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Analytix Reporter

Special Edition

Cannabis Testing

- Potency & Cannabinoids
- Moisture
- Terpenes
- Pesticides & Heavy Metals

Not with standing recent changes in certain state laws in the US and federal law in Canada, federal law in the US remains unchanged and the cultivation, possession, and/or sale of marijuana and related cannabis products continues to be illegal under such law. In addition, the distribution of marijuana to minors under the age of (i) 21 years remains illegal under state law in the US and (ii) 18 or 19 years (depending on the state) remains illegal under state law in Canada

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Cannabis Testing

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Dear Reader,

The first records of the medicinal use of cannabis by ancestral cultures date back to the 500s BC. The Greek, Roman, and Asian civilizations used it for the therapeutic treatment of various diseases like arthritis, inflammation, and pain among others.

After being considered an illicit drug for decades, cannabis has now once again gained prominence in the medical field. Several scientific studies have proved its beneficial effects in the treatment of various pathologies. This has made the countries to start reviewing the legal status of the plant.

Paraguay, Canada, and the United States have led efforts to legalize cannabis in recent years. They are now being followed by countries from Europe, Latin America, Asia, and Oceania.

Currently, the laws regulating the manufacturing practices and quality standards of cannabis source materials are few or non-existent. Besides, they vary greatly between countries or even between different states, in the case of the USA.

Cannabis quality control is mandatory to alleviate patient concerns about the efficacy and safety of cannabis-derived therapies, as well as other legal derivatives.

With the rise of cannabis and hemp legislation, the industry has seen an increased demand for accurate development and validation of data, in addition to accurate testing methods. This includes potency testing, measuring the presence and amount of various terpenes, as well as testing for contaminants such as pesticides, residual solvents, heavy metals, and mycotoxins. Besides these analyses, the measurement of moisture content is also important.

It is essential that cannabis and cannabis-related products meet the purity and safety standards as per the regulations established. This will ensure the quality and safe consumption of products released to the market. Our analytical chemistry experts and partners have developed a collection of Application Notes, which you will find in this issue, to help you optimize the existing analytical methods as well as the recently developed ones.

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Cannabinoid Certified Reference Materials (CRMs) for Improved Testing Accuracy and Traceability

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Abstract

Reference materials play a critical role in cannabis workflows. Your results are only as accurate as your reference material. We have developed a portfolio of cannabinoid Certified Reference Materials (CRMs) for use in calibration & quantitation, system suitability studies, and qualitative screening.

Introduction

The interest in cannabinoid quantitation or potency testing of marijuana and hemp continues to grow with the expanding commercialization of cannabis dietary supplements and recreational products. While most of the U.S. states have legalized marijuana for medical use and several for recreational use, it still remains federally illegal and is classified as a schedule 1 substance. The growing of hemp crops in the U.S. was federally legalized by the U.S. Agricultural Improvement Act of 2018, also known as the 2018 Farm Bill. The U.S. Department of Agriculture (USDA) final rule for hemp production published on January 19, 2021 requires the total Tetrahydrocannabinol (THC) content of plant material on a dried weight basis to be less than 0.3% for it to be legally defined as hemp. Total THC content is taken as the sum of Δ-9 THC and its biosynthetic precursor, Tetrahydrocannabinolic acid (THCA), as THC is the degradation product of THCA after its decarboxylation. A hemp producer must discard the entire lot if the sampled plant material contains more than 0.3% of THC resulting in potentially dire financial repercussions. Production of plant material containing more than 1% of total THC is defined as a negligent violation and could result in suspension or revocation of the producer's USDA license to grow hemp. These implications underscore the importance of accurate cannabinoid analytical testing. However, the final rule does acknowledge the importance of analytical variability and requires testing laboratories to report the measurement of uncertainty (MU) associated with the THC test results, in order to allow the "acceptable hemp THC level" to account for the MU.[1]

In the interim final rule (IFR), the Agricultural Marketing Service (AMS) requested inputs on the potential requirements for hemp testing laboratories to obtain ISO/IEC 17025 accreditation. However, the requirement was not included in the final rule due to the comments citing insufficient laboratory capacity currently available to accommodate all the needed hemp testing. Labs are required to meet certain standards of performance and use test methods that are fit-for-purpose, however currently there is no federal laboratory approval process in place and the state requirements vary widely. The allowed analytical test methodologies are GC-FID or HPLC with either UV or MS detection, and the methods must meet the AOAC Standard Method Performance Requirements 2019.003.[2] AOAC has published official method 2018.11 for the quantification of cannabinoids in plant materials, that has undergone a rigorous approval process and is accepted as a highly credible method.[3] The United States Pharmacopeia (USP) has noted the need for increased quality control and harmonized practices in cannabis testing for medical purposes and has published important quality attribute considerations to aid in addressing these gaps.[4] NIST offers a cannabis quality assurance program similar to a proficiency test to help laboratories evaluate their testing comparability and competence.[5] Even with these guidelines and resources in place, the industry has acknowledged the continued problem of inconsistent results obtained from different laboratories.

The accuracy of a testing laboratory's calibration standards is a critical factor that directly impacts the accuracy of the test results. The labs must manufacture or purchase a suitable raw material or solution-based reference material for use as a calibrator. Availability of cannabinoid Certified Reference Materials (CRMs) allows testing laboratories to make cost-effective in-house calibrators, traceable to the CRMs, thereby contributing to a higher level of test accuracy and reproducibility. ISO 17034 - specific to reference material producers and ISO/IEC 17025 - specific to testing laboratories provide standardization to the manufacturing and testing of CRMs. CRMs are considered to have the highest level of accuracy and traceability to the SI unit of measurement for of all the materials manufactured by ISO accredited producers.[6,7] There is a selection of cannabis related reference standards offered by USP

for medical use. But there are no cannabinoid reference standards manufactured by any national metrology institute. However, hemp plant and oil reference materials are currently being developed by NIST.[5]

We have designed and manufactured a portfolio of cannabinoid CRMs listed in **Table 1** to support the cannabis testing industry. These products are offered as single or multiple analytes dissolved in an appropriate diluent and packaged in amber flame-sealed ampoules. Appropriate handling and process controls were put in place to ensure the analyte's stability in storage and transit. The concentration of each analyte is certified in accordance with ISO 17034 and ISO/IEC 17025. The concentration uncertainty is calculated and reported along with the certified concentration in the accompanying certificate of analysis (CoA). The cannabis testing laboratories should propagate this value of unexpanded uncertainty in their own method's uncertainty calculations.

Table 1. Cerilliant® portfolio of cannabinoid CRMs

Description	Cat. No.
Single compound solutions	
Cannabidiol (CBD), 1 mg/mL in methanol	**C-045**
Cannabinol (CBN), 1 mg/mL in methanol	**C-046**
Cannabidivarin (CBDV), 1 mg/mL in methanol	**C-140**
Cannabigerol (CBG), 1 mg/mL in methanol	**C-141**
Cannabigerolic acid (CBGA), 1 mg/mL in acetonitrile	**C-142**
Cannabichromene (CBC), 1 mg/mL in methanol	**C-143**
Cannabidiolic acid (CBDA), 1 mg/mL in acetonitrile	**C-144**
Cannabichromenic acid (CBCA), 1 mg/mL in acetonitrile	**C-150**
Cannabidivarinic acid (CBDVA), 1 mg/mL in acetonitrile	**C-152**
Cannabinolic acid (CBNA), 1 mg/mL in acetonitrile	**C-153**
Cannabicyclol (CBL), 1 mg/mL in acetonitrile	**C-154**
Cannabicyclolic acid (CBLA), 0.5 mg/mL in acetonitrile	**C-171**
Δ9-tetrahydrocannabinol (Δ9-THC), 1 mg/mL in methanol	**T-005**
Δ8-tetrahydrocannabinol (Δ8-THC), 1 mg/mL in methanol	**T-032**
Exo-THC, 1 mg/mL	**T-033**
Δ9-tetrahydrocannabinolic acid (THCA), 1 mg/mL in acetonitrile	**T-093**
Tetrahydrocannabivarin (THCV), 1 mg/mL in methanol	**T-094**
Tetrahydrocannabivarinic acid (THCVA), 1 mg/mL in acetonitrile	**T-111**
THC-O-Acetate Solution, 1 mg/mL	**T-151**
Mixes	
Cannabinoid Mixture (Acids), 6 Component, 500 µg/mL each	**C-218**
Cannabinoid Mixture (Neutrals), 8 Component, 500 µg/mL each	**C-219**
Hemp Compliance Mix, 1 mg/mL CBD, 3 µg/mL THC	**C-217**
THC Cannabinoids Mixture-3, 1 mg/mL	**T-108**

Manufacturing and Certification of Cannabinoid CRMs

The cannabinoid raw materials used to prepare Cerilliant® CRMs were all synthesized in-house and certified by a predetermined test plan designed in accordance with ISO 17034 and ISO/IEC 17025. The identity of each cannabinoid was verified by ^{1}H-NMR and high-resolution liquid chromatography-mass spectrometry (LC-MS). The potency or mass fraction of each raw material was assigned as a mass balance purity factor (MBPF), calculated by subtraction of the mass of impurities from the mass of the analyte using **Equation 1**. Impurities were determined through a range of techniques accounting for those that are volatile, inorganic, and organic. The residual water content was determined by Karl Fischer coulometry. While other residual volatile content was determined by headspace gas chromatography with flame ionization detection (HS-GC-FID), the residual inorganic content was determined by residue on ignition (sulfated ash).

Organic impurities were determined by high performance liquid chromatography with ultraviolet detection (HPLC-UV) and reported as the average of two orthogonal methods. Orthogonal selectivity of the two methods was established through different chromatographic stationary and mobile phases. GC-FID was used as a confirmatory technique for analysis of organic impurities wherever appropriate. In some cases, quantitative ^{1}H-NMR was also used as a confirmatory technique to MBPF.

Formulation studies were performed to determine appropriate handling techniques, storage conditions, suitable concentrations and diluents for the proposed products. The final CRM designs were based on the results of the formulation studies. Each solution was prepared by gravimetric measurement of the analyte(s) and diluent

Equation 1. Mass balance purity factor calculation.

$$\text{Mass Balance Purity Factor} = \left[[100 - (\text{wt\% Solvents}) - (\text{wt\% } H_2O) - (\text{wt\% Inorganics})] * \frac{\text{ChromPurity}}{100} \right]$$

Where:

wt% Solvents = residual solvent content with uncertainty, u_{OVI}

wt% Inorganics = residual inorganic content with uncertainty, u_{ROI}

wt% H_2O = residual water content with uncertainty, u_{KF}

ChromPurity = Chromatographic purity with uncertainty, u_{cp}

with the concentration determined by **Equation 2**, using the actual measured mass, purity adjustment of the analyte(s), measured mass of the solution, and density of the pure diluent at 20 °C. For viscous, glassy, or hard-to-handle raw materials, a stock solution was made and analytically verified prior to the final dilution. All mass measurements are traceable to the International System of Units (SI) through qualified and calibrated analytical balances and were reported on the conventional basis for weighing in air. The mass of each solution was converted to volume by dividing the mass by the density of the solution. Density measurements were made on a density meter and are traceable to higher order standards through calibration. The prepared concentration of each analyte is reported in units of mass per volume, with expanded uncertainty and specified confidence interval. The solutions were dispensed into amber ampoules with a fill volume of not less than 1 mL and flame sealed under argon.

Equation 2. Certified concentration calculation for gravimetrically prepared CRM.

$$C = \frac{(m_{v+a}-m_v)d}{(m_{f+s}-m_f)p} \pm U$$

Where:

- C = Certified Property Value, concentration of analyte in solution in units of mass/volume
- m_{v+a} = mass of analyte + vial
- m_v = mass of empty vial
- m_{f+s} = mass of flask + solvent
- m_f = mass of empty flask
- d = density of solution
- p = purity adjustment, 100/mass balance purity factor (for the analyte)
- U = the assigned combined expanded measurement uncertainty

The concentration, homogeneity, and purity of each CRM was verified through HPLC-UV analytical testing. Sealed ampoules were selected for testing from across the batch based on a random weighted stratified sampling plan, with a higher percentage of samples taken at critical process points. The concentration was verified by comparison to an independently prepared calibration solution and calculated as the average of replicate analyses of samples. Within and between ampoules homogeneity was verified through relative standard deviation of the replicate analyses of samples. The accuracy and homogeneity acceptance criteria included allowances for uncertainty contributions from the analytical measurement, and variability from transfer and evaporative loss during preparation of samples for analysis.

Short-term transit stability was established through temperature stress studies performed at freezer (-25 to -10 °C), refrigerator (2-8 °C), room temperatures (15-30 °C) and a stressed temperature (40 °C +/-2 °C). One ampoule of each solution standard was removed from each storage condition at specified time points and moved to sub-freezer storage until analysis. The ampoules stored at stressed temperatures were evaluated for purity and/or concentration by HPLC-UV.

Long-term stability of the CRMs was assessed for 14 months following their manufacture. This was done using HPLC-UV analysis for real-time studies of solution purity and concentration. These studies are subsequently carried out for the entire shelf life of the product.

Results and Discussion

The ISO defines a reference material (RM) as a material that is homogenous, stable, and fit for its intended measurement use. A CRM must meet additional requirements to those for RMs. CRM characterization methods must be metrologically valid and traceable to the measurement unit of the certified property value.[8] The ISO guides give some flexibility to the CRM manufacturers as to how they meet these requirements. As a result, the certification process can vary widely among the manufacturers, from assigning a potency from a simple chromatographic purity to a comprehensive MBPF approach. Cannabis testing laboratories must be mindful of this and review the CRM's certificate of analysis (CoA) to ensure it is fit for their intended use. **Table 2** shows possible pitfalls associated with using only a chromatographic purity. The chromatographic purity of cannabinol is 99.5% but the potency assigned by MBPF to it is 96.0%— due to the presence of residual solvent and water.

Table 2. Calculated potency and impurity contributions for representative cannabinoid raw materials.

Compound	Chrom. Purity (%)	Residual solvent content (%)	Residual water content (%)	Trace inorganic content (%)	Content/Potency (%)
Cannabidiol (CBD)	99.3	0.85	< LOD	< LOQ	98.4
Cannabinol (CBN)	99.5	3.39	0.11	NA	96.0
(-)-Δ^9-THC	98.1	1.47	NA	NA	96.7
Cannabigerol (CBG)	99.0	< LOD	< LOQ	< LOQ	99.0
Cannabichromene (CBC)	99.0	< LOD	< LOQ	NA	99.0
Cannabidiolic acid (CBDA)	99.0	1.40	< LOQ	< LOQ	97.6
Cannabigerolic acid (CBGA)	99.3	0.16	< LOD	< LOQ	99.1
Δ^9-Tetrahydrocannabinolic acid (THCA-A)	98.4	0.41	< LOD	< LOQ	98.0
Tetrahydrocannabivarin (THCV)	98.8	1.68	< LOQ	NA	97.2
Cannabidivarin (CBDV)	98.8	0.91	< LOQ	< LOQ	97.9

In addition to the certification method, every CoA should include data to support stability during transit and long-term over the shelf life of the product. **Figure 1A** shows an example of a temperature stress study for an unstable prototype formulation, where we see a decrease of concentration with an increase in storage temperature and time. A final stable formulation of product **C-218** was developed through optimized diluent selection, material handling, and formulation process controls. **Figure 1B** shows that the concentrations of all analytes in **C-218** remain stable at multiple storage temperatures up to four weeks, with degradation only observed in the samples kept at 40 C°. **Figure 1C** shows the same accelerated stability data plotted as a line graph for two representative analytes, CBDA and THCA-A, with additional real-time stability shown up to 6 months. Continued real-time stability is assessed throughout the shelf life of the product.

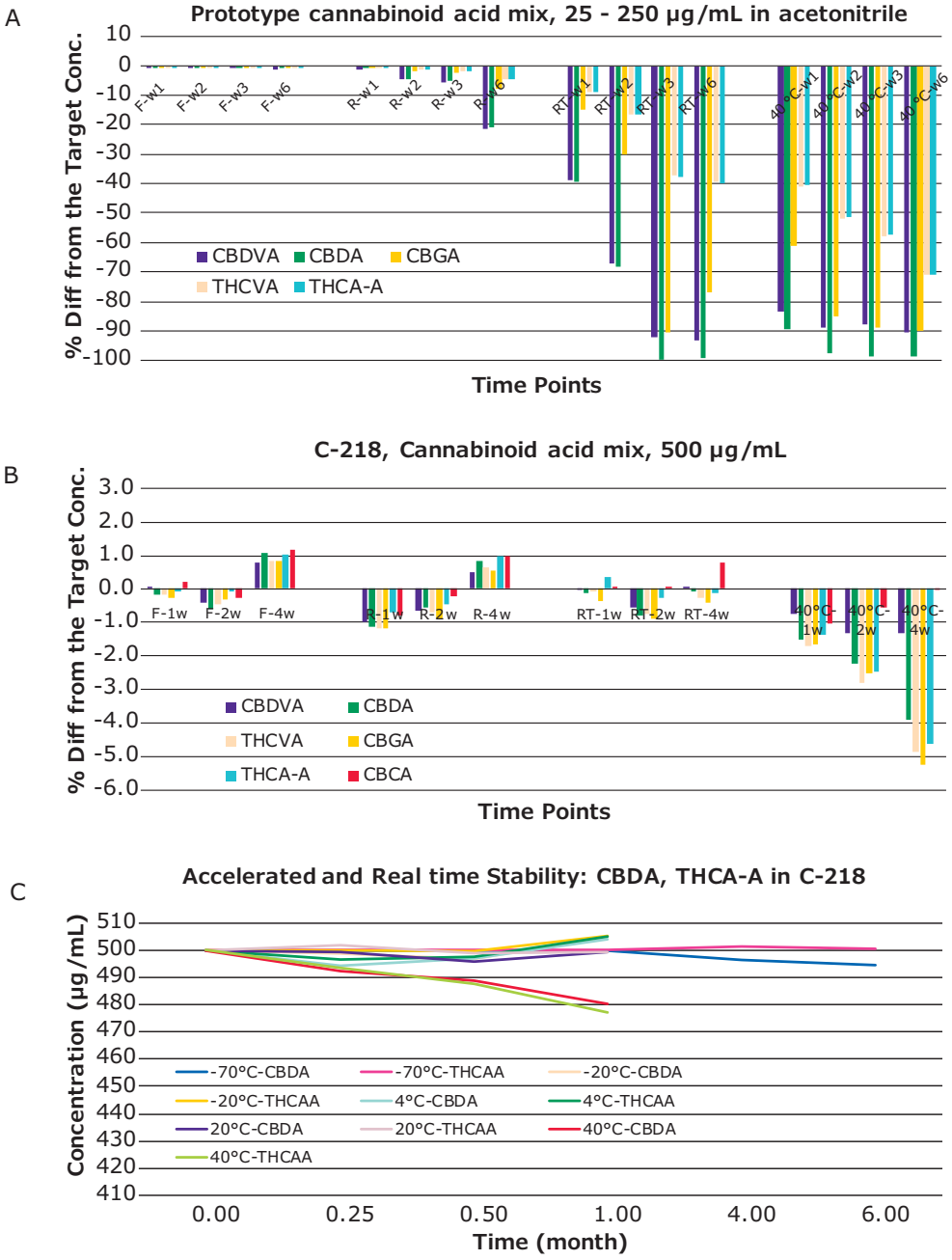

Figure 1. Bar graphs depicting the temperature stress studies of a prototype **(A)** and finalized **(B)** acidic cannabinoid mixture with bars showing the change in concentration related to time stored at varied temperatures: F: freezer (-25 to -10 °C), R: refrigerate (2-8 °C), RT: room temperature (15-30 °C) and 40 (±2) °C. The bottom graph **(C)** plots the change in concentration over six months of CBDA and THCA-A in cat. no. **C-218** stored at varied temperatures.

A CRM CoA should report the method used to determine the measurement uncertainty. **Figure 2** shows an example fishbone diagram for the sources of uncertainty associated with the certified concentration of a gravimetrically prepared solution standard. **Equation 3** shows the calculation for propagation of uncertainty. The method used to calculate the uncertainty was established in accordance with ISO 17034 and ISO Guide 35 through identification of the production process variables and risks coupled with statistical analysis. Uncertainty is expressed as expanded uncertainty at the approximate 95% confidence interval using a coverage factor of k=2. It incorporates uncertainty of the purity factor, material density, balance, weighing procedure, solution standard homogeneity, and stability. The uncertainty of the certified concentration is stated in the CoA in terms of mass per volume.

Equation 3. Uncertainty calculation for the certified concentration of a CRM including homogeneity and stability terms.

$$u_{cert} = \sqrt{(u_{char}^2 + u_{bb}^2 + u_{stab}^2)}$$

Where:

u_{cert} = Standard uncertainty of the Certified Property Value

u_{char} = Standard uncertainty of the solution standard preparation and includes u_{pf} for characterization of the analyte mass balance purity factor(pf), u_m for mass measurements, and u_d for the solvent density.

$$u_{char} = \sqrt{(2u_{pf}^2 + 4u_m^2 + u_d^2)}$$

u_{bb} = Standard uncertainty of between bottle homogeneity

u_{stab} = Standard uncertainty of stability

Determining the uncertainty value for a CRM incorporates technical studies on all aspects of a solution standard preparation process, including mass measurement, density measurement, verification, homogeneity, and stability. The studies incorporate replicate measurements under different process conditions and establish standard uncertainties for the density and mass measurements. The studies also provide validated process controls for weighing and dispensing. Homogeneity uncertainty contributions are assessed through statistical analysis of concentration accuracy data for samples pulled at critical timepoints during the dispensing of a given lot. Stability uncertainty contributions are assessed from temperature stressed or real-time stability data.

