cannabis science and technology

LC GC

Cannabinoids in Everyday Products: Keys to Successful Laboratory Testing

Agilent

Simple, Accurate, and Reliable Quantification of THC and CBD in Cannabis-Infused Chocolate Edibles, Pastries, and Candies

Simple and rugged potency testing of edibles depends on a well-designed sample preparation method, which is easier with modern sample cleanup systems.

Overview

As the size and legal status of the cannabis market grows and stabilizes, the need for reliable testing is unavoidable, including tests for potency and content uniformity. A good potency method should be able to accurately quantify the levels of cannabinoids in a given product. The growing legitimacy of the industry will depend on the establishment of clear and identifiable standards for labeling and a track record of adherence. Among the many cannabinoids present in cannabis materials, the most commonly quantified are (-)trans-Δ9-tetrahydrocannabinol (THC) and cannabidiol (CBD).

In 2015, Vandrey et al. surveyed 75 cannabis-containing edible products for labeling accuracy (1). Only 17% of the products were within +/- 10% of the label claim, 23% contained more THC than labeled, and 60% contained less (1). This could be because of poor tracking of ingredient addition but could

also be the result of incomplete mixing during production, resulting in poor content uniformity. This study highlights how challenging potency testing can be, as well as the need for more robust and consistently applied methods for potency in edibles.

From an analytical chemist perspective, any challenging application needs to be optimized on three main sections or pillars: sample preparation, chromatography, and detection. The chromatography and detection are relatively straightforward but are also dependent on the analyst's ability to render the complex food or beverage sample into a relatively simple, clean solution that is ready for injection. An optimized chromatographic method is applicable to any sample, provided that the sample has been adequately cleaned and rendered into an injectable form.

The sample processes can be complicated by the fact that edibles can vary in nature, ranging from relatively simple beverages to high sugar or high fat edible products. This article will present simple methods to address different classes of edibles that an analyst is likely to encounter.

Comparison of Sample Preparation Techniques

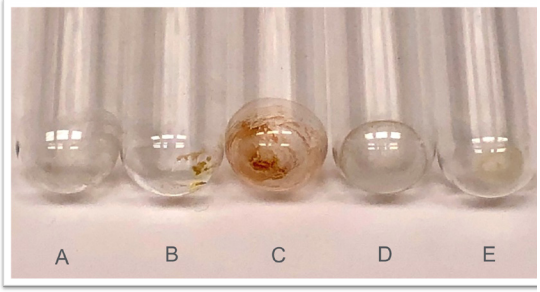
Any sample preparation technique must be able to convert the sample from a solid form into a liquid form, removing as much extraneous material as possible while leaving as much of the active ingredients as possible.

Traditional procedures, such as grinding, homogenizing, and cryomilling, can be time consuming and difficult with sticky materials such as chocolate. Methanol is often the chosen solvent for extraction but tends to produce dirty samples.

While QuEChERS (quick, easy, cheap, effective, rugged, and safe) is a popular extraction and cleanup technique—with C18 chemistry that is effective at removing lipids—high fat samples can overwhelm its capacity. Additionally, C18 does not provide sufficient selectivity between fats and cannabinoids.

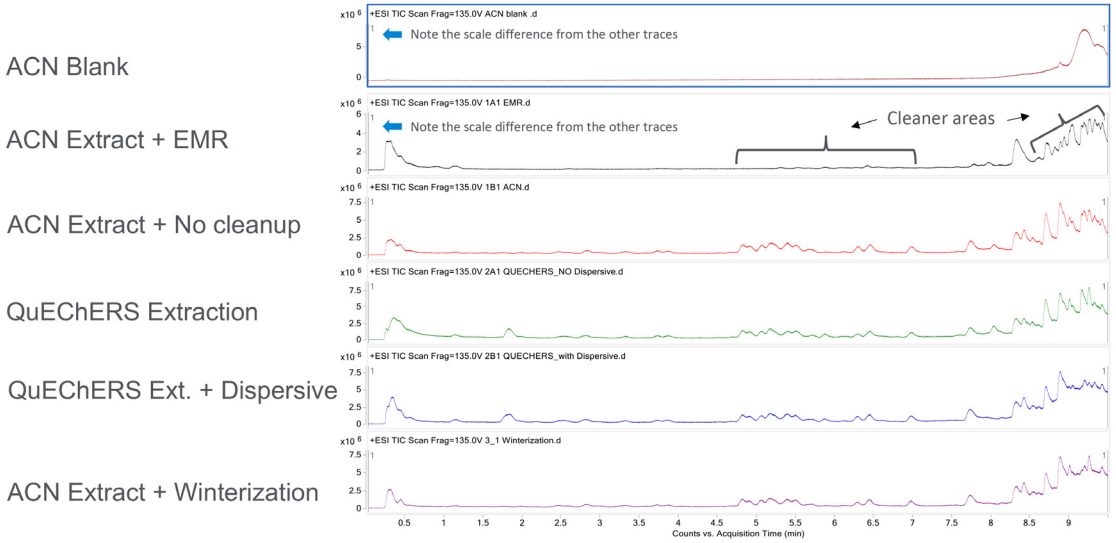
Winterization is another common technique to handle fats. This is because in this technique, the sample is frozen,

FIGURE 1. Sample cleanliness comparison after sample preparation and dry-down. Gravimetric analysis: average weight (n=3) of residuals after dry-down (in mg) for each treatment.



A: Acetonitrile extraction + Captiva EMR-Lipid cleanup (0.4 mg)
B: Acetonitrile extraction (no treatment) (30.3 mg)
C: QuEChERS extraction (26.7 mg)
D: QuEChERS extraction + dispersive for fatty samples (8.3 mg)
E: Acetonitrile extraction + cold stabilization (winterization procedure) (0.4 mg)

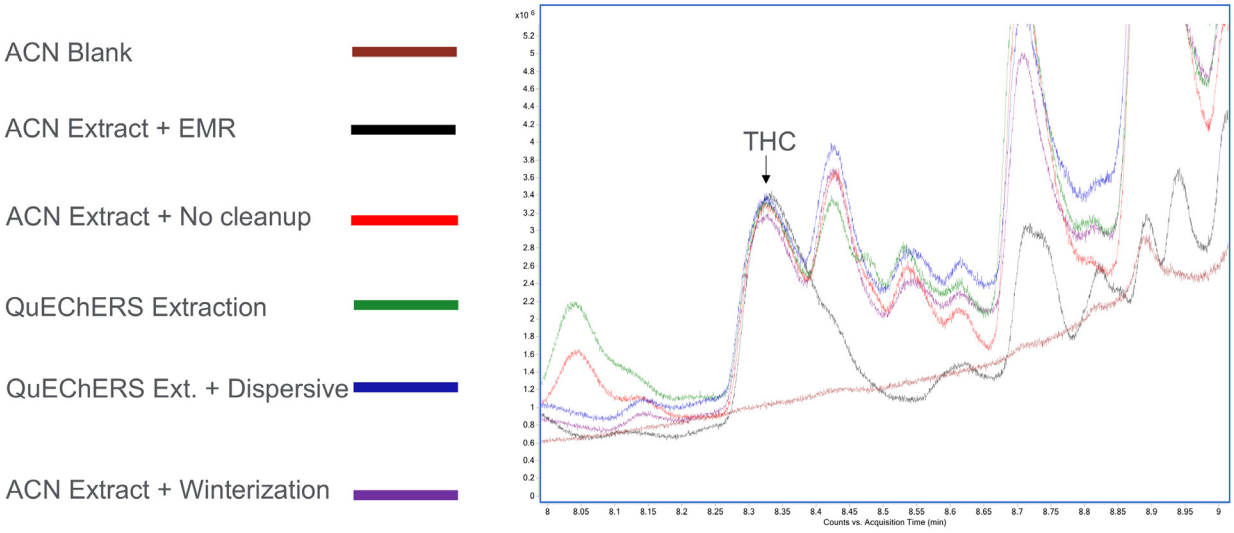
FIGURE 2. LC/Q-TOF TIC comparison of various cleanup treatments of an acetonitrile extract from a THC-infused milk chocolate.



which precipitates the fats. Unfortunately, winterization also suffers from poor selectivity and will remove some THC as well.

A full study was performed to compare sample preparation techniques for chocolate. After processing, all samples were blown to dryness. The quality of the sample cleanup was evaluated based on visual inspection (how much visible pigment remained) and by gravimetric analysis (a lighter sample indicates more matrix was removed). In addition, sample cleanliness was compared by LC/Q-TOF. The list of protocols and visual results can be seen in FIGURE 1. Based on mass, simple acetonitrile extraction produced the heaviest sample, with a straight QuEChERS extraction close behind. Visually, both extractions had significant coloration from pigments still in the sample. Combining QuEChERS with the dispersive cleanup of the fats yielded a much cleaner and lighter sample. Enhanced Matrix Removal (EMR)-Lipid cleanup provided the cleanest-looking sample and only 0.4 mg of material remained. This tied for mass with the sample that was extracted (and then winterized) to remove lipids; however, the latter still had some coloration, and the process was quite time consuming.

FIGURE 3. LC/Q-TOF TIC comparison of various cleanup treatments of an acetonitrile extract from a THC-infused milk chocolate.



The samples were subjected to LC/Q-TOF with total ion count detection. The comparisons can be seen in FIGURE 2. The trace for the EMR-Lipid sample is by far the cleanest. Also note that the scale of the Y-axis is lower for that chromatogram. The one exception to the relative flatness of the baseline is the peak at about 8.3 minutes, which is THC. There appears to have been significant loss of THC in all the other samples.

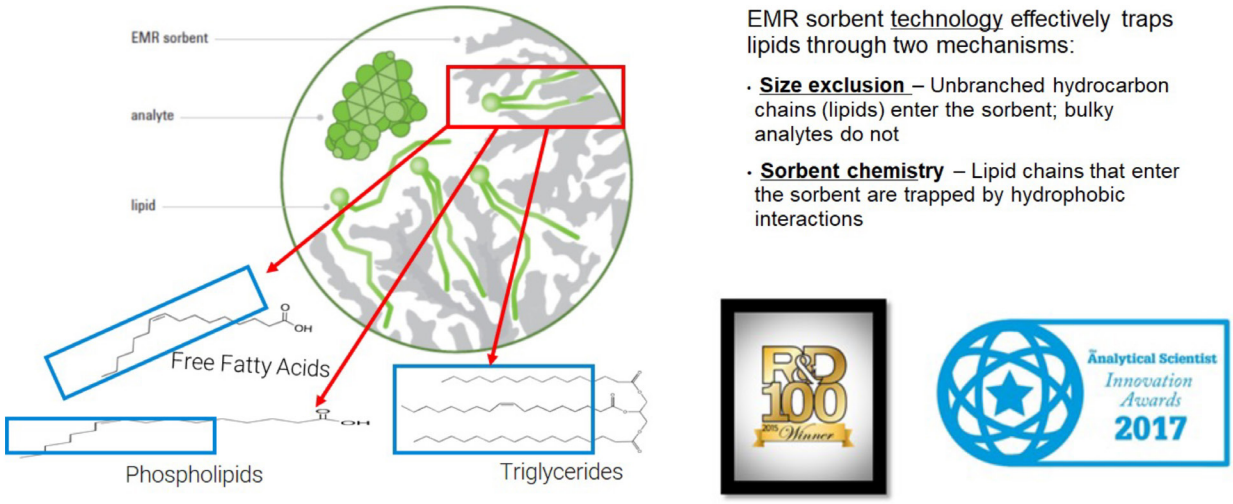
When the chromatograms are overlaid, focusing on the THC elution window (FIGURE 3), it is clear that both the size and the relative purity of the THC peak are far superior in the sample that was cleaned with EMR-Lipid. As a result of superior extraction with Captiva EMR-Lipid, quantification of THC and CBD by LC–UV consistently provided 10–20% higher signal compared to other extraction methods, with winterization having the most loss.

Optimized Chocolate Method

Chocolate can be quite challenging as a sample, as it can melt and become sticky during processing. The high fat content (30–40%) can present specific problems. For instance, if fats are not removed, they can clog the column. Removal should be done selectively, however, as cannabinoids are fat soluble and can also be removed in addition to the fat content (if proper care is not taken). Chocolate also contains flavonoids, which can interfere with quantitation.

