatography/mass spectrometry detection with multivariate curve resolution-alternating least squares. *Talanta*, 2014, *121*, 273-280.

[100] Pellegrini M., Marchei E., Pacifici R., Pichini S., A rapid and simple procedure for the determination of cannabinoids in hemp food products by gas chromatography-mass spectrometry. *J. Pharm. Biomed. Anal.*, 2005, *36*, 939-946.

[101] Baptista M.J., Monsanto P.V., Marques E.G.P., Bermejo A., Ávila S., Castanheira A.M., Margalho C., Barroso M., Vieira D.N., Hair analysis for Δ 9-THC, Δ 9-THC-COOH, CBN and CBD, by GC/MS-EI: Comparison with GC/MS-NCI for Δ9-THC-COOH. *Forensic Sci. Int.*, 2002, *128*, 66-78.

[102] Villamor J.L., Bermejo A.M., Tabernero M.J., Fernandez P., Determination of cannabinoids in human hair by GC/MS. *Anal. Lett.*, 2004, *37,* 517-528.

[103] Emidio E.S., Prata V.D., Dorea H.S., Validation of an analytical method for analysis of cannabinoids in hair by headspace solid-phase microextraction and gas chromatography-ion trap tandem mass spectrometry. *Anal. Chim. Acta*, 2010, *610,* 63-71.

[104] de Oliveira C.D.R., Yonamine M., Moreau R.L.D., Headspace solid-phase microextraction of cannabinoids in human head hair samples. *J. Sep. Sci.*, 2007, *30,* 128-134.

[105] Chou S.L., Ling Y.C., Yang M.H., Pai C.Y., Determination of Δ 9-tetrahydrocannabinol in indoor air as an indicator of marijuana cigarette smoking using adsorbent sampling and in-injector thermal desorption gas chromatography–mass spectrometry. *Anal. Chim. Acta*, 2007, *598*, 103-109.

[106] Mehmedic Z., Chandra S., Slade D., Denham H., Foster S., Patel A.S., Ross S.A., Khan I.A., ElSohly M.A., Potency trends of Δ9-THC and other cannabinoids in confiscated cannabis preparations from 1993 to 2008. *J. Forensic Sci.*, 2010, *55,* 1209-1217.

[107] Teixeira H., Proença P., Castanheira A., Santos S., López-Rivadulla M., Corte-Real F., Marques E.P., Vieira D.N., Cannabis and driving: the use of LC–MS to detect Δ 9-tetrahydrocannabinol (Δ 9-THC) in oral fluid samples. *Forensic Sci. Int.*, 2004, *146*, S61-S63.

[108] Concheiro M., Lee D., Lendoiro E., Huestis, M.A., Simultaneous quantification of Δ 9-tetrahydrocannabinol, 11-nor-9-carboxy-tetrahydrocannabinol, cannabidiol and cannabinol in oral fluid by microflow-liquid chromatography–high resolution mass spectrometry. *J. Chrom. A*, 2013, *1297*, 123-130.

[109] Andersson M., Scheidweiler K.B., Sempio C., Barnes A.J., Huestis M.A., Simultaneous quantification of 11 cannabinoids and metabolites in human urine by liquid chromatography tandem mass spectrometry using WAX-S tips. *Anal. Bioanal. Chem.*, 2016, *408*, 6461-6471.

[110] Desrosiers N.A., Scheidweiler K.B., Huestis M.A., Quantification of six cannabinoids and metabolites in oral fluid by liquid chromatography-tandem mass spectrometry. *Drug Test. Anal.*, 2015, **7**, 684-694.

[111] Laloup M., Fernandez M.D.M.R., Wood M., De Boeck G., Henquet C., Maes V., Samyn N., Quantitative analysis of Δ 9-tetrahydrocannabinol in preserved oral fluid by liquid chromatography–tandem mass spectrometry. *J. Chrom. A*, 2005, *1082*, 15-24.

[112] Sergi M., Montesano C., Odoardi S., Rocca L.M., Fabrizi G., Compagnone D., Curini R., Micro extraction by packed sorbent coupled to liquid chromatography tandem mass spectrometry for the rapid and sensitive determination of cannabinoids in oral fluids. *J. Chrom. A*, 2013, *1301*, 139-146.

[113] Abbara C., Galy R., Benyamina A., Reynaud M., Bonhomme-Faivre L., Development and validation of a method for the quantitation of Δ 9 tetrahydrocannabinol in human plasma by high performance liquid chromatography after solid-phase extraction. *J. Pharm. Biomed. Anal.*, 2006, *41*, 1011-1016.

[114] Pandohee J., Holland B.J., Li B., Tsuzuki T., Stevenson P.G., Barnett N.W., Pearson J.R., Jones O.A., Conlan X.A., Screening of cannabinoids in industrial-grade hemp using two-dimensional liquid chromatography coupled with acidic potassium permanganate chemiluminescence detection. *J. Sep. Sci.*, 2015, *38,* 2024-2032.

[115] Gul W., Gul S.W., Radwan M.M., Wanas A.S., Mehmedic Z., Khan I.I., Sharaf M.H., ElSohly M.A., Determination of 11 cannabinoids in biomass and extracts of different varieties of Cannabis using high-performance liquid chromatography. *J. AOAC Int.,* 2015, *98,* 1523-1528.

[116] Giese M.W., Lewis M.A., Giese L., Smith K.M., Method for the Analysis of Cannabinoids and Terpenes in Cannabis. *J. AOAC Int.*, 2015, *98*, 1503-1522.

[117] Ameur S., Haddou B., Derriche Z., Canselier J.P., Gourdon C., Cloud point extraction of Δ9-tetrahydrocannabinol from cannabis resin. *Anal. Bioanal. Chem.*, 2013, *405,* 3117-3123.

[118] Fischedick J.T., Glas R., Hazekamp A., Verpoorte R., A qualitative and quantitative HPTLC densitometry method for the analysis of cannabinoids in Cannabis sativa L. *Phytochem. Anal.*, 2009, *20*, 421-426.

[119] Hazekamp A., Simons R., Peltenburg-Looman A., Sengers M., van Zweden R., Verpoorte R., Preparative isolation of cannabinoids from Cannabis sativa by centrifugal partition chromatography. *J. Liq. Chromatogr. Relat. Technol.*, 2004, *27,* 2421-2439.

[120] Nascimento I.R., Costa H.B., Souza L.M., Soprani L.C., Merlo B.B., Romão W., Chemical identification of cannabinoids in street marijuana samples using electrospray ionization FT-ICR mass spectrometry. *Anal. Methods*, 2015, *7*, 1415-1424.

[121] Iwamuro Y., Iio-Ishimaru R., Chinaka S., Takayama N., Hayakawa K., Analysis of 11-nor-Δ9-tetrahydrocannabinol-9-carboxylic acid and its glucuronide in urine by capillary electrophoresis/mass spectrometry. *Biomed. Chrom.*, 2012, *26,* 1452-1456.

[122] Szabady B., Hidvegi E., Nyiredy S., Determination of neutral cannabinoids in hemp samples by overpressured-layer chromatography. *Chromatographia*, 2002, *56,* S165-S168.

[123] Eiras M.M., De Oliveira D.N., Ferreira M.S., Benassi M., Cazenave S.O.S., Catharino R.R., Fast fingerprinting of cannabinoid markers by laser desorption ionization using silica plate extraction. *Anal. Methods*, 2014, *6,* 1350-1352.

[124] Voss A., Witt K., Kaschowitz T., Poitz W., Ebert A., Roser P., Bär K.J., Detecting cannabis use on the human skin surface via an electronic nose system. *Sensors*, 2014, *14,* 13256-13272.

[125] Tsai J.S., ElSohly M.A., Tsai S.F., Murphy T.P., Twarowska B., Salamone S.J., Investigation of nitrite adulteration on the immunoassay and GC-MS analysis of cannabinoids in urine specimens. *J. Anal. Toxicol.*, 2000, *24,* 708-714.

[126] Abraham T.T., Lowe R.H., Pirnay S.O., Darwin W.D., Huestis M.A., Simultaneous GC-EI-MS determination of Δ9-Tetrahydrocannabinol, 11-Hydroxy-Δ9-tetrahydrocannabinol, and 11-Nor-9-carboxy-Δ9-tetrahydrocannabinol in human urine following tandem enzyme-alkaline hydrolysis. *J. Anal. Toxicol.,* 2007, *31,* 477-485.

[127] Purschke K., Heinl S., Lerch O., Erdmann F., Veit F., Development and validation of an automated liquid-liquid extraction GC/MS method for the determination of THC, 11-OH-THC, and free THC-carboxylic acid (THC-COOH) from blood serum. *Anal. Bioanal. Chem.*, 2016, *408*, 4379-4388.

[128] Fabritius M., Chtioui H., Battistella G., Annoni J.M., Dao K., Favrat B., Fornari E., Lauer E., Maeder P., Giroud C., Comparison of cannabinoid concentrations in oral fluid and whole blood between occasional and regular cannabis smokers prior to and after smoking a cannabis joint. *Anal. Bioanal. Chem.*, 2013, *405*, 9791-9803.

[129] Van der Linden T., Silverans P., Verstraete A.G., Comparison between self-report of cannabis use and toxicological detection of THC/THCCOOH in blood and THC in oral fluid in drivers in a roadside survey. *Drug Test. Anal.*, 2014, *6,* 137-142.

[130] Nadulski T., Sporkert F., Schnelle M., Stadelmann A.M., Roser P., Schefter T., Pragst F., Simultaneous and sensitive analysis of THC, 11-OH-THC, THC-COOH, CBD, and CBN by GC-MS in plasma after oral application of small doses of THC and cannabis extract. *J. Anal. Toxicol.*, 2005, *29*, 782-789.

[131] Han E., Park Y., Kim E., In S., Yang W., Lee S., Choi H., Lee S., Chung H., myong Song J., Simultaneous analysis of Δ 9-tetrahydrocannabinol and 11-nor-9-carboxy-tetrahydrocannabinol in hair without different sample preparation and derivatization by gas chromatography–tandem mass spectrometry. *J. Pharm. Biomed. Anal.*, 2011, *55,* 1096-1103.

[132] Lee D., Bergamaschi M.M., Milman G., Barnes A.J., Queiroz R.H., Vandrey R., Huestis M.A., Plasma cannabinoid pharmacokinetics after controlled smoking and ad libitum cannabis smoking in chronic frequent users. *J. Anal. Toxicol.*, 2015, *39,* 580-587.

[133] Milman G., Barnes A.J., Lowe R.H., Huestis M.A., Simultaneous quantification of cannabinoids and metabolites in oral fluid by two-dimensional gas chromatography mass spectrometry. *J. Chrom. A*, 2010,*1217*, 1513-1521.

[134] Weinmann W., Vogt S., Goerke R., Müller C., Bromberger A., Simultaneous determination of THC-COOH and THC-COOH-glucuronide in urine samples by LC/MS/MS. *Forensic Sci. Int.*, 2000, *113*, 381-387.

[135] Hädener M., Weinmann W., van Staveren D.R., König S., Rapid quantification of free and glucuronidated THCCOOH in urine using coated well plates and LC–MS/MS analysis, *Bioanalysis*. DOI: 10.4155/bio-2016-0281

[136] Fernández M.D.M.R., Wille S.M., Samyn N., Wood M., López-Rivadulla M., De Boeck G., On-line solid-phase extraction combined with liquid chromatography–tandem mass spectrometry for high throughput analysis of 11-nor-Δ 9-tetrahydrocannabinol-9-carboxylic acid in urine. *J. Chrom. B*, 2009, *877,* 2153-2157.

[137] Ferreirós N., Labocha S., Walter C., Lötsch J., Geisslinger G., Simultaneous and sensitive LC–MS/MS determination of tetrahydrocannabinol and metabolites in human plasma. *Anal. Bioanal. Chem.*, 2013, *405,* 1399-1406.

[138] Scheidweiler K.B., Andersson M., Swortwood M.J., Sempio C., Huestis M.A., Long-term stability of cannabinoids in oral fluid after controlled cannabis administration. *Drug test. Anal.*, 2017, *9,* 143-147.

[139] He X., Kozak M., Nimkar S., Ultra-Sensitive Measurements of 11-Nor-Delta(9)-Tetrahydrocannabinol-9-Carboxylic Acid in Oral Fluid by Microflow Liquid Chromatography-Tandem Mass Spectrometry Using a Benchtop Quadrupole/Orbitrap Mass Spectrometer. *Anal. Chem.*, 2012, *84,* 7643-7647.

[140] Kuwayama K., Miyaguchi H., Yamamuro T., Tsujikawa K., Kanamori T., Iwata Y.T., Inoue H., Micro-pulverized extraction pretreatment for highly sensitive analysis of 11-nor-

9-carboxy-Δ9-tetrahydrocannabinol in hair by liquid chromatography/tandem mass spectrometry. *Rapid Commun. Mass Spectrom.*, 2015, *29,* 2158-2166.

[141] Thieme D., Sachs H., Uhl M., Proof of cannabis administration by sensitive detection of 11-nor-Delta(9)-tetrahydrocannabinol-9-carboxylic acid in hair using selective methylation and application of liquid chromatography-tandem and multistage mass spectrometry*. Drug Test. Anal.*, 2014, *6,* 112-118.

[142] Simoes S.S., Ajenjo A.C., Dias M.J., Qualitative and quantitative analysis of THC, 11-hydroxy-THC and 11-nor-9-carboxy-THC in whole blood by ultra-performance liquid chromatography/tandem mass spectrometry. *Rapid Commun. Mass Spectrom*, 2011, *25,* 2603-2610.

[143] König S., Aebi B., Lanz S., Gasser M., Weinmann W., On-line SPE LC-MS/MS for the quantification of Δ9-tetrahydrocannabinol (THC) and its two major metabolites in human peripheral blood by liquid chromatography tandem mass spectrometry. *Anal. Bioanal. Chem.,* 2011, *400*, 9-16.

[144] Casano S., Grassi G., Martini V., Michelozzi, M., Variations in Terpene Profiles of Different Strains of Cannabis sativa L. *Acta Hortic*., 2011, *925*, 115-121.

[145] Ferioli V., Rustichelli C., Pavesi G., Gamberini G., Analytical characterisation of hashish samples. *Chromatographia*, 2000, *52*, 39-44.

[146] Nissen L., Zatta A., Stefanini I., Grandi S., Sgorbati B., Biavati B., Monti A., Characterization and antimicrobial activity of essential oils of industrial hemp varieties (Cannabis sativa L.). *Fitoterapia*, 2010, *81*, 413-419.

Chapter 2

USING A VARIETY OF ANALYTICAL TOOLS TO ASSESS COMPLEX CANNABIS-INFUSED MATRICES[1]

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2.1 Abstract

Cannabis is currently one of the most widely studied plants in the world, due to the medicinal and pharmaceutical benefits its natural products. These benefits are primarily attributed to the presence of cannabinoids and terpenes, which can elicit a myriad of molecular responses. The increased interest in cannabis has led to a deeper knowledge of modern pharmacopeia, not only with respect to the all the possible medical benefits of cannabis constituents and their respective biochemical mechanisms of action, but also in reference to a comprehensive spectrum of its components within the contexts of discovery and quality control. Cannabis products can be found in many shapes and forms ranging from the raw plant material to extracts, which can be consumed directly or incorporated into a variety of products designed for human consumption. Such edible products can encapsulate cannabis constituents in a variety of different matrices, which require different sample preparation considerations due to their variable complexity and profiles of interferences. In many cases, edibles represent a significant challenge because there are many various different types that can be prepared, each one of which may require different suitable sample preparation techniques to isolate the cannabinoids for an effective analytical determination. Here, different edibles have been considered based on their composition, and different sample preparation strategies are discussed that may be appropriate for analyzing different products. The choices for different sample preparation methods have been considered based on prior food chemistry literature. This information is adapted to provide recommendations for the targeted determination of cannabinoids within the context of each method, the matrix of interest, and subsequent choice of instrumental determination, such as liquid chromatography or gas chromatography. We focus on the different research approaches used in the past for the comprehensive analysis

of cannabis and related products. Challenges and the possible solutions will be highlighted to provide insight into what the future may require in terms of more reliable methods for the characterization of cannabinoids in complex matrices, such as edibles.

2.2 Introduction

As of 2017, 29 States plus the District of Columbia support the legal use of medicinal *Cannabis sativa* and *Cannabis indica,* 8 of which also allow recreational use [1]. These numbers illustrate the importance of performing proper quality assurance/ quality control (QA/QC) with a comprehensive analysis of all cannabis products, which should include the cannabinoids (the main components of the plant that confer its psychoactivity and medical benefits) and terpenes, but also any potential contaminants, such as pesticides, growth regulators, heavy metals, microbes, pests, and residual solvents (Table 2.1) [2–4].

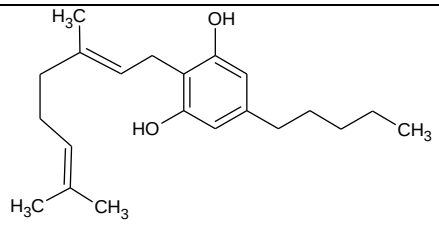
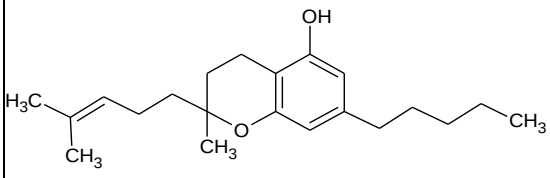
Table 2.1 List of pertinent biological and chemical contaminant that can arise during the various stages of cannabis cultivation and product management. Detection of any of these above their designated safety level renders the product in question unsafe for consumption. *CFU, Colony-Forming Units; ppm, parts-per-million.*

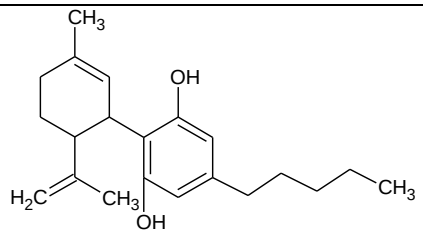
Contaminant	Classification	Safety Level
Spider mite	Biological-Insect	<1[1]
Fungus gnat	Biological-Insect	<1[1]
Leaf Miner	Biological-Insect	<1[1]
Aphid	Biological-Insect	<1[1]
Aspergillus flavus	Biological-Fungi	<1 CFU/gram[1]
Aspergillus fumigatus	Biological-Fungi	<1 CFU/gram[1]

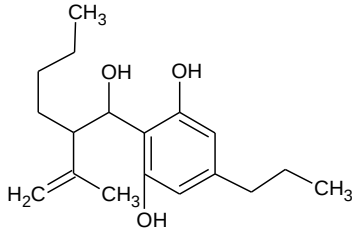
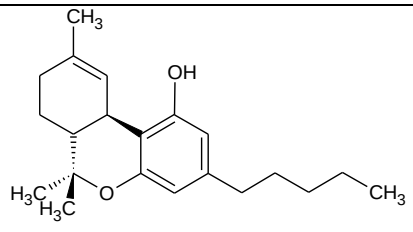
Aspergillus niger	Biological-Fungi	<1 CFU/gram[1]
Aspergillus terreus	Biological-Fungi	<1 CFU/gram[1]
Total Mold and Yeast	Biological-Fungi	<10,000 CFU/gram[1]
E. coli	Biological-Bacterium	<100 CFU/gram[1]
H7:0157 *E. coli*	Biological-Bacterium	<1 CFU/gram[1]
Pseudomonas aeruginosa	Biological-Bacterium	<1 CFU/gram[1]
Clostridium botulinum	Biological-Bacterium	<1 CFU/gram[1]
Salmonella enterica	Biological-Bacterium	<1 CFU/gram[1]
Listeria monocytogenes	Biological-Bacterium	<1 CFU/gram[1]
Myclobutanil (Eagle 20)	Chemical-Fungicide	<0.5 ppm
Quinoxyfen	Chemical-Fungicide	<0.5 ppm
Azoxystrobin	Chemical-Fungicide	<0.5 ppm
Difenoconazole	Chemical-Fungicide	<0.5 ppm
Imidacloprid	Chemical-Pesticide	<0.5 ppm
beta-Cyfluthrin	Chemical-Pesticide	<0.5 ppm
Bifenthrin	Chemical-Pesticide	<0.5 ppm
Paclobutrazol	Chemical-Growth Regulator	<0.5 ppm
Daminozide	Chemical-Growth Regulator	<0.5 ppm
Propane	Chemical-Hydrocarbon	500 ppm[2]
Isobutane	Chemical-Hydrocarbon	500 ppm[2]
n-Butane	Chemical-Hydrocarbon	500 ppm[2]
Hexane	Chemical-Hydrocarbon	290 ppm[3]

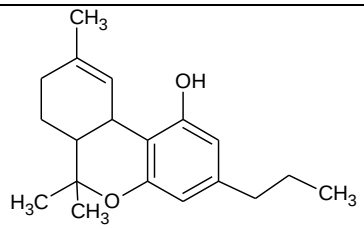
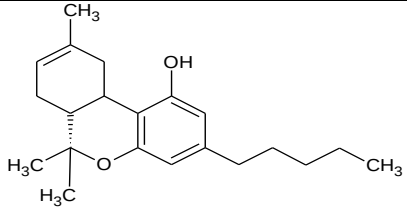
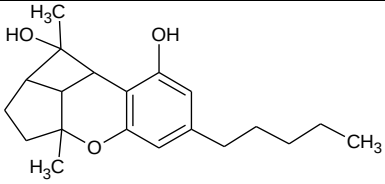
Methanol	Chemical-Alcohol	3,000 ppm[3]
Ethanol	Chemical-Alcohol	5,000 ppm
Isopropanol	Chemical-Alcohol	500 ppm[2]
Acetonitrile	Chemical-Solvent	410 ppm[3]
Acetone	Chemical-Solvent	500 ppm[2]
Chloroform	Chemical-Solvent	60 ppm[3]

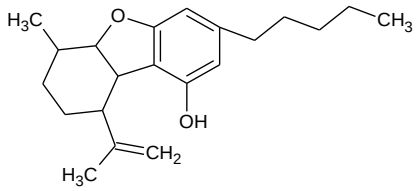
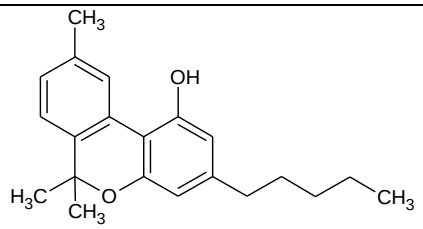
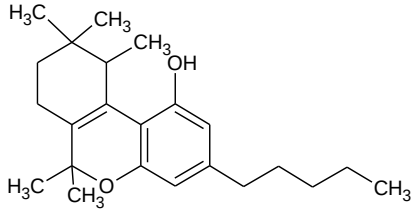
More than 100 different putative cannabinoids have been discovered in cannabis, 10 of which are ubiquitously found in numerous cultivars (Table 2.2 lists the more prevalent cannabinoids) [1,5–10].

*Table 2.2 **T**he ten classes of cannabinoids*			
Name	Abbreviation	Structure	Medical benefits
cannabigerol	CBG		Sleep aid, inhibits cancer cell growth, slows bacterial growth, promotes bone growth [5].
cannabichromene	CBC		Inhibits cancer cell growth, promotes bone growth, reduces inflammation, pain reliever, antibacterial, anti-fungal, anti-depressant [5,6,7]

cannabidiol	CBD		Antibacterial, inhibits cancer cell growth, neuroprotective, reduces seizure and convulsions, reduces both blood sugar levels, reduces inflammation, reduces risk of artery blockage, antiemetic, reduces nausea, pain reliever, anxiolytic, suppresses muscle spasms, tranquillizer, vasorelaxant, modulates THC-induced psychoactivity

			[8,9]
cannabidivarin	CBDV		Reduces hyperexcitability, reduces epilepsy, reduces nausea [5]
Δ9-tetrahydrocannabinol	Δ9-THC		Reduces vomiting and nausea, relieves pain, stimulates appetite, suppresses muscle spasms [5]

tetrahydrocannabivarin	THCV		Reduces convulsions and seizures, promotes bone growth, stimulates energy and metabolism, improves glucose intolerance, anti-inflammatory, anti-convulsant [5,10]
Δ8-tetrahydrocannabinol	Δ8-THC		Pain reliever [5]
cannabicyclol	CBL		No reported bioactivity

cannabielsoin	CBE		No reported bioactivity
cannabinol	CBN		Sleep aid, suppresses muscle spasms, pain reliever, appetite stimulant, anti-tumorigenic against some types of lung cancer [5].
cannabitriol	CBT		No reported bioactivity.

Some of these are well known, such as Δ9-tetrahydrocannabinol (Δ9-THC, the main psychoactive component) and cannabidiol (CBD, has the widest range of reported medical benefits). However, it is believed that the therapeutic effects of cannabis are not exclusively attributable to any single cannabinoid, but rather to their synergistic effects when administered in different combinations and in concert with numerous terpenes [11].

Terpenes are a class of molecules found in cannabis that are primarily responsible for odor and fragrances, while exhibiting antimicrobial, antioxidant, and anti-inflammatory properties. More than 30,000 terpenes exist in the natural world, 100 of which have been detected in cannabis. These molecules all derive from isoprene subunits and can be categorized based on the number of C atoms: hemiterpenes (C_5), monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), and sesteterpenes (C_{25}) [1,12,13]. Terpenes from the same categories may be differentiated based on the number of double bonds, but also based on the position of the double bonds or atom connectivity. This compound class is characterized by a large number of isomeric structures, which makes their individual speciation challenging. Another category of terpenes are the so-called "oxygenated terpenoids", which differ due to the presence of one or more O atoms. Some of the most common terpenes are technically terpenoids, since they contain at least an O atom (e.g. linalool belongs to this group) [12].

To satisfy consumers' different tastes, there are different ways to consume or use cannabis, with the most popular means being through smoking plant material, utilizing sublingual tinctures, and consuming edibles [14]. The latter is sometimes preferred because edibles are said to be more discreet for consumers, and the toxins linked to smoking can be avoided [14]. Another reason why edibles are becoming so important is because of their longer-lasting psychoactive effects. Edibles usually provide peak effects at 2-4 hours after ingestion, in contrast to the peak at 20-30 minutes following inhalation [14]. The reason for this difference is the pathway by which Δ9-THC is metabolized throughout the human body. After consumption and once in the liver, Δ9-THC is hydrolyzed enzymatically (mainly, cytochrome P450) to 11-hydroxy-Δ9-tetrahydrocannabinol (11-OH-Δ9-THC) [14–16]. This metabolite confers higher psychoactivity than Δ9-THC, and it is actually believed to be a more active form of the cannabinoid, since it is able to cross the

blood-brain barrier more easily [14–16]. Analysis performed on blood has shown that 11-OH-Δ9-THC is found at higher levels in the human body after cannabis ingestion, compared than inhalation [14]. However, this metabolic difference can result in undesirable effect. Due to the delayed onset of Δ9-THC during ingestion, non-experienced consumers can consume greater amounts than what is suggested, leading to adverse effects. As such, properly dosing cannabis edibles, which is guided by the accurate quantification of the psychotropic cannabinoids, is of paramount importance for individuals who wish to retain the therapeutic benefits of cannabis without the potential pulmonary impacts that can result from smoking.

The scope of this review includes an overview of traditional analytical methods for cannabis natural products and contaminants of concern. Furthermore, different methods for extracting and analyzing cannabis edibles are discussed within the context of matrices complexity. Unfortunately, only a limited amount of literature exists on the topic of cannabis edibles analysis; however, a number of insightful parallels can be drawn from available literature in the food sciences. For a systematic approach, edibles have been classified into different subcategories based on the potential interferences they can contain. For example, gummy bears contain mainly sugars and glycerin, which ought to be well differentiable from cannabinoids, but might cause issues in terms of pesticides analysis. In contrast, cannabis-infused fermented beverages can contain ethanol, which can render the quantification of cannabinoids difficult. Further to this point, baked goods can contain a multitude of fats, proteins, fiber and other ingredients, which can hamper the extraction and analysis of targeted components. Additionally, the inherent hydrophobicity of cannabinoids can drive variable binding to individual compounds of edibles products. As such different matrices, and potential for heterogeneous cannabinoid distribution through an edible product, require different sample preparations and selective analytical platforms

can also increase accuracy and precision of determinations. As the scope of products incorporating cannabinoids expands, so too must the analytical methods available to provide reliable quality control of these products.

