C-Vue® Chromatography

Standard Operating Procedure for the Measurement of Cannabidiol (CBD) and delta-9-tetrahydrocannabinol (THC) in Hemp using the C-Vue® HPLC.

Scope

This protocol is designed for use by Hemp growers and others who may need to measure the CBD and THC content in plant tissue or processed material for the purpose of compliance with regulations for the legal level of THC and for the estimation of CBD content for yield.

The target content used for this procedure is 15% by weight CBD and 0.3% by weight THC. All concentrations from this point forward are in mg/g. The protocol may be applied to the measurement of greater or lesser concentrations of cannabinoids by appropriate selection of the amount of sample and diluent.

15% = 150mg/g

0.3% = 3mg/g

Please note that the expected CBD content is 50 times greater than the maximum THC content. This causes an analytical challenge when trying to optimize the method for measuring both with one sample extraction and dilution. The method is focused on getting the best measurement for THC by using more concentrated samples. The use of higher concentrations may cause the CBD peak's areas to be in the non-linear part of the calibration curve—they are too big for the calibration. For samples with higher (than 15%) CBD content, it is recommended that a second, smaller sample of the extract be diluted to get the CBD into the linear part of the calibration curve.

How to use this document

We have written this SOP to be used in conjunction with three videos. Please read up to page 4 to **"Starting the Instrument"** and then start **video 1**. Please pause the video as necessary to follow the SOP. The goal is to help you become comfortable with the instrument. Below, each part's location and function is explained. Then the video shows the instrument as it is started up and readied for an analysis.

Video 2 shows how to build a calibration. Calibration is the process where you inject standards with known amounts (in milligrams/gram) of CBD and THC. The areas under each peak is measured and stored in a calibration file. The software establishes a relationship between the area and concentration of each peak and expresses it as an equation of a curve for CBD and a line for THC.

Video 3 shows how to use the instrument to analyze a real sample beginning with extraction of the cannabinoids from plant material or dilution of an oil. The injection the same as for standards. At the end, the software calculation corrects the analyzed sample information for the extraction and dilution and provides the concentration of CBD and THC in the sample and generates a report.

Hardware

The C-Vue® HPLC uses these parts to accomplish these goals:

-- A Spring-driven syringe pump to push the eluent--the liquid that carries the samples and standards through the injector, column and detector,

-- An injection valve to measure the same amount of samples and standards and allows you to inject them into the flowing eluent,

-- A column to separate the CBD and THC,

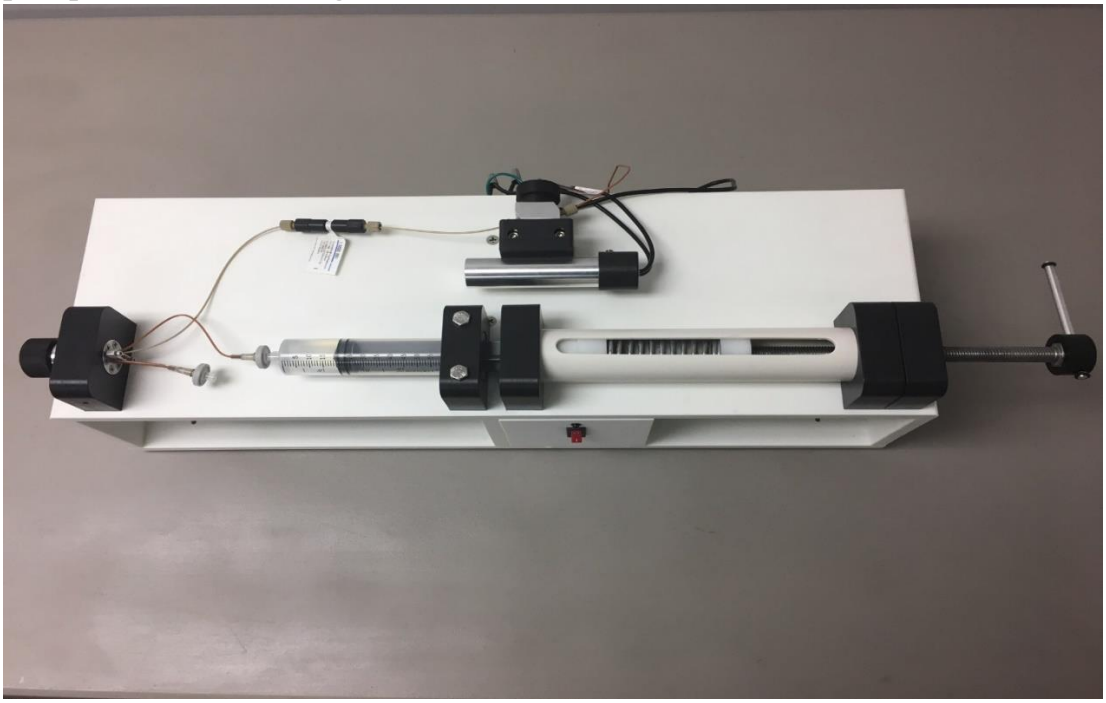
-- A detector to allow you to "see" the peaks made by CBD and THC and measure them—you "see" them because they absorb the light and stop it from getting to the detector—more CBD and THC absorb more light and make bigger peaks,

