Document Title:	**Validation of a LC-DAD-ESI/MS/MS Method for the Accurate Measurement of Δ^9-THC and Δ^9-THCA-A Among Twenty Cannabinoids in Various Products of Cannabis**
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Summary of the Project.

Major goals and objectives.

There were six major goals, which progressively dealt with higher number of cannabinoids (from twelve up to twenty) and samples with more complicated matrices (from concentrates, plant materials, edibles, to topicals) using either liquid chromatography (LC) or ultrahigh performance liquid chromatography (UHPLC) to separate the cannabinoids and DAD (diode array detector), ESI/TOFMS (electrospray ionization time-of-flight mass spectrometry), or ESI/MS/MS (electrospray ionization tandem mass spectrometry) to detect the cannabinoids.

1. Development of a validated method for high throughput quantification of twelve cannabinoids in hemp oil using LC-UV;
2. Development of a validated UHPLC-DAD method with optional ESI/TOFMS detection for rapid quantification of up to sixteen cannabinoids in hemp concentrates;
3. Development of a validated LC-DAD method with optional ESI/TOFMS detection for high throughput quantification of up to twenty cannabinoids in plant materials of cannabis;
4. Validation of a high throughput LC-ESI/MS/MS method for accurate measurement of Δ^9-THC and Δ^9-THCA among eighteen cannabinoids in plant materials of cannabis;
5. Development of a validated UHPLC-DAD method with optional ESI/TOFMS detection for rapid quantification of up to sixteen cannabinoids in hemp infused products: (a) drinks; (b) water soluble hemp oil; (c) candies; (d) snacks; (e) pet treats;
6. Validation of a high throughput UHPLC-ESI/MS/MS method for quantification of up to eighteen cannabinoids in hemp infused products: (a) drinks; (b) water soluble hemp oil; (c) candies; (d) snacks; (e) pet treats; (f) topicals.

The objectives of each goal were similar, so some of the objectives in later goals were well dealt with in earlier goals. Consequently, some protocols developed in earlier goals could be adopted without extensive study in later goals.

1. Separation optimization;
2. Detection optimization;
3. Method validation;
4. Sample preparation;
5. Sample analysis;
6. Assessment of recovery;
7. Confirmation of identity;
8. Assessment of specificity;
9. Discovery of unknown Δ^9-THC structural isomers.

Research questions.

On December 20, 2018, the Congress enacted the 2018 Farm Bill [1] that re-defined hemp as "the plant *Cannabis sativa* L. and any part of that plant, including the seeds thereof and all derivatives, extracts, cannabinoids, isomers, acids, salts, and salts of isomers, whether growing or not, with a delta-9 tetrahydrocannabinol concentration of not more than 0.3 percent on a dry weight basis". The research question of this project can be simply described as the quantitative determination of the percentage of Δ^9-THC (tetrahydrocannabinol) in various products of cannabis to differentiate marijuana from hemp, which is necessitated by the 2018 Farm Bill. However, there are a few caveats in order to properly answer the question:

1. The testing guidelines [2] require that the testing methodology must consider the potential conversion of Δ^9-THCA (tetrahydrocannabinolic acid) into Δ^9-THC and the test result must

reflect the total available Δ^9-THC derived from the sum of the Δ^9-THC and the Δ^9-THCA content.

2. For the accurate measurement of Δ^9-THC and Δ^9-THCA, interferences from other cannabinoids, many of them structural isomers of Δ^9-THC and Δ^9-THCA, must be eliminated. For DAD detection, baseline separation of all cannabinoids with content higher than LOQ (limit of quantification) was required. For ESI/TOFMS and ESI/MS/MS detection, baseline separation of structural isomers of Δ^9-THC and Δ^9-THCA with content higher than LOQ was required. According to our analysis of a broad range of samples, a total of eighteen cannabinoids were quantified with content higher than LOQ, including CBC (cannabichromene), CBCA (cannabichromenic acid), CBD (cannabidiol), CBDA (cannabidiolic acid), CBDV (cannabidivarin), CBDVA (cannabidivarinic acid), CBG (cannabigerol), CBGA (cannabigerolic acid), CBL (cannabicyclol), CBLA (cannabicyclolic acid), CBN (cannabinol), CBNA (cannabinolic acid), CBT (cannabicitran), Δ^9-THC, Δ^8-THC, Δ^9-THCA, THCV (tetrahydrocannabivarin), and THCVA (tetrahydrocannabivarinic acid). Therefore, our research was focused on the analysis of eighteen cannabinoids, which is the maximum number that have been quantified so far.
3. In the literature, many methods can be found for the analysis of cannabinoids, which have been well reviewed by a few recently published articles [3-6]. Briefly, quantification of cannabinoids was traditionally accomplished by gas chromatography (GC), but GC requires a derivatization step to avoid the decarboxylation of acidic cannabinoids in the injection port. In the last years, LC-DAD and LC-MS has emerged as the golden standard because LC can avoid thermal stress so that cannabinoids can be analyzed in their original

acidic forms [5]. Since LC-MS methods demand expensive instruments that are commonly unavailable for routine analysis by cannabis growers, commercial suppliers, and crime labs, LC-DAD methods have been especially favored. However, according to the Scientific Working Group for the Analysis of Seized Drugs (SWGDRUG), LC-DAD methods are unable to provide definitive identification like LC-MS methods. Therefore, our research used optional ESI/TOFMS to confirm identity, assess specificity, and discover unknown Δ^9-THC structural isomers for validated LC-DAD methods. In addition, LC-ESI/MS/MS methods were also developed and validated.

4. Published LC-DAD methods have been characterized by narrow calibration ranges [3]. As hemp contains high content of CBD/CBDA and very low content of Δ^9-THC/Δ^9-THCA, it was difficult to provide an accurate quantification of both pairs with a single analytical run. Therefore, two different dilutions were often prepared to get both pairs within the limits of the calibration ranges. Our research has achieved the lowest LOQ and the widest linear calibration range so far, which allowed samples to be analyzed at one concentration.
5. For the accurate measurement of Δ^9-THC and Δ^9-THCA, interferences from sample matrices must be eliminated. As our research progressed from the analysis of concentrates, plant materials, edibles, to topicals, the sample matrices became more and more complicated. Correspondingly, appropriate sample preparation procedures were developed.
6. Published methods were complicated by recovery experiments due to the unavailability of cannabinoid-free cannabis matrix. The high cost of cannabinoid standards further complicated recovery experiments because spiking was prohibitive even at a few percentage levels. In addition, published recovery experiments could not be used to track recovery in real time for each sample. Our research used an internal standard, i.e., ACBD

(abnormal cannabinoid), which is not naturally present in cannabis products and commercially available with a reasonable price, to track the recovery of sample preparation for each sample in real time.

Research design, methods, analytical and data analysis techniques.

