



New Frontiers in Cannabis and Hemp Testing, Part 2



Introduction

As the demand for the development and validation of accurate and precise testing methods that support cannabis and hemp regulations evolves, so does the need for connecting with key players who are working behind the scenes to determine more efficient ways to analyze these products.


New Frontiers in Cannabis and Hemp Testing, Part 2, features the insight of various scientists at Agilent Technologies who share their thoughts on:

- Accurate and Simple Quantification of THC and CBD in Beverages Containing Microemulsions and Nanoemulsions
- Derivatization Chemistries for the Analysis of Cannabinoids Using GC-MS
- Chemovar Characterization using Time-of-Flight Mass Spectrometry

Readers also have access to a number of resources throughout this guide, including:

- Educational videos that support best practices for cannabis testing labs
- A Brief Review of Derivatization Chemistries for the Analysis of Cannabinoids Using GC-MS
- A virtual symposium delivered by thought leaders who discuss understanding the intersection of product quality and safety

We hope the resources available in the guide will serve as innovative, reliable solutions to issues faced by those who test cannabis.



Accurate and Simple Quantification of THC and CBD in Beverages Containing Microemulsions and Nanoemulsions

Interview with Jean-Francois Roy
and Christophe Deckers

| Improving the speed and accuracy of potency testing in cannabis beverages.

How can laboratories avoid common pitfalls when doing potency testing in beverages? Here, Jean-Francois Roy, M.Sc., Application Scientist, Mass Spectrometry at Agilent, and Christophe Deckers, M.Sc., Application Scientist, Sample Preparation at Agilent, explain how accurate quantification of tetrahydrocannabinol (THC) and cannabidiol (CBD) by liquid chromatography with ultraviolet detection (LC-UV) can be negatively affected by several factors such as the stability of cannabinoids and their low solubility in water. Learn tips and tricks for streamlining the cleanup of infused beverages, increasing lab productivity, and eliminating unpredictable results in potency testing.

What are the top challenges with potency analysis of cannabis beverages? Can you use the approach for beverages as edibles?

A sample preparation method that will work for flowers or edibles most likely needs to be adapted for beverages. One reason for this is that cannabis beverages tend to contain much lower concentrations of cannabinoids than other cannabis products. Therefore, the analytical procedure must be optimized to provide adequate sensitivity. The one-size-fits-all approach may

appear to be successful at first, but we have had multiple customers reporting issues with sensitivity and robustness in the long run.

To keep the hydrophobic cannabinoids in solution, cannabis-infused beverage manufacturers use emulsions that contain oil and emulsifiers. These excipients can clog analytical columns and degrade their performance. Thus, columns require more maintenance, which increases laboratory costs. Moreover, common emulsifiers like polysorbate 80 and phospholipids will cause ion-suppression in electrospray ionization sources of LC-MS systems. A well-suited method will take these factors into account. For instance, the emulsion must be broken down as part of the sample preparation to reach the lower detection limits.

Another challenge is that plastic and aluminum beverage containers can adsorb some of the cannabinoids. This issue is less of a problem in hydrophilic glass bottles but can be significant in plastic bottle or can linings that have been coated with hydrophobic polymers. Warming the sample in the container before sampling will help to desorb the compounds.

Lastly, the solubility of cannabinoids in cannabis-infused beverages tend to be unstable. The emulsions may break over time, resulting in separation and precipitation, and could mean different quantification results depending on the age of the sample. Therefore, it's a good idea to test beverages

as soon as possible upon their arrival to remove any time-related variability. Once opened, oxidation begins, so the stability timeline from that point in time is even shorter.

What's the best procedure to prepare samples before LC-UV and LC-MS detection?

We performed a comparison of all currently reported sample preparation techniques for beverages. Agilent unbuffered QuEChERS performed the best. A beverage volume of 10 mL was warmed to start breaking down the emulsion, then degassed via sonication. 10 mL of acetonitrile were then added, and the resulting solution was briefly mixed. Agilent QuEChERS extraction salts were then added, which triggered the separation into a mostly organic phase and a mostly aqueous phase. The polar interferants are in the aqueous layer. The organic layer contains the cannabinoids of interest but also still has many of the oils.

The top (organic) layer was then subjected to various cleanup techniques, including filtering with a PTFE filter, using three different QuEChERS dispersive phase extractions (fruit and vegetable with PSA, pigmented with GCB, and fatty sample with C18), and running the sample through an enhanced matrix removal (EMR) cartridge. The EMR cleanup provided the cleanest baseline and strong UV signal.