-- A data system helps you compare the sizes of the unknown sample peaks for CBD and THC with the sizes of known standard peaks of CBD and THC,

-- All connections are made with tubing with very small holes to minimize diffusion during the separation. This keeps the things you separate from mixing back together.

Getting Started

Place the instrument on a level surface at a comfortable height with the syringe pump located to the right and on the same side as the user.



Getting Familiar with the C-Vue® HPLC.

<u>The pump</u>

1. Stainless steel bolt--provides a means to compress the spring,

2. White bearings located inside the white pipe—allow uniform pressure from bolt to spring and from the spring to the syringe plunger.

3. Spring—stores mechanical energy that is used to push the eluent through the injector, column and detector.

3. Syringe—serves as reservoir for the eluent and pushes the pressurized eluent through the injector, column and detector.

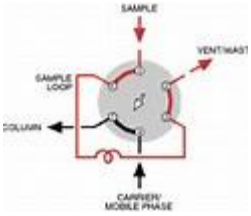
4. PVC pipe—retains the spring,

5. metal ruler—used to set the amount of compression on the spring plunger at the beginning of an analysis so that the flow rate is consistent,

7. Filter—removes particulates larger than 0.45micrions to keep from plugging the injector and column.

The injector.

1. Sample Filter--Removes particulates from samples to prevent plugging
2. 1/16" PEEK tubing—connects to the pump and to the column.
3. Sample loop. Volume is 10 microliters.
4. Knurled knob is used to rotate the valve from **loop load** position (rotated towards the user) to **inject** position (rotated away from user)**,**
5. Sample and standard loop is a small length of tubing with a fixed volume of 10 microliters (ten millionths of a liter)
6. These comprise the injector which provides the means to introduce a measured volume of standards and samples. The flow pattern is shown below:



Column

The column is the heart of the instrument. This is where the components of the standard or sample are separated.

Detector

1. UV lamp—uses a Zinc lamp which emits 214 nanometer light that is absorbed by CBD and THC. It is inside of a metal cylinder to protect the user from exposure to ultraviolet light (UV), the light is directed through the flow cell,
2. Mounting bracket—provides a means to attach the injector to the instrument,
3. Flow cell—this is where the eluent carries the components of the sample from the column through a path between the UV light and the detector, peaks are made when the CBD and THC pass between the light and the detector,

4. The detector is a light-sensitive silicon photodiode located at the back of the instrument facing the UV light. It responds to the amount of light reaching it.

Electrical connections

On the back of the aluminum box, there are connections to power the on-board computer, a USB cable to connect to the data processing computer and a port to plug in the signal from the detector.

One power cord connects 115 VAC to the lamp power supply and the other connects 115 VAC to power the on-board computer. These are located in the compartment under the column.

There is also a charger for the data processing computer.

Starting the instrument

Preparing the syringe Pump

A. Unscrew the stainless steel bolt located at the top right until the spring stops moving to the right (as you hold pressure to push the spring to follow the bolt).

