# Towards the development of a MALDI/TOF-MS Fingerprint Library for the Identification and Differentiation of Cannabis Extracts

**By**

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**A Thesis submitted to the Faculty of Graduate Studies of the**

**University of Manitoba**

**In partial fulfilment of the requirements for the degree of**

**Master of Science**

**Department of Chemistry**

# Abstract

The legalization of recreational cannabis in Canada has resulted in an increased interest for research on the plant within the scientific community. Currently in Canada, there is no requirement for the confirmation of cultivar/strain identity for cannabis that is sold in recreational distributors. Strains of cannabis differ through their chemical make-up which result in different pharmacological effects. The aim of this research is to develop a method using sequential extraction and matrix assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) and Brukers Biotyper™ software that uses cannabis extracts to fingerprint and distinguish strains purchased from the recreational market.

Cannabis was first extracted sequentially using methanol, acetonitrile and hexane to obtain a range of biomolecules from cannabis. Using a methanol extract, Biotyper™ software was optimized to generate peak lists that would include the maximum number of distinguishable peaks in the desired mass range. Then, using generated score values and dendrograms, MALDI mass spectra of strain extracts were compared.

It was found that the spectra of the methanol and acetonitrile extracts contained previously observed peaks that are consistent with cannabinoids. Also, some consistent patterns could be found using the methanol and acetonitrile extracts from cannabis. The use of hexane as an extracting solvent was less practical as it is not miscible with the optimized matrix solution. Biotyper software was sometimes consistent but overall showed discrepancies for how peaks were matched. Further work for this research includes the use of tandem mass spectrometry (MS/MS) to determine if matched pairs of peaks are truly identical. Another avenue for future work is method exploiting high

molecular weight (MW) cannabis proteins for fingerprinting instead of low MW compounds as described in this work.

# Acknowledgements

I would first like to thank my supervisor, Dr. Hélène Perreault, for her consistent support, encouragement and patience throughout this process. I would also like to thank my committee members, Dr. Gregg Tomy (Chemistry) and Dr. Jake Stout (Biology) for their time, effort and feedback in research meetings.

I would also like to thank Emy Komatsu (Chemistry) for her technical assistance, suggestions and support.

I would like to thank the members of my laboratory, Taylor Battellino and Milan Teraiya, for providing me with consistent support and encouragement.

Finally, I would like to thank my parents, Shere and Charlie Gigolyk for their consistent love, patience and support throughout this journey. I would also like to thank my grandmother, Carole Storozuk.

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# List of Abbreviations

| | |
|---|---|
| Microliter | µL |
| Atmospheric-pressure chemical ionization | APCI |
| Acetonitrile | ACN |
| Cannabichromene | CBC |
| Cannabidiol | CBD |
| Cannabidiolic acid | CBDA |
| Cannabielsoin | CBE |
| Cannabigerol | CBG |
| Cannabicyclol | CBL |
| Cannabinol | CBN |
| Cannabinodiol | CBND |
| Cannabitriol | CBT |
| α-cyano-4-hydroxycinnamic acid | CHCA |
| Dalton | Da |
| Dihydroxybenzoic acid | DHB |
| Electrospray ionization | ESI |

| | |
|---|---|
| Gas chromatography-flame ionization detection | GC-FID |
| Hexane | Hex |
| High performance liquid chromatography/ electrospray ionization-tandem mass spectrometry | HPLC-ESI MS/MS |
| High-performance thin-layer chromatography | HPTLC |
| Low molecular weight compounds | LMWC |
| Mass-to-charge ratio | m/z |
| Matrix assisted laser desorption-ionization time of flight mass spectrometry | MALDI-TOF MS |
| Methanol | MeOH |
| Main Spectrum Profile | MSP |
| Principal Component Analysis | PCA |
| Parts per million | ppm |
| Sinapinic acid | SA |
| Ultra-fast liquid chromatography | UFLC |
| (-)-Δ-8-trans-tetrahydrocannabinol | Δ8-THC |
| Δ9-tetrahydrocannabinol | Δ9-THC |
| Δ9-Tetrahydrocannabinolic | Δ9-THCA |

# Chapter 1: Introduction

## 1.1 Introduction and background

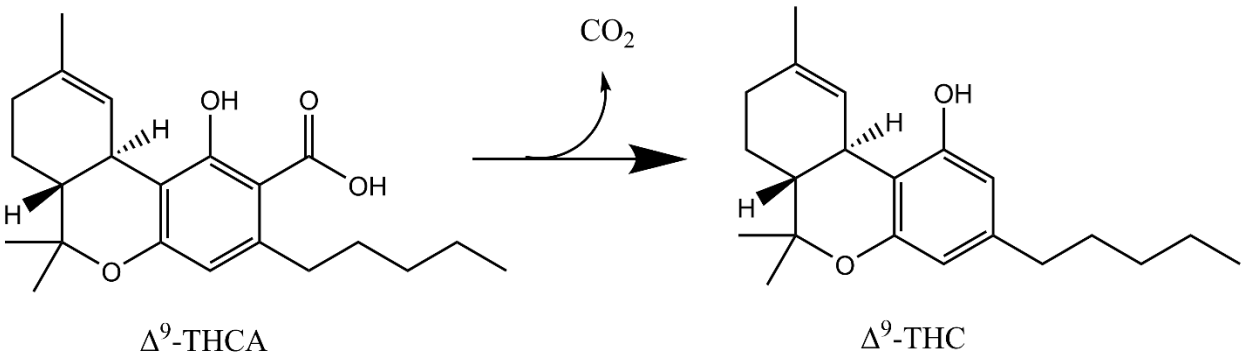
In October of 2018, Canada became the first G7 country to legalize cannabis for recreational use. Upon legalization, each province and territory were able to determine how the plant was regulated in terms of age restrictions for purchase, how cannabis was controlled and distributed, options for personal growing as well as where cannabis was allowed to be consumed. Cannabis has a long global history from both a medicinal and a recreational standpoint. In a scientific viewpoint, research and understanding of cannabis has progressed within the last 20 years such that there is an understanding of how the plant interacts with the human endocannabinoid system[1]. Pharmaceutical effects have been studied and the list includes anti-anxiety[2], anti-inflammatory[3], appetite-stimulating[4], anti-tumor[5], and anticonvulsant[6] effects. Prior to federal legalization, usage of the plant was recognized and recommended by the Canadian Pain Society for effective treatment for chronic pain in adults[7]. Recreationally, cannabis has a long history of usage dating back thousands of years with documented use for hallucinogenic effects throughout ancient Asia[8]. However, more recently and more close to home, almost half of Canadians have admitted to experimenting with the drug at some point in their lives[9].

The Canadian federal legalization of the plant is beneficial for a multitude of reasons when it comes to the welfare of citizens. For example, the legalization would result in a reduction of harms caused through the illicit market due to new competition when it comes to where and when consumers are purchasing from. This regulation allows for more control on who is purchasing the plant and therefore it becomes more difficult for underage youth to gain access to the drug. Another bonus of the legalization

is that now production of the plant is monitored and consumers can have confidence knowing that the product they obtained is indeed what the label says it is[9]. Although regulation and control of cannabis products are respectable, we are still in the beginning stages and it is expected that with more research and development of testing on the plant, the regulations will change over time. This has been seen in somewhat similar manufacturing areas such as the food, beverage, pharmaceutical and tobacco industries. This relatively new field of study allows for an abundance of research and development to be explored in order to further and completely understand the cannabis plant.

## 1.2 Constituents of Cannabis

The cannabis flower is composed of large variety of molecules including cannabinoids, terpenoids, lipids, prenylated polyketides, amino acids, and organic acids[8,10]. Cannabinoids are a group of biologically active molecules that are synthesized in Cannabis in secretory cells inside glandular trichomes, commonly found in female flowers[11]. In the plant, cannabinoids are synthesized as cannabinoid acids and when the flower is stored for long periods of time, dried, heated or exposed to light, they decarboxylate into their neutral form[12]. This is shown in Figure 1.2.1. The most common and most researched cannabinoids are $\Delta^9$-Tetrahydrocannabinol ($\Delta^9$-THCA)/ $\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC) and cannabidiol (CBDA)/cannabidiol (CBD).

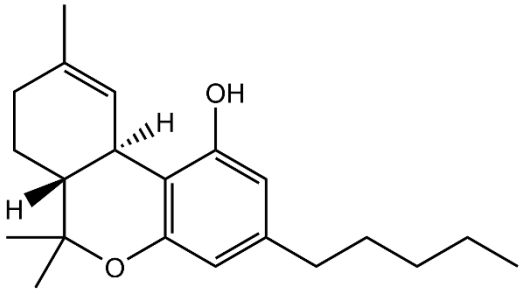
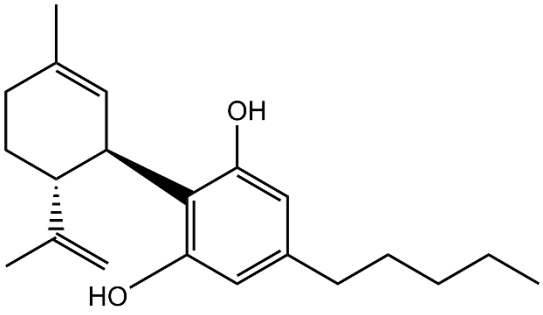



$\Delta^9$-THCA $\Delta^9$-THC

**Figure 1.2.1.** Decarboxylation reaction of Δ9-THCA to Δ9-THC.

The decarboxylated versions of Δ9-THC and CBD can be found in Table 1.2.1.

**Table 1.2.1. Structure of major cannabinoids (acidic and neutral) that exist in cannabis.**

| Cannabinoids | Structure | Molecular Weight (Da) |
|---|---|---|
| Δ9-THC |  | 314.45 |
| CBD |  | 314.45 |

Cannabinoids work through binding to receptors in the endocannabinoid system[13].The main psychoactive and psychedelic component of cannabis is Δ9-THC

which activates a G protein-coupled receptor known as cannabinoid $CB_1$ receptor[8]. This compound has also been shown to be analgesic[14], antioxidant[15] and antipruritic[16]. Cannabidiol is the other major cannabinoid found in cannabis and is not psychoactive, although it has been shown to have a number of therapeutic benefits including anti-anxiety[2,17–19], anticonvulsant[6,20,21], anti-inflammatory[22,23] and for addiction treatment[24,25]. Other than $\Delta^9$-THC and CBD, there exist over 100 cannabinoids that are derivatives of the main types. These main types that are less common than $\Delta^9$-THC and CBD include (-)-$\Delta^8$-*trans*-tetrahydrocannabinol ($\Delta^8$-THC), cannabigerol (CBG), cannabichromene (CBC), cannabinodiol (CBND), cannabielsoin (CBE), cannabicyclol (CBL), cannabinol (CBN), and cannabitriol (CBT)[8]. This group of molecules have masses in the 300-400 Dalton (Da) range. Structures of the other most commonly found types of cannabinoids can be viewed in Figure 1.2.2-1.2.4

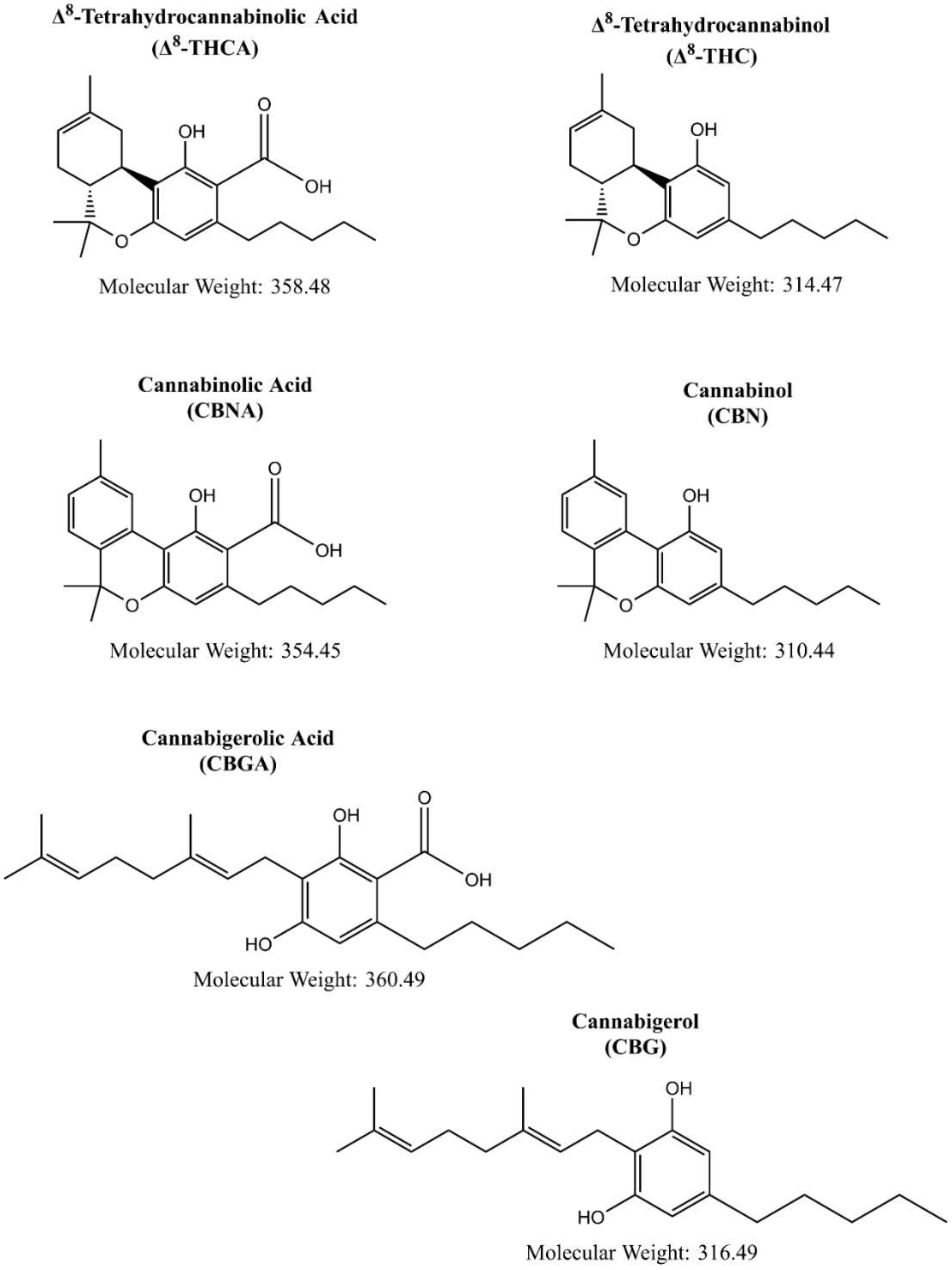
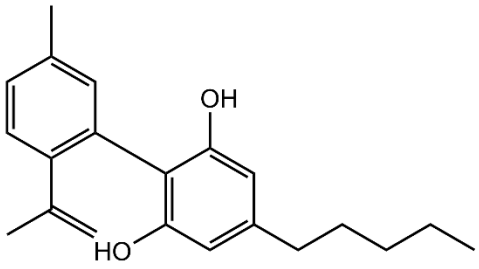


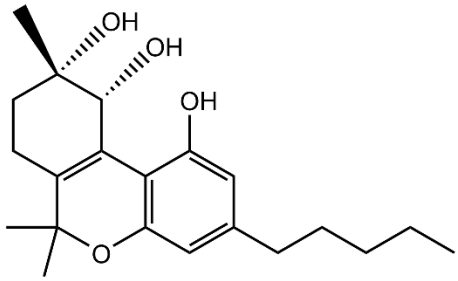

**Figure 1.2.2 Names, structures and molecular weight of major cannabinoids.** Cannabinoids shown include $\Delta^8$-Tetrahydrocannabinolic acid/ $\Delta^8$-tetrahydrocannabinol ($\Delta^8$-THCA/ $\Delta^8$-THC), Cannabinolic acid/ Cannabinol (CBNA/ CBN) and Cannabigerolic Acid/ Cannabigerol (CBGA/ CBG).

**Cannabidiol**
**(CBND)**



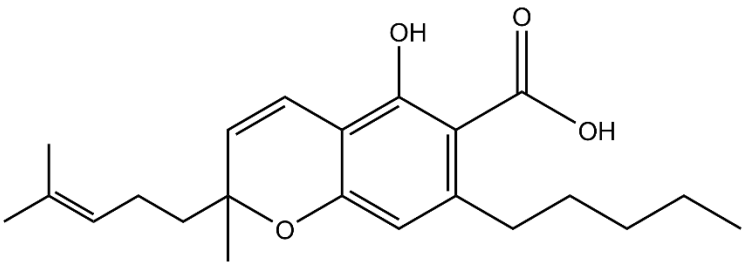

Molecular Weight: 310.44

**Cannabitriol**
**(CBT)**



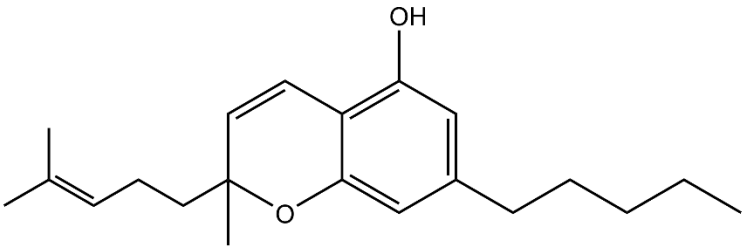

Molecular Weight: 346.47

**Cannabichromenic Acid**
**(CBCA)**




Molecular Weight: 358.48

**Cannabichromene**
**(CBC)**




Molecular Weight: 314.47

**Figure 1.2.3 Names, structures and molecular weight of major cannabinoids.** Cannabinoids shown include Cannabidiol (CBND), Cannabitriol (CBT) and Cannabichromenic acid/ Cannabichromene (CBCA/ CBC).

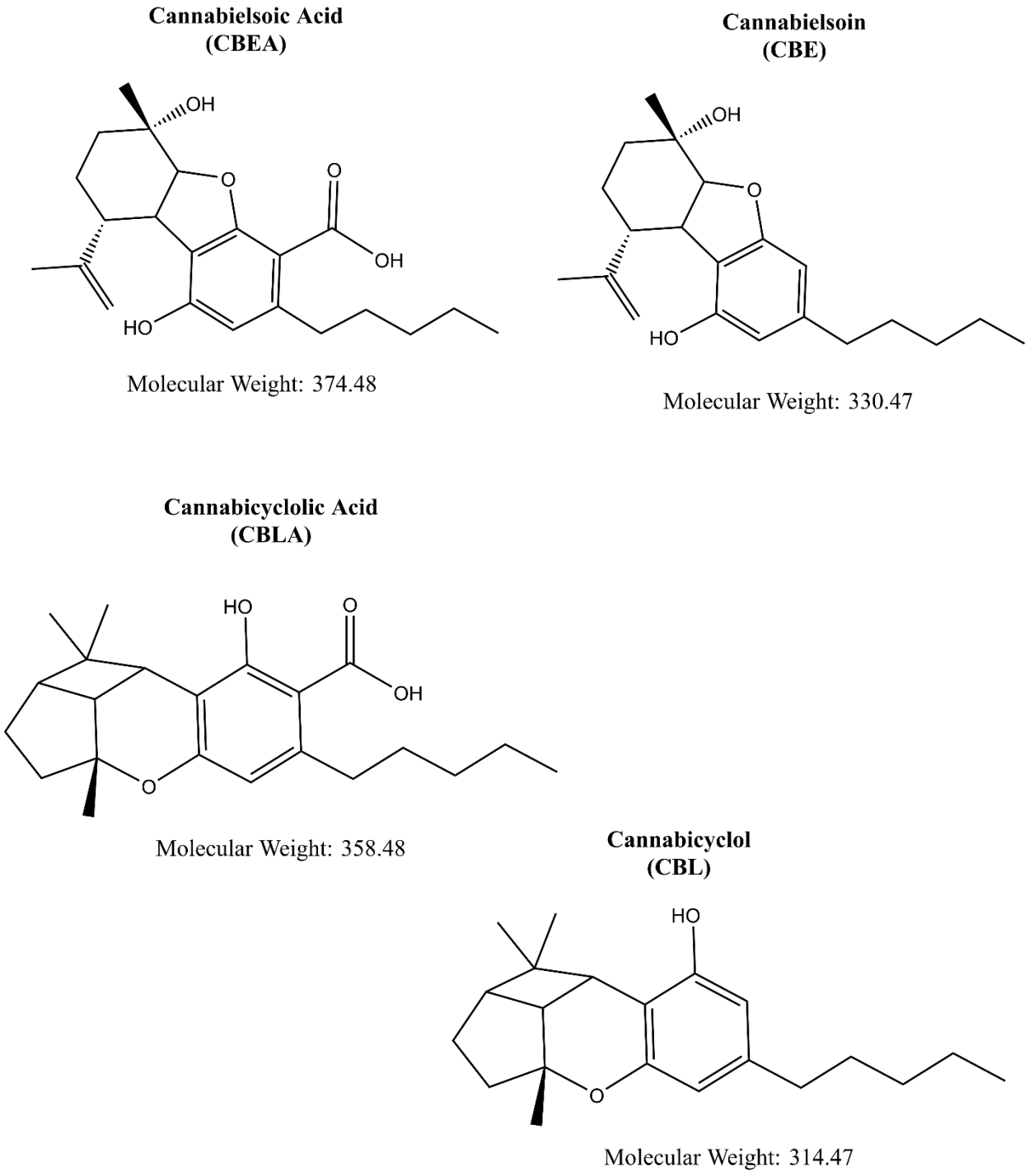



**Figure 1.2.4 Names, structures and molecular weight of major cannabinoids.** Cannabinoids shown include Cannabielsoic Acid/ Cannabielsoin (CBEA/ CBE) and Cannabicyclolic Acid/ Cannabicyclol (CBLA/ CBL).