2.3 Analysis

Currently, there are many different methods used for the characterization of cannabis, but gas chromatography (GC) and liquid chromatography (LC) dominate the scene. In particular, LC is widely used for potency testing [1]. Cannabinoids and their metabolites can be analyzed both on GC and LC, coupled with different detectors (for example, mass spectrometry, for trace analysis). LC with ultraviolet detection is often sufficient for potency testing, especially for samples of direct plant extracts that can contain carboxylated and decarboxylated constituents. However, as the matrix becomes more complicated, a more selective detection for particular potency elements may be required. Tandem mass spectrometry becomes quite attractive in this case, irrespective of whether analysis is performed by GC or LC. Recently, the potential for use of vacuum ultraviolet absorption spectroscopic detection in combination with GC (GC-VUV) has been demonstrated for cannabinoid speciation [17]. GC-VUV offers some advantages for differentiation of isomers and deconvolution of coeluting peaks.

On the other hand, terpenes, as well as residual solvents, are best analyzed by GC due to their high volatility. Different detectors may be used, and usually mass spectrometry (MS) is not required, though it does provide a high degree of qualitative information. Even so, the isomeric nature of terpenes can make them difficult to differentiate based on electron impact mass spectra. To help alleviate this problem, GC-VUV has also been demonstrated for speciating terpenes [18,19]. VUV absorption spectra

have been shown in many cases to be highly complementary to mass spectra for differentiation of isomer species [20].

A tricky class of compounds to address are pesticides. The methodology for analysis of more than 500 pesticides in a vast array of food products and matrices exist and are used routinely worldwide. Virtually an entire conference, the North American Chemical Residues Workshop (www.nacrw.org), is dedicated to discussing and advancing the state-of-the-art in pesticide analysis. In the cannabis industry, pesticide testing is dictated by state regulations, and thus, lists of required pesticides can vary from state to state, with some lists still being developed and refined. To address the full complement of pesticides of concern worldwide, both LC- and GC-based methods are required because of the wide range of physicochemical properties, such as volatility, exhibited by different pesticide classes. With that being said, even restricted lists of target pesticides will require testing labs to have both LC and GC instruments available to test for an adequate range of compounds with the necessary sensitivity [19,21,22]. While it is important to verify the lack of pesticides in the original plant if it is to be consumed directly, further thought should be given to how the plant is processed to produce other products, and how different pesticides may transfer through the process. For example, many pesticides are thermally labile, and can degrade if heat is used during the extraction process [23,24].

Heavy metals and microbes are also often tested in cannabis plants and extracts using techniques such as inductively-coupled plasma-mass spectrometry (ICP-MS) for the former, and traditional biochemical methods or matrix-assisted laser desorption/ionization – mass spectrometry (MALDI-MS) for the latter [1,25]. Heavy metals accumulated from the environment can be concentrated from plant matter into different products during processing. On the other hand, a large majority of microbes do not survive many extraction or processing steps, especially if they involve organic solvents. Contamination by different

fungi can be an alternate consideration, as these microorganisms can produce small molecule toxins, which are more robust than pesticides and can be transferred through different extraction or processing steps [26].

Therefore, due to the different components that can be found in the plant, it is important to remember that one analysis method is not enough for comprehensive speciation of the different components of interest, because each analyte requires different preparation.

2.4 Edibles

Edibles are growing in importance among cannabis consumers, not only for their easy preparation and consumption, but also for their longer-lasting effects [14]. There are many different categories of edibles, many of which are prepared with cannabis oil or butter. Cannabis oil is prepared by extracting Cannabis flowers with different solvents, such as naphtha, petroleum ether, ethanol, or olive oil [27,28]. Romano and Hazekamp [27] performed an extraction comparison with these 4 different solvents, considering not only the recoveries of both cannabinoids and terpenes, but also the residual solvents. Their conclusion was that olive oil was the most efficient extraction solvent, not only because of its lack of toxicity and non-flammability, but also because it extracted the highest amount of terpenes.

The primary issue with the characterization of cannabis natural products in edibles is due to the varying matrix effects that can associated with different final products. In edibles, all the normal food's components must be taken in consideration, including fatty acids, sugars, sugar alcohols, proteins, fiber, etc.; therefore, effective sample preparation is mandatory prior to analysis.

A primary reason for the analysis of edibles is to verify label accuracy of products. Vandrey et al. [29], in a 2015 research study, found that Δ9-THC and CBD content was within 10% of the labeling of the 75 analyzed products. 17% of the products were correctly labeled, 23% were underlabeled, and 60% were overlabeled with regard to Δ9-THC content. In earlier work, Pellegrini et al. [30] also analyzed different types of edibles (beer, liquor, oil, etc.) all derived from hemp. They extracted all the samples with hexane: isopropanol (9:1), looking exclusively at Δ9-THC, CBD, and cannabinol (CBN) content. They found that Δ9-THC had the highest concentration in all the samples, contrary to the prior literature. The authors suggested that this expression of Δ9-THC were more indicative of a cannabis than a hemp phenotype [30].

A different approach was taken by Zoller et al. [31]. They analyzed different matrices comparing two different extraction methods. Hempseed oil and hemp tea were prepared from a methanol extract, and hempseed, biscuits, and herb were prepared from a methanol: dichloromethane (9:1) extraction mixture. They compared the analysis with HPLC-UV and HPLC with fluorescence detection, with the latter being extremely selective, making this detector a viable tool for the detection of Δ9-THC [31]. Furthermore, none of the extracted samples showed interference peaks in the chromatogram, meaning that the extraction steps were effective.

Many other studies have been performed on Cannabis oil, always using a solvent extraction protocol to quantify the amount of Δ9-THC [32,33]. However, none of these studies aimed to determine impurities (e.g. pesticides) or trace cannabinoids. If the final aim is to focus more on determination of trace impurities to guarantee safety of the product, then a more sensitive analysis method and a more efficient extraction technique is needed. A valid solution is the use of "Quick, Easy, Cheap, Effective, Rugged, and Safe" kit (QuEChERS), a combined liquid-liquid extraction (LLE) and dispersive solid phase

extraction method that is already widely used for the analysis of pesticides [23,34,35]. This kit uses also an extraction salt to improve the efficiency in case of water-soluble analytes, and this also improves the cleanliness of the extract. Unfortunately though, the acetonitrile layer still contains some matrix components such as fats, sugars, and pigments. For this reason, it is important to couple the extraction step with a sensitive and specific method that will allow the detection of low concentration cannabinoids and/or pesticides. Either LC with tandem mass spectrometry detection (MS/MS) or GC-MS/MS (or both) could be viable or needed, depending on the specific targets.

In general, it could be useful to differentiate foods based on their matrix composition in order to choose the most suitable extraction technique. Tanner et al. [36] suggested the use of a triangle based on the normalized content of the three main components of food, namely fats, proteins, and carbohydrates (Figure 2.1), to describe different food compositions. This scheme was then divided into 9 different sectors, each one with different concentrations of those components. The idea is that if one extraction method is suitable for a type of food, then it would be suitable for all the foods in the same sector.

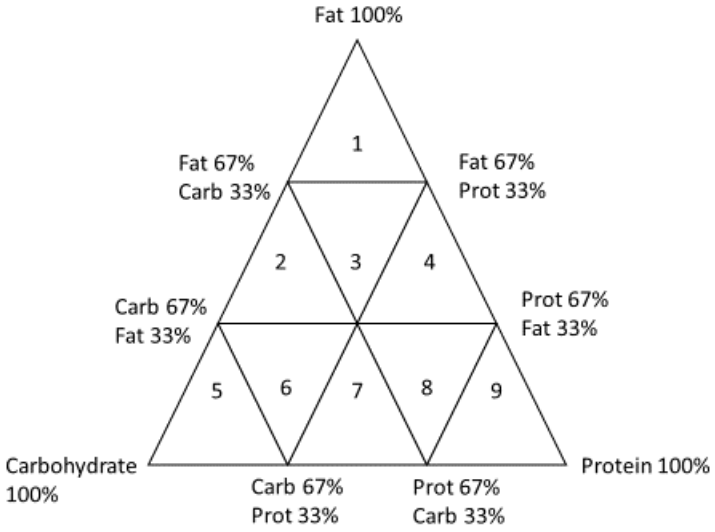


Figure 2.1 Food composition triangle based on the normalized content of the three main components of food.

Marazuela et al. [37] and Kinsella et al. [38] have provided comprehensive reviews about the different extraction methods for food analysis, ranging from off-line extraction to sample purification. One particularly promising and popular technique is pressurized liquid extraction (PLE). This automated technique is carried out at higher temperatures than the boiling point of the solvent, by keeping it in the liquid phase with high pressure. This results in increased throughput and low solvent consumption, but the required equipment is fairly expensive. Furthermore, even if the conditions are optimized, some other matrix components may be extracted as well, requiring therefore a cleanup step prior the analysis. The most used techniques in this case are solid phase extraction (SPE), pre-PLE, and matrix solid phase dispersion (MSDP). Pre-PLE could be performed with a non-polar solvent to eliminate the hydrophobic interferents, while if the sample has a high concentration of fats, fat-retaining sorbents can be used (such as alumina or silica gel) [38].

Microwave-assisted extraction (MAE) is another common food extraction technique. MAE uses microwave energy to heat a solvent and to extract the analyte from the matrix into the solvent in a confined vessel at a specified temperature [37]. An average extraction is 15 minutes, and this approach offers higher throughput (parallel processing with many systems) and low solvent consumption [38]. There are two modes of operation for MAE, focused or closed, that use open and closed vessels, respectively. The former works at atmospheric pressure, while the latter under high pressures, making it similar to PLE, since the pressure can be increased with temperature [38]. A drawback of this extraction is the difficulty of extracting analytes in matrices with more than 30% of water, due to the limited diffusion of the solvent [37]. Furthermore, even in this case an additional clean-up step is usually required.

Another extraction technique that is making a resurgence due to the renewed availability of commercial instrumentation is supercritical fluid extraction (SFE). SFE uses the properties of a supercritical fluid (solvability and density of a liquid, with the viscosity and diffusivity of a gas) to extract the compound of interest [38]. This method is useful because the solvating power can significantly improve by slightly changing the temperature and the pressure of the fluid. Usually, carbon dioxide (CO_2) is widely used as SF due to its inertness, low cost, high purity, low toxicity, and low critical point conditions, but if the analyte is strongly bound to the matrix, a more polar SF can be used. It is very common to add organic modifiers, such as methanol, to increase the extractability of more polar compounds [38]. It has been shown by Veress that this technique extracts CBD faster than Δ9-THC, because it is dissolved more easily by supercritical CO_2 [39,40]. Diaz-Maroto et al. showed that the extraction of terpenes does not require the use of any modifier, as it was expected due to the low polarity of these molecules [41].

Being one of the most widely used sample preparation techniques, SPE is available in different formats, the most common being packed particle beds. As an alternate format, sorbent-impregnated disks are available that can withstand high flow rates, but these have been primarily used for environmental analysis (e.g., water sampling). It can be difficult to obtain consistent flow for automated clean-up [37,38]. Dispersive-SPE (DSPE), where functionalized solid particles are dispersed on solution, was mentioned previously as a common first step for QuEChERS extraction [38]. DSPE can be effective on its own, but requires a filtration step after extraction to remove the particles. Balancing the functionality of the sorbent with the physicochemical nature of the analytes (or interferences) desired to be removed would be important as different edible compositions are considered.

Another type of SPE involves the use of molecularly imprinted polymers (MIPs), which are crosslinked polymers designed to exhibit high affinity towards specific compounds or a class of compounds [38]. One of the biggest drawbacks is the need to prepare the highly selective material. It has also been reported that the potential for leaking can contaminate sample extracts [37], but this is likely due to the format of the material in some specific designs. Another alternative, matrix solid phase dispersion (MDSP) was introduced in 1989. It combines homogenization, disruption, extraction, and clean-up in one process. The sample is blended with a dispersing agent, then packed into a column for extraction and clean-up [37]. As of now, this technique has been mainly used in environmental, clinical, and food analysis, and mainly for the analysis of antibacterial residues [42,43].

Restricted access material (RAMs) is another form of solid phase extraction. RAM uses porous chromatographic supports to achieve extraction of small molecules, in the presence of protein or large molecule interferences [44,45]. High molecular weight species

are excluded from the pores based on a size-exclusion mechanism; macromolecules elute with the dead volume, while small molecules penetrate the pores and are retained by functional units (e.g., reversed phase or ion exchange) contained therein. As with traditional SPE, care needs to be taken in the use of RAM regarding the co-extraction of interferences such as fats when targeting hydrophobic cannabinoid compounds.

Another molecular weight cut-off clean-up is ultra-filtration (UF), used to separate macromolecules such as proteins, peptides and lipids from the analyte of interest [38,46]. While effective, these membranes can be subject to fouling due to the precipitation of proteins, microorganisms, and fats, and therefore need to be constantly cleaned [47,48].

Turbulent flow chromatography (TFC), like RAM, has been primarily designed as an on-line sample preparation format. Thus, TFC gives both high sample throughput and high reproducibility due to automation [49,50]. It consists of columns packed with large particle size particles, with which small analytes will interact. Turbulent flow is used to limit the interactions by large molecules (slow diffusion coefficients) with the sorbent. The drawback of this technique is the consumption of high volume of solvent [38].

Finally, the last clean-up technique that was analyzed was dialysis [38]. It is not selective, but the cell is easy to build, and the technique is efficient for the removal of macromolecules, since only small molecules are allowed to pass through the membrane [51,52]. This technique can be used in edibles that have a high in protein matrix, such as protein bars and smoothies, and it is generally based on the removal of salts and sugars, but could be modified by using a membrane that is impermeable to the analyte of interest but permeable to interfering matrix components.

While sample preparation is an important step to avoid interferences, the instrumental analysis step can also be chosen so that it provides sufficient sensitivity and specificity. Due to the difficulties in removing interferences solely by sample preparation

techniques, Leghissa et al. introduced the use of gas chromatography-triple quadrupole-mass spectrometry (GC-QqQ-MS) for the analysis of 7 underivatized cannabinoids and 8 silylated cannabinoids, reaching LODs in the order of picograms on column [53]. This technique is based on the fact that the focus of the analysis is on the specific transitions that each cannabinoid creates in the MS, so that the noise and impurities (compounds of non-interest) will not be detected. The advantage of using this method is the ability to analyze low-level cannabinoids even in a complex and dirty matrix, making it a viable tool for the analysis of impurities. As it was stated previously, the analysis of cannabinoids via GC-MS is easy and leads to high sensitivity and selectivity, but it must follow a derivatization reaction in order to protect the carboxyl groups of the acidic cannabinoids, and the most common technique is silylation [53]. The study of the MRM transitions of these compounds (both underivatized and in their sylilated forms) highlighted how cannabinoids undergo the same fragmentation pathways, tool and hypothesis that may be used in the future for the characterization of unknown species (Figure 2.2).

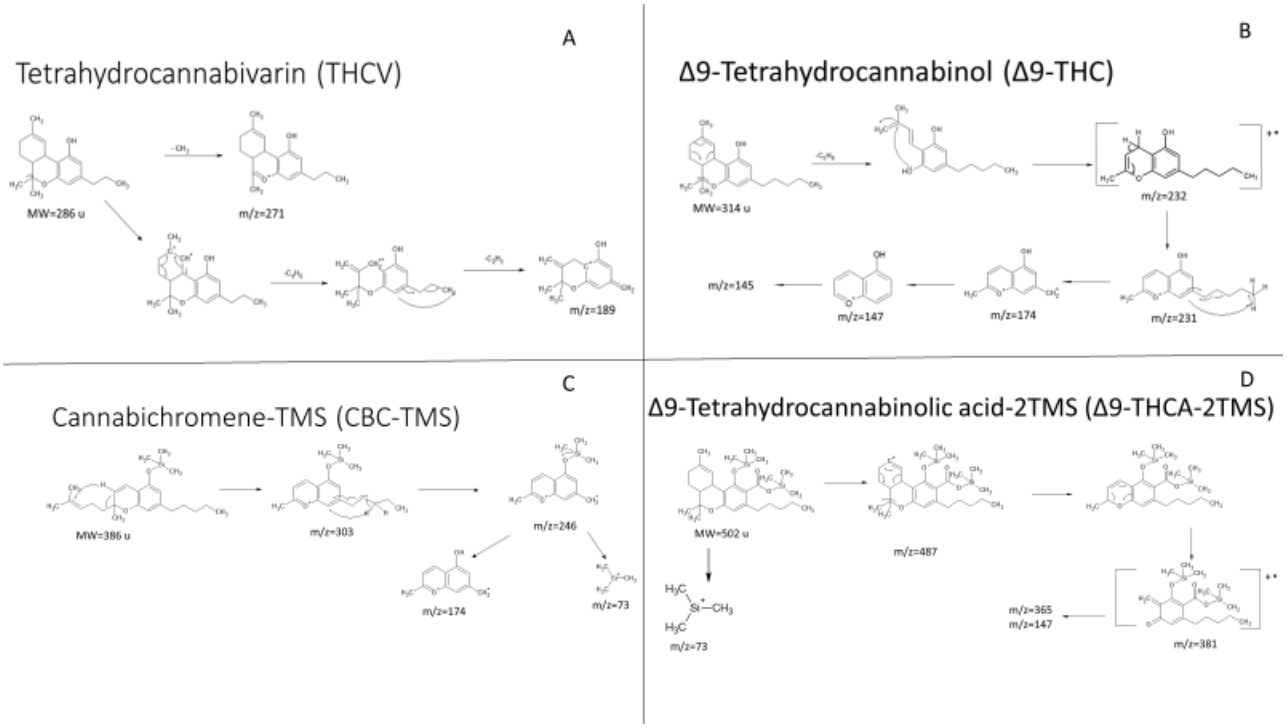


Figure 4.2 Suggested fragmentation pathways of (a) THC V, (b) Δ9-THC , (c) CBC -TMS, and (d) Δ9-THCA -2TMS via GC –MS.

As it is noticeable in Figure 2.2, the most common fragmentation that underivatized cannabinoids undergo is the Retro-Diels Alder reaction followed by the loss of the C side chain. Due to the different starting molecular weights, this reaction leads to different m/z values. For the derivatized cannabinoids, on the other hand, the most recurrent steps are the loss of a trimethylsilyl group and of the C side chain [53].

Another way of analyzing cannabinoids is via LC-MS, that does not require a derivatization reaction prior analysis, but that is not suitable for the analysis of other components of Cannabis that may be of interest (such as terpenes). Even with this instrument, high specificities can be achieved with the use of an MRM mode, but it is

important to remember that the transitions created with those type of sources (ESI and APCI) are not constant from instrument to instrument, and therefore it is impossible to create a database, but sample derivatization is not required.

Lastly, another instrument that is gaining a lot of recognition in the pharmaceutical field is SFC-MS/MS, due to its lower solvent use. When coupled with an SFE, this instrument can also be used for the simultaneous analysis of analytes with different polarities, due to the possibility to adjust the extraction capability of supercritical CO_2 by adding an organic modifier.

2.5 Future Directions

The future of cannabis analysis still has an unknown fate, partially due to the fact that each state has its own regulations, and in some cases, these regulations are still being formulated as the legality of medicinal and recreational cannabis use continues to evolve. Since edibles are becoming more and more important among cannabis consumers, it is important for producers and labs to develop standardized tests not only for their potency, but also for all the other analytes of interest. Unfortunately, as of now there are no guidelines that specifically apply to edibles, and even cannabis regulations vary from state to state. The possibility for laboratories to use an approved protocol for the quality control and potency test is the first step for the manufacturing of cannabis products; however, additional efforts should be spent on the analysis of metabolites and pharmacokinetics upon ingestion, so that a greater degree of confidence can be had with edibles versions of this new frontier of medicine.

2.6 References

[1] Leghissa A.,Hildenbrand Z. L.,Schug K. A., A Review of Methods for the Chemical Characterization of Cannabis Natural Products. *J. Sep. Sci.* 2018, *41*, 398–415 .

[2] Holmes M.,Vyas J. M.,Steinbach W.,McPartland J., Microbiological Safety Testing of Cannabis. 2015.

[3] Chapter 314-55: Marijuana Processor License Extraction Requirements. Washington State Legislature 2014.

[4] United States Pharmacopeial Convention, Chapter 467: Residual Solvents. 2007.

[5] Eisohly H. N.,Turner C. E.,Clark A. M.,Eisohly M. A., Synthesis and antimicrobial activities of certain cannabichromene and cannabigerol related compounds. *J. Pharm. Sci.* 1982, *71*, 319– 1323 .

[6] El-Alfy A. T.,Ivey K.,Robinson K.,Ahmed S.,Radwan M.,Slade D.,Khan I.,ElSohly M. A.,Ross S., Antidepressant-like effect of Δ9-tetrahydrocannabinol and other cannabinoids isolated from Cannabis sativa L. *Pharmacol. Biochem. Behav.* 2010, *95*, 434–442 .

[7] Grotenhermen F.,Müller-Vahl K., The Therapeutic Potential of Cannabis and Cannabinoids. *Dtsch. Arztebl. Int.* 2012, *109*, 495–501 .

[8] Beutler J. A.,Marderosian A. H., Chemotaxonomy of Cannabis I. Crossbreeding between Cannabis sativa and C. ruderalis, with analysis of cannabinoid content. *Econ. Bot.* 1978, *32*, 387–394 .

[9] Brenneisen R., Chemistry and Analysis of Phytocannabinoids and Other Cannabis Constituents. Humana Press 2007.

[10] Wargent E. T.,Zaibi M. S.,Silvestri C.,Hislop D. C.,Stocker C. J.,Stott C. G.,Guy G. W.,Duncan M.,Di Marzo V.,Cawthorne M. A., The cannabinoid Δ9-tetrahydrocannabivarin (THCV) ameliorates insulin sensitivity in two mouse models of obesity. *Nutr. Diabetes* 2013, *3*, E68 .

[11] Russo E. B., Taming THC: Potential cannabis synergy and phytocannabinoid-terpenoid entourage effects. *Br. J. Pharmacol.* 2011, *163*, 1344–1364 .

[12] Breitmaier E., Terpenes: Flavors, Fragrances, Pharmaca, Pheromones. John Wiley & Sons 2006.

[13] Devon T. K., Handbook of Naturally Occurring Compounds. Elsevier 2012.

[14] Barrus D.,Capogrossi K.,Cates S.,Gourdet C.,Peiper N.,Novak S.,Lefever T.,Wiley J., Tasty THC: Promises and Challenges of Cannabis Edibles. 2016.

[15] Iribarne C.,Berthou F.,Baird S.,Dréano Y.,Picart D.,Bail J. P.,Beaune P.,François Ménez J., Involvement of Cytochrome P450 3A4 Enzyme in the N-Demethylation of Methadone in Human Liver Microsomes. *Chem. Res. Toxicol.* 1996, *9*, 365–373 .

[16] Matsunagal T.,Lwawaki Y.,Watanabe K.,Yamamotol K.,Kageyama T.,Yoshimura H., Metabolism of Δ9-tetrahydrocannabinol by cytochrome P450 isozymes purified from hepatic microsomes of monkeys. *Life Sci.* 2312, *56*, 2089–2095 .

[17] Leghissa A.,Smuts J.,Qiu C.,Hildenbrand Z. L.,Schug K. A., Detection of cannabinoids and cannabinoid metabolites using gas chromatography-vacuum ultraviolet spectroscopy. *Sep. Sci. Plus* 2018, *1*.

[18] Qiu C.,Smuts J.,Schug K. A., Analysis of terpenes and turpentines using gas chromatography with vacuum ultraviolet detection. *J. Sep. Sci.* 2017, *40*, 869–877 .

[19] Schug K. A.,Sawicki I.,Carlton D. D.,Fan H.,Mcnair H. M.,Nimmo J. P.,Kroll P.,Smuts J.,Walsh P.,Harrison D., Vacuum Ultraviolet Detector for Gas Chromatography. *Anal. Chem.* 2014, *86*, 8329–8335 .

[20] Skultety L.,Frycak P.,Qiu C.,Smuts J.,Shear-Laude L.,Lemr K.,Mao J. X.,Kroll P.,Schug K. A.,Szewczak A.,Vaught C.,Lurie I.,Havlicek V., Resolution of isomeric new designer stimulants using gas chromatography – Vacuum ultraviolet spectroscopy and theoretical computations. *Anal. Chim. Acta* 2017, *971*, 55–67 .

[21] Wong J. W.,Webster M. G.,Bezabeh D. Z.,Hengel M. J.,Ngim K. K.,Krynitsky A. J.,Ebeler S. E., Multiresidue Determination of Pesticides in Malt Beverages by Capillary Gas Chromatography with Mass Spectrometry and Selected Ion Monitoring. *J. Agric. Food Chem.* 2004, *52*, 6361–6372 .

[22] Alder L.,Greulich K.,Kempe G.,Vieth B., Residue analysis of 500 high priority pesticides: Better by GC-MS or LC-MS/MS? *Mass Spectrom. Rev.* 2006, *25*, 838–865 .

[23] Anastassiades M.,Lehotay S. J.,Stajnbaher D., 18th Annual Waste Testing and Quality Symposium Proceeding. Arlington, VA 2002, pp. 231–241.

[24] Hayward D. G.,Wong J. W.,Shi F.,Zhang K.,Lee N. S.,Dibenedetto A. L.,Hengel M. J., Multiresidue pesticide analysis of botanical dietary supplements using salt-out acetonitrile extraction, solid-phase extraction cleanup column, and gas chromatography-triple quadrupole mass spectrometry. *Anal. Chem.* 2013, DOI: 10.1021/ac400481w.

[25] Santos I. C.,Hildenbrand Z. L.,Schug K. A., Applications of MALDI-TOF MS in environmental microbiology. *Analyst* 2016, *141*, 2827–2837 .

[26] Hussein H. S.,Brasel J. M., Toxicity, metabolism, and impact of mycotoxins on humans and animals. *Toxicology* 2001, *167*, 101–134 .

[27] Romano L. L.,Hazekamp A., Cannabis Oil: chemical evaluation of an upcoming cannabis-based medicine. *Cannabinoids* 2013, *1*, 1–11 .

[28] Citti C.,Ciccarella G.,Braghiroli D.,Parenti C.,Vandelli M. A.,Cannazza G., Medicinal cannabis: Principal cannabinoids concentration and their stability evaluated by a high performance liquid chromatography coupled to diode array and quadrupole time of flight mass spectrometry method. *J. Pharm. Biomed. Anal.* 2016, *128*, 201–209 .

[29] Vandrey R.,Raber J. C.,Raber M. E.,Douglass B.,Miller C.,Bonn-Miller M. O., Cannabinoid Dose and Label Accuracy in Edible Medical Cannabis Products. *J. Am. Med. Assoc.* 2015, *313*, 2491–2493 .

[30] Pellegrini M.,Marchei E.,Pacifici R.,Pichini S., A rapid and simple procedure for the determination of cannabinoids in hemp food products by gas chromatography-mass spectrometry. *J. Pharm. Biomed. Anal.* 2005, *36*, 939–946 .

[31] Zoller Otman,Rhyn Peter,Zimmerli Bernhard, High-performance liquid chromatographic determination of. *J. chromatogr. A* 2000, *872*, 101–110 .

[32] Carcieri C.,Tomasello C.,Simiele M.,De Nicolò A.,Avataneo V.,Canzoneri L.,Cusato J.,Di Perri G.,D'Avolio A., Cannabinoids concentration variability in cannabis olive oil galenic preparations. *J. Pharm. Pharmacol.* 2017, DOI: 10.1111/jphp.12845.

[33] Pacifici R.,Marchei E.,Salvatore F.,Guandalini L.,Busardò F. P.,Pichini S., Evaluation of cannabinoids concentration and stability in standardized preparations of cannabis tea and cannabis oil by ultra-high performance liquid chromatography tandem mass spectrometry. *Clin. Chem. Lab. Med.* 2017, *55*, 1555–1563 .

[34] Lehotay S. J.,Tully J.,Garca A. V.,Contreras M.,Mol H.,Heinke V.,Anspach T.,Lach G.,Fussell R.,Mastovska K.,Poulsen M. ., Determination of pesticide residues in foods by acetonitrile extraction and partitioning with magnesium sulfate: collaborative study. *J. AOAC Int.* 2007, *90*, 485–520 .

[35] Srivastava A. K.,Trivedi P.,Srivastava M. K.,Lohani M.,Srivastava L. P., Monitoring of pesticide residues in market basket samples of vegetable from Lucknow City, India: QuEChERS method. *Environ. Monit. Assess.* 2011, *176*, 465–472 .

[36] Tanner J. T.,Wolf W. R.,Horwitz W., in: Greenfield, H. (Ed.), Quality and Accessibility of Food-Related Data. AOAC INTERNATIONAL, Sydney 1993, pp. 99–104.

[37] Marazuela M. D.,Bogialli S., A review of novel strategies of sample preparation for the determination of antibacterial residues in foodstuffs using liquid chromatography-based analytical methods. *Anal. Chim. Acta* 2009, DOI: 10.1016/j.aca.2009.04.031.