B. Remove the two screws which hold the syringe block to the top of the instrument.

C .Carefully unscrew the syringe from the filter—HOLD the filter in your left hand and gently twist the syringe counter clockwise to release it.

D. Pull the plunger out of the syringe and place on the instrument with the plunger pointing up to prevent dirt from getting on the plunger tip.

E. Hold the syringe vertically and plug the tip with your finger while pouring 50-55ml of eluent into the open end where the plunger was removed.

F. Carefully re-insert the plunger into the syringe and hold the syringe with the tip facing up and gently push the plunger into the syringe about 2 cm.
G. Slowly remove your finger from the tip to release and slight pressure buildup—caution—the eluent is under some pressure so be careful not to spray anyone.

Procedure for removing air from the eluent

The eluent contains dissolved air which may cause noise in the detector as it flows out of the pressurized column to ambient pressure. To remove air from the eluent:

A. Attach the degas-syringe

B. Pull the plunger outwards and hold. This creates a vacuum above the eluent and the dissolved air will bubble out. The process goes faster if the eluent syringe is tapped lightly. This process usually takes about 5 minutes.
C. The eluent syringe bottom should be resting on a surface. This will keep the small piece of 1/16" tubing from releasing from the luer adaptors which connects the syringe to the tubing.
D. As the gas begins to evolve more slowly, the process is complete.
E. Dis-assemble the syringes and re-attach the eluent syringe to the instrument.
F. Place the eluent syringe into the syringe holder—please note that one side of the syringe holder has a recess for the syringe collar.
G. Carefully place the syringe into the filter and push slightly on the plunger before you twist the syringe into the filter—this is to displace the small amount of air trapped in the fitting.
H. At this point, gently loosen the fitting at the front of the column and allow it to leak while the syringe is reattached to the instrument.
I. Place the syringe holder over the two holes in the top plate. You may have to squeeze the syringe holder towards the block that holds the end of the spring pipe a little to get the holes to align—especially if you have put more that 52-53ml of eluent in the syringe. If this is too difficult, push some of the eluent out of the syringe…50-52ml is usually easy to reassemble.

J. After the syringe is reattached, and some pressure on the syringe is causing flow, the injector should be rotated from loop load to inject to allow any air in the injector to be flushed out.
K. To complete the process, the fitting at the front of the column should be tightened to gentle finger tight.
L. Rotate the injector so that both flow paths are filled with eluent.

Adjust the pressure on the spring by turning the stainless steel bolt until the space between the white bearings is 50mm. Use the metal ruler to check. This pressure results in a flow of about 200-250 microliters per minute which is optimum for the column. (Note: This flow rate is not critical—you may use more or less compression—BUT you must use the same flow for calibration and samples.)

Turn on the switch on the front to power the on-board computer and the lamp power switch to light the UV lamp.

CAUTION: DO NOT REMOVE THE POWERED LAMP FROM THE METAL CONTAINER. THE LIGHT EMITTED FROM THE ZINC LAMP IS VERY DANGEROUS. The metal tube holding the lamp gets hot—USE CAUTION.

Turn on the computer by pressing the power button on the left.

Launch the C-Vue software by double-clicking the icon on the desktop

You will see a message "DAQ FOUND" indicating that the computer and the instrument are connected and communicating. Select "Okay" to acknowledge.

Set the axes by selecting 5 for the top value for the "Y" axis and minus 100 for the bottom value for the "Y: axis and select "Clear Zero".

The **Gain** button shows the value used to boost the detector signal. In addition to the default 50MV/A the user may select 100, 330 and 470MV/A. The user should use the lowest Gain value that provides good signal to noise.

The X axis values are shown in white boxes at the bottom. The default value for each chromatogram is 20 minutes.

Setting the Gain and Light Level

When Clear Zero is selected, the plot area below becomes a light meter. The top of the screen indicates zero percent detector saturation; the bottom at -100

indicates 100% detector saturation. If more than 100% light reaches the detector, the DAQ Status turns RED.