CRMs may be used for identification, quantitation, system suitability and method control. **Figure 3** shows an example of a CRM dilution scheme to achieve a standard containing seventeen cannabinoids. This can initially be used as a screening tool to evaluate the presence or absence of cannabinoids in samples. Once the cannabinoids of interest are identified, the mixture can be diluted into a calibration curve spanning the expected concentration range of the constituents and used for quantitation. Alternatively, single analyte CRMs can be used to prepare the calibration curves. Example HPLC-UV chromatograms of the 17-cannabinoid mix prepared from 5 CRMs (cat. nos. **C-218**, C-219, C-153, C-154 and C-171) and hemp bud extract are compared in **Figure 3**. **Figure 4** illustrates the use of our Hemp Compliance Mix (C-217) as a system suitability test to ensure method performance for detection of THC at the maximum allowed level of 0.3%. A separate solution containing 5 µg/mL of cannabidiolquinone (CBDQ) was run using the same method. CBDQ is

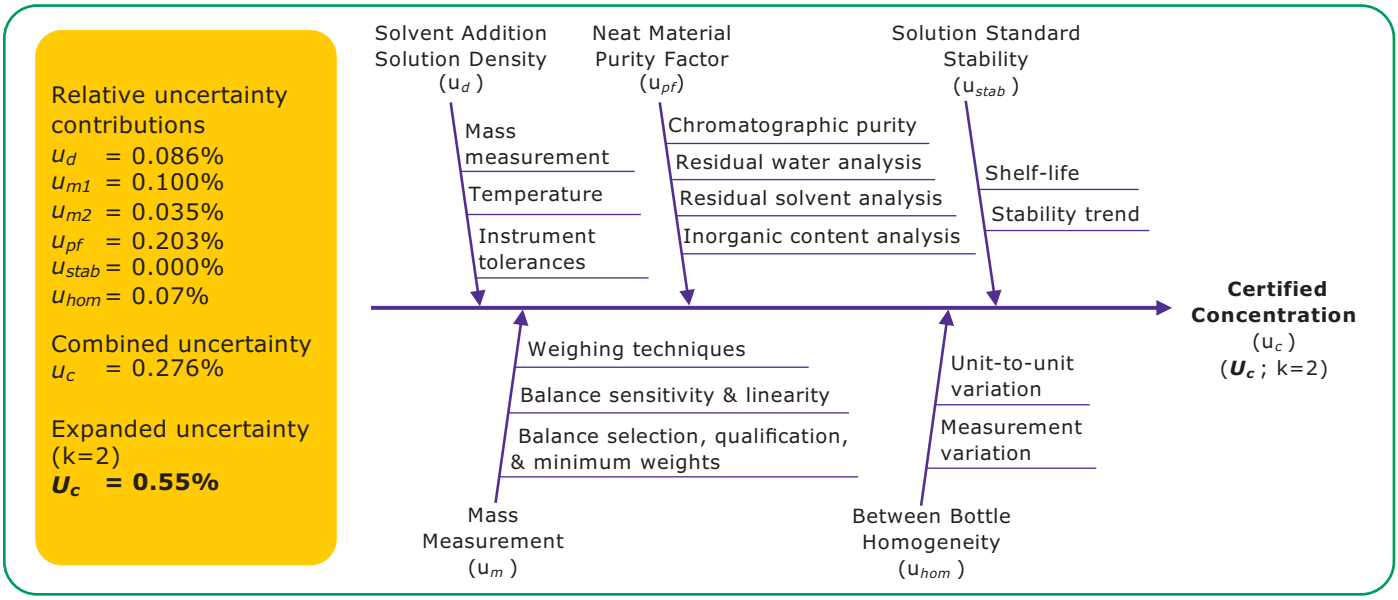

Figure 2. Example fishbone diagram of measurement uncertainty contributions for a gravimetrically prepared CRM.

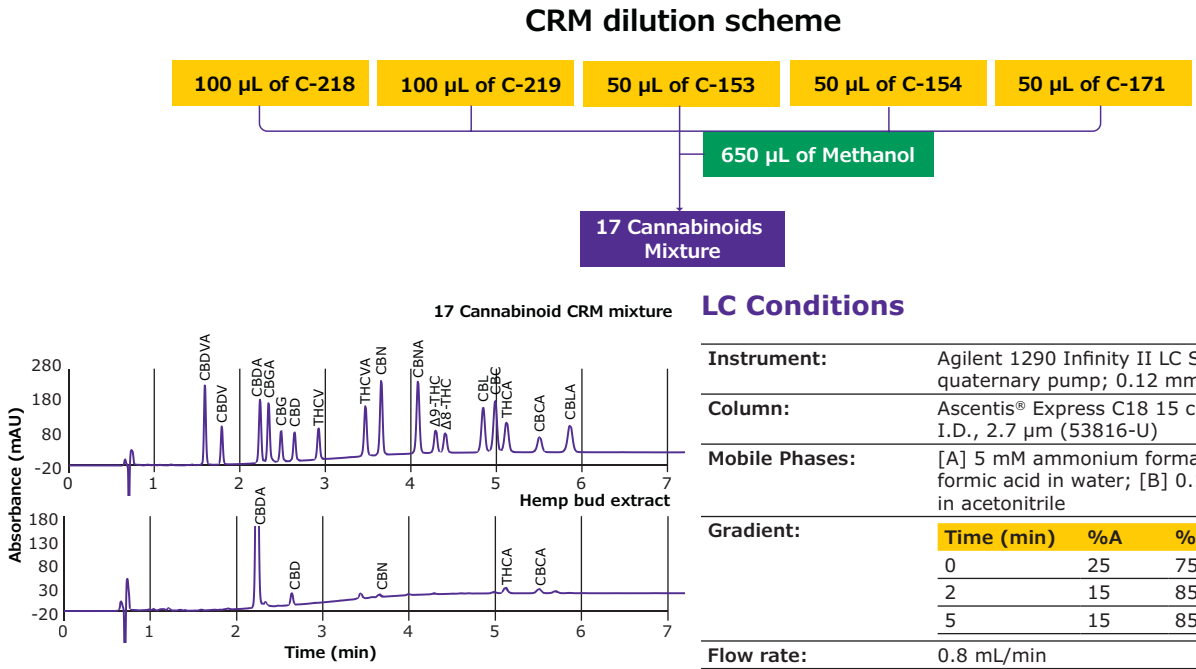

Figure 3. Dilution scheme of Cerilliant CRMs to produce a 17 cannabinoid mix with respective HPLC-UV chromatogram above a hemp bud extract chromatogram acquired with rapid gradient method.

LC Conditions

Instrument:	Agilent 1290 Infinity II LC System with quaternary pump; 0.12 mm ID tubing
Column:	Ascentis® Express C18 15 cm x 3.0 mm I.D., 2.7 µm (53816-U)
Mobile Phases:	[A] 5 mM ammonium formate in 0.1% formic acid in water; [B] 0.1% formic acid in acetonitrile
Gradient:	Time (min) / %A / %B: 0 / 25 / 75; 2 / 15 / 85; 5 / 15 / 85
Flow rate:	0.8 mL/min
Injection Volume:	5 µL
Detector:	UV 228 nm
Column Temperature:	25 ºC

one of the oxidation degradants of CBD. The overlaid chromatograms show baseline separation of THC and CBDQ by this method. If other cannabinoids or impurities coelute with THC, errors in quantitation will result." It is thus important to routinely check method performance using a mix formulated specifically for this purpose, such as illstrated in **Figure 4**.

Conclusion

We have developed CRMs of individual cannabinoids as well as mixes (**Table 1**). These can be used for potency profiling of both hemp and cannabis. Specifically, our Hemp Compliance Mix was formulated to simplify standards preparation for analysis of THC content in hemp. Using rigorous process controls and formulation studies as described here, we formulated stable cannabinoid mixtures. Our optimized raw material and packaging processes protect cannabinoids from oxidation, thus producing CRMs with long term stabilities. With the variability in testing methods and accreditation across laboratories, the use of accurate and traceable and properly certified CRMs is critical.

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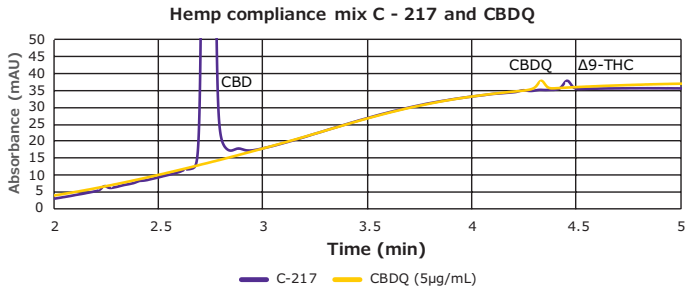

Figure 4: Overlay of HPLC-UV chromatograms of C-217 hemp compliance mix and a solution containing CBDQ.

References

1. Establishment of a Domestic Hemp Production Program. Fed. Regist., December 2021, 86 FR 5596, 5596-5691. https://www.govinfo.gov/content/pkg/FR-2021-01-19/pdf/2021-00967.pdf
2. AOAC SMPR 2019.003. Standard Method Performance Requirements (SMPRs) for Quantitation of Cannabinoids in Plant Materials of Hemp (Low THC Varieties Cannabis sp.).
3. AOAC Official Method of Analysis 2018.11. Cannabinoids in Cannabis plant materials, concentrates, and oils. www.aoac.org/
4. Sarma ND, Waye A, ElSohly MA, Brown PN, Elzinga S, Johnson HE, Marles RJ, Melanson JE, Russo E, Deyton L, Hudalla C, Vrdoljak GA, Wurzer JH, Khan IA, Kim NC, Giancaspro GI. Cannabis Inflorescence for Medical Purposes: USP Considerations for Quality Attributes. J Nat Prod. 2020;83(4):1334-1351. doi: 10.1021/acs.jnatprod.9b01200.
5. NIST Tools for Cannabis Laboratory Quality Assurance: https://www.nist.gov/programs-projects/nist-tools-cannabis-laboratory-quality-assurance (accessed 11.10.2021).
6. ISO 17034:2016, General requirements for the competence of testing and calibration laboratories, International Organization for Standardization, Geneva, Switzerland, 2016. www.iso.org
7. ISO/IEC 17025:2017, General requirements for the competence of reference material producers, International Organization for Standardization, Geneva, Switzerland, 2017. www.iso.org
8. ISO GUIDE 30:2015, Reference materials – Selected terms and definitions, International Organization for Standardization, Geneva, Switzerland, 2017. www.iso.org

Optimizing for High Throughput Analysis of Cannabinoids in Cannabis Products

Improved retention time stability and chromatographic performance using Fused-Core® technology

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With increasing cannabis and hemp legislation, there has been increased demand for development and validation of accurate and precise testing methods for potency quantitation. Cannabinoids present a number of challenges, and there is also the additional burden of dealing with a variety of matrix types. HPLC/UV is the technique most commonly used, and the HPLC parameters must be optimized to maintain good separation and stable retention over many injections and with the various sample types.

Scientists at Supra Research and Development ("SupraRnD") located in Kelowna, British Columbia, Canada (www.suprarnd.ca) have developed a high throughput and reliable method for cannabinoids that is applicable to a variety of matrices. SupraRnD's involvement in cannabis testing began in 2015 when they obtained a license from Health Canada for testing cannabis products. In 2018 they were one of the first laboratories in Canada to obtain their ISO 17025 accreditation for cannabis testing. Their potency method has evolved over time to meet the changing needs of their customers, and is now validated for several different matrices.

Experimental Conditions

Whole flower samples were frozen in hermetically sealed bags at -80 °C for a minimum of 30 minutes and then homogenized immediately.* It is critical that a representative sample is homogenized and subsampled when analyzing cannabis flower, as there can be considerable variance in phytocannabinoid concentrations between and within a given plant. The subsequent workflow involved a simple extraction of a 0.2 g sample size with methanol, followed by sonication and stabilization of the extract at -20 °C for 1 hour. The sample was then centrifuged, and the supernatant diluted 100:1 for HPLC analysis. The small sample size in combination with the pre-analysis dilution minimizes the potential for matrix-related issues (e.g., interferences, column longevity, etc.). The HPLC portion of the analysis has a cycle time of 8 minutes injection to injection. This allows 60 injections per 8-hour interval, which enables more customer samples to be run in a work shift. The cannabinoids analyzed by the method are listed in **Table 1.**

Table 1. 17 Phytocannabinoids separated by HPLC method

1.	CBDVA
2.	CBDV
3.	CBDA
4.	CBGA
5.	CBG
6.	CBD
7.	THCV
8.	THCVA
9.	CBN
10.	CBNA
11.	Δ9-THC
12.	Δ8-THC
13.	CBL
14.	CBC
15.	THCA
16.	CBCA
17.	CBLA

The final, optimized HPLC parameters are summarized in **Table 2.** When developing this method, the following were considerations:

- Chromatographic resolution of all 17 compounds.
- Cycle time (i.e. run time plus equilibration) of less than 10 minutes total.
- A rugged method with consistent performance for >1000 injections with stable retention times, while maintaining good peak shape and response.
- Suitable for use with different matrices such as flower, chocolate, ointment, oil, concentrate, etc.

* After publication of this data SupraRnD later removed the freezing step and homogenized the whole flowers at room temperature. This was done to reduce the possibility of inflating the moisture content through condensation of atmospheric water onto the cold samples.

Table 2. Optimized method HPLC parameters

Column:	Ascentis® Express C18, 15 cm x 2.1 mm I.D., 2 µm
Mobile phase:	(A) 5 mM ammonium formate in water + 0.1% formic acid; (B) 0.1% formic acid in acetonitrile
Gradient:	70 to 90% B in 3 min; held at 90% B for 2 min; to 98% B in 0.1 min; held at 98% B for 0.9 min; to 70% B in 0.1 min; held at 70% B for 0.9 min
Flow rate:	0.4 mL/min
Pressure:	533 bar
Column temp.:	30°C
Detector:	UV, 228 nm
Injection:	25 µL
Sample:	methanolic extract of cannabis derived samples (oil, concentrate, ointment, etc.)

Calibration for the method was from 0.01 µg/mL to 40 µg/mL. This required a high dilution for some samples in order to bring them within this analytical range. For calibration and spiking, Cerilliant® certified reference materials (CRMs) were used. Individual cannabinoid CRMs at 1 mg/mL (with the exception of CBLA at 0.5 mg/mL) were diluted, along with the internal standard solution, directly into HPLC mobile phase component A, to prepare a 17-component stock solution at 40 µg/mL. This stock was then diluted further into a 30:70 mixture of HPLC mobile phases A:B, for the lower concentration calibration standards.

The HPLC column used for the analysis was an Ascentis® Express C18 column, 15 cm x 2.1 mm I.D., 2 µm. Ascentis® Express columns contain Fused-Core® particles with a solid core and porous shell architecture, also referred to as superficially porous. This particle structure provides higher separation efficiency than fully porous particles of the same size, and allows for faster analysis times with lower backpressure than approaches using smaller (<2 µm) fully porous particles. The particle architecture of Ascentis® Express columns allows for the use of larger particles, making them suitable for both conventional and UHPLC systems. For this method, SupraRnD used a UHPLC system, although with proper optimization, a similar result can be achieved on a conventional system using a 15 cm x 3.0 mm, 2.7 µm Ascentis® Express C18 column. Specifically, this would involve minimization of system dispersion. This can be done by reducing tubing length and ID of the column inlet and outlet; and for UV detectors, using a flow cell with a volume of <5 µL.

Method Validation and Performance

Prior to choosing the Ascentis® Express C18 for method validation, SupraRnD screened six other columns of similar chemistry from various manufacturers. They were able to achieve chromatographic resolution and a short run time with several columns, but it was found that the Ascentis® Express C18 was the only column that provided retention time stability – especially for the acidic cannabinoids. This is illustrated in **Figure 1** which shows chromatograms of a check standard at injection #1 and injection #1140, in between which numerous sample extracts were run.

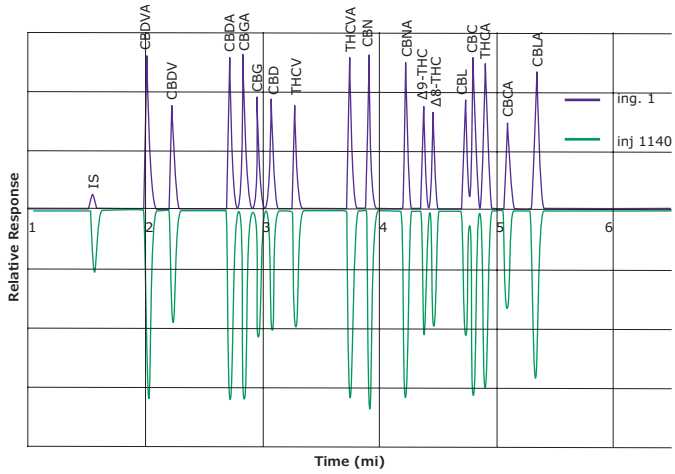

Figure 1. Cannabinoid standard on Ascentis® Express C18 column; comparison of injection #1 and injection #1140.

The method using the Ascentis® Express C18 was validated in several different matrices including hop flowers (as a surrogate matrix to cannabis), hemp seed oil, CBD concentrate, and topical ointments. Recoveries from hops ranged from 85-115% over a spiking range of 0.05 % to 20% by weight. A summary of this validation, as well as the other matrices, is summarized in **Table 3.** The method reporting limits (MRLs) achieved for the cannabinoids (except for CBDV, CBG, CBD and CBC in the concentrate) were all 0.05 wt.%. Repeatability, as %RSD, was <4% for all matrices. Further evaluation was done using proficiency testing in which the method successfully passed for samples of cannabis flower and hemp oil.

Table 3. Summary of method validation data for cannabinoid method in several matrices

	% Recovery range of all 17 cannabinoids spiked into matrix			RSD	MRL (wt%)
Spiking level (wt%)	**0.05%**	**1%**	**20%**		
Hops (surrogate matrix)	86-106	96-115	100.5-116	< 1.5%	0.05
Hemp seed oil	92-118	104-116	101.5-113	< 4%	0.05
Ointment 1					
(CBD isolate)	83-120*	80-122*	--	< 3%	0.05
Ointment 2	79-129**	86-117**	--	< 2.5%	0.05
CBD concentrate	71-123.5*	92-118*	--	< 3.5%	0.05 ***

*CBD recovery not quantitated due to high incurred levels
**Δ9-THC recovery not quantitated due to high incurred levels
***CBDV, CBG, CBD, CBC incurred in matrix led to issues preventing calculation of MRL for these compounds

Figure 2 shows example chromatograms of hop flowers spiked at 1% w/w and at the MRL concentration of 0.05% w/w. At the much lower spiking level, where matrix interference was more apparent, all 17 cannabinoids were discernable from background peaks and could be analyzed. The specific interferences eluting next to THCV and CBL were probably due to certain terpenes present in the hop sample. These peaks were not observed in cannabis flower. In a spiked ointment sample **(Figure 3)**, all cannabinoids were clearly detected at the MRL.

To date, >1,550 injections have been made on a single Ascentis® Express C18 column. SupraRnD has noted that thus far there has been no significant increase in column backpressure, or degradation in performance. Data collected on backpressure over the course of this use, showed a net increase of 2%. They also noted that retention times were stable, allowing them to identify cannabinoid peaks in samples with more confidence. An example is illustrated in **Figure 4** in which two different matrices, dark chocolate and cannabis flower, are compared. Both samples contained measurable amounts of Δ9-THC, and the difference in the retention time between the two matrices were minimal.

Conclusion

After evaluating several HPLC columns, SupraRnD has successfully developed a robust and rugged method using the Ascentis® Express C18 column for the analysis of 17 cannabinoids in a variety of matrices. Thus far, the method has been successfully applied to five different sample types including flower, ointments, chocolate, concentrates and gummies. The Ascentis® Express C18 column was chosen for the final method based on retention time stability over repeated use, and ability to maintain chromatographic performance for the cannabinoids. In addition, the column currently in use has shown minimal increase in backpressure over the course of >1,550 injections.

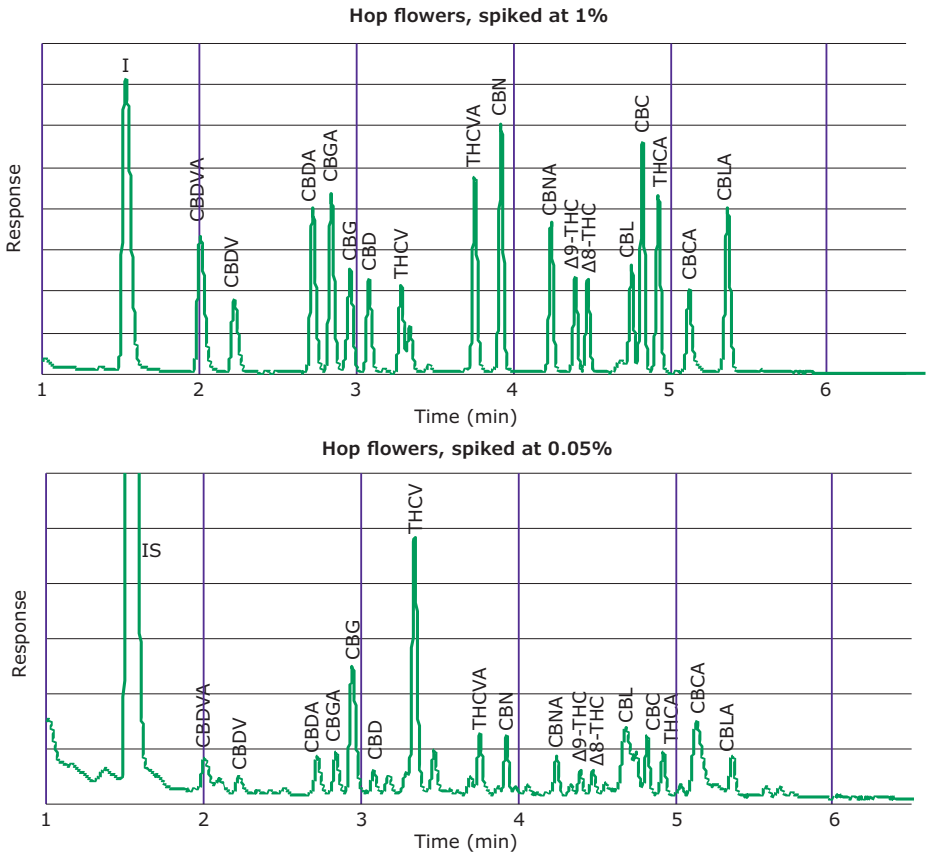

Figure 2. Hop flowers, spiked at 1% and .05% with cannabinoids.

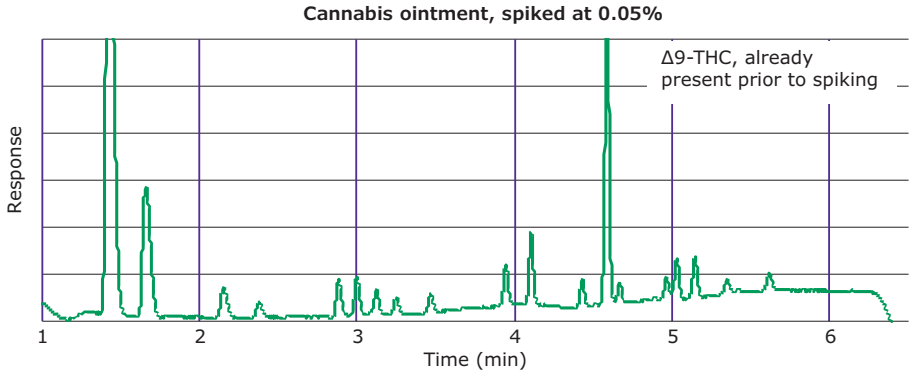

Figure 3. Ointment made from cannabis extract, spiked at 0.05% by weight.

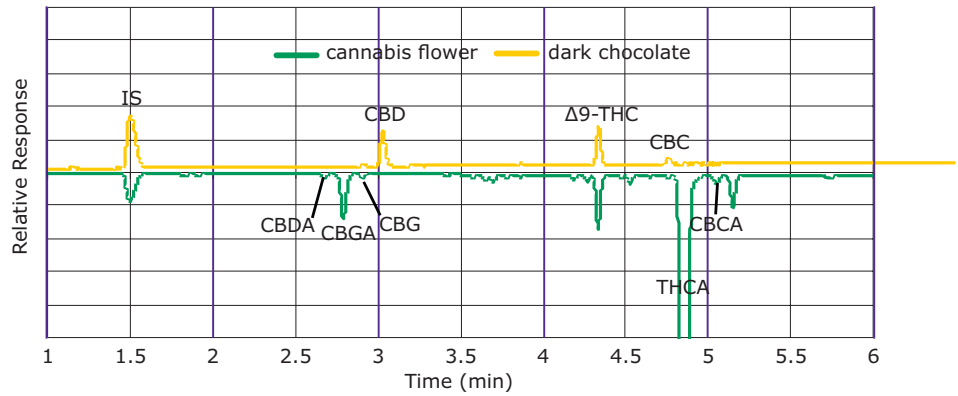

Figure 4. Comparison of elution pattern between dark chocolate and cannabis flower samples (unspiked).