[38] Kinsella B.,O'Mahony J.,Malone E.,Moloney M.,Cantwell H.,Furey A.,Danaher M.,

Current trends in sample preparation for growth promoter and veterinary drug residue analysis. *J. Chromatogr. A* 2009, *1216*, 7977–8015 .

[39] Veress T., Sample preparation by supercritical fluid extraction for quantification: a model based on the diffusion layer theory for determination of extraction time. *J. Chromatogr. A* 1994, *668*, 285–91. .

[40] Radcliffe C.,Maguire K.,Lockwood B., Applications of supercritical fluid extraction and chromatography in forensic science. *J. Biochem. Biophys. Methods* 2000, *43*, 261–272 .

[41] Díaz-Maroto M. C.,Pérez-Coello M. S.,Cabezudo M. D., Supercritical carbon dioxide extraction of volatiles from spices: Comparison with simultaneous distillation-extraction. *J. Chromatogr. A* 2002, *947*, 23–29 .

[42] Bogialli S.,Di Corcia A., Matrix solid-phase dispersion as a valuable tool for extracting contaminants from foodstuffs. *J. Biochem. Biophys. Methods* 2007, *70*, 163–179

[43] Barker S. A., Matrix solid-phase dispersion. *J. Chromatogr. A* 2000, *885*, 115–127

[44] Yang S. H.,Fan H.,Classon R. J.,Schug K. ., Restricted Access Media as a Streamlined Approach towards On-line Sample Preparation: Recent Advancements and Applications. *J. Sep. Sci.* 2013, *36*, 2922–2938 .

[45] Sadílek P.,Šatínský D.,Solich P., Using restricted-access materials and column switching in high-performance liquid chromatography for direct analysis of biologically-active compounds in complex matrices. *Trends Anal. Chem.* 2007, *26*, 375–384 .

[46] Yan-yan X. U., Extraction Methods and Techniques of Polysaccharides [J]. *J. Fujian Fish.* 2006, *3*, 8 .

[47] Kim K. J.,Sun P.,Chen V.,Wiley D. E.,Fane A. G., The cleaning of ultrafiltration membranes fouled by protein. *J. Memb. Sci.* 1993, *80*, 241–249 .

[48] Kazemimoghadam M.,Mohammadi T., Chemical cleaning of ultrafiltration

membranes in the milk industry. *Desalination* 2007, *204*, 213–218 .

[49] Xu R. N.,Fan L.,Rieser M. J.,El-Shourbagy T. A., Recent advances in high-throughput quantitative bioanalysis by LC–MS/MS. *J. Pharm. Biomed. Anal.* 2007, *44*, 342–355 .

[50] Mottier P.,Hammel Y. A.,Gremaud E.,Guy P. ., Quantitative high-throughput analysis of 16 (fluoro) quinolones in honey using automated extraction by turbulent flow chromatography coupled to liquid chromatography− tandem mass spectrometr. *J. Agric. Food Chem.* 2007, *56*, 35–43 .

[51] Herraez-Hernandez R.,Van de Merbel N. C.,Brinkman U. T., Determination of the total concentration of highly protein-bound drugs in plasma by on-line dialysis and column liquid chromatography: application to non-steroidal anti-inflammatory drugs. *J. Chromatogr. B Biomed. Sci. Appl.* 1995, *666*, 127–137 .

[52] Jönsson J. Å.,Mathiasson L., Membrane extraction in analytical chemistry. *J. Sep. Sci.* 2001, *24*, 495–507 .

[53] Leghissa A.,Hildenbrand Z. L.,Foss F. W.,Schug K. A., Determination of cannabinoids from a surrogate hops matrix using multiple reaction monitoring gas chromatography with triple quadrupole mass spectrometry. *J. Sep. Sci.* 2018, DOI: 10.1002/jssc.201700946.

Chapter 3

DETERMINATION OF CANNABINOIDS FROM A SURROGATE HOPS MATRIX USING MULTIPLE REACTION MONITORING GAS CHROMATOGRAPHY- TRIPLE QUADRUPOLE- MASS SPECTROMETRY[1]

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3.1 Abstract

Cannabinoids are the primary bioactive constituents of *Cannabis sativa* and *Cannabis indica* plants. In this work, gas chromatography in conjunction with triple quadrupole mass spectrometry in multiple reaction monitoring mode were explored for determination of cannabinoids from a surrogate hops matrix. Gas chromatography-mass spectrometry is a reasonable choice for the analysis of these compounds; however, such methods are susceptible to false positives for Δ9-tetrahydrocannabinol, due to decarboxylation of Δ9-tetrahydrocannabinolic acid, its acid precursor, in the hot injection port. To avoid this transformation, the carboxyl group of Δ9-tetrahydrocannabinolic acid was protected through a silylation reaction. Multiple reaction monitoring transitions for both unmodified and silylated cannabinoids were developed and the fragmentation pathways of the different species were assigned. Precision and accuracy were evaluated for cannabinoids spiked into hops at different levels. The developed methods provided good linearity ($R^2 > 0.99$) for all the cannabinoids with a linear range from 0.15 mg/L to 20 mg/L, and with limits of detection in the orders of low- to mid-picogram on column. The recoveries for the cannabinoids were generally between 75% and 120%. Precisions (< 6% coefficient of variation) were within acceptable ranges.

Abbreviations: **Δ9-THC**, Δ9-Tetrahydrocannabinol; **Δ9-THCA**, Δ9-Tetrahydrocannabinolic acid; **BSTFA+1%TMCS**, *N*,*O*-bis(trimethylsilyl)trifluoroacetamide; **CBC**, cannabichromene; **CBD**, cannabidiol; **CBDV**, cannabidivarin; **CBE**, cannabielsoin; **CBG**, cannabigerol; **CBL**, cannabicyclol; **CBN**, cannabinol; **CBT**, cannabitriol; **CE**, collision energy; **CV**, coefficient of variation; **DEA**, drug enforcement agency; **EI**, electron impact; **MeOH**, methanol; **MRM**, multiple reaction monitoring; **QC,** quality control; **QQQ,** triple

quadrupole; **SIM**, single ion monitoring; **THCV**, tetrahydrocannabivarin; **TMS**, trimethylsilyl; **TQ**, triple quadrupole.

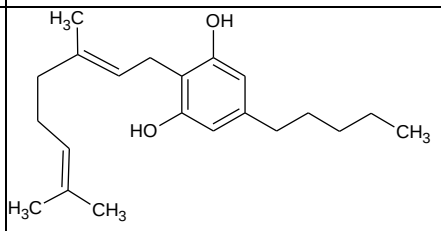
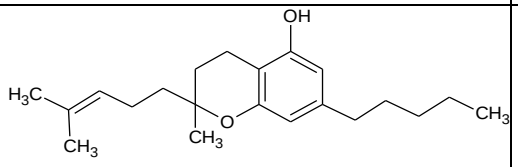
Keywords: Marijuana; *Cannabis sativa*; fragmentation; quantitative analysis; silylation.

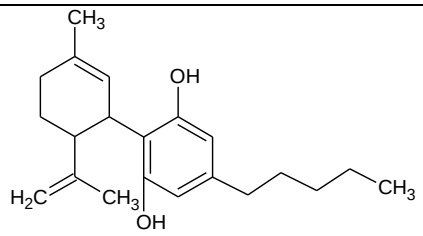
3.2 Introduction

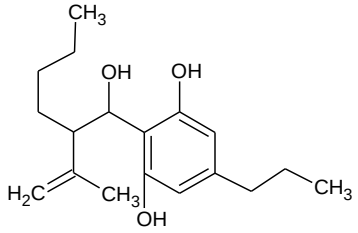
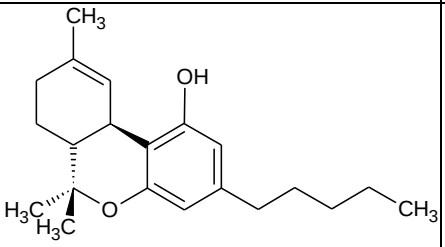
Cannabis sativa is an herbaceous annual that has its origin in the Altai mountains of Southern Siberia, Russia [1]. It can grow up to 8-12 feet tall [2], with flowers blooming from late summer to mid-fall. There are three different subspecies of cannabis (*sativa, indica,* and *ruderalis*)*,* and they differ not only in their physical appearance, but also in their chemical composition. *C. sativa* is reported to have stimulating effects, due to its high concentration of Δ9-tetrahydrocannabinol (Δ9-THC) [3],which is the main psychoactive component, while *C. indica* is reported to be sedative, due to the lower amount of Δ9-THC, in the same range of abundance as the other cannabinoids. *C. ruderalis* has no reported psychotropic effect, because the amount of Δ9-THC is much below that of the other cannabinoids.

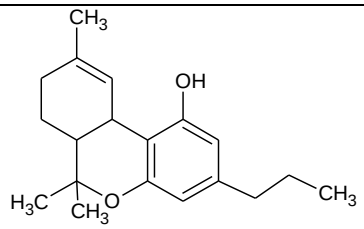
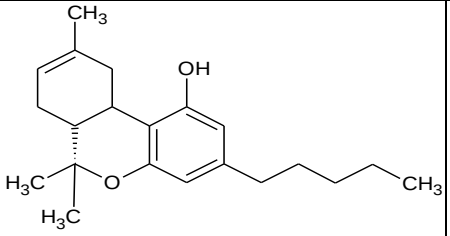
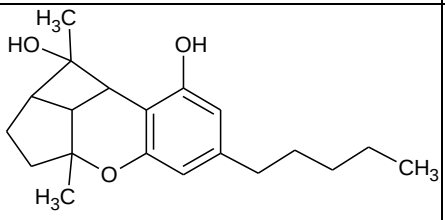
The two main classes of bioactive compounds found in cannabis are cannabinoids and terpenes. Cannabinoids are the primary bioactive components of the plant, while terpenes primarily confer odors and fragrances [4]. Currently, more than 100 cannabinoids and 100 terpenes have been identified in cannabis cultivars, but the presence or combinations of individual components can be highly variable between individual strains. The first cannabinoid to have been identified was cannabigerol (CBG) by Gaoni *et al*. in 1964 [5]. Since then, the large array of cannabinoids identified have been divided into 10 main categories [4] (Table 3.1), among which the most important ones (reported to have

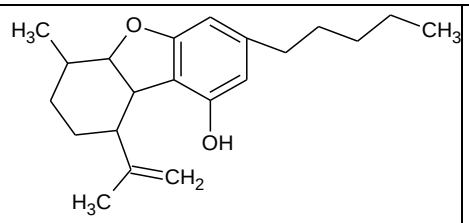
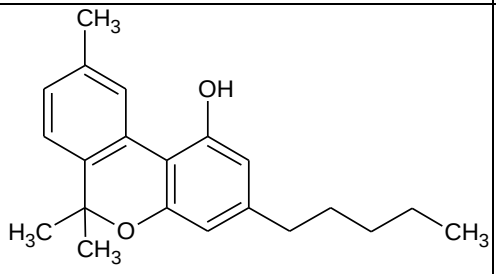
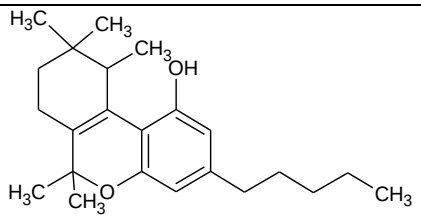
different medical benefits) are: CBG [6]; cannabichromene (CBC) [6,7,8]; cannabidiol (CBD) [9]; Δ9-THC; tetrahydrocannabivarin (THCV) [6,10]; Δ8-tetrahydrocannabinol (Δ8-THC) [6]; cannabinol (CBN) [6,11]; and Δ9-tetrahydrocannabinolic acid (Δ9-THCA) [12,13].

*Table 3.1 **T**he ten classes of cannabinoids*			
Name	Abbreviation	Structure	Medical benefits
cannabigerol	CBG		Sleep aid, inhibits cancer cell growth, slows bacterial growth, promotes bone growth [7].
cannabichromene	CBC		Inhibits cancer cell growth, promotes bone growth, reduces inflammation, pain reliever, antibacterial, anti-fungal, anti-depressant [7,8,9]

cannabidiol	CBD		Antibacterial, inhibits cancer cell growth, neuroprotective, reduces seizure and convulsions, reduces both blood sugar levels, reduces inflammation, reduces risk of artery blockage, antiemetic, reduces nausea, pain reliever, anxiolytic, suppresses muscle spasms, tranquillizer, vasorelaxant, modulates THC-induced psychoactivity

			[3,4]
cannabidivarin	CBDV		Reduces hyperexcitability, reduces epilepsy, reduces nausea [7]
Δ9-tetrahydrocannabinol	Δ9-THC		Reduces vomiting and nausea, relieves pain, stimulates appetite, suppresses muscle spasms [7]

tetrahydrocannabivarin	THCV		Reduces convulsions and seizures, promotes bone growth, stimulates energy and metabolism, improves glucose intolerance, anti-inflammatory, anti-convulsant [7,10,11,12]
Δ8-tetrahydrocannabinol	Δ8-THC		Pain reliever [7]
cannabicyclol	CBL		No reported bioactivity

cannabielsoin	CBE		No reported bioactivity
cannabinol	CBN		Sleep aid, suppresses muscle spasms, pain reliever, appetite stimulant, anti-tumorigenic against some types of lung cancer [7,13,14].
cannabitriol	CBT		No reported bioactivity.

Currently, a legalization process of cannabis is occurring in the United States, with 32 states plus the District of Columbia allowing its medical use, and among which, eight states plus the District of Columbia also allow recreational use. For this reason, there is a need to develop methods that enable reliable quantification of bioactive compounds from cannabis for the purpose of quality control.

Different methods are traditionally used for analysis of cannabinoids and terpenes. Terpenes are analyzed both using gas chromatography-mass spectrometry (GC-MS) [14], which allows for good qualification and quantification of this semi-volatile compound class in plant material and gas chromatography-flame ionization detection, mainly for qualification [2]. Cannabinoids are more commonly analyzed using liquid chromatography-mass spectrometry (LC-MS) [2,15]. Usually, the identification step coupled with LC is preferred due to the decarboxylation that occurs for acidic cannabinoids it the high temperatures of a GC injection port.

The use of gas chromatography - triple quadrupole - mass spectrometry (GC-QQQ-MS) for speciation of cannabis natural products has not been previously elaborated in the scientific literature. Importantly, the potential for use of the multiple reaction monitoring (MRM) mode of detection to enhance both sensitivity and specificity, makes it attractive to consider. GC-QQQ-MS in MRM mode provides a double mass filter, and detection of fragments specific to the analyte of interest, which results in a reduced noise level, less interferences, and lower limits of quantitation (LOQ) compared to single stage mass spectrometric detection. Given the similarities between cannabinoids, the MRM mode combined with a scan mode allows improved specificity and qualitative analysis based on the ability to monitor both tandem mass spectrometric and electron ionization fragmentation.

As mentioned previously, a problem with GC-based analysis of cannabinoids is the decarboxylation of carboxylate-containing compounds in the hot injection port. In fact, Δ9-THCA is readily decarboxylated to form Δ9-THC, and both compounds are desired to be speciated in various extracts. This phenomenon limits the independent speciation of acidic cannabinoids when using GC-MS. To avoid this interference, it is necessary to protect the carboxylate forms by silylation (trimethylsilyl derivatives). While such an extra

sample preparation step may be considered a drawback due to the additional time and resources needed, silylation of the relatively low volatility cannabinoids can provide several advantages [16]. Upon silylation, Δ9-THC and Δ9-THCA can be reliably speciated as Δ9-THC-TMS and Δ9-THCA-2TMS, respectively. Other isomers may also be differentiated by virtue of them being either singly- or multiply-derivatized. Further, as other compounds of interest, such as terpenes and some pesticides, are GC-amenable, an approach for reliably speciating cannabinoids by GC could limit the number of instruments a lab needs in order to accommodate testing.

The aim of this study was to demonstrate the ability of GC-QQQ-MS to handle the analysis of cannabinoids from complex natural product matrices by virtue of multiple reaction monitoring. Hops (*Humulus lupulus*) used in the brewing of beers were chosen to be a surrogate matrix, as hops and cannabis are both members of the cannabinaceae family, yet hops do not contain natural cannabinoids. Another goal was to highlight the differences and benefits for the analysis of silylated versus underivatized cannabinoids. General validation studies were performed to confirm that the analysis of spiked cannabinoids in hops using MRM on GC-QQQ-MS provides acceptable linearity, detection limits, precision, and accuracy for common cannabinoid determination. The elucidation of detailed tandem mass spectrometric fragmentation patterns for the different cannabinoids is also a valuable component of the presented research. Overall, combining silylation of extracts with GC-QQQ-MS and MRM provides a reliable means for analysis of cannabinoids.

3.3 Material and Methods

3.3.1 Standards and Reagents

Cannabinoid standards (CBC, CBD, THCV, CBG, CBN, Δ8-THC, Δ9-THC, and Δ9-THCA) were purchased from Cerilliant Corporation (Round Rock, TX) as drug enforcement agency (DEA)-exempt solutions (1 mg/mL) in methanol. Methanol and ethyl acetate, both LC-MS grade, were purchased from Honeywell Burdick & Jackson (Muskegon, MI). For derivatization, *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA+1%TMCS) was purchased from Restek Corporation (Bellefonte, PA).

3.3.2 Instrumental Analysis

A GCMS TQ8030 (Shimadzu Scientific Instrument, Inc., Columbia, MD) gas chromatograph coupled through electron ionization to a 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) to perform all analyses. The injection mode was splitless (1 µL injection volume; 60 sec hold time) and temperature programming was utilized: 40 °C for 1 min; raised to 200 °C at 20 °C/min; raised to 235 °C (for the underivatized cannabinoids) or to 242 °C (for the derivatized cannabinoids) at 3 °C/min; and held for 3 min. The GC was operated in constant linear velocity mode (53.4 cm/sec) to provide a column flow (helium) of 1.46 mL/min.

The procedure supplied by Shimadzu for the creation of an MRM database was followed. The MRM Smart Database Tool was used to create a product ion scan mode batch at different collision energies (CE), starting from a scan mode method. The optimized settings for the underivatized and silylated cannabinoids are shown in Table 3.2 and Table 3.3, respectively.

Table 3.2 Cannabinoids' MRM							
Compound	Retention Time	Transitions					
THCV	14.84-15.99	203.00>174.10	15 V	286.00>271.20	9 V	271.00>189.20	9 V
CBD	15.99-17.61	231.00>174.10	18 V	231.00>145.10	30 V	232.00>175.20	18 V
CBC	15.99-17.61	231.00>174.20	21 V	231.00>173.10	33 V	232.00>175.10	24 V
Δ8-THC	17.61-19.28	231.00>174.00	21 V	314.00>231.00	18 V	314.00>174.30	42 V
Δ9-THC	17.61-19.28	231.00>174.10	24 V	231.00>145.10	33 V	231.00>147.30	33 V
CBG	19.28-20.32	231.00>174.10	15 V	193.00>123.10	12 V	193.00>137.10	12 V
CBN	19.28-20.32	295.00>238.00	21 V	295.00>223.20	33 V	296.00>224.10	33 V

Table 3.3 Silylated cannabinoids' MRM							
Compound	Retention Time	Transitions					
THCV-TMS	13.08-14.96	315.00>73.10	24 V	343.00>73.10	24 V	358.00>343.20	12 V
CBD-2TMS	13.86-14.96	301.00>73.10	21 V	390.00>73.10	27 V	337.00>73.10	27 V
CBC-TMS	14.96-16.64	303.00>246.10	21 V	303.00>73.10	30 V	303.00>174.10	30 V
Δ8-THC-TMS	14.96-16.64	303.00>246.10	24 V	303.00>73.10	30 V	386.00>303.20	18 V
Δ9-THC-TMS	14.96-16.64	315.00>73.10	24 V	315.00>81.10	12 V	386.00>371.20	12 V
CBG-2TMS	16.64-19.71	337.00>73.10	27 V	338.00>73.10	27 V	391.00>73.10	27 V
CBN-TMS	16.64-19.71	367.00>310.10	24 V	367.00>295.10	30 V	382.00>367.20	21 V
Δ9-THCA-2TMS	19.71-22.07	487.00>147.20	30 V	487.00>365.10	18 V	487.00>73.20	30 V

3.3.3 Sample Preparation

Eight concentrations of standard mixtures were prepared (0.25, 0.50, 0.75, 1, 2, 5, 10, and 25 mg/L of each cannabinoid) for both the direct and derivatization-based analysis.

To perform the silylation reaction, 2 mL of the 50 mg/L solution containing the eight cannabinoids was dried with a gentle stream of N_2, and then 1 mL of ethyl acetate and 1 mL of BSTFA+1%TMCS were added. The solution was heated in a sealed vial at 70 °C for 30 minutes and allowed to cool before analysis.

Hops of the German Tettnang varietal were purchased from a local home brew store (Arlington, TX). Samples of homogenized and ground hops (10 mg for the 4 and the 15 mg/L concentration levels, and 100 mg for the 0.6 mg/L level) were spiked with cannabinoid standard solution mixtures of varying concentrations (0.6, 4, and 15 mg/L). The 0.6 mg/L solution was obtained by spiking the hops with 60 µL of a 50 mg/L mixture of the cannabinoids, the 4 mg/L by spiking them with 80 µL of the mixture, and the 15 mg/L by the spiking of 300 µL with the mixture. The spiked hops were then extracted, 3 times with 1 mL of methanol (for the 0.6 mg/L), or 3 times with 100 µL of methanol (for the 4 and 15 mg/L). The liquid extract in each case was combined and then dried under a gentle stream of nitrogen. For direct analysis, the solution was reconstituted in 5 mL of MeOH (for the 0.6 mg/L), or in 1 mL (for the 4 and 15 mg/L). For the derivatization-based analysis, the residue was reconstituted in 50:50 ethyl acetate and BSTFA+1%TMCS.

3.4 Results And Discussion

In order to analyze the cannabinoids using GC-QQQ-MS in MRM mode, it was first necessary to optimize appropriate quantification and qualification precursor to product ion transitions. Upon optimization of the MRM condisions it was clear that the different species of cannabinoids, both natural and derivatized, exhibited similar fragmentation pathways. As an example, the elucidated pathways for Δ9-THC and Δ9-THC-TMS are shown in Figure 3.1; those for the other analytes in their underivatized and derivatized forms are given in the Electronic Supplementary Information document.

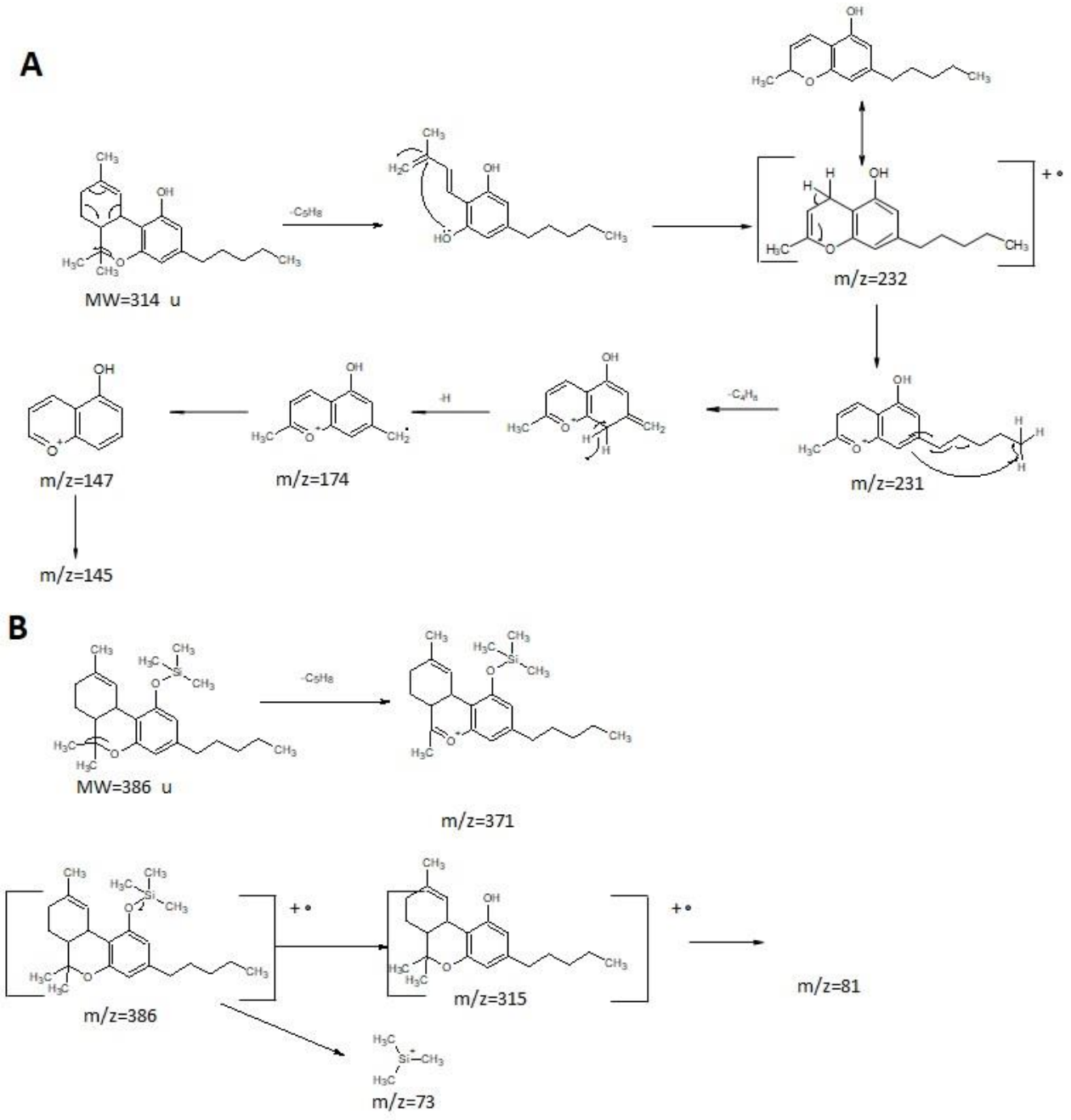

A
MW=314 u
-C5H8
m/z=232
-C4H9
m/z=231
-H
m/z=174
m/z=147
m/z=145
B
MW=386 u
-C5H8
m/z=371
m/z=386
m/z=315
m/z=81
m/z=73

Figure 3.1 Proposed mechanisms for the formation of the different ions of A) Δ9-THC and B) Δ9-THC-TMS in tandem mass spectrometry measurements leading to optimization of MRM transitions.

The most common precursor ion for underivatized Δ9-THC, Δ8-THC, CBD, and CBC was *m/*z 231, caused by a Retro-Diels-Alder reaction which results in the loss of a cyclohexene unit. Another common fragmentation pathway is the fragmentation of the C3-*n*-pentyl side chain followed by the formation of *m/z* 174 due to the loss of an H atom. The same reaction occurs for the non-isomeric cannabinoids THCV, CBG, and CBN, though different *m/z* values for this product are observed due to the different starting molecular weights. These common pathways create the need for the use of three different transitions for the differentiation of the isomeric cannabinoids. In such cases, where a large number of isomers having common fragmentation pathways are present, effective chromatographic separation becomes critical.

For the silylated analytes, the most frequent fragment is *m/z* 73*,* which corresponds to the trimethylsilyl group. However, the fragmentation of the C3 side chain and Retro-Diels-Alder pathways are also common. In fact, many of the fragmentation mechanisms correspond to the ones proposed for the underivatized cannabinoids, with the addition of observed silyl group ions. Even for the derivatized analytes, a third transition in commonly needed to differentiate isomeric analytes. However, some cannabinoids can be multiply silylated (instead of singly-silylated), which increases diffentation is some cases based on the augmentation of precursor ion mass. A disadvantage of this phenomenon could be the potential for incomplete silylation, which could lead to multiple analyte peaks depending on the number of silyl units attached.

The increased specificity with the use of MRM transitions enhances the differentiation among co-eluting cannabinoids, such as CBD/CBC and CBG/CBN. On the other hand, silylation further enhances the ability to chromatographically resolve the analytes. As shown in Figure 3.2, even if derivatization requires additional sample preparation, the silylated cannabinoids are all baseline resolved.

