RAPID CHEMOTYPING OF YOUNG CANNABIS PLANTS

Project Report

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Master of Professional Studies, Hemp

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ABSTRACT

Cannabinoids are a large section of the *Cannabis* market which has created new opportunities and challenges for industry and academia. Breeding *Cannabis sativa* L. plants to have specific chemical profiles for all aspects of the industry takes time and resources, which requires new methods to help accommodate those needs. These protocols were developed to create less resource intensive methods for testing chemotype segregation of young cannabis plants. This protocol can be used for breeding new cultivars of *Cannabis* and help cull plants with undesirable chemotypes at a younger age, along with testing plants in a less harmful manner for further growth. Creating two methods, one for HPLC and the other for a LightLab 3 Cannabis Analyzer, provides more options for chemotype determination for breeding and testing of *Cannabis* and shows the limitations of testing using these methods.

BIOGRAPHICAL SKETCH:

Kady Maser is from Au Gres, MI, a small farming town on the coast of Lake Huron. Her parents are Ross Maser, a semi-retired businessman, and Kim Anderson, who was a jeweler and jewelry fabrication teacher. She started college in 2012 as an adult after determining she wanted to go into biology by attending a community college and graduated with an associate before going to University of Michigan to double majored in Plant Biology and English Literature. Feeling that it wasn't a good fit, she transferred to Cornell in 2016, and graduated in 2018 with a BS in Plant Science with a concentration in plant breeding and genetics. After graduating, she worked at the USDA as a Biological Science Aid for the Plant Genetic Resources Unit in Geneva for horticulture crops at their germplasm repository. After the term ended, she went to work at a hemp farm and discovered what she wanted to do with her life and decided to go back to school for a graduate degree in Hemp Sciences at Cornell University. She plans to work preserving genetic diversity in cannabis and eventually go into breeding and seed production.

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LIST OF ABBREVIATIONS:

THC – Tetrahydrocannabinol

CBC - Cannabichromene

CBD - Cannabidiol

CBG - Cannabigerol

CBN - Cannabinchromene

HPLC – High performance liquid chromatography

GC – Gas chromatography

THCA – Tetrahydrocannabinolic acid

CBCA - Cannabichromenic acid

CBGA - Cannabigerolic acid

CBNA - Cannabinolic Acid

CBDA – Cannabidiolic acid

CBCV - Cannabichromevarin

CBDV - Cannabidivarin

CBGV - Cannabigerivarin

THCV - Tetrahydrocannabivarin

CBCVA - Cannabichromevarinic Acid

CBDVA - Cannabidivarinic acid

CBGVA - Cannabigerovarin acid

THCVA - Tetrahydrocannabivarinic acid

THCAS - Tetrahydrocannabinolic acid synthase

CBDAS - Cannabidiolic acid synthase

PCR – Polymerase chain reaction

INTRODUCTION

After *Cannabis sativa* L. was reclassified in the 2018 farm bill, new industries involving production and processing began to boom in the United States (*AGRICULTURE IMPROVEMENT ACT OF 2018*, n.d.). With the multitude of uses for *Cannabis*, ranging from industrial to pharmacological applications, there has been an increased need for breeding new cultivars to suite different uses and needs. However, when the USDA disposed of the last of the *Cannabis sativa* L. cultivars that they had in their germplasm repositories in the 1980s, there was a great loss of genetic diversity (Small & Marcus, 2002). Now that *Cannabis* is legal in some forms at both state and federal levels, this has driven both academia and industry to compensate for this loss by researching the genetic makeup of *Cannabis* with what is currently available and creating new cultivars as fast as possible.