The light output from Zinc lamps varies. The output at 50 MV/A may be from 0 to minus 50. If the light level is between 0 and -15, it is necessary to increase the light to the detector. Try unscrewing the screw in the top of the detector block located near the middle of the top plate and towards the back. It is immediately behind the shiny aluminum cylinder—WHICH IS VERY HOT—so use caution. The screw permits the user to adjust the light level by partially blocking the light path. If this does not give enough light, the next step is to increase the Gain to 100 and observe the light level. It should increase. If it goes to -50 or below, use the screw to adjust it to -50 and record that value. After calibration, you will need to record the Gain and the Light Level value with the name of the calibration. You will use these same Gain and Light Level settings when analyzing samples.

End of Video 1.

Begin Video 2

Collecting a Chromatogram

When the baseline stabilizes (stops drifting and the noise is less than 1% of scale), it's time to inject a standard.

Immediately before every injection, assure that the pump spring is compressed to 50mm between the white bearings and leave it until beginning the next analysis. Flush the loop with 91% isopropanol and then with air to remove the isopropanol, assure that the knurled injector knob is rotated toward the front then load the loop with sample or standard. Typically having the syringe about half full of sample or standard will fill the loop adequately. Be cautious to avoid loading any air bubbles into the loop.

While in the loop load (knob rotated towards the front) position, the sample syringe is connected to flow through the filter and the injection loop—the small semi-circle in the middle of the injection valve. The excess vents through the small tube above the syringe connection where it is collected on a paper towel. It's supposed to leak while the loop is being loaded. During the loop load step, the eluent by-passes the loop and goes directly to the column.

After the sample loop is loaded, leave the syringe attached to the filter and rotate the knurled knob towards the back of the instrument and immediately press **START** on the computer.

At this point, the eluent from the large syringe flows through the sample loop and loads the 10ul of sample from the loop into the column and the separation begins.

The Chromatogram will show several peaks that usually go off scale, up and down at about 1 ½ to 2 minutes after injection. This area is called the Vo or the Void volume. This is where un-retained components like alcohol show up. This area contains no valuable information.

The first peak of interest comes out at about 4-5 minutes. This is CBD. The THC peak will come out at approximately ten minutes.

For standards, the chromatogram is finished after the THC peak has come out and the back side of the peak rejoins the baseline. For samples, the user must allow the run to continue to assure that all components that were extracted flow out. Since the samples are natural products which contain differing amounts of cannabinoids, the user must watch and learn with each variety of Hemp until it is learned when the chromatogram is finished and all components are out.

Once the user is comfortable with the process, it is time to build a calibration. Each standard is injected using the same protocol above**, rotate the knurled knob of the injection valve towards the front of the instrument,** rinse the loop with 1 cc of 91% isopropanol, blow out the loop with 1-2 syringes filled with air, then load the loop by slowly pushing ½ to 1cc of standard through the loop.

Assure that the pump spring is compressed to 50mm and leave it, then simultaneously rotate the knurled know towards the back of the instrument and press **Start** on the computer. Collect and save chromatograms for each standard using the same procedure for each.

I suggest creating a file in Documents for C-Vue data. It may be desirable to separate standards and samples into separate files.

Once all standards have been injected and chromatograms saved, go to the **Analyze** tab and open the chromatograms for each standard one at a time and integrate the areas under the CBD and THC peaks as described below. The chromatograms are found by selecting the manila folder icon at the top and find the file where you stored it.

You will select **Raw Data** for the **Type File**. After you locate the file, double click and the file will be displayed on a new screen.

The chromatogram is usually compressed to show all peaks, adjust the Y axis by selecting -0.2 in the bottom left white window and +2 in the top white window. You should also select minimum peak height of 0.1 especially for low THC samples.

You should now have a good view of both CBD at about five minutes and THC at about ten minutes. Select the **MANU** icon by left clicking on the icon. A pop up screen will tell you how to pick the points to begin and end integration by **left clicking** the beginnings and endings of all peaks you want to integrate. I suggest that you visualize the baseline before the peak going under the peak and reforming after and **select** the points where you see that the component rises above the baseline and select these points for each peak's beginning and end. Place the cursor just under the baseline at the points you select.

