Method Application Note
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Eight Cannabinoids by HPLC-UV

Overview

Measurement of cannabinoid content, or "the potency test", is the most frequently discussed method in cannabis analysis. Many such methods have been developed in the past few years, but at the time of this writing no *published* cannabinoid method has been rigorously validated and shown to work consistently with real cannabis samples of all types. Consequently, cannabis labs are developing their own methods to measure cannabinoid content in consumer products. Some of these methods are loosely based on the guidelines of the American Herbal Pharmacopoeia's *Cannabis Inflorescence* monograph, which briefly describes a method developed by Debaker *et al.* (2009) and adapted by Swift *et al.* (2013). Still other cannabis labs are using methods developed by equipment manufacturers and chemical suppliers.

In this paper we describe the method used by Confidence Analytics routinely since 2014, which is based on the work of Swift et al. (2013). We include example instructions for sample preparation -- including our recommended means of homogenizing flower samples -- instrument requirements and method performance, important considerations, and an overview of our experience using this method for years.

A recent publication by Jikomes and Zoorob (2018) reviewed the laboratory outcomes of 6 leading cannabis laboratories in Washington state and highlighted the need for standardization between them. That research found the data reported by Confidence Analytics -- generated with the method described here -- closely agrees with previous research outside the context of compliance testing. The study discusses the value in consistent analytical sensitivity for relatively low levels of CBDA and CBD, with data graphics showing that this method also performs well in that context.

Performance Specifications

This method uses an HP/Agilent LC 1100 equipped with a quarternary pump, 100-well vial autosampler, column oven, and Diode Array Detector. This instrument was selected because it is cheap, reliable, easy to maintain, and of sufficient performance for adequate cannabinoid analysis.

Cycle time (per sample)	<12.5 minutes
Linear Dynamic Range (standards)	0.4 ug/mL – 50 ug/mL, up to 1000 ug/mL*
LC Solvent Use per 1000 samples	4.85 L HPLC-grade water, 13.7 L HPLC-grade acetonitrile
Column Lifetime	>2000 injections**
Wavelengths Monitored	220 nm, 270 nm
Analytes Measured (elution order)	CBDA*, CBGA, THCA*, CBG, CBD*, CBN, THC (delta-9)*, CBC
Sample Reporting Range (% w/w)	0.1% to 100%*
Sample Types Analyzed	Green plant matter, concentrate, marijuana-infused products

Table 1: some select method performance specifications

**some analytes have a broader calibration range due to their greater abundance in real samples*

***no guard column used for RT stability, depends on number of non-inhalable samples injected*

Method Technical Parameters

HP/Agilent LC 1100 system:
- Inline Degasser (G1322A, G1379A, or equivalent)
- Quarternary Pump (G1311A or equivalent)
- Autosampler (G1313A or equivalent) fitted with 50 uL sample loop
- Column Compartment (G1316A or equivalent)
- Diode Array Detector (G1315A or equivalent)

Solvent Gradient:
20% B Start (0 minutes)
90% B 8.5 minutes
20% B 10 minutes

Column:	Agilent ZORBAX TC-C18(2), 150 x 4.6 mm, 5 um fully porous particles
Column Temperature:	35 °C
Mobile Phase A:	25 mM ammonium formate in 50:50 water and acetonitrile, HPLC-grade
Mobile Phase B:	neat acetonitrile, HPLC-grade
Flow Rate:	1.5 mL/min
Expected back-pressure:	80 - 100 bar (initial parameters)
Injection Volume:	50 uL
Measurement Time:	9.5 minutes
Post-time:	1.7 minutes
Measured Wavelengths:	220 nm, 270 nm (4 nm band), ref. 370 nm (80 nm band), Slit Width 4 nm
Measured Spectra:	190 nm to 700 nm, step 0.5 nm