Featured Products

Description	Cat. No.
Ascentis® Express C18, 15 cm x 2.1 mm I.D., 2 µm	**50814-U**
Cerilliant® Certified Reference Materials (all 1.0 mg/ml if not noted otherwise)	
Cannabidivarinic acid (CBDVA), in acetonitrile	**C-152**
Cannabidiolic acid (CBDA), in acetonitrile	**C-144**
Cannabigerolic acid (CBGA), in acetonitrile	**C-142**
Tetrahydrocannabivarinic acid (THCVA), in acetonitrile	**T-111**
Δ9-tetrahydrocannabinolic acid (THCA), in acetonitrile	**T-093**
Cannabinolic acid (CBNA), in acetonitrile	**C-153**
Cannabichromenic acid (CBCA), in acetonitrile	**C-150**
Cannabicyclolic acid (CBLA), 0.5 mg/mL, in acetonitrile	**C-171**
Cannabidivarin (CBDV), in methanol	**C-140**
Cannabigerol (CBG), in methanol	**C-141**
Cannabidiol (CBD), in methanol	**C-045**
Tetrahydrocannabivarin (THCV), in methanol	**T-094**

Description	Cat. No.
Cannabinol (CBN), in methanol	**C-046**
Δ9-tetrahydrocannabinol (Δ9-THC), in methanol	**T-005**
Δ8-tetrahydrocannabinol (Δ8-THC), in methanol	**T-032**
Cannabichromene (CBC), in methanol	**C-143**
(±)- Cannabicyclol (CBL), in acetonitrile	**C-154**

Related Products

Description	Cat. No.
Ammonium formate, eluent additive for LC-MS, LiChropur™, ≥99.0%	**70221**
Formic acid, for HPLC LiChropur™	**5.43804**
Acetonitrile, gradient grade LiChrosolv® Reag. Ph Eur	**1.00030**

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POTENCY & CANNABINOID TESTING

Cannabinoid Analysis of Hemp: Developing an Efficient HPLC Method Workflow

Edited article, refer to SigmaAldrich.com/Analytix (Issue 8) for full version

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Abstract

Several different HPLC method options are explored for the efficient and time effective analysis of cannabinoids in a sample of hemp flower. The differences between gradient and isocratic analyses are examined, and the two methods compared in determining the weight percentage of cannabinoids.

Introduction

Consumption of both marijuana and hemp products for medical use stems from the proposed benefits of cannabinoids and other natural compounds present in the plants. In the case of marijuana, the need for cannabinoid testing, often referred to as "potency testing", is driven by the need to assure product quality and safety. Cannabinoid testing of hemp and hemp-derived products is also on the rise. Hemp, classified as a strain of *Cannabis sativa* with low THC content, was made legal on a federal level in the U.S. with the passing of the Agriculture Improvement Act of 2018, also known as the "2018 Farm Bill".[1] This resulted in the establishment of the U.S. Domestic Hemp Production Program by the United States Department of Agriculture (USDA). Guidelines issued under this program as the "interim final rule" (IFR) designate that for a product to be classified as hemp, its THC content must be < 0.3%.[2] This has subsequently driven not only quality testing of hemp for cannabinoid content, but also the need for accurate THC measurement to ensure that the product meets legal requirements.

The two major cannabinoids of interest in both marijuana and hemp are Tetrahydrocannabinol (THC) and Cannabidiol (CBD), which are commonly reported on product information labels. However, there are many more cannabinoids present, and some states are increasing their requirements for the number that must be reported. Thereby, testing must be able to distinguish and provide accurate results for multiple cannabinoids.

In the case of marijuana and hemp flower, a common approach to cannabinoid testing incorporates a simple liquid extraction with methanol or ethanol, followed by HPLC-UV analysis. UV detector is preferred as it is easier and less expensive to operate than a mass spectral (MS) detector; however, it requires peaks to be separated chromatographically for proper identification and accurate quantitation.

Many HPLC methods for cannabinoid analysis utilize a gradient elution. But this often adds to the overall run time as an equilibration to initial conditions is required for every sample. Using an isocratic method eliminates the need for repeat equilibration, which may in turn allow more samples to be run per unit time. However, the tradeoff can be a loss in resolution during some portions of the run. In this work, we demonstrate both gradient and isocratic conditions capable of separating 17 cannabinoids on Ascentis® Express HPLC columns. For each set of conditions, the overall elution time was maintained at <8 minutes. We then applied these methods to the analysis of dried hemp flower. The cannabinoids targeted for testing consisted of those listed by the AOAC in standard method performance requirements (SMPRs) for dried plant material, chocolates, and concentrates, plus three additional cannabinoids of interest.[3,4,5]

Experimental

The sample preparation method used was taken from a first action AOAC method for the quantitation of cannabinoids in cannabis.[6] A 5 g sample of hemp flower of unknown origin, donated by an external source, was homogenized to a particle size of <1 mm using a Cryo-Cup™ grinder. In order to homogenize the sample without degradation of the cannabinoids, use of a technique in which the sample is cooled is preferred. The low temperature prevents analyte degradation and produces uniform particle sizes.

A 500 mg sample of homogenized hemp flower was weighed into a 50 mL centrifuge tube. A 20 mL aliquot of ethanol was added, and the tube was vortexed briefly to mix. The tube was then shaken at 250 rpm for 30 min and centrifuged at 4000 rpm for 5 min to pelletize the plant material. The supernatant was carefully decanted into an amber 50 mL volumetric flask and set aside. A second extraction was performed from the plant material in the same 50 mL centrifuge tube using an additional 20 mL aliquot of ethanol. The resulting supernatant was then combined with the first and brought to a final volume of 50 mL with ethanol. To allow quantitation of high and low levels of cannabinoids in the hemp sample, the extract was diluted 1:10 and 1:100 with methanol. The diluted extract was filtered directly into a glass autosampler vial using a Mini-Uniprep™ G2 filter vial with a 0.20 µm PTFE membrane.

A peak identification standard at 50 µg/mL (CBLA at 25 µg/mL) containing 17 cannabinoids was prepared from certified reference materials of each cannabinoid. A separate mix containing the cannabinoids that were subsequently quantitated in the sample was prepared at 100 µg/mL and then diluted down to produce six calibration standards ranging from 0.25 to 100 µg/mL. HPLC analysis was performed on the same set of hemp flower extracts using two different sets of conditions (**Table 1**), that are described in more detail in the following sections. The instrumentation used for the analyses was a modern UHPLC, with a low-pressure mixing system and a 1 µL UV flow cell.

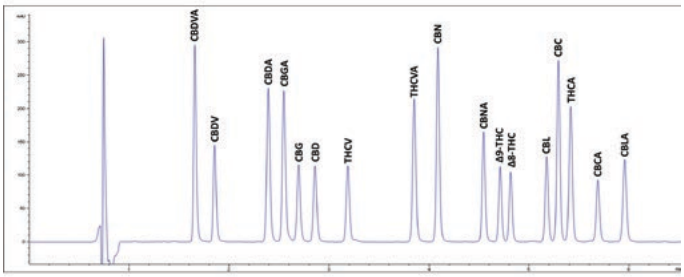

Figure 1. Separation of Cannabinoids in the Peak Identification Mix Using a Gradient on a 2 µm Ascentis® Express C18 Column

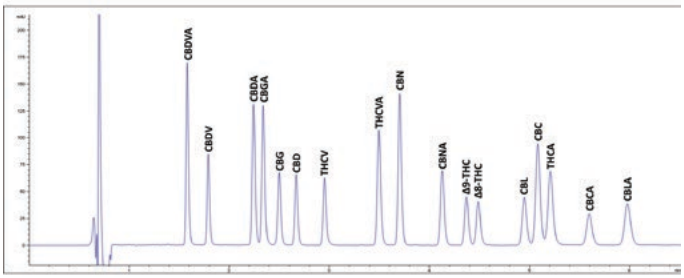

Figure 2. Separation of Cannabinoids in the Peak Identification Mix Using a Gradient on a 2.7 µm Ascentis® Express C18 Column

Results & Discussion

HPLC method optimization. Several factors considered when developing the HPLC methods for this workflow included: separation efficiency, speed, mobile phase composition, flow rate, and isocratic vs. gradient conditions.

The columns chosen for this work were from the Ascentis® Express line. This family of columns contains particles with a solid core/porous shell architecture (also referred to as superficially porous), which provides higher separation efficiency for a given particle size, compared to conventional fully porous particles.

Gradient methods. Gradient methods were developed on C18 columns with two different particle sizes: 2 µm and 2.7 µm. The gradient conditions and injection size were optimized for each column, as noted in **Table 1**. Formic acid and ammonium formate were added to the mobile phase to improve peak shape and resolution. Chromatograms showing the separation of the 17 cannabinoids in the peak identification mix on the two columns are shown in **Figures 1** and **2**. Separation is achieved in a little over 6 minutes. As expected the resolution is better on the 2 µm column. Another advantage of the 2 µm C18 method is the lower flow rate (0.4 mL/min vs. 0.8 mL/min), which uses less mobile phase per run and will ultimately result in cost savings both in solvent usage and waste disposal. However, with the smaller 2 µm particles there is higher backpressure, which requires the use of a UHPLC system.

Isocratic method. Isocratic HPLC methods are simpler than gradient methods and can be faster since no pre-run equilibration is required between samples. An isocratic method was developed for the 17 cannabinoids on an Ascentis® Express C8 column (**Table 1**). The organic and aqueous constituents of the mobile phase were of the same composition as those used for the gradient methods, with the ratio optimized for the

Table 1. HPLC Method Conditions

	Ascentis® Express C18 15 cm x 2.1 mm, 2.0 µm	Ascentis® Express C18 15 cm x 3 mm, 2.7 µm	Ascentis® Express C8 15 cm x 3 mm, 2.7 µm
Column	**(50814-U)**	**(53816-U)**	**(53853-U)**
mobile phase:	[A] 5 mM ammonium formate + 0.1% formic acid in water [B] 0.1% formic acid in acetonitrile		
mobile phase conditions:	75% to 90% B in 2 min; held at 90% B for 5 min	75% to 85% B in 2 min; held at 85% B for 5 min	Isocratic: 27:73, A:B
flow rate:	0.4 mL/min	0.8 mL/min	0.7 mL /min
pressure:	530 bar	250 bar	220 bar
column temp.:	25 °C	25 °C	30 °C
detector:	UV, 228 nm	UV, 228 nm	UV, 228 nm
injection:	3 µL	5 µL	5 µL

application. The resulting separation (**Figure 3**) yielded broader peaks as compared to the gradient methods, especially for Δ^8- and Δ^9-THC, and the run time was 2 minutes longer. This was expected since the gradient method utilizes an increased solvent strength to speed up elution of more retained analytes. In optimizing the isocratic method, a C8 phase was used to help counter this to some degree, as retention would have been even longer using C18 under the same conditions. The C8 phase also showed different selectivity towards some of the acidic cannabinoids compared to the C18 phase. Specifically, the retention of THCVA, CBNA, THCA, CBCA, and CBLA shifted relative to their neutral counterparts, resulting in a different elution order for two of these cannabinoids. Note: After the last peak, an additional 6 minutes (8 min + 6 min) proved sufficient for washing the column. The method uses a high ACN composition, which also helped reduce the wash time. However, to prevent any further build-up on the column, it's good practice to perform a high organic wash after each sample batch.

Analysis of hemp flower for cannabinoids. Analysis of the hemp sample was performed using both the 2.7 µm C8 and 2 µm C18 stationary phases. The hemp extract prepared as previously described was first screened by comparison to the 17-component peak ID mix. The six most prevalent cannabinoids were then quantitatively analyzed on both columns, yielding similar results (**Table 2**). This dual approach confirmed a high abundance of total CBD, which is characteristic of hemp. Total THC and CBD contents were calculated as a sum of their neutral and acidic forms after applying a conversion factor of 0.877 to account for the mass difference from decarboxylation. This dual column approach confirmed a high abundance of total CBD, which is characteristic of hemp. However, the total THC content exceeded the 0.3% limit to qualify as hemp, as per the USDA Interim Final Rule.

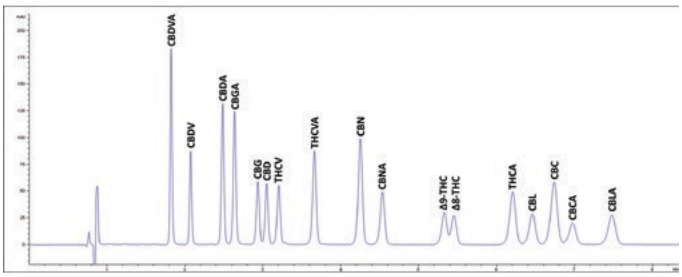

Figure 3. Separation of Cannabinoids in the Peak Identification Mix Using an Isocratic Method on a 2.7 µm Ascentis® Express C8 Column.

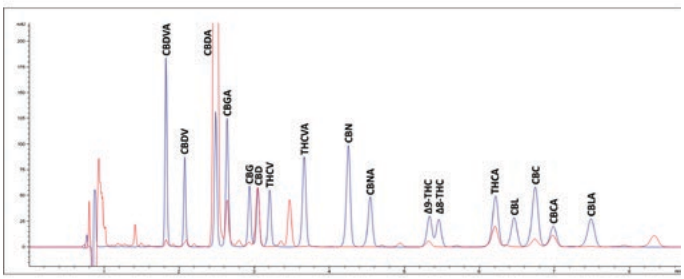

Figure 4. Overlay of Hemp Sample and Peak Identification Mix, Isocratic Method on Ascentis Express C8 2.7 µm Column

Table 2. Results of Cannabinoid Analysis of Hemp Sample on C8 and C18 Columns

	w/w % (Dry Weight)	
Analyte*	C8, 2.7 µm	C18, 2 µm
CBDA	18.20	18.10
CBG	0.13	0.10
CBD	1.40	1.40
CBN	ND	ND
Δ^9-THC	0.22	0.24
THCA	0.46	0.50
Total THC	0.62	0.68
Total CBD	17.36	17.26

*For analyte abbreviations, see featured products table on the next page

Conclusion

A complete HPLC workflow for the analysis of 17 cannabinoids was developed using both gradient and isocratic HPLC methods. The total THC content of a hemp sample was evaluated by two different HPLC methods, and was found to significantly exceed the 0.3% total THC limit necessary to be classified as hemp by the USDA Interim Final Rule. The results confirmed the high total CBD content characteristic of hemp, with the majority detected in the acidic form (CBDA). Results from both methods were in agreement and demonstrated a high degree of selectivity despite the abundance of matrix components. The workflow can easily be adopted to quantitate cannabinoids in plant material and concentrates with concentrations spanning 0.05 – 100% by weight. The short run times and low solvent consumption of both methods also makes them cost-effective for high-throughput potency testing.

References

1. Hemp: USDA.gov/topics/hemp, accessed Apr. 6th,2020.
2. Establishment of a Domestic Hemp Production Program. Fed. Regist., 2019, 84 (211), 58522-58564.
3. AOAC SMPR 2017.002. Standard Method Performance Requirements (SMPRs) for Quantitation of Cannabinoids in Dried Plant Materials
4. AOAC SMPR 2017.019. Standard Method Performance Requirements (SMPRs) for Quantitation of Cannabinoids in Edible Chocolate
5. AOAC SMPR 2017.001. Standard Method Performance Requirements (SMPRs) for Quantitation of Cannabinoids in Cannabis Concentrates
6. Vaclavik, L.; Benes, F.; Fenclova, M.; Hricko, J.; Krmela, A.; Svobodova, V.; Hajslova, J.; Mastovska, K. Quantitation of Cannabinoids in Cannabis Dried Plant Materials, Concentrates, and Oils Using Liquid Chromatography-Diode Array Detection Technique with Optional Mass Spectrometric Detection: Single-Laboratory Validation Study, First Action 2018.11. J. AOAC 2019, 6, 1822-1833.

Featured Products

Description	Cat. No.
HPLC Columns	
Ascentis® Express C18, 15 cm x 2.1 mm, 2 µm	**50814-U**
Ascentis® Express C18, 15 cm x 3.0 mm, 2.7 µm	**53816-U**
Ascentis® Express C8, 15 cm x 3.0 mm, 2.7 µm	**53853-U**
Solvents & Reagents	
Ultrapure water from Milli-Q® System e.g. IQ 7005 or bottled water	**Milli-Q® or 1.01262**
Acetonitrile Solution, contains 0.1 % (v/v) formic acid, for UHPLC, for MS	**900686**
Water Solution, contains 0.1 % (v/v) formic acid, for UHPLC, for MS	**900687**
Ammonium Formate, eluent additive for LC-MS, LiChropur®, ≥99.0%	**70221**
Formic Acid, for LC-MS LiChropur®	**5.33002**
Ethyl Alcohol, HPLC/spectrophotometric grade*	**459828**
Methanol, UHPLC, for mass spectrometry	**900688**
Certified Reference Materials (Packs of 1 mL)	
Cannabidivarinic acid (CBDVA), 1 mg/mL in acetonitrile	**C-152**
Cannabidiolic acid (CBDA), 1 mg/mL in acetonitrile	**C-144**
Cannabigerolic acid (CBGA), 1 mg/mL in acetonitrile	**C-142**
Tetrahydrocannabivarinic acid (THCVA), 1 mg/mL in acetonitrile	**T-111**
Δ^9-tetrahydrocannabinolic acid (THCA), 1 mg/mL in acetonitrile	**T-093**
Cannabinolic acid (CBNA), 1 mg/mL in acetonitrile	**C-153**
Cannabichromenic acid (CBCA), 1 mg/mL in acetonitrile	**C-150**
Cannabicyclolic acid (CBLA), 0.5 mg/mL in acetonitrile	**C-171**
Cannabidivarin (CBDV), 1 mg/mL in methanol	**C-140**
Cannabigerol (CBG), 1 mg/mL in methanol	**C-141**
Cannabidiol (CBD), 1 mg/mL in methanol	**C-045**
Tetrahydrocannabivarin (THCV), 1 mg/mL in methanol	**T-094**
Cannabinol (CBN), 1 mg/mL in methanol	**C-046**
Δ^9-tetrahydrocannabinol (Δ^9-THC), 1 mg/mL in methanol	**T-005**
Δ^8-tetrahydrocannabinol (Δ^8-THC), 1 mg/mL in methanol	**T-032**
Cannabichromene (CBC), 1 mg/mL in methanol	**C-143**
Cannabicyclol (CBL), 1 mg/mL in acetonitrile	**C-154**

Description	Cat. No.
Sample Preparation Hardware, Tubes and Flasks	
Corning® 50 mL centrifuge tubes, polypropylene, conical bottom w/ CentriStar cap	**CLS430828**
BRAND® BLAUBRAND® Volumetric Flask, Glass Stopper, Amber Glass, 50 mL	**Z327050**
BRAND® BLAUBRAND® Volumetric Flask, Glass Stopper, Amber Glass, 20 mL	**Z327026**
BenchMixer™ Shaker/Vortexer, 115V, US 2-pin plug	**Z742705**
BenchMixer™ Shaker/Vortexer, 230V, Schuko plug	**Z742706**
BenchMixer™ Shaker/Vortexer, 230V, UK Plug	**Z742707**
Corning® LSE™ Compact Centrifuge, 120 V	**CLS6755**
Corning® LSE™ Compact Centrifuge, 230 V, EU plug	**CLS6759**
Corning® LSE™ Compact Centrifuge, 230 V, UK plug	**CLS6758**
Corning® LSE Rotor fixed angle rotor for 6 x 50 mL tubes	**CLS480136**
Whatman® Mini-UniPrep G2 Filter Vial Starter Kit, Pk.100	**WHAGN203 APEORGSP**

*Not Available in all countries. Please visit SigmaAldrich.com to check for alternatives.

Related Products

Description	Cat. No.
Ascentis® Express Guard Cartridge Holder, Pk.1	**53500-U**
Ascentis® Express C18, 5 mm × 2.1 mm, 2 µm Guard Cartridge, Pk.3	**50822-U**
Ascentis® Express C18, 5 mm × 3 mm, 2.7 µm Guard Cartridge, Pk.3	**53504-U**
Ascentis® Express C8, 5 mm × 3 mm, 2.7 µm Guard Cartridge, Pk.3	**53511-U**
BRAND Seripettor Bottle-top Dispenser	**Z627577**
Certified Vial Kit - Amber	**29653-U**
Millex® Syringe Filter, Hydrophilic PTFE Filters, 0.20 ®µm	**SLLG033**
Samplicity® G2 Filtration System	**SAMP2SYSB**
Millex® Samplicity® Hydrophilic PTFE Filters, 0.20 µm, Pk.96	**SAMPLG001**
Millipore® Chemical Duty Pump, 115 V/60 Hz	**WP6111560**
Millipore® Chemical Duty Pump, 220 V/50 Hz	**WP6122050**
Corning Glass Media Storage Bottles (1L)	**CLS13991L**

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Did you know ...

…..that High-Performance Thin-Layer Chromatography (HPTLC) coupled to MS enables both, qualitative and quantitative analyses of CBN, CBD and Δ9-THC in CBD oils of different matrices such as hempseed oil, olive oil or sunflower oil?

Reference: Schmidt T, Stommel J, Kohlmann T, Kramell AE, Csuk R, Separating the true from the false: A rapid HPTLC-ESI-MS method for the determination of cannabinoids in different oils, Results in Chemistry 3 (2021) 100234 https://doi.org/10.1016/j.rechem.2021.100234

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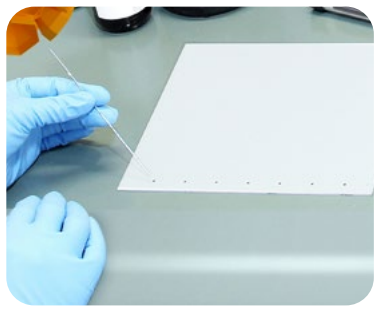


Analysis of Active Cannabis Compounds in Edible Food Products: Gummy Bears and Brownies

Olga Shimelis, Principle R&D Scientist; Kathy Stenerson, Principle R&D Scientist; Analytix@milliporesigma.com
Margaret Wesley, 2016 R&D Summer Intern from Pennsylvania State University, State College, PA

Introduction

Potency testing in marijuana-infused edibles is a problematic task that analytical labs are facing due to the complexity of the involved matrices. Among the most popular matrices are gummy bear candies and brownies. According to one laboratory site, the concentration of active ingredients in the edibles can range from a few parts per million to 3.5 parts per thousand.[1] In this application, a procedure was developed to extract active cannabinoid compounds from gummy bears and brownies. The procedure included a simple and fast extraction of the active compounds from the studied foods, and analysis by HPLC-UV using a biphenyl stationary phase chemistry.

Experimental

Cerilliant® cannabinoid standards, available as 1 mg/mL solutions in either methanol or acetonitrile, were used for this experiment. The concentration of cannabinoids allowed for the spiking of both gummy extract and brownies at about 40 ppm with all compounds. The following compounds were included in this study: cannabidivarinic acid (CBDVA), cannabidivarin (CBDV), cannabigerolic acid (CBGA), cannabigerol (CBG), cannabidiolic acid (CBDA), cannabidiol (CBD), tetrahydrocannabivarin (THCV), cannabinol (CBN), (-)-Δ^9-Tetrahydrocannabinol (Δ^9-THC), (-)-Δ^8- Tetrahydrocannabinol (Δ^8-THC), and (-)-Δ^9-Tetrahydrocannabinolic acid A (THCAA). This list of 11 different cannabinoids includes several acidic forms; thus HPLC analysis was used in order to quantitate these in their native forms.

The HPLC column used was Ascentis® Express Biphenyl, 2.7 µm particle size, which gave the best separation of all 11 compounds in under 13 minutes. The use of this column with Fused-Core® particle architecture resulted in low back pressure, thus a standard pressure HPLC system could be used during this experiment.