Here's a breakdown of the optimal method: put the plastic bottle or aluminum can in

warm tap water for 10 minutes and then open the container. Sonicate at approximately 50°C for 20 minutes to degas and start to break the emulsion, then transfer 10 mL into a 50 mL centrifuge tube and put on a mechanical shaker for 3 minutes (1500 RPM). Add Agilent Original extraction salts (PN 5982-5550), shake and degas, then shake for another minute in the mechanical shaker. Centrifuge at 3000-5000 RPM for 5 minutes. Remove 2 mL of the supernatant and dilute with 500 µL water. Pour into a Captiva EMR-Lipid tube (PN 5190-1003), and let drip through, or use an SPE manifold for faster elution and to fully empty the cartridge. The fully detailed procedure can be found in App Note 5994-3791EN.

Do we need to control the tap water temperature for sample warm up?

Not really. The samples are subsequently incubated and sonicated at well-controlled temperatures, which promotes cannabinoid desorption from the walls of the container.

Are there any downsides to using QuEChERS for THC beverages?

Using QuEChERS salts as part of a liquid/liquid extraction is an excellent way of breaking the emulsion and extracting

the unwanted hydrophilic compounds. The QuEChERS dispersive phases are less useful, however. They do not provide sufficient selectivity between the cannabinoids and the fats and surfactants found in the sample. Separating the lipids from the cannabinoids requires much higher selectivity than can be obtained from C18 or other classical dispersives.

Can you explain enhanced matrix removal (EMR)?

EMR is a filtration and selective chemical capture system. The sorbent material is highly porous, with nanopores that are sized to selectively capture long and straight alkane chains like in long chains fatty acids, phospholipids, detergents and triglycerides. Other molecules, such as cannabinoids, do not fit and are not captured. In practice, the EMR format is a tube with the material in the bottom. The sample is poured in the top and flows through the EMR bed by gravity to be collected below. The flow can be driven faster with pressure, but effectiveness can decrease if it flows too quickly. Using a Positive Pressure Manifold (PPM) helps to fully empty EMR cartridges after the sample was filtered.

Why is it important to optimize your LC method to save solvents and separate some critical isomers?

The optimal LC-UV method can achieve baseline resolution of all compounds of interest in a minimum amount of time. Less time means higher throughput and lower solvent

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Less time means higher throughput and lower solvent consumption and disposal costs.

consumption and disposal costs. When using LC-MS, it is possible to sacrifice some LC resolution when compounds of different mass are being measured, but when dealing with isomers, as is the case with cannabinoids, it is essential that the chromatography provides baseline separation.

Why can't we just dilute in methanol and inject?

Methanol extraction and direct injection is a common method for raw materials. Unfortunately, while methanol extracts cannabinoids very well, it also can extract a lot of matrix compounds, which can lead to dirty samples. Beverages have the added problem that they are mostly water. Adding methanol creates a water:methanol mixture that is not optimal for the solubility of cannabinoids.

What effect does temperature have on the stability of THC and CBD?

At the temperatures typically observed in the lab, we demonstrated the stability of samples for up to a week or more. However,

elevated temperatures may shorten the sample's shelf life.

Have you considered SPE extraction to remove surfactants?

Yes, we have. SPE is a useful and effective technique to clean and concentrate analytes. It can be very helpful when a low detection limit is needed. Our first instincts were that SPE would be needed for beverages due to the low concentrations in beverages, but we found that it was not required. EMR provides a similar cleaning ability to SPE and is more selective for the separation of cannabinoids from lipids and surfactants.

What are the lowest THC and CBD concentrations you managed to test using this procedure?

In our tests with matrix-matched samples, we were able to show linearity down to 0.5 $\mu\text{g/mL}$ using LC-UV detection.

Why do you need to warm up the bottle or can before opening?

In the aqueous environment of beverages, the highly hydrophobic cannabinoids will tend to adsorb to the walls of the container, especially if those walls are of organic source, like plastic bottles or the polymeric linings of aluminum cans. Warming the contain initiates the desorption of the compounds from the walls of the container. It can also provide a head start in breaking the emulsion.

You mentioned labs should not make calibration curves using methanol or ethanol. Do we need to add THC to a beverage with no cannabis and make a calibration curve?

The best-case scenario is always to matrix-match the calibration curves, when possible. We have seen different response levels to THC and CBD in matrix vs neat solvents using LC-UV detection. Matrix-matched calibration allows to account for interferences like potentially coeluting peaks or ion suppression.