Besides these main groups of cannabinoids there also exist miscellaneous types of cannabinoids that are not derivatives of the types listed in Figure 1.2.2[26]. Overall, these molecules are present in varying concentrations in the flowering bud of the plant but are less discussed than $\Delta^9$-THC and CBD in the literature.

Other constituents of cannabis include terpenes, flavonoids, steroids, nitrogenous compounds, non-cannabinoid phenols, amino acids, proteins, enzymes, glycoproteins, sugars and related compounds, hydrocarbons, simple alcohols, simple aldehydes, simple ketones, simple acids, fatty acids, simple esters, lactones, steroids, vitamins and pigments[27]. Depending on the technique used, all of these compounds can potentially contribute to the distinct and unique mass spectral fingerprints.

## 2.1 Classification systems

Terminology and classification of cannabis have been derived and grandfathered into the Federal government system from methods developed prior to legalization. Names for the cannabis plant are endless (marijuana, pot, weed, ganja, etc) and then there also exist many classification systems within cannabis to describe varieties of the plant. Currently, consumers shopping at dispensaries can browse cannabis products through different criteria including the advertised type (sativa, indica or hybrid) and through $\Delta^9$- THC and CBD percentages that are found in the bud. As shown, there are a large variety of describing factors for cannabis. With a growing community of recreational users, the ability to clearly distinguish between varieties is becoming more important.

The terms “sativa” and “indica” are used to describe different cannabis cultivars and this specific terminology was introduced in the 1800’s by Jean-Baptiste Lamark[28].

Lamark used physical properties to describe differences in two varieties. He declared that sativa variety plants had stems and leaves that were taller and leaner. Lamark was presented with samples from India which were short and bushy in comparison and this species was classified as Indica variety. In current day, cannabis retailers and consumers still describe different varieties through the sativa and indica nomenclature system[29]. Sativa variety plants are marketed for their uplifting, energetic, stress reducing and increasing creativity effects. Indica variety plants are associated with relaxation, insomnia reduction and an overall sense of calming.

Within the recreational and scientific community, it is acknowledged that cannabis is one species of plant that is highly variable, both morphologically and chemically[30,31]. Although the sativa/indica nomenclature is still used in the recreational world, cannabis is now classified through different "strains" where this is reference to different chemovars of the plant[32]. Different strains (chemovars) of cannabis are described through the content of the two major cannabinoids- $\Delta^9$-THC and CBD as well as different pharmacological effects[33–36]. The word strain is properly used to describe varieties for microorganisms and it is still widely used to describe chemovars of cannabis, even though this is not a biologically correct term Therefore, throughout this thesis, strain will be used to describe different cannabis cultivars.

Names of strains for cannabis are more-so a representation of the producers' vocabulary creativity and not a description of chemical profile. This can be demonstrated through strain names such as "Skittlez", "Slurricane", "Blue Dream", and "White Widow". However, another contributing factor to strain differentiation is terpene content. Terpenes have been reported to contribute to pharmaceutical

properties of cannabis through the interaction with cannabinoids known as an entourage effect[37,38]. Terpenes are responsible for aromas and flavours of cannabis[39,40]. Strain names can sometimes be an outcome of terpene content such as “Pineapple Express”, “Watermelon”, “Lemon Skunk” and “Cherry Pie”. Chemical make-up of the cannabis plant is further discussed in the next section.

Although not scientifically accepted but useful to the general public, there are popular online resources for discussion of strains such as Leafly[41]. Leafly is a forum with a large descriptive catalogue of different strains. Information that can be shown about a strain includes suggestions of where it originates from, its parent strains, scents, as well as feelings and effects that it causes. It also lists weight percentages of THC and CBD within a strain and major terpenes that exist within the strain. However, it is unclear from the website (https://www.leafly.ca/) if amounts and/or presence of cannabinoids/terpenes are determined scientifically, are suggested by producers or are suggested by interested cannabis users. This type of input-based library of various strains allows for ideas on how cannabis can be adopted into the scientific community. Currently in Canada, there is no in-depth detailed federal regulated library for the chemical composition of different cannabis strains.

Current quality assurance protocols in place for Canadian federal approval of cannabis are in place for safety measures. Different categories tested are potency (% weight of $\Delta^9$-THC and CBD), biological contaminants, chemical (mycotoxins, pesticides and metals) solvents, and encapsulation degradants[42]. Brown et al[43] used liquid chromatography as well as gas chromatography to quantify cannabinoids, terpenes and alflatoxins from recreational cannabis samples. Fischedick et al[44] used high-

performance thin-layer chromatography (HPTLC) to analyze seven major cannabinoids from cannabis. Liquid chromatography methods are more common for cannabinoid analysis because derivatization is not necessary. This is often coupled with mass spectrometry or ultraviolet detectors[44–49]. Gas chromatography is the preferred method for analysis of terpenes due to their volatility[51–53].

Ideally, over time, recreational cannabis sold in Canada will include a more complete description of its complete chemical make-up. Chemical composition of an individual strain can be described through its chemical fingerprint. Using matrix assisted laser desorption-ionization time of flight mass spectrometry (MALDI-TOF MS), chemical fingerprint libraries are commonly used to map and classify and map large quantities of like samples such as microorganisms including bacteria, viruses and fungi by using molecular markers[54]. A spectral fingerprint library that has the ability to identify a specific strain is comparable to a barcode that is placed on any retail item for purchase. Ideally, using MALDI-TOF-MS, a barcode would be developed for each strain of cannabis that is available for purchase in the Canadian market. The development of a cannabis spectral fingerprint library, similar to fingerprint libraries that have been developed in the past, would be useful for quality assurance of samples, tracking of inventory and to contribute to the scientific understanding of cannabis.

## 2.2 Cannabinoid Extraction

Pharmacologically, the molecules responsible for distinction between cannabis cultivars is quantities of cannabinoids (mainly $\Delta^9$-THC and CBD) as well as terpenes[37]. With legalization, cannabis research and the development of quality assurance methods

have sparked the interest of researchers. Therefore, there exist many different analytical methods to quantify naturally occurring molecules in cannabis. For the extraction of cannabinoids, variation rises in solvent choice and overall extraction technique. Other factors are the quantity of cannabinoid required to be extracted, the stability of the cannabinoids, cost, if there exist environmental impacts or if purification steps are required[55]. However, change in regulations on cannabis results in a need for a different types of method development for accurate analysis. Methods need to be fast, safe, simple and precise to meet demand for high-throughput analysis.

The most commonly used solvent combination for cannabinoid extraction is methanol/chloroform in a 9:1 ratio (v/v). This ratio has been shown to produce the highest extraction efficiency[56]. Methanol alone as an extraction solvent has shown to produce similar results for extraction of cannabinoids and this reduces the risk of toxicity due to chloroform[57].

Overall, one of the purposes of this research is to perform a fast and effective extraction of plant material. Developing a sequential that is quick, easy, cheap, effective, rugged and safe (QuEChERS)- like extraction method would be most suitable.

Sequential extraction (SE) analysis is an analytical technique applied to solid samples which has gained popularity in extraction of solid waste and soil, in search of trace metals[58]. The basis of this technique is the application of a series of solvents that increase in strength to typically extract and determine potentially toxic elements. The general idea of SE can be applied to cannabis samples in hopes to extract molecules of varying polarity. General SE extractions range from three to nine different solvent systems. Through the addition of solvents with differing polarity indices and

characteristics, it is assumed that each extract from a sequential extraction will contain different substances, useful towards a cannabis fingerprinting experiment.

Considerations for SE analysis of cannabis products should include the choice of solvents, temperature and further washing/clean-up of the extract is necessary prior to MALDI analysis. The first solvent of choice is methanol as it has well documented extraction capabilities for cannabis plant material. A solvent that is similar to methanol but differs in its hydrogen-bonding capabilities is acetonitrile, which is to be explored as the second solvent system in the sequential extraction. Finally, hexane is a solvent to be explored as it is completely non-polar and therefore the contents of its extraction is expected to differ greatly from those of the first two solvents selected.

Other considerations to take into account are the temperature of the sequential extraction and the choice of clean-up technique for extracts prior to analysis. Cannabinoids are known to decarboxylate when heated to above 100°C[56,59]. In order to maintain integrity of the original material coming from the plant, extraction should not occur at a high temperature. Finally, in order to maintain a "whole picture" analysis and to make the method time effective, clean-up of samples should be avoided. MALDI-MS is tolerant of impurities and leads itself well to freshly extracted samples.

## 2.3 Matrix Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry

MALDI-TOF MS[60] is a technique used for the identification and/or determination of mass-to-charge ratio (m/z) values of $[M+H]^+$ ions of selected

compounds, typically peptides or proteins[60]. In MALDI, the sample is co-crystalized with an acidic organic matrix onto a target plate. The target plate is then inserted into the ion source and a UV laser beam is pulsed at the target. The matrix absorbs the energy, is vaporized along with the sample, and protein exchange reactions in the gas phase to produce $[M+H]^+$ analyte ions. A schematic representation of this phenomenon can be observed in Figure 1.5.1. It is also possible to obtain $[M]^+$ ions by desolvation. The released ions are accelerated down a flight tube, separated by their m/z value according to their time of flight and then detected.

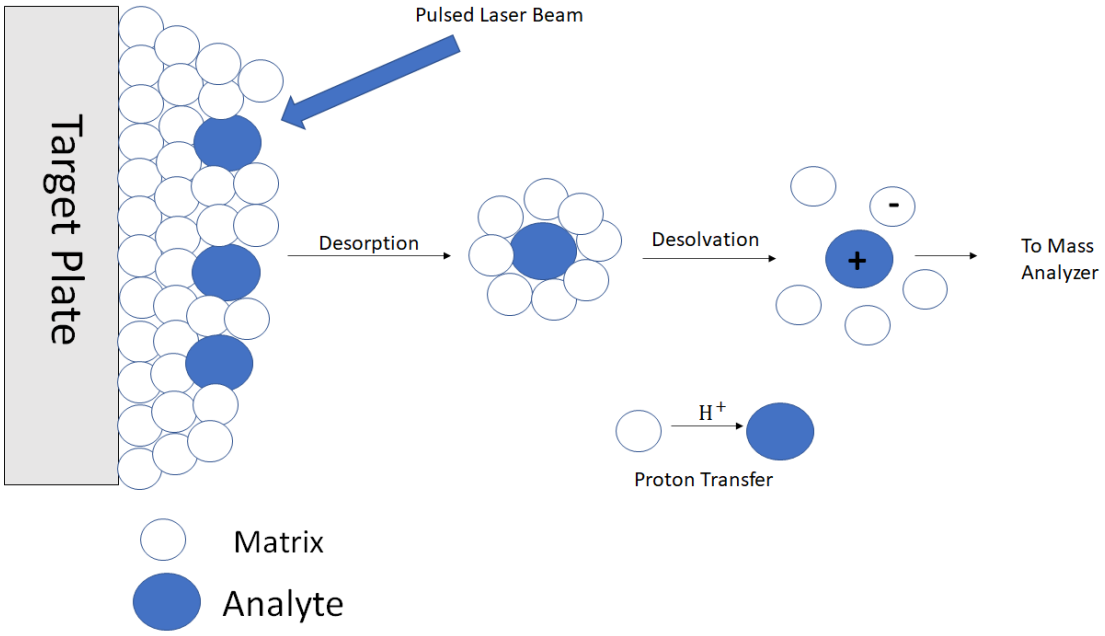



**Figure 2.3.1 Diagram showing ionization in a MALDI source.** Figure adapted from Ref No. [61].

MALDI has proven useful for the identification of samples including large ions, e.g.- intact proteins. Microorganisms are such samples, and have been investigated through two methods including fingerprinting and bioinformatics. The fingerprinting of microorganisms using MALDI is done through measuring sample spectra that can be compared back to a library of previously collected fingerprints[62]. In this method, specific peaks of proteins and other biological molecules from the sample are used to differentiate at the genus, species and strain levels. In a second identifying method based on fingerprinting, single specific peaks with known masses associated with a specific component are used to identify the sample. These specific peaks are assumed to originate from particular proteins or biomolecules which can then undergo tandem MS-MS analysis to further confirm their identity. The first approach is more of a whole-picture method as it looks at the entire mass spectra to identify and compare samples, while the second approach looks at single peaks to identify and distinguish varying organisms.

Analysis of low molecular weight compounds (LMWC) using MALDI-TOF-MS is rather uncommon compared to the use of other techniques such as atmospheric-pressure chemical ionization (APCI). The unpopularity for MALDI-MS for the analysis of LMWC is due to the interference caused by matrix molecules in the 0-500m/z region[63]. However, MALDI offers advantages, e.g- a high tolerance towards contaminants, is high-throughput and is a soft ionization technique. Moreover, it generates spectra with mostly singly charged ions and it is highly sensitive[64]. The high throughput capabilities would result in a large quantity of samples being tested without a lot of sample workup. Also, the soft ionization technique will maintain integrity for

small molecule analysis i.e.-little fragmentation occurs. Maintaining sample integrity is important as there exist a large number of naturally occurring molecules in cannabis. The generation of singly charged peaks using MALDI makes it possible for fast comparison between samples when each sample has many different ions present. Spectral libraries can keep track of a large quantity of samples, which would be useful for cannabis, as a single retailer could easily list over 100 different strains available for purchase. Given a potentially large number of samples that in turn contain a large variety of molecules in unique distributions, a spectral library would be very valuable. As many cannabinoids do have the same mass, it is hoped that the immense number of compounds present in cannabis with different concentrations will give details to strain identity. Due to these advantages, this project was chosen with MALDI-TOF-MS in mind with the aim of creating a quick and simple protocol to compare cannabis samples.

The high-throughput capabilities of MALDI-TOF analysis are appealing for the generation of a spectral library to compare samples. Reproducible fingerprint patterns of different samples allow for programs such as Bruker's Biotyper™ to compare samples using characteristics such as peak intensity, peak frequency and peak symmetry. This program has been used extensively for fast and reliable identification of bacteria, yeasts and fungi using spectral fingerprints using proteins[65]. Also, in combination with simple sample preparation techniques, this program has been used in various fields including clinical diagnostics, environmental and taxonomical research as well as food-processing quality control[54].

## 2.4 Taxonomy of Cannabis

Bruker's Biotyper™ software has been used for fingerprinting of various sample

types including bacteria, fungi and viruses. However, this type of analysis is uncommon for the identification of samples that do not have large molecular weight proteins or peptides as there do not exist markers that can distinguish samples from one another based on evolutionary differences. Also, matrix interfering peaks would make identification more difficult comparing samples with low molecular weight molecules, such as cannabis.

The description of an analytical method for the differentiation and verification of cannabis strain identity has not been reported. Currently in Canada, suppliers are not required to provide confirmation of a strain identity. Consumers are simply required to trust that what they purchased is indeed what it is labelled to be. This missing component in the cannabis industry could result in strains being sold that are mislabeled. Also, strains from unknown origin could be sold or perhaps strains could be renamed. Although there exist methods to test the safety of cannabis in Canada, there does not exist a chemical profile of each individual strain for verification of identity.

Determination of cannabis taxonomy and differentiation between cannabis samples is a complex topic[30,66–68]. Recent research has been conducted to try and differentiate strains from one another using cannabinoids and terpenoids found in the flower. In 2015, Fischedick et al[69] used principal component analysis (PCA) to study 35 different cannabis strain flower samples to investigate the existence of distinct cannabis chemotypes using 31 compounds including cannabinoids and terpenes. This study used 5-7 g of ground cannabis flower placed into a centrifuge tube with zirconia beads and extraction solvent to extract. The extraction was centrifuged and aliquots of the supernatant were collected for terpene analysis using GC-FID and cannabinoid analysis

using HPLC-Diode Array Detector. A highlight of this study is that most strains did not have reproducible results for chemical composition analysis therefore were not grouped together in PCA clustering. Five out of the 25 strains showed relatively high concentrations of specific terpenes and were clustered together upon repeat analysis which suggests strains are more identifiable by smell than psychoactive effects. Other studies by Fischedick et al show that chemical profiles of cannabinoids and terpenoids for the same strain are reproducible if growing conditions are controlled and identical[32,70]. In 2020, Delgado-Provedano et al[10] used both GC-TOF/MS and LC-QTOF MS/MS for the comparison of cannabinoid and terpenoid content from the identical cultivars in greenhouse and field growing conditions. This work showed that quantity of cannabinoids was variable and not necessarily a reflection of optimized growing conditions through that of a greenhouse. However, terpenoid content was related directly to optimized growing conditions and was more abundant from greenhouse cultivars. In 2021, Bakain et al[71] used GC-FID to generate chemical profiles of cannabis samples from 23 different American states using a total of 45 different cannabinoids and terpenoids. They found three different clusters of strains were created. The first cluster was $\Delta^9$- THC dominant, the second cluster was $C_1$ (undetermined cannabinoid) dominant and the last cluster was CBN/Fenchol dominant. When clusters were created excluding all other biomolecules (i.e.- basing clustering on only quantities of $\Delta^9$-THC, $C_1$ and CBN) different clusters were created. This suggested that strains can be grouped together based on these molecules but less concentrated contents are also a factor. Geographical location did not contribute to clustering as, for example, strains from Alaska and Nevada were grouped together. Elzinga et al[72] attempted to use ultra-fast liquid chromatography (UFLC) and gas chromatography-flame ionization detection

(GC-FID) for cannabinoids and terpenes to generate chemotypes of different strains. They found no a distinct clustering of chemotypes, but instead a continuum of varying chemical composition. Although beyond the scope of this work, there exists an increasing amount of genetic work to classify cannabis[29,31,73–77]. While there have been a lot of studies on quantification of cannabinoids and other constituents, there is not a lot of work on the use of a full picture of the chemical makeup to differentiate cannabis strains.