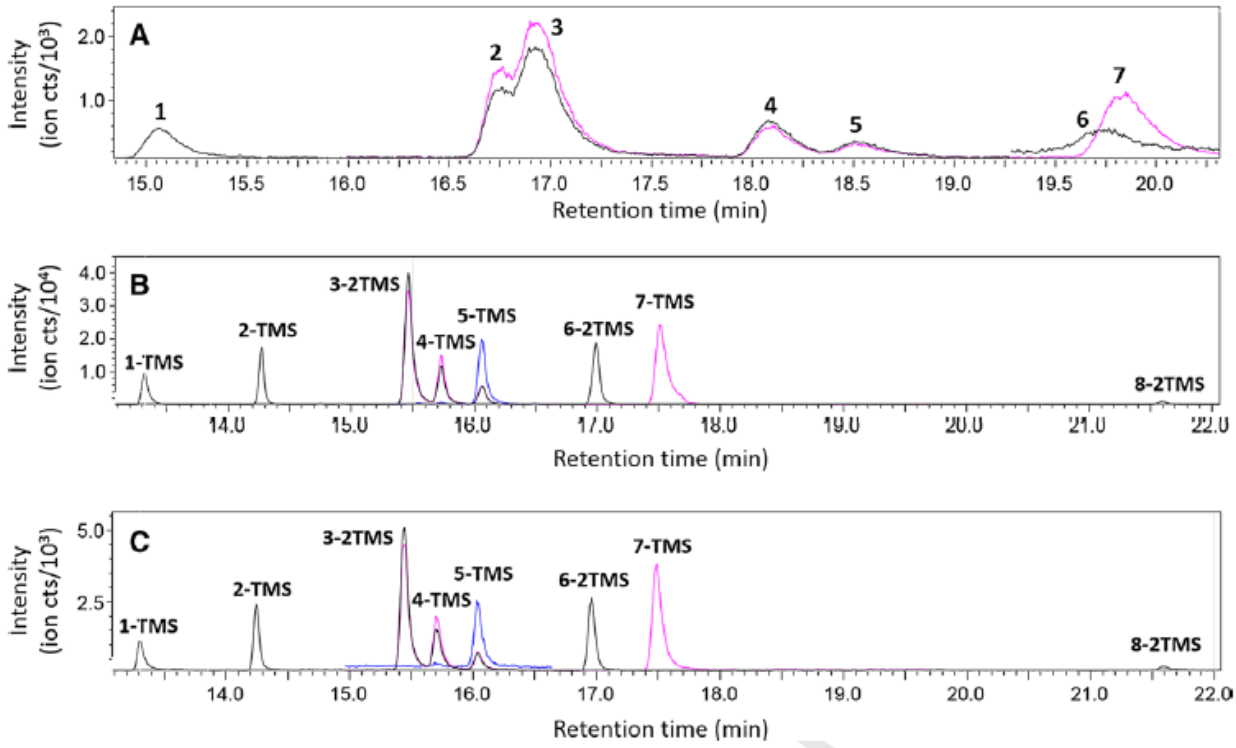


Figure 3.2 Representative chromatograms for A) 0.5 ppm underivatized cannabinoids, B) 10 ppm derivatized cannabinoids, and C) hops spiked with 4 ppm cannabinoids, extracted, derivatized, and analyzed. (1, THCV; 2, CBC; 3, CBD; 4, Δ8-THC; 5, Δ9-THC; 6, CBG; 7, CBN; 8, Δ9-THCA.)

Again, this is aided by the fact that some analytes are multiply silylated. Another advantage of the silylation is the possibility to analyze Δ9-THCA, which otherwise would be decarboxylated in the hot injection port and converted into Δ9-THC.

Different standards, quality controls, and spiked hops were extracted and analyzed in triplicate, along with external standard calibration. The use of hops as a surrogate for cannabis was necessary due to the federal restrictions on cannabis research and the lack of a DEA license. Hops were chosen because the plant material is similar to cannabis, and the challenges in the analysis due to interferences from the essential oils and waxes would be extremely similar; the use of the MRM mode to target specific compounds and to limit interferences also helps to ameliorate this problem. In fact, the linearity was always greater than 0.99, and the limits of detection of the method were consistently lower than 100 pg on-column, excepting Δ9-THC-TMS which showed 101 pg on-column. Table 3.4 and table 3.5 show the results for calibration and the determinations of precision, accuracy, limit of detection, and recovery for the quality controls and the spiked hops.

Table 3.4 Cannabinoids validation method. [a]Mean ± SD, *n*=3

Cannabinoid	**Linearity (R^2)**	**LOD (pg)**	**Prepared Concentration (mg/L)**	**QC Measured Concentration (mg/L)[a]**	**Spiked Measured Concentration (mg/L)[a]**	**Recovery (%)**	**Precision (CV %)**	**Accuracy (%)**
THCV	0.9931	12.4	0.60	0.557 ± 0.005	0.62±0.04	103	6.80	3.10
			4	3.7±0.2	3.9±0.1	98	2.7	-1.6
			15	6.4±0.4	14.44±0.04	96	0.30	-3.71
CBC	0.9913	6.33	0.60	0.580±0.004	0.66±0.03	109	4.22	9.52

			4	1.38±0.09	3.8±0.2	95	4.3	-5.2
			15	12±2	14±3	96	22.6	-4.5
CBD	0.9902	24.0	0.60	-0.022±0.007	0.46±0.01	77	2.79	-22.78
			4	3.54±0.09	4±1	93	24	-7
			15	16±2	18±6	119	23	19
Δ8-THC	0.9974	25.2	0.60	0.38±0.07	0.45±0.02	75	3.76	-25.23
			4	3.9±0.2	4.5±0.2	113	4.9	12.8
			15	14.1±0.1	13.5±0.7	90	5.4	-9.8
Δ9-THC	0.9907	68.6	0.60	0.629±0.006	0.67±0.04	112	5.32	11.53
			4	3.68±0.02	3.7±0.2	91	4.9	-8.6
			15	15.1±0.1	11.5±0.1	77	1.0	-23.3
CBG	0.9932	34.3	0.60	0.694±0.004	0.719±0.002	120	0.329	19.865
			4	3.56±0.03	3.3±0.2	83	6.5	-17.5
			15	15±1	15.6±0.4	104	2.5	4.3
CBN	0.9929	6.04	0.60	0.581±0.002	0.701±0.008	117	1.109	16.843
			4	3.7±0.6	3.7±0.1	93	2.9	-7.2
			15	15±2	16.0±0.6	107	3.9	7.0

Table 3.5 Silylated cannabinoids validation method

Cannabinoid	**Linearity (R^2)**	**LOD (pg)**	**Prepared Concentration (mg/L)**	**Measured Concentration (mg/L)[a]**	**Recovery (%)**	**Precision (CV %)**
THCV-TMS	0.9938	42	0.60	0.69±0.04	115	5.61
			4	3.58±0.04	90	1.08

			15	13±2	89	17
CBD-2TMS			0.60	0.662±0.003	110	0.477
			4	3.69±0.08	92	2.11
	0.9905	34	15	11.3±0.3	75	2.2
CBC-TMS			0.60	0.71±0.02	118	2.76
			4	4.1±0.1	104	2.8
	0.99	36	15	12.5±0.3	83	2.5
Δ8-THC-TMS			0.60	0.75±0.02	125	3.16
			4	2.916±0.005	73	0.165
	0.9923	42	15	12.0±0.2	80	1.7
Δ9-THC-TMS			0.60	0.692±0.004	115	0.579
			4	3.6±0.1	91	2.9
	0.9937	101	15	13.4±0.3	89	2.3
CBG-2TMS			0.60	0.71±0.01	118	0.84
			4	4.0±0.1	99	3.6
	0.9912	30	15	13.15±0.08	88	0.64
CBN-TMS			0.60	0.69±0.02	115	2.81
			4	4.2±0.2	104	5.2
	0.9938	30	15	10.86±0.07	72	0.67
Δ9-THCA-2TMS			0.60	1.066±0.001	177	0.060
			4	3.96±0.01	99	0.36
	0.992	1.8	15	16.3±0.8	109	5.1

The LODs obtained for the derivatized cannabinoids are an order of magnitude higher than the equivalent natural version, as stated in Table 3.4 and 3.5, and they require

a higher elution temperature, as seen in Figure 3.2. On the other hand, even if the LODs are higher for the silylated cannabinoids, they are still lower than the expected levels naturally found in *C. sativa* cultivars.

Overall, this method provides better sensitivities and specificities compared to other methods on GC-MS that only used scan or selected ion monitoring mode [17,18], or even those using MRM mode with LC-MS [19]. Another advantage of using the GC-MS for the analysis of these compounds is their constant fragmentation through the electron impact (EI) source. This ensures consistent precursor ion generation, and further ensures consistency of resulting MRM transitions produced therefrom. The additional fragmentation obtained through initial ionization, as well as during tandem mass spectrometric fragmentation, also provides additional qualitative information for confident qualitative assignment of the analytes.

3.5 Conclusions

To the authors' knowledge, this is the first work performed on GC-QQQ-MS using an MRM mode for cannabinoid analysis. Although this mode can be used solely on a triple quadrupole, and the quantification should be performed only after the silylation reaction (to avoid false positive of the Δ9-THC), it enhances both the sensitivity and the linearity. These qualities lead to an easier quantification and qualification of the cannabinoids of interest, relative to analysis of underivatized cannabinoids or the use of other analytical approaches. Additionally, the silylation provides not only a greater differentiation in the transitions of the naturally isomeric cannabinoids, due to the different numbers of silylation sites, but also a baseline separation of the cannabinoids that are not fully separated in the direct analysis mode.

This method was shown to be reliable for the analysis of the cannabinoids from a plant material matrix, but it is possible that this method could also be applicable for the analysis of other cannabis products, such as edibles, given the high specificity of an MRM approach. It would also be possible to use this method to determine the abundance of cannabinoids in different parts of the plant, which may not have been previously detected with other methodologies due to their low abundance. The main advantage of this method, in fact, is that the extraction step does not have to be perfectly efficient because the risk of interferences is lessened. This leads to potentially less laborious sample preparation steps, such as solid phase extraction, and to the possible detection of lower abundance cannabinoids.

3.6 Acknowledgements

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3.7 Conflicts of interest

The authors declare no conflict of interest.

3.8 References

[1] Sharma O.P., Plant Taxanomy. Second Edition, Tata McGraw-Hill Education, New Dehli 2011.

[2] Recommended Methods for the Identification and Analysis of Cannabis and Cannabis Products. Laboratory and Scientific Section United Nations Office on Drugs and Crime, New York 2009.

[3] Beutler J.A., Marderosian A.H., Chemotaxonomy of Cannabis I. Crossbreeding between Cannabis sativa and C. ruderalis, with analysis of cannabinoid content. *Econ. Bot*. 1978, *32,* 387-394.

[4] Brenneisen, R., in: ElSohly, M.A. (Eds), Marijuana and the Cannabinoids. Humana Press, New York 2007, pp. 17-49.

[5] Gaoni Y., Mechoulam R., The structure and synthesis of cannabigerol, a new hashish constituent. Chem. Soc., London 1964.

[6] Watson S.J., Benson J.A., Joy J.E., Marijuana and medicine: assessing the science base: a summary of the 1999 Institute of Medicine report*. Arch Gen Psychiatry*, 2000, *57,* 547-52.

[7] Eisohly H. N., Turner C. E., Clark A. M., Eisohly, M. A., Synthesis and antimicrobial activities of certain cannabichromene and cannabigerol related compounds. *J. Pharm. Sci.*, 1982*, 71*, 1319-1323.

[8] El-Alfy A. T., Ivey K., Robinson K., Ahmed S., Radwan M., Slade D., Khan I., ElSohly M.A., Ross, S. Antidepressant-like effect of Δ 9-tetrahydrocannabinol and other cannabinoids isolated from Cannabis sativa L. *Pharmacol. Biochem. Behav.*, 2010, *95*, 434-442.

[9] Grotenhermen F., Müller-Vahl K., The Therapeutic Potential of Cannabis and Cannabinoids. *Dtsch Arztebl Int.*, 2012, *109,* 495–501.

[10] Wargent E.T., Zaibi M.S., Silvestri C., Hislop D.C., Stocker C.J., Stott C.G., Guy G.W., Duncan M., Di Marzo V., Cawthorne, M.A., The cannabinoid Δ9-tetrahydrocannabivarin (THCV) ameliorates insulin sensitivity in two mouse models of obesity. *Nutr.Diabetes*, 2013, *3,* e68.

[11] Farrimond J.A., Whalley B.J., Williams C.M., Cannabinol and cannabidiol exert opposing effects on rat feeding patterns*. Psychopharmacology (Berl)*, 2012, *223,* 117-29.

[12] Ruhaak L.R., Felth J., Karlsson P.C., Rafter J.J., Verpoorte R., Bohlin, L., Evaluation of the cyclooxygenase inhibiting effects of six major cannabinoids isolated from Cannabis sativa. *Biol. Pharm. Bull.*, 2011, *34*, 774-778.

[13] Moldzio R., Pacher T., Krewenka C., Kranner B., Novak J., Duvigneau J.C., Rausch W.D., Effects of cannabinoids Δ (9)-tetrahydrocannabinol, Δ (9)-tetrahydrocannabinolic acid and cannabidiol in MPP+ affected murine mesencephalic cultures. *Phytomedicine*, 2012, *19*,.819-824.

[14] Hillig K.W., Mahlberg P.G., A chemotaxonomic analysis of cannabinoid variation in Cannabis (Cannabaceae)*. Am. J. Bot.*, 2004, *91*, 966-75.

[15] Aizpurua-Olaizola O., Omar J., Navarro P., Olivares M., Etxebarria N., Usobiaga A., Identification and quantification of cannabinoids in Cannabis sativa L. plants by high performance liquid chromatography-mass spectrometry. *Anal. Bioanal. Chem.*, 2014, *406*, 7549-7560.

[16] Turner C.E., Hadley K.W., Henry J., Mole M.L., Constituents of Cannabis sativa L. VII: use of silyl derivatives in routine analysis. *J. Pharm. Sci.*, 1974, *63*, 1872-1876.

[17] Romano L.L., Hazekamp A., Cannabis Oil: chemical evaluation of an upcoming cannabis-based medicine. *Cannabinoids*, 2013, *1*, 1-11.

[18] Musshoff F., Lachenmeier D.W., Kroener L., Madea B., Automated headspace solid-phase dynamic extraction for the determination of amphetamines and synthetic designer drugs in hair samples. *J. Chromatogr. A*, 2002, *958*, 231-238.

[19] Grauwiler S.B., Scholer A., Drewe J., Development of a LC/MS/MS method for the analysis of cannabinoids in human EDTA-plasma and urine after small doses of Cannabis sativa extracts*. J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci.*, 2007, *850,* 515-522.

Chapter 4

DETERMINATION OF THE METABOLITES OF Δ9-TETRAHYDROCANNABINOL IN URINE AND PLASMA USING MULTIPLE REACTION MONITORING GAS CHROMATOGRAPHY-TRIPLE QUADRUPOLE- MASS SPECTROMETRY[1]

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4.1 Abstract

Once introduced into the human body, only minimal amounts of cannabinoids are excreted in their original forms, due to various complex metabolic pathways. For this reason, it is less desirable to quantify the amount of parent cannabinoids in urine, plasma, or faeces, as opposed to the pertinent cannabinoid metabolites. Δ9-tetrahydrocannabinol is reported to be the most potent and psychoactive among natural cannabinoids. Here, two known metabolites, 11-hydroxy-Δ9-tetrahydrocannabinol and 11-nor-9-carboxy-Δ9-tetrahydrocannabinol, plus their precursor Δ9-tetrahydrocannabinol, were targeted for analysis. We describe the creation of a method using gas chromatography – triple quadrupole – mass spectrometry and multiple reaction monitoring (MRM) mode for these two metabolites. The analytes were silylated prior to analysis. The validation of the methods using samples of urine and plasma spiked with known amounts of the metabolites was shown to provide recoveries between 80 and 110%, LODs were 75 pg for both Δ9-THC and Δ9-THC-OH, and 25 ng for 11-Nor-9-carboxy-THC, in their silylated forms.

Abbreviations: **11-OH-Δ9-THC**, 11-hydroxy- Δ9-tetrahydrocannabinol; **11-Nor-9-carboxy-Δ9-THC,** 11-nor-9-carboxy-Δ9-tetrahydrocannabinol; **Δ9-THC**, Δ9-Tetrahydrocannabinol; **APCI,** atmospheric pressure chemical ionization; **BSTFA+1%TMCS**, *N*,*O*-Bis(trimethylsilyl)trifluoroacetamide; **EI,** electron impact; **ESI**, electrospray ionization; **MRM**, multiple reaction monitoring; **QC,** quality control; **QQQ,** triple quadrupole; **S/N,** signal-to-noise ratio; **TMS**, trimethylsilyl.

Keywords: *Cannabis sativa,* metabolism, silylation.

4.2 Introduction

A wide variety of products can be made from the *Cannabis sativa* and *Cannabis indica* plants, including herbal cannabis, cannabis resin or hashish, liquid cannabis, and cannabis seeds [1]. The absorption of cannabinoids varies due to the mode of administration, with inhalation being the most common route amongst cannabis consumers. Yet, only 3% of the consumed Δ9-Tetrahydrocannabinol (Δ9-THC), the main psychoactive cannabinoid, is detected in its free form in blood [2]. Once this cannabinoid reaches the liver, the lungs, or the intestine, it is metabolized through allylic oxidation, epoxidation, aliphatic oxidation, and conjugation reactions [2]. Studies have shown that Δ9-THC is mainly metabolized into 11-hydroxy-Δ9-tetrahydrocannabinol (Δ9-THC-OH) (Figure 4.1) by a series of hepatic microsomal enzymes. This form is considered to be a highly bioactive metabolite [3,4]. At this point, Δ9-THC-OH can be oxidized, yielding the relatively inactive carboxylic acid form, 11-nor-9-carboxy-Δ9-tetrahydrocannabinol (11-Nor-9-carboxy-THC) (Figure 4.1) [5].

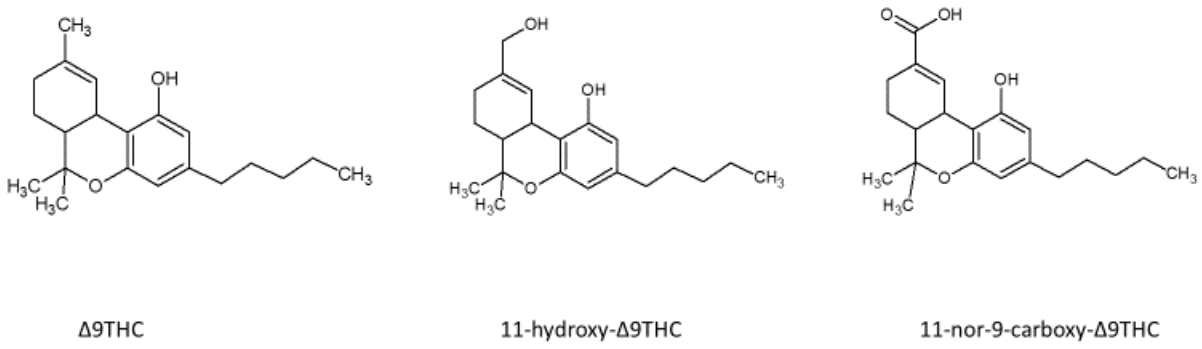

Figure 4.1 Structures of Δ9-tetrahydrocannabinol (Δ9-THC), 11-hydroxy- Δ9-tetrahydrocannabinol (11-0H- Δ9-THC), and 11-nor-9-carboxy- Δ9-tetrahydrocannabinol (11-nor-9-carboxy- Δ9-THC).

Cannabinoids are mainly excreted in faeces (68%), with only 13% excreted in urine [6]. The primary metabolite, Δ9-THC-OH, is detected in both sample types, while 11-Nor-

9-carboxy-THC is mainly found in faeces. Both of these components are easily detectable also in blood plasma, which is an easier matrix to analyze and handle than faeces.

Currently, there are a few reported methods for the analysis of these two natural cannabinoid metabolites [7]. The main separation technique that has previously been utilized for the separation of Δ9-THC metabolites has been gas chromatography-mass spectrometry (GC-MS) [8,9]. However, these analytes are also highly suitable for liquid chromatography analysis. The use of a GC coupled with a triple quadrupole-mass spectrometer (GC-QqQ-MS) has not been reported for the analysis of these compounds. Compared to single stage GC-MS, GC-QqQ-MS allows the development of a tandem mass spectrometry-based multiple reaction monitoring (MRM) mode method to detect the targeted analytes, providing higher signal-to-noise (S/N) ratios (greater sensitivity), as well as improved specificity.

One issue that occurs with the GC analysis of acidic cannabinoids is decarboxylation. In this case, 11-Nor-9-carboxy-THC presents a carboxyl group that is degraded in the high temperatures of the GC injection port. To avoid this reaction, it is necessary to protect the carboxyl group by silylation, to form its trimethylsilyl (TMS) derivative. Upon silylation, Δ9-THC, 11-Nor-9-carboxy-THC, and Δ9-THC-OH can be reliably analyzed as Δ9-THC-TMS, 11-Nor-9-carboxy-THC-2TMS, and Δ9-THC-OH-2TMS, respectively; the different numbers of silylating sites is caused by the different numbers of available carboxyl and -OH groups in the molecule.

The aim of this study was to create a sensitive method for the qualification and quantification of Δ9-THC metabolites using GC-QqQ-MS in MRM mode. This approach allows for the specific analysis of these components in complex matrices like urine and plasma. This work should be of particular interest given that forensic analysis of Δ9-THC

exclusively may not be sufficient to determine the consumption of cannabis, considering its fast metabolism.

4.3 Experimental

4.3.1 Standards and Reagents

Standards (Δ9-THC, Δ9-THC-OH, and 11-Nor-9-carboxy-THC) were purchased from Cerilliant Corporation (Round Rock, TX) as DEA-exempt solutions (1 mg/mL) in methanol. For derivatization, *N*,*O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA+1%TMCS) was purchased from Restek Corporation (Bellefonte, PA). Bovine plasma was purchased from Innovative Research, Inc. (Novi, MI) and human urine was from CST Technologies, Inc. (Great Neck, NY).

4.3.2 Instrumental Analysis

A GCMS-TQ8030 (Shimadzu Scientific Instruments, Inc., Columbia, MD) gas chromatograph coupled through electron ionization to a 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) to perform all analyses. The injection mode was splitless (1 µL injection volume; 60 sec hold time) and temperature programming was utilized: 40 °C for 1 min; raised to 300 °C at 20 °C/min and held for 3 min, for a total run time of 19 minutes. The GC was operated in constant linear velocity mode (53.4 cm/sec helium) to provide a column flow of 1.46 mL/min.

The procedure supplied by Shimadzu for the creation of an MRM database was followed. The MRM Smart Database Tool was used to create a product ion scan mode batch at different collision energies (CE), starting from a scan mode method analyzed with

the Shimadzu software LabSolutions. The optimized MRM transitions are given in Table 4.1.

Table 4.1 Metabolites' MRM

Compound	Retention Time	Transitions								
Δ9-THC-TMS	11.89-12.70	*315>73*	24 V	100	*315 >81*	12 V	84	*386>371*	12 V	81
Δ9-THC-OH-2TMS	12.70-14.02	*371>73*	27 V	100	*371>67*	21 V	77	*371>289*	18 V	61
11-nor-9-carboxy-Δ9-THC-2TMS	12.70-14.02	*473>355*	21 V	100	*371>67*	30 V	73	*371>73*	27 V	66

4.3.3. Sample Preparation

Eight concentrations of standard mixtures were prepared (0.25, 0.50, 0.75, 1, 2, 5, 10, and 25 mg/L of each cannabinoid) for the derivatization-based analysis. To perform the silylation reaction, 2 mL of the 50 mg/L solution containing the Δ9-THC and its two metabolites was dried with a gentle stream of N_2, and then 1 mL of ethyl acetate and 1 mL of BSTFA+1%TMCS were added. The solution was heated in a sealed vial at 70 °C for 30 minutes and allowed to cool before analysis.

Plasma and urine were spiked with the mixture of underivatized cannabinoids to obtain 3 different concentrations (0.6, 4, and 15 mg/mL). The 0.6 mg/mL solution was obtained by spiking plasma and urine with 12 µL of a 25 mg/mL mixture of the cannabinoids

(for a total of 500 µL), the 4 mg/mL by spiking them with 16 µL of the mixture (for a total of 100 µL), and the 15 mg/mL by the spiking of 60 µL with the mixture (for a total of 100 µL). The spiked matrices were then extracted 3 times with ethyl acetate, and the solution silylated as explained above.

4.4 Results And Discussion

The first step in a GC-QQQ-MS analysis is the creation of an MRM database. Table 4.1 shows the three transitions (one quantitative and two qualitative) for the three analytes investigated in this study. Some significant commonalities exist between the fragment ion generated from the different analytes. Fragment *m/z* 73 corresponding to the loss of a trimethylsilyl group was observed in each case. While it was the quantifier product ion for both Δ9-THC-TMS and 11-hydroxy-Δ9-THC-2TMS, the precursor ion for each transition was different, ensuring specificity can be maintained. Overall, the optimized quantifier and qualifier transitions provide for significant differentiation of the analytes, despite similarities

in their structures. Proposed fragmentation pathways, corresponding to primary observed ions in the mass spectra and those assigned to MRM transitions are given in Figure 4.2.

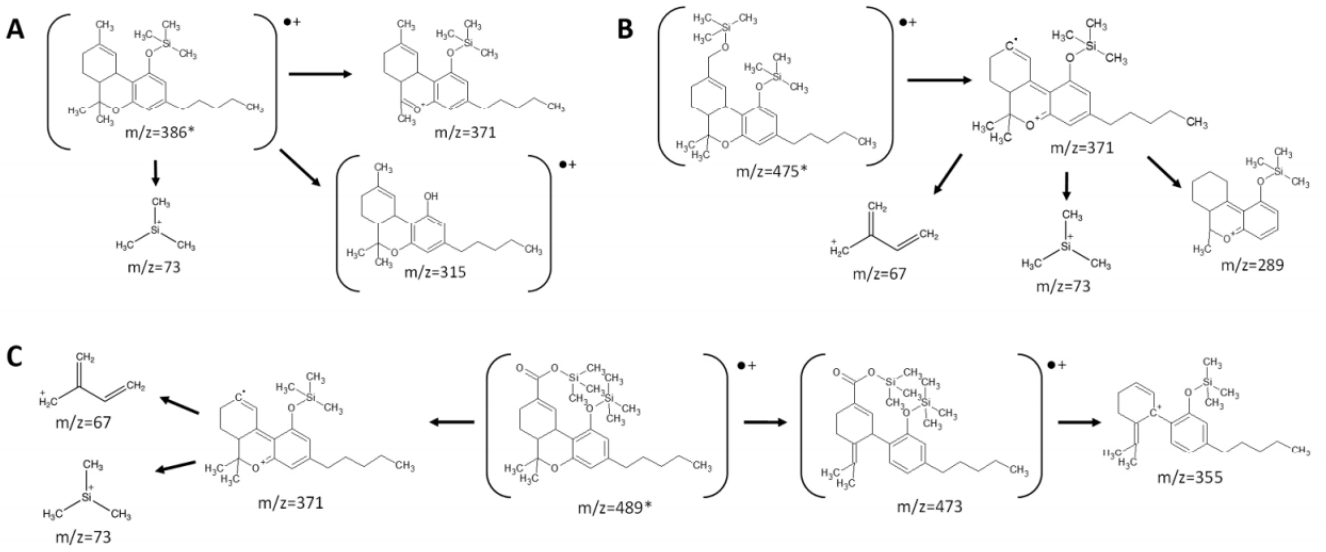

Figure 4.2 Fragmentation patterns for A) Δ9-THC-TMS; B) Δ9-THC-OH-2TMS; and C) 11-Nor-9-Carboxy-Δ9-THC silylated analytes in the GC-QQQ-MS. An * indicates the molecular ion for each analyte.

Due to the different numbers and positions of silyl groups placed on the analytes through the derivatization process, chromatographic resolution of each for the analysis of standard mixtures and extracted spiked matrices was fairly straightforward. Figure 4.3

shows the separation of the analytes in the standard mixture, and in the urine and plasma extracts.