Separation optimization. The separation optimization was based on our discovery in **Goal 1** that the acetonitrile and water eluting system had enough selectivity to separate a complicated mixture of either neutral or acidic cannabinoids. However, neutral cannabinoids could co-elute with acidic ones. Fortunately, the retention of acidic cannabinoids could be manipulated by adjusting the pH of the mobile phase; therefore, good separations of a complicated mixture of both neutral and acidic cannabinoids could still be achieved. The use of gradient elution was excluded due to baseline problems because the maximum UV absorbance of most neutral cannabinoids are approximately at 210 nm. Our separation optimization led to two separations, i.e., a baseline separation of sixteen cannabinoids plus ACBD by UHPLC and a baseline separation of twenty cannabinoids plus ACBD by LC. The UHPLC separation used a Phenomenex Luna Omega Polar C18 1.6 µm 150 mm × 2.1 mm column at 30°C. The mobile phase consisted of (A) 2 mM ammonium formate with 0.011% (v/v) formic acid at pH 3.6 and (B) acetonitrile at a flow rate of 0.3 mL/min. The separation was achieved using isocratic elution with 73% (v/v) B. It was later used for the analysis of eighteen cannabinoids by LC-ESI/MS/MS because ESI/MS/MS was able to distinguish cannabinoids that coeluted but were not structural isomers, i.e., THCVA/CBG and CBLA/CBL. The LC separation used two Restek Raptor ARC-18 150 × 2.1 mm, 2.7 µm columns that were sequentially connected, also at 30°C. The mobile phase consisted of (A) 0.5 mM ammonium formate plus 0.02% (v/v) formic acid (pH 3.0) and (B) acetonitrile at a flow rate of 0.4 mL/min. The separation was achieved using isocratic elution with 75% (v/v) B.

DAD optimization. All the cannabinoids, excluding CBCA and CBNA, had a major peak of UV absorption with its apex at wavelength of lower than 210 nm, so they were quantified at 230 nm to be consistent with other published methods, except for CBT at 223 nm to achieve improved detection which was not needed by other cannabinoids. CBCA and CBNA had only one major peak of UV absorption each with their apexes at 251 and 261 nm, respectively, so they were quantified with these corresponding wavelengths in addition to 230 nm. The practice to quantify a cannabinoid with two wavelengths increased the method specificity because interference could be identified if the two results were statistically different. Other acidic cannabinoids had two additional peaks of UV absorption with the apexes at wavelengths of approximately 269 and 305 nm, respectively, with the absorption higher at 269 nm, so they were also quantified at 269 nm in addition to 230 nm. Unlike other neutral cannabinoids with just one peak of UV absorption, CBN and CBC had a second peak of UV absorption with their apex at a wavelength of approximately 285 nm, so they were also quantified at two wavelengths, i.e., 230 and 285 nm.

ESI/TOFMS and ESI/MS/MS optimization. Both positive and negative-ion ESI were examined for the ionization of cannabinoids. While neutral cannabinoids could only be effectively ionized by positive-ion ESI, acidic cannabinoids could be effectively ionized by both positive and negative-ion ESI. Eventually, positive-ion ESI was chosen because it achieved better sensitivity for not only neutral but also acidic cannabinoids. Optimized MS conditions were as follows: MS acquisition mass range, 100-1000 *m/z*; MS acquisition rate, 5 spectra/s; drying gas temperature, 325°C; drying gas flow, 10 L/min; nebulizer pressure, 20 psi; sheath gas temperature, 400°C; sheath gas flow, 12 L/min; capillary voltage, 3000 V; nozzle voltage, 600 V; fragmentor, 120 V; skimmer, 45 V; Oct1 RF Vpp, 750 V; MS reference mass ions, 121.0509, 922.0098 *m/z*. Optimized MS/MS parameters common for nineteen cannabinoids and seven isotopically labelled

cannabinoids were as follows: MS/MS acquisition mass range, 50-400 *m/z*; MS/MS acquisition rate, 5 spectra/s; precursor ion isolation width, narrow (~1.3 *m/z*). Optimized MS/MS parameters specific to individual cannabinoids and isotopically labelled cannabinoids, including precursor ion, collision energy (CE), quantifier ion, and qualifier ion, are listed in **Table 1**.

Table 1. Optimized MS/MS parameters specific to individual cannabinoid.

Cannabinoid	Precursor	CE	Quantifier	Qualifier
CBDV	287.2006	25	165.089	231.133
THCV	287.2006	25	165.087	231.133
CBN	311.2006	25	223.111	293.187
CBD	315.2319	25	193.120	259.163
ACBD	315.2319	15	221.152	259.164
Δ^9-THC	315.2319	25	193.120	259.169
Δ^8-THC	315.2319	25	193.120	259.169
CBL	315.2319	25	235.170	81.070
CBC	315.2319	25	193.120	259.169
CBT	315.2319	25	193.120	259.169
CBG	317.2475	15	193.120	207.136
CBDVA	331.1904	15	313.181	191.070
THCVA	331.1904	15	313.181	191.070
CBGA	343.2268	15	219.100	261.146
CBNA	355.1904	15	337.176	311.202
CBDA	359.2217	15	341.211	219.102
CBCA	359.2217	15	341.211	219.102
Δ^9-THCA	359.2217	15	341.211	219.102
CBLA	359.2217	15	341.211	261.149
CBN-d_3	314.2194	25	223.111	296.207
CBD-d_3	318.2507	25	196.142	262.184
ACBD-d_3	318.2507	15	224.172	262.184
Δ^9-THC-d_9	324.2883	25	202.177	268.220
Δ^8-THC-d_9	324.2883	25	202.177	268.220
CBC-d_9	324.2883	25	202.120	268.220
CBG-d_9	326.3034	15	202.177	216.194

Preparation of calibration solutions. A mixture solution containing eighteen individual cannabinoids was first prepared in methanol at 50 µg/mL individual concentration. It was then serially diluted with methanol to obtain nine solutions with the following concentration of individual cannabinoids: 25, 10, 5, 2, 1, 0.4, 0.2, 0.08, and 0.04 µg/mL. Afterwards, each of the mixture solution was mixed with a 1 µg/mL ACBD solution, which was also prepared in methanol, in a 1:1 ratio (v/v), for LC-DAD calibration. Therefore, the ten calibration solutions for LC-DAD calibration contained 0.5 µg/mL ACBD and the following concentration of individual cannabinoids: 25, 12.5, 5, 2.5, 1, 0.5, 0.2, 0.1, 0.04, and 0.02 µg/mL. For LC-ESI/MS/MS calibration, each mixture solution for LC-DAD calibration was further mixed with a mixture solution in methanol containing seven isotopically labelled cannabinoids at 1 µg/mL individual concentration in a 1:1 (v/v) ratio. Therefore, the final ten calibration solutions for LC-ESI/MS/MS calibration contained 0.25 µg/mL ACBD, seven isotopically labelled cannabinoids at 0.5 µg/mL individual concentration, and eighteen cannabinoids at the following concentration of individual cannabinoid: 12.5, 6.25, 2.5, 1.25, 0.5, 0.25, 0.1, 0.05, 0.02 and 0.01 µg/mL.