## 2.5 Research Goals

The development of a cannabis library using MALDI-TOF-MS would be useful for quality assurance and control, following the legalization of recreational cannabis in Canada. With a large variation in strain names and classification of cannabis, a library would be useful in ensuring that products are labelled and marketed for what a retailer says they are. The goal of this project is to create a spectral library to help in the identification of different cannabis strains using MALDI-TOF MS fingerprinting. The preliminary development of this library is based on the sequential extraction of six different cannabis samples using three different solvents. It is hoped that each solvent will give different identifying chemical fingerprints to create an overall full picture of each strain. As previously mentioned, cannabinoids are highly soluble in methanol, a polar protic solvent. It is assumed that methanol extracts will give the most detail in differentiation of strains. The next solvent of choice for sequential extraction is acetonitrile. Acetonitrile has a similar polar index but differs from methanol as it is aprotic. The assumption is that choosing an aprotic solvent as second in the sequential extraction will allow to remove any cannabis products that were not soluble in

methanol. Hexane is to be used last as it is completely non-polar. It is assumed that any non-polar constituents left would be extracted within this solvent.

Samples were compared using Bruker's Biotyper™ software. First, this software must be optimized to identify samples that produce many peaks and in the low-mass range (0-1000 *m/z*). Optimization is to be done using one strain's methanol extracts for reasons previously stated. For each extract, three spectra are collected and averaged in the Biotyper program. Once the program is optimized, it will be possible to use the software to generate scores on the likeness of varying strains based on the m/z values of peaks generated. It is hoped that these scores will be consistent over multiple trials and that patterns of differentiation of strains can be developed.

# Chapter 2: Materials and Methods

## 3.1 Materials

For comparative purposes in this study, the following cannabis sample varieties were purchased from Mota Cannabis at http://motacannabisproducts.biz: Blueberry (Indica), Charlotte's Web (Sativa), Four Star General (Hybrid), Island Banana (Hybrid), Lake of Fire (Hybrid), and Skookie (Sativa). Methanol (MeOH) was purchased from Fisher Scientific (Fair Lawn, NJ). Acetonitrile (ACN) and Hexane (Hex) was purchased from Sigma (St. Louis, MO). α-cyano-4-hydroxycinnamic acid (CHCA) matrix was purchased from Sigma. Sinapinic acid (SA) was purchased from Fluka (St. Louis, MO). Dihydroxybenzoic acid (DHB) was purchased from Fluka. Trifluoroaceic acid was purchased from Sigma. $\Delta^9$-tetrahydrocannabinol and cannabidiol standards (1 mg/mL) were purchased from Toronto Research Chemicals (Toronto, Canada). MALDI matrix solutions were prepared as follows: CHCA was diluted to 5 mg/mL in 50:50 water:ACN, SA to 10 mg/mL in 50:50 water:ACN, and DHB to 5 mg/mL in 90:10 water:MeOH.

## 3.2 Sequential solvent extraction

Cannabis sample buds (0.0350 g – 0.0450 g) were broken up by pulling buds apart manually. Samples were placed into a 1.5-mL Eppendorf tube with 1.00 mL of a selected solvent (MeOH, ACN or Hex, starting with MeOH) and allowed to shake on vortex mixer for 15 min. The extraction solvent was collected into another Eppendorf tube. Bud samples were allowed to air dry inside the extraction vessel until the next sequence solvent was added. Liquid extracts were spotted onto a MALDI target immediately after extraction, then stored in the freezer at -20°C until further use.

## 3.3 Determination of appropriate MALDI matrices

Different matrices were used to identify which one would cause the least interference while producing the best ionizing efficiency for specific cannabinoid standards. Matrices tested include CHCA, DHB, and SA. Cannabinoid standards $\Delta^9$-THC and CBD were obtained individually as 1 mg/mL solutions. Different ratios of matrix and standard were spotted on the MALDI target plate as shown in Table 2.3.1.

**Table 3.3.1 Different matrices and ratios used for testing of cannabinoid standards.** Ratios are represented as volume of solution. Sandwich spotting technique refers to mixing a 1:1 volume ratio of matrix and standard and allowing it to dry, then spotting the matrix solution again on top of the pre-dried mixture.

| Matrix: Standard Ratio | Ratios tested | | | |
|---|---|---|---|---|
| DHB: $\Delta^9$-THC | 1:1 | 2:1 | 1:2 | Sandwich |
| DHB: CBD | 1:1 | 2:1 | 1:2 | Sandwich |
| CHCA: $\Delta^9$-THC | 1:1 | 2:1 | 1:2 | Sandwich |
| CHCA: CBD | 1:1 | 2:1 | 1:2 | Sandwich |
| SA: $\Delta^9$-THC | 1:1 | 2:1 | 1:2 | Sandwich |
| SA: CBD | 1:1 | 2:1 | 1:2 | Sandwich |

## 3.4 Preparation of samples for MALDI MS analysis

Matrix (0.2 µL) and sample (0.2 µL) were mixed directly onto the stainless steel MALDI target plate and allowed to dry. Figure 2.4.1 shows a MALDI plate being irradiated by a laser beam, followed by desorption of ions and TOF analysis.

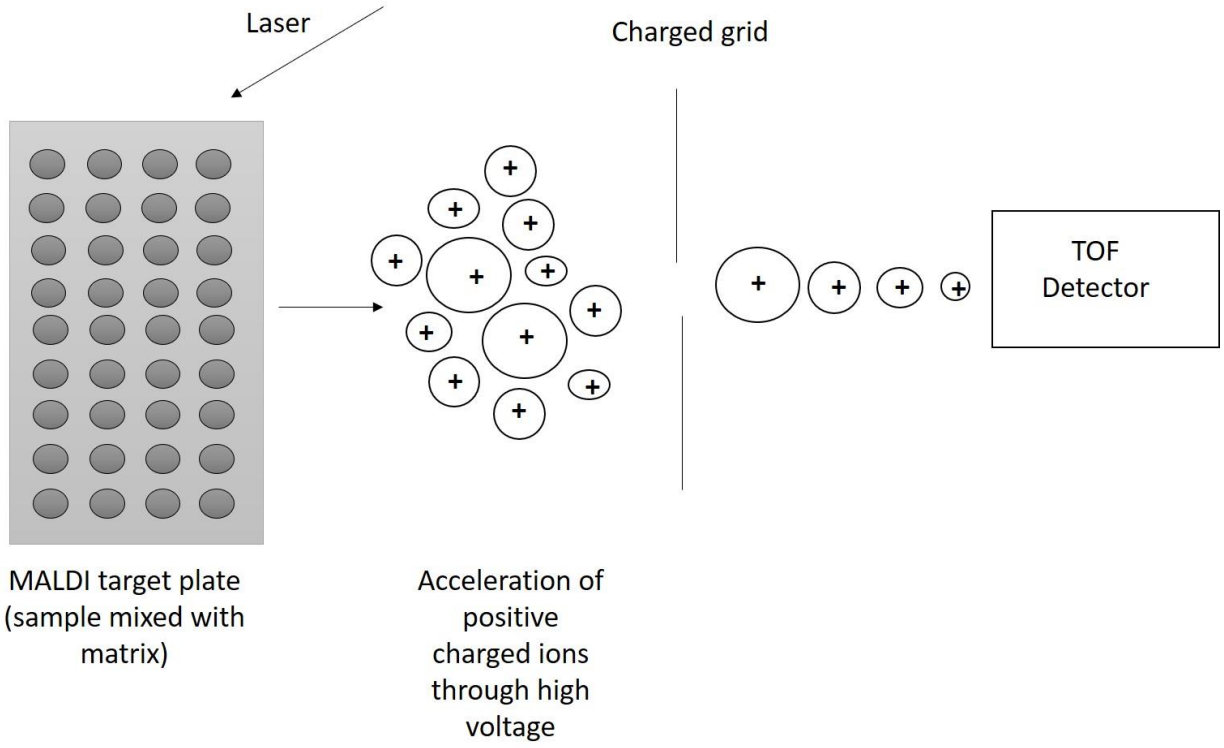



**Figure 3.4.1. A schematic of a MALDI target plate.** This shows how ions are accelerated to the TOF detector.

## 3.5 MALDI-TOF MS

All MS analyses were performed on a Bruker UltraFleXtreme™ mass spectrometer (Bruker Daltonics, Billerica, MA). The samples were analyzed in positive reflector mode. The acceleration voltage was 20 kV. The beam intensity was set to 30% and 40% of its full intensity. The m/z range measured was 0-1200. Matrix suppression (0-500 m/z), normally used for high molecular weight measurements, was turned off. To create a sum spectrum, eight laser shots on each individual sample were summed up. Each sample was spotted separately three times. Measurements were calibrated using a three-point system with the CHCA matrix peaks of $[M+H]^+$, $[M+Na]^+$ $[2M+H]^+$ at

190.04987Da, 212.03181 and 379.09246Da. Figure 2.5.1 shows a schematic representation of the Ultraflex mass spectrometer used in this study.

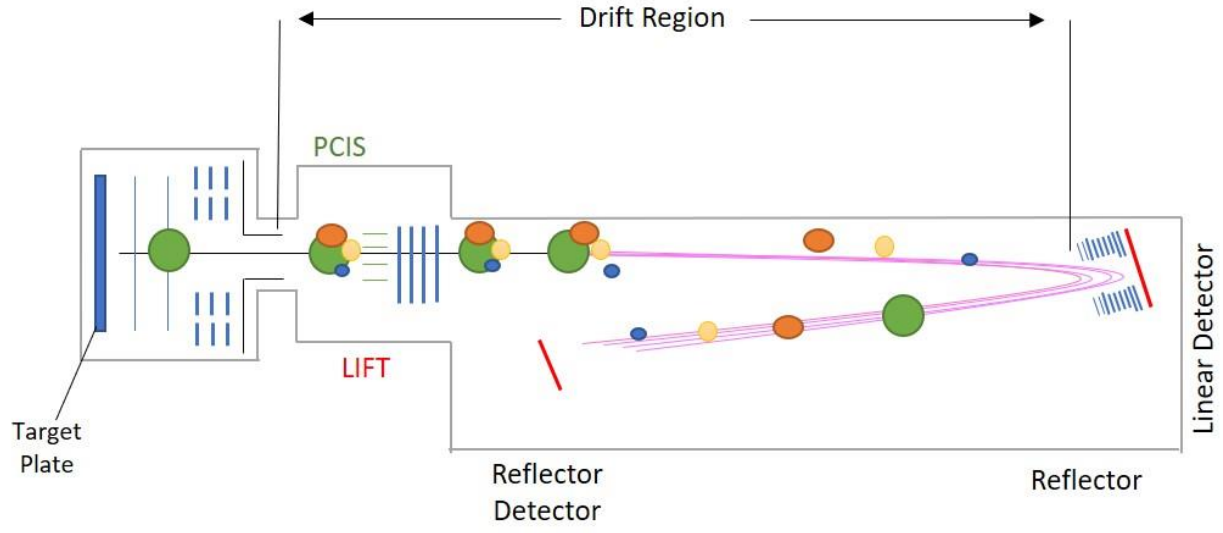



**Figure 3.5.1. Schematic of the Bruker UltraFleXtreme™ MALDI-MS instrument used in this work.** Software used for data acquisition was FleXControl V 3.4, and FlexAnalysis V 3.4 was used for data analysis.

## 3.6 Library preparation

The library was generated using Bruker Biotyper® software (V 3.1) with six different cannabis strains from Mota Cannabis including; “Banana Kush”, “Blueberry Indica”, “Charlotte’s Web”, “Four Star General”, “Girl Scout Cookies” and “Lake of Fire”. Three MALDI-MS spectra were collected for each fraction for each sample, and from a reference/main spectrum profile (MSP) was generated with modifications to the parameters of preprocessing, MSP creation and identification. Adjustments to the parameters were used as the data collected are not the typical data that Biotyper is capable of processing. The adjustment of these parameters results in different peak lists and can alter the identification. Therefore, different parameters were tested in order to

create peak lists that covered the entire peak range (0-1200 Da) and to include as many visually relevant peaks as possible. Parameters were tested and adjusted using a "Blueberry" strain methanol extract. Different parameters result in the same sample having different classification results when compared to itself. The effect of different Biotyper parameters is described in Chapter 3.

## 3.7 Preprocessing of data

Preprocessing methods are used to optimize and speed up analysis through the reduction of a data set size by generating peak lists from raw spectra. Parameters that can be adjusted include mass adjustment, smoothing, baseline subtraction, normalization and peak picking. The following parameters were used: Start (lower bound) and end (upper bound) of the m/z range for the peak list were 0 and 1000 Da. The minimum distance between two peaks along the x-axis which is necessary to separate the peaks from each other (resolution) was 1 Da. The spectra were compressed using a factor of 5. Smoothing was done using the Savitzky-Golay filter with a frame size of 10 Da. Baseline subtraction was done using a multipolygon method with a window of 5 Da and 2 runs. Spectra were normalized using the maximum norm method. The maximum number of peaks to pick (max. peaks) was 100. To include the largest possible number of peaks, peak picking adjustments were made such that the peaks must have an intensity of at least 0.25% of the highest peak (threshold: 0.0025). To detect peaks, the local-maxima with a width of 1-10Da was tested with a final value of 5 Da selected.

## 3.8 Main Spectrum Profile (MSP) creation

Modifications of parameters of the MSP creation section need to be considered in order to optimize the use of the program for identification. MSP's needed to be created to maximize the inclusion of all relevant peaks of a sample, including those of low intensity. The max. mass error for each single spectrum of a MSP, which is the recalibration of peaks from individual spectra based on a mass range recalibration window, was adjusted from 200 to 20000 ppm with a final value of 2000 selected. The desired mass error for the MSP after recalibration was adjusted from 200 to 4000ppm and the desired peak number was set to 200. The desired peak frequency minimum (%) was set to 25% and therefore a peak only needs to occur in 1 out of 4 of the individual spectra to be included in the MSP.

## 3.9 Main Spectrum Profile (MSP) Identification

The minimum frequency of occurrence for a peak from the spectra set (frequency threshold for score calculation) was set to the standard 50%. The frequency threshold for score calculation (minimum frequency of occurrence (%) of a peak for use in the classification procedure) was set to 30%. The mass tolerance for initial peak adjustment before recalibration (max. mass error of the raw spectrum) was set to 2000 ppm to remain consistent with the creation parameters. The desired mass tolerance for any peak after adjustment (des. mass tolerance of the adjusted spectrum- inner mass window) was set to 200 ppm to remain consistent with the creation parameters. The additional mass tolerance for any peak after adjustment (accepted mass tolerance of a peak) was set to 600 ppm.

The software calculates a unit score value between 0 and 3 such which indicates the similarity between a reference spectra and other samples. The results were displayed into “detected species” window as specified by the manufacturer with an identification score value of >2.0 for a probable identification, and 2.0> was unreliable. The score is generated through an algorithm for three separate values, including:

1) Number of signals in the reference with a closely matching partner in unknown spectrum are calculated. A value of 0 indicates no matches and a value of 1 indicates a complete match.
2) The number of signals in the unknown that have a closely matching partner in the reference spectrum are calculated. A value of 0 indicates no matches and a value of 1 indicates a complete match.
3) The symmetry of said matching signals is compared. If high intensity signals of the unknown correspond with high intensity signals of the reference and low intensity signals of the unknown correspond with the low intensity signals of the reference then a high symmetry and results in a value closer to 1. If the matching pairs have no symmetry then this results in a score closer to 0.

The three values are multiplied together and this result is then normalized to 1000 which then results in the log score used to characterize samples. A score of 3.000 is considered a perfect match.

# Chapter 3: Library Preparation in Biotyper

## 4.1 General Considerations

The goal of this project was to determine if MALDI-TOF MS is a suitable tool for the identification and analysis of different cannabis flowers through comparison of extracts resulting in fingerprint spectra. As mentioned in Chapter 1, the cannabis flower contains a highly complex mixture of over 568 unique molecules with over 100 of those being compounds called cannabinoids[8]. It is generally acknowledged that different strains of the plant have been used for different intended purposes with particular strains being known to cause varying therapeutic side effects. The large mixture of constituents found in the cannabis plant make the plant a respectable candidate to create a fingerprint spectral library to aid in identification and comparison.

There are a few reasons why MALDI would not be the ideal choice for cannabis analysis and these include matrix interference, the fact that MALDI is a dry technique and cannot be paired with a liquid separation technique, and the impossibility to determine concentrations of components precisely as MALDI absolute peaks areas are not reproducible from run to run. The overall spectra may look the same, i.e. with same relative intensities between peaks, however absolute numbers can differ. Another consideration is that cannabis is unique through cannabinoid content as well as terpene content.

Matrix interference is perhaps the most pressing issue facing the identification of strains owing to the type/amounts of cannabinoids present, - as these compounds have low molecular weights (<1000 Da). Common matrices like DHB, CHCA and SA are also low molecular weight compounds, with masses below 300 Da, which results in interferences when analyzing cannabinoids. Matrix interference peaks are generally in

the 0-600 *m/z* range and could include $[M+H]^+$, $[2M+H]^+$ and $[M-H_2O+H]^+$, where M is the molecular weight of the matrix. Therefore, when analyzing a sample containing masses smaller than 1000 Da, matrix peaks typically cause interference and can make it difficult to properly analyze a sample.

The second reason MALDI is not an ideal candidate for cannabis extract analysis is due to the isomeric nature of cannabinoids. The two most found cannabinoids, $\Delta^9$-THC and CBD, have monoisotopic masses of 314.2246 g/mol. As there is no liquid separation prior to analysis, these molecules would have the same m/z value and would be indistinguishable. Previous literature has shown that $\Delta^9$-THC undergoes laser induced rearrangement and daughter ions are also produced[78]. However, it was unclear through literature search how CBD and other common cannabinoids behave when induced with a laser by MALDI. Due to varying amounts of $\Delta^9$-THC and CBD, as well as other cannabinoids, extracts could contain distinguishing peaks that could be used to recognize and identify different cannabis strains.

Finally, the analysis of volatile compounds is not easily achieved using MALDI. Terpenes are present in cannabis are responsible for the aroma and could be used to differentiate strains from one another. MALDI requires co-crystallization of the analyte with a matrix compound for it to then be induced by a laser. Due to the highly volatile characteristics of terpenes, it is likely that any terpene present in the sample will evaporate with the solvent and therefore not be detected[79]. It would be ideal to have terpene content also part of a spectral fingerprint but because of their volatile nature, this is unlikely.

## 4.2 Matrix Selection

The first task of development towards a cannabis library is the determination of an appropriate MALDI matrix. The chosen matrix should first have the ability to ionize cannabinoids in positive mode while not producing interfering peaks. Common matrices for analysis include CHCA, DHB and SA. These matrices were used and tested at different ratios with $\Delta^9$-THC and CBD standards which were at the concentration of 1 mg/mL as shown in Table 3.2.1. Both $\Delta^9$-THC and CBD have the same chemical formula of $C_{21}H_{30}O_2$ and share the monoisotopic molecular weight of 314.2246 g/mol and it is expected that both of the $[M+H]^+$ monoisotopic peaks would be 315.2324 m/z. None of the matrices mentioned had a major peak in this range, which indicated that ionization capabilities would need to be considered more so than interferences.

Previous analysis of cannabinoids using MALDI by Beasley et al[78] have shown that the THC molecule undergoes a rearrangement from the ionization caused by the MALDI laser. A possible explanation of this phenomenon is an increase in conjugation of the molecule from loss of hydrogens as free radicals and therefore the molecule becomes more stable through rearrangement. Their research showed a linear relationship between the ionization energy and the ratio of the 313/315 m/z peaks, suggesting the rearrangement of the THC molecule was a result of the ionization energy. The Bruker UltraFleXtreme™ used in this study also uses a neodymium-yttrium aluminum garnet (Nd:YAG) laser with a 355nm wavelength. The proposed rearrangement of the THC molecule in positive reflector mode is shown in Figure 3.2.1 below[78].