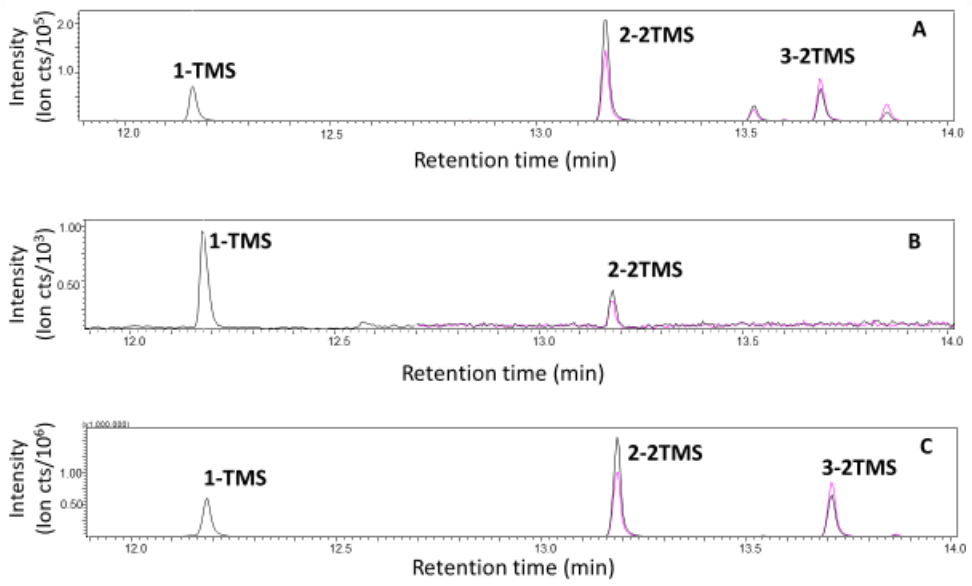

Figure 4.3 Representative chromatograms for: A) a 4 ppm mixture extracted from urine matrix; B) 0.6 ppm mixture extracted from a plasma matrix; and C) a mixture of standards at 15 ppm. Analytes correspond to the indicated silylated forms of: 1, Δ9-THC; 2, Δ9-THC-OH; and 3, 11-Nor-9-Carboxy- Δ9-THC.

It is noticeable how 11-nor-9-carboxy-Δ9-THC was less detectable at lower concentrations, and this may be due to the higher difficulty in forming stable and detectable fragments. The standards and spiked matrices were analyzed in triplicate following external standard calibration. Table 4. 2 summarizes the results of the validation experiments.

Table 4.2 Validation method. * Mean ± SD, *n*=3

Cannabinoid	Δ9-THC-TMS	Δ9-THC-OH-2TMS	Δ9-THC-COOH-2TMS

R^2	0.9906			0.9903			0.9969		
LOD (pg)	75			75			250		
Prepared Concentration (mg/L)	0.6	4	15	0.6	4	15	0.6	4	15
Spiked Measured Concentration (mg/L)* in Urine	0.6±0.1	4.4±0.2	15±1	0.70±0.03	4.5±0.1	15.5±0.6	0.59±0.06	3.8±0.4	15.0±0.3
Recovery (%)	103	109	98	116	114	104	98	94	100
Precision (CV %)	11.6	5.2	12.5	3.91	2.88	4.17	10.2	9.9	2.2
Spiked Measured Concentration (mg/L)* in Plasma	0.54±0.05	4.58±0.05	13.81±0.04	0.65±0.01	4.5±0.1	15±1	0.6356±0.0002	3.87±0.02	14.81±0.01
Recovery (%)	89	114	92	109	114	101	106	97	99
Precision (CV %)	4.4	1.1	0.3	1.75	2.89	7.52	0.0387	0.63	0.10

The linearity was always greater than 0.99, and the limits of detection of the method were 75 pg for Δ9-THC-TMS and Δ9-THC-OH-2TMS, and 25 ng for 11-nor-9-carboxy-Δ9-THC-2TMS. Recovery ranged from 89% to 116% and precision ranged from 0.037% to 12.5%, indicating acceptable performance for this method for analytes extracted from two complex biological fluid matrices.

Considering these results and the ability to perform the analysis on these metabolites by always using the transitions obtained in Table 4.1, it is understandable how

the GC-QQQ-MS is a reliable tool for this purpose; the only drawback is the required silylation prior the analysis. The results obtained in the two different matrices were comparable, but between the two different metabolites, Δ9-THC-OH-2TMS was more detectable. This is consistent with previous analysis of these components reported in the literature; 11-Nor-9-carboxy-THC was previously shown to exhibit worse LODs [10]. This difference is useful because, not only is Δ9-THC-OH the primary metabolite of Δ9-THC, but it is also found to be concentrated in urine, while 11-Nor-9-carboxy-THC is found primarily in faeces.

4.5 Conclusions

To our knowledge, this is the first work performed on GC-QQQ-MS using an MRM mode to demonstrate the potential for determining the metabolites of Δ9-THC from biological fluids. This method enhances both the sensitivity and the linearity of the analysis compared to basic scan or SIM modes on standard GC-MS instruments. Many reported methods use GC-MS as a technique for the analysis of cannabinoids and their metabolites, but also LC-MS is widely employed. The advantages of the former is the use of EI as ionization source, which fragments the analytes in a constant way, allows for the use of a library to aid qualitative analysis. Furthermore, cannabinoids and their metabolites are not easily ionized by LC-MS ionization sources. For this reason, LC-MS is considered to be less sensitive for the analysis of cannabinoids than GC-MS; however, LC-MS, does not require the derivatization of the analytes, because of the lower temperatures used in this technique.

The next important step towards improving the speed of the GC-MS analysis would be to develop a one-step extraction/silylation procedure, in order to decrease the preparation time. It is worth remembering that new species of cannabinoids are growing in

importance due to their therapeutic uses; therefore, the further study of their metabolites will be inevitable. This study of the fragmentation pathways of these Δ9-THC metabolites using GC-QQQ-MS may lead to an easier identification of metabolites of other cannabinoid compounds, because it is predictable that they will undergo similar transformations.

4.6 Acknowledgements

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4.7 References

[1] Recommended Methods for the Identification and Analysis of Cannabis and Cannabis Products. Laboratory and Scientific Section United Nations Office on Drugs and Crime, New York 2009.

[2] Teixeira H.M., Reis F., in Vieira D.N. (Ed). Forensic Medicine - From Old Problems to New Challenges. InTech, Rijeka2011, pp. 215-250.

[3] Iribarne C., Berthou F., Baird S., Dréano Y., Picart D., Bail J.P., Beaune P., Ménez J.F., Involvement of cytochrome P450 3A4 enzyme in the N-demethylation of methadone in human liver microsomes. *Chem. Res. Toxicol.*, 1996, *9,* 365-373.

[4] Matsunaga T., Iwawaki Y., Watanabe K., Yamamoto I., Kageyama T., Yoshimura H., Metabolism of Δ9-tetrahydrocannabinol by cytochrome P450 isozymes purified from hepatic microsomes of monkeys. *Life Sci.,* 1995, *56*, 2089-2095.

[5] Lemberger L., Weiss J.L., Watanabe A.M., Galanter I.M., Wyatt R.J., Cardon P.V., Delta-9-tetrahydrocannabinol: Temporal correlation of the psychologic effects and blood levels after various routes of administration. *New Eng. J. Med.*, 1972, *286*, 685-688.

[6] Perez-Reyes M., Di Guiseppi S., Mason A.P., Davis K.H., Passive inhalation of marihuana smoke and urinary excretion of cannabinoids. *Clin. Pharmacol. Ther*., 1983, *34*, 36-41.

[7] Leghissa A., Hildenbrand Z.L., Schug K.A. A Review of Methods for Chemical Characterization of Cannabis Natural Products. J. Sep. Sci. 2018 (In Press).

[8 Kemp P.M., Abukhalaf I.K., Manno J.E., Manno B.R., Alford D.D., McWilliams M.E., Nixon F.E., Fitzgerald M.J., Reeves R.R., Wood, M.J., Cannabinoids in humans. II. The influence of three methods of hydrolysis on the concentration of THC and two metabolites in urine. *J. Anal. Toxicol.*, 1995, *19,* 292-298.

[9] McBurney L.J., Bobbie B.A., Sepp L.A., GC/MS and EMIT analyses for Δ9-tetrahydrocannabinol metabolites in plasma and urine of human subjects. *J. Anal. Toxicol.,* 1986, *10,* 56-64.

[10] Teixeira H., Verstraete A., Proença P., Corte-Real F., Monsanto P., Vieira D.N., Validated method for the simultaneous determination of Δ 9-THC and Δ 9-THC-COOH in oral fluid, urine and whole blood using solid-phase extraction and liquid chromatography–mass spectrometry with electrospray ionization. *Forensic Sic. Int.*, 2007, *170*, 148-155.

Chapter 5

DETECTION OF CANNABINOIDS AND CANNABINOID METABOLITES USING GAS CHROMATOGRAPHY- VACCUM ULTRAVIOLET SPECTROSCOPY[1]

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5.1 Abstract

Relatively new technology, including vacuum ultraviolet (VUV) absorption spectroscopy (120 – 240 nm) detection for gas chromatography (GC) was evaluated and shown to successfully differentiate cannabinoids, their metabolites, and derivatives thereof. The possibility to deconvolve co-eluting peaks due to additivity of overlapping absorbance spectra of the analytes was also demonstrated. This feature allows the use of faster temperature ramps, faster analysis time, and reduces the need to baseline resolve all analytes, without sacrificing sensitivity. The combination of VUV with GC allows for the analysis of cannabinoids that have similar structures and molecular weight, and whose chromatographic separation can be difficult to obtain.

Abbreviations: **Δ9-THC**, Δ9-Tetrahydrocannabinol; **Δ9-THCA**, Δ9-Tetrahydrocannabinolic acid; **BSTFA+1%TMCS**, *N*,*O*-Bis(trimethylsilyl)trifluoroacetamide; **CBC**,cannabichromene; **CBD**, cannabidiol; **CBDV**, cannabidivarin; **CBE**, cannabielsoin; **CBG**, cannabigerol; **CBGA,** cannabigerolic acid; **CBL**, cannabicyclol; **CBN**, cannabinol; **CBT**, cannabitriol; **MeOH**, methanol; **MRM**, multiple reaction monitoring; **QC,** quality control; **THCV**, tetrahydrocannabivarin; **TMS**, trimethylsilyl; **VUV,** vacuum ultraviolet detector.

Keywords: Cannabinoids; *Cannabis sativa*; deconvolution; gas phase absorption spectroscopy; silylation.

5.2 Introduction

Cannabis sativa and *Cannabis indica* are extremely important plants that have been used both in the making of basketry and cordage, and as a medicine since the early Gravettian Settlements in Eastern Europe, 30,000 years ago [1]. Cannabis is an herbaceous annual plant whose major constituents are cannabinoids, a group of psychotropic and bioactive compounds that are gaining more notoriety for their medicinal properties. In fact, cannabis is not only the most extensively used drug worldwide, with 4% of the population aged between 15 and 60 using it, but it is also widely used as a medicine due to the bioactivity of cannabinoids [2]. The United States is currently expanding the legalization process for the medical (29 states and the District of Columbia) and recreational (8 states and the District of Columbia) use of *Cannabis sativa* and *Cannabis indica*. Collectively, due to the societal relevance and medical significance of cannabis use, the development of efficient qualificative and quantitative methods for pertinent cannabinoids is becoming increasing more crucial.

The most medically relevant cannabinoids found in *cannabis* cultivars are tetrahydrocannabivarin (THCV), cannabidiol (CBD), cannabidivarin (CBDV), cannabichromene (CBC), Δ8-tetrahydrocannabinol (Δ8-THC), Δ9-tetrahydrocannabinol (Δ9-THC), cannabigerol (CBG), cannabinol (CBN), and the acidic precursors cannabidiolic acid (CBDA), and Δ9-tetrahydrocannabinolic acid (Δ9-THCA). CBGA is a progenitor molecule, which can be converted into THCA, CBDA, or CBCA through a series of enzymatic reactions [3]. Subsequent decarboxylation reactions occur with the addition of heat, which in the case of Δ9-THC, results in an equilibrium shift towards psychoactivity. Furthermore, upon ingestion, either through inhalation or through food, Δ9-THC, is metabolized to 11-Hydroxy-Δ^9-THC, a cannabinoid that crosses the barrier between blood and brain easier. As a result, the 11-Hydroxy-Δ^9-THC metabolite is considered to be more potent than Δ9-THC [4]. Subsequently, 11-Hydroxy-Δ^9-THC is metabolized into 11-nor-9-

carboxy-Δ9-THC, which does not have any psychoactive effects, but still provides the therapeutic activity against inflammation and pain [4]. The analysis of these metabolites is extremely important to carry out because only minimal amounts of Δ9-THC are excreted in its free form [5].

A wide range of instruments have been used to detect cannabinoids, among which the most common are gas chromatography [6] and liquid chromatography [2,7] coupled with mass spectrometry. The reason why the latter may be preferred is the ability to differentiate between the cannabinoids and their acidic precursors, as the high temperature of the GC inlet causes decarboxylation of the acidic forms. This reaction can be prevented through the protection of the group via silylation [8]. However, in both GC and LC, the similarity (and isomeric nature) among cannabinoids require a good chromatographic separation prior the identification, which usually leads to more extensive analyses. To overcome this problem, the use of new technologies, such as gas chromatography-vacuum ultraviolet spectroscopy (GC-VUV), may provide a viable alternative.

The VUV detector analyzes compounds in the UV/VUV spectral range (120-240 nm), where virtually all chemical compounds absorb light [9,10]. In this range, photons excite electrons in chemical bonds and non-bonded electrons to excited energy states ($\sigma \rightarrow \sigma^*$ and high energy $\pi \rightarrow \pi^*$ transitions can be observed, among others). Because excitation energies are highly dependent on atom connectivity and molecular structure, absorption events are very sensitive for differentiating isomers (positional and diastoreomers), an area where MS has problems. Another benefit of using the GC-VUV for the detection of cannabinoids is its ability to deconvolve overlapping spectra [11-14]. The differentiation between two or more cannabinoids can rely less on chromatographic separation because of good spectral discrimination. This results in greater distinction amongst all species, and the potential for faster chromatographic separations [15].

The goal of this work was to demonstrate the potential for use of GC-VUV in cannabinoid analysis, and to compare the analysis of underivatized cannabinoids versus their silylated versions. Considering the growing importance of both *C. sativa* and the GC-VUV, this qualitative work was carried out to report the absorbance spectra of these compounds, so that it could be used in the future for further uses and applications.

5.3 Materials and Methods

5.3.1 Standards and reagents

Cannabinoid standards [THCV (1), CBD (2), CBC (3), Δ8-THC (4), Δ9-THC (5), CBG (6), CBN (7) and Δ9-THCA (8)], and their metabolites [11-Hydroxy-Δ^9-THC (9), and 11-nor-9-Carboxy-Δ^9-THC (10),] were purchased from Cerilliant Corporation (Round Rock, TX) as DEA-exempt solutions (1 mg/mL) in methanol. For derivatization, *N*,*O*-Bis(trimethylsilyl)trifluoroacetamide (BSTFA+1%TMCS) was purchased from Restek Corporation (Bellefonte, PA). Methanol and ethyl acetate, both LC-MS grade, were purchased at Honeywell Burdick & Jackson International (Muskegon, MI).

5.3.2 Instrumental Analysis

A GC 2010 (Shimadzu Scientific Instruments, Inc., Columbia, MD) gas chromatograph coupled to a VGA-100 (VUV Analytics, Inc., Cedar Park, TX) VUV detector was used in conjunction with a Rtx-5 column (30 m L x 0.25 mm i.d. x 0.25 µm d_f) (Restek) to perform all analyses. The injection mode was splitless (1 µL injection volume; 60 sec hold time, 250°C) and temperature programming was used: 40 °C for 1 min; raised to 220 °C at 30 °C/min; raised to 260 °C at 10 °C/min; and held for 5 min. The GC was operated in constant linear velocity mode (34.7 cm/sec) to provide a column flow (helium) of 1.58

mL/min. The temperature of both the transfer line and the flow cell were set at 250°C, the make-up gas (N_2) pressure was 0.25 psi, and the acquisition rate was 5 Hz.

5.3.3. Sample Preparation

In order to speciate cannabinoids that possess a carboxyl group (Δ9-THCA and 11-nor-9-Carboxy-Δ^9-THC), they must be protected to avoid decarboxylation in the high temperature GC injection port. For this reason alone, many analysts prefer HPLC analysis, but the protection step is not overly cumbersome. Standard mixtures of the cannabinoids were prepared for both direct (underivatized) and derivatization-based analysis, while the metabolites were analyzed solely as their derivatized adducts. To perform the silylation reaction, 1 mL of the solutions were dried with a gentle stream of N_2, and then 0.50 mL of ethyl acetate and 0.50 mL of BSTFA+1%TMCS were added. The solution was heated in a sealed vial at 70 °C for 30 minutes and allowed to cool before analysis. Different concentrations were prepared to detect the LODs (ranging from 1 ppm to 25 ppm).

5.4 Results And Discussion

Figure 5.1 shows representative chromatograms for the separations of underivatized and silylated cannabinoids.

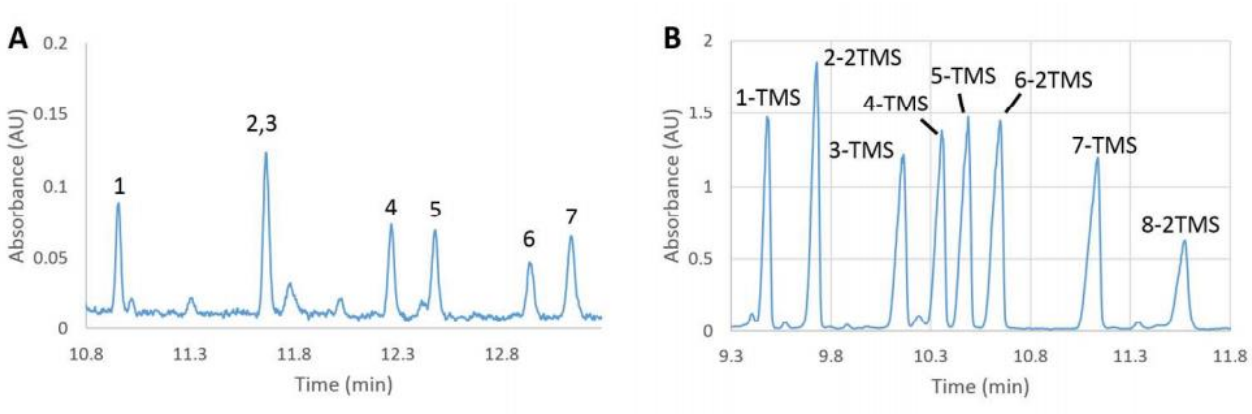

Figure 5.1 Representative chromatograms of (A) underivatized and (B) silylated cannabinoids. Shown are signals filtered for response in the 125 – 160 nm wavelength range.

In comparison, the latter approach provides for baseline separation of all the compounds of interest. This is facilitated in some cases by the fact that while most analytes are singly-silylated, others are doubly-silylated (CBD-2TMS, CBG-2TMS, and Δ9-THCA-2TMS). Comparing the elution times of the underivatized cannabinoids with their silylated counterparts, the silylated cannabinoids elute first because they are more volatile, making for a slightly shorter analysis time. With that being said, even if not complicated, the silylation reaction does require additional chemicals and time, that are not required from the direct injection of the underivatized cannabinoids. Care must be also taken to fully silylate analytes extracted from complex sample matrices.

Silylation also slightly alters the absorption spectra for the analytes relative to the unmodified forms, as shown in Figure 5.2.

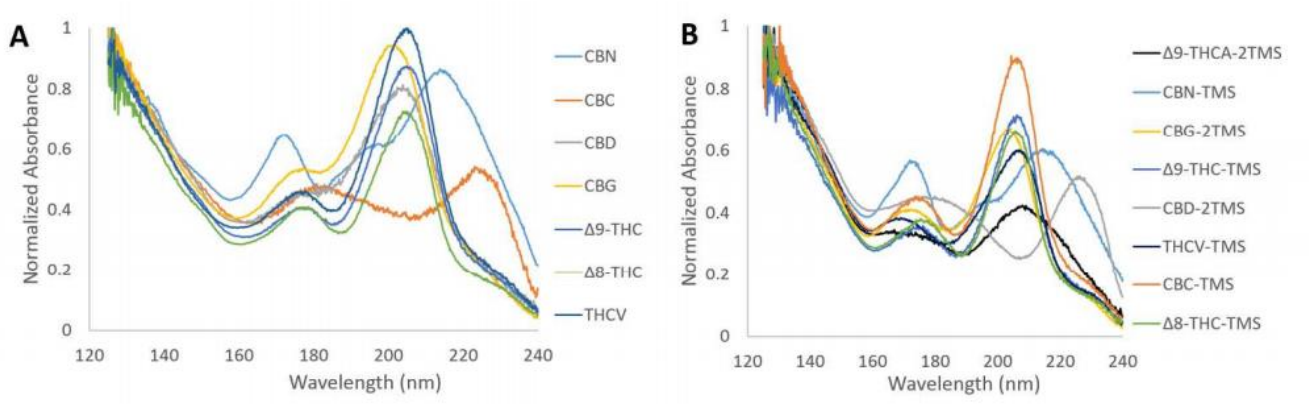

Figure 5.2. Normalized gas phase VUV/UV absorbance spectra for (A) underivatized and (B) silylated cannabinoids.

In general, absorption band shapes are consistent with chemical structures that bear aromaticity and significant functionality. These features provide good chromophores for absorption of VUV/UV light. Of note, aromatic structures are characterized by intense absorption bands above 185 nm. Oxygenation and general unsaturation gives rise to absorption features between 160 – 185 nm. Saturated portions of the molecule contribute most to absorption below 160 nm.

In Figure 5.1A, peaks 2 and 3 (CBC and CBD) co-elute. This is not a problem for the GC-VUV analysis, as the individual contribution of each peak to the total response can be easily deconvolved. Figure 5.3A shows the combined absorption spectrum measured, overlaid with the pure spectra for each of the individual compounds CBC and CBD.

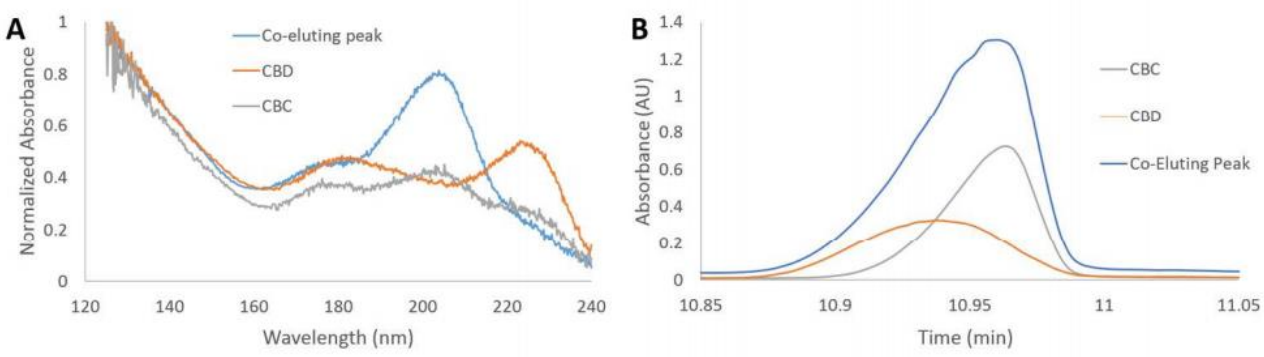

Figure 5.3 Deconvolution of coeluting underivatized cannabinoids CBC and CBD. In (A) are the spectra for the combined signal and the individual pure spectra; in (B) is shown the combined chromatographic signal deconvolved into the contributions from the individual components.

The combined absorption spectrum is a simple linear combination of the pure spectra, weighted by the abundance of each compound [11-14]. It is noticeable how in Figure 5.2A almost all analytes (being isomers) show the same maxima at 200 nm and 170 nm, with no overlap among the different cannabinoids. On the other hand, figure 5.2B shows how these compounds are more differentiated after silylation. In Figure 5.3B, the chromatogram for the co-eluting peak is shown, along with the deconvolved individual contributions of each component contributing to the measured signal. Further chromatographic separation of the overlapping components need not be pursued.

This general principle can also be utilized to assess peak purity in samples that might contain interferences from other cannabinoids or sample constituents. A spectrum obtained for a peak can be searched against the VUV library. Assuming the peak is pure and the component is in the library, then a comparison of the spectrum with the corresponding library spectrum will yield a very small residual difference between the two.

If another component were present, then that would be apparent based on a poor match (large residual) between the experimental and library spectra, a deconvolution or further separation can be pursued.

The cannabinoid metabolites were only evaluated in their derivatized form, in order to avoid decarboxylation of the analytes. Figure 5.4 shows the obtainable baseline separation of the components, along with Δ^9-THC, and the respective absorbance spectra of the analytes. 11-nor-9-Carboxy-Δ^9-THC was less responsive to the method than 11-Hydroxy-Δ^9-THC and Δ^9-THC. This is likely due to incomplete silylation, which is also likely attributable to the additional minor peaks seen in Figure 5.4A.

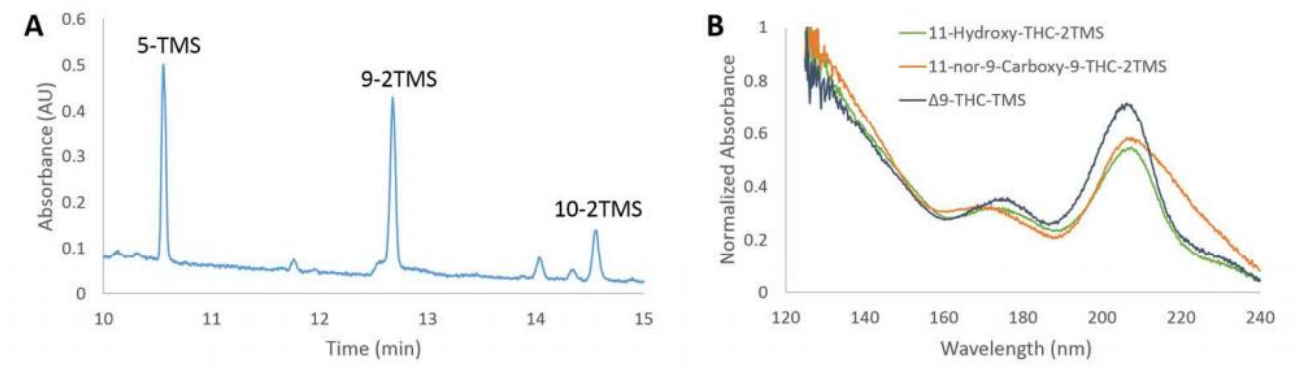

Figure 5.4 (A) Chromatogram and (B) normalized absorbance spectra for Δ9-THC and its two major metabolites.

The different absorbance profiles of all the analytes can be found in the supplemental information.

An evaluation of limits of detection (LOD) for the various cannabinoids was performed. Sequential dilutions were performed until the amount determined to provide a signal approximately three times the noise level was determined. For the underivatized cannabinoids, the LOD was 5 ng on column. For the silylated cannabinoids, the result was

slightly better, but very comparable; the analysis yielded a LOD of 3 ng on column (3 ppm). The LOD of silylated Δ^9-THC and the silylated metabolites were 10 (10 ppm) and 5 ng (5 ppm) on column, respectively. These values are an important point that must be considered when performing analysis on real samples. In fact, even though the LODs for these compounds are relatively high, various sample preparation techniques are common when preparing samples of biological fluid for analysis. As a reference, one study reported the concentrations of cannabinoid metabolites in dried blood spots to range between 0.13 to 15.75 ppb [16], while Schwilke et al reported an amount of metabolites in plasma between 3.8 and 196.9 ppb after multiple oral Δ9-THC doses [4].

5.5 Concluding Remarks

The qualification of cannabinoids and their metabolites is fast and simple with a GC-VUV system, due to the possible deconvolution of constituents. This new method may be used for a rapid determination of these analytes in different types of matrices, and due to its short analysis time, may help facilitate routine analysis, without necessarily requiring a baseline separation of similar cannabinoids for their correct quantification. In fact, the analysis of the silylated components could be accelerated to the point of significant overlap, and the individual constituents could still be spectrally isolated. The only drawbacks of this method are the high LODs, that will not allow the detection of these analytes in biological matrices, but that are suitable for the analysis of cannabinoids in plants.

Considering that the VUV is a universal detector, it is plausible that the high interference from the background of more complex samples could lead to a complicated differentiation and analysis of the compounds of interest from real samples. However, since all of the cannabinoids and their metabolites have an aromatic backbone, it could be possible to select specific absorbance regions (longer wavelengths), to increase the

specificity of the analysis. The primary goal of this application note was to report the different VUV/UV absorption features of different cannabinoids, as a potential additional tool for analysis in a fast-growing market.

5.6 Acknowledgements

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5.7 Conflict of Interest

Disclaimer: KAS is a member of the Scientific Advisory Board for VUV Analytics, Inc.

5.8 References

[1] Hanus L.O., Pharmacological and Therapeutic Secrets of Plant and Brain (Endo) Cannabinoids. *Med. Res. Rev.*, 2009, *29*, 213-271.

[2] Recommended Methods for the Identification and Analysis of Cannabis and Cannabis Products. Laboratory and Scientific Section United Nations Office on Drugs and Crime, New York 2009.