When you talk about matrix match for standard curves, do you mean using a matching blank beverage for the standard curve? What if your lab is testing a variety of different beverages at the same time? Does a “generic” beverage exist?

Each beverage will have its own list of excipients, and different choices and concentrations for the fats and surfactants to make the emulsion. Ideally, when a sample is quantified, it should be against a

calibration curve that is based on the same matrix as that sample. We believe there is no all-purpose beverage that can serve as a matrix blank. Each matrix is potentially different and neat solvents don't behave the same. For good accuracy, its best to add standards in sample matrix before the EMR cleanup.

Would normal phase chromatography work better for oily samples?

In theory, yes, but there are challenges. Without a similar degree of sample cleanup, the high concentration and number of fats will result in a crowded chromatogram and likely co-elutions with the compounds of interest. Because water is the strongest possible mobile phase component, normal phase separations are also very sensitive to even small amounts of water in the sample. Even oily edibles will have a fair amount of water in the sample and must be thoroughly dried.

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Getting it Right: From Sample Preparation Through Data Analysis, Best Practices for Cannabis Testing Labs

In this series of brief videos, Agilent cannabis testing experts define the dos and don'ts for robust and reliable sample preparation, testing methods for cannabinoids in challenging matrices, customized tips for edibles sample preparation, and residual pesticide analyses using both LC/MS/MS and GC/MS/MS.

We invite you to get a cup of coffee, relax, and watch these light-hearted but highly educational videos with your colleagues. Enjoy!

Watch these videos now: www.agilent.com/chem/cannabis-tips

Agilent products and solutions are intended to be used for cannabis quality control and safety testing in laboratories where such use is permitted under state/country law.

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Topics presented:

Potency Testing in Fatty Edibles, Like Chocolate and Baked Goods

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Derivatization Chemistries for the Analysis of Cannabinoids Using GC-MS

Interview with Anthony Macherone, PhD

| Best practices for analyzing derivatized cannabinoids.

To quantify the total amount of Δ^9 -tetrahydrocannabinol (THC) and other cannabinoids in cannabis samples, many analytical laboratories turn to high performance liquid chromatography (HPLC) with ultraviolet detection. Gas chromatography (GC) can also be used, but the laboratory must determine if it will chemically modify (derivatize) the target cannabinoids or analyze them in their natural state—a choice that affects sample preparation procedures and GC system conditions.

Here, Anthony Macherone, PhD, Strategic Technical & Content Scientist at Agilent Technologies, Inc., addresses some commonly asked questions about the use of GC-mass spectrometry (MS) for the analysis of derivatized cannabinoids in hempseed oil matrix. He also discusses sample preparation and synthetic conditions for silylation.

Why must the laboratory decide whether to do the analysis with derivatized or underivatized cannabinoids?

If the analyst wants to perform analysis by GC, it is necessary to cap the polar moieties like carboxylic acids and hydroxyls. This reduces the polarity of the molecules, resulting in an increased volatility, shorter run times,

and better peak shapes. Functionalization may also facilitate other ionization methods. For example, functionalizing with a trifluoroacetic acid would give the compound a strong signal under negative chemical ionization conditions.

Derivatization is particularly important for phytocannabinoids because their acid forms are very easily decarboxylated. The process can take place during drying, upon exposure to light, and in the hot inlet of a GC system. In fact, there might even be further degradation of THC to cannabinol. Derivatization mitigates degradation and allows the analyst to obtain a full cannabinoid profile. If an analyst wishes to differentiate the acid and neutral forms by GC analysis, then derivatization is essential.

The most common derivatization chemistry is via silylation. Adding heavy moieties like silylation typically means that the silylated compounds will often have a higher boiling point. However, their overall polarity will be substantially reduced. The net effect is that their mobility on the GC column is increased, and they elute faster. The decision of whether to derivatize is ultimately based on the analytical goals.

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A Brief Review of Derivatization
Chemistries for the Analysis of
Cannabinoids Using GC-MS

What is the difference between hemp and marijuana?

From a biological perspective, they are the same species, and in some cases, even the same strain grown under different conditions. The legal definition set by the US Drug Enforcement Administration (DEA) comes down to Δ^9 -THC concentration. A sample over 0.3% is considered marijuana. Below that, it is considered hemp. The US Federal Register similarly defines hemp as any part or derivative of the plant *Cannabis sativa L.* in which there is no more than 0.3% of "tetrahydrocannabinols." This definition includes any salts and isomers of Δ^9 -THC. The USDA who governs the production of hemp in the U.S., requires that all tests for potency must be in a DEA-registered laboratory. The standards were set at a time when cannabis varieties were typically much less potent. It is now not uncommon to find samples with potencies of 10–30%. Although the stipulations of the Farm Bill do not specifically mention some isomers like Δ^8 -THC, they still fall under the classification as an isomer of Δ^9 -THC.