Principle of sample preparation. Methanol was used to extract cannabinoids from a broad range of samples, including concentrates, plant materials, drinks (sparkling water and tea), water-soluble oils, coffee, gummies, hard candies, snacks (all in solid or semi-solid forms), pet treats (all in solid or semi-solid forms), and tablets. All extractions were based on the following principle: a sample, whether it was liquid, solid, or semi-solid, whether it was brittle or sticky, should be completely and homogenously suspended into methanol for efficient recovery of cannabinoids. Therefore, solid and liquid samples that could be readily dissolved or suspended into methanol, including concentrates, drinks (sparkling water and tea), and water-soluble oils, were simply added into methanol under ultrasonication. Brittle samples, such as plant materials and tablets, were

ground under pulverization, then approximate amount was weighed into methanol under ultrasonication. Solid samples that are sugar-rich, including coffee, gummies, and hard candies, were first completely and homogenously suspended into water under pulverization, then the suspension was added into methanol under ultrasonication at a 5/95 (v/v) ratio. Solid or semi-solid samples that are sticky themselves or sticky under moisture, including snacks and pet treats, were completely and homogenously suspended into methanol under pulverization followed by ultrasonication.

Sample preparation of concentrates for LC-DAD analysis. Using an analytical balance, approximately 100 mg sample was weighed into a 15 mL centrifuge tube and dissolved with 75 µg/mL ACBD in methanol to make a 25 mg/mL solution of the sample. The centrifuge tube was sonicated for 20 minutes and then serially diluted as a solution containing 0.06 µg/mL ACBD and 20 µg/mL sample. Therefore, the content of spiked ACBD in the sample was 0.3% and the linear calibration range of the cannabinoids were 0.04 to 125% (an over 100% upper limit was necessary for hemp concentrates due to random errors of measurements with a possible 100% sample). The extract was centrifuged at 13,000 rpm before analysis.

Sample preparation of plant materials for LC-DAD analysis. In order to homogenize the sample, approximately ¼ oz. or 7 g sample was first ground for 2 minutes using a Waring lab blender. Then, approximately 0.5 g cuttings were powdered by shaking at 3000 rpm for 2 minutes using a Spex Genolyte 1200 after it was put into a 7 mL SPEX Sample Prep tube containing two ¼-inch stainless steel balls. In order to extract the cannabinoids, approximately 100 mg powdered sample was weighed into a 15 mL centrifuge tube and suspended into 75 µg/mL ACBD in methanol to make a 25 mg/mL solution of the sample. The centrifuge tube was sonicated for 5 minutes and then vortexed briefly to wash samples up on the walls down to the bottom if there was

any. After four cycles of ultrasonication and vortexing, approximately 2 mL of the supernatant was centrifuged at 13,000 rpm for 10 minutes, which was followed by filtration of approximately 1 mL of the supernatant of the centrifuged sample with a 0.2 µm syringe filter. Afterwards, the sample was serially diluted as a solution containing 0.15 µg/mL ACBD and 50 µg/mL sample. Therefore, the content of spiked ACBD in the sample was 0.3% and the linear calibration range of the cannabinoids were 0.016 to 50%.

Sample preparation of liquid edibles for LC-DAD analysis. For homogenization, a sample was ultrasonicated for 10 minutes. Then, approximately 100 mg was weighed and mixed with appropriate volume of a 75 µg/mL ACBD solution in methanol to make a 25 mg/mL mixture of the sample. Therefore, the spiked ACBD content into the liquid edibles was calculated to be at 0.3% (w/w) level. After ultrasonication for 20 minutes, the mixture was centrifugated at 13,000 rpm for 10 minutes. Then, the mixture was filtered with a 0.2 µm PTFE syringe filter. Afterwards, the filtrate was diluted with methanol for 100 times, obtaining a sample solution containing 0.75 µg/mL ACBD and 250 µg/mL sample. Therefore, the calibration range for each individual cannabinoid was calculated to be 0.008 to 10% (w/w).

For liquid edibles with cannabinoid levels lower than 0.008%, e.g., drinks, a 0.75 µg/mL ACBD solution in methanol was used to dissolve approximately 100 mg sample to make a 25 mg/mL mixture. Therefore, the spiked ACBD content into the sample was calculated to be at 0.003% (w/w) level. After the same ultrasonication, centrifugation and filtration process, a 10-times dilution of the 25 mg/mL sample solution was carried out. Therefore, the calibration range for each individual cannabinoid was calculated to be 0.0008 to 1% (w/w).

Sample preparation of gummies, hard candy, and coffee for LC-DAD analysis. After a sample was chopped using a lab blender, approximately 1 g of the sample was weighed into a 7

mL SPEX Sample Prep tube containing two ¼-inch stainless steel balls. After appropriate volume of water was added into the tube so that a 250 mg/mL mixture of the sample was made, the 7 mL SPEX Sample Prep tube was shaken at 3000 rpm for 5 minutes using a SPEX Genolyte 1200 to obtain a uniform water-dispersion. To extract cannabinoids, 1800 µL methanol was first added into a 15 mL centrifuge tube under ultrasonication, which was followed by the addition of 2000 µL 75 µg/mL ACBD in methanol, and then 200 µL uniform water-dispersion of the sample at 250 mg/mL, making a sample solution containing 37.5 µg/mL ACBD and 12.5 mg/mL sample in a 95/5 methanol/water solvent. After the same ultrasonication, centrifugation and filtration process, the filtrate was diluted with 95/5 methanol/water for 50 times, obtaining a sample solution containing 0.75 µg/mL ACBD and 250 µg/mL sample in 95/5 methanol/water. Therefore, the spiked ACBD content into the sample was calculated to be at 0.3% (w/w) level and the calibration range for each individual cannabinoid were calculated to be 0.008 to 10% (w/w).

Sample preparation of snacks and pet treats for LC-DAD analysis. After a sample was chopped using a Waring lab blender, approximately 1 g of the sample was weighed into a 7 mL SPEX Sample Prep tube containing two ¼-inch stainless steel balls. Then, appropriate volume of a 0.25 mg/mL ACBD solution in methanol was added into the tube so that a 250 mg/mL mixture of the sample could be made. After the 7 mL SPEX Sample Prep tube was shaken at 3000 rpm for 5 minutes using the SPEX Genolyte 1200 to obtain a uniform water-dispersion, it was immediately ultrasonicated for 10 minutes. The shaking and ultrasonication process was repeated for one more time, then the mixture was centrifugated at 13,000 rpm for 10 minutes, filtered with a 0.2 µm PTFE syringe filter, and serially diluted with methanol for 1000 times, obtaining a sample solution containing 0.25 µg/mL ACBD and 250 µg/mL sample in methanol. Therefore, the spiked ACBD

content into the sample was calculated to be at 0.1% (w/w) level and the calibration range for each individual cannabinoid were calculated to be 0.008 to 10% (w/w).