A sample processing method has been developed that can be applied to dark, milk, and white chocolates (which counterintuitively

FIGURE 4. Captiva EMR-Lipid technology.



can be the hardest to clean). During the first step in this method, the bars were chopped with scissors into small pieces. One gram of chopped chocolate was then placed in a 50-mL centrifuge tube. Using a disposable tube eliminates the risk of sample carryover. The sample was then chilled for 20 minutes in a -20 °C freezer to ensure the chocolate was solidified. 10 mL of cold, acidified (2% formic acid) acetonitrile was then added, as the presence of the formic acid disrupts the binding of cannabinoids with proteins. After 5 minutes at high speed on a mechanical shaker, the sample was sufficiently homogenized. Centrifugation at 3600–5000 rpm for 5 minutes yields a clear supernatant.

However, at this point in the method, the supernatant was not fully clean yet. Next, 2 mL were transferred to a 15-mL polypropylene centrifuge tube, a 500 µL of water was added, and the sample was vortexed. This starts the precipitation of proteins, as evidenced by the solution turning milky again. The sample was then poured into a 3-mL Captiva EMR-Lipid tube with a new disposable centrifuge tube beneath it. The sample was allowed to flow through the cartridge under gravity, and then an additional 1.5 mL of 4:1 ACN:water was used as a rinse to fully elute the THC. The result was a clean sample free from potential interferants. To ensure homogeneity, the collection tube should be vortexed before injection.

The Captiva EMR-Lipid system provides a reliable way to remove the remaining proteins and lipids. The system is a relatively new technology, which uses both hydrophobic interactions and

stearic interference to selectively capture lipids from a sample while leaving other molecules behind. A diagram of the EMR sorbent technology can be seen in FIGURE 4 . The sorbent contains many pores that can accommodate lipid chains but not lower aspect ratio molecules, such as cannabinoids. The capacity is high enough to clean high lipid samples.

This procedure has been shown to be applicable to any high-fat edible product. One caveat is that if the product has nuts, chocolate chips, or other chunks in it, it needs to be ground to a homogeneous powder before weighing out the sample. This sample preparation method has been shown to provide linear results (R^2 > 0.999) over the range 0.5–100 µg/mL.

Hard Candies and Gummies

Candies are generally much easier to work with than chocolate because candies typically do not contain as much fat, but they still present challenges. One such challenge involves gummies, which are sticky and difficult to process. Although cryomilling will break down and homogenize gummies, the resulting powder will become sticky and difficult to handle immediately upon warming. By contrast, hard candies are relatively easy to grind.

Another challenge is that the sugars and pectin that make up candies and gummies are not fully soluble in either methanol or acetonitrile. Extraction requires water to start. Once the sample is solubilized, organics can be added.

For these kinds of samples, QuEChERS is an excellent cleanup choice. The typical procedure for candies involves first grinding, and then transferring the powder to a 50-mL centrifuge tube. Then 10 mL of water is added to dissolve the sugars, followed by 10 mL of acetonitrile. After vigorous shaking, the salt packet from the QuEChERS kit is added. As the salt dissolves under shaking, it triggers an organic/aqueous phase separation. A short period of centrifugation results in a clear stratification into a layer of excess salt at the bottom, then a water layer, then a layer of organic materials and particulates, and finally an organic layer at the top containing the compounds of interest.

The final step of QuEChERS is typically a dispersive step, but we found that that extra step added cost with little benefit. Instead, we filter the supernatant with an Agilent PTFE syringe filter. The resulting solution is ready for injection for LC–UV, or may require some dilution for liquid chromatography–mass spectrometry.

There was a slight modification of the procedure for gummies. First, the gummies were chopped with scissors before being weighed into a 50-mL centrifuge tube with two ceramic homogenizers. They are then shaken for 3 minutes with 10 mL

of water before 10 mL acetonitrile with 2% ammonia was added. The high pH is necessary to dissolve the gelatin or pectin in the gummies. Another 5 minutes on the shaker fully dissolved the sample. Then the QuEChERS kit salt was added and the sample was shaken for 1 minute and centrifuged at 3600 rpm for 5 minutes. The dispersive step of the QuEChERS process was also not necessary for gummies. The only added treatment was filtration with a 0.2µm PTFE filter just prior to injection.

CONCLUSION

The widely variable nature of edibles requires some adaptability of sample preparation procedures. Fortunately, most edibles fall within a few categories. Those containing high fats are best cleaned using EMR-Lipid system. Those with sugar and pectin/gelatin are best first dissolved in water, and then cleaned up with QuEChERS. Both chromatography and detection depend on an adequate sample cleanup procedure. That said, a good chromatography method can be applied to almost any sample if the sample preparation is sufficient.

Cleaning samples of strongly retained compounds or particulates will prevent premature changes in the column, or increased maintenance of the instrumentation. Removing potentially interfering species is also important so that integration and quantitation is straightforward. Detection can also be negatively affected by an incompletely cleaned sample. Noisy baselines can cause problems with quantization, and coeluting species may interfere with ionization, even if they are not detected. Having a robust cleanup method is the most important part of having a robust quantitation method.

References

1. R. Vandrey, et al., *JAMA*. 313(24), 2491–2493 (2015).

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FAQs: Simple, Accurate, aand Reliable Quantification of THC and CBD in Cannabis-Infused Chocolate Edibles, Pastries, and Candies

By: Jean-Francois Roy, Christophe Deckers

> *The following questions were asked during the Cannabis Science and Technology/LCGC webcast, "Simple, Accurate, and Reliable Quantification of THC and CBD in Cannabis-Infused Chocolate Edibles, Pastries, and Candies," sponsored by Agilent Technologies.*

LCGC: Can the Captiva Enhanced Matrix Removal (EMR) cartridge be used on an oil matrix? And which can be used—tinctures or bombs?

ROY: We have not tested it for potency testing in an oil matrix yet. However, we've used EMR on multiple oil matrices for pesticide testing, and we found it to be very effective at removing lipids. EMR is powerful enough to work on pure oils, so we're hopeful that in the future we will be able to test it on oil-based concentrates too.

DECKERS: With chocolate, we have proven that Captiva is highly effective at removing fatty matrix, but it does not interfere with cannabinoids. So as long as the work is geared toward potency testing, Captiva EMR will be an efficient tool no matter what matrix is used.

LCGC: How does your procedure for chocolate compare with an extraction with methanol followed by filtration?

ROY: Methanol is a great solvent for cannabinoids; many cannabinoid reference solutions are shipped in methanol. However, methanol is a protic sample, just like water, and captures more polar interferences from the cocoa.

In the application note, we demonstrate with Liquid Chromatography-Time of Flight (LC-TOF) mass spectrometry that acetonitrile has very similar recoveries for cannabinoid extraction compared to methanol, but produces much cleaner extracts than methanol—the mass spec baseline is much lower when we use acetonitrile. This means that the injected extract is much cleaner.

DECKERS: We removed a significant percentage of late-eluting, hydrophobic components from the matrix, as seen from the data. That's a big difference—acetonitrile and the extra cleanup makes a huge difference in the quantification. As a result, we consistently got 20% more UV signal for the cannabinoids, showing how effective it is.

LCGC: For these methods to work, do you have to use them exactly as you describe them—for example, using the specific columns and mobile phases you mentioned—or can a sample prep for chocolates and gummies be used on the method we're currently using?
DECKERS: As long as the sample prep is geared toward removing as many matrix interferences as possible, the sample prep will work with your chromatography because the main goal of this sample preparation is to reduce the amount of interference being loaded on the column.

So, yes, if you are happy with your current chromatographic method, then stick to it. But we definitely recommend the sample preparation because it has been proven to greatly reduce the amount of matrix that is loaded onto the column at each injection.

LCGC: Do you have a recommendation for CBD quantification in coffee?
DECKERS: It's not something we have tried before. I expect it to be challenging because, most likely, coffee contains a lot of flavonoids, and those flavonoids may interact or co-elute with CBD if not removed during sample preparation, potentially influencing its quantitation.

With that being said, a solution here would be to use acetonitrile versus methanol. This is because with methanol, we have seen a difference in the coloration of chocolate (e.g., dark chocolate). With acetonitrile, it does not have the dark coloration that could potentially interfere. So, again, I would advise to try with acetonitrile first.

LCGC: Was the calibration curve prepared using a blank matrix? And if so, how is the blank matrix chosen?
DECKERS: Fortunately, it's easy to find gummies and chocolates that are not infused with cannabinoids—that's the matrix we're using. We're using the same procedure, same extraction, and EMR cleanup, and doing our pre- and post-spikes on that matrix.

LCGC: How do you achieve a homogenous mixture if you're cutting the sample with scissors? I have found in testing that servings are not homogenous.
DECKERS: For chocolate, it's not an issue. We know that you have a lot to run—productivity is important; time is money—so our goal was to have the best procedure, both in laboratory economics and scientific output. For the economics aspect of it, chopping with scissors enabled us to process more samples per hour, especially with chocolate.

Gummies are a challenge. Gummies are finely chopped (and very sticky); then with a spatula, they are put on the side of the tube. But again, this way you can process more samples per hour.

If you use a cryomilling device, it only allows you to do two samples at a time, depending on which one you have. When you return to room temperature, it can jellify and start to be sticky.

While using the Geno/Grinder and processing many samples at the same time with scissors, you can do more samples per hour and dissolve them effectively using water first, then solvent. Optimized pH is key, especially to dissolve pectin gummies. Warm water and sonication have also shown to help.

LCGC: What kind of cleaning procedure would be suitable for cleaning scissors between samples? Cannabis products tend to be very sticky, and I'd be concerned about cross contamination.

DECKERS: I take my chocolate directly out of the fridge so that it's cold and doesn't stick to the scissors. Also, you can rinse with a swab of methanol, which cleans the scissors quickly. If you're using a different device such as a mortar and pestle, or if you're using a cryomilling device, you have to clean the pestle, but cleaning scissors is faster.

LCGC: Why is it important to use the middle or inner part of the gummies for analysis?

DECKERS: The outside of the gummies are sometimes sugar-coated or coated with palm oil, beeswax, and coconut oil, so they aren't too sticky to handle. However, those waxes and oils interfere with cannabis detection—the more oil, the less accurate your results are.

For smaller gummies, it's a bit more of a challenge. In this case, you want the middle of the gummy because it gives you a more accurate quantification of cannabinoids.

LCGC: You mentioned an alternate chromatography for the gummy work that showed good resolution between the cannabidiol (CBD) and tetrahydrocannabinol (THC) peaks. Did you try to separate other cannabinoids with this alternate chromatography?

ROY: Yes, we did. We are able to separate 17 cannabinoids at the baseline level with this alternate chromatography. We have used it for our gummy work, and we are currently using it for some work on cannabis-infused beverages.

LCGC: Can you elaborate on the work you're doing on cannabis-infused beverages?

DECKERS: You would think working with beverages would be easier; unfortunately, because THC and the other cannabinoids are not water-soluble, they have nano- and micro-emulsifiers to keep them in solution, and they build up on the instrumentation and interfere with detection of the cannabinoids.

Right now, we are working on a procedure using a combination of the QuEChERS (quick, easy, cheap, effective, rugged, and safe) method and EMR to remove those emulsifiers selectively without losing the cannabinoids. Thus far, we're getting great results, and we plan to publish these results soon.

LCGC: Does the sample prep work for both THC and THC acid (THCA)?

DECKERS: Our potency work on edibles has focused on CBD and THC because manufacturers typically infuse edible matrix with CBD/THC distillates and concentrates that contain very little CBDA/THCA. We however expect that this sample prep will work great with THCA.

LCGC: You said with QuEChERS, there's an exothermic reaction involved. Does this lead to conversion of THCA to THC?

DECKERS: QuEChERS treatment will lead to a small temperature increase in the solution, not warm enough and not for long enough to induce TCHA decarboxylation.

LCGC: Are all the data presented with Diode Array Detection (DAD)?

DECKERS: Yes, all the quantitative data was generated using UV detection through a DAD detector. If you have a mass spectrometer (MS), either a quadrupole-based or TOF-based detector, it is also possible to use it. Of course, this sample prep will also help MS-based detection for the same reasons—removing interferences and ease of use. The difference with LC–MS is that you'll need to further dilute your sample, from 200 to 1000 times depending on the model.

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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

Topics presented:

Potency Testing in Fatty Edibles, Like Chocolate and Baked Goods

Potency Testing in Cannabis Infused Beverages

Potency Testing Cosmetics

Potency Testing in High Sugar and Low-Fat Edibles

Troubleshooting tips for analysis of Captan

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.