The need for new cultivars has created multiple markets and businesses centered around breeding *Cannabis* with different desired chemical profiles. These profiles, called chemotypes, are separated into multiple categories depending on the presence or absence and ratio of cannabinoid profiles and concentrations (de Meijer et al., 2003). Research on the unique cannabinoid profiles of *Cannabis* has been conducted for decades which has resulted in a system segregating *Cannabis* into five major chemotypes with new chemotypes being described as more research on cannabinoids is being conducted (de Meijer & Hammond, 2005). *Cannabis* plants with the predominant cannabinoid of tetrahydrocannabinol (THC) are classified as Type I plants. Plants with both cannabidiol (CBD) and THC are considered and intermediate type and are considered Type II (Pacifico et al., 2008). Plants with predominantly CBD and low THC are considered Type III. Type IV are plants with cannabigerol (CBG) as the dominant cannabinoid, which is the precursor to THC and CBD, but also containing CBD, are considered Type IV (de Meijer, 2005). Plants with minimal to no detectable cannabinoids are considered Type V (Mandolino & Carboni, 2004). Previous studies indicate that the young leaf tissue contains cannabinoids can hold true to their chemotype as they mature, so developing a method with

young plants and as little tissue as possible shows great potential and could be used for early breeding selection and cannabinoid detection (de Meijer et al., 2009; Pacifico et al., 2008).

Breeding for specific chemical profiles has led to an increased interest in developing affordable and efficient *Cannabis* breeding methods to reliably create new cultivars. One of the solutions to this issue has been the development and use of genetic markers to identify specific cannabinoids in a breeding population to efficiently select plants with desired chemotypes. For grain and fiber, this means selecting plants with little to no THC production, but for *Cannabis* grown for cannabinoids, this means easier selection of specific cannabinoid chemotypes. These markers have been developed recently for the more common and marketable cannabinoid producing genes, such as tetrahydrocannabinolic acid synthase (THCAS) and cannabidiolic acid synthase (CBDAS) precursors (Toth et al., 2020). With the increased interest in the unique chemical profiles that *Cannabis* has to offer, however, there's been a growing interest in developing cultivars with unique chemotypes and target cannabinoids. These cannabinoids, such as CBG and cannabinchromene (CBC), have fewer genetic markers, if any at all, available to use for identification and selection. Therefore, development of other methods and protocols has been created to supplement breeding more viable *Cannabis* for the intended markets. Such methods involve testing the cannabinoid content and profile of a plant via high performance liquid chromatography (HPLC), and even the development of portable testing machines specifically made for *Cannabis*.

HPLC is a method used for systematic profiling of complex plant samples while focusing on their identification and evaluation of identified compounds, which makes it a powerful and rapid analysis technique (Kumar, 2017). It is unique in that it can test simultaneously for both the acid and neutral forms of each cannabinoid, where the acid forms retain their form instead of degrading. New methods are being developed to shorten the run time on processing these samples through HPLC as well, providing results within minutes for each individual sample

(Patel et al., 2017). It also does not need multiple kinds of testing equipment like genetic markers need, such as a PCR machine, which takes even more time to run and complete (Allan & Max, 2010). It is used often in analytical laboratories to analyze multiple kinds of samples, and there are many companies who offer these kinds of analytical services for a price. For those who want to have these kinds of testing capabilities in house, these machines do need lab training, have high costs ($85,195+), making them potentially prohibitive to those who are just starting in the *Cannabis* industry or who may not have as much capital (Valdes-Donoso et al., 2020). That's why companies have created portable and more easily affordable machines ($13,800) specifically meant for *Cannabis* that have a more limited number of cannabinoids that can be tested and be used as an alternative method to use expensive testing equipment or having to send your sample to an off-site lab(Orange Photonics, 2020). Having these multiple testing options opens new avenues for the *Cannabis* industry and is why both industry and academia are developing new protocols.

This project was conducted to create a protocol for testing young *Cannabis* plants for chemotype segregation using the leaves of the plant via HPLC and using a product from Orange Photonics called the LightLab 3 Cannabis Analyzer. This can be applied for breeders and researchers to differentiate young *Cannabis* plants using minimal plant tissue, a leaf, so that the plants that show the desired chemotype can continue to grow to maturity with minimal damage. This protocol for HPLC and LightLab testing will offer chemotyping not only the main five types and could be adapted for rare cannabinoid chemotype development.