[3] Degenhardt F., Stehle F., Kayser O., in Preedy V.R. (Ed). Handbook of Cannabis and Related Pathologies: Biology, Pharmacology, Diagnosis, and Treatment. Academic Press, Cambridge 2016 pp. 13-23

[4] Schwilke E.W., Schwope D.M., Karschner E.L., Lowe R.H., Darwin W.D., Kelly D.L., Goodwin R.S., Gorelick D.A., Huestis M.A., Δ9-tetrahydrocannabinol (THC), 11-hydroxy-THC, and 11-nor-9-carboxy-THC plasma pharmacokinetics during and after continuous high-dose oral THC. *Clin. Chem.,* 2009, *55,* 2180-2189.

[5] Hunt C.A., Jones R.T., Tolerance and disposition of tetrahydrocannabinol in man. *J. Pharmacol. Exp. Ther.*, 1980, *215*, 35-44.

[6] Hillig K.W., Mahlberg P.G., A chemotaxonomic analysis of cannabinoid variation in Cannabis (Cannabaceae)*. Am. J. Bot.*, 2004, *91*, 966-75.

[7] Aizpurua-Olaizola O., Omar J., Navarro P., Olivares M., Etxebarria N., Usobiaga A., Identification and quantification of cannabinoids in Cannabis sativa L. plants by high performance liquid chromatography-mass spectrometry. *Anal. Bioanal. Chem.*, 2014, *406*, 7549-7560.

[8] Turner C.E., Hadley K.W., Henry J., Mole M.L., Constituents of cannabis sativa L. VII: Use of silyl derivatives in routine analysis. *J. Pharm. Sci*., 1974, *63*, 1872–1876.

[9] Schug K.A., Sawicki I., Carlton Jr D.D., Fan H., McNair H.M., Nimmo J.P., Kroll P., Smuts J., Walsh P., Harrison D. Vacuum ultraviolet detector for gas chromatography. *Anal. Chem.*, 2014, *86*, 8329-8335.

[10] Santos, I.C., Schug. K.A., Recent advances and applications of gas chromatography vacuum ultraviolet spectroscopy. *J. Sep. Sci,* 2016, *1,* 138-151.

[11] Fan H., Smuts J., Bai L., Walsh P., Armstrong D.W., Schug K.A., Gas-chromatography-vacuum ultraviolet spectroscopy for analysis of fatty acid methyl esters. *Food Chem.,* 2016, *194*, 265-271.

[12] Fan H., Smuts J., Walsh P., Schug K.A., Gas Chromatography - Vacuum Ultraviolet Spectroscopy for Multiclass Pesticide Identification. *J. Chrom. A*, 2015, *1389*, 120-127.

[13] Schenk, J, Mao X, Smuts J, Walsh P, Kroll P, Schug KA., Analysis and Deconvolution of Dimethylnaphthalene Isomers Using Gas Chromatography Vacuum Ultraviolet Spectroscopy and Theoretical Computations. *Anal. Chim. Acta.,* 2016, *945*, 1-8.

[14] Walsh P., Garbalena M., Schug K.A., Rapid Analysis and Time Interval Deconvolution for Comprehensive Fuel Compound Group Classification and Speciation using Gas

Chromatography – Vacuum Ultraviolet Spectroscopy. *Anal. Chem.,* 2016, *88,* 11130-11138.

[15] Santos I.C., Smuts J., Schug K.A., Rapid profiling and authentication of vanilla extracts using gas chromatography - vacuum ultraviolet spectroscopy. *Food Anal. Methods*, 2017 (in Press).

[16] Mercolini L., Mandrioli R., Sorella V., Somaini L., Giocondi D., Serpelloni G., Raggi M.A., Dried blood spots: Liquid chromatography–mass spectrometry analysis of Δ 9-tetrahydrocannabinol and its main metabolites. *J. Chrom. A*, 2013, *1271*, 33-40.

Chapter 6

EXTRACTION, QUALIFICATION AND QUANTIFICATION OF TERPENES IN HOPS VIA HEADSPACE- GAS CHROMATOGRAPHY-VACUUM ULTRAVIOLET SPECTROSCOPY

6.1 Introduction

Terpenes are a classification of naturally occurring compounds that derive from isoprene (C_5H_8) [1–4]. The 55,000 different species have been categorized into different subgroups based on their carbon number: hemi- (C5), mono- (C10), sesqui- (C15), di- (C20), sester- (C25), tri- (C30), tetra- (C40), and polyterpenes $(C5)_n$ with $n > 8$ [1–4]. The main reason why terpenes are synthesized by plants is either to attract insects for pollination, or to repel microorganisms and predators. However, these aromatic molecules also play important roles as signal compounds and phytohormones [4]. Approximately 1,500 different types of monoterpenes have been identified, and due to their high volatility, they are the main causes for the odor and fragrances of the plant [4].

Terpenes are mainly hydrocarbons, but they can also include alcohols, aldehydes, ketones, ethers, and esters [2]; these compounds are usually called terpenoids, but nowadays the terms “terpenoid” and “terpene” are colloquially used interchangeably.

Different methods have been used for the full characterization of these volatile compounds, including gas chromatography (GC) coupled with either a flame ionization detector (FID) or mass spectrometry (MS). One major issue that arises during the qualitative analysis of various terpene mixtures is the structural similarities that can render certain terpenes difficult to distinguish. In some cases, this ultimately leaves their distinction solely based on retention index (RI) [3]. Opstaele et al. [5] used a headspace-solid phase

microextraction (HS-SPME) method coupled with GC-MS for the analysis of terpenes in hops, using a 100 µm polydimethylsiloxane (PDMS)- coated fiber for a 30 min extraction. The GC method was straightforward, using a 1.0 mL/min flow on a Rtx-1 column. Their findings stated that monoterpene hydrocarbons had the highest area percentage, with β-myrcene being the most abundant (927 ± 23 µg/mL), and α-pinene the least abundant (0.28 ± 0.02 µg/mL) [5]. A similar approach was taken by Hamm et al. [6], using a PDMS-coated fiber for the HS-SPME extraction of various olibanum samples (but the fiber was 65 µm, and the process was carried out for 60 minutes), and using a DB5 column on the GC-MS [6]. They were able to set the starting point for the creation of a database to determine the botanical origin of the olibanum samples. On the other hand, Aberl et al. [7] coupled a GC-MS with an HS-trap, extracting the terpenes from the hop plant with the use of ethanol (EtOH) at first, and then mixing 20 mg of the supernatant with 5 mL of water in an HS vial. The column used for the separation was a DB-5MS 60m x 0.25 mm x 0.25 µm, with a temperature ramp that reached 300 °C [7]. Alternatively, they compared the HS-trap with the "European Brewery Convention" (EBC) method, which includes steam distillation, and they found that the former is more suitable for thermolabile compounds, such as caryophyllene oxide, which may degrade during distillation. Leghissa et al. [8] carried out a SIM analysis, as opposed to using the SCAN mode on the GC-MS, for the quantification of 6 main terpenes that can be found in cannabis (linalool, exo-fenchol, caryophyllene, terpineol, guaiol, and α-bisabolol).

Currently, a number of studies have been performed using the new vacuum ultraviolet detector (VUV), for the analysis of petroleum products [9–11], naturally occurring substances [2,12,13], and compounds of human making (such as pesticides and stimulants) [14,15]. The benefits of this new technology include the ability to measure all compounds that absorb light in the range of 125-240 nm [16], and the possibility of being

connected to any GC system. The reason why the VUV system is a valuable detector for the analysis of terpenes is the fact that isobaric and isomeric compounds react differently to light; therefore, their absorbance spectra is going to be different, even if their structures are similar [2,16]. This is extremely important for qualitative analysis, because all of the other detectors, such as MS and FID, perform well quantitatively, but the identification of terpene usually relies on RI, due to their similar response [3]. Another advantage offered by the VUV is the ability to deconvolve co-eluting peaks due to the different absorbance spectra given by each analyte, and their simple additivity when they pass together through the flow cell. This deconvolution process is performed post run and it is based on the principle that the absorbance spectrum of a co-eluting peak is equal to the abundance-weighted sum of the two co-eluting compounds [16]. A reference absorbance spectrum for each compound to be deconvolved must be present in the VUV library.

The goal of the current work was to evaluate the use of room temperature ionic liquids (RITLs) as extraction solvents for the analysis of terpenes via HS-GC-VUV. These chemicals constitute a class of salts with low melting points (below room temperature (RT)) [17–19]. Most RTILs are made by organic cations, such as ammonium and pyridinium, and anions that could also be inorganic, such as Cl^- and PF_6^- [17]. Due to the high number of possible combinations, up to 10^{18} RITLs are currently available [17]. RITLs recently gained a lot of interest in various fields for their many uses, including as a solvent for extractions [19–24], capillary electrophoresis (CE) [17,25], organic reactions [26], matrices for mass spectrometry [17], and stationary phases for gas chromatography [17,25,27,28]. They are considered excellent HS solvents due to their properties, such as high viscosities, negligible vapor pressures at RT, and good thermal stabilities. Because of this, they can be used for extended periods for HS extractions at higher T than conventional solvents [17,19]. Moreover, these RTILs can mitigate matrix effects caused by extremely complex

samples, due to their potential to help homogenize sample matrices, their capabilities to trap chemically similar analytes, and repel the opposite analytes of interest.

In this research, extractions were carried out with three hydrophilic RTILs, 1-ethyl-3-methylimidazolium diethyl phosphate ([EMIM][DEP]), 1-ethyl-3-methylimidazolium ethyl sulfate ([EMIM][ESO_4]), and tris(2-hydroxyethyl) methylammonium methylsulfate ([MTEOA][$MeOSO_3$]), one hydrophobic RTILS, 1-ethyl-3-methylimidazolium bis(trisfluoromethanesulfonyl)imide ([EMIM][NTf_2]), water (H_2O), MeOH, cyclohexane, hexane, and no solvent. The preliminary comparison was purely qualitative, and once [EMIM][ESO_4] was chosen as the best headspace co-solvent, other qualitative experiments were carried out to establish the optimum incubation time and T, the syringe T, the amount (g) of hops to solubilize with the RTIL, and the amount (mL) of HS to inject into the GC-VUV instrument.

6.2 Materials and Methods

6.2.1 Standards and Reagents

RTILs 1-ethyl-3-methylimidazolium diethyl phosphate ([EMIM][DEP]) (≥95%), 1-ethyl-3-methylimidazolium ethyl sulfate ([EMIM][ESO_4]) (≥95%), and tris(2-hydroxyethyl) methylammonium methylsulfate ([MTEOA][$MeOSO_3$]) (≥95%) were purchased at Sigma-Aldrich (St. louis, MO), while 1-ethyl-3-methylimidazolium bis(trisfluoromethanesulfonyl)imide ([EMIM][NTf_2]) (99%) was purchased at Solvionic (France). Water (LC-MS grade) and Methanol (LC-MS grade) were purchased at Honeywell Burdick & Jackson (Muskegon, MI), Hexanes (HPLC grade) at EMD Millipore Corporation (Germany), and Cyclohexane (HPLC grade) (99%) at Alfa Aesar (Haverhill, MA). The two Cannabis Terpenes Mix (1 and 2), were purchased at SPEX CertiPrep (Metuchen, NJ), while the three different varieties of hop were purchased at Austin

Homebrew Supply (Austin, TX). Amarillo, distinguished by its high Myrcene concentration, and a citrusy aroma, Columbus, with a pungent flavor, and high in co-humulene, and finally Magnum, without a particular aroma and used as a clean bittering hop due to the high humulene concentration.

6.2.2 Instrumental Analysis

Headspace sampling was performed with an AOC-5000 Plus (Shimadzu Scientific Instruments, Inc., Columbia, MD) unit. Samples were incubated and agitated at 120 °C for 20 min. A 5 mL 1005 GF HDHT Headspace SYR, Glue Free NDL, 26 ga, point style 5 (200 °C) syringe (Hamilton Robotics, Reno, NV) was used to inject 1 mL of sample with an injection speed of 500 µL/sec, at 120 °C. The samples were agitated for 20 min at 120 °C. A GC 2010 (Shimadzu Scientific Instruments) gas chromatograph coupled to a VGA-101(VUV Analytics, Inc., Cedar Park, TX) VUV detector was used in conjunction with a Rtx-5 column (30 m L x 0.25 mm i.d. x 0.25 µm d_f) (Restek Corp., Bellefonte, PA) to perform all analyses. The injection mode was splitless (1 mL injection volume; 60 sec hold time, 300 °C) and temperature programming was used: 60°C for 1 minute, then raised to 230°C at 20°C/min, and held for 0.5 minutes. The GC was operated in constant linear velocity mode (62.5 cm/sec) to provide a column flow (helium) of 3 mL/min. The temperature of both the transfer line and the flow cell were set to 275 °C, and the make-up gas (N_2) pressure was 0.5 psi, while the system pressure was set at 2 psi.

6.2.3 Sample Preparation

Approximately 0.68 mg of RTIL (approximately 0.58 mL) were pipetted into a 20 mL HS vial (Restek Corp.), along with 0.5 g of hops. The vials were immediately closed

with a magnetic screw and PTFE/silicone septa based cap (Restek Corp.). The sample was vortexed for 10 s, allowing the RTIL to totally wet the hops.

6.3 Results and Discussion

6.3.2 Optimization of Solvent

The first step was determining the best solvent for extraction, comparing both liquid and HS injection. The 3 hydrophilic RTILs, the hydrophobic RTIL, water, and no solvent were compared for HS extraction, using the same variety of hops, in the same quantity, and with the same amount of solvent, due to the high viscosities of RTILs. All of the analyses were carried out using 80 °C for agitation and syringe temperature, and 20 minutes was used as the agitation time. For the liquid injection, hexane, cyclohexane, and methanol were used and compared. The comparison was first carried out qualitatively, by determining the number of terpenes detected. The overall results showed that the extraction followed by liquid injection lead to the characterization of 1 unique specie of terpenes, while HS sampling extracted 10 unique terpenes using hydrophilic RTILs, 3 unique terpenes with the Hydrophobic one, and 2 unique ones with water and no solvent. This assessment indicated that the hydrophilic [EMIM][ESO_4] led to the best results.

6.3.3 Optimization of Incubation and Syringe Temperature

Once the hydrophilic RTIL was chosen as the solvent, the incubation and syringe temperature was then optimized. Usually, the two temperatures are equivalent or with few °C difference, in order to avoid the re-condensation of the analyte in the syringe. The chosen parameters were 80°C, 100°C, 110°C, and 120°C, using 20 minutes as agitation time, and the results show not only a higher amount of terpenes extracted at higher

temperatures, but also greater peak areas and therefore higher concentrations. The best analysis was obtained using 120 °C for both incubation and syringe temperature.

6.3.4 Optimization of Agitation Time

The third parameter to be optimized was the agitation time, the time the HS vial was heated before injection. The evaluated times were 10, 15, 20, 30, and 40 minutes, all with 120 °C syringe and incubation temperature. After 15 minutes (optimized value), the peak areas for determined terpenes was reaching a plateau.

6.3.5 Amount of Grinded Hops

The amount of plant material to analyze was then evaluated, comparing 0.1, 0.5, and 1.0 g of hops. Of course, the amount of relative RTIL to use varied based on the wetting capability (all of the plant material should be covered by the RTIL). It is surprising how there is no quantitative difference between 0.5 and 1.0 g of hops, therefore 0.5 g was chosen as final parameter in order to use less hops.

6.3.6 Amount of Injected HS Volume

Lastly, the amount of mL of HS to be injected in the GC-VUV system were evaluated, using 1.0, 3.0, and 5.0 mL as standard values. Even though 5 mL lead to higher peak areas than the other volumes, the following chromatographic parameters required a higher split ratio to avoid the saturation of the detector and the overloading of the column, therefore 1 mL was chosen as final parameter.

6.4 Conclusions

Different parameters were evaluated in this research, to optimize not solely the chromatographic separation of terpenes, but also their extraction from the plant matrix. The comparison between different injection methods showed that headspace was more satisfying than liquid injection, and that the use of a highly hydrophilic RTIL, [EMIM][ESO_4] in this case, lead to the extraction of a higher amount of terpene specimens. Considering the hydrophobic nature of terpenes, it is plausible that the use of a hydrophilic RTIL leading to better results is caused by the salting out effect during the process, meaning that hydrophilic interferents are trapped in the similar solvent, rather than extracted at the high temperature and sampled via headspace.

This method still needs to be validated on different types of hops, to assess the extraction and chromatographic efficiency, as well as the recovery, by spiking them with terpene standards at known concentrations. Three different types of hops were chosen, based on the flavor description given by the brewery, to evaluate the efficiency of this novel method at different terpene concentrations.

6.5 References

[1] Devon T. K., Handbook of Naturally Occurring Compounds. Elsevier 2012.

[2] Qiu C.,Smuts J.,Schug K. A., Analysis of terpenes and turpentines using gas chromatography with vacuum ultraviolet detection. *J. Sep. Sci.* 2017, *40*, 869–877 .

[3] Leghissa A.,Hildenbrand Z. L.,Schug K. A., A Review of Methods for the Chemical Characterization of Cannabis Natural Products. *J. Sep. Sci.* 2018, *41*, 398–415 .

[4] Breitmaier E., Terpenes: Flavors, Fragrances, Pharmaca, Pheromones. John Wiley & Sons 2006.

[5] Van Opstaele F.,De Causmaecker B.,Aerts G.,De Cooman L., Characterization of

novel varietal floral hop aromas by headspace solid phase microextraction and gas chromatography-mass spectrometry/olfactometry. *J. Agric. Food Chem.* 2012, *60*, 12270−12281 .

[6] Hamm S.,Bleton J.,Connan J.,Tchapla A., A chemical investigation by headspace SPME and GC-MS of volatile and semi-volatile terpenes in various olibanum samples. *Phytochemistry* 2005, *66*, 1499–1514 .

[7] Aberl A.,Coelhan M., Determination of volatile compounds in different hop Varieties by headspace-trap GC/MS-in comparison with conventional hop essential oil analysis. *J. Agric. Food Chem.* 2012, *60*, 2785−2792 .

[8] Leghissa A., Method Development for Qualification and Quantification of Cannabinoids and Terpens in Extracts by Gas Chromatography-Mass Spectrometry, University of Texas at Arlington, 2016.

[9] Bai L.,Smuts J.,Walsh P.,Fan H.,Hildenbrand Z.,Wong D.,Wetz D.,Schug K. A., Permanent gas analysis using gas chromatography with vacuum ultraviolet detection. *J. Chromatogr. A* 2015, *1388*, 244–250 .

[10] Walsh P.,Garbalena M.,Schug K. A., Rapid analysis and time interval deconvolution for comprehensive fuel compound group classification and speciation using gas chromatography–vacuum ultraviolet spectroscopy. *Anal. Chem.* 2016, *88*, 11130–11138 .

[11] Schenk J.,Mao J. X.,Smuts J.,Walsh P.,Kroll P.,Schug K. ., Analysis and deconvolution of dimethylnaphthalene isomers using gas chromatography vacuum ultraviolet spectroscopy and theoretical computations. *Anal. Chim. Acta* 2016, *945*, 1–8 .

[12] Leghissa A.,Smuts J.,Qiu C.,Hildenbrand Z. L.,Schug K. A., Detection of cannabinoids and cannabinoid metabolites using gas chromatography-vacuum

ultraviolet spectroscopy. *Sep. Sci. Plus* 2018, *1*, 37–42 .

[13] Schenk J.,Nagy G.,Pohl N. L. B.,Leghissa A.,Smuts J.,Schug K. A., Identification and deconvolution of carbohydrates with gas chromatography-vacuum ultraviolet spectroscopy. *J. Chromatogr. A* 2017, *1513*, 210–221 .

[14] Skultety L.,Frycak P.,Qiu C.,Smuts J.,Shear-Laude L.,Lemr K.,Mao J. X.,Kroll P.,Schug K. A.,Szewczak A.,Vaught C.,Lurie I.,Havlicek V., Resolution of isomeric new designer stimulants using gas chromatography – Vacuum ultraviolet spectroscopy and theoretical computations. *Anal. Chim. Acta* 2017, *971*, 55–67 .

[15] Fan H.,Smuts J.,Walsh P.,Harrison D.,Schug K. A., Gas chromatography-vacuum ultraviolet spectroscopy for multiclass pesticide identification. *J. Chromatogr. A* 2015, *1389*, 120–127 .

[16] Schug K. A.,Sawicki I.,Carlton D. D.,Fan H.,Mcnair H. M.,Nimmo J. P.,Kroll P.,Smuts J.,Walsh P.,Harrison D., Vacuum Ultraviolet Detector for Gas Chromatography. *Anal. Chem.* 2014, *86*, 8329–8335 .

[17] Sun P.,Armstrong D. W., Ionic liquids in analytical chemistry. *Anal. Chim. Acta* 2010, *661*, 1–16 .

[18] Anderson J. L.,Ding J.,Welton T.,Armstrong D. W., Characterizing ionic liquids on the basis of multiple solvation interactions. *J. Am. Chem. Soc.* 2002, *124*, 14247–14254 .

[19] Varona-Torres E.,Carlton D. D.,Hildenbrand Z. L.,Schug K. A., Matrix-effect-free determination of BTEX in variable soil compositions using room temperature ionic liquid co-solvents in static headspace gas chromatography mass spectrometry. *Anal. Chim. Acta* 2018, *1021*, 41–50 .

[20] Peng J. F.,Liu J. F.,Jiang G. Bin,Tai C.,Huang M. J., Ionic liquid for high temperature headspace liquid-phase microextraction of chlorinated anilines in environmental

water samples. *J. Chromatogr. A* 2005, *1072*, 3–6 .

[21] Liu J. F.,Li N.,Jiang G. Bin,Liu J. M.,Jönsson J. Å.,Wen M. J., Disposable ionic liquid coating for headspace solid-phase microextraction of benzene, toluene, ethylbenzene, and xylenes in paints followed by gas chromatography-flame ionization detection. *J. Chromatogr. A* 2005, *1066*, 27–32 .

[22] Vidal L.,Psillakis E.,Domini C. E.,Grané N.,Marken F.,Canals A., An ionic liquid as a solvent for headspace single drop microextraction of chlorobenzenes from water samples. *Anal. Chim. Acta* 2007, *584*, 189–195 .

[23] Ye C. L.,Zhou Q. X.,Wang X. M., Headspace liquid-phase microextraction using ionic liquid as extractant for the preconcentration of dichlorodiphenyltrichloroethane and its metabolites at trace levels in water samples. *Anal. Chim. Acta* 2006, *572*, 165–171 .

[24] Han X.,Armstrong D. W., Ionic liquids in separations. *Acc. Chem. Res.* 2007, 1079–1086 .

[25] Anderson J. L.,Armstrong D. W.,Wei G.-T., Ionic Liquids in Analytical Chemistry. *Anal. Chem.* 2006, 2893–2902 .

[26] Welton T., Room-Temperature Ionic Liquids. Solvents for Synthesis and Catalysis. *Chem. Rev.* 1999, 2071–2083 .

[27] Anderson J. L.,Armstrong D. W., High-stability ionic liquids. A new class of stationary phases for gas chromatography. *Anal. Chem.* 2003, 4851–4858 .

[28] Armstrong D. W.,He L.,Liu Y. S., Examination of ionic liquids and their interaction with molecules, when used as stationary phases in gas chromatography. *Anal. Chem.* 1999, *71*, 3873–3876 .

Chapter 7

ANALYSIS OF THE FRAGMENTATION PATHWAYS OF CANNABINOIDS; A COMPARISON BETWEEN ELECTRON IONIZATION (EI), ELECTROSPRAY IONIZATION (ESI) AND ATMOSPHERIC PRESSURE CHEMICAL IONIZATION (APCI) FOLLOWED BY FRAGMENTATION BY QQQ-MS.

7.1 Abstract

Cannabinoids, the main components of *Cannabis sativa,* are the chemicals that confer the primary medicinal and pharmaceutical properties to the plant. Therefore, there is a strong desire to optimize methods for their characterization and quantification. Because this class contains a significant number of isomers, obtaining detailed mass spectrometry fragmentations patterns may be key to differentiating different compounds. Using eight different cannabinoids, tetrahydrocannabinol (THCV), cannabichromene (CBC), cannabidiol (CBD), Δ8-tetrahydrocannabinol (Δ8-THC), Δ9-tetrahydrocannabinol (Δ9-THC), cannabigerol (CBG), cannabinol (CBN), and Δ9-tetrahydrocannabinolic acid (Δ9-THCA)), and the 2 main metabolites of Δ9-THC (11-hydroxy-Δ9-tetrahydrocannabinol (11-OH-Δ9-THC) and 11-nor-9-carboxy-Δ9-tetrahydrocannabinol (11-nor-9-carboxy-Δ9-THC)), we compared the structure and identity of the three most abundant fragmentation ion products produced by electron ionization – mass spectrometry (EI-MS), electrospray ionization – tandem mass spectrometry (ESI-MS/MS), and atmospheric pressure chemical ionization – tandem mass spectrometry (APCI-MS/MS). The fragments were divided into different categories based on the common fragmentation pathways, and it was noticeable that some of them were formed not only in both positive APCI and ESI, but also in EI. Furthermore, some fragments may have different m/z but same pathway of formation, due

to the non-isomeric nature of the cannabinoids, and even more surprisingly, some of them are observed in both positive and negative mode.

7.2 Intruduction

Interest in *Cannabis sativa* and its main chemical components, cannabinoids, is rapidly growing in a variety of fields, including in analytical chemistry, medicinal chemistry, biochemistry, and pharmaceutical science. New methods for fast analysis of cannabinoids are being reported regularly. In the laboratory, these methods focus mainly on the use of gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS). These techniques offer the appropriate sensitivity and specificity to qualitatively and quantitatively characterize complex cannabinoid natural products extracted from plants, extracts, and other cannabis-infused products [1].

As of now, cannabinoids have been divided into 10 different categories [1,2], but the interest of this research is mainly focused on 8 of them, due to the standards' availability. The structures for each of these compounds is shown in Figure 7.1, which includes tetrahydrocannabinol (THCV), cannabichromene (CBC), cannabidiol (CBD), Δ8-tetrahydrocannabinol (Δ8-THC), Δ9-tetrahydrocannabinol (Δ9-THC), cannabigerol (CBG), cannabinol (CBN), and Δ9-tetrahydrocannabinolic acid (Δ9-THCA)), and on the metabolites of Δ9-THC (11-hydroxy-Δ9-tetrahydrocannabinol (11-OH- Δ9-THC), and 11-nor-9-carboxy- Δ9-tetrahydrocannabinol (11-nor-9-carboxy- Δ9-THC).

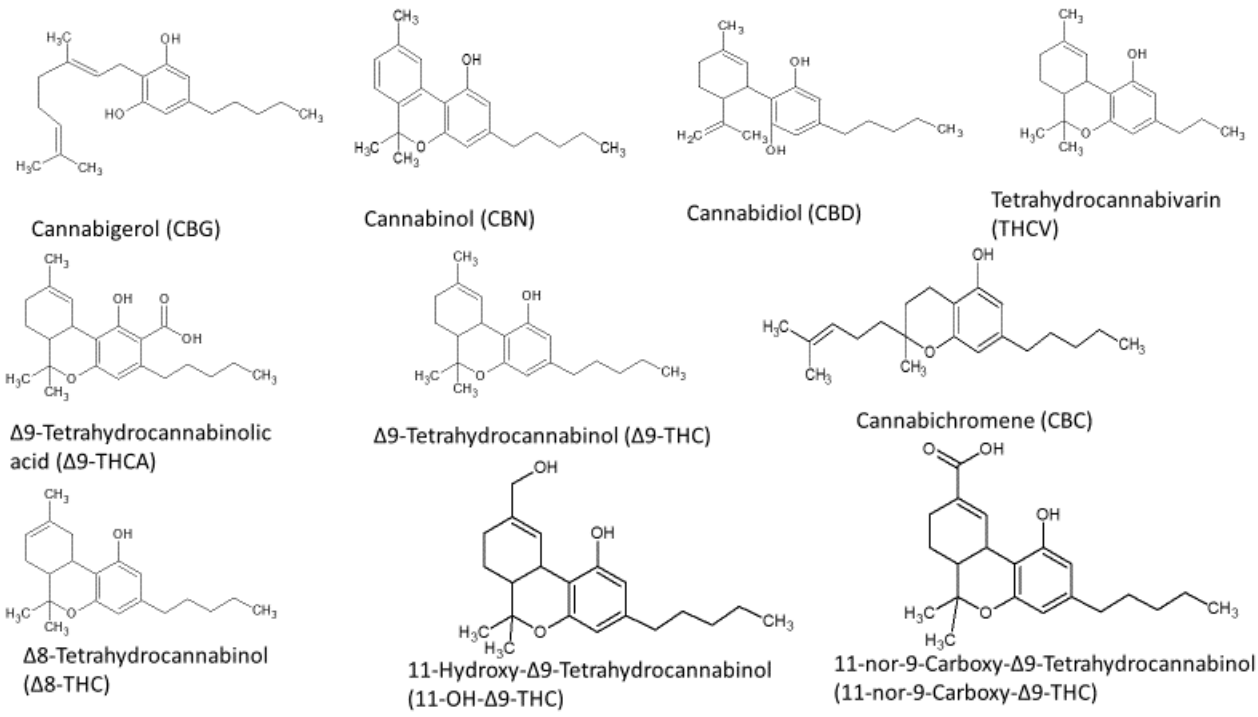


Figure 7.5 The 8 different cannabinoids and 2 metabolites studied in this research.