Accurate and Simple Quantification of THC and CBD in Beverages Containing Micro and Nano Emulsions

Sample prep is really key for success, and it all works hand-in-hand with chromatography and detection. It provides robustness, less instrument maintenance, prevents LC column clogging, and results in the best accuracy.

Accurate quantifications of THC and CBD by LC-UV in beverages like beer, iced tea, sodas, and fruit drinks can be negatively impacted by several factors. The first issue lies with the stability of cannabinoids. Analytes like THC and CBD tend to bind to the inner surface of bottles and cans.

As a result of their low solubility in water, cannabinoids require the presence of oils and emulsifiers to keep them in solution during storage and shipping. Oils and emulsifiers like palm oil, glycerin, lecithin, and polysorbate 80 impact accuracy and tend to build up, contaminate and clog LC columns. Concentrations of cannabinoids in beverages are much lower compared to other edibles, and require an optimized extraction procedure for robust and accurate quantification.

This article provides tips and tricks for simple and fast cleanup of infused beverages that can increase lab productivity and eliminate unpredictable results. This article will review the hydrophobic nature of cannabinoids and its implications when manufacturing THC and CBD infused beverages, and the importance of having a complete and well-aligned analytical procedure for potency testing in beverages, from sample preparation all the way to compound detection.

CANNABINOID POTENCY

Potency testing can be defined as the accurate quantification of cannabinoid levels in various hemp and cannabis products. It is a universal test required for almost all products within the cannabis market. Testing will confirm expected levels of cannabinoids, as well as confirm low levels, or even the absence of some cannabinoids. An example would be to confirm the concentration of total THC below 0.3% in hand products. The expanding cannabis market includes an edibles product category, and the two most common cannabinoids being tested for potency in this category are THC and CBD.

THE CHEMISTRY OF CANNABINOIDS

Cannabinoids are highly hydrophobic (water-fearing). The logP value is a measurement used to indicate the degree for which a compound is hydrophobic. The higher the logP value, the more hydrophobic a compound is considered.

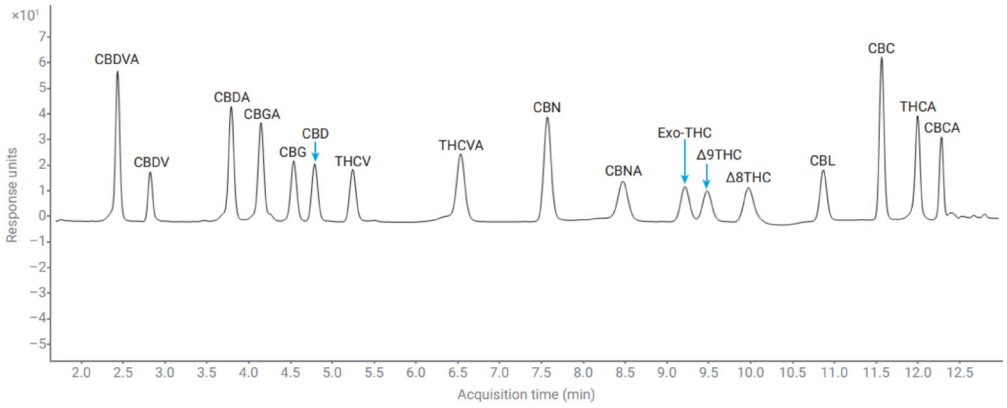
For example, fructose has a logP of -2.8, and formic acid has a logP of -0.5. These negative values indicate that these compounds are highly hydrophilic (affinity to water) and very water soluble. In contrast, THC having a logP of 5.7 and CBD with a logP of 6.5, are highly hydrophobic. This is why they are commonly sold in oil-containing or fat-containing products. The hydrophobic characteristic of cannabinoids plays a role in bioavailability for ingestion, as well.

CANNABINOIDS IN WATER-BASED BEVERAGES

Water-based beverages contain about 90% water. The challenge with infusing THC and CBD products into these beverages is that oils and emulsifiers must be utilized in that remaining 10% of beverage constituents. Emulsifiers typically used in the food industry include lecithin, fatty acid derivatives, mono- and diglycerides, and polysorbate 80. Antioxidants, nitrogen, and carbon dioxide are frequently added to beverages for stability, which present additional challenges.

In addition to manufacturing challenges, potency testing is a huge chemical challenge. Product mislabeling in the market is an issue. A report published in 2015, by Vandrey et al, revealed that of 75 different edibles tested for potency, only 17% were accurately labeled (1). This supports the need for testing methods designed specifically for edibles.

FIGURE 1. Baseline separation of 17 cannabinoids



THE CHALLENGE WITH POTENCY ANALYSIS IN EDIBLES

A well-aligned procedure includes three main interconnected aspects, or pillars. These include sample preparation, chromatography, and detection. All must work together to achieve accuracy and robustness. Sample prep must be aligned with chromatography, chromatography with detection, and detection with sample prep.

In the past, a uniform single preparation approach had been used for all products. However, the preparation regime used for dry flowers, as an example, is not one that can be used for edibles, as their constituents are drastically different. Since many cannabinoids basically share the same chemical formula and mass, attention needs to be focused on their baseline separation, as demonstrated in FIGURE 1.

SOLVING THE CHEMISTRY CHALLENGE WITH STATE-OF-THE-ART SAMPLE PREPARATION

Sample preparation is key for potency testing of beverages. Potency testing in beverages is very challenging for many reasons, including low levels of THC and CBD in beverages, the presence of emulsions to accommodate solubility, the wide variety of beverages, and instrumentation maintenance concerns.

THC AND CBD MICROEMULSIONS: ANALYTICAL CHALLENGES

Emulsifiers are unique in that they have a polar side that is water soluble and a nonpolar side that is oil soluble. The nonpolar side coats the oil droplets containing the cannabinoids, while the polar side helps them to mix with the water portion of the beverage. However, the oils and emulsifiers create problems in the analytical lab. They may clog columns, increasing instrument

downtime. In addition, oils and emulsifiers can interfere with UV and MS detection. Removing the oils and emulsifiers results in a more robust chromatography and reduces instrument maintenance.

To detect THC and CBD trapped in emulsions, it is necessary to break down emulsions, thereby freeing cannabinoids. This can be accomplished using heat, sonication, solvents, or QuEChERS. The solution to this challenge is to selectively remove oils and emulsifiers to get accurate quantification of cannabinoids.

As previously mentioned, concentrations of THC and CBD in beverages are much lower than in other edibles. For example, 10 mg of THC in 355 mL of liquid is much more diluted compared to 10 mg of THC in 10 g of chocolate. Those differences impact the detection limit. The effects of these differences can be resolved by optimizing sample preparation using Agilent's DAD detectors to enable better sensitivity. This approach also eliminates the need for LC-MS/MS for routine potency testing.

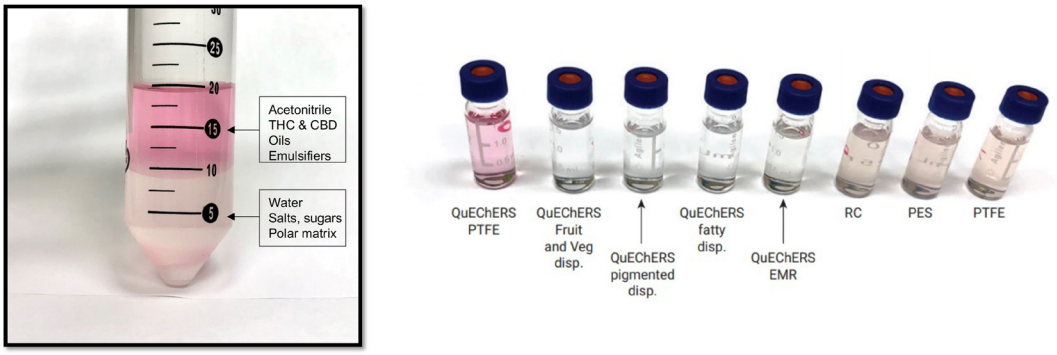
VARIETY OF BEVERAGES AND INGREDIENTS, AND EFFECTS ON STABILITY

There is a great variety of beverages on the market, and the extraction method needs to be applicable to all of these beverage types (ie, sodas, beer with or without alcohol, fruit punches, etc). The list of ingredients, colorings, emulsifiers, and preservatives varies greatly as well. Even when the same ingredients are used, there are different ratios of each to contend with. The solution to best handle all of these variables is to have a robust and universal sample preparation method.

Sometimes beverages are stored at room temperature. More often, they are stored at 4°C. Therefore, it is important to warm up the sample bottle before testing. This can typically be done in a sink of warm tap water. This will prevent the loss of cannabinoids that may stick to the inside of plastic bottles or metal containers. Hydrophobic polymers are often sprayed in the inside of aluminum cans and can potentially bind THC and BCD. Glass bottles, on the other hand, are hydrophobic and have less unwanted interactions with cannabinoids than plastic bottles made from PET (polyethylene terephthalate).

Cannabis-infused beverages are unstable. Oxidation can degrade cannabinoids and the quality of emulsion changes its shelf life. Therefore, it's important to test beverage samples promptly, before they can precipitate. Testing parameters such as temperature and air exposure should remain constant throughout testing.

FIGURE 2. Visual comparison between various treatments of a carbonated fruit beverage infused with a THC Microemulsion



Sample after QuEChERS

*Cannabinoids are more stable and soluble in acetonitrile vs water, especially when emulsions are disrupted

FINDING THE SIMPLEST AND MOST ADVANCED SAMPLE PREPARATION METHOD

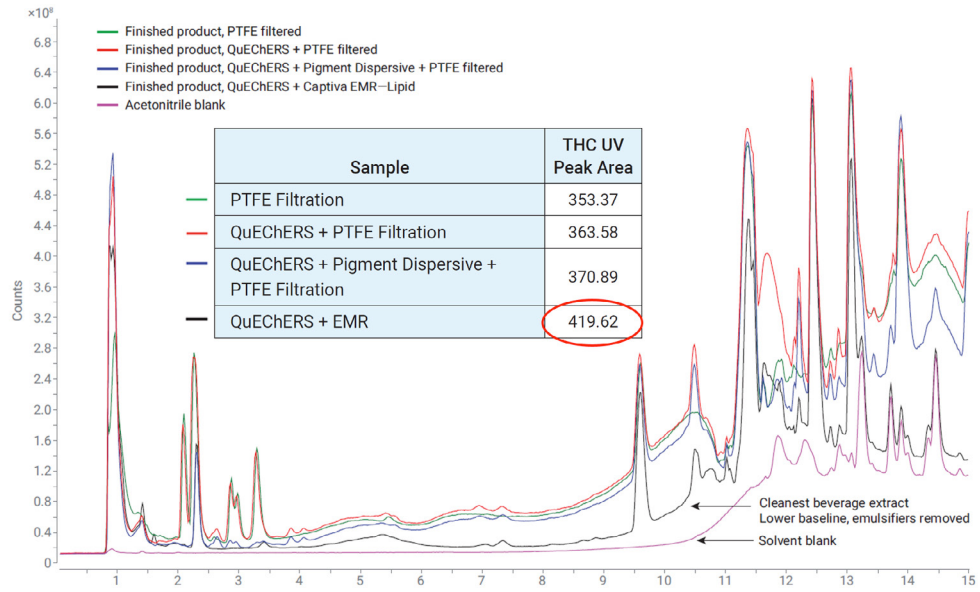
FIGURE 2 demonstrates a visible comparison between various treatments of a carbonated fruit beverage infused with a THC microemulsion. Sonication degasses the sample and starts to disrupt the emulsions, which releases cannabinoids. Then, 10 mL of that sample is poured into a fresh tube and acetonitrile is added. Here, the solvent starts to further disrupt this emulsion, liberating more cannabinoids. Next, Agilent QuEChERS extraction salts are added. Visibly seen at the bottom is water, with polar interferences and the excess salts. At the top is acetonitrile, with all cannabinoids.

In the separate vials, going from left to right, are the results from different prep steps taken from the top layer. The first is filtered with a PTFE filter. For the next three steps, different QuEChERS dispersives are applied, including PSA, graphitized carbon black, and C18. An EMR (Enhanced Matrix Removal) filter has been applied to the fifth. The last three samples show the results of straight beverage filtered using three different types of filters. With the RC (Regenerated Cellulose), PES, and PTFE. Some samples appear to be cleaner than others. Cannabinoids are more stable and soluble in acetonitrile, as compared to water, especially when emulsions are disrupted.