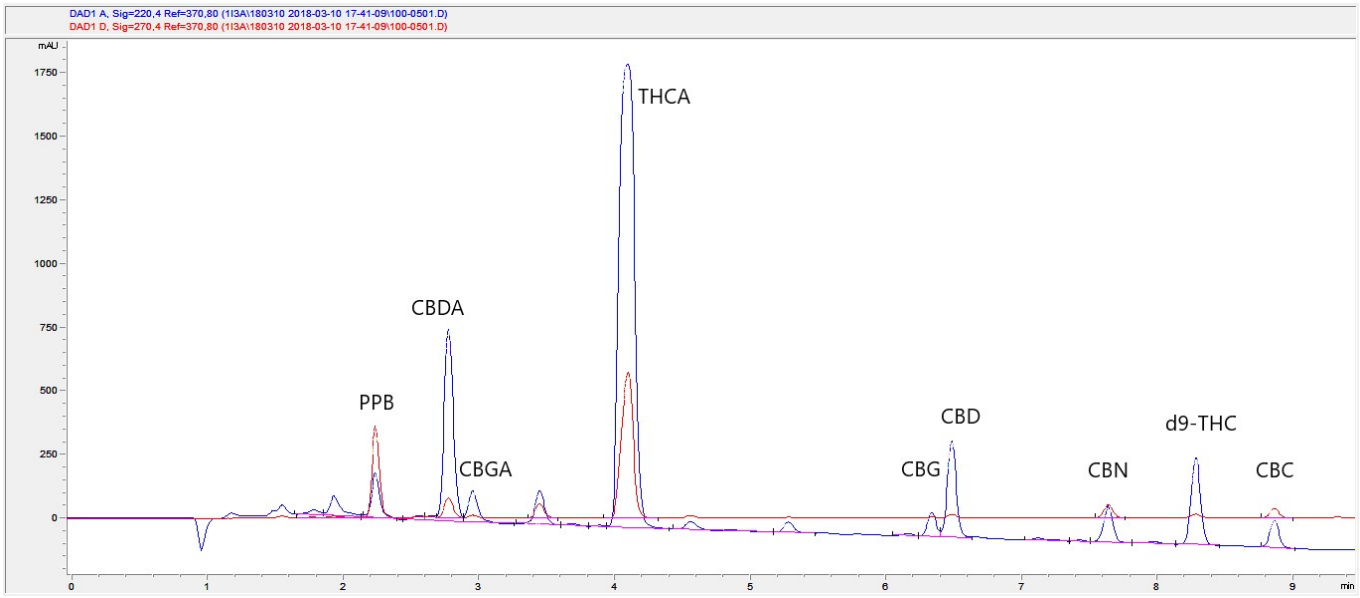


Figure 1: Typical LC chromatogram of a qualitative retention time standard. This is a real sample solution spiked with reference standards. The column Peaks are annotated where they match target analytes; note that not all peaks are known analytes.

Background and Discussion

Analyte Selection

Of the 8 analytes listed in Table 1, some are required by law to be part of the cannabinoid analysis. The analytes cited in Washington state regulations are CBDA, CBD, THCA, and THC (both assumed as delta-9). We call these “the Big 4” cannabinoids, so named because most other analytes aren’t usually present in samples at concentrations exceeding 5% w/w. CBGA is relevant because it is the enzyme substrate for THCA and CBDA biosynthesis, and CBGA content may have value to farmers and processors in optimizing harvest. CBG, the decarboxylated “neutral version” of CBGA is also present in cannabis products. Because of the many chemical similarities between CBGA and CBDA (and CBD/CBG), the chromatographer must be careful to resolve them to avoid over-reporting of CBDA content where CBGA is present in abundance. These three “acid/neutral pairs” for CBDA/CBD, THCA/THC, and CBGA/CBG are the most important cannabinoids to measure in cannabis products intended for inhalation. Additionally, CBN is useful to measure as it is a known degradation product of THC – and therefore a quality marker – and seems to modify pharmacological activity of cannabis materials. CBC is the last compound to elute on this method, and is convenient to add to calibrations because its CRM standards are cheap, and making sure CBC elutes in the acquisition window is useful for determining system suitability. Other cannabinoids may elute with resolution on this method.

Sample Preparation

Cannabis flower samples need to be homogenized in a way that preserves cannabinoid content and minimizes any fractionation of trichome parts from the rest of the green plant matter. Mortar and pestle, blender, and herb grinder -- before or after drying -- have all been experimented with as methods of homogenization. No method of cannabis flower homogenization has been found to work as well as frozen ball-milling for its ability to produce uniform particle size without static-charge tendency. Concentrates are easier to homogenize, and many such samples are presented in a homogeneous form. As with marijuana-infused products, the appropriate means of homogenization depends on the sample itself.