Even though the analysis of cannabinoids can be performed on both GC and LC, due to their chemical stability and volatility, it is important to notice that Δ9-THCA, 11-OH-Δ9-THC, and 11-nor-9-carboxy-Δ9-THC present a carboxyl group that is detached at the high temperature reached in the GC injection port. Therefore, they require a silylation derivatization prior to analysis, to protect and maintain their original structure [3,4]. The sylilation reaction for Δ9-THC is proposed in Figure 7.2.

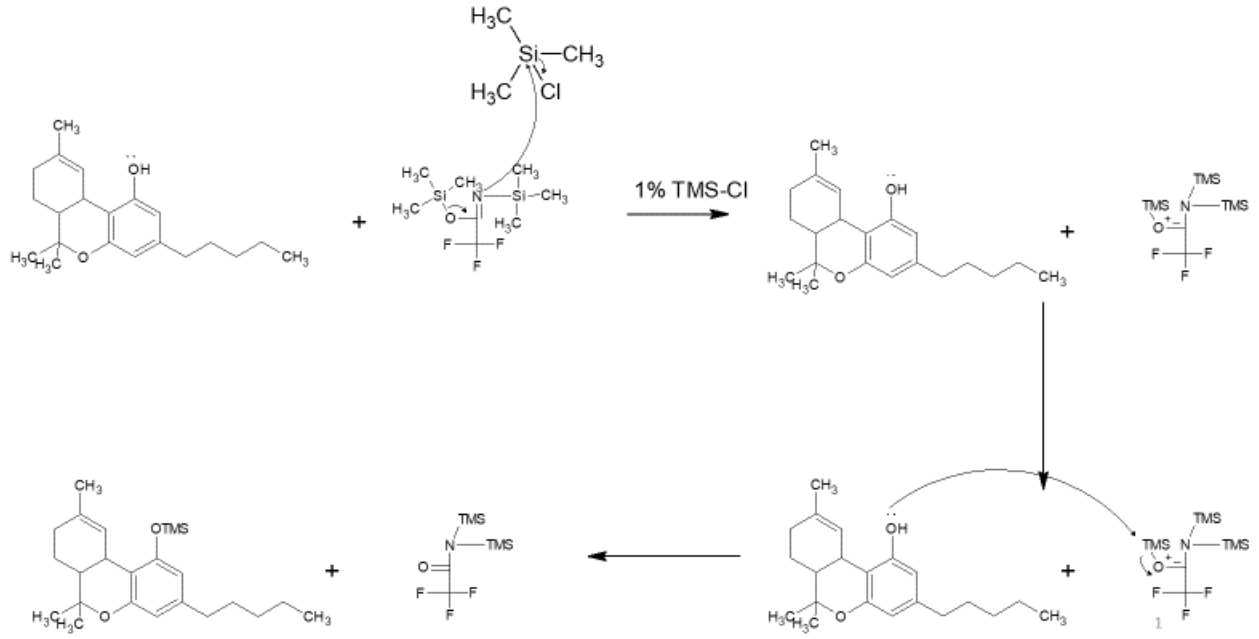


Figure 7.6 Proposed silylation mechanism for Δ9-THC.

As of today, mass spectrometry (MS) is one of the most widely used detector coupled with both GC and LC. Its popularity is due to its high sensitivity and its near universality, the capacity of analyzing almost any type of samples [5]. Each mass spectrometer contains two primary differentiating components, the ionization source and the mass analyzer, which dictate their performance in different applications [6,7]. Both of them exist in a wide variety of types, depending on the final goal of the analysis and the type of sample to be analyzed[8].

For samples introduced to the MS in the gaseous phase (e.g. by a GC or direct insertion probe), the most common ionization source is electron ionization (EI) [9]. EI was first developed in 1918 by Dempster et al. [9], making it one of the oldest ionization sources, and it is widely used with analytes with molecular weight (MW) lower than 600 amu. Once the gaseous molecules reach the source, they are bombarded with a beam of electrons in

a low pressure area (10^{-5}-10^{-6} Torr), and these collisions cause the analyte (M) to lose an electron, creating a positive ion with an odd number of electrons (i.e., a radical cation, or an odd-electron ion). EI is often referred to as an hard ionization source due to the large number of diagnostic fragment ions it produces when operated at its standard 70 eV ionization energy [5,9,10].

When the analyte of interest is in liquid phase, other ionization modes need to be used, in order to evaporate the high amount of solvent that accompanies the analytes. The two main modes of achieving this are through the use of either electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) [5].

ESI was first developed in the 1960s and 1970s, but its application towards analysis of biological molecules was popularized by Fenn [11,12], who was awarded the Nobel Prize in Chemistry in 2002 after discovering the potential for ESI to impart multiple charges on protein molecules. Even though the exact mechanism occurring in the ESI source is still cause for debate, it is believed that it happens in 3 stages; charged droplet formation, droplet shrinkage, and ion evaporation/desolvation [5,13]. Due to the high voltage applied at the entrance of the source, a partial separation of charges in the liquid occurs. When working in positive mode, the cations accumulate on the tip of the capillary and migrate towards the counter electrode, while anions migrate away from the tip; the opposite happens in negative mode. The movement of the cations towards the counter electrode cause the formation of the so-called Taylor cone at the tip of the capillary. This process is caused by the counterbalance between the movement of ions and the surface tension of the liquid. At this point, due to the voltage, the cone breaks into a mist of fine droplets from which the solvent is evaporated, leading to droplet shrinkage. The continuous shrinkage of this charged droplets is followed by an increase in the charge density of the particles, until the Rayleigh limit is reached; at this point, the repulsive forces are higher

than the droplets surface tension, causing a Coulombic explosion into second-generation droplets. The result of this technique is the formation of mainly protonated or deprotonated pseudo-molecular ions ($[M+ nH]^{n+}$ or $[M-nH]^{n-}$). These are even electron ions because all electrons are paired. ESI is said to facilitate transfer of ions formed into solution, into the gas phase. Further, ESI is a soft ionization technique, which does not impart a significant amount of excess energy into the molecule during ionization; thus, fragmentation upon ionization is minimal. To interrogate the ion further in the gas phase, tandem mass spectrometry techniques are need to induce further fragmentation [5,13].

The other common ionization source for ionization of samples from liquid sample studied in this research is APCI; this technique, in contrast to ESI, is widely used for the analysis of less polar and thermally stable compounds, with a maximum MW of 1500 amu [5,14]; no multiple charging is observed. This source was first developed by Horning at al. [15,16], and it is made by 3 main parts, a heated nebulizer probe (350°-500°C), a corona discharge ionization site, and an intermediate-pressure ion-transfer region [5]. The sample reaches the APCI from the LC system through a capillary tube, where the nebulizer and make-up gas flow coaxially, creating a mist of fine droplets. The heat applied to the gas in the heated nebulizer probe transforms these droplets into a gas stream, before reaching the ionization area. In this part, a corona discharge electrode with a potential between 2-3 kV ionizes the solvent molecules surrounding the analyte of interest. These reagent ions then transfer their charge to the analyte, and the resulting ions are then taken to the analyzer through a series of skimmer cones [15,17]. APCI generally produces even-electron ions, but some od-electron ion forms are also possible to be observed. APCI is a little less soft than ESI, and some unusual ion forms, even some odd-electron ions, can also be observed [5].

The aim of this work was to analyze and study the main fragmentation pathways occurring for the 10 cannabinoids when coupling these 3 ionization sources with a triple quadrupole analyzer, that allowed for a further fragmentation of the ions of interest with a collision induced gas (Ar). The first step was the selection of the most abundant precursor ions, and then the 3 major transitions were isolated and studied. Only three transitions were chosen because previous studies [3] showed that this was the number needed to adequately speciate between isobaric cannabinoids via GC-MS, and therefore this amount was kept consistent even with LC-MS. Collision energies were not optimized, because the focus was mainly on the m/z obtained and the relative structure, independently of the voltage applied.

7.3 Materials and Methods

7.3.1 Standards and Reagents

Cannabinoid standards (CBC, CBD, THCV, CBG, CBN, Δ8-THC, Δ9-THC, Δ9-THCA, Δ9-THC-OH, and 11-Nor-9-carboxy-THC) were purchased from Cerilliant Corporation (Round Rock, TX) as drug enforcement agency (DEA)-exempt solutions (1 mg/mL) in methanol (MeOH). MeOH and ethyl acetate (EtAc), both LC-MS grade, were purchased from Honeywell Burdick & Jackson (Muskegon, MI). For derivatization, *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA+1%TMCS) was purchased from Restek Corporation (Bellefonte, PA).

7.3.2 *Instrumental analysis*

A GCMS TQ8040 (Shimadzu Scientific Instrument, Inc., Columbia, MD) gas chromatograph coupled through electron ionization to a 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) to perform the first set of analyses.

The injection mode was splitless (1 µL injection volume; 60 sec hold time) and temperature programming was utilized: 40 °C for 1 min; raised to 200 °C at 20 °C/min; raised to 235 °C (for the underivatized cannabinoids) or to 242 °C (for the derivatized cannabinoids) at 3 °C/min; and held for 3 min. The GC was operated in constant linear velocity mode (53.4 cm/sec) to provide a column flow (helium) of 1.46 mL/min [3]. To perform the silylation reaction for the GC-MS analysis, 2 mL of the 50 mg/L solution containing the eight cannabinoids was dried with a gentle stream of N_2, and then 1 mL of ethyl acetate and 1 mL of BSTFA+1%TMCS were added. The solution was heated in a sealed vial at 70 °C for 30 minutes and allowed to cool before analysis [3].

For liquid sample introduction, a Shimadzu NEXERA UC (Shimadzu Scientific Instrument, Inc.) was used with no column, and MeOH as isocratic mobile phase, with 0.25 mL/min as column flow. The nebulizing gas flow was set at 2 L/min, while the heating gas flow at 10 L/min. the different temperatures were set as; Interface 300°C, DL 250°C, and heat block at 400°C. Finally, the drying gas flow was set at 10 L/min.

7.4 Results and Discussion

The first comparison that was drawn was between cannabinoids with same starting MW under the same ionization mode, but after studying all the different transitions, it was noticeable that the same fragmentation pathway was undergone by cannabinoids with different starting MW, and therefore with different final m/z, but also among different sources.

The first example is m/z 193 (Figure 3), who foresees the loss of the 2 cyclohexanes connected to the central phenol, and that occurs with Δ9-THC, Δ8-THC,

CBG, CBD, CBC, and 11-OH-Δ9-THC in both positive ESI and APCI, with 11-nor-9-Carboxy-Δ9-THC is positive ESI, and in CBG also with EI. The interesting fact is that also Δ9-THCA-2TMS creates the equivalent fragment in EI, but with final m/z 381 (Figure 7.3), due to the trimethylsilyl groups attached to the alcohol groups. At the same way, THCV forms the same type of fragment with final m/z 165 (Figure 7.3) on both positive APCI and ESI, while CBN forms m/z 179 (Figure 7.3) solely with positive ESI. This one is the most common fragment obtained from these cannabinoids, meaning that it could be used in the future as starting point for the discovery of new species of cannabinoids.

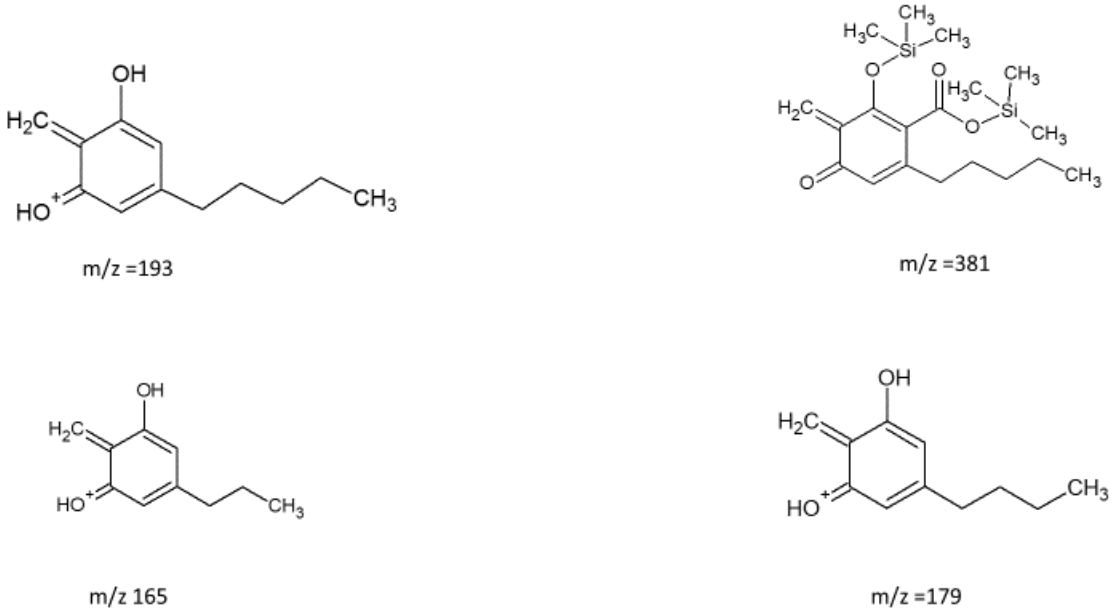


Figure 7.7. Proposed structures for m/z 193, m/z 381, m/z 165, and m/z 179.

Another common fragment obtained in negative APCI and ESI, therefore not obtainable with EI, is m/z 245 (Figure 7.4). This fragment is given by Δ9-THC, CBD, and Δ9-THCA in both ESI and APCI, by 11-nor-9-Carboxy-Δ9-THC solely by ESI, and by Δ8-

THC by APCI. Just like for the previous m/z, also THCV undergoes this fragmentation pathway, forming a final m/z 217 (Figure 7.4) with negative APCI.

Figure 7.8. Proposed structures for m/z 245, and m/z 217

Always in negative APCI and ESI mode, the fragment m/z 191 (Figure 7.5) is commonly formed. Δ9-THC, CBG, and CBC foresee it in both APCI and ESI, while 11-nor-9-Carboxy-Δ9-THC solely by ESI, and Δ8-THC by APCI, just like for m/z 245. THCV, on the other end, undergoes the same pathway on negative APCI, forming a final m/z 163 (Figure 7.5). Interestingly, Δ9-THCA creates the same m/z 191 with negative APCI, but two possible structures have been identified; both shown in Figure 7.5.

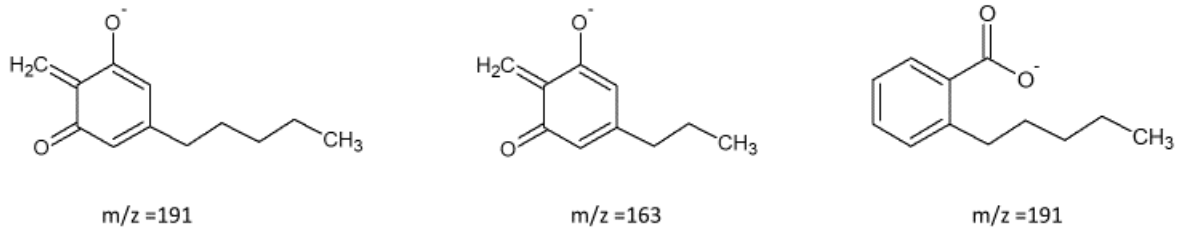


Figure 7.9. Proposed structures for m/z 191, and m/z 163.

A fragment that is formed on both positive APCI and ESI, but also on EI, is m/z 123 (Figure 7.6); Δ9-THC, Δ8-THC, CBD, and THCV create it with positive APCI, while CBG on EI, APCI, and also ESI.

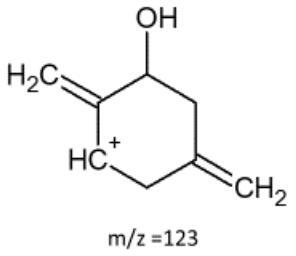


Figure 7.10. Proposed structure for m/z 123.

This fragment is extremely interesting because, together with m/z 193, is the only one formed with all three sources, whereas m/z 223 is formed solely by EI and positive APCI by CBN.

Other fragments, such as m/z 43, 102, 135, 203, and 111, on the other hand, are formed by different cannabinoids but only in one source; m/z 43 is given from CBN and 11-OH- Δ9-THC by positive ESI, m/z 111 by Δ8-THC, CBD, and CBC solely by negative ESI, m/z 102 by Δ9-THC, Δ8-THC, and CBC with positive ESI, m/z 135 by Δ9-THC, Δ8-THC, and THCV by positive APCI, and m/z 203 by Δ8-THC and CBC under negative ESI.

There is another type of fragments, such as m/z 107, 299 and 69, that are formed in both positive and negative mode, leading to different final structures. m/z 107 is given by Δ8-THC, CBD, THCV, and Δ9-THCA under negative ESI, but also by Δ9-THCA and CBD by positive ESI, whereas m/z 69 by Δ9-THCA in negative ESI, and by 11-OH- Δ9-

THC by positive ESI. m/z 299, on the other hand, is formed solely by the metabolite 11-nor-9-carboxy- Δ9-THC, but in both positive and negative APCI and ESI.

The last class of fragments are those created with both ESI and APCI but from only one cannabinoids, such as m/z 219 and 279; the former is obtained from Δ9-THCA in negative mode, while the latter from CBN, in negative mode as well.

7.5 Conclusions

Whereas it was predictable that the same source would produce similar fragments for isobaric cannabinoids, the creation of same m/z among different types of sources, and especially when comparing hard and soft ones, is what makes the study of cannabinoids extraordinary. These similarities are important because they could be used in the future for the determination of new species that naturally occur in plants but are at extremely low concentrations, and therefore have never been detected, but also for having an easier database to use for the study of new synthetic cannabinoids. This last possibility, in fact, has to be taken in consideration because it was observed that even if starting from different MW and finishing with different m/z, non-isobaric cannabinoids may undergo the same types of fragmentation.

It would be interesting also to perform a study to confirm whether this degradation happens in absence of CID, but for instance in a biological environment, and it could help

better understanding the synergistic effect between cannabinoids and terpenes, but also how cannabinoids bind to the cannabinoid receptors in the human body.

7.6 References

[1] Leghissa A.,Hildenbrand Z. L.,Schug K. A., A Review of Methods for the Chemical Characterization of Cannabis Natural Products. *J. Sep. Sci.* 2018, *41*, 398–415 .

[2] ElSohly M. A.,Slade D., Chemical constituents of marijuana: The complex mixture of natural cannabinoids. *Life Sci.* 2005, *78*, 539–548 .

[3] Leghissa A.,Hildenbrand Z. L.,Foss F. W.,Schug K. A., Determination of cannabinoids from a surrogate hops matrix using multiple reaction monitoring gas chromatography with triple quadrupole mass spectrometry. *J. Sep. Sci.* 2018, *41*, 459–468. .

[4] Leghissa A.,Hildenbrand Z. L.,Foss Jr F. W.,Schug K. A., Determination of the metabolites of Δ9-tetrahydrocannabinol in urine and plasma using multiple reaction monitoring gas chromatography with triple quadrupole mass spectrometry. *Sep. Sci. Plus* 2018, *1*, 43–47 .

[5] Dass C., Fundamentals of Contemporary Mass Spectrometry. John Wiley & Sons, Hoboken, New Jersey 2007.

[6] Dole M.,Mack L. L.,Hines R. L.,Mobley R. C.,Ferguson L. D.,Alice M. B., Molecular beams of macroions. *J. Chem. Phys.* 1968, *49*, 2240–2249 .

[7] Iribarne J. V.,Thomson B. A., Field induced ion evaporation from liquid surfaces at atmospheric pressure. *J. Chem. Phys.* 1979, *71*, 4451–4463 .

[8] de Hoffman E.,Stroobant V., Mass Spectrometry: Principles and Applications. John Wiley and Sons 2001.

[9] Dempster A. J., Positive Ray Analysis of Lithium and Magnesium. *Phys. Rev.* 1921, *18*, 415–422 .

[10] Traeger J. C., The Development of Ion Production Methods The Development of Electron Ionization. *Encycl. Mass Spectrom.* 2015, 77–82 .

[11] Fenn J. B.,Mann M.,Meng C. K.,Wong S. F.,Whitehouse C. M., Electrospray ionization for mass spectrometry of large biomolecules. *Science (80-.).* 1989, *246*, 64–71 .

[12] Fenn J. B.,Mann M.,Meng C. K.,Wong S. F.,Whitehouse C. M., Electrospray ionization–principles and practice. *Mass Spectrom. Rev.* 1990, *9*, 37–70 .

[13] Kebarle P., A brief overview of the present status of the mechanisms involved in electrospray mass spectrometry. *J. mass Spectrom.* 2000, *35*, 804–817 .

[14] Andrade F. J.,Shelley J. T.,Wetze W. C.,Webb M. R.,Gamez G.,Ray S. J.,Hieftje G. M., Atmospheric pressure chemical ionization source. 1. Ionization of compounds in the gas phase. *Anal. Chem.* 2008, DOI: 10.1021/ac800156y.

[15] Horning E. C.,Horning M. G.,Carroll D. I.,Dzidic I.,Stillwell R. N., New picogram detection system based on a mass spectrometer with an external ionization source at atmospheric pressure. *Anal. Chem.* 1973, *45*, 936–943 .

[16] Carroll D. I.,Dzidic I.,Stillwell R. N.,Horning M. G.,Horning E. C., Subpicogram detection system for gas phase analysis based upon atmospheric pressure ionization (API) mass spectrometry. *Anal. Chem.* 1974, *46*, 706–710 .

[17] Carroll D. I.,Dzidic I.,Stillwell R. N.,Haegele K. D.,Horning E. C., Atmospheric pressure ionization mass spectrometry: Corona discharge ion source for use in liquid chromatography-mass spectrometer-computer analytical system. *Anal. Chem.* 1975, *47*, 2369–2373 .

Chapter 8

SUMMARY AND FUTURE WORK

GC coupled with two different detectors (MS and VUV) was found to provide adequate tools for the analysis of *C. sativa* components and their metabolites, ranging from high to low concentration targets. While direct application to C. sativa was limited due to lack of a DEA license, it was possible to develop and test methods using DEA-exempt standards and hops, as a matrix surrogate.

In the MS detector, the use of an MRM mode was used to enhance sensitivity and specificity, in order to detect and quantify components and metabolites at low concentrations, and with minimum interference from the plant or biological matrices, leading to great specificity for the analytes of interest. The downside of using GC as separation technique is the necessity to derivatize the different cannabinoids prior analysis, because the high temperatures of the injector port will decarboxylate and degrade the acidic analytes. The need for this preparation step is the plausible reason why GC has not been widely used in the past for the analysis of cannabinoids, and it would be therefore interesting to study and compare the different specifics of GC and LC towards these analytes.

This method and the study of the different fragmentation pathways of the molecules were used as a starting point for the creation of a method for the potential discovery of new species. This concept would be based on the fact that different cannabinoids have many similarities, and that these similarities, as manifested in tandem MS fragmentation patterns would allow one to identify a new species. The information that would be used to develop this approach was further augmented beyond just GC-EI-MS/MS-based fragmentation patterns, but also using LC-MS with the 2 different sources

ESI and APCI. Many fragments involve Retro Diels-Alder reactions with the opening of side cycles, and the loss of the C side chains; what is interesting is that even non-isobaric cannabinoids undergo the same types of fragmentation, leading though to different final m/z due to the different initial molecular weight. Because of this, a Precursor Ion Scan may be used in the future for this type of discovery study, identifying new cannabinoids based on these suggested fragmentation patterns. Furthermore, it would be interested to use this information for the discovery of human metabolites of all the other cannabinoids, by running human plasma and urine with PIS. .

With the VUV detector, a fast and easy chromatographic separation was obtained even thanks to the use of the deconvolving capabilities of the instrument. Because of this, in fact, faster flow and temperature ramp could be used without worrying about co-eluting isomers, that were separated post-run. This method is not as sensitive as the MS, therefore it is indicated for potency testing and characterization of the main cannabinoid species. Considering the wide number of techniques and methods that are now being used for potency testing, it needs to be considered that GC-VUV may not be as sensitive as LC-UV, but the main advantage of this instrument is the ability to eventually analyze more than one type of analyte found in *C. sativa,* such as terpenes, solvent residues, and some pesticides.

The GC-VUV is also an excellent tool for the analysis of terpenes, due to its ability to differentiate among isomers that have even only minimal differences in their absorbance spectra. The search for the best extraction and chromatographic conditions for these analytes is still being investigated, due to the high number of species that each plant carries. In this research, we explored the ability of using RTILs as possible co-solvents for headspace sampling, arriving to the conclusion that they work more efficiently than the other solvents due to their ability to resist higher temperatures during the sampling. Once

this method has been validated on hops, it would be interesting to test it using chiral columns, with the purpose to differentiate enantiomeric terpenes. The same technique may be used also for complex *C. sativa* matrices, such as edibles, hoping that the use of these c-solvents would eliminate the necessary and long preparation steps to clean the complex samples.

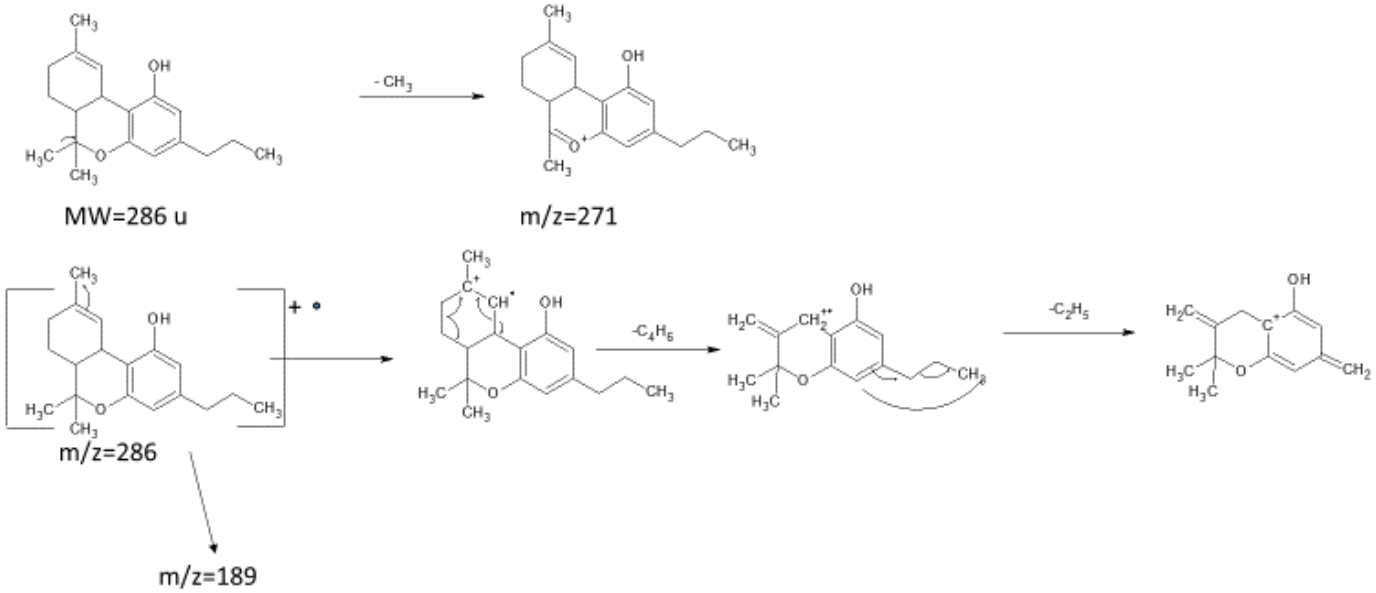
The future for *C. sativa* research is still wide and full of possibilities,and considering the great legalization process that is going on in the United States, more efforts are going to be put daily for the total understanding of the plant and its effects. The next important steps to be taken are the chiral analysis of both cannabinoids and terpenes, that confer different benefits to each specimen, and they can be carried out with the use of a chiral stationary phase such as cyclodextrins. Furthermore, discovering how the entouragic effect works is going to be a breakthrough in this field, helping both patients and doctors to better assess different cases and possible cures for each disease that needs to be treated. Another important step to be taken due to the increasing use of *C. sativa* as medical treatment, is the characterization of all cannabinoids' metabolites, and how these molecules interact with the receptors in the human body. The same method used for the discovery of new species in the plant can be used for this purpose, using liver cells in vitro to produce metabolites and then characterize them on the LC or GC, and from there understand the biochemistry behind the action mechanism.