Sample preparation of tablets for LC-DAD analysis. To homogenize a sample, two ¼-inch stainless steel balls were placed into a 7 mL SPEX Sample Prep tube, then a few tablets were put into the tube; afterwards, the 7 mL SPEX Sample Prep tube was shaken at 3000 rpm for 5 minutes using the SPEX Genolyte 1200. The rest of the sample preparation followed the exact procedure for liquid edibles to obtain the recovery of sample preparation at 0.3% (w/w) and achieve calibration range for each individual cannabinoid between 0.008 to 10% (w/w).

Calibration for LC-DAD analysis. External standard calibration was chosen over internal standard calibration due to simple procedure and good performance. Linear calibration curves were constructed between 0.02 to 25 µg/mL by plotting peak area of each cannabinoid versus its concentration after a weighing factor of $1/x^2$ was applied.

Assessment of recovery/accuracy by LC-DAD analysis. Before extraction, ACBD, a cannabinoid not naturally present in hemp, was spiked into each sample in order to track extraction recovery of cannabinoids in real time. After extraction, the extract was diluted to an appropriate concentration to be analyzed by LC-DAD. An ACBD solution in solvent at the same concentration was similarly diluted and subjected to the same LC-DAD analysis as the extract. Therefore, the recovery of ACBD can be calculated by using the following equation where PA represents peak area:

$$Recovery\ (\%) = \frac{PA_{ACBD\ in\ extract}}{PA_{ACBD\ in\ solvent}} \times 100\%$$

Sample preparation of plant materials, liquid edibles, gummies, hard candy, coffee snacks, pet treats, and tablets for LC-ESI/MS/MS analysis. Sample preparation followed the

exact procedure as described above, except that at the end the sample solutions were mixed with a mixture of seven isotopically labelled cannabinoids at 1 µg/mL individual concentration in methanol in a 1:1 ratio (v/v). In addition, the samples of drinks were prepared at a concentration 10 times higher, i.e., at 12.5 mg/mL.

Sample preparation of topicals for LC-ESI/MS/MS analysis. The procedures of sample preparation for terpene-rich topical serum were identical to water-soluble oils due to their liquid nature and similar contents of cannabinoids. For CBD body lotion and CBD face cream, homogenization of a sample was carried out by an initial stirring which was followed by shaking at 3000 rpm for 5 minutes using a SPEX Genolyte 1200 after approximately 1 g of the sample was weighed into a 7 mL SPEX Sample Prep tube containing two ¼-inch stainless steel balls. For CBD lip balm, homogenization of the sample was carried out by vortexing for 30 seconds a couple of times after it was melted into a 15 mL centrifuge tube which was put inside a 60 ^{o}C water bath for 10 minutes. The rest of the sample preparation for the topicals followed identical procedures for water-soluble oils due to their similar contents of cannabinoids.

Calibration for LC-ESI/MS/MS analysis. Linear internal calibration curves were constructed by plotting the peak area ratio of each cannabinoid and its isotopically labelled analogue versus the corresponding concentration ratio using $1/x^2$ as a weighing factor. As isotopically labelled analogues of some cannabinoids were not obtained, other isotopically labelled cannabinoids were used instead, due to close retention time and chemical structure, e.g., CBD-d_3 for CBDV, THCV and CBDA, Δ^9-THC-d_9 for Δ^9-THCA, and CBC-d_9 for CBT, respectively.

Assessment of recovery/accuracy by LC-ESI/MS/MS analysis. Before extraction, ACBD was spiked into each sample in order to track extraction recovery of cannabinoids in real time. After extraction, the extract was diluted to an appropriate concentration to be analyzed by LC-

ESI/MS/MS and before analysis ACBD-d_3 was spiked into each extract at 0.5 μg/mL in order to compensate for matrix effect, also known as ion suppression/enhancement of ESI, on extraction recovery of ACBD. An ACBD solution in solvent at the same concentration was similarly diluted, spiked with ACBD-d_3, and subjected to the same LC-ESI/MS/MS analysis as the extract. Therefore, the recovery of ACBD and the ion suppression/enhancement of ESI on ACBD can be calculated by using the following equations:

$$Recovery\ (\%) = \frac{(PA_{ACBD}/PA_{ACBD-d3})_{in\ extract}}{(PA_{ACBD}/PA_{ACBD-d3})_{in\ solvent}} \times 100\%$$

$$Matrix\ Effect\ (\%) = \frac{(PA_{ACBD-d3})_{in\ extract}}{(PA_{ACBD-d3})_{in\ solvent}} \times 100\%$$

Expected applicability of the research.

Our research was designed to have a wide applicability. The advantage to use DAD and QTOF in our research was that the developed methods can be easily adopted by crime laboratories with different instrumentation because they can be easily dissected after our comprehensive study, e.g., a LC-UV method using a traditional UV detection with single or dual wavelength monitoring, a LC-DAD method using multiple wavelength monitoring, a LC-ESI/MS method using a single quadrupole mass spectrometer, or a LC-ESI/MS/MS method using a triple quadrupole mass spectrometer.

Outcomes.

Results and findings.

Goal 1: Development of a validated method for high throughput quantification of twelve cannabinoids in hemp oil using LC-UV. In this study, the LC separation of twelve cannabinoids, including CBC, CBD, CBDA, CBDV, CBDVA, CBG, CBGA, CBN, Δ^9-THC, Δ^8-THC, Δ^9-THCA, and THCV, has been systematically optimized using a Phenomenex Luna Omega 3 µm Polar C18 150 mm × 4.6 mm column with regard to the effects of the type of organic solvent, i.e., methanol and acetonitrile, the content of the organic solvent, and the pH of the mobile phase. The optimization has resulted in three LC conditions at 1.0 mL/min able to separate the twelve cannabinoids: 1) a mobile phase consisting of water and methanol, both containing 0.1% formic acid (pH 2.69), with a gradient elution at 75% methanol for the first 3 minutes and then linearly increase to 100% methanol at 12.5 minutes; 2) a mobile phase consisting of water and 90% (v/v) acetonitrile in water, both containing 0.1% formic acid and 20 mM ammonium formate (pH 3.69), with an isocratic elution at 75% acetonitrile for 14 minutes; and 3) a mobile phase consisting of water and 90% (v/v) acetonitrile in water, both containing 0.03% formic acid and 20 mM ammonium formate (pH 4.20), with an isocratic elution at 75% acetonitrile for 14 minutes; that can be selected for further investigation. An LC-UV method is further validated for the high-throughput and simultaneous analysis of twelve cannabinoids. The method used the mobile phase at pH 3.69 with a run time of 14 minutes, which resulted in significant improvement in throughput compared to other validated LC-UV methods published so far. The linear calibration range of all the cannabinoids were between 0.1 to 25 µg/mL with $R^2 \geqslant 0.9993$. The validation used a hemp oil containing 3.2% (w/w) CBD and no other cannabinoids, which was reported by the vendor with a certificate of analysis, as the matrix to prepare control

samples: the hemp oil was first extracted with methanol; cannabinoids were then spiked into the extract at both 0.5 and 5 µg/mL level. Afterwards, the recovery, precision (RSD, relative standard deviation) and accuracy (relative error) of the control samples were assessed and the results met the requirements of the ISO 17025. Although this study was meant to be preliminary, which did not result in a peer-reviewed article, further separation optimization was based on its discovery that the acetonitrile and water eluting system had enough selectivity to separate a complicated mixture of either neutral or acidic cannabinoids. To further separate neutral and acidic cannabinoids, the pH of the mobile phase can be adjusted. On the other hand, the use of gradient elution was prohibitive due to baseline drifts because the maximum UV absorbance of most neutral cannabinoids are substantial at the low wavelength, e.g., 210 nm.