Why do I need to chromatographically resolve/separate Δ^8 from Δ^9 ?

THC has several isomers that involve the movement of a carbon-carbon double bond around the ring. All these isomers have the same mass and UV response. If they are not separated chromatographically, a UV detector will not be able to differentiate them. With GC-MS, Δ^8 -THC and Δ^9 -THC are readily

identified by unique fragmentation patterns and ion ratios, but chromatographic separation is still required.

What are common artifacts that may appear in my analysis?

If derivatizing, then excess silyating reagents will probably be seen, and reactions with trace water or acids will cause hydrolysis products to be present. If dichloromethylene (DCM) is being used as a solvent, some amylene will likely be seen, which is used as a free radical scavenger. It is however, recommended not to use DCM as a solvent since it can become acidic as it ages and can react with cannabidiol for synthesize Δ^8 -THC. Artifacts can also result from the septa and liners, especially if they have not been handled meticulously. Last, some column and septum bleed is almost inevitable, especially as they age. Inlet septum and vial septum bleed are usually identifiable as regularly spaced peaks (representing siloxane oligomers) in the total ion chromatogram while column bleed tends to manifest as a rising baseline as the oven temperature increases.

Can we use the autosampler on our GC to perform derivatization reactions using a default work list, and do we need to purchase additional software?

Modern autosamplers can perform several different operations as part of their standard programming. It's completely possible to program an autosampler to do the reagent additions and mixing necessary for some

derivatizations. This works best if the reaction is fast. The benefit of the automation tends to be lost if the reaction is slow enough to become the rate-limiting step in the analysis. In an optimized system, the derivatization reaction will be started while the previous separation is running and will ideally be done in time for the next injection. In terms of instrumentation, you might want a second tower on your autosampler with a larger syringe.

What's the approximate variance in THC levels between GC-MS and HPLC?

If you have two properly controlled analytical methods, ideally using well-chosen internal standards, and best practices with quality controls, an acceptable variation would be on the order of 5–10% between the two systems. With multiple injections, however, the mean and the standard deviation should be very close. Most of the variance that is seen in the industry is seen between labs and is usually down to a failure of one or more labs in performing the appropriate quality controls or variability in sample preparation.

How stable are TMS cannabinoid derivatives? Is there noticeable peak area drift for longer sequences?

The derivatives are fairly stable, as long as they are kept dry. They will generally be stable while sitting in vials waiting to be injected for the first time. Once the vial septum has been pierced, however, atmospheric water can make its way in and artifacts may start to show up within hours. With some septa,

septum bleed may also start to show up. Septa with PTFE on the underside are a good choice. Tray chillers will also preserve the life of a derivatized sample. If a sample has been injected, and you wish to keep it overnight for reanalysis, the best bet is to recap it and refrigerate it.

Do you have a recommended vial/vessel types to perform derivatization?

First and foremost, the vessel, sample and all solvents must be dry, and only polar aprotic solvents be used. If the sample is in a protic solvent, like methanol or ethanol, it must be dried down and exchanged into a dry polar, aprotic solvent like ethyl acetate.

The material or form of the vessel is not critical but maintaining dryness and preventing contamination are both important. Derivatizing directly in the sample vial minimizes transfers, and so helps prevent contamination and exposure to moisture in the air. When using a sample vial, screw cap or crimp caps are both acceptable, but having a septum with a Teflon inner surface is preferable to minimize septum bleed.

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Chemovar Characterization using Time-of-Flight Mass Spectrometry

Interview with
Anthony Macherone, PhD

| *How best to characterize cannabis chemovars.*

Many cannabis species are complex, comprising more than 500 identified chemicals. As a result of decades of interbreeding and the complexity of the plants and their secondary metabolites, differentiating plants by strain identifications like *Cannabis sativa L.* or by chemotype is much less relevant for contemporary cannabis—especially when you're interested in looking at specific properties of the plant.

More recently, the concept of *chemovars* has been introduced. Chemovars consider cannabinoid, terpene, and flavonoid chemical profiles as chemical markers for a better overall characterization of the plant. A primary analytical methodology for chemovar identification is quadrupole time-of-flight mass spectrometry (qTOF), which offers high resolution accurate mass information for feature identification, structure elucidation, and annotation.