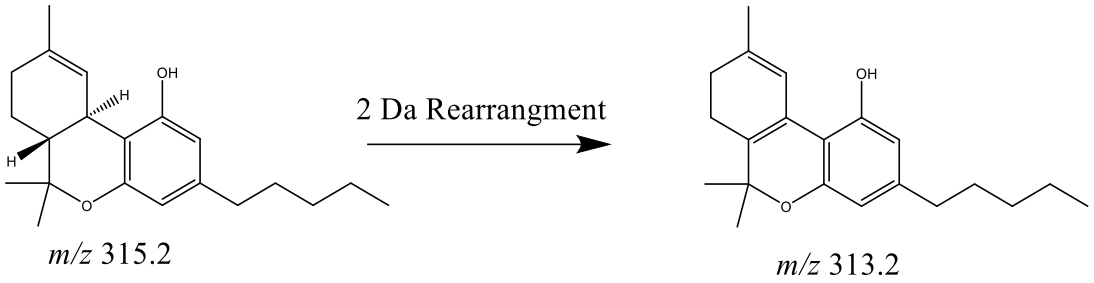



**Figure 4.2.1 A proposed rearrangement of THC using positive ionization mode MALDI.** Adapted from Ref No.[78]. The laser and wavelength used in the study was a neodymium-doped yttrium aluminum garnet (Nd:YAG) at 355nm (1 kHz).

Three popular matrices used for general MALDI analysis include DHB, CHCA and SA. These three matrices were used for with $\Delta^9$-THC and CBD standards to see which offered the best ionization efficiency for cannabinoids in positive reflector mode. Peaks used to determine the matrix ionization capability were 313, 314 and 315 m/z. Different ratios of matrix: sample were tested and a sandwich spotting technique with ratios of 1:1:1 was also tested[80]. The recorded relative intensities of peaks are shown in Tables 3.2.1 and 3.2.2.

**Table 4.2.1. $\Delta^9$-THC (1 mg/mL) standard peaks with DHB, CHCA and SA.**
The intensities listed are the average sums of eight laser shots.

| Matrices and liquid ratios | 313 m/z intensity | 314 m/z intensity | 315 m/z intensity |
|---|---|---|---|
| 1 DHB: 1 THC | 229777 | 163685 | 329190 |
| 2 DHB: 1 THC | 74093 | 57266 | 136196 |
| 1 DHB: 2 THC | 46471 | 30947 | 68429 |
| 1 DHB: 1 THC: 1 DHB (sandwich) | 19001 | 11468 | 24858 |
| 1 CHCA: 1 THC | 159493 | 74299 | 109952 |
| 2 CHCA: 1 THC | 312911 | 128910 | 323059 |
| 1 CHCA: 2 THC | 362187 | 160269 | 384067 |
| 1 CHCA: 1 THC: 1 CHCA (sandwich) | 257099 | N/A | 203131 |
| 1 SA: 1 THC | 584 | N/A | 519 |
| 2 SA: 1 THC | 428 | N/A | 694 |
| 1 SA: 2 THC | 117 | N/A | 76.1 |
| 1 SA: 1 THC: 1 SA (sandwich) | 544 | 537 | 2541 |

**Table 4.2.2 CBD (1 mg/mL) standard with DHB, CHCA and SA.** The intensities listed are the average sums of eight laser shots.

| Matrices used and molar ratios | 313 intensity | 314 intensity | 315 intensity |
|---|---|---|---|
| 1 DHB: 1 CBD | 3194 | 2834 | 33459 |
| 2 DHB: 1 CBD | 2922 | 3814 | 31786 |
| 1 DHB: 2 CBD | 3880 | 3588 | 38802 |
| 1 DHB: 1 CBD: 1 DHB (sandwich) | 646 | N/A | 2847 |
| 1 CHCA: 1 CBD | 65999 | 35217 | 142560 |
| 2 CHCA: 1 CBD | 26871 | n/a | 82562 |
| 1 CHCA: 2 CBD | 32314 | N/a | 155678 |
| 1 CHCA: 1 CBD: 1 CHCA (sandwich) | 33945 | N/A | 161251 |
| 1 SA: 1 CBD | N/A | N/A | 972 |
| 2 SA: 1 CBD | N/A | N/A | 1561 |
| 1 SA: 2 CBD | N/A | N/A | 4468 |
| 1 SA: 1 CBD: 1 SA (sandwich) | N/A | N/A | 788 |

First, it is possible to see from Table 3.2.1. that SA has poor ionization capability of the THC and CBD ion and therefore was not considered to be the matrix for this project. The work of Beasley[78] suggests that the 313 m/z peak should be the most intense, 315 m/z being the second most intense and 314 m/z being the least intense peak of the three. This was observed using the CHCA matrix. This is observed in the Figure 3.2.2.

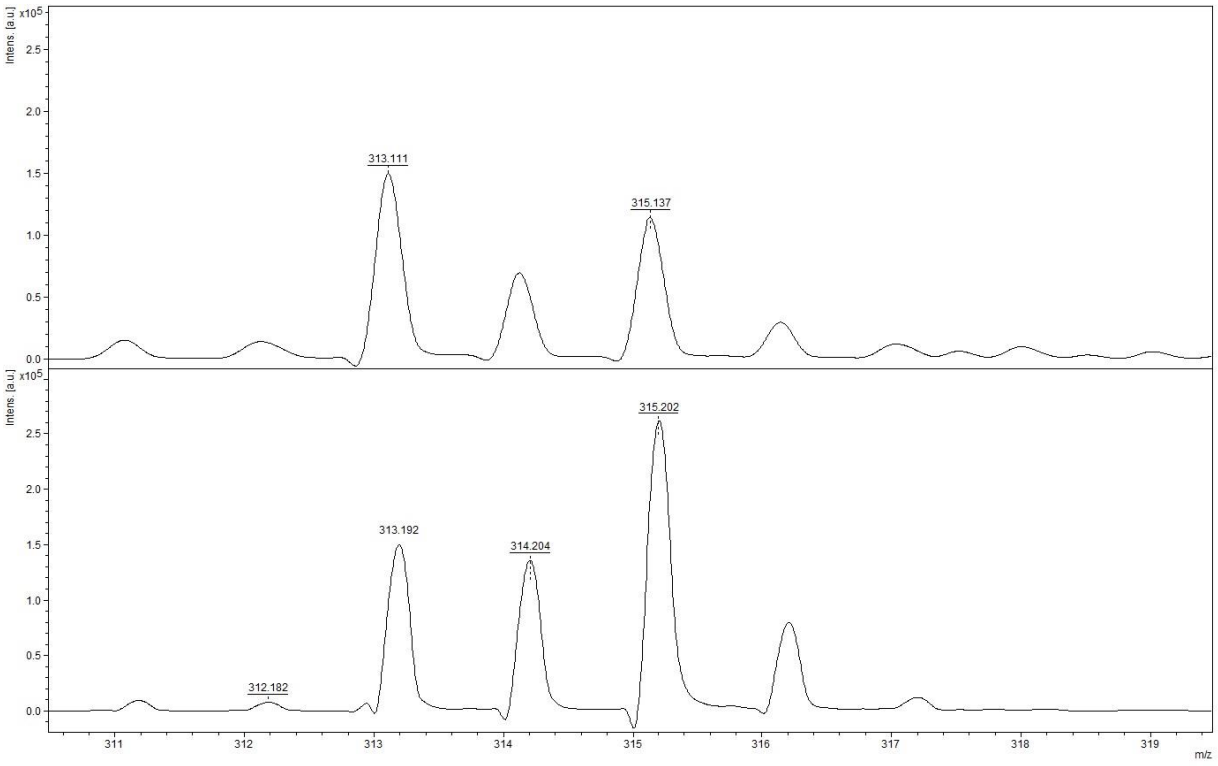



**Figure 4.2.2 Ionization pattern of Δ⁹-THC using CHCA (top) and DHB (bottom).** CHCA ionization match with previously observed ionization patterns of the molecule[78].

CHCA was therefore chosen as the best matrix for the work as it was consistent with previous ionization patterns observed[78], and was also the best at ionizing the cannabinoid standards. Spectra of all six strains with sequential extraction methods were collected using CHCA.

## 4.3 Determination of m/z range to use

The goal of this project was to create a library of cannabis strains using solvent extracts collected from multiple strains to determine if MALDI and the Bruker Biotyper

software is capable of consistent distinction between them. When creating a mass spectral fingerprint library, it is recommended that multiple spectra are collected for a single sample, which can then be grouped together using the Biotyper software. Collecting multiple spectra ensures that the whole sample is represented and also to detect variations which can be accounted for when the spectra are averaged in the software.

For each strain, an extract was collected through sequential extraction in order of methanol, acetonitrile and hexane. This sample was directly combined with CHCA matrix on the target plate and a spectrum was collected.

As there has not been a previous study to determine at a fingerprint-like spectra from cannabis, it is unclear on what exactly will be extracted from the sample and then capable of undergoing positive ionization under MALDI conditions. Therefore, the spectra of the extracts were first collected over a relatively large range (0-1300 m/z) to determine the mass range that should be used for further collection. It was expected that the molecules collected would primarily be cannabinoids ranging in the 300-400 m/z range, fragments of cannabinoids and possibly other simple biomolecules with molecular weights less than 1000 Da. The raw spectra of extracts can be viewed in Figure 3.3.1.

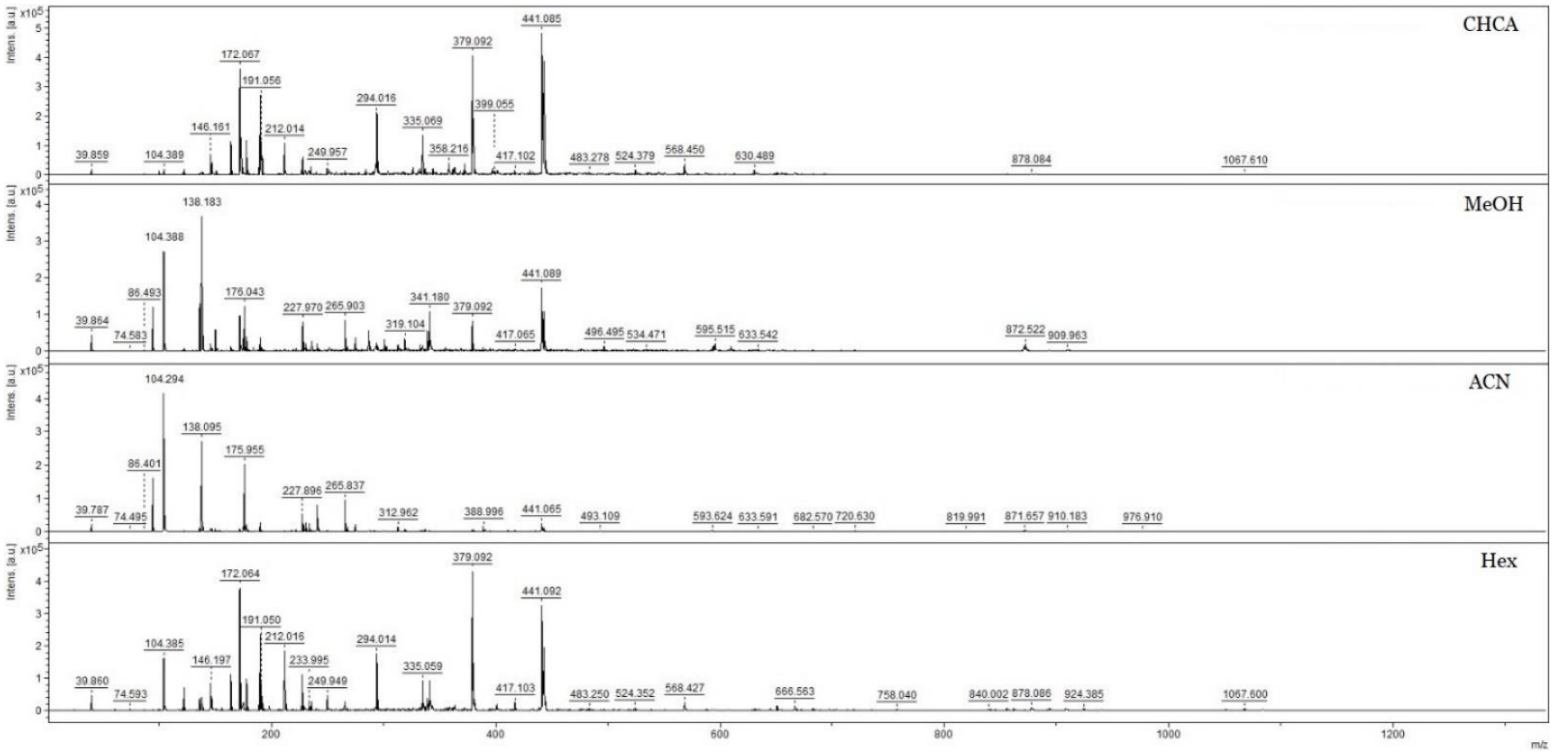



**Figure 4.3.1**. **Extracts collected from the Blueberry strain**. The spectra collected show the matrix CHCA alone (top) followed by the methanol, acetonitrile and hexane extracts combined with CHCA.

It is demonstrated in Figure 3.3.1 that overall the extracts contain constituents with m/z values that are less than 1000 Da. In the hexane extract there exists a peak at 1067 m/z that can also be observed in the matrix. This peak is not considered to be something crucial to distinguish sample strains from one another and can therefore be ignored.

## 4.4 Raw Spectra of all Strains from Sequential Extraction

Prior to building a library using Biotyper™ software, multiple spectra of single extracts must be collected. In this project, each extract from a strain was spotted three

times on the MALDI plate and each spot was shot eight times to ensure a full grasp of the contents of the extract was shown on the spectra.

The purpose of the sequential extraction and the order of it (order of MeOH, CAN and Hex) was to attempt to get a full picture of constituents of cannabis by using solvents with differing characteristics. Previous work has shown that an efficient extraction solvent for cannabinoids is 9:1 methanol/chloroform (v/v)[81]. The use of chloroform is not ideal as it is toxic to humans and destructive to the environment and therefore in this work, the sequential extraction was started with methanol alone. Also, the use of a single solvent slightly increases the ease and quickness of this step. Methanol alone has previously shown to have decent extraction yields of cannabinoids[82] and as this project is dealing with a fingerprint representation instead of quantitative amount, this solvent was chosen. Spectra of methanol extracts from all cannabis strains can be observed in Figure 3.4.1.

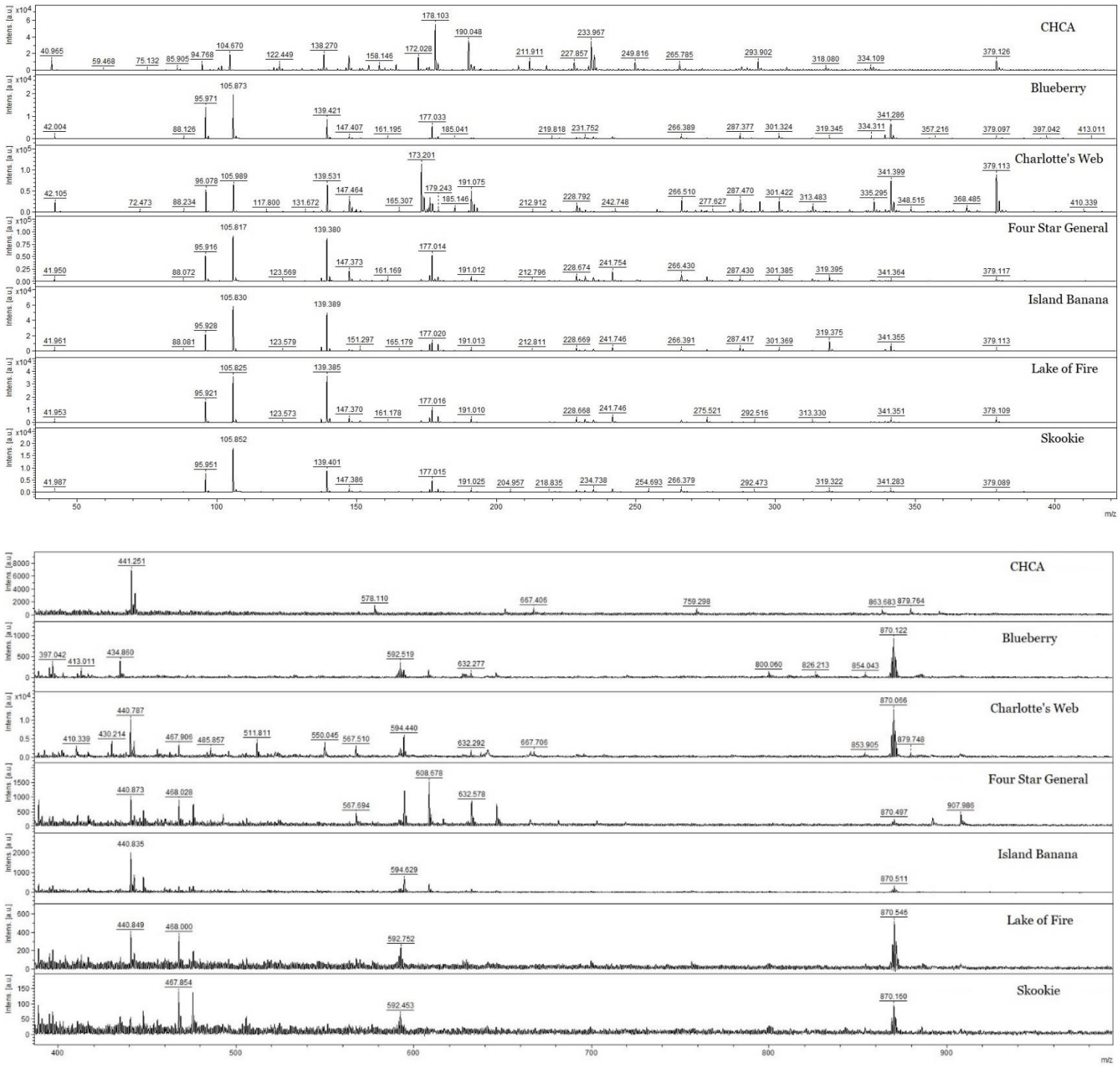



**Figure 4.4.1 MALDI spectra of methanol extracts of all strains as well as CHCA matrix alone.** The top shows the lower range of the spectra (0-400 m/z) and te bottom shows the upper range (400-1000 m/z).

This project does not deal with identification of peaks but instead the acknowledgement that peaks exist and that MALDI spectra of samples differ from matrix spectra. There are numerous peaks in the varying cannabis strains spectra that are not present in the matrix spectra and previous work has shown that these peaks are consistent with cannabinoids and the degradation of such[78,83]. Overall, the important takeaway from the raw data is that the sample spectra are visibly different from the matrix spectra. Further discussion on the possible identification of cannabis peaks and then comparison of said peaks will be further discussed in Chapter 4.

The next solvent choice for the sequential extraction was acetonitrile. This solvent is not an overly popular choice when it comes to cannabinoid extraction but was selected for a couple of reasons. First, it is polar but possess aprotic properties instead of protic properties such as methanol. As both solvents are polar but have differing characteristics, it was assumed that using acetonitrile as the second sequential solvent would extract any leftover cannabinoid type molecules and could give a different representation of a MALDI fingerprint relative to methanol. A second reason acetonitrile was selected is its miscibility with water. As the MALDI matrix is usually dissolved in an acetonitrile-water mixture, acetonitrile fractions can be directly deposited onto the target and mixed with the matrix. Therefore, it is assumed that the extract contents would have a larger chance to be ionized. Overall, the selection of acetonitrile as the second solvent was in hopes of creating different fingerprint spectra of cannabis in aim to get ample data to identify and differentiate samples by MALDI. The spectra collected from the acetonitrile extracts can be seen in Figure 3.4.2.