Goal 2: Development of a validated UHPLC-DAD method with optional ESI/TOFMS detection for rapid quantification of up to sixteen cannabinoids in hemp concentrates. A UHPLC-DAD method for rapid quantification of up to sixteen cannabinoids has been developed, validated and used in the analysis of hemp concentrates. The effects of various LC parameters, including pH of the aqueous solvent, acetonitrile content of the mobile phase, and temperature of the column oven, were systematically investigated on the retention of cannabinoids, leading to baseline separation of the well-known critical pairs of cannabinoids, e.g., CBG/CBD, CBD/THCV, and Δ^9-THC/Δ^8-THC, while published LC-UV methods were often for twelve and fewer cannabinoids and might not meet the required minimum resolution of 1.5 for appropriate method validation. Method validation met the precision and accuracy requirements of the ISO 17025 guidelines. The linear calibration range of all cannabinoids were between 0.02 to 25 µg/mL in methanol, leading to the quantification of 0.1 to 125% (w/w) individual cannabinoids in hemp concentrates after they were mixed with methanol at 20 µg/mL and analyzed after ultrasonication, centrifugation and

filtration (because a hemp concentrate may contain only one cannabinoid at 100% (w/w), the calibration range was beyond 100% (w/w) in consideration of measurement uncertainty). Due to a low LOQ, standard cannabinoids with a concentration of 1 mg/mL or lower, which are widely available commercially as exempt preparations, were used, while published methods might have to use standard cannabinoids with concentration higher than 1 mg/mL, leading to the requirement of a DEA (Drug Enforcement Administration) license for their purchase (many cannabinoids are regulated as Schedule I substances in the USA). Due to a wide linear calibration range, samples were analyzed at one concentration, i.e., 20 μg/mL in methanol, while published methods might have to analyze the same sample at more than one concentration. Nine samples of hemp concentrates were analyzed in triplicate. The analytical results showed that none of the nine analyzed samples contained any of the seven acidic cannabinoids due to extensive decomposition, but all the nine neutral cannabinoids were detected, with average content ranging from 0.10 to 93.23% (w/w) and RSD values from 0.3 to 11.2% in triplicate. Particularly, two hemp concentrates, i.e., Δ^8-THC distillate and Δ^8-THC shatter contained 9.35 and 11.33% (w/w) Δ^9-THC, respectively. While published recovery experiments were limited by the unavailability of cannabinoid-free matrix and the high cost of cannabinoid standards, this problem was solved by spiking ACBD, a cannabinoid not naturally present in cannabis plants and commercially available with a reasonable price, into the samples. The obtained average recovery ranged from 94.8 to 103.6% with RSD values from 1.5 to 9.0% in triplicate for the nine analyzed samples. ESI/TOFMS detection confirmed that the method had good specificity, i.e., without any false positive identification of individual cannabinoids during the analysis of hemp concentrates. ESI/TOFMS detection also discovered six untargeted cannabinoids that were structural isomers of Δ^9-THC in the hemp concentrate samples.

Goal 3: Development of a validated LC-DAD method with optional ESI/TOFMS detection for high throughput quantification of up to twenty cannabinoids in plant materials of cannabis. A LC-DAD method has been developed, validated, and applied in analysis of cannabinoids in nine samples of plant materials of marijuana, six samples of marijuana cigarettes, five samples of hemp flowers, one sample of hemp cigarette, and two samples of Δ^8-THC fortified hemp flowers. The method has achieved significant improvements over published methods, which was characterized by twenty targeted cannabinoids, eighteen quantified cannabinoids, baseline resolution among quantified cannabinoids, a low LOQ (0.02 μg/mL), a wide linear range (0.02 to 25 μg/mL or 0.04 to 50% (w/w)), a unique experiment to track the recovery of sample preparation in real time by spiking CBD (the recovery ranged from 93.6 to 106.1% while the RSD values ranged from 1.1 to 7.0%), and a well-assessed specificity by ESI/TOFMS. Precision and accuracy were assessed using QC (quality control) samples at three concentration levels, i.e., 0.02, 0.5, and 12.5 μg/mL, in triplicate with inter-day and intra-day precision of less than 15% RSD and accuracy of less than ±15% relative error, therefore meeting the requirements of the ISO 17025 standard. A total of twenty-one samples were analyzed in triplicate with the average content of the eighteen individual cannabinoids ranged from 0.04 to 26.97% (w/w) and the RSD values ranged from 0.9 to 19.6%, with higher RSD values usually for lower contents. The method also had overall good specificity with only a few minor interferences from compounds in the samples, which was verified by ESI/TOFMS detection. While possible interferences to the quantification of CBDVA, CBNA and CBC at close to their LOQ levels could be identified by the method itself through two-wavelength quantification, the same could not be done for CBDV and Δ^8-THC because the interfering compounds had similar UV absorption. Therefore, cutoff values, i.e., 0.12% (w/w) for CBDV and 0.05% (w/w) for Δ^8-THC, were recommended to avoid false positive quantification. Additionally,

ESI/TOFMS has discovered seven unknown cannabinoids, including one structural isomer of CBG, one structural isomer of CBNA, four structural isomers of Δ^9-THC, and one structural isomer of Δ^9-THC acetate. Furthermore, it uncovered that one of the two samples of Δ^8-THC fortified hemp flowers contained 5.16% (w/w) Δ^9-THC, an alarm to the current Δ^8-THC craze by the public.