After you complete the selections, **Right** click and you will see the points you have selected—the screen shrinks so you will have to expand it again. If you want to change the points, select the **Erase** icon and try again.

The next step is to select **Name/Cal Peaks** at the bottom left of the screen. Here you will **name** the peaks by selecting peak 1 in the peak number box and calling it CBD and, using the drop box to the right, select peak 2 and name it THC. I recommend that you use the **AREA** as the means for measuring the peak's amplitude. The use of peak height is used with other type detectors.

Record the amount (concentration in mg/g) and areas for each component in each standard in a table in a book that stays with the instrument.

Select the Create External Calibration Tab and copy the areas and amounts (concentrations in mg/g) into the table. Add the component name and, select AREA. I suggest selecting ANALYZE at the bottom of the table after each standard amount and area are added to the table. If a point is not a good fit, it will show up best when it is first added.

You should see a good, best-fit line through the data points. The equation for the best fit line is shown in the Results window to the right. The closer the r^2 is to 1, the more linear the fit.

To save the calibration, select the icon under the manila folder. I suggest naming the calibration for each component with the date completed and include the **Gain and Light Level** settings that were employed.

After the calibration process is complete, you will use this same box to label CBD and THC in samples and use the **Load Ext. Std**. beneath the Peak Name box to select the appropriate calibration for each component.

If you connect a printer to the computer, you can print out the calibrations and sample reports.

End of Video 2.

Start Video 3.

Sample extraction and Dilution

Since the C-Vue® HPLC is sold in several states and there may be differing rules for sample handling, I recommend that you contact the appropriate authority at the governing agency (probably the Department of Agriculture) for guidance regarding sample collection and sample extraction.

If you cannot reach an authority with a sample protocol, I suggest the following;

Sample according to the convention in your area, which may mean flowers from all over the plant or flowers from the top foot and separate according to your situation i.e. do you need to know about an entire field, the sunny side vs, the shady side, one green house as compared to another. These are individual decisions that are yours. You may want to analyze before or after drying or decarboxylation. Again, your decision.

Once you have collected a sample that you believe meets the requirements for the question at hand, I suggest that you homogenize the sample in a coffee grinder or equivalent and spread it uniformly over an area—a paper plate or an 8” x 11” sheet of paper—then divide the sample into halves, then quarters then eighths. I use the metal ruler used for pump control to divide the sample.

I suggest that you collect an equal amount from each eighth, homogenize and take from that sample for analysis.

I have had success extracting the sample as follows:

Weigh 0.2-0.4g of the sample to the nearest milligram into a tared sample bottle. Record the sample weight. Add 17-20g of 190 proof Ever Clear, record the total weight and cap, seal and label the bottle with the sample name, date and weights. Do not tare the balance between the sample weight and the weight of Ever Clear—you need the total of both.

The extraction step will need to be investigated as to how long to extract before you sample the extractant. Unless you can get a procedure that explains the process used by your governing agency, I suggest that you shake (or ultrasonic) the sample/Ever Clear mixture for 5-10 minutes then take a 0.2-0.5g sample and dilute with 17 to 18g of 70:30 Ever Clear: Distilled water. You will not get a good chromatogram if you inject the sample in straight Ever Clear. Again, record the weights of sample and the Ever Clear to the nearest milligram.

Cap the sample to minimize evaporation. If you lose solvent during the analysis, you will get erroneously high numbers.

The diluted sample should be injected the same way as the standards.

After the analysis is complete for the first sample, repeat the process after the extraction has proceeded and compare the amount of CBD and THC in the two samples. It is likely that you will find more CBD and THC in the second extraction. Repeat until the CBD and THC values are similar. Record the extraction time that it took to get to full extraction. Use this time for future extractions of similar samples.

Daily Maintenance

When the cannabinoids that are to be measured are extracted from plant tissue, there are other components that are extracted at the same time. These include proteins, fats and carbohydrates that do not absorb at the same wavelength as the cannabinoids so the detector does not respond to these as it does to the cannabinoids.