Calibration Standards and Sample Mass Ranges

Concentrations of available DEA-exempt certified reference materials (CRMs) are a significant constraint on any cannabinoid measurement method. The ceiling for reference standard concentrations is generally 1,000 ug/mL. Using an extraction solvent volume of 10.0 mL, 100 mg of a highly purified isolate of a cannabinoid generates a solution concentration of 10,000 ug/mL – 10-fold higher than the highest afforded by reference standards. The maximum capacity of the column used in this method, with respect to peak shape, is about 25 ug per analyte (50 uL injection of 500 ug/mL). Detector response remains constant when computed by peak **area** - but not peak **height** - to at least 50 ug of injected analyte, despite peak fronting. Considering this constraint – that there is about a 20-fold difference in concentration between the largest reasonable injection of reference standard and the largest reasonable injection of prepared isolate sample – this method applies a dilution factor of 20 to all inhalable cannabis products, using a diluent of 50:50 water and acetonitrile. Extending the calculation for a typical flower at 25% of one analyte, we find a maximum acceptable sample mass of 400 mg in 10.0 mL extraction solvent. To exceed the calibration minimum of 0.4 ug/mL, a sample containing 0.1% of one analyte requires at least 80 mg in 10.0 mL extraction solvent. Therefore, we use a sample mass range of 80 to 100 mg for concentrates and 200 to 400 mg for flower. Sample mass, extraction solvent volume, and dilution ranges for marijuana-infused products can be computed similarly within the boundaries of extraction protocols demonstrated to recover a satisfactory amount of target cannabinoids.

Linearity in Calibration and Dynamic Range Extension

Detector response for each analyte is a value generated during analyte calibration. In order to maintain the 3-order dynamic range necessary to maintain a reporting range of 0.1 to 100% w/w in sample, more than one detector wavelength must be used. Typically, wavelength maxima for spectral features of each analyte would be selected for quantitative monitoring. Given the variety of UV spectra among the 8 analytes listed, it is convenient to use two wavelengths where analyte response factors differ by at least several-fold between them for all analytes. The wavelength of 220 nm is effective for measuring low levels of analytes up to 50 ug/mL in prepared sample solution, and the wavelength of 270 nm is effective for measuring higher levels of analytes between 50 and 1000 ug/mL in prepared sample solution. Utilizing the difference in response factor between these two wavelengths, the method described here very accurately reports quantities of all target analytes through the stated reporting range of 0.1% to 100 % w/w in sample.

Internal Standard

To achieve high replicate precision, use of an internal standard is necessary. This method uses propyl paraben (PPB), a fairly safe material available in high purity at a low cost. Unlike a true internal standard calibration, where a response ratio between analytes and the internal standard is computed at calibration, this method uses external standard calibration with internal standard adjustment. Extraction solvent methanol is infused with propyl paraben at a concentration of 0.500 g/L and thoroughly homogenized before use. Method blanks are prepared by filling an empty extraction tube with the internal standard-infused methanol (PPB-MeOH). The internal standard area of four method blanks is measured and averaged with each run, to provide an adjustment factor for all other samples in the run. Quantitative precision has been demonstrated up to to +/- 20% PPB area deviation from the average. The adjustment factor provided by the internal standard allows for correcting errors that arise from evaporation of solvent, pipette inaccuracies, and other sources of error that cause incorrect concentration in the instrumental injection bolus.

Chromatographic Robustness

Mixing acetonitrile and water is quite endothermic. Water buffered at a neutral or basic pH is known to absorb ambient CO2 from the air, making the buffered solution more acidic over time and impacting the quality of analysis. Pre-mixing aqueous buffered solutions with an organic solvent is a well-known means to mitigate the pH drift problem (Crawford Scientific). To ensure this method is as robust as possible, the aqueous mobile phase is blended 50:50 with acetonitrile, mixed thoroughly, and allowed to warm to room temperature before it is used for analysis. Conveniently, each mobile phase is consumed in roughly equal amount with each sample, further streamlining workflows. Because of the resilience to pH change, 4-liter bottles of mobile phase are safe to use for at least a week. It also appears to minimize inconsistency of mobile phase mixing in the pump, further improving chromatographic robustness.

For this method, typical retention time precision is +/- 0.5% over a whole run of 100 injections, with even tighter back-to-back precision. For THCA at about 4.1 minutes, a typical variance is all peaks between 4.08 and 4.12 for a run of 100 injections. Column-age drift is gradual, expected to advance retention times, and can be adequately compensated for with a qualitative retention time standard in each run. Columns last at least 2000 injections before showing chromatographic problems, and have exceeded 3000 injections while still passing QC parameters. Columns are typically replaced when system back-pressure has risen by more than 30% since the previous installation and column conditioning.