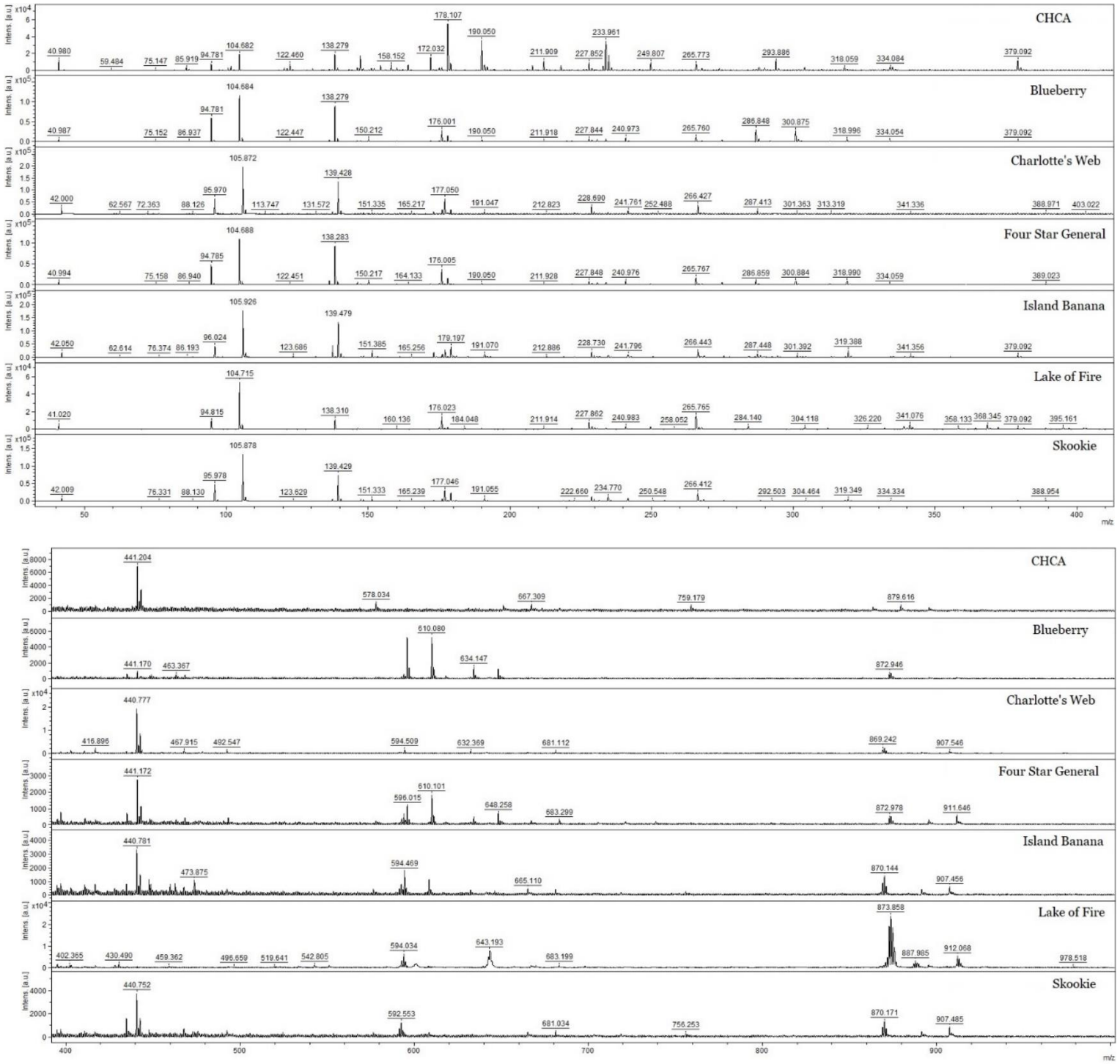



**Figure 4.4.2 MALDI spectra of acetonitrile extracts of all strains as well as the CHCA matrix alone.** The top shows the lower range of the spectra (0-400 m/z) and the bottom shows the upper range (400-1000 m/z).

It can be seen that the cannabis acetonitrile extract spectra differ from the matrix spectrum. It is also important to note that the acetonitrile spectra differ from the methanol spectra and therefore at first glance, the goal of collecting differing fingerprints from a single sample is, to a certain degree, effective.

Finally, the last solvent selected was hexane. When discussing the methanol extraction step, it was mentioned that chloroform was commonly used in combination with methanol but was removed to decrease the environmental impact. The use of this solvent was to determine if any non-polar molecules could be retrieved in the extract. The results can be observed in Figure 3.4.3.

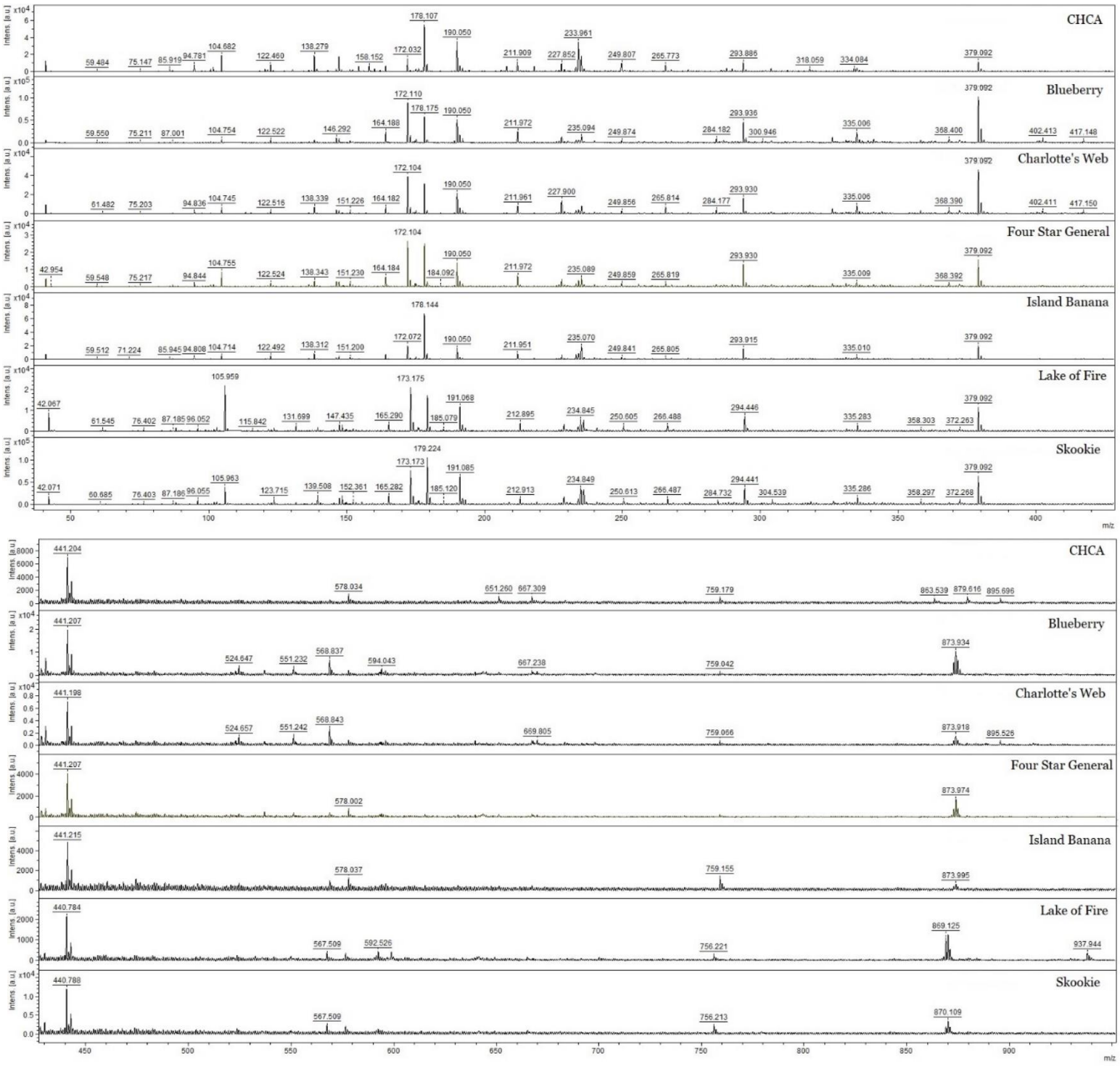



**Figure 4.4.3 MALDI spectra of hexane extracts of all strains as well as the CHCA matrix alone.** The top shows the lower range of the spectra (0-400 m/z) and the bottom shows the upper range (400-1000 m/z).

Overall, the attempt at using this solvent as an extractant was rather unsuccessful. The spectra generated did not differ greatly from that of the matrix and therefore there is no added fingerprint view of cannabis. The use of this solvent was therefore disregarded.

## 4.5 Biotyper Parameter Optimization

The Biotyper software offers the advantage to enable the optimization of a large number of parameters including preprocessing, MSP creation and MSP identification. This results in the ability to optimize them for a given data set. Optimization of Biotypers parameters was based on the comparison of the generated averaged peak lists from the raw data collected. By changing each parameter one by one and holding others constant, it was possible to determine parameter values that would make cannabis strain differentiation most attainable with simple fingerprinting of low molecular weight compounds from various extracts.

Optimization was done using three spectra collected using the one methanol extract of the blueberry strain. This strain was chosen to optimize parameters with because the raw data have a large peak m/z range, peaks in this range are consistent with those previously observed and singly charged cannabinoid peaks[78,83]. Also, there exist unique peaks, with varying intensity, in the cannabis extract sample which are not found in the matrix spectra. All of these factors are important to determine if the Biotyper software is capable of differentiating cannabis strains consistently.

### 4.5.1 Preprocessing Parameter Adjustments

Adjustment of parameters in the preprocessing section of Biotyper resulted in the generated MSP's having different peak lists (different ranges, different peaks included) and therefore each with different views at the "fingerprint" of the sample. Differences in peak lists from the adjusted parameters could be the *m/z* range of the peak list, the number of peaks included and which peaks were included. Overall, the m/z assignments of peaks in the spectrum do not change but the choice of peaks used in the fingerprint does change and therefore this is what needs to be optimized.

The three raw spectra were imported into the Biotyper software and parameter adjustments were made. Then, the three spectra were averaged to create an MSP peak list. Peak lists of raw data from FleXanalysis were compared with MSP peak lists in order to determine if the MSP was a true average of the raw spectra collected. The goal is to adjust these parameters to create a large and inclusive peak list from the raw spectra as well as to optimize the data set. The adjustments were made to one parameter while others were held constant in order to see how the peak list changed. The MSP creation and MSP identification parameters were maintained at the standard software inputs, as adjustment of these at the same time would not make it possible to detect the effect that the preprocessing parameters had. MSP were created using the Biotyper MSP creation standard method in order to see how changing individual preprocessing parameters affected the peak lists. Although to also be optimized and defined in further detail in section 3.3.2, the standard MSP creation method inputs consisted of Max mass error of each single spec: 2000, Desired mass error for the MSP: 200, Desired peak frequency: 25%, max number of peaks in the MSP: 100.

### 4.5.2 Mass Adjustment

The mass adjustment parameter defines whether and how to adjust the masses from raw spectra. The parameters that are available to adjust in this section include mass adjustment (lower and upper), resolution and the method used for mass adjustment. The mass adjustment parameter was adjusted to a range of 0-1000 m/z as this was the range where unique sample peaks were observed. Raw spectra were collected up to 1200 m/z, but it was observed that peaks above the 1000 m/z were consistent with peaks found in the matrix spectra. In various methanol and acetonitrile extracts from all cannabis strains, peaks in the 870-911 m/z range with considerable intensity (intensity of 1000-5000 detector counts) were observed. Although it is unknown what these peaks could represent, their varying intensities and presence in multiple samples indicated they should be included within the fingerprint comparison, but that anything above 1000 m/z should not be included.

Resolution is defined by the program as the peak resolution threshold and it is stated that it is not relevant for MSP spectra preprocessing. The resolution was chosen to be 1 Da which is also the default value used by the program. The software manual does not go into further detail on how resolution is calculated.

The method of mass adjustment chosen was spectra compressing with a compression factor of 5. There also exists a “no compression” option and when this was selected, the program was not capable of producing an MSP and therefore this parameter option was not chosen. As defined by the program, the chosen method of spectra compression “performs a partial integration of the data points collected using compression factor of 5” and also, “reduces the number of data points and removes any

high frequency peaks". From these definitions, it is assumed that this function takes data points from the peak area and transforms them into intensity related values so that the generated MSP peak list consists of peaks that all have the same width and can therefore be compared solely on *m/z* values. This is important as the program uses the intensity of the signals to determine the score of a match such that a higher score is achieved if high intensity signals in an unknown have a closely matching high intensity signal in the reference spectrum and the same with low intensity signals. Also, the term "frequency" used in the software for this parameter is not further explained. It is assumed that it means the number of times a peak returns.

In the spectra compressing parameter, the program suggests a compression value of 10 but the values of 5 and 20 were also tested in this work. In all the MSP peak lists generated from this sample, the most intense peak is 139.31 m/z and when the compression values were changed, so did the percent intensity of the peak. These values of percent change can be observed in Table 3.5.1.

**Table 4.5.1. Compression factor adjustments.** This shows how the variation of the compression factor value changes how many peaks are generated in the MSP.

| Compression Factor | Relative Intensity (%) of largest peak- 139.31 m/z |
|---|---|
| 5 | 100 |
| 10 | 97.22 |
| 20 | 96.81 |

The 139.31 *m/z* peak consistently remained the most intense peak in all of the MSP peak lists generated from changing this parameter. When the compression value was set to 5, the intensity of this peak was 100.00% and when the compression value

increased, the relative intensity of this peak decreased. This relationship makes sense as an inverse relationship as increasing compression would decrease the intensity and vice versa. The compression value of 5 was chosen as this gives the largest peak a relative intensity of 100%, or 1, and all of the other peak intensities are then compared to a value of 1. If a larger compression factor was chosen, peak intensities would instead be compared to a value less than 1 which decreases simplicity and is not necessary.

### 4.5.3 Smoothing, Baseline subtraction and Normalization

All of these parameters were left as the default software supported parameters and adjustment tests were not done in this work. These parameters include a smoothing method of Savistsky-Golay, a baseline subtraction method of multipolygon and a normalization method of maximum norm.

### 4.5.4 Peak Picking

Peak picking adjustments define how to pick peaks in spectra during the preprocessing. The parameters available for adjustment in this section include; max peaks to be included in the peak list, threshold and method of peak picking.

The max number of peaks observed in the raw spectra using FleXanalysis was 200 using a threshold value of 0.001 and the method for peak picking used was local maximum with a window width of 5 Da.

The threshold value in preprocessing corresponds to the % relative intensity of the highest peak that a peak must exceed in order to be detected. For example, a

threshold value of 0.01 means that the peak must have an intensity of at least 1% of that of the highest peak in the spectrum. Also, the max number of peaks set to 150 indicates that the first 150 peaks meeting the threshold value will be chosen even if there are other peaks within the range that meet the threshold value. This parameter must be optimized to include all relevant peaks within the desired range but not be too inclusive that it creates peaks that are not visually present in the raw spectra. Comparisons of the peak list to the raw spectra were done in order to ensure that the peaks being generated were not from a large amount of noise in an area, i.e.- were actual peaks. This parameter was adjusted by factors of ten (0.0001, 0.001, 0.01 and 0.1) as observed in Table 3.5.2.

**Table 4.5.2 Threshold value adjustments.** This shows how variation of the threshold value changes the number of peaks produced in the MSP.

| Threshold | Range | Number of Peaks |
|---|---|---|
| 0.1 | 95.83-301.46 | 10 |
| 0.01 | 41.86-908.82 | 36 |
| 0.001 | 41.86-858.26 | 99 |

The largest threshold value of 0.1 is too strict as it was only able to pick the ten most intense peaks generated. It is not inclusive to all visually relevant peaks with lower intensity. When the value is decreased by a factor of ten to 0.01, the m/z range is increased to the ideal mass range however the number of peaks within this range was still too small when compared to the raw data and therefore the threshold needed to decreased again. The threshold value of 0.001 included peaks that were generated from areas of noise instead of actual low intensity peaks. This results in peaks in the MSP list

that would not truly be considered a possible peak. Also, the max number of peaks was reached before the desired m/z range was reached. When threshold value was increased to 0.0025 (peaks must have 0.25% intensity relative to the largest peak), the peak list became more consistent with peak values in the raw data and therefore this threshold value was used. The number of peaks generated in this peak list was 71 with a range from 41.86-908.82 m/z. The signal to noise ratio in the raw data was set to a value of eight and the total peaks was 81 with a range from 42.608- 884.011 m/z. Using a threshold value of 0.0025 made the MSP peak list most consistent with the raw data as the number of peaks and the m/z range was more aligned. This is observed in Figure 3.5.1.

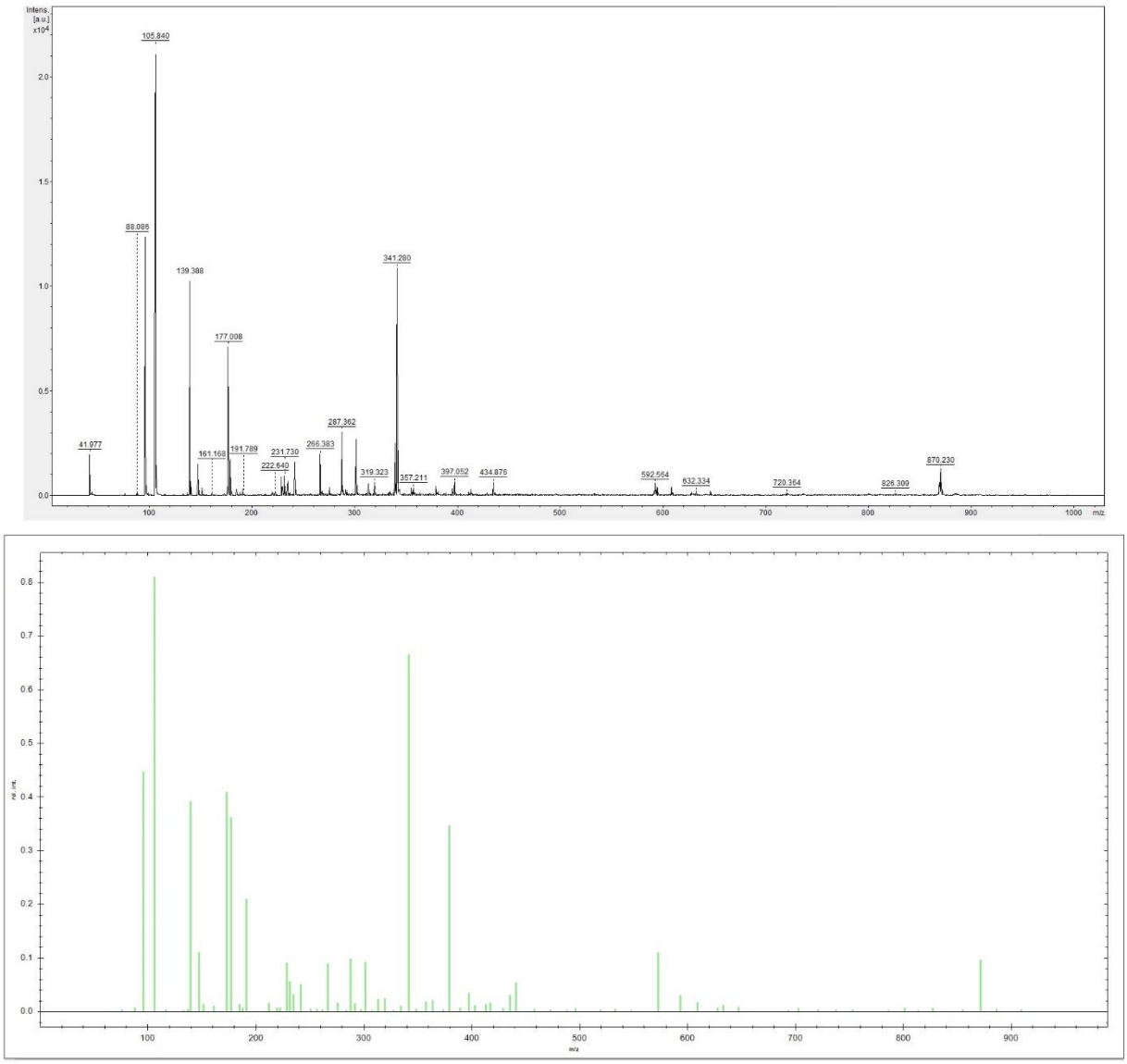



**Figure 4.5.1. Peaks generated in the raw spectra (top) compared to the MSP generated in the Biotyper program (bottom) with a threshold value of 0.0025).** The raw data taken from FleXanalysis contains one spectrum collected of a methanol extract from the Blueberry strain.