These components must be removed from the inside of the injector, column and detector because they will coat the column, tubing and the optics in the detector so the result is reduced separation and reduced signal. They will also induce detector noise and drift.

These components may be removed by flushing the system by the repeated injection of 91% isopropanol which may be purchased from a drug store or grocery. The injection syringe is filled with alcohol and the injection valve loop filled—with the knurled knob rotated toward the front. The alcohol is injected after the syringe plunger is compressed about 1/4 ". This process is repeated for two syringes filled with alcohol. The baseline usually drops indicating more light is passing through the detector after cleaning. Test the effectiveness of cleaning by selecting **Clear Zero** on the **Acquire Screen**. If there was debris on the optics, that was limiting the light to the detector, this should help. If the light level remains low, it may be time to replace the Zinc lamp. Contact your C-Vue® Representative to get a replacement.

Calculations

The process of completing an analysis of an unknown consists of the following process.

First a good calibration must be stored in **External Standards** for each component to be measured.

Next, about 0.2 to 0.3g of a representative sample must be weighed into a container—preferably UV-impermeable glass with 20ml capacity--on a recently

verified balance (using standard weights) to the nearest milligram (mg) and the weight recorded. The recommended extractant is 190 proof (95% ethanol) Ever Clear. Approximately 17-18g of Ever Clear is weighed into the same container and the weight recorded to the nearest milligram and the sample cap replaced immediately to minimize evaporation errors.

The extraction begins as soon as the alcohol and sample are mixed. The time it takes to complete the process must be determined by the user. The process for establishing this has been covered above. The extraction will be faster if the sample is shaken or placed into an ultrasonic bath.

After the extraction is determined to be complete, a sample of the extract is diluted by weighing approximately 0.3 to 0.5g of the extractant to the nearest milligram into another UV-impermeable bottle and the weight recorded.

This sample is immediately diluted with 17-18g of 70% 190 proof Ever Clear/ 30% Distilled water and mixed by slowly inverting 10m to 20 times.

After the sample is homogenized, it is ready for injection. It is critical that one of the round tube filters be attached on the sample and eluent inlets to the injector because the filterable debris that is in the extract and eluent will cause problems of plugging and inaccurate volumes if they are permitted into the injector.

After the chromatogram is collected and saved, the CBD and THC peaks are located by comparison with the standard.

Select the **Analyze** tab, select **Raw Data** as the file type and open the sample file. Expand the Y axis to get a better view of the peaks—typical range of -0.2 to 2 usually works. Select minimum peak height of 0.1, select the **MANU** icon and follow the on screen directions to select the peak beginning and ending points for each peak. If you want to change the points, select the erase icon and start over.

Once you are satisfied with the manner the peaks were integrated, select **Analyze** in the bottom left of the screen. You will be asked "**Is the data correct**?" If you previously entered the data in the **Data Entry** tab at the top left of the screen, the data should be correct for the sample. If not, then answer **No** and fill in the correct data on the screen. When you select "**Yes**", the table will display the **Name, Retention Time** and the **concentration** of the CBD and THC in mg/g in the **Amount** column.

Validation of the calibration

The calibration should be useful for several days as long as the same **Gain** and **Light Level** values are used. But this must be verified each day. The ideal situation would be to recalibrate each day. It may be acceptable to re-validate the calibration by re-injecting one of the mid-point standards and correcting for any variation by using the third factor in the weight/dilution page to correct for differences in peak area.

This is done by placing the Area for the CBD that was used in the calibration in the box for weight 3 and the Area for the CBD on the day for which we are

validating the calibration in the box for dilution 3. This process is to validate the data given by the calibration on a later date.

It is recommended that the validity of this step be established before data is used externally.

C-Vue HPLC training links.

Video 1 covers setup, connections and starting the pump

https://youtu.be/Ph7qxlWMYPI

Video 2 covers the process of building a calibration

https://youtu.be/vx5kinid7-I

Video 3 covers running a sample and generating a report

https://youtu.be/hHXii4PoRB8

Please provide feedback regarding the effectiveness of these in making the C-Vue HPLC useful.

Terry