In this article, Anthony Macherone, PhD, Strategic Technical & Content Scientist at Agilent Technologies, Inc., answers key questions about chemovar identification in cannabis and hemp extracts and the use of qTOF.

What are the differences among cultivars, strains, and chemovars?

The cannabis species such as *Cannabis sativa* or *Cannabis indica* have been highly cultivated, selectively bred, and interbred. Some plants are bred for specific properties and therapeutic purposes such as to produce high psychoactive Δ^9 -tetrahydrocannabinol (THC), cannabidiol (CBD), or cannabigerol (CBG) concentrations. When just looking at the concentrations of THC, the strains available have changed from those containing 1% to 2% a few decades ago, to modern strains with as much as 30% THC. Moreover, selective breeding for fragrance or flavor has affected the levels of other compounds, including terpenes. In addition to the plant genetics, growing conditions, and a version of terroir (more often associated with wine) will influence the chemical makeup.

Generally speaking, a *cultivar* is a family of plants, produced by sexual reproduction and grown from seeds. They are generally identified by their phenotype (i.e., the outward appearance and measurable features). Unlike other cultivated plants, the breeding of cannabis has been mostly

performed informally with little record keeping or tracking of parentage.

When a particularly preferable plant is found, a strain is often created. A *strain* is a set of plants that have been produced via cuttings. They are all genetically identical clones of each other.

More recently, the concept of chemovar has been introduced. A *chemovar* considers the cannabinoid, terpene, and flavonoid chemical profile of the plant to provide the overall characterization and classification. This is essentially a fingerprinting technique that does not directly consider the medicinal or flavor properties of the compounds being measured.

Are there really 500+ cannabinoids given that there are just eight phytocannabinoid acids synthesized in the plant?

The genome of *C. sativa* only encodes for the enzymes that produce a total of eight cannabinoid acids. Downstream processes, including decarboxylation, oxidation, irradiation, or isomerization can produce far more cannabinoid compounds. Most of these processes take place after harvest and might depend on storage and processing conditions. The total may reach into the hundreds, but whether it reaches 500 is unclear. When cannabinoids are processed, often isomerization takes place, producing a wide collection of isomers. Another consideration is stereochemistry. For each chiral center, 2^n diastereomers can exist, as well as epimers and enantiomers. Given

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these facts, the probability of hundreds of unique cannabinoids is high. Of course, absolute identification is hampered by the fact that there are only about 30 commercially available cannabinoid reference materials.

To summarize, 500+ cannabinoids may be a stretch, but if one also considers other important flavor components, like terpenes and flavonoids, the count of measurable compounds can easily exceed 500.

Among cannabinoid, terpene, and flavonoid profiles, which is best suited for liquid chromatography (LC), and which is best suited for gas chromatography (GC)?

Having both available is the ideal situation, as it provides full coverage as well as some orthogonal verification where there is overlap. Cannabinoids are generally best separated by LC methods since it conserves the acid forms. Terpenes tend to be more volatile. While some can be readily separated by LC, GC is generally more applicable. LC is more appropriate for flavonoids (polyphenols) but there is some overlap with GC.

The above dialogue only considers chromatography. Mass spectrometry (MS) must also be considered. There are rationales for electrospray ionization (ESI) with LC/MS or electron ionization (EI) with GC/MS. For example, many terpenes are hydrocarbons (C_5H_8 isoprenoids) and do not possess a molecular moiety amenable to form a quasi-

molecular ion in ESI. Therefore, EI is better suited for this analysis. As another example, flavanoids have oxygen and hydroxyl moieties amenable to ESI in both positive and negative modes and although many do respond to EI, ESI with LC/MS systems is a better choice.

What can you do with qTOF in terms of identifying chemovars?

One advantage of time of flight is high resolution, accurate mass (HRAM) information. This capability allows the system to discriminate between true analytical signals, matrix, interferences, and noise. It further facilitates elemental analysis to generate putative empirical formulae and chemical structure information using HRAM MS/MS. A qTOF system can provide a broad range of information about a sample in a single run. For example, an LC-qTOF system can readily identify cannabinoids, flavonoids, and some terpenoids in a sample and provide a chemovar fingerprint. Coupled with GC-qTOF, a full cannabinoid, terpene, and flavonoid profile can be obtained.

What about identifying contaminants, adulterants, or pesticides? Which instrument(s)/platform(s) are best suited?