Goal 4: Validation of a high throughput LC-ESI/MS/MS method for accurate measurement of Δ^9-THC and Δ^9-THCA among eighteen cannabinoids in plant materials of cannabis. A LC-ESI/MS/MS method for the quantification of eighteen cannabinoids has been developed, validated and applied in the analysis of eight samples of marijuana plant materials and three samples of hemp flower. A linear regression using $1/x^2$ as a weighing factor showed that the residual values in the middle of the calibration curves were below 80% for most of the cannabinoids. This problem was solved by breaking one linear regression using $1/x^2$ as a weighing factor into two, one from 0.01 to 0.25 µg/mL and the other one from 0.25 to 12.5 µg/mL. While all eighteen cannabinoids had a lower LOQ of 0.01 µg/mL, nine of them, including CBDVA, CBGA, CBD, THCV, CBN, CBNA, Δ^8-THC, CBCA and CBLA, had an upper LOQ of 12.5 µg/mL. The rest of the eighteen cannabinoids had an upper LOQ of 6.25 µg/mL. QC samples at 0.02, 0.25 and 2.5 µg/mL were analyzed in triplicate in each day, consecutively in three separate days. All the eighteen cannabinoids met this requirement of precision and accuracy set by the ISO 17025 guidelines. A total of eleven samples were analyzed and thirteen of the eighteen targeted cannabinoids were identified and quantified in the samples. The contents of individual cannabinoids ranged from 0.04 to 13.6% with RSD from 1.3 to 15.6%. The total Δ^9-THC contents ranged from 0.065 to 6.92% with RSD from 1.4 to 13.7%. The total CBD contents ranged from 2.64 to 14.01% with RSD from 2.8 to 8.5%. ACBD was spiked into all the samples at 0.3% level for the assessment of recovery.

Our assessment results showed that the recovery ranged from 92.5 to 107.9% with RSD 1.6 to 11.8% in triplicate.

Goal 5: Development of a validated UHPLC-DAD method with optional ESI/TOFMS detection for rapid quantification of up to sixteen cannabinoids in hemp infused products: (a) drinks; (b) water soluble hemp oil; (c) candies; (d) snacks; (e) pet treats. A LC-DAD method has been developed and validated for rapid quantification of up to sixteen cannabinoids. A separation optimization led to the baseline separation of the well-known two critical pairs of cannabinoids, i.e., CBG/CBD and Δ^9- /Δ^8-THC, while published LC-DAD methods were for thirteen and less cannabinoids and might not meet the required minimum resolution of 1.5 for appropriate quantification. Even though the inclusion of CBT into the list due to its presence in hemp products added approximately 6 minutes to the separation, it still completed within 18 minutes. The method was assessed according to the ISO 17025 guidelines and met the requirements. The LC-DAD method was applied for the analysis of twenty hemp-infused edibles encompassing a broad range of complex matrices. An alternative sample preparation procedure was developed: samples or their uniform water-dispersions were extracted by methanol under homogenization through pulverization and/or ultrasonication. Liquid samples were simply added into methanol under ultrasonication; sugar-rich samples were first suspended into water under pulverization and then the suspension was added into methanol under ultrasonication at a ratio of 5/95 (v/v); solid and semi-solid samples were first suspended into methanol under pulverization and then subject to ultrasonication for two cycles; brittle samples were ground under pulverization and then simply added into methanol under ultrasonication. All acidic cannabinoids were found below the LOQ level, indicating that they were decomposed during food processing and/or storage. Seven of the nine neutral cannabinoids, excluding Δ^8-THC and THCV, were above the LOQ level, with average

contents and RSD values of triplicate measurements ranging from 0.0040 to 6.068% (w/w) and 0.5 to 14.7%, respectively. By spiking ACBD into each hemp-infused edible, recovery of sample preparation was tracked in real time for the first time. The average recovery of ACBD in triplicate and RSD values ranged between 89.8 to 108.3% and 0.5 to 6.5%, respectively. ESI/TOFMS detection confirmed that the method had good specificity; i.e., without any false positive identification of individual cannabinoid. Nevertheless, based on the concentration LOQ of 0.02 µg/mL, it was preferable to analyze hemp-infused edibles using a 250 µg/mL solution and a percentage LOQ of 0.008% (w/w) so that matrix peaks would not become intense enough to cause false positive identifications. For drinks (sparkling water and tea), however, a 2.5 mg/mL solution and a percentage LOQ of 0.0008% (w/w) were used. The corresponding linear calibration range was 0.008-10% and 0.0008-1% (w/w), respectively.

Goal 6: Validation of a high throughput UHPLC-ESI/MS/MS method for quantification of up to eighteen cannabinoids in hemp infused products: (a) drinks; (b) water soluble hemp oil; (c) candies; (d) snacks; (e) pet treats; (f) topicals. A UHPLC-ESI/MS/MS method for the quantification of up to eighteen cannabinoids has been developed, validated and applied in the analysis of eighteen samples of hemp-derived edibles and topicals with a broad range of matrices. A thorough study of published LC-ESI/MS/MS methods using triple quadrupole mass spectrometers revealed a misconception that multiple reaction monitoring (MRM) was able to differentiate structural isomers of cannabinoids, e.g., Δ^8-/Δ^9-THC, which explained why many of them were developed for limited number of cannabinoids, as small as two, and did not include Δ^8-THC. In this study, the use of QTOFMS for targeted analysis revealed that MRM was unable to differentiate structural isomers of cannabinoids, especially Δ^8-/Δ^9-THC, so for accurate quantification their baseline separation was achieved through UHPLC. Accuracy and precision

were assessed using quality control samples at three concentration levels, i.e., 0.01, 0.25, and 6.25 μg/mL, in triplicate. The intraday and interday accuracy were within 80-120% for low QCs and 85-115% for medium and high QCs, while the intraday and interday precision were below 20% for low QCs and 15% for medium and high QCs, therefore meeting the requirements of the ISO 17025 standard. The method was further applied for the analysis of eighteen hemp-derived products, including drinks, water-soluble oils, topical serum, body lotion, face cream, lip balm, gummies, hard candy, coffee, snacks, and pet treats. A LOQ of 0.01 μg/mL was achieved, which was equivalent to 0.00008 % (w/w) for drinks with the analysis of 12.5 mg/mL extracts and 0.008% (w/w) for other samples with the analysis of 125 μg/mL extracts due to higher contents of cannabinoids. For the first time, extraction recovery and matrix effect of each sample being analyzed were tracked in real time due to the use of a novel approach, i.e., spiking ACBD and ACBD-d_3 into each sample before and after extraction, respectively, obtaining 92.9 to 106.3% and 91.3 to 120.2% in triplicate measurements, respectively. It is expected that the optimized LC-ESI/MS/MS parameters, e.g., precursor ion, CE, quantifier ion and qualifier ion can be used not only by MS/MS analysis using QTOFMS but also by MRM analysis using triple quadrupole MS. In addition, the method should be applicable for the analysis of hemp plant materials and concentrates due to less complex matrices.

Limitations.

All the developed methods have been validated according to the ISO 17025 guidelines in terms of selectivity, calibration linearity, calibration range, LOQ, accuracy, precision, recovery, specificity, and matrix effect, if applicable. Therefore, we do not foresee any unexpected hurdles that will prevent the success of their adoptation. However, please note that detailed information may not be included in this report for proper adoption due to content limitations. For proper

adoption, parameters extensively described in peer-reviewed articles that are listed in **Outcomes** are recommended to be followed.

Artifacts.