A peak picking method of local maxima was chosen as this generated the greatest number of peaks within the desired range. This method looks for local maxima within the spectrum to detect peaks with width window (units: Da) adjustments in which local

maxima are detected. Adjustments to the local maxima width resulted in different peak ranges as well as different number of peaks, as observed in Table 3.5.3.

**Table 4.5.3 Window width adjustment effects on the number of peaks in MSP.**

| Window Width (Da) | m/z Range | Number of peaks in MSP |
|---|---|---|
| 4 | 41.86-525.16 | 89 |
| 5 | 41.86-908.81 | 70 |
| 6 | 41.86-908.81 | 66 |

A local maxima value of 5 was chosen as it produced the largest number of peaks within the desired m/z range. As the window width increases above 6, the number of peaks within the desired range begins to decrease and therefore the peak list is optimized with a window width of 5 Da.

## 4.6 MSP creation parameters

MSP is considered to be the basis of classification in the Biotyper program and is a generated reference peak list from raw spectra. The software generates peak lists from the entire data set- i.e multiple raw spectra of one sample- and creates a MSP by using information on peak m/z value, peak frequency, and peak distribution. Once the preprocessing parameters were optimized, it was possible to then optimize the MSP creation parameters as well. Optimization of MSP creation parameters are used to determine how peaks from the raw spectra can be grouped together and shown in the

final peak list. This is done using mass error of a peak and how often the peak occurs (out of the three spectra collected from one extract).

### 4.6.1 Max. Mass error of Each Single Spectrum

The mass error of each single spectrum is the mass error window (ppm) to be applied along the whole spectrum for recalibration. The recalibration is performed using peak lists that were generated in the preprocessing segment.

This value was adjusted by factors of ten with values of 200, 2000, 20000 and 200000 being tested, as shown in Table 3.6.1.

**Table 4.6.1 Mass error and how it effects the mass range and number of peaks in the MSP.**

| Mass Error (ppm) | Mass range | Number of peaks in range |
|---|---|---|
| 200 | 41.86-908.81 | 70 |
| 2000 | 41.86-908.81 | 70 |
| 20000 | 41.86-908.81 | 73 |
| 200000 | 90.46-908.97 | 48 |

When the max mass error increases, the MSP peaks are generated with a larger +/- window of what could be accepted as one specific peak and therefore the software has more leeway for grouping peaks together. There are more peaks because with a larger area to group together, small intensity peaks can combine together to form a possible peak, hence the extra three peaks when the mass error is 20000 ppm. When the mass error is smaller, there is not enough area in the combined peaks to make a possible peak in the MSP list. The extra peaks created are at 137 m/z, 348 m/z and 517 m/z.

Upon analysis of these peaks in the raw spectrum it was observed that they were miniscule and should not be included in the main spectrum. The default value of 2000 ppm was used.

### 4.6.2 Desired Mass error for the MSP

The desired mass error for the MSP is defined as the maximum mass error (ppm) for each peak after recalibration. This value was changed by factors of ten using values of 200, 2000 and 20000 ppm. Overall, this did not drastically change the peak list by a considerable amount as shown in Table 3.6.2.

**Table 4.6.2. Mass error and how it effects the mass range and number of peaks in the MSP.**

| Mass error (ppm) | Mass range | Number of peaks |
|---|---|---|
| 200 | 41.85-908.81 | 72 |
| 2000 | 41.86-908.81 | 70 |
| 20000 | 41.86-908.81 | 75 |

As the desired mass error increased, so did the number of peaks. As seen during max mass error parameter adjustments, the increasing number of peaks that result as mass error increases is a due to the addition of slightly more noise which can accumulate just enough for the program to believe it is a justified peak. However, this is not the case and therefore this parameter value was left at the default software value of 200 ppm for this reason.

## 4.7 MSP Identification Parameters

Parameters available for adjustment include: frequency threshold for spectra adjusting, frequency threshold for score calculation, max. mass error of the raw spectrum, desired mass tolerance of the adjusted spectrum and also the furthermore accepted mass tolerance of a peak.

### 4.7.1 Frequency Threshold for Spectra Adjusting

This parameter is defined as the minimum frequency of occurrence (%) of a peak within a set of spectra for use in calibration of an unknown spectrum to MSP's. This value remained at 50% and was not possible to adjust.

### 4.7.2 Frequency Threshold for Score Calculation

This parameter is defined as the minimum frequency of occurrence (%) of a peak within a set of spectra for use in the classification procedure. The software recommended value for this parameter is 5% however the number of spectra collected per strain was three. This value was therefore set to 30% as this would indicate that the peak must be present in one of the three spectra collected in order to be used in the classification procedure.

### 4.7.3 Maximum Mass Error of the Raw Spectrum

This parameter is defined as the mass tolerance (ppm) for the initial peak adjustment before recalibration. To remain consistent with the MSP creation parameters, this value was set to 2000 ppm.

### 4.7.4 Desired Mass Tolerance of the Adjusted Spectrum

This parameter is defined as the desired mass tolerance (ppm) for any peak after adjustment using the inner mass window. To remain consistent with the MSP creation parameters, this value was set to 200 ppm.

### 4.7.5 Furthermore Accepted Mass Tolerance of a Peak

The addition mass tolerance window (ppm) for any peak after adjustment using the outer mass window was set to 600 ppm. The value of 600 ppm is the software recommended value and when adjusted from this to 1000 ppm, there was minimal effect on the MSP peak list.

# Chapter 4: Results Using Biotyper™ Software

## 5.1 General Considerations

The goal of this work was to use MALDI-TOF analysis for the distinction and recognition of varying cannabis strains obtained from the recreational market. Currently, the Canadian federal government does not have method in place for the differentiation and confirmation of cannabis strain identity. Instead, cannabis quality assurance testing more-so focuses on potency so that consumers can know what to expect when using the product[83,84]**.** Fingerprinting of microorganisms using MALDI-TOF is a technique that has been well documented for identification of bacteria, fungi and viruses[85–89]. Using Bruker's Biotyper software, a pattern algorithm uses peak positions, intensity distributions and frequencies to find matches between unknown samples and reference samples. Biotyper was designed and is traditionally used for the identification of microorganisms and relies on small mutations of large molecular weight compounds like proteins to accurately differentiate strains of microorganisms. The algorithm that the software uses relies on peak lists generated in MSP's that focuses on factors such as peak position, peak intensity distributions and peak frequencies. Ideally, this work would be used to differentiate and confirm identity of cannabis strains.

The difference between chemical fingerprinting of cannabis and microorganisms- as the Biotyper software is traditionally used for- is that this work focuses on the low molecular weight molecules produced in abundance in the flower and not large proteins. Instead, this work is relying on the large variation of natural products found in cannabis flower to create a chemical fingerprint of each strain that can be used for comparison. The development of a MALDI-TOF fingerprint library for various cannabis strains

would be useful for quality assurance of cannabis as it is a fast, easy to use, highly accurate and highly throughput technique.

This chapter focuses on the results obtained using MALDI-TOF and the Bruker Biotyper software. As previously mentioned in Chapter 3, each strain underwent a sequential extraction using methanol, acetonitrile and hexane that was repeated multiple times and all of which occurred at room temperature. Once collected, each solvent extract was spotted onto the MALDI target plate three times using CHCA matrix. Each spot was shot eight times with the laser and individual spectra were collected. Raw spectra were imported into the Biotyper software and these were then combined together to form a MSP. Therefore, to summarize, each MSP contains a total of 24 laser shots from one single solvent extract of a particular cannabis strain.

## 5.2 Comparison of same extracts on different days

Using the program optimized settings outlined in Chapter 3, cannabis strain extracts are to be compared to one another in the Biotyper software. However, prior to that, the program was used to determine score values for the matrix MSP's on days that extracts were also analyzed. The purpose of this was to determine how much scores can vary for a well documented low molecular weight sample using the same method that is to be used for cannabis. Also, the matrix was used for internal calibration of cannabis samples and therefore the variation of scores for the standard are useful for comparison. As mentioned in Chapter 2, the 3-point internal calibration was done using matrix peaks of 190.04987, and 379.09246 m/z corresponding to $[M+H]^+$, $[M+Na]^+$ and $[2M+H]^+$. The comparison of matrix MSP's was done by setting each day as the reference and all

others were separately set as the unknown. By doing this with six different MSP's, it is possible to obtain 30 different score values that can be used to determine how much variation there is within the matrix MSP lists. Score values are defined in the program as how much likeliness an unknown is to a reference and uses the following criteria;

1) Number of signals in the reference spectrum that have a closely matching partner in the unknown spectrum are calculated. No matches score=0, complete match score=1
2) Number of signals in the unknown spectrum that have closely matching partner in the reference are calculated. No matches score=0, complete match score =1
3) Symmetry of matching signals is calculated. If high-intensity signals of unknown correspond with high-intensity signals of the reference and low-intensity signals also correspond, the value is close to 1. If matching pairs have no symmetry, the value is close to 0.

These values are multiplied together and the result is normalized to 1000 and the score value obtained is the log of this result. An absolute perfect match of spectra results in a score of 3.000. The Biotyper program uses the score value of ≥2.000 to be considered as a probable microorganism classification/match. The scores of various matrix MSP's compared to one another can be seen in Table 4.2.1.

**Table 5.2.1 Matrix CHCA MSP scores on various days when extracts of cannabis strains were collected.** The reference MSP are listed along the horizontal and the unknown MSP are listed along the vertical.

| CHCA | | Reference MSP | | | | |
|---|---|---|---|---|---|---|
| | | **1** | **2** | **3** | **4** | **5** |
| **Unknown MSP** | **1** | 3.000 | 2.661 | 2.568 | 2.248 | 2.567 |
| | **2** | 2.773 | 3.000 | 2.766 | 2.506 | 2.753 |
| | **3** | 2.738 | 2.913 | 3.000 | 2.668 | 2.790 |
| | **4** | 2.515 | 2.627 | 2.693 | 3.000 | 2.691 |
| | **5** | 2.625 | 2.577 | 2.627 | 2.552 | 3.000 |
| | | Mean score (Using antilog score values to average, then take log of this number, also excluding compared to self): 2.663 | | | | |

In Table 4.2.1, the MSP that is used as the reference is set on the horizontal top axis and the unknown is set on the vertical left side axis. In the first column, a score is obtained for each being compared to MSP 1 as the reference and all others as the unknown and this pattern is followed in each column of the table. When an MSP is compared to itself, a perfect score of 3.000 is obtained, as expected. The lowest value obtained for the comparisons was using day 4 as the reference and day 1 as the unknown for a score of 2.248. The largest score obtained for the comparisons, other than 3.000, was obtained using MSP 2 as the reference and MSP 3 as the unknown for a score of 2.913. The average score for all comparisons was 2.643 with a standard deviation of

0.1359 and a coefficient of variation (100*standard deviation/mean) of 5.14%. To calculate the mean, score values of 3.000 are not included as this would increase the average and would not show the true comparison of peak lists. Overall, the average scores for same-sample comparisons were higher than the minimum value for MSP's to be classified as a match. Figure 4.2.1 shows comparison of the MSP spectra for the highest and lowest score values.

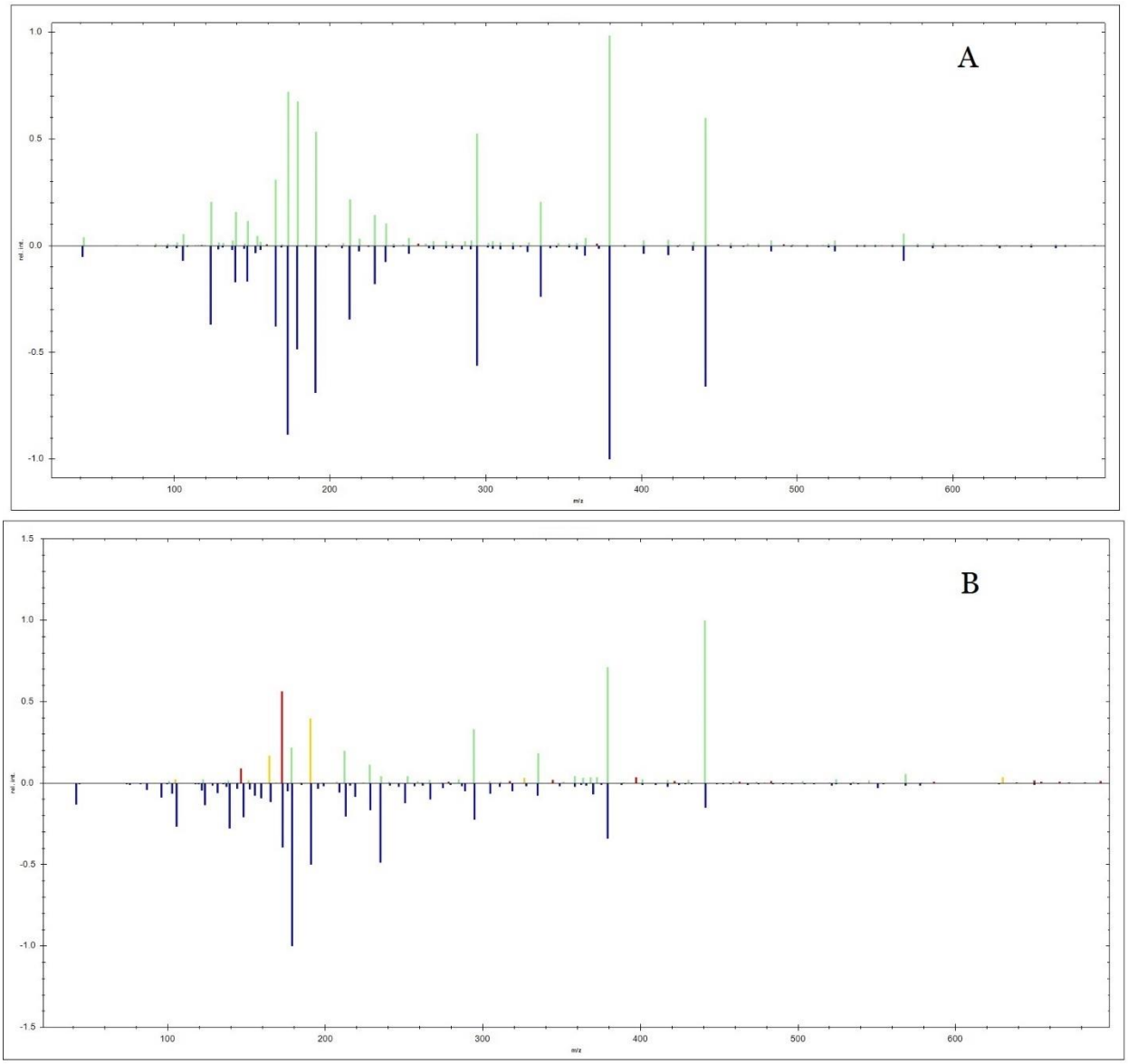



**Figure 5.2.1. MSP identification spectra comparing the closest and furthest related matrix MSP peak lists.** The top comparison (A) shows MSP 3 as the reference and MSP 2 as the unknown with a score of 2.913 and the bottom comparison (B) shows the lowest score obtained with MSP 1 as the reference and MSP 4 as the unknown with a score of 2.248. When comparing MSP, the unknown has positive relative intensity while the reference has negative relative intensity. Green peaks indicate the peak is a full match, yellow indicates a partial match and red indicates no match.

An interesting aspect of the scores obtained is the changing of values dependent on which is set as the reference and which is set as the unknown. For example, in Table

4.2.1, it can be seen that for MSP 1 as the reference and MSP 2 as the unknown, the score value is 2.773 but when the reference and unknown are switched, the score obtained is 2.661. These comparisons are shown in Figure 4.2.2.

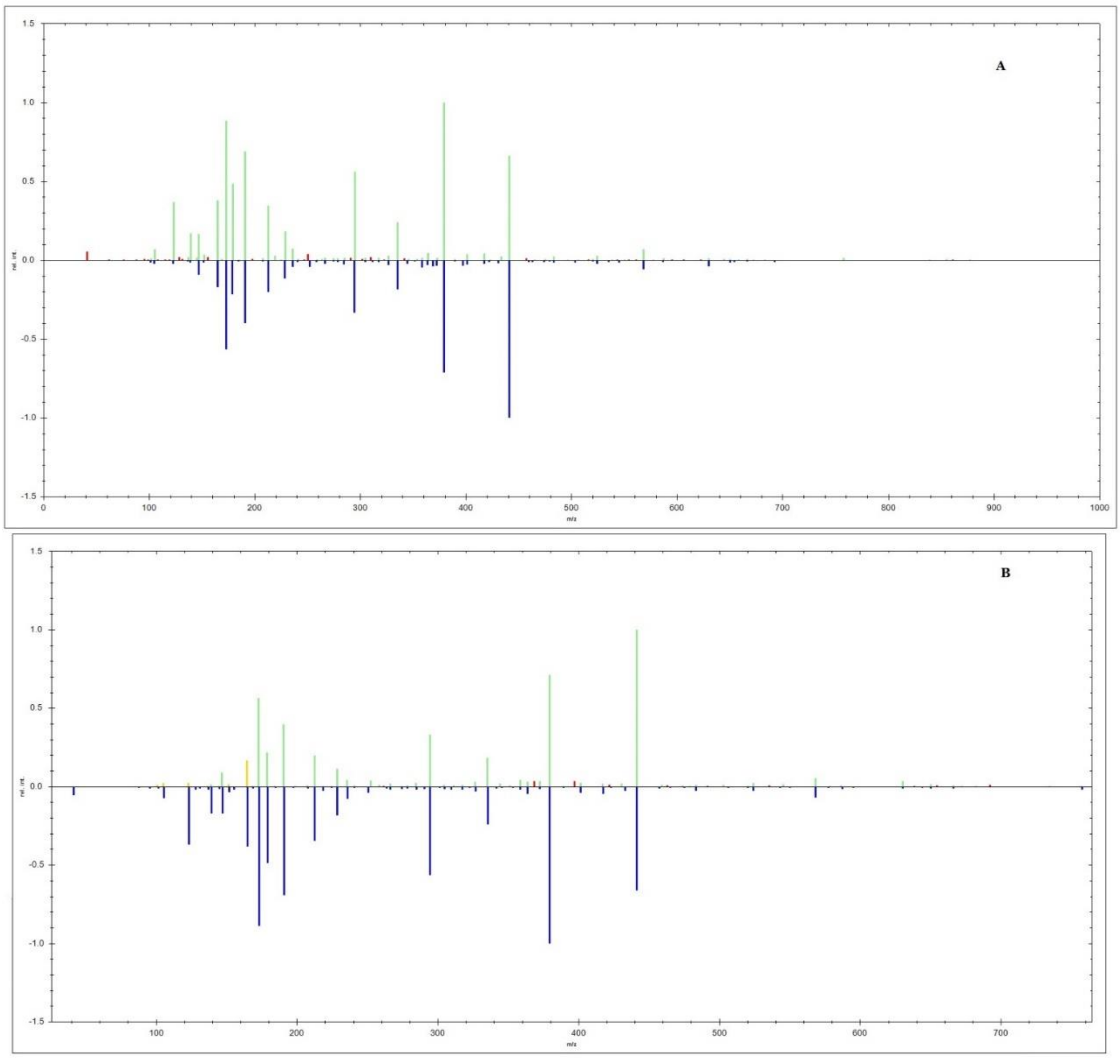



**Figure 5.2.2**. **MSP identification spectra showing the comparison of MSP 1 and MSP 2 of the matrix.** The top comparison (A) shows MSP 1 as the reference and MSP 2 as the unknown with a score of 2.773 and the bottom comparison (B) shows MSP 2 as the reference and MSP 1 as the unknown with a score of 2.661. When comparing MSP, the unknown has positive relative intensity while the reference has negative relative intensity. Green peaks indicate the peak is a full match, yellow indicates a partial match and red indicates no match.