Sample Type Robustness

This method performs well for all cannabis flowers and concentrates. The use of a high-organic-fraction LC pump program affords useful resolution between matrix components of marijuana-infused products (MIPs) and the target analytes for most sample types. Beverages, liquid emulsions, hard candies, dairy-containing candies, gummies, baked

goods, cooking oils, and salty snacks are all handled deftly by this method, *granted proper sample cleanup during preparation and **adequate recovery** of target analytes*. Analyte retention times are essentially unaffected by various sample matrices. The various preparations for such infused products are outside the scope of this document.

Additional Data Collection

A variety of detector systems can be used for UV-Vis measurement. This method was developed using a Diode Array Detector (DAD) that permits recording of a spectrochromatogram for each sample – the DAD spectrum output for the whole chromatogram. We strongly encourage any analyst adopting this method to also record spectrochromatograms for each injection. A number of common unknown peaks are resolved with this method, and spectral confirmation of unknown peaks between different labs using this method greatly increases the potential value of the resulting data set.

Limitations

This method features two important limitations.

- First, and most importantly, this method does not resolve an array of abundant peaks -- including isomers of THC not made by the cannabis plant -- in heat-treated concentrate samples. Some of these compounds tend to elute around CBC, and at least 3 unknown compounds have been observed shouldered into each other on heavily heat-treated distilled samples. Other methods are known to suffer this flaw, as well, and the nature and identity of these unknown compounds is an area of active research. Ostensibly, CBC cannot be reported in these samples due to interference from unknown peaks.
- Second, this method has a hard time handling some MIPs, depending on the matrix of the product and how the sample is prepared. Topical products especially are known to contain very lipophilic constituents that can retain on the column and elute in the next injection as ghost peaks. Such products are also a risk to column longevity and can cause system over-pressure and the associated retention time problems.

To address the first limitation, different mobile phase or stationary phase conditions are needed. This is also an area of active research.

The second limitation can be addressed with careful sample preparation and running a blank injection after each topical injection to avoid ghost peaks in regular data. An extended pump program with a larger volume of organic “flush” would mitigate this problem, too.

An additional limitation exists for any LC method for this application, and that is MIPs with interfering constituents that co-elute with analytes or the internal standard. This problem is rare. Since propyl paraben is an FDA-approved food additive, other internal standards are desirable for MIPs.

Sample Preparation Procedure

Equipment

- HPLC with 50 uL sample loop, calibrated to the LC method described below
- Analytical balance, calibrated to NIST-traceable standard weights
- SPEX CertiPrep GenoGrinder
- Freezer capable of -25 C or lower, high-speed or “flash” freezer preferred for thru-put
- Vortexer, platform-style preferred for thru-put
- NIST-traceable calibrated bottle-top dispenser for 4L jug
- NIST-traceable calibrated aspirating pipettes with disposable tips
- NIST-traceable calibrated positive-displacement pipettes with disposable tips
- 1.00 L graduated cylinder or equivalent

Supplies (for each sample, unless otherwise specified)

- GenoGrinder polycarbonate ball-mill tube, 50 mL capacity, with cap
- Vortex tube, 15 mL capacity, with cap
- 2 stainless steel ball bearings
- GenoGrinder aluminum tube holder blocks
- Receptacle for homogenized sample (weigh boat, bag, etc.)
- Means of membrane filtration at 0.45 um or smaller pore size
- Glass autosampler vial, 2 mL capacity, with cap
- Small glass test tube
- Means of labeling tubes and vials
- Disposable pipette tips as needed for aspiration and positive-displacement pipettes

Chemicals

- HPLC-grade methanol, infused at 0.500 g/L with propyl paraben (extraction solvent “PPB-MeOH”), an empty 4L methanol jug labeled
- Propyl paraben, >99% purity
- HPLC-grade water, an empty 4L acetonitrile or water jug labeled for Mobile Phase A
- HPLC-grade acetonitrile, an empty 1 L bottle

Steps - Solvents Preparation

Extraction Solvent - Methanol with propyl paraben internal standard

1. Weigh 2.000 +/- 0.005 grams of propyl paraben on a weigh paper. Carefully transfer all of the weighed material into an empty 4L methanol jug labeled “PPB Methanol”.
2. Carefully measure 4 1.00 L aliquots of HPLC-grade methanol into the 4L jug with propyl paraben.
3. Mix the jug contents by shaking, inverting, swirling, etc. until all of the propyl paraben has dissolved.
4. Attach the bottle-top dispenser, and purge the line until solvent flows with no bubbles. Verify volume dispensed is accurate. This solution is now ready for use.