List of products (e.g., publications, conference papers, technologies, websites, databases), including locations of these products on the Internet or in other archives or databases.

Peer-reviewed articles:

1. Meyer, G., Adisa, M., Dodson, Z., Adejumo, E., Jovanovich, E., Song, L.G.: An ultrahigh performance liquid chromatography electrospray ionization tandem mass spectrometry method for quantification of up to eighteen cannabinoids in hemp-derived products. J. Pharm. Biomed. Anal. 238, 10 (2024)
2. Song, L.G., Meyer, G., Adejumo, E., Jovanovich, E., LeBlance, L., Provis, J.: Potency testing of up to sixteen cannabinoids in hemp-infused edibles using liquid chromatography diode array detector with optional confirmation of identity by electrospray ionization time-of-flight mass spectrometry. Food Chemistry. 417, 9 (2023)
3. Song, L.G., Valenzuela, G., Carlson, S., Dodson, Z., Adisa, M.: Potency testing of up to twenty cannabinoids by liquid chromatography diode array detector with optional electrospray ionization time-of-flight mass spectrometry. Anal. Chim. Acta. 1207, 9 (2022)
4. Song, L.G., Carlson, S., Valenzuela, G., Chao, M.D.S., Pathipaka, S.B.: Development of a validated method for rapid quantification of up to sixteen cannabinoids using ultra-high-performance liquid chromatography diode-array detector with optional electrospray ionization time-of-flight mass spectrometry detection. J. Chromatogr. A. 1670, 10 (2022)

Conference Papers (*M.S. graduate student; **Undergraduate student):

1. Song, L., Carlson, S., Valenzuela, G., Chao, M., Development of a UHPLC-DAD-ESI/TOFMS method for simultaneous quantification of up to sixteen cannabinoids and analysis of hemp concentrates, in: HPLC 2022, San Diego, California, USA, 2022.
2. Song, L., Valenzuela, G., Carlson, S., Dodson, Z., Adisa, M., Validation of a LC-DAD method with optional ESI/TOFMS detection for the accurate measurement of delta-9-THC and delta-9-THCA among twenty cannabinoids in cannabis, in: 2023 NIJ Forensic Science R&D Symposium, Orlando, FL, 2023.
3. Song, L., Carlson, S., Valenzuela, G., Chao, M., Pathipaka, S.B., Development of a validated UHPLC-DAD method with optional ESI/TOFMS detection for rapid quantification of delta 9-THC and delta 9-THCA-A among sixteen cannabinoids in hemp concentrates, in: 2022 NIJ Forensic Science R&D Symposium, Virtual, 2022.
4. Meyer*, G., Whyte**, J., Song, L., Quantification of Cannabigerol among Sixteen Cannabinoids in Hemp Oil by Liquid Chromatography Ultraviolet Detection in: ACS Fall 2022, Chicago, IL, 2022.
5. Carlson*, S., Adejumo*, E., Song, L., Quantification of Delta-9-Tetrahydrocannabinol among Sixteen Cannabinoids in Cannabis by Liquid Chromatography Ultraviolet Detection in: ACS Fall 2022, Chicago, IL, 2022.
6. Adisa*, M., Cooper**, S., Song, L., Quantification of Cannabidiol among Sixteen Cannabinoids in Hemp Oil by Liquid Chromatography Ultraviolet Detection, in: ACS Fall 2022, Chicago, IL, 2022.
7. Weseloh, A., Meyer*, G., Song, L., Potency Testing of Cannabidiolic Acid in Dried Hemp Flowers among Sixteen Cannabinoids by Liquid Chromatography Ultraviolet Detection in: 2023 Illinois State Academy of Science Annual Meeting, Peoria, IL, 2023.

8. Provis, J., Adejumo*, E., Song, L., Quantification of Δ9-Tetrahydrocannabinol among Nineteen Cannabinoids in Delta 8 Concentrate by Liquid Chromatography Ultraviolet Detection in: 2023 Illinois State Academy of Science Annual Meeting, Peoria, IL, 2023.
9. Meyer*, G., Adejumo*, E., Song, L., Quantification of Cannabichromene in Cannabichromene Isolates of Hemp among Nineteen Cannabinoids by Liquid Chromatography Ultraviolet Detection in: 2023 Illinois State Academy of Science Annual Meeting, Peoria, IL, 2023.
10. LeBlanc, L., Meyer*, G., Song, L., Quantification of Cannabigerolic Acid among Sixteen Cannabinoids in Dried Hemp Flowers by Liquid Chromatography Ultraviolet Detection in: 2023 Illinois State Academy of Science Annual Meeting, Peoria, IL, 2023.
11. Jovanovich, E., Adejumo*, E., Song, L., Potency Testing of Δ8-Tetrahydrocannabinol in Delta 8 Concentrate among Nineteen Cannabinoids by Liquid Chromatography Ultraviolet Detection in: 2023 Illinois State Academy of Science Annual Meeting, Peoria, IL, 2023.
12. Fabien*, K.J., Meyer*, G., Song, L., Potency Testing of Cannabinol in Cannabinol Isolates of Hemp among Nineteen Cannabinoids by Liquid Chromatography Ultraviolet Detection in: 2023 Illinois State Academy of Science Annual Meeting, Peoria, IL, 2023.
13. Dodson*, Z., Meyer*, G., Song, L., Quantification of Cannabidiol in Hemp-infused Water by Ultra High Performance Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry, in: 2023 Illinois State Academy of Science Annual Meeting, Peoria, IL, 2023.
14. Brownlee*, G., Adejumo*, E., Song, L., Quantification of Cannabichromene in Cannabichromene Isolates of Hemp among Nineteen Cannabinoids by Liquid

Chromatography Ultraviolet Detection in: 2023 Illinois State Academy of Science Annual Meeting, Peoria, IL, 2023.

15. Adejumo*, E., Meyer*, G., Song, L., Quantification of Cannabigerol among Nineteen Cannabinoids in Hemp-infused Gummies by Liquid Chromatography Ultraviolet Detection, in: 2023 Illinois State Academy of Science Annual Meeting, Peoria, IL, 2023.
16. Valenzuela*, G., Dodson*, Z., Song, L., Quantification of Cannabigerol in Hemp Oil by Ultra High Performance Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry, in: 2022 Illinois State Academy of Science Annual Meeting, Virtual, 2022.
17. Valenzuela*, G., Carlson*, S., Song, L., Quantification of Cannabinol in Hemp Oil by Ultra High Performance Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry, in: 2022 Illinois State Academy of Science Annual Meeting, Virtual, 2022.
18. Carlson*, S., Valenzuela*, G., Song, L., Quantification of Delta-9-Tetrahydrocannabinolic Acid among Sixteen Cannabinoids in Cannabis by Liquid Chromatography Ultraviolet Detection in: 2022 Illinois State Academy of Science Annual Meeting, Virtual, 2022.
19. Ayer*, B., Jovanovich**, E., Song, L., Quantification of Cannabinol among Sixteen Cannabinoids in Hemp Oil by Liquid Chromatography Ultraviolet Detection in: 2022 Illinois State Academy of Science Annual Meeting, Virtual, 2022.