This is a demonstration of how the software and description for peak matching is unclear. The program user manual suggests that a score is calculated through the determination of three separate values that are multiplied together, which would suggest that the order of operations for this function is irrelevant. The score values are quite similar although it would be expected that they would match if values multiplied together. However, this does not appear to be the case. One reason for the different scores achieved would be that the reference and unknown spectra are treated differently. Another reason this could occur is due to the peak intensities. For example, in a pair of peaks, the peak in the unknown is more intense than the peak in the reference. When the spectra are switched, this pair of peaks would now have the less intense peak in the unknown. It could be that the score is lowered when the more intense peak is in the unknown or even the opposite of that occurs. The lack of detail provided by the user manual leaves this as an unanswered question.

By using sequential extracting and multiple raw spectra averaged together, it is expected that the extracts will differ enough that the Biotyper program will be able to differentiate that the program will be able to identify cannabis product as such in the same solvent system but perhaps have difficulty differentiating strains from one another as all cannabis contains cannabinoids that are all mostly isomers of one another. However, with a large variation of natural products produced by the plant, it is possible that strain differentiation will be possible using this application.

The first comparison that was done for the cannabis product was comparing the different extracts of a cannabis product to themselves. The purpose of this was to determine if the Biotyper software would consistently be able to recognize the same

strain over different collection days. As done with the matrix shown above, an MSP of an extract from a strain, for example, the methanol extract from the Blueberry strain, was compared to itself over six different collection periods. Therefore, MSP 1 of Blueberry methanol was set as the reference and each of MSP 2-6 were all set as the unknown and the score values of these comparisons were recorded. Then, MSP 2 of Blueberry methanol would be set as the reference and each of MSP 1, 3-6 would be set as the unknown and so on. This resulted in 30 different score values which were used to show how well Biotyper was able to identify the extracts collected from a single cannabis product. This procedure was done for all strains using each of the methanol and acetonitrile extracts. Once all of these values were recorded, the average, standard deviation and coefficient of variation was determined. These data can be seen in Table 4.2.2.

**Table 5.2.2. Cannabis extracts averaged MSP scores when compared to itself over various collections.** Scores were calculated by setting a single MSP from one strain extract as the reference and all others of matching strain and extracting solvent as unknowns. The mean, standard deviation and coefficient of variation were calculated from 30 scores. The values of 3.000 corresponding to a sample compared to itself was not used in these calculations.

| Strain | Methanol | Acetonitrile |
|---|---|---|
| | Log Score Value | Log Score Value |
| Blueberry | 2.577 | 2.599 |
| Charlotte's Web | 2.560 | 2.655 |
| Four Star General | 2.640 | 2.604 |
| Island Banana | 2.623 | 2.574 |
| Lake of Fire | 2.608 | 2.619 |
| Skookies | 2.564 | 2.631 |

Table 4.2.2 shows the average score values for comparison of the same extracts collected at various times. Similar to the matrix, the score values for an MSP compared to itself- i.e., 3.000, were excluded from the calculations in order to show a true comparison. The mean values for both methanol and acetonitrile extracts are all ≥2.5. Compared to the matrix, the average values obtained for single strain comparison were slightly lower. One factor that needs to be considered when comparing cannabis product spectra is that matrix peaks are still present and therefore are contributing to the score. It would be expected that the average scores would be slightly larger than that of the matrix as there exist more peaks that would have matching m/z values and intensities that would contribute to an increasing score value. However, the data show the opposite trend and therefore, the peak list and intensities of the cannabis peaks must vary enough to result in decreasing the score value. The average score values for methanol and acetonitrile extracts are similar.

Small variations of the MSP's between collection days for a single sample could be a result of a few factors including the calibration method used and also just the variation of intensity of the peaks that are present. The calibration method used was done using three peaks from the matrix. Although the internal calibration using the matrix was fast, it is quite possible that a more in-depth calibration method was required. Peaks that are suspected to be the same as they follow the same pattern in two different MSP spectra, are off by small m/z amounts which would result in lowering of a score value as the peaks are not perfectly matching.

Another factor that results in lowering score value would be the difference of peak intensities. It has been previously discussed that each sample undergoes the same

extraction method and MALDI analysis. One important factor for MALDI analysis is that each spectrum was generated using the same amount of laser shots to be summed together- eight. Therefore, all spectra and MSP lists are generated in the same way and any intensity differences between extracts are attributed to differences in the sample on that particular extraction collection day. However, in some aspects, it is unclear on how the program determines peaks to be a match which would increase the score and how it deems peaks to not be a match. For example, using the Blueberry methanol extracts MSP 6 and MSP 3, two sets of peaks can be compared, seen in Figure 4.2.3.

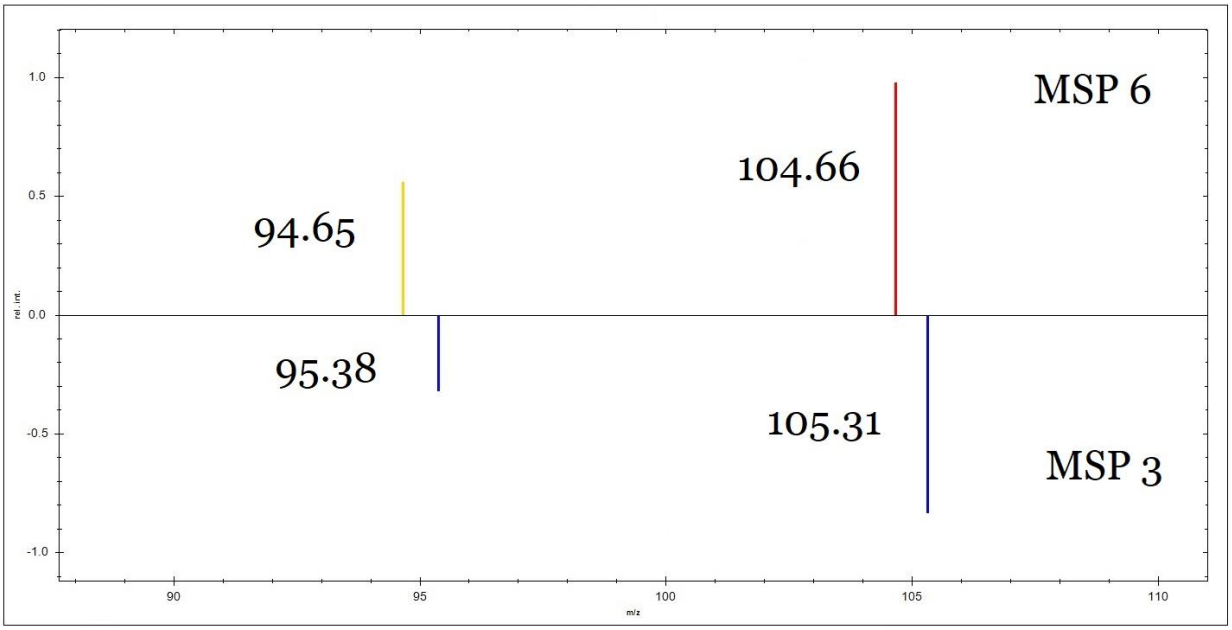



**Figure 5.2.3**. **MSP identification spectra showing the comparison of MSP 6 (unknown, positive rel. intensity) and MSP 3 (reference, negative rel. intensity).** This shows a partial match pair of peaks and a non-matching pair of peaks. The partial match has a yellow peak at 94.65 m/z and the non-match has a red peak at 104.66 m/z. The reference peaks that these are being compared to are the blue peaks with peaks at 95.38 m/z and 105.31 m/z.

There exists a peak in the unknown, MSP 6, at 94.65 m/z with a rel. intensity of 0.56 and a peak in the reference, MSP 3, at 95.38 m/z with a rel. intensity of 0.32 and the difference between these two peaks is 0.73 Da. There also exist a peak in the unknown, MSP 6, at 104.66 m/z with a rel. intensity of 0.98 and a peak in the reference, MSP 3, at 105.31 m/z with a rel. intensity of 0.83 and the difference between these peaks is 0.65 Da. The program uses colour coordination for how much of a match the peak in the unknown is to the corresponding peak in the reference. In this example shown in Figure 4.2.3, the peaks at ~95 m/z are a partial match, resulting in the unknown peak being coloured yellow. The peaks at ~105 m/z are not a match, resulting in the unknown peak being coloured red. The difference in the m/z value and the relative intensity is smaller in the 105 m/z matching peaks yet these peaks are not deemed as a match. It is unclear if these pairs of peaks are truly considered to be identical compounds. Further investigation would require the use of MALDI MS/MS to observe fragmentation patterns of each of these ions to help determine if they are the same molecule.

Small inconsistencies in the software such as those listed above result in scrutiny of the validity of its capabilities for analysis and comparison of cannabis extracts. Small changes in score values for the comparison of samples that are so similar is not ideal. However, this work is not to judge the matching algorithm designed by Bruker but instead use it for possible identification and comparison of different cannabis strain extracts. These irregularities show that finding consistencies within the score values is not as simple as it would initially appear.

## 5.3 Comparison of same strain in different solvents

To determine relationships for how closely related cannabis strains are, Biotyper is able to generate dendrograms. The closeness of the strains relationship to one another is reflected through an arbitrary distance level. Distances in a given dendrogram are normalized to a maximum value of 1000 which means that distances are relative for one particular dendrogram and cannot be compared to other dendrograms. For this work, dendrograms are used to group strains together and to view consistencies between strains likeliness by observing which are consistently grouped together or if there is the groupings are random. Using data from collection day 2, a dendrogram provided by Biotyper show that extracts collected from different solvents can somewhat be grouped together. This dendrogram is shown in Figure 4.3.1.

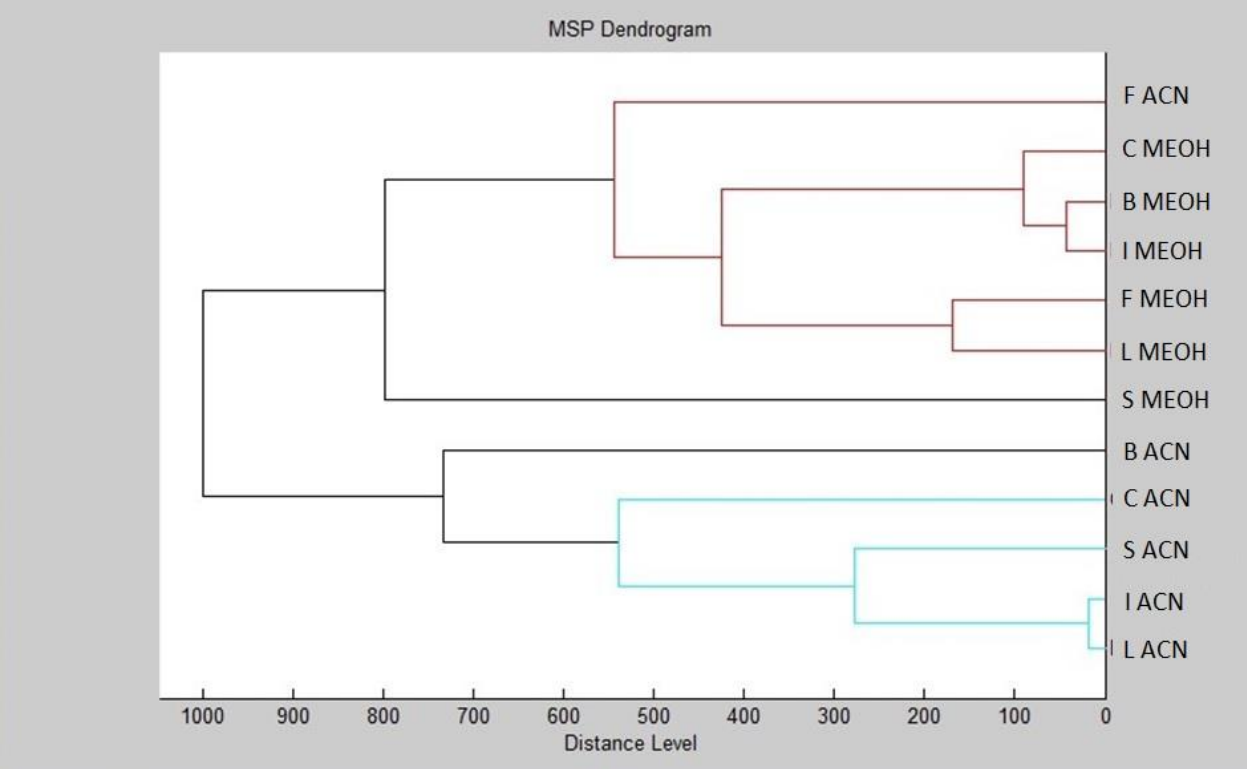



**Figure 5.3.1**. **A dendrogram from Biotyper using collection day 2 data showing grouping of cannabis strains in different solvent systems.** Each strain is abbreviated to the first letter of its name and methanol extracts are abbreviated as MEOH while acetonitrile extracts are abbreviated as ACN. Each strain is abbreviated to the first letter of its name (B=Blueberry, C=Charlotte's Web, F= Four Star General, I=Island Banana, L=Lake of Fire, S=Skookies).

In Figure 4.3.1, it can be seen that for the most part, methanol extract samples are grouped together and acetonitrile extract samples are grouped together. The exclusion to this is the acetonitrile extract of F (Four Star General), which is shown to be grouped with the methanol extracts. It is unclear why this single sample is not grouped with the other acetonitrile extracts from other samples the software denotes that peaks in this sample are more correlated to other methanol extracts. Although this grouping is decent at separating different solvent extracts, dendrograms from other collection days

are a bit more random and do not follow a nice pattern. Using data from collection day 6, patterns of grouping extracts in such as Figure 4.3.2 are not as distinctly observed.

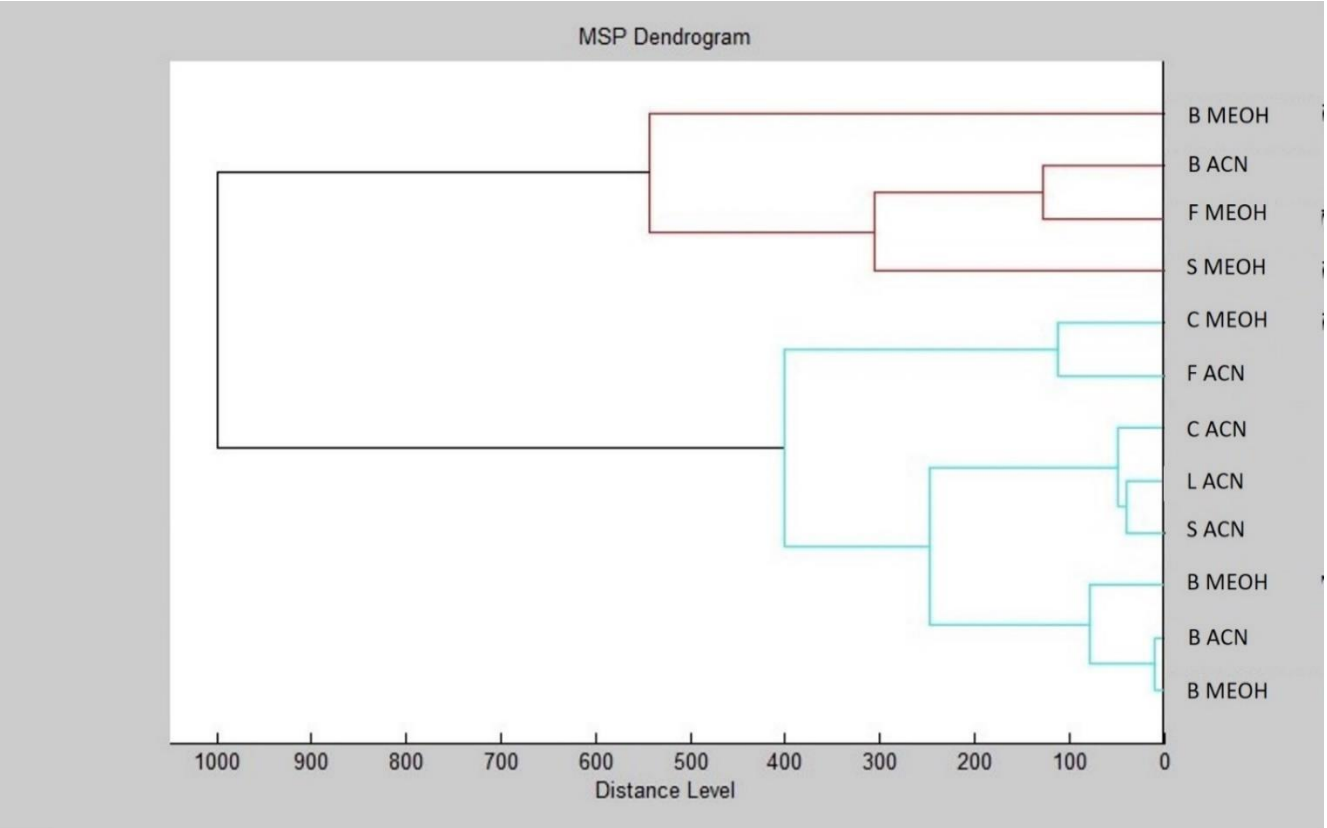



**Figure 5.3.2. A dendrogram from Biotyper using collection day 6 data showing grouping of cannabis strains in different solvent systems**. Each strain is abbreviated to the first letter of its name and methanol extracts are abbreviated as MEOH while acetonitrile extracts are abbreviated ACN. Each strain is abbreviated to the first letter of its name (B=Blueberry, C=Charlotte's Web, F= Four Star General, I=Island Banana, L=Lake of Fire, S=Skookies).

Out of all of the collection days, data from day 2 is the most organized dendrogram in terms of a grouping like-solvent extracts together. A few possibilities for the reasoning behind this could be involved. First, a reason could be that the cannabis sample was not allowed to fully dry from methanol residue and the acetonitrile solvent was then added, resulting in crossovers. The second reason could be that the solvent

systems are too similar. This would mean that extracts collected in the acetonitrile would not be distinguishable from those collected in the methanol extract.

## 5.4 Likeness of strains to each other in same solvent system

The initial goal of this work was to develop a chemical fingerprint library of various cannabis strains. The last set of comparison that will be made for this work is to determine if there are any relationships between strains. In the previous section, it was shown that the Biotyper algorithm can only sometimes detect difference of MSPs in solvent systems for which a sample was extracted with. For the purpose of this section however, only same solvent extracts are to be compared. Also, this section will focus on the analysis of cannabis likeliness patterns through dendrograms of samples collected on the same day and those that are in the same solvent. Therefore, for example, a dendrogram shown in this section will feature only methanol extracts from a single collection day. Dendrograms from each collection day for both methanol and acetonitrile extracts can be viewed below in Figure 4.4.1 and Figure 4.4.2.