MATERIALS AND METHODS

There were two sets of hemp plants used for testing the methods of this project. The first set was comprised of hemp plants selected from seeds from a grain and fiber trial at Cornell University in 2020, where the seed parent was a USO-31 cultivar that was open pollinated with other cultivars in the trial that may have included a range of chemotypes. Analysis of USO-31 found minimal production of cannabinoids, especially THC, which makes it an ideal candidate for fiber and grain (de Meijer et al., 2009; Pacifico et al., 2006). Fifty seeds from the USO-31 seed parent were collected randomly and planted in January of 2021 in greenhouses. They were grown for four weeks before being harvested at the base of the plant above the soil line, placed in brown paper bags, and dried in a freeze dryer. The second set of plants comprised of 10 cultivars containing rare cannabinoid profiles, such as CBGA, cannabichromenic acid (CBCA), and cannabinolic acid (CBNA), along with varin forms of cannabinoid acids such as cannabichromevarinic acid (CBCVA), cannabidivarinic acid (CBDVA), cannabigerovarin acid (CBGVA), and tetrahydrocannabivarin acid (THCVA).

The second set of plants were still being grown for another project and only a few leaves from the middle of the plant were harvested for method development. The plants from the first set had at least three fully expanded sets of leaves, where one whole leaf from the middle set was taken for the HPLC, and 100mg of the remaining expanded leaves was used for tests on the Orange Photonics LightLab 3 Cannabis Analyzer. The second set used tissue ranging from 2 leaflets to a whole leaf for the HPLC method, and 100mg of leaf material was used for tests on the LightLab 3 Cannabis Analyzer. The LightLab is a portable liquid chromatography machine specifically designed for testing *Cannabis* and *Cannabis* products for a select number of cannabinoids.

Plant samples in their brown paper bags were placed in a Harvest Right freeze dryer to lower the moisture content while preserving cannabinoids. After the plants were finished in the

freezer dryer, each plant was weighed whole. Of the three expanded leaf sets on the plant, one full middle leaf was taken and weighed separately, with a range of tissue weight of 18.1-74.9mg. After the leaf samples was weighed, they were placed into a 2ml centrifuge tube with four 2.4mm grinding steel beads. The tubes were placed onto a VWR Vortexer 2 on high speed, shaken at room temperature to homogenize the plant material, then filled with 1.5ml of MeOH after being fully homogenized for 10 minutes. After the 10 minutes of shaking the samples were placed in a centrifuge for 5 minutes at 5000x. The supernatant was filtered through a Captiva 0.45 µm Regenerated Cellulose Filter and directly subjected to HPLC analysis (Agilent 1220 Infinity II LC System) using a Agilent Infinity Lab Poroshell 120 3x50mm 2.7µm column heated at 50°C. Samples were injected and eluted at 1ml/minute at 60% MeOH+0.05% Formic Acid and 40% Infinity Lab Ultrapure HPLC grade water +0.1% Formic acid for the first 60 seconds, followed by a 6-minute gradient to 70% MeOH, then an additional 90 second gradient to 95% MeOH. Absorbance was measured at 230 nm. The following standards were used as calibrants: THCA, Δ^9-THC, CBDA, CBD, CBDV, THCV, CBC, CBGA, CBG, CBN, and Δ^8-THC. The standards had a range of cannabinoid potency for calibration ranging from 1µg to 250µg.

For analysis via the LightLab, 100mg of dried leaf material was placed into a 15ml conical tube with a grindstone and shaken until homogenized. Followed the prompts on the LightLab unit, inputting 0% for moisture content and 0.5g for amount of plant material. When prompted by the LightLab unit, took a 10ml syringe and measured 5ml of the Orange Photonics Solvent meant to be used with this machine and filled the 15ml conical tube with the solvent. The conical tube with plant material and solvent was shaken for the appointed 3 minutes as prompted on the LightLab unit. The supernatant was poured into a 10ml syringe with a 0.45 µm filter attached to the end and slowly injected into the syringe port of the LightLab unit. Results were shown after waiting the appointed 8 minutes and 30 seconds as the LightLab processed the sample.