Sample Preparation

One gummy bear candy, non-spiked, (2.3 g) was dissolved in 20 mL of warm water. This solution was then spiked with cannabinoids and extracted using a QuEChERS procedure. The average spiking level in each gummy bear was 45 ppm for each compound. Bears of four different colors were tested – orange, yellow, red, and green. After spiking, the water/candy solution was

transferred to a 50 mL plastic QuEChERS extraction tube (55248-U). Acetonitrile (10 mL) was added, and the tube was shaken for one minute by hand. Supel™ QuE non-buffered salts (55295-U) were added, and the samples were shaken for 5 minutes on an automated QuEChERS shaker. Post-shaking, the samples were centrifuged for 5 minutes at 5000 rpm. The top layer was collected and injected directly into the HPLC.

For brownies, a 2.5 g sample of a non-spiked brownie with frosting was added to the QuEChERS extraction tube. This sample was spiked with cannabinoids and allowed to sit for 30 minutes prior to extraction. The average spiking level for the brownies was 40 ppm. The QuEChERS extraction was performed as previously described for gummy bears. Post-extraction, the top acetonitrile layer was collected into a vial and kept under refrigeration for a minimum of 3 hours to remove fats prior to HPLC analysis.

A calibration curve was constructed in acetonitrile bracketing the expected concentration of 10 µg/mL in the final extracts. The following calibration points were included: 2 µg/mL, 5 µg/mL, 10 µg/mL, 20 µg/mL and 25 µg/mL.

Results and Discussion

For the gummy bear samples, it was found that neither the red, yellow, nor green color interfered with detection of cannabinoids at 220 nm. The red color was partially extracted into acetonitrile, while the green and yellow colors stayed in the aqueous layer upon extraction. However, the orange color from the gummy bear, when extracted into acetonitrile, was found to have an interfering peak that co-eluted with CBDVA. Thus, for the orange gummy bear, quantitation of CBDVA was done at 280 nm, where CBDVA has significant absorbance free of interference. Quantitation was done at 220 nm for the rest of compounds in this study (**Figure 1**).

While no cleanup was required for gummy bear samples post-extraction, the co-extractives in the brownie were found to decrease the recoveries of the analytes if the brownie extract was injected into HPLC without further processing. The brownie extract was cleaned by refrigeration to remove the co-extracted fats.

The ruggedness of the method for brownies was tested by injecting the brownie extract (**Figure 2**) multiple times followed by the injection of the 10 µg/mL standard. After 7 injections of the brownie extract, it was found that the peak retention times were not affected, indicating that the column was being thoroughly cleaned between injections. The peak areas for the standards showed a slight decrease of 4 %.

Excellent recovery values of above 90 % for gummies and above 80 % for brownies were achieved with good accuracies (**Table 1**).

Table 1. Recoveries From Spiked Gummy Bears and Brownies

Peak No.	Compound	Yellow Gummy	Orange Gummy	Red Gummy	Average Gummy and RSD	Average Brownie and RSD
1	CBDVA	90 %	92 %*	92 %	91 % (2 %)	91 % (1 %)
2	CBDV	93 %	100 %	100 %	98 % (3 %)	93 % (5 %)
3	THCV	87 %	93 %	90 %	90 % (3 %)	87 % (1 %)
4	CBDA	94 %	90 %	95 %	94 % (3 %)	95 % (1 %)
5	CBGA	87 %	91 %	89 %	91 % (4%)	90 % (2 %)
6	CBD	95 %	100 %	98 %	97 % (3 %)	89 % (5 %)
7	CBG	93 %	99 %	98 %	96 % (4 %)	91 % (5 %)
8	CBN	88 %	95 %	97 %	95 % (6 %)	84 % (4 %)
9	Delta-9-THC	93 %	99 %	100 %	97 % (3 %)	82 % (4 %)
10	Delta-8-THC	91 %	97 %	98 %	95 % (3 %)	80 % (4 %)
11	THCA-A	89 %	89 %	89 %	92 % (7 %)	91 % (2 %)

*The orange gummy was done at 280 nm due to the interfering background peak quantitation.
Note: THCA is the abbreviation used by AOAC

Conclusion

A method was developed for analysis of active cannabinoid compounds in both brownies and gummy bears. The extraction procedure involved a salting out step into acetonitrile and did not require intensive cleanup. The separation of eleven compounds was achieved on a biphenyl stationary HPLC phase and was completed in 13 minutes. The active compound CRMs are available from Cerilliant® through **SigmaAldrich.com**.

Reference

1. Analytical 360, Test Results, Sour Gummy Bears. http://analytical360.com/m/archived/216628, (accessed July 2016).

To learn more about our HPLC columns & phases based on Fused-Core® technology, visit **SigmaAldrich.com/express**

If you would like to learn more on cannabis testing, visit us at **SigmaAldrich.com/cannabis**

Designed to your needs - Millex® syringe filter **SigmaAldrich.com/onemillex**

Figure 1. HPLC Chromatogram of Orange Gummy Bear Extract at (a) 220 nm and (b) 280 nm **Figure 2**. HPLC of a Brownie Extract at 220 nm. The peak elution order is listed in **Table 1**.

column:	Ascentis® Express Biphenyl, 10 cm × 2.1 mm I. D., 2.7 µm particles (64065-U)
mobile phase:	(A) 0.1 % TFA in water; (B) 0.1 % TFA in acetonitrile
gradient:	at 47 % B, to 50 % B in 13 minutes, to 100 % B in 0.1 min, 100 % B for 3 minutes, to 47 % B in 0.1 min, at 47 % B for 2.5 minutes
flow rate:	0.70 mL/min
column temp.:	35 °C
detector:	UV, 220 nm and 280 nm
injection:	5 µL
pressure:	340 bar
instrument:	Agilent® 1200, with UV detector

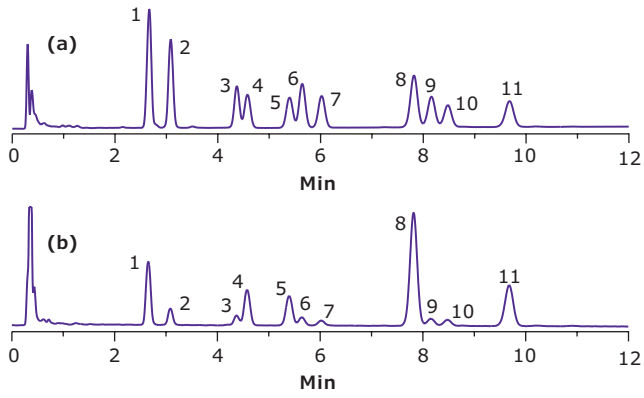
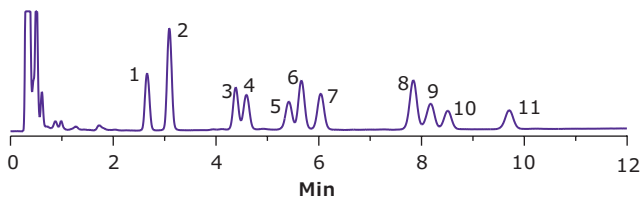


Figure 2. HPLC of a Brownie Extract at 220 nm
The peak elution order is listed in **Table 1**.

Conditions same as **Figure 1**.



Featured Products

Description	Cat. No.
Supel™ QuE QuEChERS Products	
Non-buffered Extraction Tube 2, 12 mL, pk of 50	**55295-U**
Empty Centrifuge Tube, 50 mL, pk of 50	**55248-U**
Ascentis® Express Biphenyl HPLC Column	
10 cm × 2.1 mm I.D., 2.7 µm particle size	**64065-U**
Cerilliant® Certified Reference Materials	
Cannabidivarinic acid (CBDVA), 1 mg/mL in acetonitrile, CRM	**C-152**
Cannabidivarin (CBDV), 1 mg/mL in methanol	**C-140**
Cannabigerolic acid (CBGA), 1mg/mL in acetonitrile	**C-142**
Cannabigerol (CBG), 1 mg/mL in methanol	**C-141**
Cannabidiolic acid (CBDA), 1 mg/mL in acetonitrile	**C-144**
Cannabidiol (CBD), 1 mg/mL in methanol	**C-045**
Tetrahydrocannabivarin (THCV), 1 mg/mL in methanol	**T-094**
Cannabinol (CBN), 1 mg/mL in methanol	**C-046**
(-)-Δ^9-Tetrahydrocannabinol (Δ^9-THC), 1 mg/mL in methanol	**T-005**
(-)-Δ^8-Tetrahydrocannabinol (Δ^8-THC), 1 mg/mL in methanol	**T-032**
(-)-Δ^9-Tetrahydrocannabinolic acid A (THCA-A), 1 mg/mL in acetonitrile	**T-093**
Accessories	
QuEChERS Shaker and Rack Starter Kit, USA compatible plug	**55278-U**
QuEChERS Shaker and Rack Starter Kit, EU Schuko plug	**55438-U**
Certified Vial Kit, Low Adsorption (LA), 2 mL, pk of 100	**29653-U**

MOISTURE ANALYSIS

Determination of Water in Cannabis & Hemp by Karl Fischer Titration

Anita Piper, R&D Scientist; Bruce Herzig, R&D Scientist;
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Introduction

Hemp and cannabis are becoming important agricultural products, and are being increasingly used in medicinal products, cosmetics, foods, oils, and textile fibers around the world. The cannabis market is growing rapidly, mainly due to the use as therapeutics for medicial treatments by the pharmaceutical industry.

Water in cannabis and hemp impacts the determination of the potency and must be determined accurately to calculate the correct delta-9 tetrahydrocannabinol (THC) content of the plant. Although both plant types share similar characteristics, cannabis contains a higher amount of THC compared to hemp. However, to be legally classified as hemp, the United States Department of Agriculture (USDA) set a limit for the total delta-9 tetrahydrocannabinol (THC) concentration. The limit is set to contain not more than 0.3 % THC on a dry-weight basis (see definition 7 CFR Part 990 Oct 2019).[1] European Union guidlines currently define a limit ≤ 0.2% for the total THC concentration on a dry-weight basis for industrial hemp.(status Jan. 2022).[2]

To calculate the dry-weight delta-9 THC concentration, an accurate analytical method must be employed for determining the exact water content. Currently most laboratories use loss on drying methods (LOD) which measure all volatile components by heating. This, however, can overstate the water content of the sample, which in turn would lead to an incorrect delta-9 THC concentration in the dry weight, resulting in with wrong classification as hemp. This could potentially lead to penalties for a farmer or processor, or the forced destruction of their product.

The purpose of this application is to demonstrate a moisture determination method for hemp and cannabis flower which is selective for water, and will also provide rapid and accurate test results.

Methods to Determine the Water Content

Three methods for determining water in hemp and cannabis were evaluated:

- Loss on drying (LOD)
- Karl Fischer oven method with coulometry
- Direct volumetric Karl Fischer titration with external extraction

Loss on drying is a simple weighing-based technique that removes water by heating. The equipment needed is reasonably priced, but the method can be time consuming. Nowadays, this method is commonly used in several different industries. However, the loss on drying method is not specific for water and the test results obtained can include amounts of other volatile compounds too. Depending on the conditions chosen, this could lead to an incorrect water value, which in turn would affect the accuracy of the reported dry weight THC concentration result.

Karl Fischer titration based methods are simple to run, but the equipment is a bit more expensive than that used for LOD determination. The advantage of the Karl Fischer titration method is its specificity for water. As a result, the reported water value does not include amounts of other volatile compounds. Two methods based on the Karl Fischer titration were evaluated — coulometry with a Karl Fischer oven and a direct volumetric titration with an external extraction. The coulometric method is best suitable for samples with low water content in the range of 10 ppm to 10,000 ppm (1%) or when only little sample material is available. In contrast, the volumetric titration is used for solid and liquid samples with water contents from 0,01% to 100%. Samples for direct volumetric Karl Fischer titration must be soluble in the Karl Fischer solvent. Organic plant materials like hemp and cannabis are not suitable for direct measurement, so either an external extraction with a suitable solvent or a Karl Fischer oven method is employed. The Karl

Fischer oven method is ideal for this measurement as it completely evaporates the water from the sample and directly transfers it to the titrator. Both approaches of direct volumetric titration after external extraction and the Karl Fischer oven method have been examined. Water content of finely ground hemp flower samples was determined using the methods described below. For any hemp or cannabis analysis, proper preparation is important to provide a representative and well homogenized sample. A method of sample homogenization that is frequently applied in the industry is cryogenic ball milling. This thoroughly homogenizes the sample and leads to a particle size of 100 µm or smaller.

Experimental

Karl Fischer instrumentation used

- Karl Fischer coulometer
- Karl Fischer oven with sample processor
- Karl Fischer volumeter with 5 mL burette

Experimental conditions— Karl Fischer oven method with coulometry

Table 1. Reagents used in the determination of water by Karl Fischer oven method with coulometry

Ground hemp flower sample Reagents & Sample	
Standard:	Water standard oven 1%, solid water standard for Karl Fischer oven method Aquastar® (**1.88054**)
Cell type:	Cell without diaphragm
Reagent type: (Anolyte)	CombiCoulomat fritless; Karl Fischer reagent for coulometric water determination for cells with and without diaphragm Aquastar® (**1.09257**) or Anolyte; Karl Fischer reagent for coulometric water determination for cells without diaphragm Aquastar® (**1.88079**)

Table 2. Titration parameters for water determination by Karl Fischer oven method with coulometry

Coulometer settings for cell without diaphragm, e.g.:	
I(pol):	10 µA
Generator current:	400 mV
Endpoint:	50 mV
Drift stop:	Relative < 10 µg/min
Stirring time:	5 s
Sample size:	20 – 50 mg

Table 3. Oven settings for water determination by Karl Fischer oven method with coulometry

Oven settings	
Temperature:	150 °C
Extraction time:	5 min
Gas flow:	60 – 70 mL/min

Experimental conditions — Direct Karl Fischer volumetric titration with external extraction

Table 4. Reagents used for the water determination by Karl Fischer volumetric titration with external extraction

Reagents & Sample	
Sample:	Hemp methanol extract (from external extraction), 0.5 - 1.0 g (depending on expected water content)
Standard:	Water standard 1%, standard for volumetric Karl Fischer titration 1 g ≙ 10 mg H_2O Aquastar® (**1.88052**)
Titrant:	CombiTitrant 2 Aquastar® (**1.88002**)
Solvent:	CombiMethanol Aquastar® (**1.88009**)

Results and Discussion

Experiment 1 – Water determination by Karl Fischer oven method with coulometry

The water content of a hemp sample was determined by coulometric Karl Fischer titration combined with a Karl Fischer oven. A temperature ramp was run prior to the analysis for evaluating the optimum temperature at which the water is completely and efficiently released without decomposition of the sample. The optimal temperature for the sample used was determined to be 150 °C. Samples were weighed into sealed vials for use in the Karl Fischer oven. An empty vial was used as a blank to determine any water which may have adhered to the vial. The value obtained for the blank vial was subtracted from each sample's value as determined by the instrument.

The sample was analyzed in quintuplicate, and the measured values were averaged to obtain the result (see results with 2 different coulometric reagents, CombiCoulomat and Anolyte, in **Tables 5** and **6**).

Table 5. Karl Fischer oven titration results with Aquastar® CombiCoulomat fritless

Sample	Weight (g)	Start drift (µg/min)	Time (min)	Water content (%)
1	0.0255	6.8	16	7.59
2	0.0222	6.2	14	7.51
3	0.0271	6.1	19	7.69
4	0.0250	5.4	19	7.79
5	0.0303	5.7	21	7.78
Mean				**7.67**
Standard Deviation				**0.12**
(%) RSD				**1.60**

Table 6. Karl Fischer oven titration results with Aquastar® Anolyte

Sample	Weight (g)	Start drift (µg/min)	Time (min)	Water content (%)
1	0.0239	5.4	15	7.42
2	0.0265	5.1	19	7.67
3	0.0237	5.2	18	7.67
4	0.0244	4.7	19	7.75
5	0.0333	4.9	24	7.59
Mean				**7.62**
Standard Deviation				**0.13**
RSD				**1.65**

Experiment 2 – Water determination by volumetric titration with external extraction

1 g hemp was extracted with 25 g of methanol by stirring in septum sealed vials. Different extraction conditions (extraction times and temperature) were applied. The water value of the methanol determined was used as a blank value for use in the final calculations. The solid hemp was allowed to settle, and an exact weight aliquot of the mixture (methanol/extracted water) was taken using a syringe, and injected into the titration cell of a volumetric Karl Fischer titrator. The exact sample weight was determined by back weighing. The titrator then measured the water content of the injected sample. The exact value of water content of the hemp sample was then calculated using the following equation:

$$W_1 = [W_3 \times (m_1+m_2) - W_2 \times m_2]/m_1$$

Where: W_1 is the result in %

W_2 is the % water of the methanol used for extraction

W_3 is the % water determined for the extracted methanol aliquot

m_1 is the mass of the sample extracted

m_2 is the mass of the extraction methanol

The results of the analysis were found to be insufficiently reproducible and highly dependent on the chosen extraction conditions. And hence are not presented here in detail. The external extraction technique was found to be disadvantageous in comparison to the KF oven technique and thereby cannot be recommended for water determination in hemp.

Experiment 3 - Water determination by loss on drying (LOD)

In this experiment, the water content of the sample was determined by loss on drying until a constant mass was reached. The sample was heated for 2 h at a temperature of 150 °C. The weight of the sample was determined before and after heating, to calculate the weight lost during the experiment — the loss on drying. The sample was analyzed in duplicate and the values were averaged to obtain the result (see results in **Table 7**).

Table 7. Loss on drying results

Sample	Starting weight (g)	Weight after 2 h at 150 °C (g)	Weight loss (%)
1	1.7093	1.5307	10.45
2	2.0872	1.8748	10.18
Mean			**10.31**

Comparison of Karl Fischer oven method and loss on drying

The Karl Fischer oven method with coulometry was compared to the loss on drying method (see **Table 8**). Results for the latter (LOD) were about 35% higher than for the Karl Fischer oven method. This requires to consider the measument of other volatile compounds by the loss on drying method to avoid wrong water content results.

Table 8. Comparison between the water content measured by Karl Fischer coulometry method with oven and loss on drying

Samples	Water content (%) Karl Fischer oven + Coulometry CombiCoulomat fritless	Water content (%) Karl Fischer oven + Coulometry Anolyte	Water content (%) Loss on drying (LOD)
1	7.59	7.42	10.45
2	7.51	7.67	10.18
3	7.69	7.67	
4	7.79	7.75	
5	7.78	7.59	
Mean	7.67	7.62	10.31

Conclusion

Water content of hemp samples was determined using Karl Fischer titration techniques (coulometry with oven; volumetric titration with external extraction) and compared to loss on drying.

The Karl Fischer coulometric titration in combination with a Karl Fischer oven provides reproducible results. It prevents an overestimation of water content caused by volatile compounds, as to be considered for the loss on drying method. In addition it requires only a small amount of sample and reagent. The volumetric Karl Fischer titration with external extraction did not produce reproducible results and is therefore not recommended. However, the volumetric Karl Fischer method in combination with a Karl Fischer oven can be employed, but since volumetry is not as sensitive as coulometry, the sample size needs to be increased to get reliable results.

Therefore, it is recommended to use the Karl Fischer oven method with coulometry for water determination in hemp and cannabis to achieve the most accurate results. This enables the exact and precise calculation of the dry weight delta-9 THC concentration.

References

1. Establishment of a Domestic Hemp Production Program Department of Agriculture, Agricultural Marketing Service, 7 CFR Part 990 (Oct 2019) 84 FR 58522 https://www.federalregister.gov/d/2019-23749 (accessed 14.01.2022)
2. European Commission - Hemp: https://ec.europa.eu/info/food-farming-fisheries/plants-and-plant-products/plant-products/hemp_en (accessed 14.01.2022)

Featured Products

Description	Cat. No.
Reagents	
CombiCoulomat fritless, Karl Fischer reagent for the coulometric water determination for cells with and without diaphragm, Aquastar®	**109257**
Anolyte, Karl Fischer reagent for the coulometric water determination for cells without diaphragm, Aquastar®	**188079**
CombiTitrant 2, one component reagent for volumetric Karl Fischer titration 1 ml @ ca.2 mg H_2O, Aquastar®	**188002**
CombiMethanol Solvent for volumetric Karl Fischer titration with one component reagents max. 0.01% H_2O, Aquastar®	**188009**
Reference Materials	
Water standard 0.1%, Standard for coulometric Karl Fischer Titration 1 g = 1 mg H_2O, Aquastar®	**188051**
Water standard oven 1%, solid standard for Karl Fischer oven, Aquastar®	**188054**

Analyzing Terpenes in Cannabis

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Abstract

An efficient and easy workflow was developed for the extraction and GC-MS analysis of 31 terpenes in cannabis. The method utilized a simple solvent extraction followed by a fast and efficient GC-MS analysis on an SLB®-5ms capillary column.

Introduction

Terpenes are a class of compounds responsible for the aroma and fragrance of the cannabis flower. Labeling of terpene content on cannabis products is important to many consumers in that different varieties exhibit very different and characteristic profiles. At the time of this article, no consensus test method exists for terpene testing. Currently there are two popular approaches – headspace or solvent extraction followed by GC analysis. Headspace analysis is a "cleaner" technique than solvent extraction in that nonvolatile matrix components will not be co-extracted with the terpenes. However, traditional headspace analysis can require special instrumentation in the form of a headspace analyzer. Headspace analysis by solid phase microextraction (SPME) offers similar advantages as traditional headspace analysis, often even more sensitivity, and it can be performed manually or with an appropriate autosampler.[1,2]

Solvent extraction also does not require special instrumentation and has been used effectively to determine terpene profiles.[3] In this work, we demonstrate a solvent extraction method in combination with certified reference materials and GC-MS analysis for the identification and quantitation of terpenes in hemp flower. The method used included a simple and quick solvent extraction followed by analysis on a highly efficient 20 m x 0.18 mm x 0.18 µm SLB®-5ms column. The short length of this column in combination with the small ID allowed for separation and elution of 31 targeted terpenes in under 17 minutes. GC-MS in full scan mode allowed for spectral identification via library match to be used in combination with retention time to verify the identity of the targeted terpenes in the hemp sample. With additional reference materials, the method could potentially be expanded to include more terpenes.

Sample preparation and analytical methods

An extract of coarse ground hemp was prepared following the scheme shown in **Figure 1**. After the centrifugation step, the supernatant was removed and placed into a 2 mL amber autosampler vial for GC analysis. GC-MS analysis proceeded following the conditions listed in **Table 1**. A calibration curve ranging from 0.75 to 200 ppm (compound dependent) was prepared from two separate cannabis terpene CRM mixes. Tridecane (100 ppm) was added as an internal standard (IS) to both samples and standards upon injection using a sandwich injection technique. To aid in identification as part of the MS method, the CRMs were used to define specific retention times for each terpene and to generate reference spectra along with corresponding ratios for quantitation and qualification ions.