Diluent - 50:50 Acetonitrile and Water

1. Carefully measure 500 mL of HPLC-grade acetonitrile into an empty 1 L bottle labeled “Diluent”.

2. Carefully measure 500 mL of HPLC-grade water into the 1 L bottle.
3. Cap the bottle, mix the contents by shaking, inverting, swirling, etc. The bottle should get cold to the touch.
4. This solution is now ready for use.

Mobile Phase A - 50:50 Acetonitrile and Water, 25 mM ammonium formate

1. Weigh 6.305 +/- 0.005 grams of ammonium formate on a weigh paper. Carefully transfer all of the weighed material into the 1.00 L graduated cylinder. Move quickly, as ammonium formate is hygroscopic.
2. Fill the graduated cylinder about half-way with HPLC-grade water, and swirl until the ammonium formate has dissolved. Fill the graduated cylinder up to 1.00 L and transfer into the empty 4L jug labeled “Mobile Phase A”.
3. Carefully measure another 1.00 L aliquot of HPLC-grade water into the 4L jug.
4. Carefully measure 2 1.00 L aliquots of HPLC-grade acetonitrile into the 4L jug.
5. Cap the jug and mix the contents by shaking, inverting, swirling, etc. until the jug feels cold to the touch. Allow to warm to room temperature before use as Mobile Phase A.

Steps - Flower Dry Preparation

1. Intake the sample as required, including visual observation for foreign matter, sample photography, etc.
2. Pack a GenoGrinder tube in order with 1.5 - 2 grams cannabis flower, two ball bearings, then another 1.5 - 2 grams. Cap the GenoGrinder tube, and label with the sample information.
3. Place filled and unground GenoGrinder tubes in GenoGrinder aluminum tube holder blocks, and place these blocks in the -25 C freezer until they are at equilibrium with the freezer temperature.
4. Moving quickly to limit warming of samples, place GenoGrinder aluminum blocks with unground, frozen sample tubes on the GenoGrinder. Grind the samples at 1500 rpm for 1 minute.
5. Remove ground samples from the GenoGrinder. Leave them capped until sampling to limit volatiles loss.
6. Sample homogenate may cake in the tube, and may require tapping to remove. *Held-up homogenate stuck to the ball bearings, the inside of the tube, and the cap have the same composition as the loose homogenate.*
7. Measuring cannabinoids from **as-received weight**, empty tubes one at a time, stir the homogenate to disperse clumps, then quickly place a sub-sample of 0.2 to 0.4 grams in a 15-mL extraction tube and record the sample weight. Cap the tube, and label with the sample information.

Steps - Concentrate Dry Preparation

1. Intake the sample as required, including visual observation for foreign matter, sample photography, etc.
2. Determine if, and how much, homogenization is needed for the concentrate sample. Kief, dry-sift and bubble hashes, and some solvent extracts like “honeycomb wax” have a dry, granular consistency and only need stirring. Shatters and similar glassy consistencies may need to be frozen and crushed. Heterogeneous consistencies, like “crystal sauce” (cannabinoid crystals mixed with cannabinoid-saturated high-terpene oil) may need a mixture of stirring and crushing to homogenize.
3. Place a sub-sample of 0.08 to 0.10 grams into a 15-mL extraction tube and record the weight. Cap the tube, and label with sample information.

Steps - Sample Wet Preparation

1. Fill an empty tube with 10 mL of extraction solvent - this is a method blank.
2. For each sample, uncap, add 10 mL extraction solvent, and recap. Vortex filled extraction tubes for 10 minutes. (Other means of extraction aides should work - shaking like with the GenoGrinder, ultrasonic bath, simple steeping - if >99.5% recovery can be validated)
3. Inspect extraction tubes. Some types of concentrate consistencies - like “raw” CO2 oil - resist dispersion and may require additional vortex time.

4. Membrane-filter at least 2 mL sample solution into a small glass test tube.
5. Add 950 uL of Diluent to a labeled autosampler vial. Add 50 uL of filtered extract solution to the autosampler vial, cap, and shake the vial.
6. This sample vial is now ready for analysis by the HPLC method described below.
7. Prepare method blanks with the same dilution as samples using the highest-accuracy measurement possible.

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