DATA ANALYSIS

The HPLC results showed us quantitative amounts of the cannabinoids within each sample, and those results were used to compare to the literature thresholds of cannabinoid amount and ratios within the cannabinoids present to establish a chemotyping protocol. Plants were assigned by their cannabinoid ratios and total cannabinoid content into different chemotypes (de Meijer et al., 1992). Plants with a total cannabinoid content lower than 0.15% were classified as Type V. From there, we used the ratio of CBG:total cannabinoids of at least 75% to identify chemotype 4 plants (de Meijer & Hammond, 2005). The remaining samples were classified based on the CBD and THC amounts/ratios. If they had a CBD:THC ratio above 10 they were classified as Type III plants, if between the ratio was between 10 and 1, then Type II plants, and if the ratio was below 1, then the plants were classified as type 1 (no Type I plants were found in this project).

The chromatograms were retrieved from the LightLab and compared to the HPLC results to identify the peaks on the chromatogram that were consistently associated with specific cannabinoids, such as CBGA, CBDA, and THCA. The peaks were identified by comparing LightLab chromatograms to known sample types after relativizing each sample, followed by chemotyping based on peak ratio thresholds. Further details of the process cannot be shown or stated due to being proprietary information for future licensing.

RESULTS

The HPLC method of determining chemotype via cannabinoid content in young leaves resulted in the first set of hemp plants to be labeled as chemotypes II-V, where no Type I THC dominant plants was found. Of the first set of hemp plants, seven were categorized as chemotype 2, twenty-two plants were Type III, one was Type 4, and nineteen were chemotype 5. Chemotype determination was not dependent on sample mass (Fig. 1). These results were used to determine representative plants to be tested on the LightLab unit to determine a chemotyping method. The plants chosen to test on the LightLab from this first set were four Type II, six Type III, one Type IV, and six Type V.

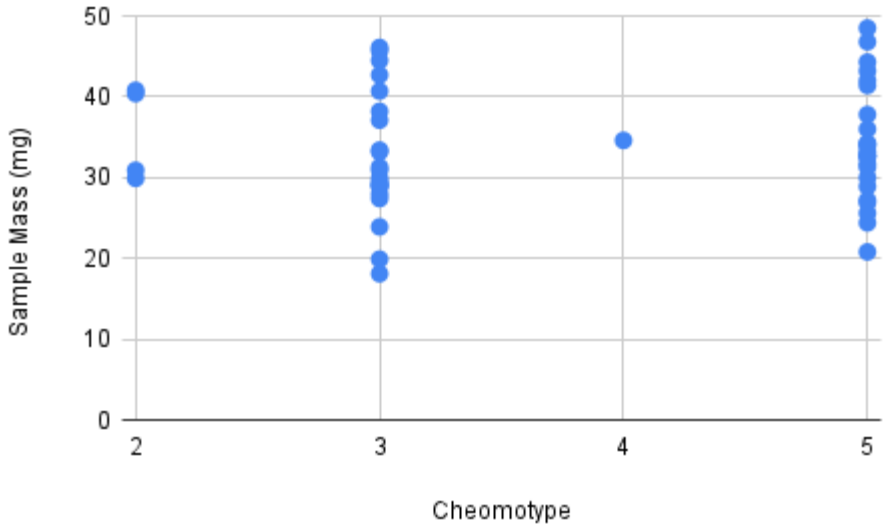

Fig 1: Sample mass of leaf samples in relation to chemotype via HPLC

From the second set of hemp plants tested, due to not having standards for the acid forms or varin forms of the rarer cannabinoids (CBCA, CBNA, CBCVA, CBDVA, THCVA, and CBGVA) for the HPLC, it was more difficult to develop a method and test for these cannabinoids. While the LightLab unit was able to test for certain cannabinoids that the HPLC could not, such as CBCA and CBNA, none were detected when tested on the LightLab. Additionally, the leaves of these plants were more mature than that of the younger four-week-old

plants from the first set of hemp plants, which resulted in tests that had too high of a concentration of cannabinoids for the HPLC to accurately test for cannabinoid content. The levels of cannabinoids in the tissue of these plants were outside the range of our standards. However, CBGA was found in three of the ten plants from the second set of hemp plants when tested on the HPLC and were categorized as Type IV. These three plants were chosen as representatives for the LightLab testing method. This resulted in twenty plants being tested on the LightLab for chemotyping comparisons.