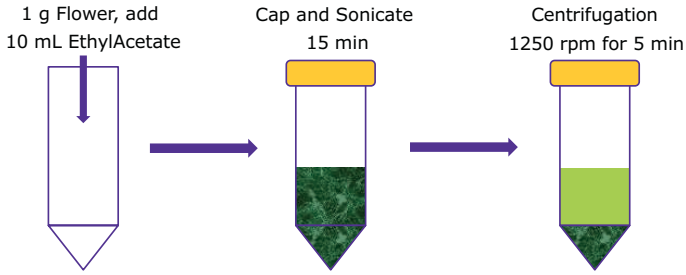


Figure 1. Sample Preparation Scheme

Table 1. Instrument Conditions

Gas Chromatograph Conditions	
column:	SLB®-5ms 20 m x 0.18 mm ID; 0.18 µm (**28564-U**)
oven:	45 °C (2 min), 10°C/min to 140°C (0.5 min), 30°C/min to 300°C (2 min)
inj. temp.:	300° C
carrier gas:	helium, 0.75 mL/min constant Flow
detector:	MSD
injection:	2.0 µL – pulsed split 50:1
liner:	Single Taper FocusLiner™ with wool (**2879905-U**)
sample:	Hemp extract (1 g flower in 10 mL ethyl acetate)

MS Conditions	
tuning:	Auto-tune
acquisition:	Full Scan Mode (EI); 40-400 amu
solvent delay:	4 min
MS source temperature:	300°C
MS quad temp.:	150°C
MS transfer line temp.:	300°C

Results

The GC method eluted the 31 targeted terpenes in under 17 minutes, with excellent peak shape and resolution, as shown in **Figure 2**.

The GC-MS method showed excellent linearity for all analytes (**Table 2**). In addition, retention time stability was evaluated. With the presence of terpenes with

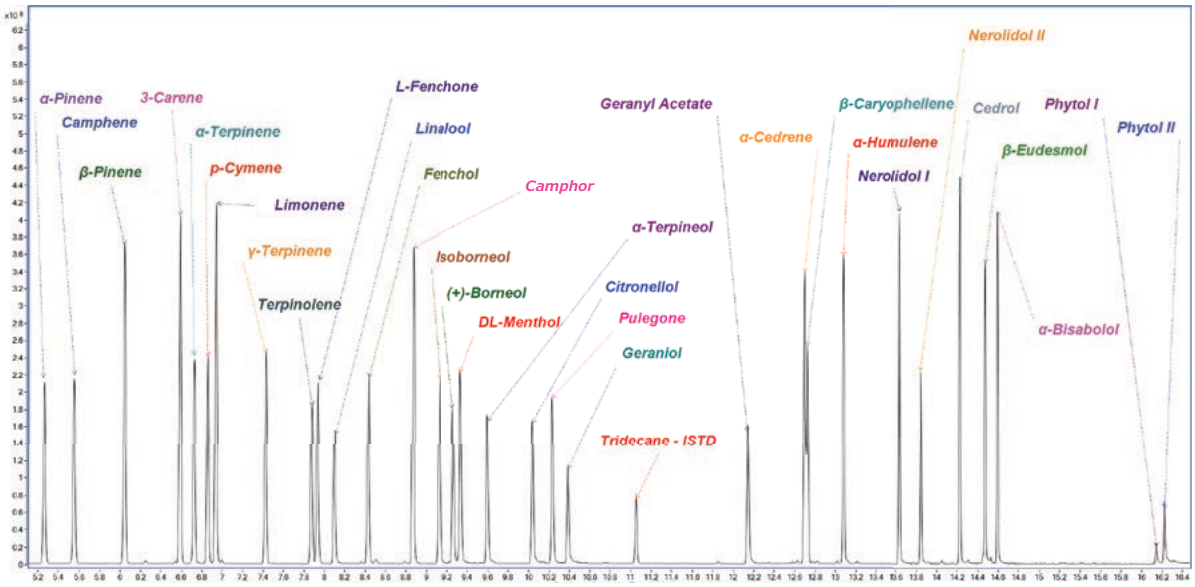


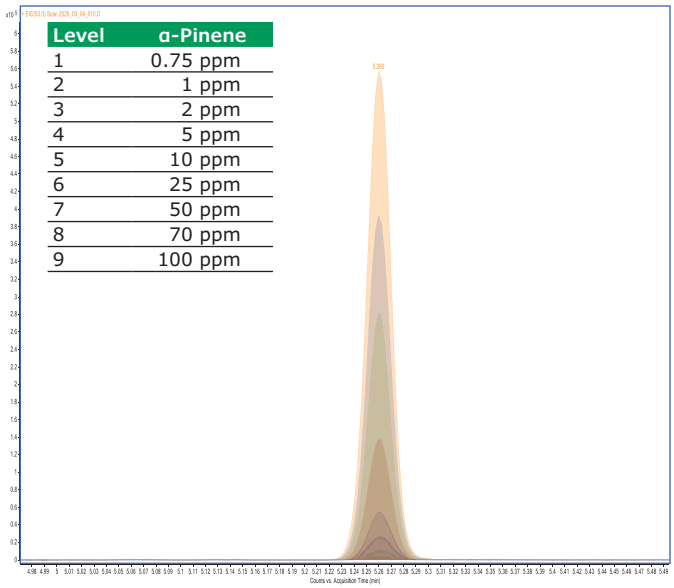
Figure 2. Standard Injection of Cannabis Terpene Standard Analyzed by GC-MS (Retention Time Range 5 to 16.5 min)

Table 2. Tabulated Results for 31 Cannabis Terpenes and IS

Peaks	Compound	CAS #	Ret. Time [min]	Lib Match Factor	r²	Range [µg/mL]	Detected in hemp sample?
1	α-Pinene	80-56-8	5.257	98.99	0.99991	0.75-100	Y
2	Camphene	79-92-5	5.553	99.00	0.99990	0.75-100	Y
3	β-Pinene	127-91-3	6.049	99.17	0.99995	1.50-200	Y
4	3-Carene	13466-78-9	6.589	99.15	0.99992	1.50-200	Y
5	α-Terpinene	99-86-5	6.726	99.62	0.99987	0.75-100	Y
6	p-Cymene	99-87-6	6.856	99.58	0.99987	0.75-100	Y
7	Limonene	138-86-3	6.938	99.21	0.99997	1.50-200	Y
8	γ-Terpinene	99-85-4	7.422	99.35	0.99994	0.75-100	Y
9	Terpinolene	586-62-9	7.872	99.21	0.99990	0.75-100	N
10	L-Fenchone	7787-20-4	7.930	99.47	0.99989	0.75-100	Y
11	Linalool	78-70-6	8.101	99.37	0.99915	0.75-100	Y
12	Fenchol	2217-02-9	8.426	99.43	0.99996	0.75-100	Y
13	Camphor	76-22-2	8.866	99.57	0.99997	1.50-200	Y
14	Isoborneol	124-76-5	9.126	99.45	0.99983	0.75-100	Y
15	(+)-Borneol	464-43-7	9.259	99.36	0.99968	0.75-100	Y
16	DL-Menthol	89-78-1	9.331	99.52	0.99988	0.75-100	Y
17	α-Terpineol	10482-56-1	9.596	99.34	0.99942	0.75-100	Y
18	Citronellol	106-22-9	10.036	99.05	0.99961	0.75-100	Y
19	Pulegone	89-82-7	10.234	99.55	0.99956	0.75-100	Y
20	Geraniol	106-24-1	10.386	98.21	0.99946	0.75-100	Y
21	Geranyl Acetate	105-87-3	12.145	98.64	0.99980	0.75-100	Y
22	α-Cedrene	469-61-4	12.699	99.62	0.99997	0.75-100	N
23	β-Caryophellene	87-44-5	12.728	99.68	0.99953	0.75-100	Y
24	α-Humulene	6753-98-6	13.082	98.90	0.99975	0.75-100	Y
25	Nerolidol I	7212-44-4	13.636	98.73	0.99913	1.50-200	Y
26	Nerolidol II	7212-44-4	13.845	99.19	0.99905		Y
27	Cedrol	77-53-2	14.226	99.46	0.99978	0.75-100	Y
28	β-Eudesmol	473-15-4	14.471	99.60	0.99945	0.75-100	Y
29	α-Bisabolol	23089-26-1	14.594	99.62	0.99965	0.75-100	Y
30	Phytol I	7541-49-3	16.145	92.91	0.99975	0.75-100	Y
31	Phytol II	7541-49-3	16.225	92.91	0.99950		Y
ISTD	**Tridecane**	**629-50-5**	**11.050**	**96.17**	******	**100 ppm**	

similar MS spectra, it is important that retention times do not vary with concentration, or in the presence of matrix components. An example of the retention time stability provided by the SLB®-5ms column is demonstrated in **Figures 3** and **4** for α-pinene. **Figure 3** is an overlay of the extracted ion chromatogram (EIC) of the quantitation ion from the 9 calibration standards used. No retention time shift is discernable with the change in concentration. Similarly, in **Figure 4**, an overlay of the α-pinene peak from 3 injections of a 50 ppm standard show no difference in retention or response. Retention time stability in matrix was evaluated also and will be discussed later in this article.

Analysis of extracted hemp samples identified the presence of 29 of the 31 terpenes targeted for this analysis (**Table 2** & **Figure 5**). The concentrations of the different terpenes present in the hemp varied,



Level	α-Pinene
1	0.75 ppm
2	1 ppm
3	2 ppm
4	5 ppm
5	10 ppm
6	25 ppm
7	50 ppm
8	70 ppm
9	100 ppm

Figure 3. Overlaid EIC of Nine Alpha-Pinene Standards.

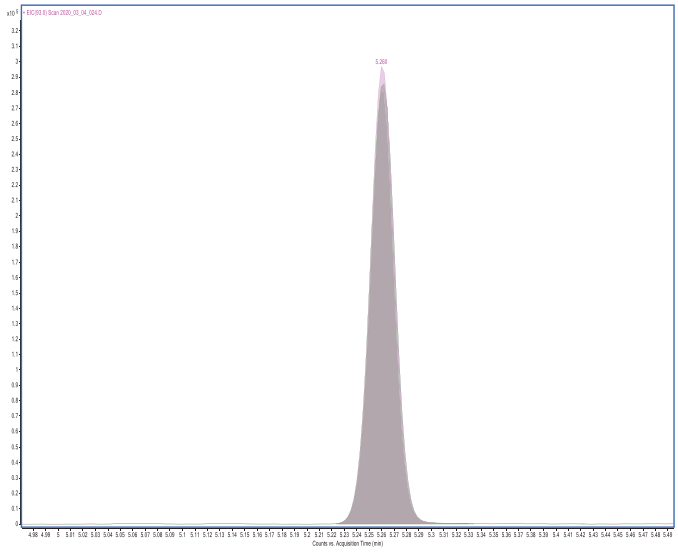


Figure 4. Three Injections of 50 µg/mL Alpha-Pinene Standard.

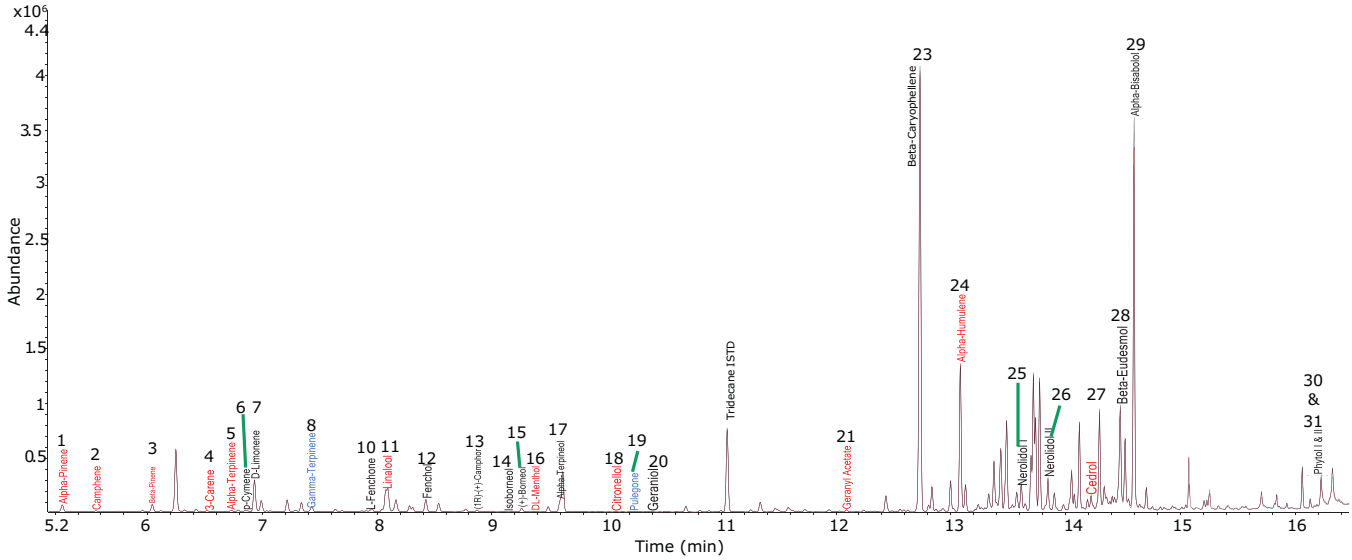


Figure 5. Chromatogram of GC-MS Analysis of Hemp Extract.

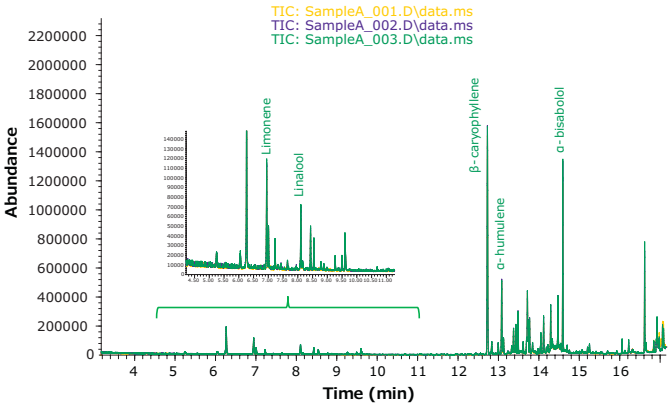


Figure 6. Overlay of TICs from 3 injections of hemp extract. Several prominent terpenes are noted.

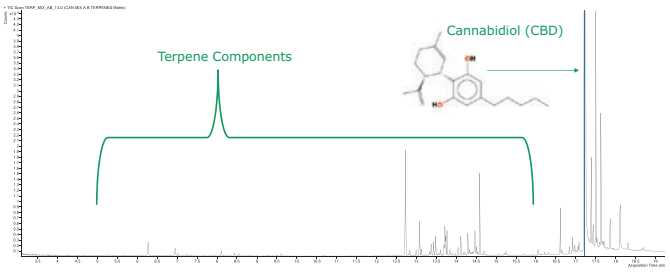


Figure 7. TIC of hemp extract showing elution of co-extracted cannabinoids relative to terpenes.

with several terpenes such as β-caryophyllene and α-bisabolol being at much higher levels than others. The approx. 100-fold calibration range of the method made it possible to quantitate the varied concentrations of terpenes present in the hemp. In addition, retention time stability from matrix, in combination with spectral ID, helped with peak identification. **Figure 6** illustrates this with overlaid total ion chromatograms (TICs) of multiple injections of a hemp extract. As with the calibration standards, no retention shifts were observed. It was noted that the solvent extraction method did result in co-extracted cannabinoids (**Figure 7**), however these eluted late enough in the run so as not to interfere with the terpenes.

Conclusion

The utility of a simple solvent extraction method in combination with GC-MS was demonstrated for the analysis of targeted terpenes in hemp flower, with identification of 29 terpenes. The use of certified reference materials in combination with MS spectra provided for proper identification in matrix, and the 20 m x 0.18 mm I.D. x 0.18 µm SLB®-5ms column provided a combination of both speed and efficiency for the analysis. While this method targeted 31 specific terpenes, it could be expanded to more by using additional CRMs.

References

1. Halpenny, M.; Stenerson, K.K. Analysis of Terpenes in Cannabis Using Headspace Solid-Phase Microextraction and GC–MS. *LCGC North America* - Special Issue, 2017, 35 (5), 27-32.
2. Stenerson K.K. Headspace SPME-GC/MS Analysis of Terpenes in Hops and Cannabis. *Analytix Reporter- Issue 3*, 2018, 9-12
3. Giese, M.W.; Lewis, M.A.; Giese, L.; Smith, K.M. Development and Validation of a Reliable and Robust Method for the Analysis of Cannabinoids and Terpenes in Cannabis. *J. AOAC Int.,* 2015, 98 (6), 1503-1522.

Featured Products

Description	Cat.No
Reference Materials & Solvents	
Cannabis Terpene Mix A, TraceCERT® certified reference material, 2000 µg/mL each component in methanol, 1 mL	**CRM40755**
Cannabis Terpene Mix B, TraceCERT® certified reference material, 2000 µg/mL each component in methanol, 1 mL	**CRM40937**
Tridecane analytical standard	**91490**
Ethyl acetate for gas chromatography MS SupraSolv®	**1.00789**
GC	
SLB®-5ms Capillary GC Column L × I.D. 20 m × 0.18 mm, df 0.18 µm	**28564-U**
Inlet Liner, Split/Splitless Type, Single Taper FocusLiner™ Design (wool packed), Pk.5	**2879905-U**
Molded Thermogreen® LB-2 Septa, solid discs diam. 11 mm, Pk.50	**28676-U**
Hamilton® Microliter™ syringe, cemented needle 701 ASN, volume 10 µL, needle size 23s ga (cone tip), needle L 43 mm (1.71 in.), Pk.6	**21317**
Accessories	
BenchMixer™ XLQ QuEChERS Shaker/Vortexer AC/DC input 115 V AC, US 2-pin plug	**Z742705**
BenchMixer™ XLQ QuEChERs Shaker/Vortexer AC/DC input 230 V AC, Schuko plug	**Z742706**
BenchMixer™ XLQ QuEChERs Shaker/Vortexer AC/DC input 230 V AC, UK plug	**Z742707**
Certified Vial Kit, Low Adsorption (LA), 2 mL, pk of 100 volume 2 mL, amber glass vial (with marking spot), natural PTFE/silicone septa, thread for 9 mm	**29653-U**
Corning® 50 mL centrifuge tubes 50 mL centrifuge tubes, polypropylene, conical bottom w/ CentriStar cap, rack packed, sterile, natural, 25/rack, 500/cs	**CLS430828**
Eppendorf® epT.I.P.S. box volume range 2-200 µL	**Z640220**
Eppendorf® epT.I.P.S. box volume range 50-1000 µL	**Z640247**
Eppendorf® epT.I.P.S. box volume range 100-5000 µL	**Z640271**

Headspace SPME-GC/MS Analysis of Terpenes in Hops and Cannabis

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In this application, headspace-SPME combined with GC/MS was used to analyze some of the terpenes present in both common hops and cannabis.

Terpenes are small molecules synthesized by some plants. The name terpene is derived from turpentine, which contains high concentrations of these compounds. Terpene molecules are constructed from the joining of isoprene units in a head-to-tail configuration (**Figure 1**). Classification is then done according to the number of these isoprene units in the structure (**Table 1**). The configurations of terpenes can be cyclic or open, and can include double bonds, and hydroxyl, carbonyl or other functional groups. If the terpene contains elements other than C and H, it is referred to as a terpenoid.[1]

Figure 1. Isoprene Unit

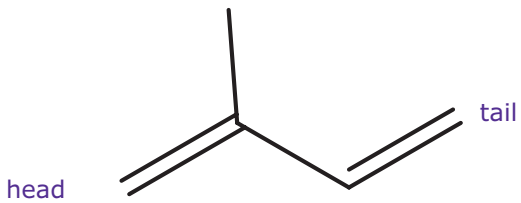


Table 1. Classification of Terpenes

Classification	Number of Isoprene Units
Monoterpene	2
Sesquiterpene	3
Diterpene	4
Triterpene	6
Tetraterpene	8

Terpenes are present in essential oils derived from plants and often impart characteristic aromas to the plant or its oil. For example, d-Limonene, which is found in lemon, orange, caraway and other plant oils, has a lemon-like odor. Essential oils, with their component terpenes and terpenoids, have been applied in therapeutic use known as aromatherapy to aid in the relief of conditions such as anxiety, depression, and insomnia.[2] This has led to the use of plants which contain these compounds in preparations such as oils, teas, and tonics.

Using Terpene Profile for Plant Identification

The *cannabis sativa* (cannabis or marijuana) plant contains over 100 different terpenes and terpenoids, including mono, sesqui, di, and tri, as well as other miscellaneous compounds of terpenoid orgin.[3] Although the terpene profile does not necessarily indicate geographic origin of a cannabis sample, it can be used in forensic applications to determine the common source of different samples.[4] In addition, different cannabis strains have been developed which have distinct aromas and flavors; a result of the differing amounts of specific terpenes present.[5] *Humulus lupulus* (common hops) and cannabis are both members of the family Cannabaceae.[6] Consequently, there are similarities in the terpenes each contains. Terpenes give both plant commodities characteristic organoleptic properties and, in the case of cannabis, produce characteristic aromas when the buds are heated or vaporized.[7]

Experimental

Dried cannabis sample was obtained courtesy of Dr. Hari H. Singh, Program Director at the Chemistry & Physiological Systems Research Branch of the United States National Institute on Drug Abuse at the National Institute of Health. The extract strain of the sample was not known. Hop flowers of an unknown variety were purchased from an on-line source. Pelletized of Cascade and US Golding hop varieties were purchased at a local home-brew supply shop. Chromatographic separation was performed on an Equity®-1 capillary GC column, and identification was done using retention indices and spectral library match. Final analytical conditions appear in the figures.

SPME Method Optimization

The SPME method was developed using a sample of dried hops flowers (0.2 g in 10 mL vial). The initial SPME parameters were based on previously published work.[8] The GC/MS results of this analysis are shown in **Figure 2**. This initial set of parameters used the 100 µm PDMS fiber, a 1 g sample size, and 60 minute equilibration at room temperature prior to extraction. The sample size was then scaled down to 0.2 g, and the equilibration temperature increased to 40 °C. This increased temperature allowed the equilibration time to be decreased from 60 to 30 minutes without a loss

Figure 2. Headspace SPME-GC/MS Analysis of Dried Hops Flowers (100 µm PDMS Fiber, 1 g Sample)

Sample/matrix:	1 g ground hop flowers
SPME fiber:	100 µm PDMS (**57341-U**)
Sample equilibration:	60 min, room temperature
Extraction:	20 min, headspace, 40 °C
Desorption process:	3 min, 270 °C
Fiber post bake:	3 min, 270 °C
Column:	Equity®-1, 60 m x 0.25 mm I.D., 0.25 µm (28047-U)
Oven:	60 °C (2 min), 5 °C/min to 275 °C (5 min)
Inj. temp.:	270 °C
Detector:	MSD
MSD interface:	300 °C
Scan range:	full scan, m/z 50-500
Carrier gas:	helium, 1 mL/min constant flow
Liner:	0.75 mm ID SPME

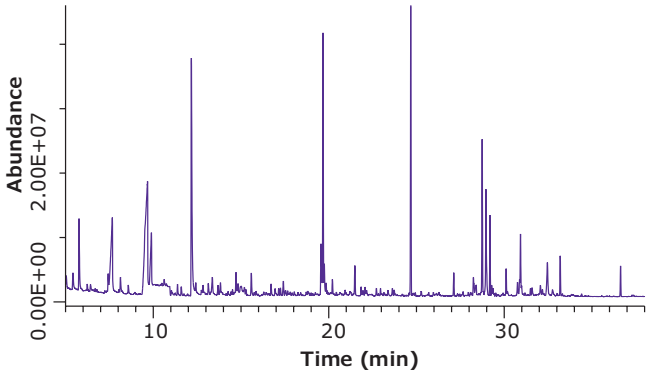
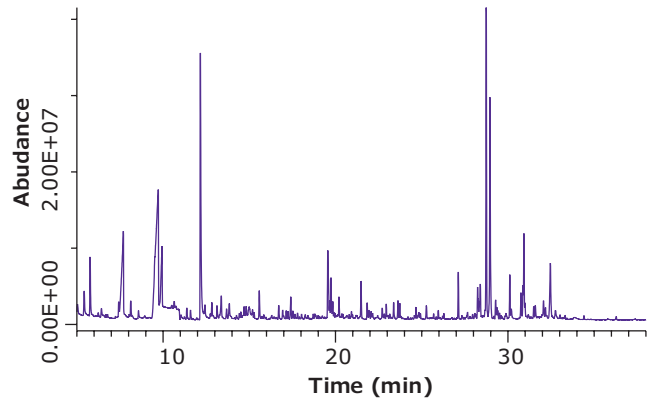


Figure 3. Headspace SPME-GC/MS Analysis of Dried Hops Flowers (100 µm PDMS Fiber, 0.2 g Sample)

Conditions same as **Figure 2** except:
sample/matrix: 0.2 g ground hop flowers



in sensitivity (**Figures 3 and 4**). The initial extraction time used was 20 min, and a shorter extraction time of 10 minutes was evaluated. However a loss in sensitivity was noted, thus extraction time was maintained at 20 minutes. The DVB/CAR/PDMS fiber was then evaluated (**Figure 5**). As expected, this fiber extracted more of the lighter compounds, which by MS spectral match, were identified as short chain alcohols and acids.