Emulsifiers and oils that need to be removed, visually lack coloration. To further access cleanliness and compare removal of those emulsifiers and oils, further testing using LC/Q-TOF in total ion count is necessary.

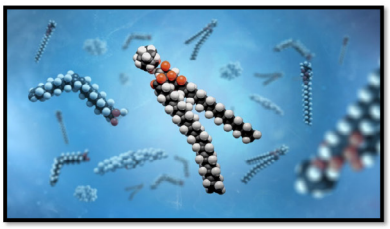
Looking at the chromatogram, shown in FIGURE 3, is a pink trace at the very bottom, which is the pure acetonitrile blank. The second lowest line is QuEChERS plus EMR.

FIGURE 3. LC/Q-TOF TIC comparison of various cleanup treatments of a THC-infused Drink



Sample	THC UV Peak Area
PTFE Filtration	353.37
QuEChERS + PTFE Filtration	363.58
QuEChERS + Pigment Dispersive + PTFE Filtration	370.89
QuEChERS + EMR	419.62

FIGURE 4. Captiva EMR-Lipid cleanup



Lipids and Emulsifiers are Removed

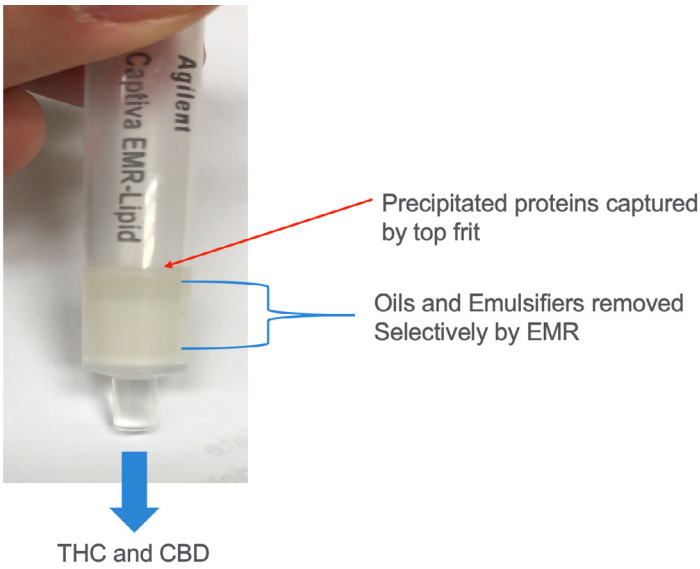
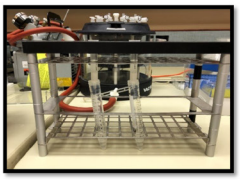
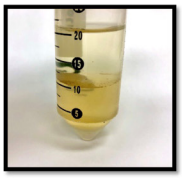


FIGURE 5. Optimized sample prep procedure for Beverages

1. Take bottle of infused beverage. Warm the can/bottle with warm tap water for 10 minutes.
2. Sonicate at approximately 50 Celsius for 20 minutes to degas and dislodge cannabinoids from emulsifiers.
3. Put 10 mL of sonicated cannabis-infused beverage into 50 mL conical-bottom centrifuge tube. Add 10 mL of acetonitrile and shake aggressively for 3 minutes on mechanical homogenizer (1500 RPM).
4. Add Agilent Original extraction salts PN 5982-5550. Shake immediately for 10 seconds and degas.
5. Shake for 1 minute, aggressively, on mechanical homogenizer (1500 RPM)
6. Spin at 3000-5000 RPM for 5 minutes.
7. Take 2 mL of supernatant and put in 15 mL conical-bottom centrifuge tube. Add 500 ul of water and mix by inversion.
8. Place 3 mL cartridge rack (part # 5191-4101) on top of waste rack (part # 5191-4112).
9. Place a 3 mL Captiva EMR—Lipid tube PN 5190-1003 in the cartridge rack, and a clean 15 mL PP centrifuge tube directly under the EMR tube in the waste rack.





10. Pour all contents from Step 7 into the 3 mL Captiva EMR—Lipid tube. This will flow by gravity.
11. After complete elution of the initial 2.5 mL portion, pour an additional 1.5 mL of 80:20, acetonitrile:water to the Captiva EMR—Lipid tube, also flowing by gravity.
12. Use Vortex 15 mL collection tube and put aliquot in vials PN 5182-0553 prior to injection. Your final dilution factor is 2X.

This is the cleanest sample of all because of the relatively low baseline and fewer/smaller peaks. The other sample prep techniques had much higher baselines. They didn't remove the oils and emulsifiers well, resulting with a much dirtier sample.

AWARD WINNING CAPTIVA EMR LIPID TECHNOLOGY

The cleanest sample appears after using Captiva EMR technology. Winning the R&D Award in 2015 and the Analytical Scientist Award in 2017, this very powerful filter enables the separation of cannabinoids from oils and emulsifiers. The Captiva EMR filter captures oils and emulsifiers that have long, straight aliphatic chains. Cannabinoids don't have these long aliphatic chains and do not get captured in the filter, allowing those to pass through.

FIGURE 4 shows results of using the Captiva EMR filter to separate THC and CBD from precipitated proteins (top layer), as well as oils and emulsions (bottom layer). The THC and CBD flow through and are now super clean.

THE PROCEDURE FOR OPTIMIZING SAMPLE PREPARATION FOR BEVERAGES

The optimized sample prep and EMR cleanup procedure for beverages provides cleaner samples, higher UV signal, and greater accuracy. The detailed procedure can be found in Agilent's Application Note 5994-3791EN, as well as in FIGURE 5. It should be noted that in the photo, on the right side at the 10 mL line, there is a darker ridge. This darker ridge is a layer of precipitated proteins. Removing protein contaminants from the sample results in a cleaner sample. The tubes

FIGURE 6. Visual Intraday accuracy and interday accuracy and precision

Calibrator 1	CBD			THC		
(0.5 µg/mL CBD, 0.5 µg/mL THC)	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
Calibrator 1: First Preparation	100.9	103.3	103.4	99.8	101.9	103.1
Calibrator 1: Second Preparation	103	102.4	103.4	101.7	103	104.5
Calibrator 1: Third Preparation	102	101.1	104.9	102.3	102.4	104
Intraday Average Accuracy (n = 3)	102.0	102.3	103.9	101.3	102.4	103.9
Interday Average Accuracy (n = 6)		102.7			102.5	
Interday Standard Deviation (n = 6)		1.3			1.4	
Interday Precision (%RSD, n = 6)		1.2			1.3	

at the end of the procedure should always be vortexed to ensure an even concentration.

METHOD PERFORMANCE

Now that the challenges impacting accuracy for potency testing and the optimized sample prep method have been outlined, the next step is to examine method performance. This is done by reviewing data.

When doing a calibration curve, do not use solvents (or pure solvents), methanol, acetonitrile, or water. Especially with edibles, we believe the best approach is to use a matrix match. In a representative calibration curve prepared by Agilent, six calibrators—from 0.5 to 100 ug/mL—were used. The average fit was zero. Recovery studies also revealed recoveries being excellent—100%-110%—showing that the EMR cartridge used did not remove or lose any cannabinoids.

A mini validation by Agilent over three days in triplicates revealed that at the very lowest concentration of 0.5 ug/mL, the average intraday accuracy was excellent—0.2%. This was outstanding, considering the complexity of the matrix. The standard deviation was only 1.3-1.4. Intraday precision was excellent as well—1.2-1.3. This was exactly what was desired—a very robust method and accurate quantification. The complete data for the intraday accuracy, deviation, and precision can be seen in FIGURE 6.

Often, identical samples sent to two different testing labs reveal very different results. This is generally due to sample preparation not working optimally. The procedure outlined will help achieve the best accuracy and precision.

Sample prep is really key for success, and it all works hand-in-hand with chromatography and detection. It provides robustness, less instrument maintenance, prevents LC column clogging, and results in the best accuracy. Agilent provides high-quality consumables, including Agilent QuEChERS salts, solvents,

standards, vials, filters, and notably, the Captiva EMR filter.

SUMMARY

In the long run, the one-sample-prep-for-all approach results in decreased performance. Potency testing in beverages requires dedicated, optimized sample preparation. Sample preparation is the key for success and works hand-in-hand with chromatography and detection. It provides robustness, reduces instrument maintenance, reduces LC column clogging, and results in the best accuracy.

A chromatography example resulted in 17 cannabinoids being resolved at baseline. The LC-UV in this example provided very good sensitivity for potency testing in beverages when coupled with optimized sample preparation. Optimized LC-UV method decreases solvent usage and enables separation of critical isomers.

With fatty edibles like chocolate and cookies, removing fats in fatty matrices provides accuracy with considerably less maintenance. Microemulsions in beverages can cause a lack of robustness and inaccuracies during potency testing. QuEChERS extraction followed by Agilent's Captiva EMR-Lipid filtration provided cleaner samples, higher UV signal, and greater accuracy.

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How to Ensure Laboratory Compliance in Your Cannabis Testing Lab

By Matthew Abrahms and Donna Payne

Laboratories ensuring the quality of Cannabis derived product should anticipate increased regulatory stringency as the industry continues to mature. To comply, it is recommended that companies implement various processes to meet current and future requirements.

OVERVIEW

Cannabis is a new and popular industry with potential for exponential growth over the coming decade. As the industry continues to mature, regulations surrounding product quality are expected to become more stringent. Basic principles are needed to ensure that laboratory data are compliant and defensible according to ISO 17025 and current Good Manufacturing Procedures (cGMP).

UNITED STATES CANNABIS LABORATORY COMPLIANCE OVERVIEW

The USDA 2018 Farm Bill effectively separates federal law surrounding the Cannabis sativa plant into two articles: hemp (≤ 0.3% THC) and cannabis (marijuana). Various products can be made or derived from both articles, which all have a diverse set of legal and quality requirements. As a result,

depending on the product that a laboratory is testing, regulations may differ, making this landscape incredibly complex.

The diagram in **FIGURE 1** illustrates the two Cannabis sativa plant articles, as defined by the United States Department of Agriculture (USDA) 2018 Farm Bill, as well as the many products that can be produced.

A Cannabis testing facility may be subjected to three different types of laboratory compliance regulations: state, external accreditation (e.g., ISO 17025), or federal requirements (e.g., cGMP). State requirements are relatively new and because of that, are frequently changing. As a result, most states rely on a common laboratory accreditation, ISO 17025, for their compliance requirements. However, it should be noted that ISO (International Organization for Standardization) requirements are designed for labs of all industry types, so their requirements are quite general. Testing facilities that ensure the quality of Food and Drug Administration (FDA) regulated products must comply with current Good Manufacturing Practices (cGMP). The difference between the two is that ISO requirements are prescriptive, while cGMP requirements are interpretive. As a result, a cGMP lab must always be ready to demonstrate compliance, as well as continually be seeking improvement. Regardless of which regulation is being followed, all the above regulations require method validation, equipment qualification, and data integrity controls.

FIGURE 1. Cannabis laboratory compliance: spectrum of different products and complex regulatory landscape.