Thesis:

1. Meyer, G., A liquid chromatography electrospray ionization tandem mass spectrometry method for quantification of up to eighteen cannabinoids in hemp-infused gummies and topicals, in: Chemistry, Western Illinois University, 2023, pp. 54.

2. Dodson, Z., Quantification of up to eighteen cannabinoids in hemp-infused beverages and snacks by ultra-high-performance liquid chromatography electrospray ionization tandem mass spectrometry, in: Chemistry, Western Illinois University, 2023, pp. 54.
3. Adejumo, E., Potency testing of up to sixteen cannabinoids in liquid edibles using liquid chromatography diode array detector with optional confirmation of identity by electrospray ionization time-of-flight mass spectrometry, in: Chemistry, Western Illinois University, 2023, pp. 54.
4. Adisa, M., Development of a validated method for high throughput quantification of up to twenty cannabinoids in cannabis cigarettes using liquid chromatography diode array detector with optional electrospray ionization time-of-flight mass spectrometry, in: Chemistry, Western Illinois University, 2022, pp. 43.
5. Valenzuela, G.M., Potency testing of up to twenty cannabinoids by liquid chromatography diode array detector with optional electrospray ionization time-of-flight mass spectrometry, in: Chemistry, Western Illinois University, 2022, pp. 54.
6. Carlson, S.T., Development of a validated method for rapid quantification of up to sixteen cannabinoids using ultra-high-performance liquid chromatography diode-array detector with optional electrospray ionization time-of-flight mass spectrometry detection, in: Chemistry, Western Illinois University, 2022, pp. 41.

Lab Experiment:

1. One lab experiment, i.e., Quantification of cannabinoids in hemp oil by high performance liquid chromatography (HPLC), has been developed for CHEM 351 Application of Forensic Chemistry.

Data sets generated (broad descriptions will suffice).

Separation optimization.

1. Data sets for LC-UV of twelve cannabinoids included effect of methanol content (80, 85, 90, and 95% (v/v)) in the mobile phase (pH 2.69) on the separation, effect of methanol gradient (initial content, final content, gradient slope, and initial hold time) on the separation, effect of acetonitrile content (80, 85, 90, and 95% (v/v)) in the mobile phase (pH 2.69) on the separation, and effect of pH of the mobile phase (2.69, 3.26, 3.69 and 4.20) on the separation.
2. Data sets for UHPLC-DAD of sixteen cannabinoids plus ACBD included effect of the content of formic acid (0.300, 0.200, 0.100, 0.050, 0.028, 0.022 and 0.020% (v/v)) in the aqueous solvent of the mobile phase on the separation, effect of acetonitrile content (68, 70, 73, 75, and 78% (v/v)) in the mobile phase on the separation, and effect of column oven temperature (25, 30, 35, 40, and 45^{o}C) on the separation.
3. Data sets for LC-DAD of twenty cannabinoids plus ACBD included effect of the content of formic acid (0.0062, 0.0085, 0.010, 0.012, 0.015, 0.018, 0.021, 0.022, 0.025 and 0.030% (v/v)) in the aqueous solvent containing 1 mM ammonium formate on the separation, and effect of the content of acetonitrile (68, 70, 73, 75 and 78% (v/v)) in the mobile phase on the separation.
4. Data sets for UHPLC-DAD of sixteen cannabinoids plus ACBD later also included effect of the content of formic acid (0.056, 0.044, 0.030, 0.018, 0.012, 0.011 and 0.010% (v/v)) in the aqueous solvent containing 2 mM ammonium formate on the separation.

DAD optimization. Data sets for twenty cannabinoids plus ACBD included detection at 223, 230, 251, 261, 269 and 285 nm with 4 nm bandwidth using reference wavelength at 360 nm with 100 nm bandwidth and recording of UV spectra from 190.0 to 400.0 nm with 2.0 nm step.

ESI/TOFMS and ESI/MS/MS optimization. Data sets for eighteen cannabinoids plus ACBD included optimization of source dependent parameters and compound dependent parameters for Δ^9-THC and Δ^9-THCA using flow injection analysis of 1 µg/mL solution in methanol. Briefly, capillary voltage between 1000 to 3500 V with a step of 500 V, nozzle voltage between 0 to 1200 V with a step of 200 V, and fragmentor voltage between 80 to 200 V with a step of 100 V were examined. Under optimized LC-ESI/TOFMS conditions, CE were further optimized between 5 to 40 V with a step of 5 V for MS/MS experiments.

Method validation. Each method validation was carried out for the analysis of ten calibration and three QC samples in triplicate each day, consecutively in three separate days. Therefore, data sets for each method validation included the analysis of thirteen samples for nine times. A total of six method validations were performed, with each one corresponding for each study described by the six **Goals**.

Sample analysis. Each sample was analyzed in triplicate. A total of eighty samples were analyzed by the six studies described by the six **Goals**.

Dissemination activities.

The PI and his students have presented their research results in international, national, regional, state and university conferences including the 2022 and 2023 NIJ Forensic Science Research and Development (R&D) Symposium, the 2022 International Symposium on High Performance Liquid Phase Separations and Related Techniques (HPLC 2022), the ACS (American

Chemical Society) Fall 2022 Meeting and Expo, the 2022 and 2023 Illinois State Academy of Science Annual Meeting (ISAS 2022 and 2023), the 2022 and 2023 WIU Graduate Research Conference, the 2022 and 2023 WIU Natural Sciences Research Symposium, and the 2022 and 2023 WIU Thomas E. Helm Undergraduate Research Day

The project has been described in the Mass Spec Center website in an effort to reach scientists in crime labs and cannabis testing labs, cannabis stake holders, and cannabis growing farmers.

The project has been used to effectively attract students into the WIU B.S. Forensic Chemistry program through two hands-on activities: 1) Presumptive identification of marijuana by Duquenois–Levine test and immunoassay; and 2) Presumptive differentiation of hemp from marijuana using thin-layer chromatography. There are four major events at WIU.

- The monthly “Discover Western” event serves as a student recruitment vehicle by hosting prospective students and their family members, which include a tour to the research and teaching facilities.
- The CAS summer camp program “Discovering the World Through Science” hosts high school students for one week to conduct hands-on scientific experiments.
- The CAS “College Day” program hosts high school students and their families for a day so that they can experience lectures and demonstration, connect with faculty, and become familiar with their research.
- The WIU-WIS “Girl Scout STEM program” hosts Girl Scouts from kindergarteners to high school seniors so that they develop an interest in STEM disciplines.

A lab experiment, entitled “Quantification of cannabinoids in hemp oil by high performance liquid chromatography (HPLC)” has been developed and incorporated into one of the PI’s courses, i.e., CHEM 351 Application of Forensic Chemistry in fall 2022.

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