Figure 4. Headspace SPME-GC/MS Analysis of Dried Hops Flowers, Increased Sample Equilibration Temperature (100 µm PDMS Fiber, 0.2 g Sample)

Conditions same as **Figure 2** except:	
sample/matrix:	0.2 g ground hop flowers
sample equilibration:	30 min, 40 °C

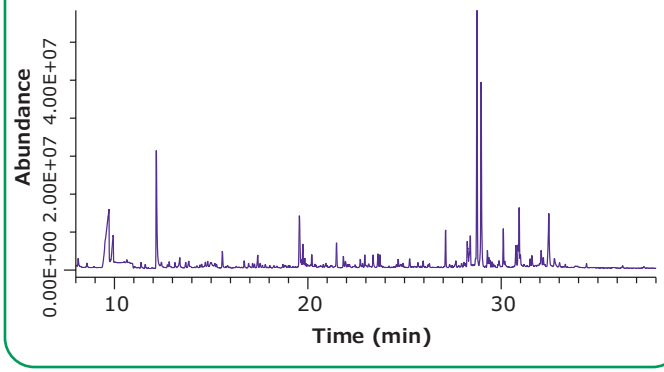
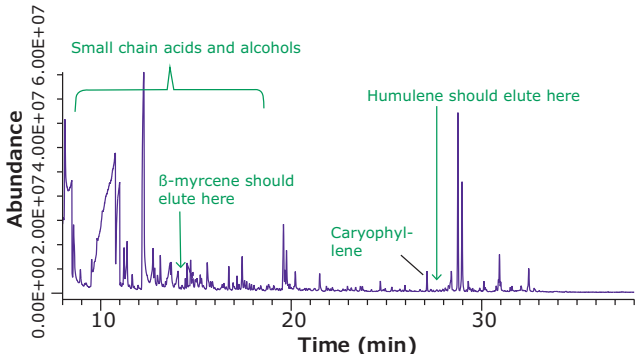


Figure 5. Headspace SPME-GC/MS Analysis of Dried Hops Flowers, Increased Sample Equilibration Temperature (DVB/CAR/PDMS Fiber, 0.2 g Sample)

Conditions same as **Figure 2** except:	
sample/matrix:	0.2 g ground hop flowers
SPME fiber:	50/30 µm DVB/CAR/PDMS (57298-U)
sample equilibration:	30 min, 40 °C



Identification of Terpenes Using GC/MS

Using the DVB/CAR/PDMS fiber, samples of hops and cannabis were analyzed using the optimized SPME method. Peak identifications were assigned using MS spectral matching against reference spectra in the Wiley and NIST libraries. Confirmatory identification was done based on retention index. Retention indices were calculated for the compounds identified in each sample using an *n*-alkane standard analyzed under the same GC conditions. This data was compared with published values (**Tables 2 and 3**), and final identifications were assigned, as shown in **Figures 6 and 7**.

Terpenes in Hops Samples

For the dried hop flower sample (**Figure 5**), the terpene profile should have shown a predominance of β-myrcene,

Table 2. Terpenes in Hops Pellets Identified by MS Spectral Library Match and Retention Index

Peak No.	RT (min)	Name	RI (calculated)	RI (literature)	Reference
1	8.58	Hexanal	—	780	11
2	12.84	α-Pinene	939	942	11
3	13.28	Camphene	953	954	11
4	13.71	6-Methyl-5-hepten-2-one	966	968	11
5	14.1	β-Pinene	979	981	11
6	14.41	β-Myrcene	988	986	11
7	15.32	Cymene	1018	1020	11
8	15.65	d-Limonene	1030	1030	11
9	15.98	β-Ocimene	1041	1038	11
10	16.72	*cis*-Linalool oxide	1066	1068	11
11	17.49	Linalool	1089	1092	11
12	21.86	Geraniol	1239	1243	11
13	25.28	Geranyl acetate	1363	1364	11
14	25.85	α-Ylangene	1384	1373	8
15	25.97	α-Copaene	1388	1398	11
16	27.22	Caryophyllene	1437	1428	11
17	27.4	*trans*-α-Bergamotene + unknown	1445	1443	12
18	17.63	*trans*-β-Farnesene	1454	1450	8
19	28.11	Humulene	1473	1465	11
20	28.41	γ-Muurolene	1484	1475	11
21	28.45	γ-Selinene	1486	1472	12
22	28.68	Geranyl isobutyrate	1495	1493	11
23	28.79	β-Selinene	1499	1487	8
24	28.94	α-Muurolene	1505	1500	11
25	28.97	α-Selinene	1507	1501	12
26	29.31	γ-Cadinene	1521	1518	11
27	29.37	Calamenene	1524	1518	11
28	29.45	Δ-Cadinene	1527	1524	11
29	30.93	Caryophyllene oxide	1590	1584	8
30	31.5	Humulene oxide	1614	1599	12

humulene, and caryophyllene, which are typical aroma compounds in hops and hop oil.[9] While caryophyllene was identified, both β-myrcene and humulene were not present at levels high enough to be detected by a library search. This may be due to the condition of the sample or the actual variety of hops analyzed since terpene profiles are known to vary between different hop varieties[10]. The variety of the hop flowers analyzed is unknown, as the identity was not indicated on the packaging. For comparison, samples of two different varieties of pelletized hops were analyzed after grinding. These samples appeared green in color, and had a much more characteristic hops-like odor than the dried flowers. Analysis of these samples showed a characteristic terpene profile, with high levels of β-myrcene, caryophyllene, and humulene present in both (**Figure 6**). The SPME method was able to detect differences in the terpene profiles between the two hops varieties. For example, farnesene (peak 18) was identified in the Cascade hops, but was too low to be confirmed in the US Goldings sample. The level of farnesene in Cascade hops is expected to be 3-7% of total oils, while in US Goldings the level should be <1%.[13]

Figure 6. Headspace SPME-GC/MS Analysis of Hops Pellets Using Final Optimized Method

The peak elution order is listed in **Table 2**.	
Conditions same as **Figure 2** except:	
sample/matrix:	0.5 g ground hop flowers (hops pellets)
SPME fiber:	50/30 µm DVB/CAR/PDMS (**57298-U**)
sample equilibration:	30 min, 40 °C

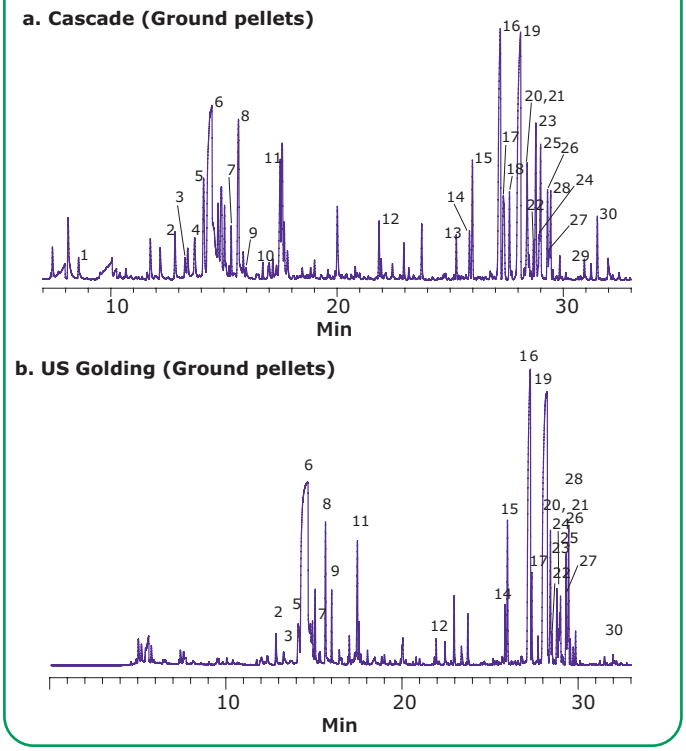
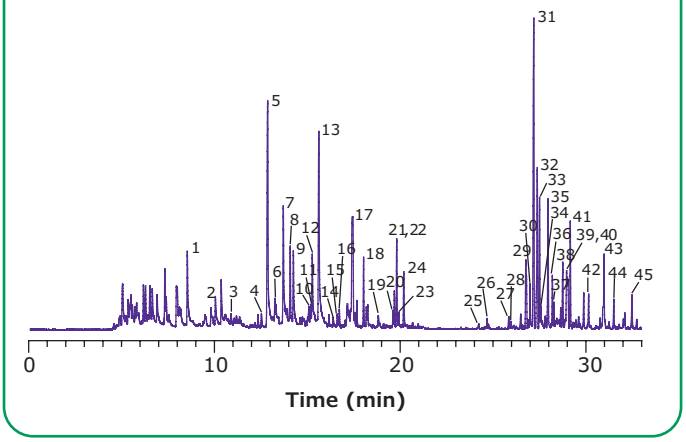


Figure 7. Headspace SPME-GC/MS Analysis of Dried Cannabis Using Final Optimized Method

The peak elution order is listed in **Table 3**.	
Same as **Figure 2** except:	
sample/matrix:	0.5 g dried, ground cannabis
SPME fiber:	50/30 µm DVB/CAR/PDMS (57298-U)
sample equilibration:	30 min, 40 °C



Terpenes in Cannabis Sample

The terpenes identified in the cannabis sample (**Figure 7**) are indicated in **Table 3**. The profile was similar to those found previously in the analysis of dried cannabis.[4,8] Peaks 1-27 in **Figure 7** (with the exception of peak 7) were monoterpenes and monoterpenoids. The later eluting peaks consisted of sequiterpenes and caryophyllene oxide, which is a sequiterpenoid. The most abundant terpene was caryophyllene. The predominance of this compound could be due to the specific strain of cannabis tested, and/or the nature of the sample tested, which was dried. Previous studies have shown the level of this compound to increase significantly relative to other terepenes and terpenoids with drying.[4] Consequently, the levels of the more volatile monoterpenes and terpenoids would be expected to be less, and this was observed to some degree. Among the monoterpenes and terpenoids the most abundant were α-pinene and d-Limonene.

Table 3. Terpenes in Dried Cannabis Identified by MS Spectral Library Match and Retention Index

Peak No.	RT (min)	Name	RI (calculated)	RI (literature)	Reference
1	8.57	Hexanal	—	—	—
2	10.05	Hexene-1-ol	—	—	—
3	10.89	2-Heptanone	—	—	—
4	12.56	α-Thujene	928	938	11
5	12.86	α-Pinene + unknown	939	942	11
6	13.27	Camphene	953	954	11
7	13.69	6-Methyl-5-hepten-2-one	966	968	11
8	14.09	β-Pinene	979	981	11
9	14.27	β-Myrcene	984	986	11
10	15.09	δ-3-Carene	1010	1015	12
11	15.2	α-Terpinene	1014	1012	12
12	15.29	Cymene	1018	1020	11
13	15.6	d-Limonene	1028	1030	11
14	16.42	γ-Terpinene	1056	1057	11
15	16.6	*trans*-Sabinene hydrate	1062	1078	11
16	16.72	*cis*-Linalool oxide	1066	1068	11
17	17.43	Linalool	1087	1092	11
18	18.04	d-Fenchyl alcohol	1107	1110	11
19	18.82	*trans*-Pinocarveol	1135	1134	12
20	19.59	Borneol L	1161	1164	11
21	19.81	1,8-Methandien-4-ol	1168	1173	8
22	19.81	*p*-Cymen-8-ol	1168	1172	12
23	19.92	Terpinene-4-ol	1172	1185	11
24	20.22	α-Terpineol	1181	1185	11
25	24.2	Piperitenone	1322	1320	12
26	24.76	Piperitenone oxide	1344	1352	12
27	25.85	α-Ylangene	1384	1373	8
28	25.97	α-Copaene	1388	1398	11
29	26.76	γ-Caryophyllene	1419	1403	12
30	27.01	α-Santalene	1429	1428	12
31	27.16	Caryophyllene	1435	1428	11
32	27.36	*trans*-α-Bergamotene + unknown	1443	1443	12
33	27.49	α-Guaiene	1448	1441	8
34	27.56	*trans*-β-Farnesene	1451	1446	12
35	27.98	Humulene	1467	1465	11
36	28.17	Alloaromadendrene	1475	1478	11
37	28.25	α-Curcumene	1478	1479	12
38	28.75	β-Selinene	1497	1487	8
39	28.97	α-Selinene	1507	1497	8
40	28.97	β-Bisobolene	1507	1506	8
41	29.13	α-Bulnesene	1514	1513	12
42	30.12	Selina-3,7(11)-diene	1556	1542	12
43	30.94	Caryophyllene oxide	1590	1595	12
44	31.5	Humulene oxide	1614	1599	12
45	32.48	Caryophylla-3, 8(13)-dien-5-ol A	1658	1656	12

Conclusion

A simple headspace SPME-GC/MS method was used in the analysis of the terpene/terpenoid profiles of both hops and cannabis. The method was able to detect the characteristic terpenes and terpenoids of both, and to distinguish between different hops varieties.

References

1. Fessenden, R.J; Fessenden, J.S. Lipids and Related Natural Products. *Organic Chemistry*, 2nd Edition; Willard Grant Press: Boston, MA, 1979; pp 897-904.
2. Bauer, B., M.D. What are the Benefits of Aromatherapy? mayoclinic.org/healthy-lifestyle, accessed 9/22/15.
3. Turner, C.E.; Elsohly, E.G.; Boeren, E.G. Constituents of Cannabis Sativa L., XVIII. A Review of the Natural Constituents. *J. Nat. Prod.*, 1980, *43*, 169-234.
4. Hood, L.V.S.; Barry, G.T. Headspace Volatiles of Marihuana and Hashish: Gas Chromatographic Analysis of Samples of Different Geographic Origin. *J. Chrom. A.*, 1978, *166*, 499-506.
5. Rahn, B. Terpenes: The Flavors of Cannabis Aromatherapy. 2/12/2014. www.leafly.com/news, accessed September 18, 2015.
6. Plants Database. www.usda.gov, accessed September 14, 2015.
7. Kemsley, J. Cannabis Safety. *CEN*, 2015, April 20, 27-28.
8. Marchini, M.; Charvoz, C.; Dujourdy, L.; Baldovini, N. Multidimensional Analysis of Cannabis Volatile Constituents: Identification of 5,5-dimethyl-1-vinylbicyclo[1.1.1]hexane as a Volatile Marker of Hashish, the Resin of Cannabis Sativa L. *J. Chrom A.*, 2014, *1370*, 200-215.
9. Wolfe, Peter H. MS Thesis, Oregon State University, 2012.
10. Hop varieties. BarthHaasGroup.com, September 15, 2015.
11. Jennings, W.; Shibamoto, T. *Qualitative Analysis of Flavor and Fragrance Volatiles by Glass Capillary Gas Chromatography,* Academic Press, 1980.
12. NIST Chemistry WebBook. www.webbook.nist.gov/chemistry.
13. Variety Manual USA Hops. Hop Growers of America, www.USAhops.org, accessed September 22, 2015.

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Description	Cat. No.
Capillary GC column	
Equity®-1, 60 m × 0.25 mm I.D., 0.25 µm	**28047**
SPME Fibers and Accessories	
SPME fiber assembly Divinylbenzene/Carboxen®/Polydimethylsiloxane (DVB/CAR/PDMS), d_f 50/30 µm, needle size 23 ga, StableFlex™, for use with autosampler, pk of 3	**57298-U**
SPME fiber assembly Polydimethylsiloxane (PDMS), d_f 100 µm (nonbonded phase), needle size 23 ga, for use with autosampler, pk of 3	**57341-U**
SPME fiber holder for CTC autosampler	**57347-U**
SPME fiber holder for manual sampling	**57330-U**
Accessories	
Inlet Liner, Direct (SPME) Type, straight design, 0.75 mm I.D. for Agilent® GC	**2637501**
Molded Thermogreen® LB-2 Septa, with injection hole, 11 mm, pk of 50	**28336-U**
Headspace Vial, screw top, rounded bottom, 10 mL, clear glass, pk of 100	**SU860099**
Magnetic Screw Cap for Headspace Vials, PTFE/silicone septum, pk of 100	**SU860103**

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Simplified Terpene Analysis in Air using the Carbotrap® T420 Thermal Desorption Tube

An Optimized Solution for Sampling and Analysis of Terpenes in Air with TD-GC/MS

Kristen Schultz, Global Product Manager Cannabis & Psychedelics Analytical Workflow, Analytix@milliporesigma.com

Terpenes are highly aromatic natural compounds found in many living plants, fruits and herbs that provide the characteristic scent or fragrance often recognized by the sense of smell. In nature, terpenes function to protect the plants from insects, animal grazing and plant diseases. As these plants are dried and cured, the terpenes oxidize and become terpenoids. Many consumer products are derived from the vibrant aromas of terpenes such as from the cannabis sativa plant for medicinal and recreational use; and also, from plants such as hemp, hops, juniper berries, for essential oils used in aromatherapy and characteristic flavorings in food and beverages. Terpene and terpenoid compounds are highly studied in research to evaluate their potential medicinal and therapeutic benefits in both animals and humans. In order to analytically profile the levels of terpenes in these plants, a flowering portion of the plant must be removed and then dried for qualitative analysis at a cost to the grower in reduced plant yield. The Carbotrap® T420 Thermal Desorption (TD) tube can provide the grower with a non-destructive profiling tool of their growing and flowering plants and plant materials with a simple grab sample of the air surrounding the plant.

People who work in the agriculture side of the growing and cultivation of cannabis and hemp in indoor greenhouse environments are exposed to high levels of terpenes and other volatile organic compounds (VOCs) during their work shift and may experience unpleasant respiratory health effects. However, there are currently no federal or state government mandated workplace exposure limits associated with terpenes emissions in these environments and no reliable air sampling method optimized for measuring these terpenes. The Carbotrap® T420 TD tube (**Figure 1**) was designed to solve the problem for sampling terpenes in air in high humidity environments for industrial hygiene, indoor air quality and ambient air emission monitoring.

Communities that neighbor the cannabis and hemp agricultural growing operations often complain of the persistent unpleasant odors, but there are few government mandates related to odor emissions and mitigation strategies. The Carbotrap® T420 TD tube can provide regulators with a tool to actively measure the fence line around these growing operations and neighborhoods to study and monitor environmental emissions, access terpene drift, and associated odors to develop their mitigation strategies and set future terpene odor limit thresholds.

Figure 1. Carbotrap® T420 stainless steel and glass tube.

Features & Benefits of the Carbotrap® T420 TD Tube for Sampling Terpenes in Air

- Efficiently retains and releases terpenes associated with cannabis, hemp, hops and other terpene fragrant plants
- Optimized for sampling a wide-range of applications such as: industrial hygiene, environmental emissions and terpene drift; terpene odor concentration; non-destructive live plant profiling and headspace air of plant materials
- Designed for sampling in high humidity environments such as indoor greenhouse environments
- Available in both glass-fritted and stainless steel TD tubes—¼ in. O.D. x 3.5 in. L (6.35 mm O.D. x 89 mm long).
- Preconditioned & QC tested to ensure low background levels
- Easy sample identification and tracking—a unique number with corresponding durable barcode on each tube

Guidelines for Air Sample Volume Collection by Application Type

To quantitate the terpene concentration from air depending on your application, it is important to know the total volume of air pulled through the tube with an air sampling pump during the sampling event. The sample volume is calculated by multiplying the flow rate (L/min) by the sampling time (minutes). The recommended sample collection volume is between 0.10 to 10 liters to prevent breakthrough and/or overloading the analytical system. The optimal sample volume is dependent on the terpene concentration levels that are present during sampling and the analytical instrument parameters of the thermal desorption and gas chromatograph parameters (TD-GC/MS). **Table 1** provides the recommended sample volumes based on various sampling conditions. Additional experimentation may need to be performed for your specific conditions.

Table 1. Recommended sample collection volumes by application

Application	Observed Terpene Odor	Recommended Sampling Volume
Testing of live plants and headspace of the actual plant material	Very Strong	0.1 to 0.2 Liters
Indoor sampling of greenhouse	Strong	1 to 2 Liters
Outdoor sampling near growing location	Detectable	2 to 5 Liters
Outdoor sampling	Undetectable	5 to 10 Liters

Note: Terpene odor thresholds will be different among users so keep this in mind when choosing a sample volume for your application.

Figure 2. Outdoor measurement of terpenes from hemp growing operations using a glass and a stainless steel Carbotrap® T420 TD tube and a Spectrex PAS-500 Personal Air Sampling pump.

Figure 3. Indoor profile measurement of cannabis terpenes (flowering plant) from cannabis greenhouse growing operations using a glass Carbotrap®T420 TD tube and detector tube pump.

Figure 4. Indoor profile measurement of cannabis terpenes using headspace on dried plant materials from cannabis greenhouse growing operations placed in a bag (e.g. Tedlar® gas sampling bag) using a stainless Carbotrap® T420 TD tubes and large syringe to pull the sample through the tube. (when using a gas sampling bag, the tube in a TDS³™ container - equipped with sampling caps - can tightly be connected to the headspace through the bags foil/septa using a luer connector and luer needle to withdraw a sample).

Examples of actual sampling set ups are displayed in **Figures 2-4** using different pumps and collection approaches.

Products for Sample Collection

Description	Qty.	Cat. No.
Carbotrap® T420, Glass-Fritted TD Tube, Preconditioned	10	**28689-U**
Carbotrap® T420, Stainless Steel TD Tube, Preconditioned	10	**28687-U**
Air Sampling Pumps and Flow Calibration Equipment		
Spectrex PAS-500 Battery-Operated Personal Sampling Pump	1	**24865**
Zefon Escort ELF® Personal Air Sampling Pump	1	**28160-U**
Battery Charger for Zefon Escort ELF®, 110 VAC	1	**28157-U**
Battery Charger for Zefon Escort ELF®, 240 VAC	1	**28158-U**
Zefon Gemini® Twin-Port Sampler	1	**28118-U**
A.P. mini-Buck™ M-5 Flow Calibrator	1	**24843**
Battery Charger for M-5 Flow Calibrator, 110 VAC	1	**24844**
Battery Charger for M-5 Flow Calibrator, 220 VAC	1	**24846**
Ellutia 7000 GC Flowmeter, includes universal charger	1	**29597-U**
TDS3 Storage Container & Sampling Apparatus		
Replacement TDS³™ Storage Container for 3.5 in. L, 89 mm TD Tubes	1	**25097-U**
TDS³™ Sampling Caps Set	1	**25069**
Male Luer Fitting for 1/8 in.	1	**21016**
Male Luer Fitting for ¼ in. Tubing	1	**24586**
Replacement TDS³™ Septa	50	**25073**
Male Luer coupler	20	**25064-U**
Needles for luer lock syringes, needle size 23 ga, needle L × O.D. 50 mm × 0.63 mm, point style, 2	5	**26270-U**

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Methods for Analysis of the California List of Pesticides in Cannabis

Geoffrey Rule, Principal Scientist, Analytix@milliporesigma.com

Abstract

Methods to quantify the California list of pesticides from dried cannabis (hemp), using both GC-MS/MS and LC-MS/MS are described with a special focus on evaluation of matrix effects and use of internal standards.