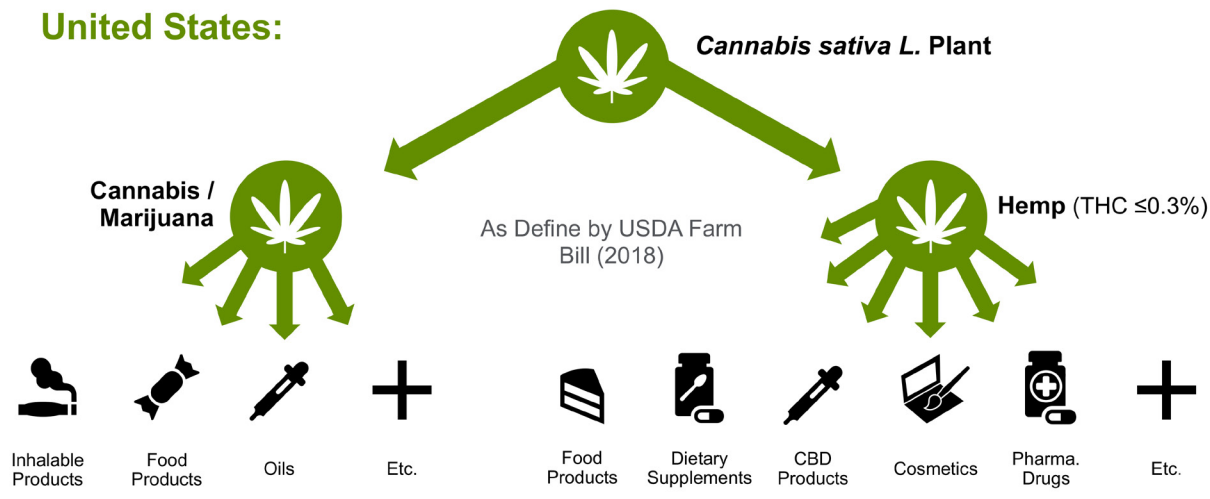
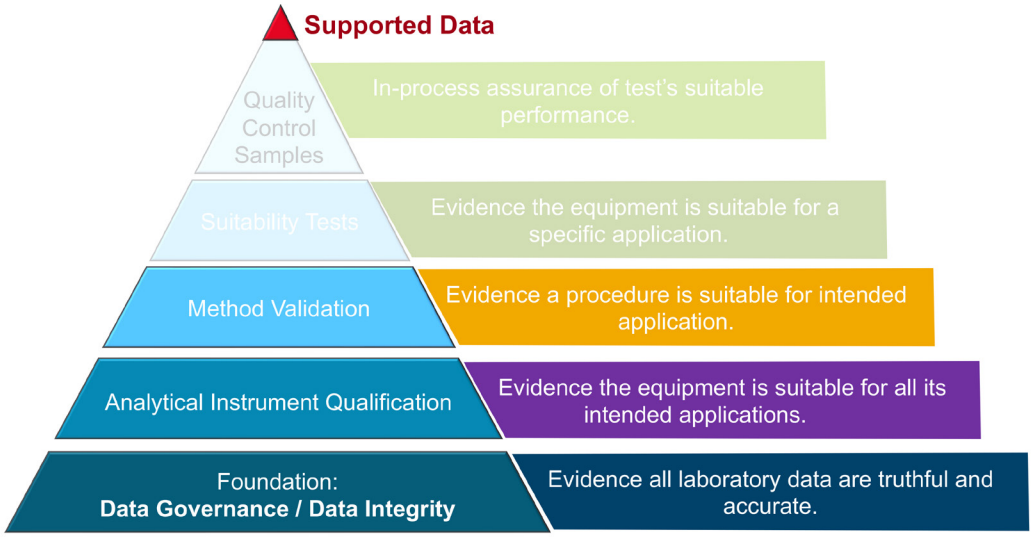


FIGURE 2. Cannabis laboratory compliance: data quality triangle from USP <1058>.

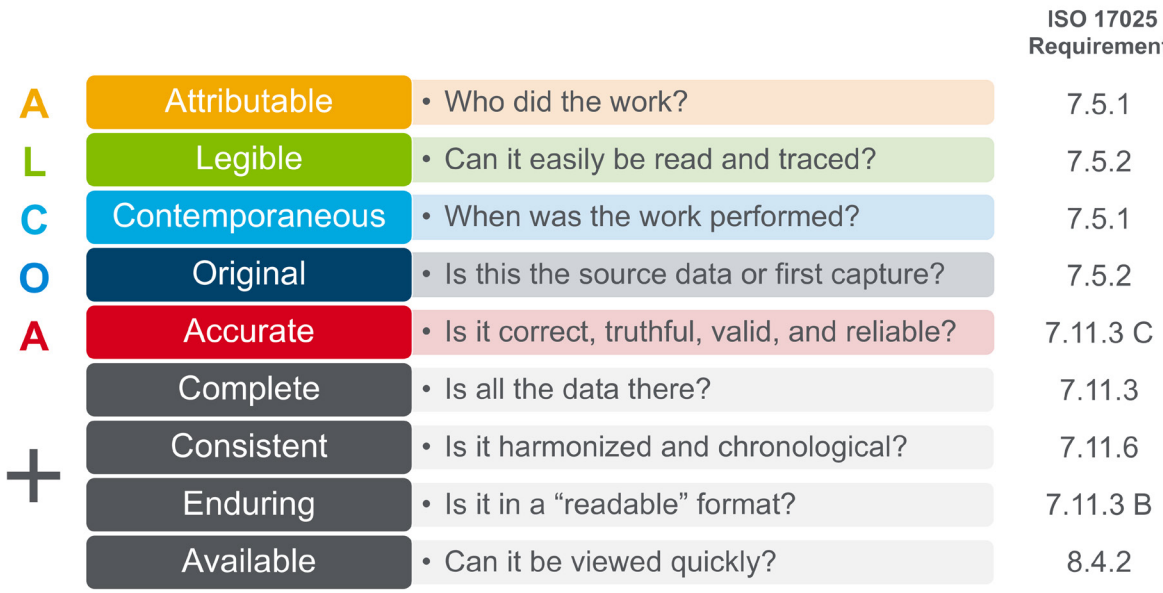


The United States Pharmacopeia's (USP) Data Quality Triangle, shown in FIGURE 2, illustrates the importance of these three fundamentals. The triangle lays down a foundation for laboratories to demonstrate that their data is meaningful. At the top are supported data. These are any data that a laboratory wishes to argue is scientifically sound or defensible. Underneath are the various activities a laboratory must take to exhibit the validity of these data/records. As a result, this pyramid demonstrates the importance of these activities (QC samples, suitability tests, method validation, analytical instrument qualification, and data integrity). Notably, all stages below a particular stage require the activities below to claim them as defensible. For example, a method cannot be validated unless there is confidence that the equipment performing the method validation is working or qualified.

DATA INTEGRITY

Data integrity is the foundation to the Data Quality Triangle, and all laboratory activities are dependent on it. To maintain this integrity, a piece of data or record must meet all the principles of the acronym ALCOA+, as illustrated in FIGURE 3. The list of these principles is on the left, with their corresponding descriptions in the center, and the ISO 17025:2017 sub-clause requirements for each on the right. At the top of the list is "attributable," meaning "does it show who did the work?". Working down the list, "can the work be traced?", "Show when it was

FIGURE 3. Data integrity: Data maintain ALCOA+.



performed?", and "Is it reliable, complete, and readily available?". Data that do not meet all these principles lack the necessary characteristics to properly defend.

To ensure that all laboratory data meet the principles of ALCOA+, various controls exist. These controls differ between paper and electronic records. Paper controls are not nearly as strong as electronic or technical controls, because they require people to remember them and willingly comply. Whereas electronic controls force people to comply through technical means. For example, to ensure attributability paper records may use ink signatures, while electronic records may use an electronic signature with a user ID and private password. As a result, electronic controls are inherently stronger, but they come with some unique challenges. These include making sure files are not overwritten, protecting the computer's clock, and assigning the correct user rights and privileges for individuals. Regardless of the data's medium (paper or electronic), laboratories must safeguard the integrity of these records.

Two types of data formats exist in the industry: static and dynamic data. Static data are any data that are fixed, such as a paper record or image. Dynamic data includes electronic records, which allow for interaction by the user (e.g., chromatography file). Most forms of source data (raw data) today come in dynamic format since they are generated by computerized systems. A general rule regarding static vs. dynamic data is that static data can be made from dynamic data, but not

the reverse. For example, a chromatography data file can be turned into a report, but not the other way around. It is therefore crucial that a lab must protect their dynamic data to support laboratory conclusions.

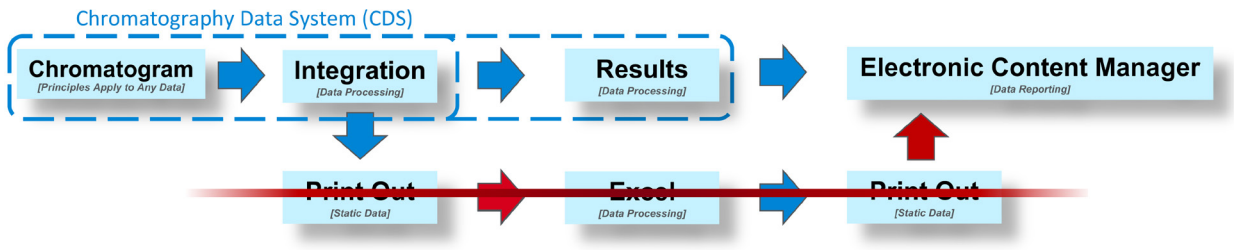
Ultimately processes should be created to maximize technical data integrity controls. The example in **FIGURE 4** is a typical workflow used in many labs worldwide. The example uses a chromatography data file, however the principles can apply to any data file. In this example workflow, the laboratory is generating and proceeding to integrate a chromatogram into a report, print it out, and then transcribe it into an Excel spreadsheet. Then, the spreadsheet is printed and either uploaded or transcribed to a content management system.

The risk in this example is that this lab uses several manual data entry steps, beginning with transcribing data from one record to another. This is not only time consuming, but also increases the risk for errors (whether unintentional or on purpose). To be done correctly, a second person should review all manual entries.

The second risk with this method is the use of Excel for performing laboratory calculations. Although a great program, Excel was never designed to have data integrity controls in place, as it is very easy to have files overwritten and have calculation changes made without transparency.

The third largest risk, in this example, is the use of multiple printouts. Every printout that

FIGURE 4. Data integrity: processes should maximize technical controls



Risks:

- Manual Entry: Prone to error/fraud as has weak and burdensome controls (2nd person reviewer)
- Excel: Difficult to validate and control (Requires lots of procedural controls)
- Print Outs: Controls and storage are burdensome (Requires traceability to electronic record)

Data Integrity Remediation: Maximise electronic controls and functionality in CDS.

a lab creates is considered a piece of critical data and must be protected and archived.

These risks can be eliminated by utilizing a Chromatography Data System (CDS) or other data acquisition software to perform the required calculation steps. By doing this all through the lab's acquisition software, the processing steps are done within a single platform as opposed to using an external program (Excel).

SPONSORED CONTENT

United States Cannabis Testing: Laboratory Compliance White Paper

In summary, this article introduces three simple ways to reduce a lab's data integrity risk. The first is to not delete or fail to safeguard source data (paper or electronic). The second is to ensure that all laboratory data used to support a lab's conclusions meet the principles of ALCOA+. Lastly, a lab should maximize the use of their equipment acquisition software when performing analysis or reporting steps.

ANALYTICAL INSTRUMENT QUALIFICATION

There is a vast spectrum of vocabulary used by different regulatory bodies regarding analytical instrument qualification. ISO uses "calibrate", USP uses "qualify", and some state regulations use "verification". For consistency, this section uses "calibrate" to define correcting an equipment's response with a known value, and "qualify" to confirm a piece of equipment is working and accurate. It is important to note there is a difference between correcting an equipment's response and proving that equipment is suitable for its intended application. The steps needed to demonstrate that a system is working accurately depends on the complexity of the equipment and the data it generates.

For example, a balance is relatively simple due to the fact that it only records one parameter (weight). Due to the equipment's simplicity, it is easy to justify that the calibration activity alone is sufficient to justify that the balance is working as intended. However, for a LC, this justification becomes much more difficult. A LC is much more complex than a balance, as it sets up and records multiple variables (e.g., response, temperature, flow, etc.).

As a result, for complex systems (e.g., chromatography), calibration alone is not sufficient to demonstrate that a system is working as intended. A separate qualification activity would be required to establish the instrument is fit for a laboratory's intended use. To accomplish this, a well-designed qualification will be made up of both modular and holistic based tests to verify the performance of all aspects of the instrument.

METHOD VALIDATION

Method validation is required to ensure that an instrument is capable of the desired analysis and that the method is appropriately designed

and scientifically sound. For method validation to be successful, appropriate and proficient resources and personnel must be in place. In other words, validation provides proof of a lab's capabilities. Not only is it required by regulatory bodies, but is also a requirement to have a scientifically sound analysis.

Method validation can be sub categorized into three different activities: "validation", "verification", and "modification". It is important to understand the differences between these activities as they are triggered situationally. "Validation" is used for establishing characteristics of newly developed methods, "verification" for new implementations of standard validated methods, and "modification" for an alteration of a validated method.

Validation of a method is broken down into two segments, validation of the instrumental method and then validation of the sample preparation method. The instrumental method must be validated first, and then the sample preparation method must be validated for each matrix. This article will specifically focus on validation of the instrumental method, which assures that the instrument is capable of accurately analyzing real samples.