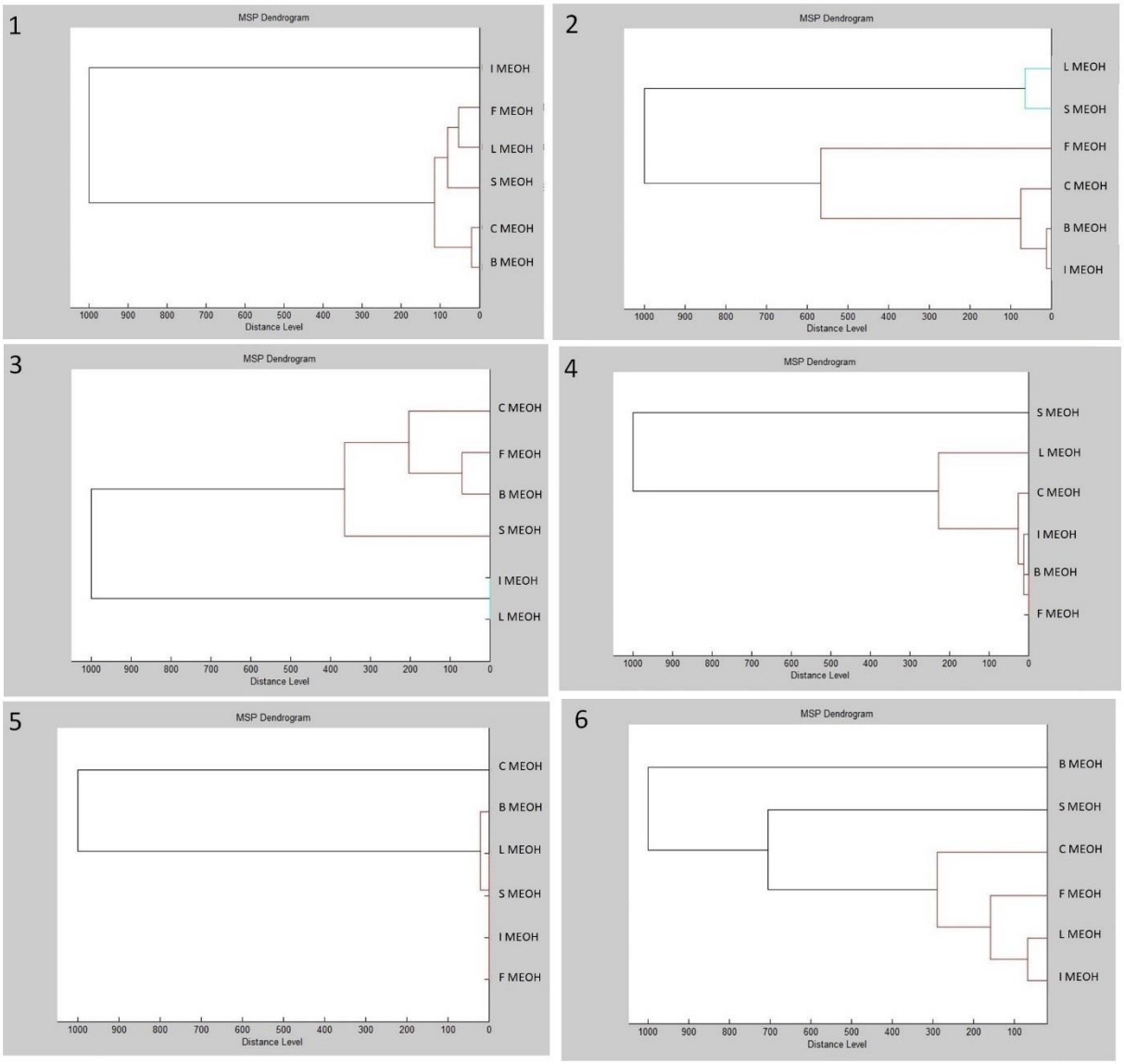



**Figure 5.4.1. Dendrograms of each collection days (1-6) showing likeness of methanol extracts from cannabis strains.** Each strain is abbreviated to the first letter of its name (B= Blueberry, C= Charlotte's Web, F= Four Star General, I= Island Banana, L= Lake of Fire, S= Skookies). Distance level is arbitrary and cannot be used for comparison between two different dendrograms.

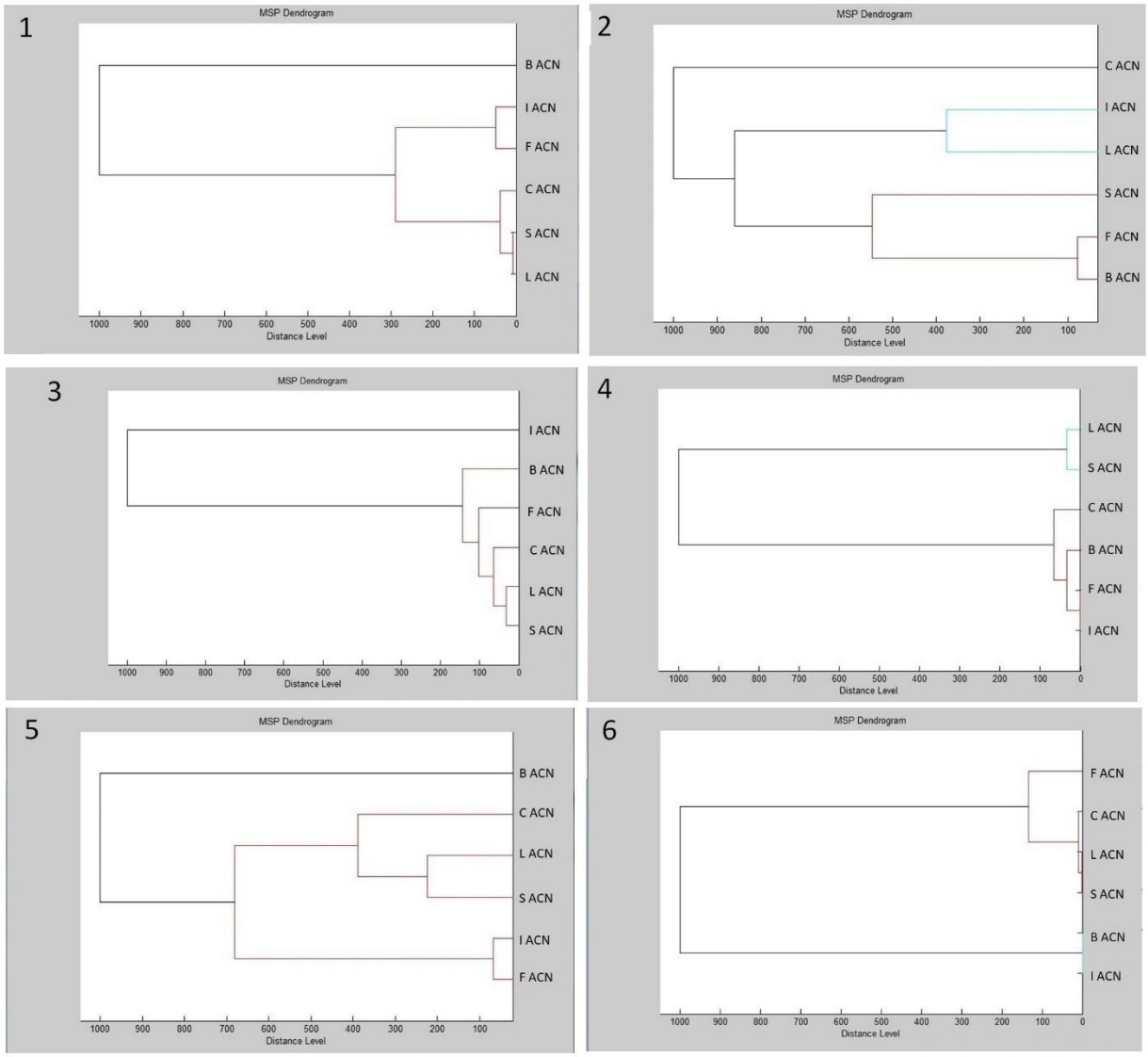



**Figure 5.4.2 Dendrograms of each collection days (1-6) showing likeness of acetonitrile extracts from cannabis strains.** Each strain is abbreviated to the first letter of its name (B= Blueberry, C= Charlotte's Web, F= Four Star General, I= Island Banana, L= Lake of Fire, S= Skookies). Distance level is arbitrary and cannot be used for comparison between two different dendrograms.

Typically, dendrograms are used to determine the hierarchical relationship between objects or organisms. Cannabis product was purchased from recreational suppliers and therefore there was no known distinction regarding any relationships

between strains. Therefore, the goal of creating these dendrograms from the random assortment of purchased strains was to determine if any particular strains were commonly grouped together. As mentioned in the Introduction chapter of this thesis, blogs that discuss cannabis, such as Leafly, commonly describe a new strain by telling consumers which strains it was crossed from to create. Although it is unclear if any of these strains are 'related', one common pattern that can be seen is the grouping of Blueberry and Charlotte's Web methanol extracts in dendrograms 1-4 from Figure 4.4.1. These dendrograms show that although a new pattern is achieved in each collection, it is more common than not that Charlotte's Web and Blueberry are commonly closely related. When comparing these two strains in the acetonitrile extract dendrograms however, they are not commonly grouped together. Another observable pattern is that between the Lake of Fire strain and the Skookies strain. Particularly in the acetonitrile extract dendrograms of Figure 4.4.2, it can be viewed that these strains are commonly grouped together in all except #2. In the methanol extract dendrograms, the Lake of Fire and Skookie strains are somewhat found together in #1 and #2 in Figure 4.4.1. The introduction chapter of this thesis noted that the Indica/Sativa nomenclature is still used but not scientifically accurate. It is accepted that cannabis is one species that is highly variable by its chemical makeup[30,31]. Upon purchase, Blueberry was labelled as Indica, Charlotte's Web was labelled as Sativa, Lake of Fire was labelled as Hybrid and Skookies was labelled as Sativa. Using these data, there was not a correlation between how strains were grouped and if they were labelled as Sativa or Indica.

These dendrograms are useful to get a picture of how strains can be related but overall, they carry a lot of variation. As mentioned, dendrograms are created using score

values of MSP's that are compared to one another. For a closer look at how different strains are related, it is possible to use data from each collection day to summarize the likeness of one strain to another. Similar to what was done with the matrix MSP's and data shown in Table 4.2.1, each strain can be set as the reference MSP and all others can be compared against it. This was done to determine if any other relationships can be confirmed using the likeness of samples. For each table, one particular strain was set as the reference MSP and another was set as the unknown. A score value was generated for this comparison for each of the collection days, 1-6. The average of the score values from a single relationship were then calculated. This data can be seen in Tables 4.4.1-4.4.6.

**Table 5.4.1. Score values from all collection days using Blueberry as the reference strain.** To determine an average score, values from all collection days (1-6) with Blueberry as a reference MSP and all other strains were set to unknowns. Values from individual comparisons of the two identified strains were used from each collection day. All unknowns are abbreviated to the first letter of their name (B=Blueberry, C=Charlotte's Web, F= Four Star General, I=Island Banana, L=Lake of Fire, S=Skookies).

| | Reference MSP: **Blueberry** | **MeOH** | **ACN** |
|---|---|---|---|
| Unknown MSP | **CW** | 2.720 | 2.737 |
| | **FSG** | 2.739 | 2.763 |
| | **IB** | 2.704 | 2.761 |
| | **LoF** | 2.702 | 2.719 |
| | **S** | 2.672 | 2.753 |
| | **Matrix** | 2.509 | 2.460 |

**Table 5.4.2. Score values from all collection days using Charlotte's Web as the reference strain.** To determine an average score, values from all collection days (1-6) with Blueberry as a reference MSP and all other strains were set to unknowns. Values from individual comparisons of the two identified strains were used from each collection day. All unknowns are abbreviated to the first letter of their name (B=Blueberry, C=Charlotte's Web, F= Four Star General, I=Island Banana, L=Lake of Fire, S=Skookies).

| | Reference: **Charlotte's Web** | **MeOH** | **ACN** |
|---|---|---|---|
| **Unknown MSP** | **B** | 2.714 | 2.723 |
| | **FSG** | 2.680 | 2.742 |
| | **IB** | 2.669 | 2.735 |
| | **LoF** | 2.667 | 2.774 |
| | **S** | 2.614 | 2.790 |
| | **Matrix** | 2.531 | 2.494 |

**Table 5.4.3. Score values from all collection days using Four Star General as the reference strain.** To determine an average score, values from all collection days (1-6) with Blueberry as a reference MSP and all other strains were set to unknowns. Values from individual comparisons of the two identified strains were used from each collection day. All unknowns are abbreviated to the first letter of their name (B=Blueberry, C=Charlotte's Web, F= Four Star General, I=Island Banana, L=Lake of Fire, S=Skookies).

| | Reference: **Four Star General** | **MeOH** | **ACN** |
|---|---|---|---|
| **Unknown MSP** | **B** | 2.726 | 2.790 |
| | **CW** | 2.692 | 2.764 |
| | **IB** | 2.760 | 2.766 |
| | **LoF** | 2.750 | 2.741 |
| | **S** | 2.732 | 2.761 |
| | **Matrix** | 2.493 | 2.462 |

**Table 5.4.4. Score values from all collection days using Island Banana as the reference strain.** To determine an average score, values from all collection days (1-6) with Blueberry as a reference MSP and all other strains were set to unknowns. Values from individual comparisons of the two identified strains were used from each collection day. All unknowns are abbreviated to the first letter of their name (B=Blueberry, C=Charlotte's Web, F= Four Star General, I=Island Banana, L=Lake of Fire, S=Skookies).

| | **Reference: Island Banana** | **MeOH** | **ACN** |
|---|---|---|---|
| **Unknown MSP** | **B** | 2.679 | 2.740 |
| | **CW** | 2.656 | 2.733 |
| | **FSG** | 2.752 | 2.752 |
| | **LoF** | 2.732 | 2.846 |
| | **S** | 2.681 | 2.722 |
| | **Matrix** | 2.516 | 2.366 |

**Table 5.4.5. Score values from all collection days using Lake of Fire as the reference strain.** To determine an average score, values from all collection days (1-6) with Blueberry as a reference MSP and all other strains were set to unknowns. Values from individual comparisons of the two identified strains were used from each collection day. All unknowns are abbreviated to the first letter of their name (B=Blueberry, C=Charlotte's Web, F= Four Star General, I=Island Banana, L=Lake of Fire, S=Skookies).

| | Reference: **Lake of Fire** | **MeOH** | **ACN** |
|---|---|---|---|
| **Unknown MSP** | **B** | 2.715 | 2.722 |
| | **CW** | 2.681 | 2.787 |
| | **FSG** | 2.769 | 2.727 |
| | **IB** | 2.755 | 2.719 |
| | **S** | 2.741 | 2.852 |
| | **Matrix** | 2.564 | 2.549 |

**Table 5.4.6. Score values from all collection days using Skookie as the reference strain.** To determine an average score, values from all collection days (1-6) with Blueberry as a reference MSP and all other strains were set to unknowns. Values from individual comparisons of the two identified strains were used from each collection day. All unknowns are abbreviated to the first letter of their name (B=Blueberry, C=Charlotte's Web, F= Four Star General, I=Island Banana, L=Lake of Fire, S=Skookies).

| | Reference: Skookie | MeOH | ACN |
|---|---|---|---|
| Unknown MSP | B | 2.660 | 2.749 |
| | CW | 2.620 | 2.784 |
| | FSG | 2.713 | 2.736 |
| | IB | 2.676 | 2.712 |
| | LoF | 2.708 | 2.846 |
| | Matrix | 2.594 | 2.531 |

A common pattern that can be observed from the tables above that cannot be observed in the dendrograms is that when Four Star General is the unknown, it is the most like most other strains, excluding Charlotte's Web. When going back to look at the methanol dendrograms of all strains in Figure 4.4.1, it can be seen that the Four-Star General strain consistently bounces around and cannot be pinned into a particular pattern. In the acetonitrile extracts however, the pattern of Four-Star General being most like other strains is not observed. A pattern that is observed in the acetonitrile extract dendrograms is that Lake of Fire and Skookies are commonly grouped together. When examining the tables above, it can be seen that Lake of Fire and Skookies are most

alike with one another as when they are compared in Table 4.4.5 (Lake of Fire reference) and Table 4.4.6 (Skookie reference), these scores are the highest in the table.

The tables above include score comparisons to the matrix in order to determine how much it was contributing to the score. In section 4.2 of this chapter and specifically Table 4.2.1, the matrix MSP's from different days are compared. In this table, it is shown that the average score value for matrix MSP's is 2.643. In the tables presented above for strain-to-strain comparison, scores are typically in the 2.65 and up range. When strains are compared to the matrix MSP, the score is typically around 2.5. This data shows that MALDI is capable of ionizing cannabis extracts collected from a solid-liquid extraction method. Also, Biotyper software is capable of distinguishing cannabis extract MSP from matrix MSP.

The goal of this work was to use MALDI-TOF MS and Bruker Biotyper software for the comparison of cannabis strains using chemical fingerprinting of extracts collecting from a sequential extraction. Due to restrictions of testing in Canada, there is not currently a standardized regulation method in place to confirm strain identity or to distinguish cannabis strains from one another. Currently, with the results provided by this thesis, it is unclear if MALDI-TOF MS can be used to distinguish cannabis samples on a consistent basis.

# Chapter 5: Conclusion and Future Work

## 6.1 Conclusion

The first goal of this work was to develop an extraction technique that would remove cannabinoids and other naturally occurring constituents from cannabis. A sequential extraction method was selected for the speed, simplicity and thoroughness. The methanol and acetonitrile extracts of all cannabis strains showed cannabinoid peaks that have been previously observed in the literature[78]. Both methanol and acetonitrile also showed unidentified unique peaks (i.e. peaks that were observed in cannabis extracts but were not observed in the matrix spectrum and were not identified through MALDI). It can be concluded that both methanol and acetonitrile were successful at extracting cannabis flower material that was ionized and detected using MALDI. The hexane extracts were not miscible with the matrix solution (acetonitrile and water) and the spectra collected did not have distinctly differing peaks other than those of the matrix spectrum. Hexane as an extracting solvent was not successful for MALDI analysis with CHCA as a matrix in the conditions described.

The second goal of this work was to optimize Biotyper software for differentiation of low molecular weight compounds that were found in cannabis extracts. Using a Blueberry methanol extract, each parameter was optimized with a goal to include as many visible peaks as possible that were found in the raw spectra as these could aid in identification of strains. By comparison with the raw spectra with generated MSP, the optimization of the program appeared to be as successful as it could be.

The third goal of this work was to then use Biotyper software to differentiate and identify cannabis strains. First, the matrix was used as a control to determine how much variation could occur. Scores achieved for comparison showed that different MSP

generated over time were consistently able to be recognized with little discrepancies between the score values. With closer investigation of matching between different matrix MSP did it show that it was not always clear when two relatively similar peaks (m/z value and intensity) were a match and when they were not. The spectra of methanol and acetonitrile extracts were compared through dendrograms. The program showed that there was sometimes consistency in grouping like-solvent extracts together while most of the time there was not consistency. It is unclear if this is due to experimental collection differences or to random features of the program. Dendrograms of methanol extracts were compared for different cannabis strains to determine if any strains were consistently matched together. It was found that the Blueberry and Charlotte's Web methanol extracts were consistently similar according to the software. It was also found that Lake of Fire and Skookies acetonitrile extracts were consistently similar.

Overall, it did not appear that MALDI-TOF MS was consistently able to confirm and differentiate cannabis strains using extracts of low molecular weight compounds from cannabis flower.

## 6.2 Future Work

Future experimentation for continuation of this project would involve the use of MALDI MS/MS. This technique would use fragmentation patterns to identify peaks that were found in cannabis extracts but were not observed previously in the literature. This technique could also be used to determine if questionable peak matches were well attributed by acquiring fragmentation patterns of each and comparing them.

It is apparent that MALDI-MS is not the ideal method for identification of strains of cannabis using their low molecular weight components. Instead, using a genomics approach for identification and differentiation of cannabis strains would potentially be a better option. In 2004, Raharjo et al[90] described a proteomics approach for studying cannabis plant tissues. Proteins were extracted from flower and gland material and underwent two-dimensional gel electrophoresis and MALDI-TOF/MS. The data showed more than 800 proteins extracted from each of flower and leaf material, but that leaf material appeared to have a larger variety of proteins than the flower. Fewer than 100 proteins were identified in this study. More recently in 2019, Vincent et al[91] developed an optimized protocol for the study of cannabis proteomics. Also in 2019, Jenkins and Orsburn[92] used electrospray ionization (ESI) to begin the creation of a proteome draft map of cannabis. They optimized protein extraction protocol by Vincent and the idea developed by Jenkins, using both high performance liquid chromatography/ electrospray ionization-tandem mass spectrometry (HPLC/ESI-MSMS) as well as MALDI-TOF-MS. This method for identification and differentiation of cannabis strains could be well suited as proteins could contain mutations that are unique through generations. Also, spectral libraries for lineage relatedness would be easier to manage in this case as there would not be the problem of dealing with isomeric compounds but instead relatively large molecular weight proteins that would be easier distinguished using subsequent proteolytic enzyme digestion and peptide sequencing.

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