Introduction

Growers and processors of cannabis must show their products to be safe as per individual state requirements. Generally, requirements include testing to ensure that the cannabis flower is free of pesticides.[1] In this application note we demonstrate how Supelco analytical standards, instrument consumables, and reagents can be used to analyze low levels of pesticides in cannabis and, in particular, the 66 pesticides required by the State of California.

QuEChERS extraction has been widely adopted for preparation of samples in the analysis of pesticides from a variety of agricultural matrices. But it shows some limitations due to the broad range of physical and chemical properties of the pesticides. As a consequence, there is a trend of minimal sample clean-up by instrument vendors, and a simple solvent extraction proposed by some. In other cases, a simple flow-through or chemical filtration type clean-up is proposed where the solvent extract is allowed to pass through an SPE cartridge of some type to remove unwanted matrix material. While these goals are admirable, they may or may not always be successful based on the number of analytes required, reporting limits, the instrumentation available as well as the matrix being extracted. This makes proper understanding of matrix effects, extraction recovery, and use of isotope labelled internal standards critical in many cases.

In this article we describe the determination of 66 pesticides from the California list of pesticides, in a locally obtained hemp sample using both LC-MS/MS and GC-MS/MS. A flow-through, interference removal clean-up procedure is utilized for the analysis. Methods are also outlined to evaluate the matrix effects and extraction recovery — two essential aspects of developing rugged methods. In addition, we describe the use of analyte protectants for compounds best analyzed by gas chromatography. The use of stable isotope labelled (SIL) internal standards is also discussed with a focus on the advantages provided by them.

Methods

One gram of coarse ground hemp was weighed into a 50 mL centrifuge tube. Two ceramic homogenization pellets were added along with 15 mL of acetonitrile. The sample was extracted manually, with vigorous shaking for 5 min, and then centrifuged for 10 min at 2800 rpm. The entire supernatant was then removed and passed through a Discovery® DSC-18 solid phase extraction cartridge (6 mL, 500 mg) by gravity flow. This was followed by two additional extractions, each with 5 mL acetonitrile. The eluents from the three extractions were combined and the final volume was brought up to 25 mL with acetonitrile. Aliquots of this solution were then placed in separate autosampler vials for both LC-MS/MS and GC-MS/MS analysis.

The conditions developed for both the LC-MS/MS and GC-MS/MS analysis methods are shown in **Tables 1** and **2**. Calibration curves were prepared using both the blank matrix extract, that had gone through the extraction procedure, and pure solvent or mobile phase. To cover the range for California requirements, a total of nine calibration standards were utilized at 0.02, 0.05, 0.1, 0.2, 0.5, 1.0, 1.5, 3 and 5 µg/g hemp equivalent. To evaluate analyte recovery, samples are prepared by "spiking" hemp with a pesticide solution pre-extraction, to best represent actual plant samples. In our case, we spiked hemp samples at concentrations of 0.1 and 3 µg/g for extraction recovery experiments. Experiments can be performed in the same fashion for both LC-MS and GC-MS evaluation of suppression or enhancement effects, and determination of extraction recovery (**Figure 1**). A comparison of the solvent based curve with the one prepared in the blank matrix extract (post-extract spike) reveals the extent to which matrix components are suppressing or enhancing chromatographic peak intensities. Comparison of the post-extract spiked curve with the samples prepared by spiking prior to extraction (pre-extract spike), provides a means of evaluating analyte losses from the extraction, or sample clean up procedure.

Table 1. LC-MS/MS Analysis Conditions

LC-MS/MS Conditions	
instrumentation:	Agilent 1290 series HPLC and autosampler with 6460 QQQ
column:	Ascentis® RP-Amide, 10 cm x 2.1 mm, 3 µm particles (**565301-U**) with RP-Amide guard column, 2 cm x 2.1 mm I.D., 5 µm (**565372-U**)
mobile phase:	[A] 2 mm ammonium formate, 0.1% formic acid, 2% methanol in Milli-Q water
	[B] 2 mm ammonium formate, 0.1% formic acid, 5% Milli-Q® water in acetonitrile
gradient	

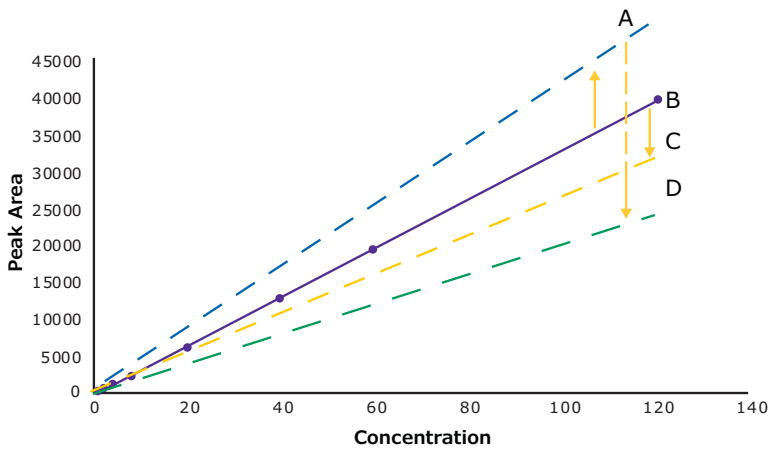
Time (min)	A (%)	B (%)
0.0	100	0
1.0	100	0
14.0	0	100
17.0	0	100
17.5	100	0
20.0	100	0

flow rate:	0.4 mL/min
column temp:	40 ° C
detector:	MS/MS, ESI (+) dMRM Acquisition Mode*
injection:	12 µL

* For a list of transitions used please contact the author

Table 2. GC-MS/MS Analysis Conditions

GC-MS/MS Conditions	
column:	SLB®-5ms 30 m × 0.25 mm, 0.25 µm (**28471-U**)
oven:	60°C (1 min), 40 °C/min to 170 °C, 10 °C/min to 310 °C (3 min)
injector:	solvent vent mode: 60 °C (0.35 min), 600 °C/min to 300 °C; 5 psi until 0.3 min, split vent flow 50 mL/min at 1.5 min
carrier gas:	helium, 1.2 mL/min, constant flow
detector:	MS/MS
injection:	2 µl, solvent vent splitless injection with 0.2 µL sandwich of analyte protectant solution
liner:	4 mm ID dual tapered liner
sample:	hemp extract in acetonitrile



A Post-extract curve showing enhancement
B Curve in pure solvent
C Post-extract curve showing suppression
D Pre-extract curve showing extraction recovery

B to A Enhancement
B to C Suppression
A-C to D Extraction Recovery

(Peak area A/B -1)x(100) = % Enhancement
(1- Peak area C/B)x(100) = % Suppression
(D/C)x(100) or (D/A)x(100) = % Extraction Recovery

Figure 1. Matrix Enhancement, Suppression and Recovery Evaluation

An additional means of evaluating suppression effects in LC-MS is through use of a "tee-infusion" experiment.[2] In this experiment a syringe pump is used to infuse a solvent solution of the analytes of interest into a tee fitting placed between the column and the mass spectrometer (**Figure 2**). The infusion flow rate and concentration are typically quite low, in the order of 10 µL/min or so, and 100-200 pg/µL. A blank matrix extract is prepared and injected to the LC system while the analytes are monitored over the course of the LC gradient. Comparing injections of the blank matrix extract with a similar injection of mobile phase indicates where matrix components elute during the run and their impact on analyte signal intensity.

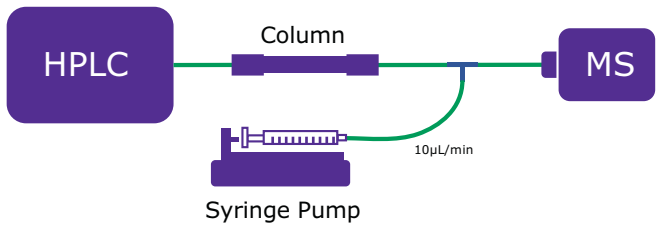


Figure 2. Illustration of Tee-Infusion Experimental Set-Up

Use of analyte protectants in gas chromatography

Suppression or enhancement effects may be observed in GC-MS also. The causes are different than electrospray ionization (ESI) LC-MS but the effects can be studied in a similar fashion. In GC, the suppression or enhancement effects generally result from the loss or degradation of analytes in the hot injection port, liner, and column inlet when injected in relatively clean extracts or pure solvent. In case of more complex sample extracts, the matrix components can protect analytes from this degradation by blocking the active sites present in these regions of the GC. The matrix therefore causes an enhancement effect, and presents as an improved analyte peak shape and intensity. To ameliorate the situation, several compounds have been identified that will reduce analyte degradation if injected simultaneously with the analyte.[3, 4] Compounds such as sorbitol and gulonic acid lactone, gluconic acid lactone, shikimic acid, and 3-ethoxy-1,2-propanediol are examples of compounds found to reduce analyte degradation. Some protectants are also shown to be most effective during specific periods of a chromatographic run, for example during early, middle, or late stages of the run, and for particular analytes. In our work, a solution of two compounds was prepared for use (**Table 3**). The solution was placed on the autosampler and an injection method was created to "sandwich" the sample extract within the protectant solution.

Table 3. Preparation of Analyte Protectant Solution

Step	Procedure
1	Weigh ~500 mg of D-Sorbitol into a 10 mL volumetric flask and add 6 mL of LC-MS grade acetonitrile. Bring to volume with Milli-Q® water (Solution A).
2	Weigh ~500 mg of L-Gulonic acid γ-lactone into a 10 mL volumetric flask and add 5mL of LC-MS grade acetonitrile. Bring up to volume with Milli-Q® water (Solution B).
3	Add 2 mL of Solution A with 4 mL of Solution B in a 10 mL volumetric flask and bring to volume with LC-MS grade acetonitrile
4	Place into appropriate autosampler vial for making sandwich injection with 0.2 µL of air gap above and 0.2 µL of the analyte protectant solution

With a study of extraction recoveries and matrix effects on peak intensities, additional efforts can be directed at either sample clean-up or in adjusting chromatographic conditions where necessary. The choice of appropriate internal standards can also be made to generate reliable methods for any given matrix.

Results

With the chromatographic and instrument conditions shown (**Tables 1** and **2**), we successfully met the California requirements for 57 of the 66 pesticides by LC-MS/MS. The HPLC conditions developed provided the separation of analytes as shown in **Figure 3** with daminozide being the earliest eluting compound and acequinocyl the latest.

Not surprisingly, results of the tee-infusion experiment indicate that acetonitrile extraction of hemp yields a large amount of cannabinoid material in the extract (**Figure 4**). The cannabinoids come off in the course of each chromatographic run and can cause variable degrees of suppression depending on the amount of each cannabinoid present. Samples expected to be high in THC and THCA might therefore benefit from slightly different chromatographic conditions than those for samples high in CBD and CBDA.

The GC-MS/MS conditions developed provided the separation of analytes as shown in **Figure 5** and allowed the successful determination of 40 compounds from the California list. The two instrumental methods (LC & GC) provide an overlap of 32 compounds, which may be considered advantageous for situations where one or more analyte-matrix combinations may be challenging on one instrument type but not the other. A tabulation of results for each pesticide, and by each method, is given in **Table 4**.

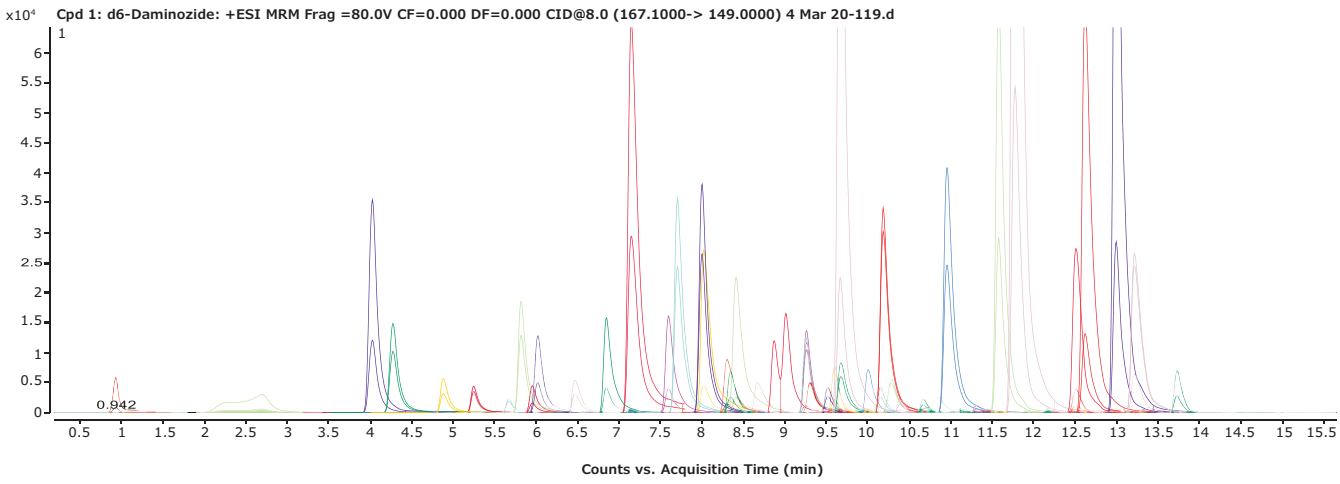


Figure 3. Standard Injection of California Pesticides Prepared in Hemp and Analyzed by LC-MS/MS

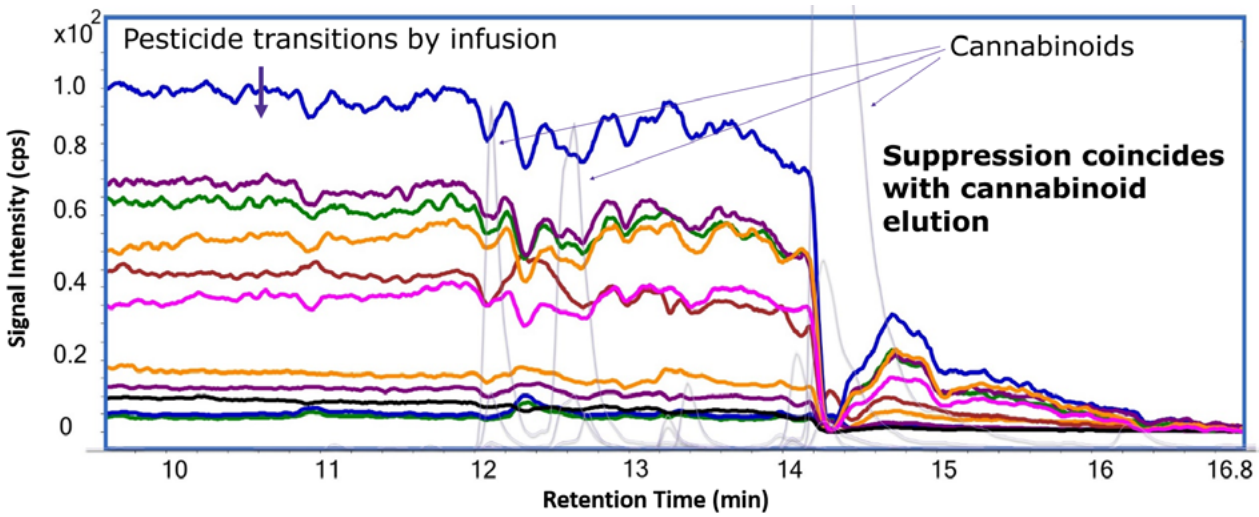


Figure 4. A portion of the Chromatographic Run in a Tee-Infusion Experiment - The colored traces indicate the signal intensity of several pesticides being infused over the course of a run. The cannabinoid peaks (grey) were collected as a separate chromatographic run and have been overlaid with the infusion data for illustration. The suppression occurring after 15 minutes is due to unidentified matrix components eluting from the column.

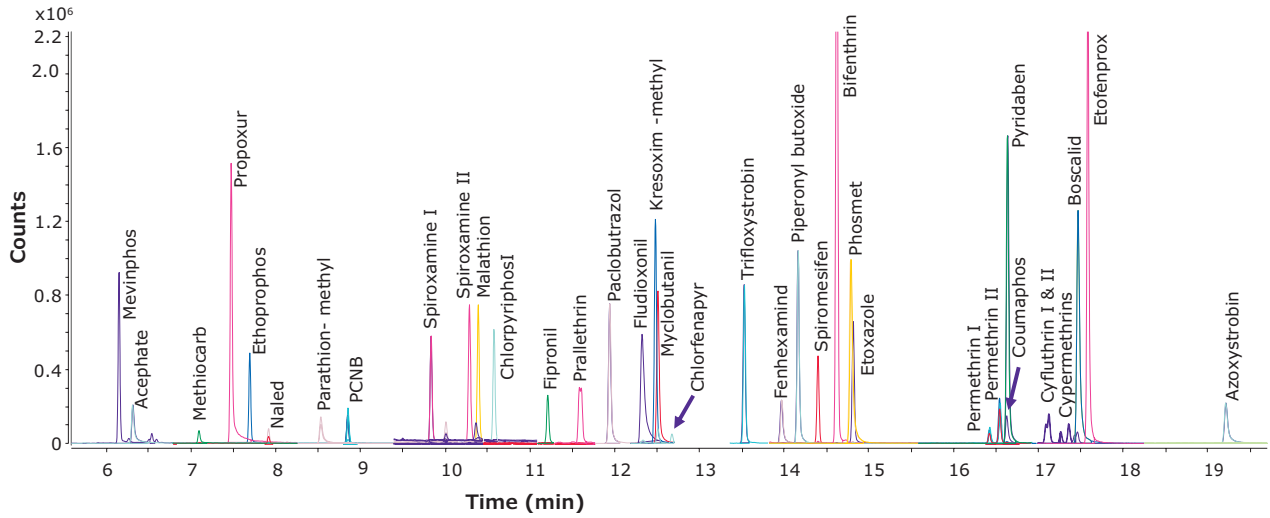


Figure 5. Standard Injection of California Pesticides Prepared in Hemp and Analyzed by GC-MS/MS

Table 4. Tabulated Results: California Pesticides at Minimum Action Level (MAL)[5]

Analyte	GC-MS/MS	LC-MS/MS
	MAL met?	MAL met?
Acephate	YES	YES
Acequinocyl	NO	NO
Acetamiprid	NO	YES
Aldicarb	NO	YES
Avermectin	NO	YES
Azoxystrobin	YES	YES
Bifenazate	NO	YES
Bifenthrin	YES	YES
Boscalid	YES	YES
Captan	YES	YES
Carbaryl	NO	YES
Carbofuran	NO	YES
Chlorantraniliprole	NO	YES
Chlordane I	YES	NO
Chlordane II	YES	NO

Analyte	GC-MS/MS	LC-MS/MS
	MAL met?	MAL met?
Chlorfenapyr	YES	YES
Chlorpyrifos	YES	YES
Clofentezine	NO	YES
Coumaphos	YES	YES
Cyfluthrin I & II	YES	NO
Cypermethrin I II III IV	YES	NO
Daminozide	NO	YES
Diazinon	NO	YES
Dichlorvos	YES	YES
Dimethoate	NO	YES
Dimethomorph	NO	YES
Ethoprop	YES	YES
Etofenprox	YES	YES
Etoxazole	YES	YES
Fenhexamid	YES	YES

Table 4. (cont.) Tabulated Results: California Pesticides at Minimum Action Level (MAL)[5]

Analyte	GC-MS/MS MAL met?	LC-MS/MS MAL met?
Fenoxycarb	NO	YES
Fenproximate	NO	YES
Fipronil	YES	YES
Flonicamid	NO	YES
Fludioxonil	YES	YES
Hexythiazox	NO	YES
Imazalil	NO	YES
Imidacloprid	NO	YES
Kresoxim-methyl	YES	YES
Malathion	YES	YES
Metalaxyl	NO	YES
Methiocarb	YES	YES
Methomyl	NO	YES
Mevinphos	YES	YES
Myclobutanil	YES	YES
Naled	YES	YES
Oxamyl	NO	YES
Paclobutrazol	YES	YES
Parathion-methyl	YES	NO
PCNB	YES	NO

Analyte	GC-MS/MS MAL met?	LC-MS/MS MAL met?
Permethrins	YES	YES
Phosmet	YES	YES
Piperonyl butoxide	YES	YES
Prallethrin	YES	YES
Propiconazole	NO	YES
Propoxur	YES	YES
Pyrethrins	NO	YES
Pyridaben	YES	YES
Spinetoram J	NO	YES
Spinosyn A	NO	YES
Spinoteram L	NO	YES
Spiromesifen	YES	YES
Spirotetramat	NO	YES
Spiroxamine I	YES	YES
Spiroxamine II	YES	YES
Tebuconazole	NO	YES
Thiacloprid	NO	YES
Thiamethoxam	NO	YES
Trifloxystrobin	YES	YES

Use of stable isotope labeled internal standards

The potential benefit of using stable isotope labeled (SIL) internal standards (IS) should always be considered when developing methods for challenging matrices or for particular analytes. While they do add to the cost of sample analysis, they make up for it in providing more accurate and rugged methods, even in the presence of matrix effects and recovery losses. Analog ISs, meaning compounds that are chemically only similar to the analyte, cannot guarantee the same advantages as SIL ISs due to differences in retention time and ionization efficiency (**Figure 6**). A SIL IS, on the other hand, is essentially identical to the analyte itself but differing only in mass. This means, once added to a sample the ratio of analyte to SIL IS will not vary through the sample preparation, chromatography, and analysis stages. Use of this ratio in quantitation therefore results in excellent accuracy. (See our isotope labeled pesticide standards at **SigmaAldrich.com/ilspesticides**)

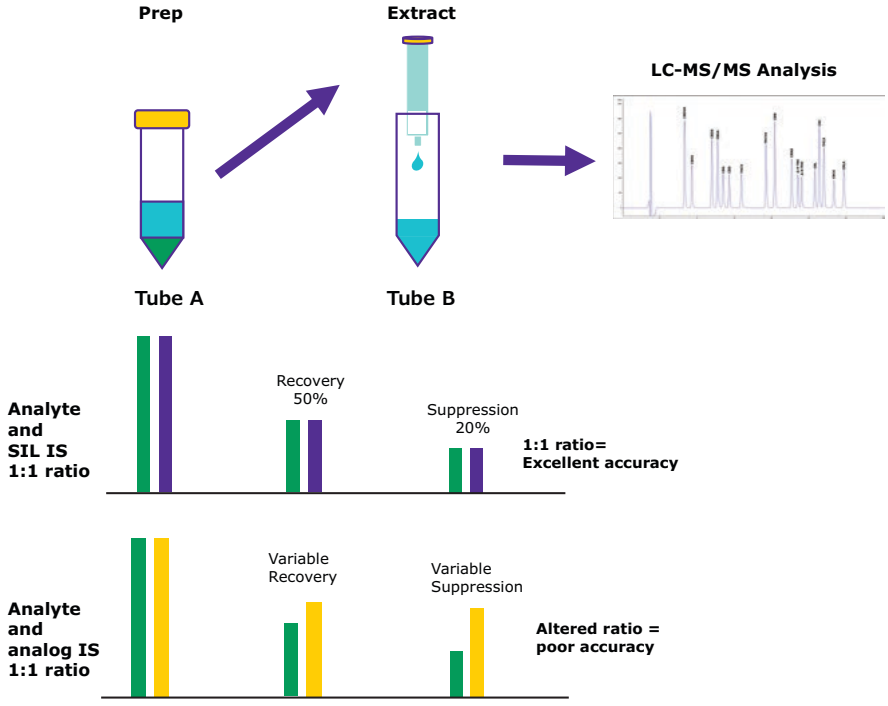


Figure 6. A Depiction of the Advantages Obtained by Use of SIL IS Over Analog IS in LC-MS/MS Analysis.