One of the first elements of the validation process is checking a method's precision and accuracy. Precision is repeatability, the ability of the method to measure the same sample with consistent results. However, just because an instrumental method is precise, does not mean its accurate. Therefore, in the validation process, it is necessary to check both precision and accuracy. **FIGURE 5** and **FIGURE 6** graphically illustrate the difference between precision and accuracy.

An example of a precision experiment would be to analyze 15 (or more) samples of a reference standard from the same vial. While there may be some natural variability, the calculated concentrations should all be consistent.

Another element that must be evaluated is the method's linearity. This is the method's ability to obtain test results that are directly proportional to the concentration of the analyte in the sample. To accomplish this, a

FIGURE 5. What is Precision?

Repeatability

- The ability of the instrument to measure the same sample with the same results
- Just because an instrumental method is precise **DOES NOT** mean it is accurate!

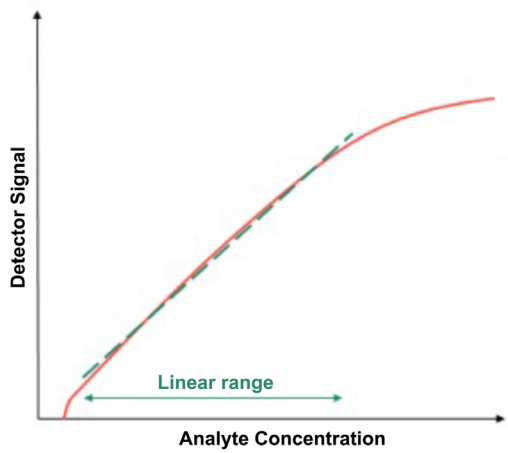
FIGURE 6. Accuracy defined.

Accuracy

- The degree to which the result of a measurement, calculation, or specification conforms to the correct value or a standard.
- 20 samples of differing known concentrations of standards are analyzed and mean accuracy is calculated
- Can get the instrumental uncertainty contribution from accuracy studies

wide range of analyte concentrations should be selected. The known concentrations for each sample are measured and plotted, then the areas of the curve where accuracy falls off are discarded (e.g., at the low and high ends). The resulting curve is classified as the working range, and it should have a linearity coefficient of 0.995 or greater. An example of the acceptable linearity range is illustrated in the graph in **FIGURE 7**.

FIGURE 7. Linear Range.

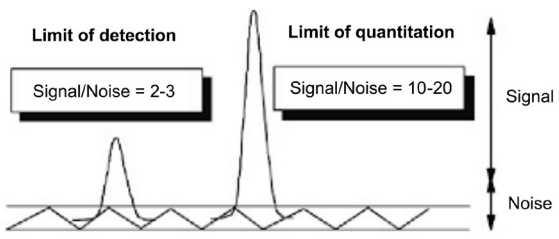


In addition to linearity, it is also important to know the limits of a method's detection capabilities. To do so, the Limit of Detection (LOD) and Limit of Quantitation (LOQ) must be calculated. The LOD is the lowest concentration that the instrument and method can detect and the LOQ is the lowest concentration that can be accurately quantitated for the instrument and method.

There are several ways to determine the LOD and LOQ. The first is an estimation by visually determining the presence of a peak and whether it can be measured and quantitated. A second more quantitative method would be to determine the signal-to-noise ratio. The concentration of the analyte that results in a signal-to-noise ratio of 3 or 10 would be the LOD or LOQ respectively. An example of LOD and LOQ signal-to-noise ratios is shown in **FIGURE 8**.

Another important facet of validation is a method's robustness. This tests the ability of the instrument and method to remain accurate in the face of minor changes. Some examples of minor changes are as follows, performing a like-for-like column replacement with a different serial or lot number, changing the mobile phase composition or pH, and minor changes to a method's flow rate or temperature. Instrumental methods that are robust will yield similar quantitative results in the face of these changes.

FIGURE 8. LOD and LOQ.



Matrix effects and interferences are another crucial part of method validation. They are the alteration of ionization efficiency by the presence of coeluting substances. Examples include cases for which matrix components prevent analytes from gaining access to the charge at the surface of the droplet, or where matrix components compete with the analyte to gain charge. Matrix effects and interferences can result in signal suppression or amplification, reducing the method's accuracy. To check for matrix effects, many different approaches can be taken, such as spiking a blank matrix with a standard and calculating its recovery rate.

Regardless of a lab's method validation strategy, all documentation related to validation, verification, and modification studies must be readily available for regulatory inspection. This should include an overall validation summary report for all elements presented in this article (precision, accuracy, linearity, range, robustness, and matrix effects). The source data, method synopsis, standards, reagents, equipment, certificates of assurances, calibration records, and everything else that went into running a validation (including training records of personnel) should all be documented and readily available for review.

SUMMARY

The Cannabis sativa plant is classified as two separate articles in the 2018 Farm Bill: hemp and cannabis (marijuana). From these articles multiple products can be derived, each subject to varying regulatory guidelines (State, Federal, and/or ISO). To comply, laboratories that ensure the quality of these products must establish that their systems and workflows meet the components listed in USP's Data Quality Triangle: data integrity, analytical instrument qualification, and method validation.

The most critical component is data integrity, as it is a requirement for laboratory conclusions to be drawn. It is the degree in which data or records are traceable and trustworthy. To maintain a record's integrity, Cannabis laboratories must establish sound data integrity controls to ensure both paper and electronic records meet the principals of ALCOA+ (attributable, legible, contemporaneous, original, accurate, complete, consistent, enduring, and available).

The second core component is instrument qualifications, which are needed to demonstrate and document that a system is accurate and working correctly. This activity varies based on equipment complexity and shouldn't be mistaken for calibration.

Building on these two foundational components is method validation. This is the totality of steps taken to demonstrate and document that a laboratory workflow is scientifically sound. Validation should be performed whenever a new workflow is adopted, or current workflow is modified, and verify the method's precision, accuracy, linearity, range, robustness, and possible matrix effects.

Laboratories ensuring the quality of Cannabis derived product should anticipate increased regulatory stringency as the industry continues to mature. To comply, it is recommended that companies implement the above topics to meet current and future requirements.

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Characterizing Xenobiotic Hemp Metabolites in the Raw Honey of Western Honey Bees (Apis mellifera) by UHPLC–MS/MS

By Edward A. Palumbo II

Administering high-CBD hemp extract to honey bees to produce CBD-infused honey is a promising new hemp-based dietary supplement.

OVERVIEW

Currently, there is a US patent granted for the process of administering high-CBD hemp extract to Western honey bees (Apis mellifera) through their food. The process results in raw honey infused with low-milligram quantities of phytocannabinoids.

This article presents a fascinating new dietary supplement format that is hemp-based, highlighting the method development process, sample preparation techniques, and instrument selection creating sensitive and fast dMRM methods with natural product stereoisomers using the Agilent 6470 UHPLC Triple Quadruple Mass Spectrometer and the Agilent MassHunter Molecular Optimizer.

THE CHARLOTTE'S WEB ANALYTICAL TEAM AND COLLABORATIONS

Charlotte's Web manufactures dietary supplements that are derived from hemp plants. The company's roots come from the development of a hemp oil that helps children with treatment-resistant epilepsy. Charlotte was one of the first children to respond very positively from CBD supplements and she bears the name of the company now.

The R&D team at Charlotte's Web (Yvonne DePorre, Edward Palumbo, and Alexander McCorkle) have worked on everything from method development to method validation, to chemical engineering projects, to optimizing the manufacturing processes at their 137,000 ft^2 CGMP-compliant facility in Colorado. They continue to build analytical chemistry expertise through collaborations with private companies like Agilent, as well as research partners throughout Colorado and the Northeast. The goal of this research collaboration is to create a novel dietary supplement or food product made from honey bees that is naturally infused with natural products. A worldwide patent issued to Avner Ben-Ahron at PhytoPharma International inspired this research collaboration.

SPONSORED CONTENT

Agilent Cannabis and Hemp Testing Solutions

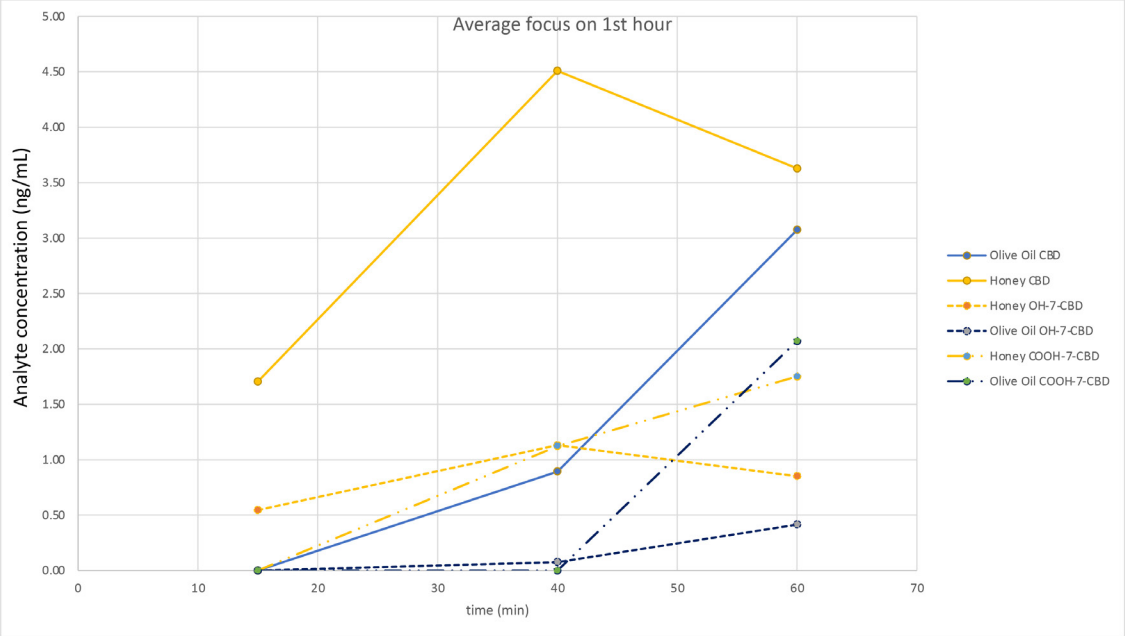
The first application of Avner's patent includes feeding honey bees with high-CBD cannabis oil that he emulsifies in water with non-GMO beet sugar extract. The bees consume the product and produce both pink honeycomb and pink raw honey containing milligram quantities of phytocannabinoids. Charlotte's Web is evaluating this product for its promise within the food and dietary supplement industries.

PHYTOPHARMA INTERNATIONAL'S MALE SPRAGUE DAWLEY RATS PK STUDY

PhytoPharma International initiated a preclinical research study to better understand the bioavailability of CBD in the hemp honey and compared the pharmacokinetic profile to a more conventional CBD tincture made with olive oil. It was found that the C_{max} detected in the blood of rats was several times higher in the honey. The same preclinical laboratory studied the concentration of CBD metabolites in the plasma of the rats. The CBD from the honey resulted in a much higher C_{max} with a more rapid onset than the CBD in the olive oil.

The results of the study, shown in FIGURE 1, indicate the primary metabolite of CBD, 7-hydroxy CBD, is readily detected in the blood of the rats following just 15 minutes of oral administration of the honey, which is not the case for the olive oil. These findings questioned whether primary and secondary CBD metabolites already present in the honey and fed to these male Sprague Dawley rats potentially exist.

FIGURE 1. Male Sprague Dawley Rats PK Study



The study concluded that honey bees have evolved very efficient detoxification mechanisms to metabolize compounds and inherently create a safety profile around these xenobiotics. Although honey bees possess the same first-pass metabolism enzymes that humans do, examining both honey bee and human consumption of nicotine, each utilizes a different isoform of P450 enzymes to create the same metabolite, cotinine. Given that the homology of these enzymes are found not only in humans and in honey bees but throughout the animal kingdom, the Charlotte's Web team asked, could honey bees that consume CBD in the their food-base be producing the same primary and secondary metabolites of CBD that humans do—namely, 7-hydroxy CBD and 7-carboxy CBD?

Instrument Selection

The Charlotte's Web team, possessing a full Agilent analytical suite of instrumentation, needed to determine which instrument would best meet the needs of the project. After considering several viable possibilities, the ideal candidate for this method development project was determined to be Agilent's UHPLC triple quadruple mass spec. This process involves using low temperatures to skip any derivatization techniques required on the front end of sample preparation. High sensitivity and high specificity of a

triple quadruple mass spec is retained, as well. The available Agilent Technologies instrumentation selection is shown in FIGURE 2, and indicates the low temperature, high sensitivity, and high specificity of their UHPLC-MS/MS (far right).