The great thing about qTOF mass spectrometry in either the liquid or the gas phase is the rich amount of chemical information in the data.

Once the chemical features are distinguished from the noise, matrix, and artifacts, exogenous compounds like adulterants and xenobiotics like pesticides can be putatively identified using commercially available mass spectral databases.

What is a common workflow when analyzing cannabis?

Analysis of cannabis always starts with sample preparation. In most cases, sample preparation differs for the various cannabis or cannabinoid product matrices like chocolates, gummies, candies, beverages, and other edibles. It cannot be over-emphasized how important sample preparation is. If done well, everything downstream including data acquisition, data analysis, and reporting will be robust and repeatable. If sample preparation is done poorly, it will quickly foul the instruments and increase maintenance needs which is a euphemism for downtime and lost revenue.

Can you talk about the significance of your findings when analyzing CBD oil for pet supplements?

In this study, we obtained five lots of six different brands of CBD oil pet supplements. We performed untargeted analysis using GC-TOF. Both standard EI and low energy EI were used. We performed feature extraction and annotation on the resulting data. Compounds detected included fatty

acids and their esters, di- and triglycerides, terpenoids, cannabinoids, tocopherols, steroids, and sterols, and more. We statistically differentiated each product and identified commonalities and unique chemical profiles. Interestingly, we found that the THC content for two products exceeded the 0.3% by weight threshold defined by the U.S. DEA. This was confirmed quantitatively with HPLC-UV.

Are there now known cannabis matrix interferences that can be background-subtracted out?

That may depend on how you define an interference. Some chemovars will have naturally occurring compounds that coelute with other compounds of interest. There are also high-concentration compounds like chlorophyll that you'll want to minimize with a good sample preparation method. Because of the high variability among samples, it would be hard to establish a good matrix blank. It will be more reliable to use high-efficiency separations and high-resolution mass spectrometry to differentiate the compounds of interest from potential interferences.

Can you point to literature on how much variation to expect between samples of a single lot of flower?

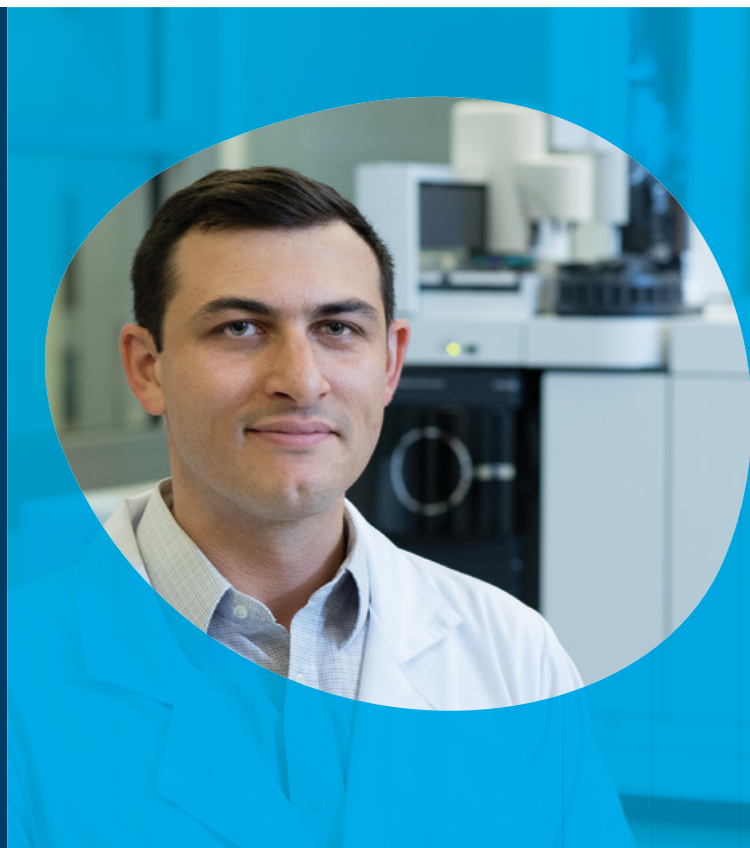
There is some good work from the hemp program of the University of Kentucky

(<https://hemp.ca.uky.edu>). The chemical makeup of hemp can be affected by both genetics and growing conditions. Even within a single lot, there can be variation among plants at the front or the back of a lot based on sun/shade or watering conditions.

Agilent products and solutions are intended to be used for analytical testing of cannabis in laboratories where such use is permitted under state/country law.

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