Overall, the twenty plants were tested on the LightLab, and the chromatograms that resulted showed that none tested as Type I, four plants were classified as Type II, six tested as Type III, four tested as Type IV, and six tested as Type V. Of the twenty plant samples tested, all but one plant was assigned to the same chemotype determined by HPLC. This resulted in a 95% level of accuracy for the LightLab being able to qualitatively identify correct chemotypes for young hemp plants. Of the one that was mis-typed, it was from a Type II on the HPLC reading as a Type III on the LightLab chromatogram.

		LightLab Chemotypes			
		Type II	Type III	Type IV	Type V
HPLC Chemotypes	Type II	75	25	0	0
	Type III	0	100	0	0
	Type IV	0	0	100	0
	Type V	0	0	0	100

Table 1: Confusion matrix of the chemotype results for the HPLC method and the LightLab method, showing one difference in chemotyping of the same plant

DISCUSSION

The *Cannabis* industry is starting to flourish with the advent of legalization in different levels and forms, creating new demands for *Cannabis*-based products. With new cannabinoids being researched and a greater demand for different cannabinoid profiles to enter the market, chemotyping *Cannabis* plants accurately and rapidly is needed to help in the breeding and cultivating process to meet those demands. Having multiple options to chemotype *Cannabis* plants accurately will provide more opportunities for people in academia and industry to research and develop new cultivars with different cannabinoid profiles.

I tried to identify the reason for the discrepancy between the HPLC and the LightLab for one sample (HPLC Type II, LightLab Type III). I explored total cannabinoids as measured by HPLC to see if this was a factor in accurately chemotyping young cannabis plants for the HPLC method results and compared that to the LightLab results. For the one mistype of Type II to Type III seen in the LightLab, total cannabinoids did not play a factor into the mistyping. Neither did cannabinoid ratios, nor the quantification of each cannabinoid detected. The sample that was tested did show signs of disease, which may have contributed to the misclassification. Although we can't determine the cause, this was only in one sample out of twenty and resulted in 95% accuracy of chemotyping young plants with minimal tissue.

The methods developed for the HPLC and for the LightLab showed potential for accurate chemotyping with less tissue, younger plants, and in a portable *Cannabis* analyzer unit that wasn't originally designed for chemotyping in very young plants. This can provide more avenues for those in industry and academia to work on rapid chemotyping for their breeding and other research opportunities. However, there were issues with high concentrations of cannabinoids in the leaves when using the HPLC method on more mature leaves, and further testing should be done to determine the dilution rate and what would be the minimal tissue threshold on accurate cannabinoid testing, therefore accurate chemotyping, for *Cannabis* leaves at different ages. Further testing needs to be done to provide a more accurate picture of not only chemotyping, but also to better understand how cannabinoid accumulation happens for specific cannabinoids over the lifetime of the *Cannabis* plant. For example, research of the rare

cannabinoids, like CBC, CBN, the varins, and their acid forms, on whether the inflorescence tissue needs to be tested instead of the leaves since this may indicate that not enough of these cannabinoids accumulate within the leaves for accurate chemotyping. Another example of potential research would be if the leaves needed for testing need to be from a more mature plant, most likely closer to harvesting, instead of from younger leaves.

CONCLUSION

Here we develop a method for an HPLC method for chemotyping young plant material. Currently, there are few chemotyping methods that don't rely on sending samples to a lab, using genetic markers, or expensive and technical equipment. The methods developed in this paper, while not ideal for rare chemotypes, show that there is potential for testing on younger plants with minimal tissue loss on a lower cost and portable machine meant for testing cannabinoids. These methods can provide those in academia and industry other, more accessible, efficient methods for testing and chemotyping their plants with accurate results. Being able to test on younger plants with minimal tissue will help reduce the cost of production for research and breeding, which also means providing more sustainable and efficient options for the *Cannabis* world.

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