A paper published in 2019 by Brighenti et al. investigating the levels of phytocannabinoids in the raw honey produced by bees pollinating hemp and cannabis plants in the wild found that CBDA was present, along with THCA and some other acidic minor cannabinoids. The researchers developed a triple quadruple mass spec method that proved incredibly successful in reaching lower levels of detection and quantification.(1)

Charlotte's Web adopted this sample preparation technique, injecting reference material into the Agilent 6470B LC triple quadruple mass spec, then utilized Agilent's MassHunter Optimizer in positive mode and generated both precursor and daughter ions at relatively high abundance. The positive ion mode was used for analysis based on a 2019 application note published by Agilent for analyzing THC metabolite levels in rodent plasma.

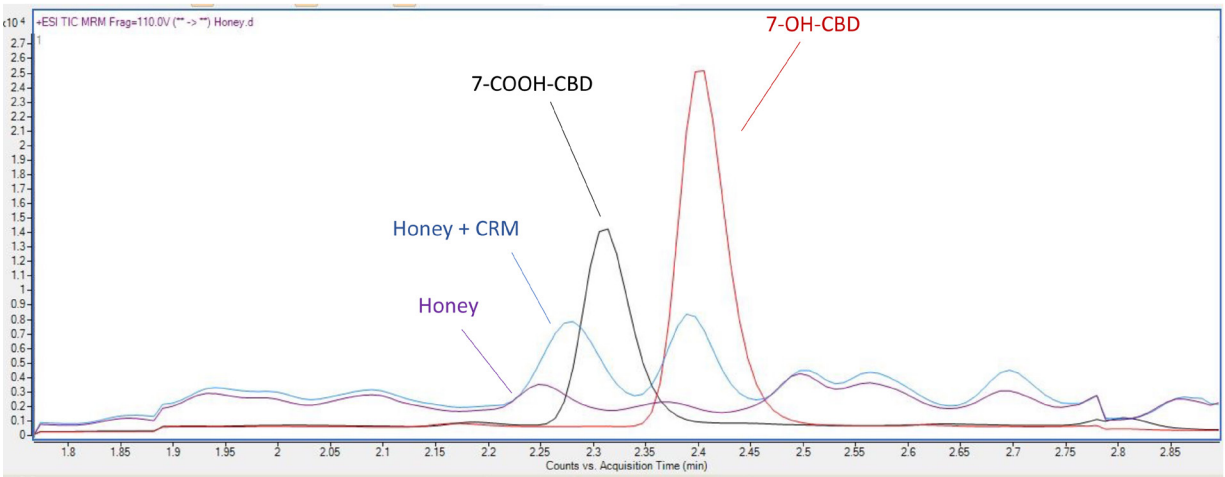
Spike Recovery Experiment - Reference Standard TIC Chromatograms

In a quick spike recovery experiment using a phenyl-hexyl column, two analytical standards were injected with a modified Agilent protocol and method. The primary

FIGURE 2. Instrument Selection

UHPLC-UV	GC-MS/FID	GC-MS/MS	UHPLC-MS/MS
(+) Low Temp	(+) Medium Specificity	(+) High Specificity	(+) Low Temp
(-) Low Specificity	(+) Medium Sensitivity	(+) High Sensitivity	(+) High Sensitivity
(-) Low Sensitivity	(-) Derivatization	(-) Derivatization	(+) High Specificity

FIGURE 3. Enzyme Catalytic TurnoverReference Standard TIC Chromotgrams



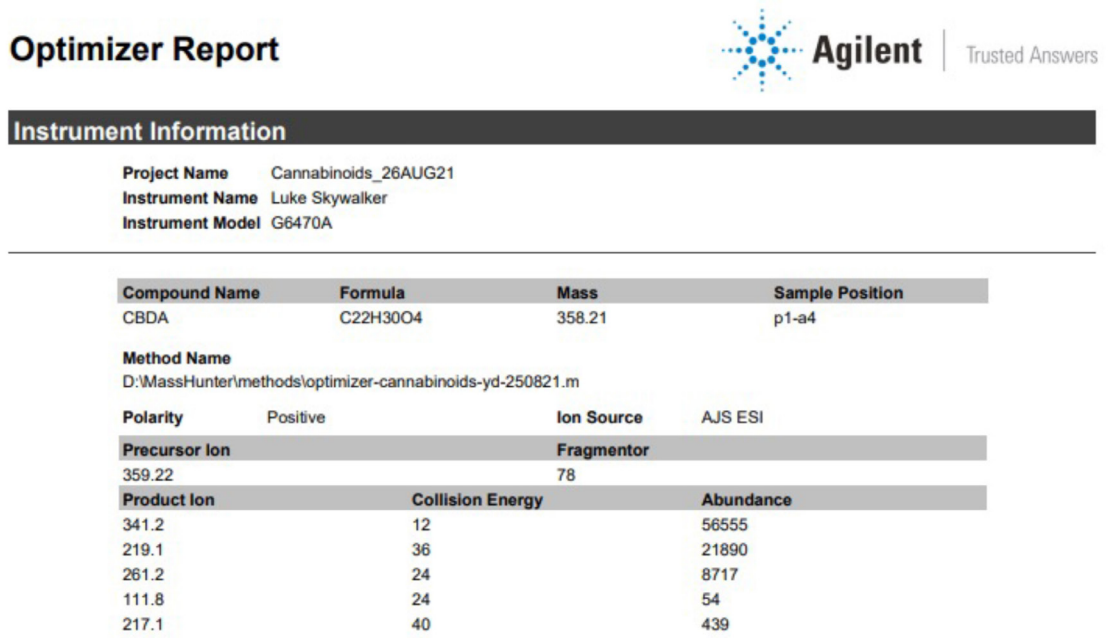
and secondary CBD metabolites are shown in FIGURE 3, with their transitions and an overlay of the total ion count chromatograms with the standards, the honey, and the honey spiked with the CRM.

Using methanol for the mobile phase, the resulting chromatograms indicated that the retention time peaks of the honey and honey with CRM were not lining up. This was most likely due to a strong solvent effect. The QuEChERS (quick, easy, cheap, effective, rugged, and safe) sample preparation technique was repeated using acetonitrile, a stronger solvent than methanol. However, there were some issues with strong solvent effects. Based on this observation, it was determined that there was, indeed, 7-carboxy CBD and 7-hydroxy CBD MRMs present with the method scan. Ultimately, the team decided to scan the entire chromatogram for those transitions.

Agilent's MassHunter Molecular Optimizer

Then, a comprehensive phytocannabinoid method on the LC triple quadruple was prepared. The first step was to determine the precursor and product ions produced by each phytocannabinoid being analyzed within the honey. Agilent's MassHunter Molecular Optimizer generated a comprehensive report eliminating many of the painstaking calculations using resonance structures, molecular masses, and fragmentation patterns that had been nightmares in the past. It was now possible to decipher what would be the major precursor and major product ions just by injecting a neat

FIGURE 4.



Optimizer Report

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Instrument Information

Project Name Cannabinoids_26AUG21
Instrument Name Luke Skywalker
Instrument Model G6470A

Compound Name	Formula	Mass	Sample Position
CBDA	C22H30O4	358.21	p1-a4

Method Name
D:\MassHunter\methods\optimizer-cannabinoids-yd-250821.m

Polarity	Positive	Ion Source	AJS ESI
Precursor Ion		**Fragmentor**	
359.22		78	

Product Ion	Collision Energy	Abundance
341.2	12	56555
219.1	36	21890
261.2	24	8717
111.8	24	54
217.1	40	439

reference standard onto the LC and running this software package. An example Optimizer Report from Agilent's MassHunter Molecular Optimizer is shown in FIGURE 4.

Using a mass spec can be somewhat of a problem if relying on just the mass of parent and daughter ions. In FIGURE 5, several molecular structures are all shown to have the same molecular formula and weight. Their only differences are stereoisomers.

Different quantifier ions and qualifier ions were developed through Agilent's MassHunter Molecular Optimizer software to ensure the same parent, precursor, daughter, and product ions throughout the mass spec portion of the method were being produced. About 18 total phytocannabinoids resulted using this triple quadruple mass spec method, demonstrating one to three orders of magnitude higher abundance when the negative ion mode versus positive ion mode was used. It was, therefore, decided to move towards making this a negative ion mode-specific method.

A sample chromatogram, FIGURE 6, includes a super mix of all 18 phytocannabinoids that were analyzed with this negative ion mode method. With an abundance of negative ion modes for all of our analytes, the same source conditions were used for the mass spec. The

FIGURE 5.

Cannabinoid	Polarity	MW	Parent ion	Fragmentor (V)	Daughter ion	CE (V)	Abundance
CBG	+	316.2	317.2	98	192.9	16	1.20E+03
					123	36	4.68E+02
	-		315.23	124	191.1	28	2.80E+04
					136	28	1.88E+04
CBN	+	310.19	311.2	110	223.1	20	1.10E+04
					293.1	16	4.43E+03
	-		309.18	139	279.2	36	4.12E+05
					222.1	52	2.20E+05
CBGA	-	360.23	359.5	130	315.2	24	
					341.2	24	
CBDVA	-	330.18	329.17	116	311.1	20	
					217.1	28	
CBNA	-	354.18	353.17	127	309.2	24	
					279.1	44	
CBCA	-	358.21	357.2	136	313.2	24	
					339.2	20	
THCVA	-	330.18	329.17	127	285.2	24	
					217.1	32	
D10THC	+		315.2	127	193.1	28	4.52E+04
					93.1	36	2.93E+04
	-	314.22	313.21	153	255.1	40	1.98E+05
					298.2	28	1.23E+05
CBL	-	314.22	313.21	206	191.1	24	
					311.2	16	
CBDV	+	286.19	287.2	89	165	24	3.80E+02
					123.1	32	1.75E+02
	-		285.18	107	217.1	20	2.16E+04
					283.2	16	9.39E+03
CBC	+	314.22	315.2	75	193.1	20	4.83E+02
					81.1	16	3.33E+02
	-		313.21	139	191.1	20	8.52E+04
					311.2	16	2.90E+04

Specificity II

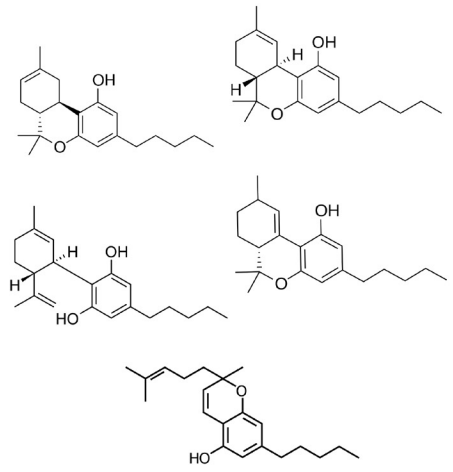
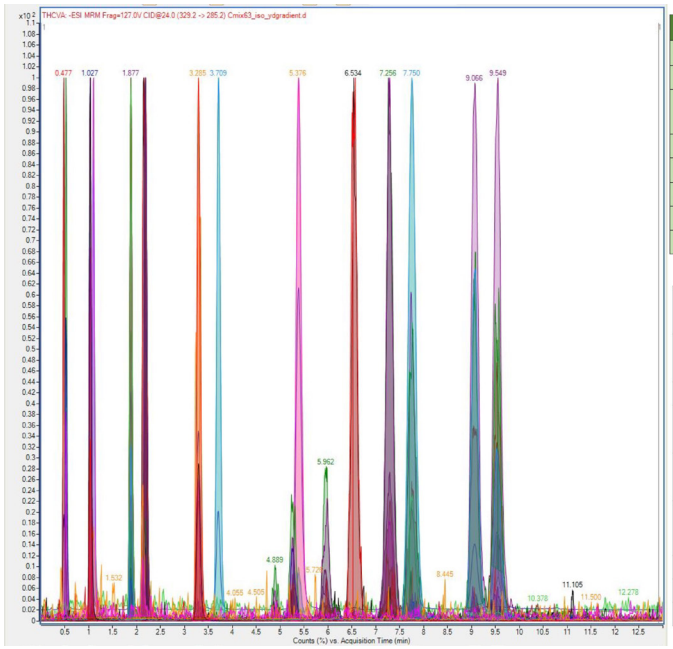


FIGURE 6.