Appendix A

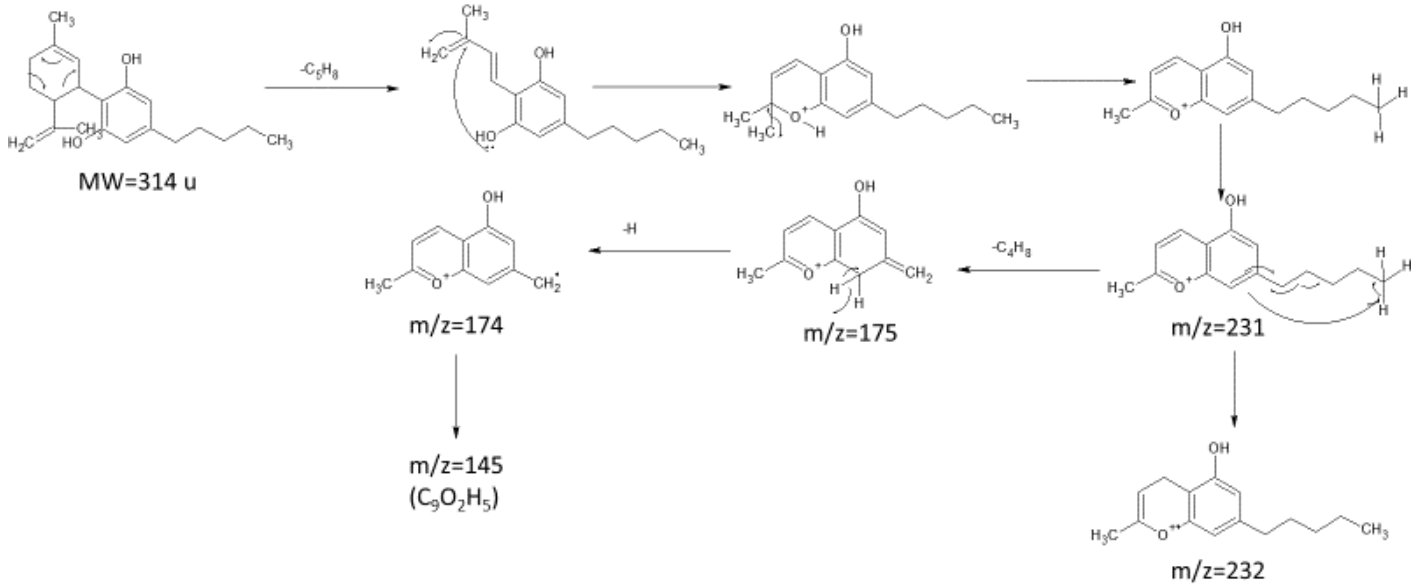
Suggested Fragmentation Pathways of 7 Underivatized and 8 Silylated Cannabinoids

Included in the electronic supplementary information are the fragmentation pathways proposed for 6 underivatized cannabinoids, and for 7 silylated versions. Fragmentation pathways for Δ9-THC are given in the main text.

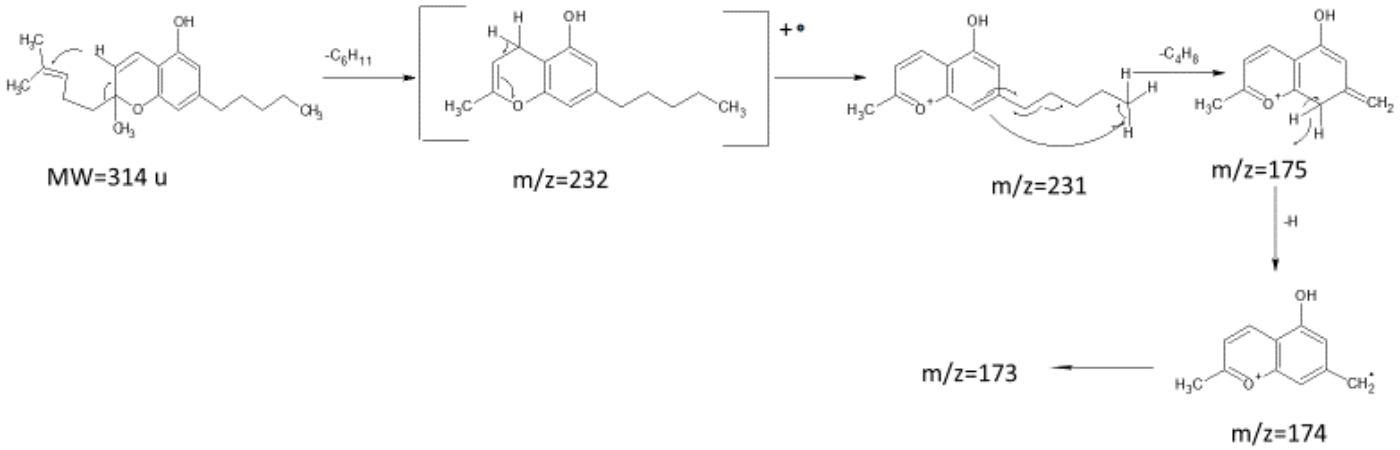
Tetrahydrocannabivarin



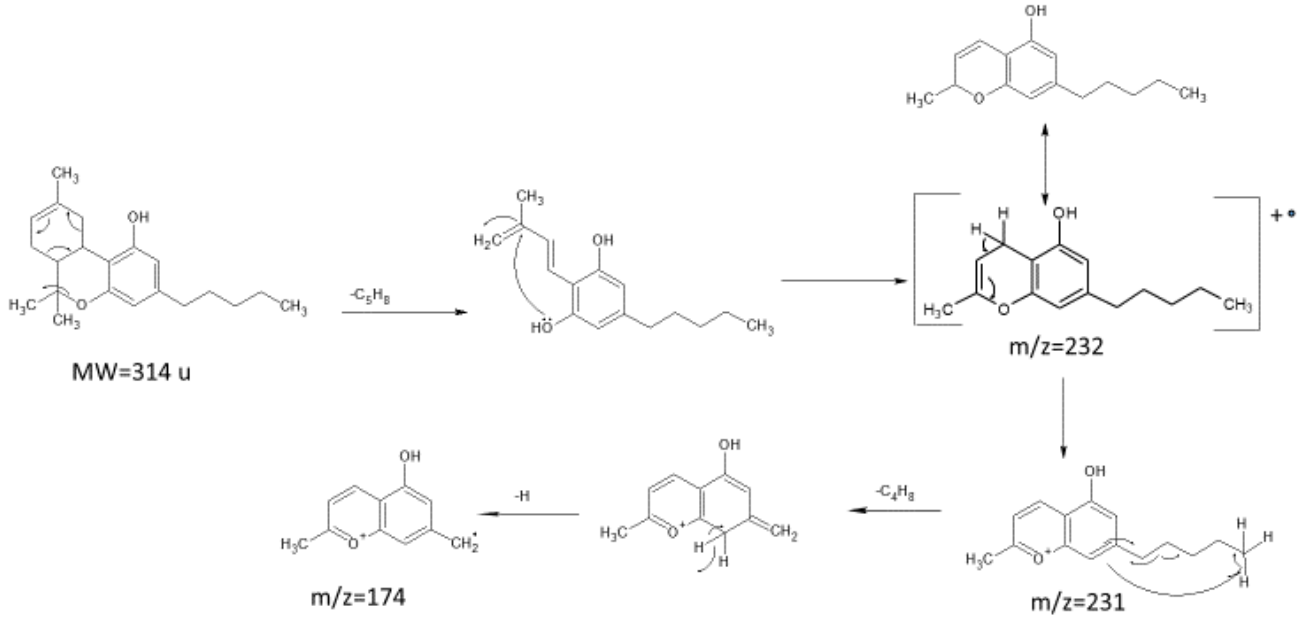
Cannabidiol



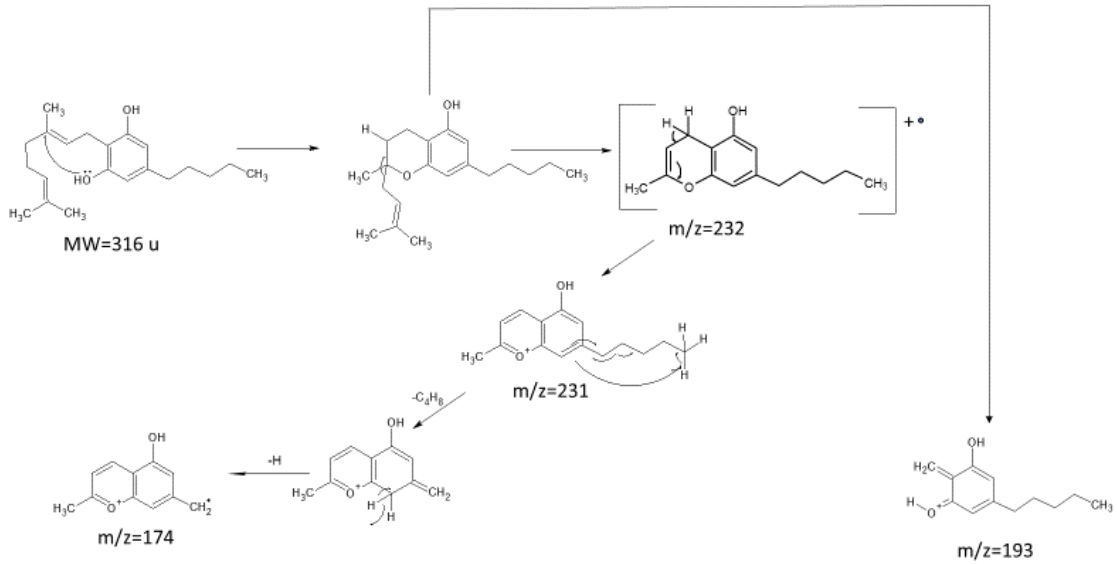
Cannabichromene



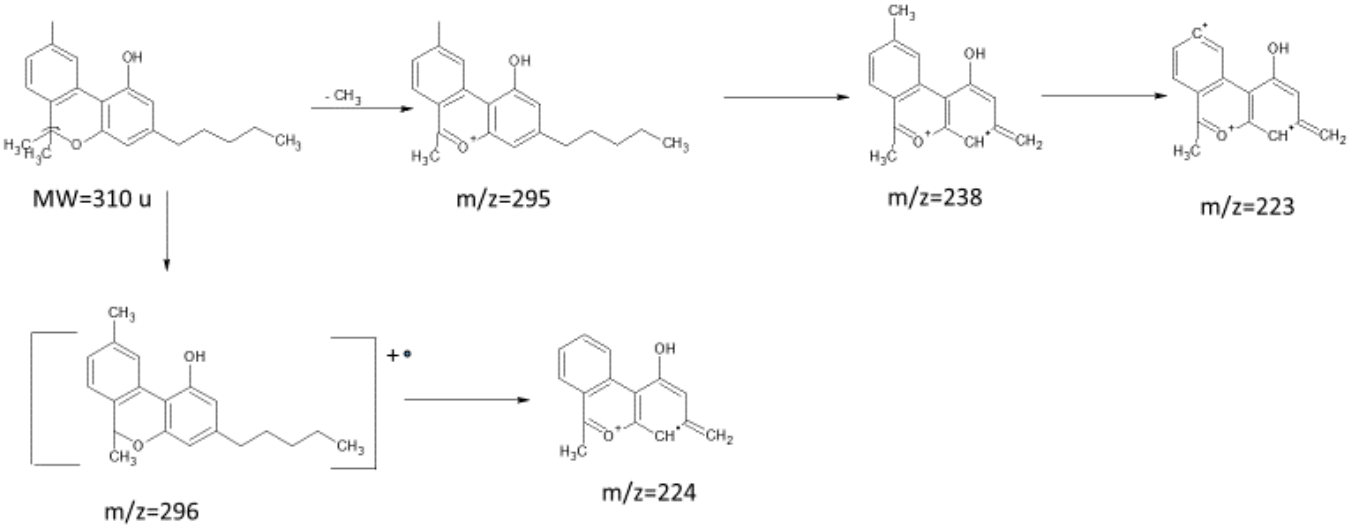
Δ8-Tetrahydrocannabinol



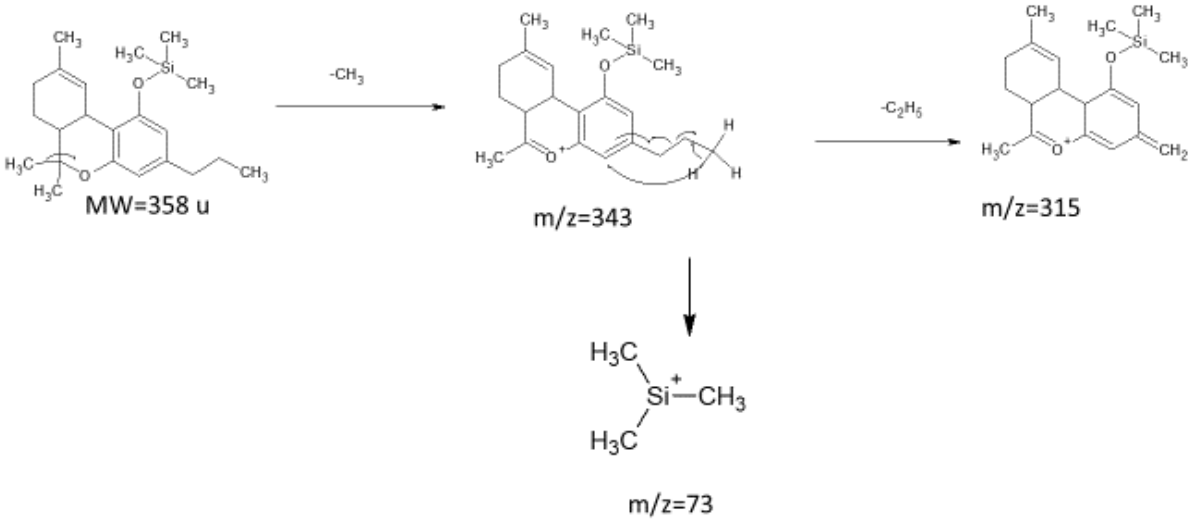
Cannabigerol



Cannabinol



Tetrahydrocannabivarin-TMS



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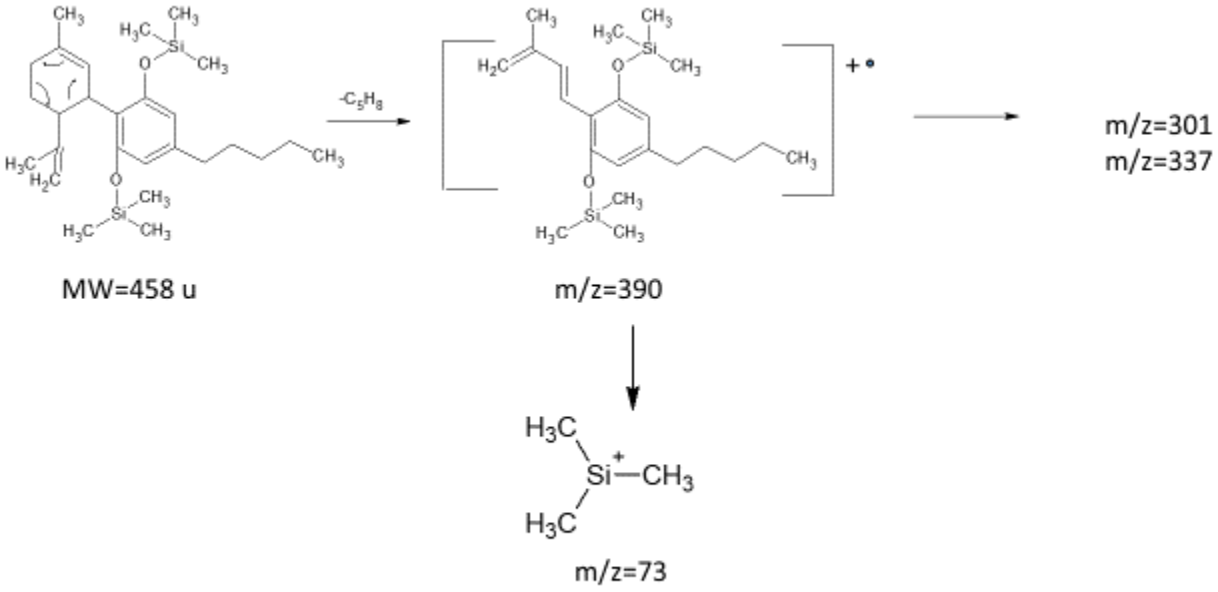

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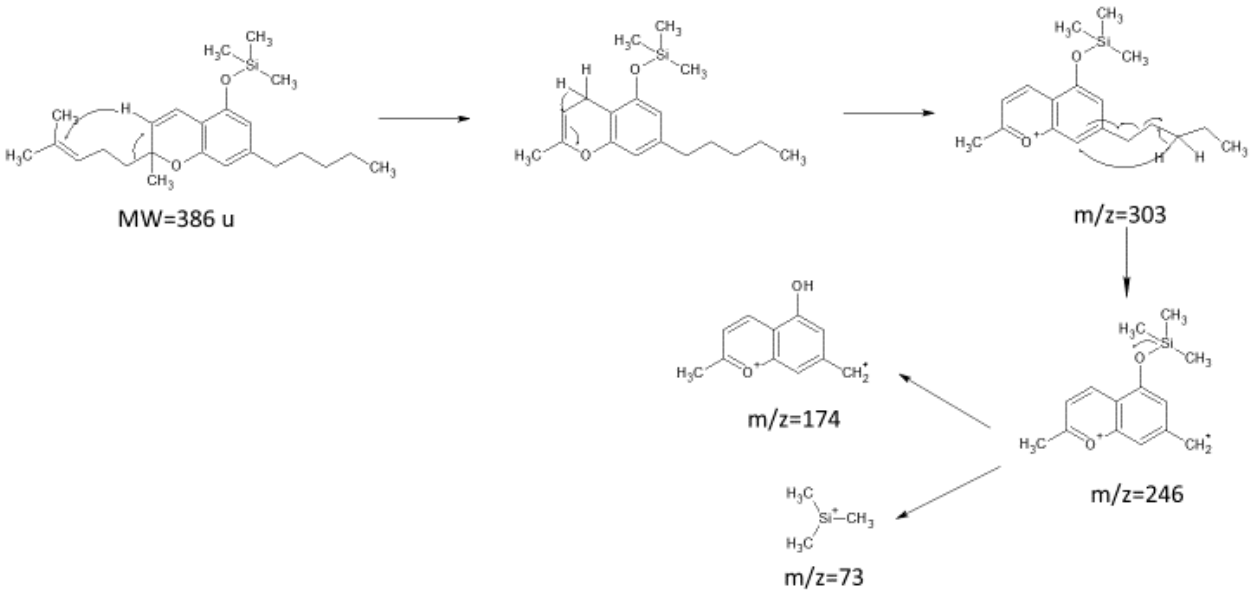
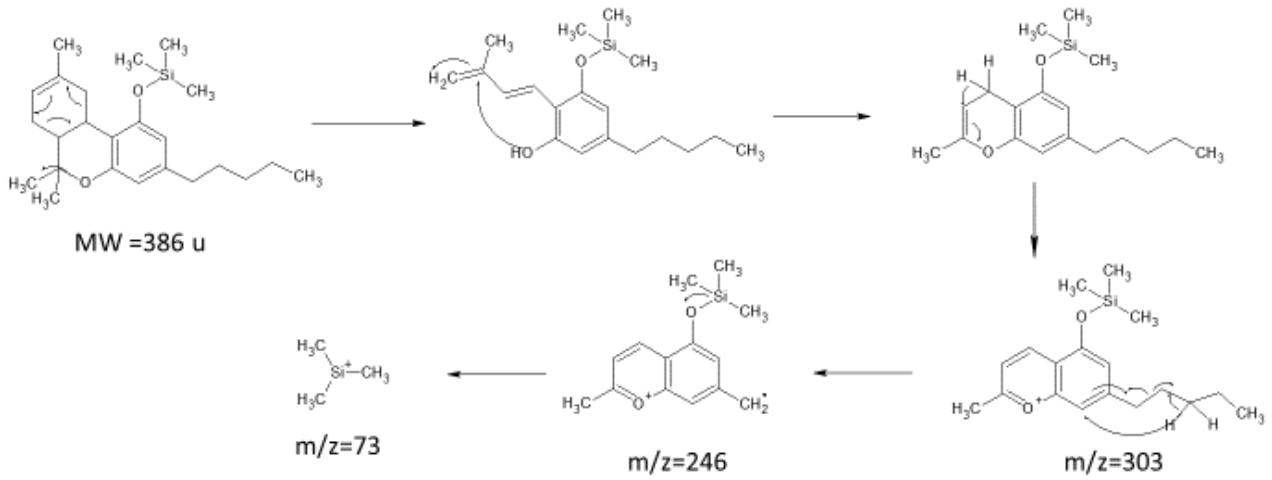
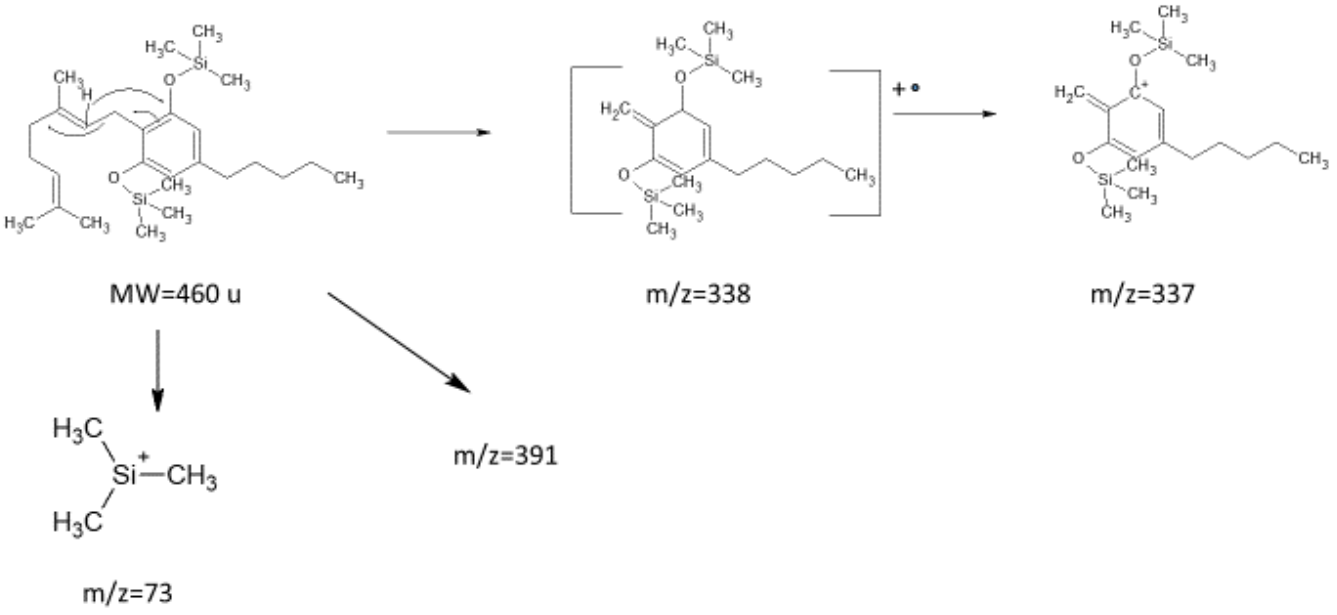


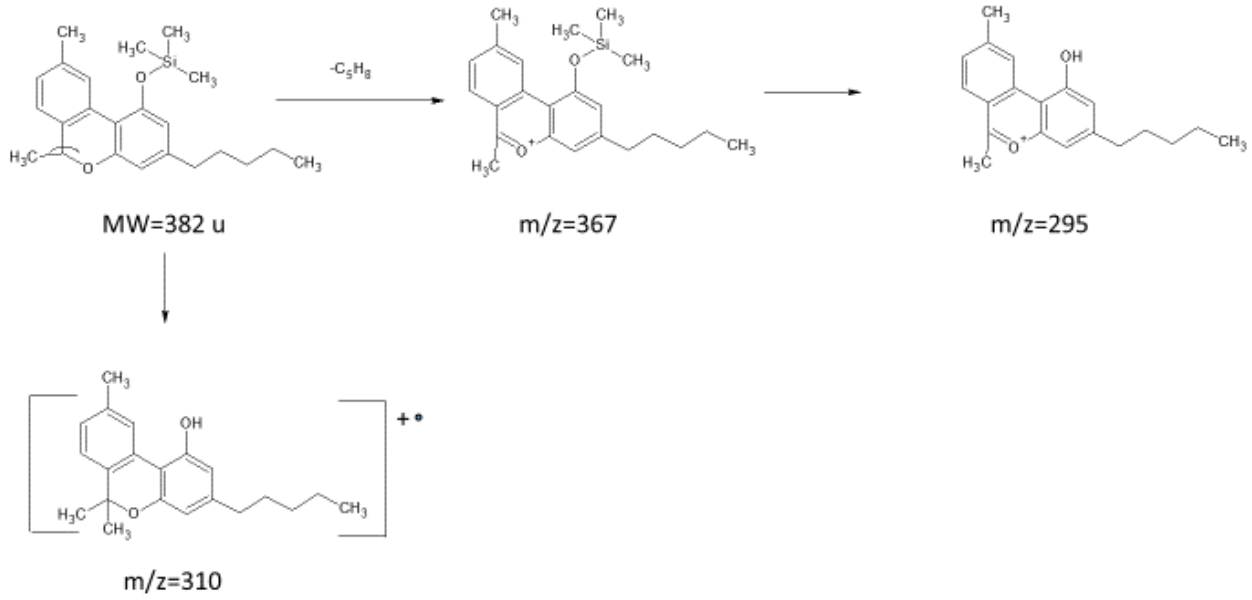
Δ8-Tetrahydrocannabinol-TMS



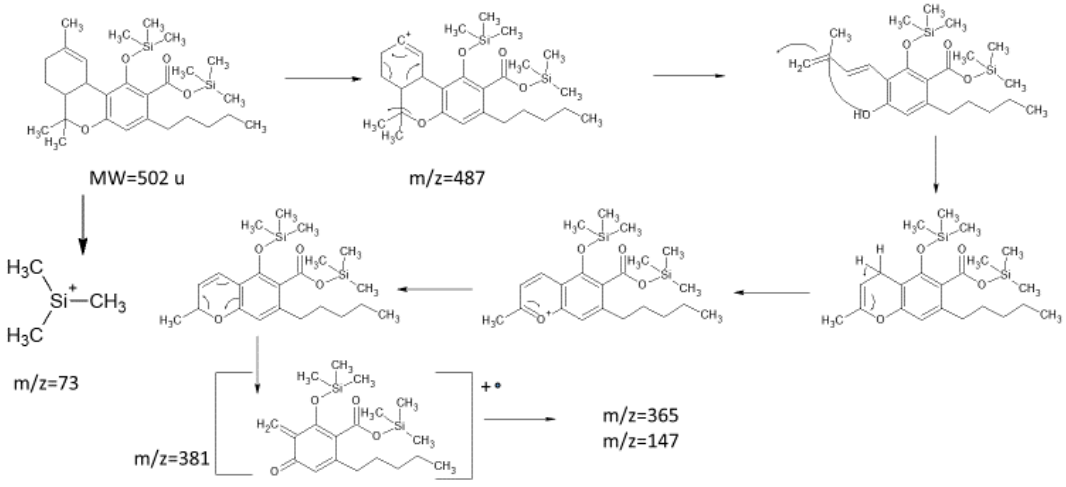
Cannabigerol-2TMS



Cannabinol-TMS



Δ9-Tetrahydrocannabinolic acid-2TMS



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Biographical Information

Allegra Leghissa obtained her bachelor degree in Chemistry and Material Chemistry at the University of Bologna in July 2014. She then decided to pursue her master degree in Analytical Chemistry at the University of Texas at Arlington, working under the supervision of Prof. Kevin A. Schug, from whose lab she graduated in July 2016. Her thesis was based on the analysis of *Cannabis sativa* components, such as cannabinoids and terpenes. She later applied for the Doctorate program in the same lab, and she kept working on the same project until July 2018, when she graduated.

She has decided that her future involves working in academia, reason why she is going to start a post-doctorate in September 2018, always under the supervision of Prof. Kevin A. Schug.

Allegra highly enjoyed working on her project, and she hopes that someday people will understand the importance of research in the Cannabis world, overcoming all the stereotypes.