UHPLC Conditions	
Column	Zorbax RRHD Bonus-RP C18 2.1 mm x 50 mm, 1.8 µM
Detection	MRM in negative ion mode
Mobile Phase	**A:** 0.01% formic acid H_2O **B:** 0.01% formic acid ACN
Pump Conditions	Gradient
Column Temperature	55 °C
Sample Temperature	4 °C, prepared at ambient
Flow Rate	0.575 mL/min
Injection Volume	5.0 µL

MS Source Conditions	
Gas Temperature	300 °C
Gas flow	10 L/min
Sheath Gas Temperature	375 °C
Sheath Gas Flow	12 L/min
Capillary Voltage	+3500 V to -3000 V
Nozzle Voltage	500 V
Dwell Time	18 msec
Nebulizer	45 psi

Time (min)	Mobile Phase B (%)
0.0	63.0
7.0	69.0
7.1	100.0
10	100.0
11.0	63.0
13.0	63.0

mobile phase was converted from methanol to acetonitrile to bypass any potential strong solvent effects during the chromatographic portion of our method.

The certified reference material mixtures were designed, keeping in mind that they contain stereoisomers that could possibly elute closely to one another. For this reason, each analyte was separated out based on retention time into four separate mixtures to reduce ion suppression or ion enhancement effects.

Raw honey purchased from a grocery store was spiked with 2-20 ppm each of each CRM (certified reference material) mix. Three separate spike recoveries were calculated, demonstrating good recoveries for every analyte, except for the metabolites. It is believed that these metabolites are very thermally labile compared to the other cannabinoids. It was also found that the phytocannabinoid composition of the honey mirrored the composition extract used to feed the honey bees.

However, the dynamic MRM (multiple reaction monitoring) method revealed that neither 7-hydroxy CBD nor 7-carboxy CBD was found in the honey. Those MRMs are not present within the same retention time window established using the certified reference material used for these two metabolites.

When the very large amount of CBD was fed to the honeybees, intact CBD was measured and detected at milligram quantities. It is therefore believed that not finding 7-hydroxy CBD or 7-carboxy CBD does not necessarily conclude there are no cannabinoid metabolites present in the honey. In fact, they could be present as different stereoisomers of these hydroxy and carboxy metabolites, or be conjugated to other macromolecules like glucose.

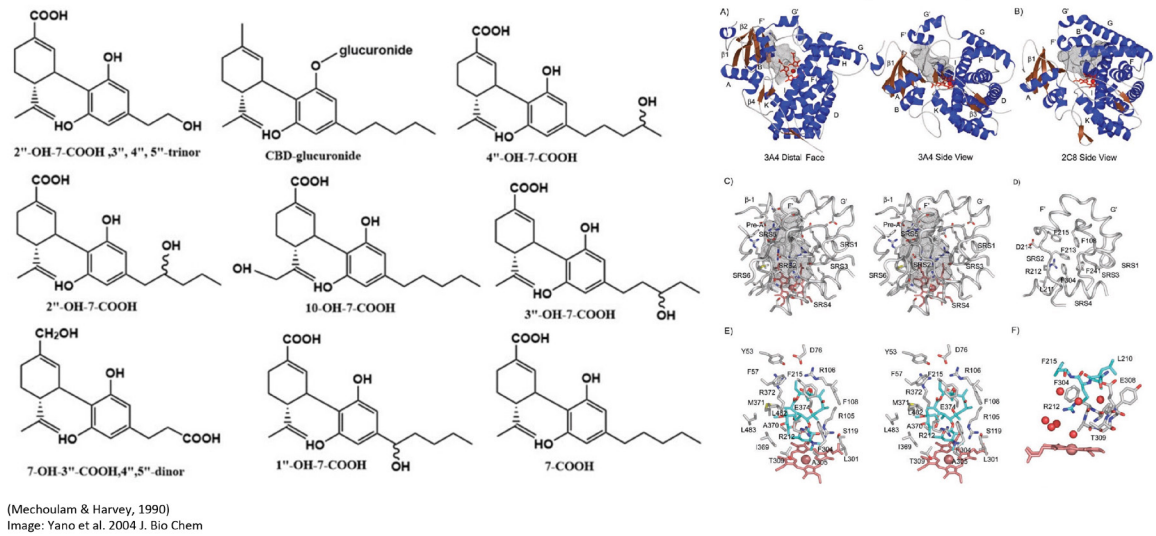
Enzyme Catalytic Turnover

Formation of glucose macromolecules is a very common detoxification mechanism used by honey bees. To examine that within a biochemistry perspective, enzyme efficiency needs to be considered. The cytochrome P450 enzymes that humans and honey bees contain are represented in the X-ray crystallography images shown in FIGURE 7.

A study was conducted in 1990 by Raphael Mechoulam in Israel of a terminally-ill patient who was consuming approximately 600 milligrams of CBD per day. By comparison, most people typically consume less than 100 mg/day. Analyzing all of the metabolites produced by this patient using a GC mass spec, Mechoulam's group was able to characterize 34 distinct metabolites in the urine. As a result, they determined that 20% of the cannabinoids in this person's urine was indeed intact CBD.(2)

Studies also found that the application of CBD being fed to honey bees resulted not only in getting more bioavailable honey, but also that the honey bees fed CBD had a much lower incidence of colony collapse disorder compared to honey bee hives not

FIGURE 7. Enzyme Catalytic Turnover



(Mechoulam & Harvey, 1990)
Image: Yano et al. 2004 J. Bio Chem

fed CBD. Through the collaboration with several research partners, the first step would be to conduct a full metabolomic analysis of the raw honey to try to fill in the gaps for what is not understand in the honey from a compositional aspect. Next would be to better investigate how CBD consumed by the honey bees affects their physiology, including the transcriptome, proteome, glycome, and lipidome.

ADDITIONAL OBSERVATIONS, CHALLENGES, AND GOALS

Environmentally, there is a link to be found between CBD consumption in honey bees and enhanced survival. This enhancement could be accomplished by manufacturing a type of hemp-based honeybee aspirin which could possibly boost the longevity of honeybee hives.

There was a potential degradation of target compounds using QuEChERS as a sample preparation step since it is very exothermic in nature. Ideally, it might be better to switch to a liquid-liquid extraction, eventually eliminating the QuEChERS technique. Another possibility is to find some middle ground where the vessel that contains the QuEChERS, salt, and solvents could be cooled. This would reduce some of the heat produced during the mixing of the reagents.

The Charlotte's Web group worked to have a very large, dynamic range—from low ppm quantities of CBD to high ppb values of potential metabolites. The linearity portion development of this method found that there were mostly quadratic fits for the line of best fit or second order natural logs.

Lastly, there were some challenges finding what would be the best ion mode for the source, when looking at analytes of interest. It is extremely important to have very fresh standards when running the MassHunter Optimizer package.

CONCLUSION

Administering high-CBD hemp extract to honey bees to produce CBD-infused honey is a promising new hemp-based dietary supplement.

Charlotte's Web manufactures dietary supplements that are derived from hemp plants. The company's roots come from the development of a hemp oil that helps children with treatment-resistant epilepsy.

Research studies presented in this article are spearheaded by the Charlotte's Web R&D analytical team at their 137,0000 ft^2 CGMP-compliant facility in Colorado. Their research and experiments include collaborations and partnerships with Temple University, Agilent Technology, and the University of Colorado, as well as research papers previously published from other sources.

PhytoPharma initiated a preclinical research study to better understand the bioavailability of CBD in the hemp honey and compared the pharmacokinetic profile to a more conventional CBD tincture made with olive oil. It was found that the C_{max} of CBD detected in the blood of rats was several times higher in the honey.

Important method development tools include the Agilent 6470 UHPLC triple quadruple mass spec and Agilent's MassHunter Optimizer. These instruments are ideal, as the resulting method operates at low temperature while providing high sensitivity and high specificity.

Researchers at Charlotte's Web believe there is great potential for utilizing living organisms to produce natural product compounds. To date, they have focused on the honey bee and how CBD consumed affects their physiology.

In collaboration with Agilent and Temple University, researchers at Charlotte's Web would like to investigate and better understand the entire "ome" of the honey bee—the transcriptome, proteome, glycome, and lipidome … the full "ome" of the honey bee.

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1. Brighenti V, Licata M, Pedrazzi T, et al. Development of a new method for the analysis of cannabinoids in honey by means of high-performance liquid chromatography coupled with electrospray ionisation-tandem mass spectrometry detection. Journal of Chromatography A. 2019. https://doi.org/10.1016/j.chroma.2019.03.034.
2. Harvey DJ, Mechoulam R. Metabolites of cannabidiol identified in human urine. *Xenobiotica*. 1990. doi:10.3109/00498259009046849.

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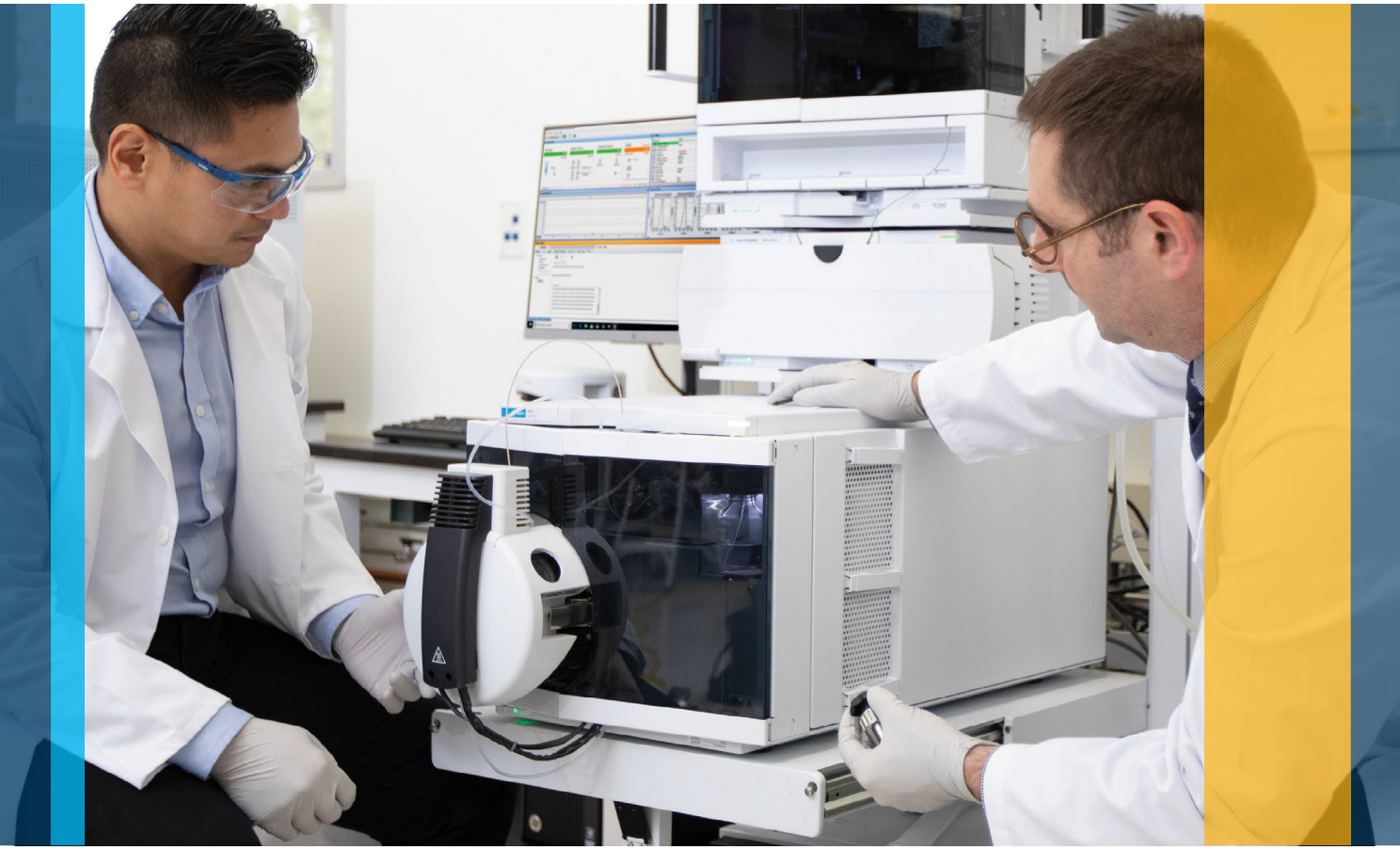

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