Conclusion

A method has been developed to quantify the California list of pesticides from dried cannabis (hemp), in accordance with the state requirements, utilizing a combination of both LC-MS/MS and GC-MS/MS. A single step, flow-through (interference removal) solid phase extraction cleanup is used to prepare sample extracts for both instrumental techniques. The linearity, recovery, and precision were satisfactorily achieved (not shown) and schemes for performing calibration, extraction recovery, and suppression/enhancement studies are provided.

A total of 57 pesticides were reported by LC-MS/MS and 40 using GC-MS/MS (**Table 4**). Due to high levels of interfering CBDA, one analyte, acequinocyl, was not detectable at minimum levels with the existing instrumentation. All other pesticides were reported with one or the other analytical technique to meet or exceed current California regulatory limits for each category.[5]

It is shown that a combination of GC-MS/MS and LC-MS/MS instrumentation provides an efficient way to analyze cannabis for pesticides.

Literature

1. Seltenrich, N. Into the Weeds: Regulating Pesticides in Cannabis. Enviro Health Persp. 2019; 127(4) doi.org/10.1289/EHP5265
2. Bonfiglio R, King RC, Olah TV, Merkle K. The effects of sample preparation methods on the variability of the electrospray ionization response for model drug compounds. Rapid Commun Mass Spectrom. 1999;13(12):1175-1185. doi:10.1002/(SICI)1097-0231(19990630)13:12<1175::AID-RCM639>3.0.CO;2-0
3. Mastovská K, Lehotay SJ, Anastassiades M. Combination of analyte protectants to overcome matrix effects in routine GC analysis of pesticide residues in food matrixes. Anal Chem. 2005;77(24):8129-8137. doi:10.1021/ac0515576
4. Anastassiades M, Mastovská K, Lehotay SJ. Evaluation of analyte protectants to improve gas chromatographic analysis of pesticides. J Chromatogr A. 2003;1015(1-2):163-184. doi:10.1016/s0021-9673(03)01208-1
5. California Bureau of Cannabis Control, Text of Regulations; https://bcc.ca.gov/law_regs/cannabis_order_of_adoption.pdf

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SigmaAldrich.com/ilspesticides

For more such information on testing of cannabis visit us at **SigmaAldrich.com/cannabis**

Featured Products

Description	Part Number
LC-MS/MS	
Discovery® C18 SPE Cartridges, 500 mg, 6 mL, Pk.30	**52604-U**
Ascentis® RP-Amide 100 x 2.1 mm, 3 µm	**565301-U**
Ascentis® RP-Amide Supelguard™ Guard Cartridge, 2 cm x 2.1 mm I.D., 5 µm, PK.2	**565372-U**
Ultrapure water from Milli-Q® system or bottled water	**Milli-Q® IQ 7005 or 1.15333**
Acetonitrile with 0.1% (v/v) Formic acid hypergrade for LC-MS LiChrosolv®	**1.59002**
Methanol hypergrade for LC-MS LiChrosolv®	**1.06035**
Water with 0.1% (v/v) Formic acid hypergrade for LC-MS LiChrosolv®	**1.59013**
Ammonium Formate, eluent additive for LC-MS, LiChropur™, ≥99.0%	**70221**
Formic acid 98% - 100%, for LC-MS LiChropur™	**5.33002**
50 mL Centrifuge Tubes, Pk.500	**T2318**
15 mL centrifuge tube, Pk.500	**T1818**
Certified Vial Kit – Amber (Autosampler vials), Pk.100	**29653-U**
Guard frit with holder	**803410**
Replacement frits	**803411**
GC-MS/MS	
SLB®-5ms, 30 m × 0.25 mm, df 0.25 µm	**28471-U**
Acetonitrile for gas chromatography ECD and FID SupraSolv®	**1.00017**
Molded Thermogreen® LB-2 Septa, solid discs, diam. 11 mm, Pk.50	**28676-U**
Inlet Liner Splitless Type, Dual-Taper Design (unpacked), Pk.5	**2048505**
Certified Vial Kit, Low Adsorption (LA), 2 mL, amber glass vial, natural PTFE/silicone septa (with slit), thread for 9 mm, Pk. 100	**29654-U**
Hamilton® syringe701N, volume 10 µL, needle size 26s ga (bevel tip), needle L 51 mm (2 in.)	**20734**
L-Gulonic acid γ-lactone, 95%	**310301**
D-Sorbitol, 99%	**240850**
Supelco® Helium Purifier, stainless steel fittings, 1/8 in	**27600-U**
OMI®-2 Purifier Tube, Pk.1	**23906**
OMI®-2 Purifier Holder, Pk.1	**23921**

Approaches to the Analysis of the Oregon List of Pesticides in Cannabis Using QuEChERS Extraction and Cleanup

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Introduction

Consumption of cannabis and / or cannabis-based products is currently legal in some form in 33 US states plus the District of Columbia. Testing of the plant materials and products is required by many of these states; however, the specific test methods and target compound lists are not mandated in all cases. In October of 2016, the state of Oregon took a major step forward by requiring that all labs testing cannabis be accredited by the Oregon Environmental Laboratory Accreditation Program (ORLEP) and licensed by the Oregon Liquor Control Commission (OLCC).[1] Consequently, Oregon Administrative Rules (OAR) list specific contaminants to be tested in marijuana samples, along with action levels.[2] The pesticides on this list include carbamate, organophosphorus, macrocylic lactone, neonicotinoid, pyrethroid, and triazole fungicides as well as others. Action levels per OAR vary from 0.2 to 1 µg/g, depending on the specific pesticide. In addition, the state of California, which legalized recreational cannabis in 2016, requires testing for a list of pesticides similar to that on the OAR list, plus 8 additional.[3]

Due to its ease of use and applicability to a wide range of pesticides, the "quick, easy, cheap, effective, rugged & safe" (QuEChERS) approach has been adopted by many testing laboratories for use on cannabis. After extraction, incorporation of a cleanup step is important for removing pigments, as well as other contaminants. QuEChERS cleanup using a mixture of primary secondary amine (PSA), C18 and graphitized carbon black (GCB) is often chosen for this purpose. PSA will remove acidic interferences, C18 hydrophobic interferences and GCB retains some pigments – specifically the green color imparted by chlorophyll. This mixture of sorbents thus retains a wide range of contaminants; however it also has potential to reduce recoveries of target pesticides which are susceptible to hydrophobic retention on C18, or planar enough in structure to be strongly retained by GCB. In previous work done by the author in 2015 with cannabis, an alternative sorbent mix, Supel™ QuE Verde, was evaluated for cleanup in the analysis of various pesticides, and found to offer an advantage with regards to background reduction and recovery.[4] This sorbent mix contains PSA, Z-Sep+, and ENVI-Carb Y. Z-Sep+ is a zirconia coated silica functionalized with C18. The zirconia retains by Lewis acid/base interactions, and has been found to retain certain fatty compounds as well as some pigments. ENVI-Carb Y is a specially manufactured graphitized carbon that is engineered to have weaker retention of small, planar molecules such as certain pesticides. This mixture offers a better balance than traditional PSA/C18/GCB with regards to removal of pigmentation and pesticide recovery. In this application, the pesticide list tested in 2015 has been expanded to include many of those on the OAR list described above. Supel™ QuE Verde was compared directly to PSA/C18/GCB for cleanup and analysis of spiked replicates of cannabis plant material analyzed by LC-MS/MS and GC-MS/MS. Column and mobile phase selection for LC-MS/MS was done based on several factors, which will be described. For the additional pesticides included on the OAR list, Supel™ QuE Verde was found to yield better overall recovery than PSA/C18/GCB.

Experimental

Extraction

Dried cannabis* was pulverized using a IKA T10 Ultra Turrax mixer. 1.9 g was weighed into a 50 mL centrifuge tube and spiked with pesticides at 50 ng/g. After a 10 min equilibration time, the sample was mixed with 10 mL of deionized water and allowed to sit for 30 minutes. 10 mL of acetonitrile was added, and the sample was shaken at 2500 rpm for 30 minutes. The contents of the Supel™ QuE Citrate tube (55227-U) were added, and the sample shaken for 1 minute. The sample was then centrifuged at 5000 rpm for 5 min, and the supernatant removed for cleanup.

**Dried cannabis was supplied courtesy of Dr. Hari H. Singh, Program Director at the Chemistry & Physiological Systems Research Branch of the National Institute on Drug Abuse at the National Institute of Health.*

Cleanup

1 mL of extract was added to a 2 mL tube containing the mixture of cleanup sorbents. Two different sorbent mixtures were used:

1. PSA/C18/GCB/MgSO4 (400 mg/400 mg/ 400 mg/1200 mg)
2. Supel™ QuE Verde (**55447-U**)

Samples were shaken for 1 minute, centrifuged at 5000 rpm for 3 minutes, and the supernatant removed for analysis.

Analysis

Samples were analyzed by LC-MS/MS and GC-MS/MS using the conditions listed in **Tables 1** and **2**. The same extracts were run on both systems. Pesticides that did not yield response by LC-MS/MS were attempted by GC-MS/MS. Quantitation was done against a 5-point matrix-matched calibration curve prepared in unspiked cannabis extract. Separate curves were prepared for each cleanup. No internal standards were used, thus all recovery values reported are absolute.

Table 1. LC-MS/MS conditions

column:	Ascentis® RP-Amide, 10 cm x 2.1 mm I.D., 3.0 µm (**565301-U**) with RP-Amide Supelguard™ cartridge, 2 cm x 2.1 mm I.D., 5 µm (565372-U)
mobile phase:	[A] 5 mM ammonium formate, 0.1% formic acid in 95:5 water:acetonitrile;
	[B] 5 mM ammonium formate, 0.1% formic acid in 5:95 water:acetonitrile
gradient:	10 % B held for 1 min; to 100 % B in 13 min; held at 100 % B for 6 min; to 10 % in 0.5 min; held at 10 % B for 6 min
flow rate:	0.4 mL/min
column temp.:	30 °C
detector:	MRM*
injection:	5 µL
sample:	QuEChERS extract in acetonitrile

Table 2. GC-MS/MS analysis conditions

column:	SLB®-5ms, 20 m x 0.18 mm I.D., 0.18 µm (**28564-U**)
oven:	50 °C (2 min), 8 °C/min to 325 °C (10 min)
inj. temp.:	250 °C
carrier gas:	helium, 1.2 mL/min constant flow
detector:	MRM*
MSD interface:	325 °C
injection:	1 µL, pulsed splitless (50 psi until 0.75 min, splitter open at 0.75 min)
liner:	4 mm I.D. FocusLiner™ with taper

*Detailed MRM listings can be requested from the author.

Results and Discussion

HPLC column and mobile phase selection. Typical cannabis samples analyzed by testing labs contain high levels of cannabinoids, often in the range of 20-25% by weight. These compounds will coextract with the pesticides during the QuEChERS process. The acidic forms can be partially retained by some cleanup sorbents (specifically PSA and Z-Sep+), however the neutral forms are not retained well by cleanup sorbents used for pesticide testing. In the case of LC-MS/MS analysis, these co-extracted cannabinoids can build up on the detector, requiring more frequent system maintenance. In this application, column and mobile phase selection were based on conditions that would force elution of the cannabinoids as late as possible in the run, ideally after the pesticides. Under these conditions, the diverter valve on the LC-MS/MS system could be set to flow to waste after elution of the last pesticide. This will then prevent a majority of the cannabinoids from entering the detector.

To facilitate the appropriate HPLC conditions, a screening experiment was designed to study elution of the major cannabinoids compared to the targeted pesticides on several different column chemistries, and using both acetonitrile and methanol based gradients. The columns screened were as follows:

1. Ascentis® Express C18
2. Ascentis® Express RP-Amide
3. Ascentis® Express Phenyl-Hexyl
4. Ascentis® Express Biphenyl
5. Ascentis® Express F5

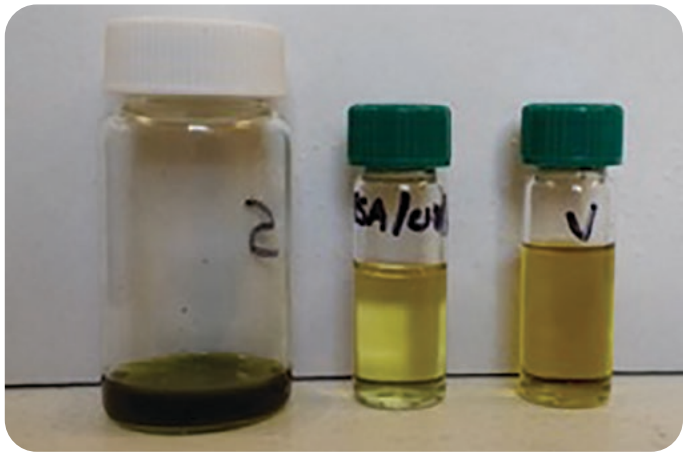
All columns were 10 cm x 2.1 mm I.D., 2.7 µm. The Ascentis® Express Fused-Core version of these chemistries was initially chosen for both efficiency and speed. The HPLC conditions were similar to those listed in **Table 1**, with UV used for detection, and ammonium formate omitted from the mobile phase. Samples were injected in 100% acetonitrile, to emulate samples resulting from the QuEChERS extraction, and as expected, this resulted in poor peak shapes of the earliest eluting pesticides on all five columns. Using an acetonitrile gradient, the Ascentis® Express RP-Amide yielded the least amount of overlap between the pesticide and cannabinoid elution ranges. In addition, comparing acetonitrile to methanol, using the former in the gradient eluted the pesticides faster, resulting in less overlap with the cannabinoids.

To simplify the method as much as possible, the same QuEChERS extract was analyzed by both HPLC and GC. However, as indicated previously in the column screening experiment, injection of 100% acetonitrile into the high aqueous starting conditions of the gradient produced poor peak shapes for the early eluting pesticides. To improve the peak shapes of these compounds, a 3 µm Ascentis® RP-Amide was substituted for the 2.7 µm Ascentis® Express RP-Amide. Installation of a guard column further improved peak shape most likely due to increased retention and improved mixing of the sample with the

mobile phase. (For chromatograms see online version of article on **SigmaAldrich.com/analytix** - Issue 5.) In addition, when working with high background samples, use of a guard column is highly recommended to extend the life of the analytical column.

Background reduction. A comparison of the cannabis extracts before and after cleanup with PSA/C18/GCB and Verde is shown in **Figure 1**. As expected, the co-extracted chlorophyll generated an extract with a deep green color. After cleanup, a majority of the green color was removed, with the extracts appearing yellowish in color. The Verde cleaned extract was slightly darker than the PSA/C18/GCB cleaned extract. Analysis of the extracts by GC/MS in full scan mode is shown in **Figure 2**. The data showed a similar peak pattern between uncleaned and cleaned extracts (both cleanups), but a difference in the amplitude of background peaks (indicated in shaded regions). The predominant peaks eluting in these regions are terpenes (earlier) and cannabinoids (later). Overall reduction in background was compared by summation of total peak area for each chromatogram. Compared to no cleanup, Verde was slightly better than PSA/C18/GCB (35% vs. 31% reduction in background). Specifically in the highlighted regions, Verde showed lower peak amplitudes.

Figure 1. QuEChERS extracts of cannabis before and after cleanup



Elution of cannabinioids. Using the optimized HPLC conditions described previously in the final LC-MS/MS analysis of the cannabis extracts, minimal overlap was observed between two of the major cannabinoids present in the samples and the later eluting pesticides. **Figure 3** shows an EIC of m/z 314.5, taken from a full scan LC/MS analysis of a cannabis extract compared to a TIC of the pesticides of interest in the analysis. The EIC represents the molecular ion of the two major cannabinoids detected in the sample extract; tetrahydrocannabinol and cannabidiol. As indicated, the last pesticide analyzed, pyridaben, eluted just before cannabidiol. The most abundant cannabinoid present, THC, eluted well after. Column flow could be switched to waste after elution of pyridaben, preventing some of the CBD and all of the THC from entering the MS. Other cannabinoids; specifically CBG, CBN, CBDA, CBC, CBGA, and THCAA, are known to elute after CBD on the RP-Amide phase. Thus, if present in the cannabis sample, all of these could also be diverted to waste as well.

Pesticide recoveries. The pesticides included in this study represented a majority of those on the OAR list. Two pesticides from this list, avermectin B1a and naled

Figure 3. EIC, m/z 314.5 from full scan analysis of cannabis extract (green), compared to a TIC of MRM analysis for pesticides (purple).

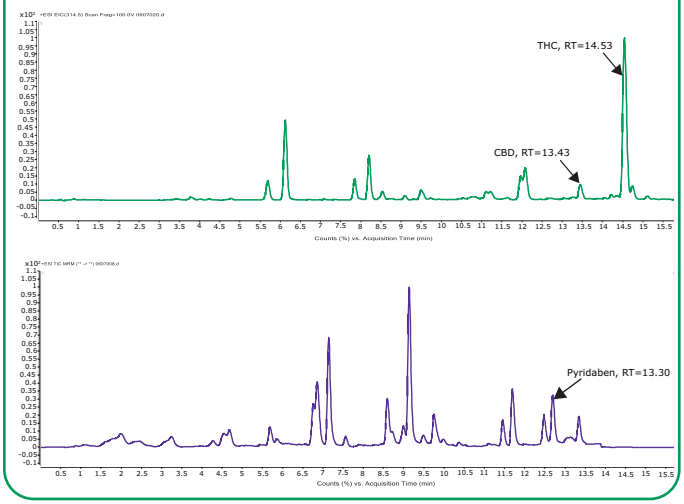
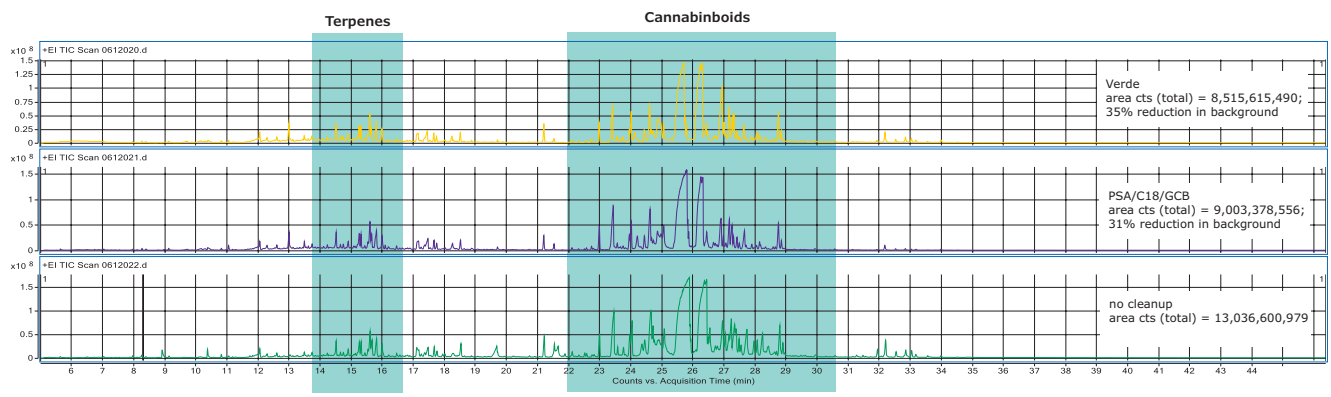


Figure 2. GC/MS scan comparisons of the cannabis extracts



were not analyzed due to lack of response. Avermectin is prone to sodium and potassium adduct formation. The presence of ammonium formate in the mobile phase should reduce this occurrence (as it is monitored as an ammonium adduct). However, even with these measures, others have also reported issues with low level detection of this compound.[4,5] Naled is susceptible to adsorption by PSA, and thus did not make it through the cleanup process with either sorbent mix.

Comparing spike data from the two cleanup methods (**Figure 4**) Supel™ QuE Verde exhibited better overall performance than PSA/C18/GCB. Several pesticides (**Table 3**), specifically bifenthrin, chlorantraniliprole, clofentezin, fenproximate, fludioxinil and hexythiazox showed notably better recoveries using Verde. Although none of these are completely planar in structure, it is possible that recovery was reduced using PSA/C18/GCB

Figure 4. Overall method performance for pesticides spiked at 50 ng/g in cannabis; Supel™ QuE Verde compared to PSA/C18/GCB.

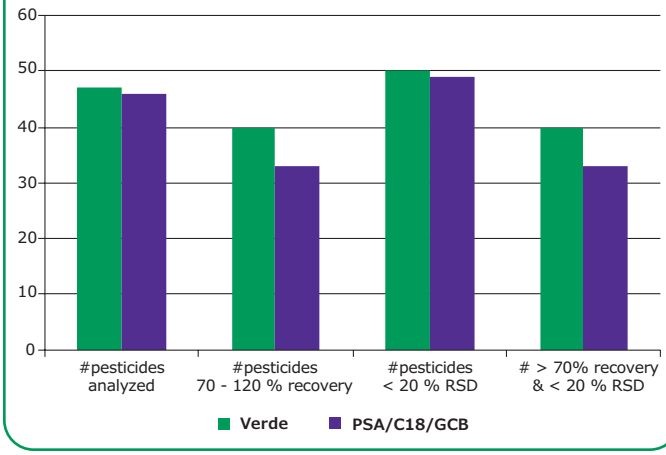


Table 3. Recovery and reproducibility summary; 50 ng/g spiked replicates

Cleanup:	Verde		PSA/C18/GCB		
	% Rec	% RSD	% Rec	% RSD	Analysis
Acephate	72%	12%	82%	6%	LC
Acetamiprid	87%	5%	86%	0.9%	LC
Aldicarb	85%	15%	82%	7%	LC
Azoxystrobin	90%	4%	88%	2%	LC
Bifenazate (D 2341)	50%	6%	50%	2%	LC
Bifenthrin*	74%	4%	57%	9%	GC
Boscalid (Nicobifen)	85%	3%	82%	5%	LC
Carbaryl	87%	7%	87%	2%	LC
Carbofuran	89%	5%	88%	2%	LC
Chlorantraniliprole	87%	6%	72%	1%	LC
Chlorfenapyr	72%	3%	69%	16%	GC
Chlorpyrifos*	87%	5%	71%	5%	GC
Clofentezin	77%	6%	58%	4%	LC
Cyfluthrin*	72%	6%	112%	11%	GC
Cypermethrin*	77%	16%	49%	25%	GC
Daminozide	4%	45%	3%	64%	LC
Diazinon*	92%	3%	88%	10%	GC
Dichlorvos	31%	23%	matrix		LC
Dimethoate	87%	4%	86%	0.4%	LC
Ethoprop (Ethoprophos)	82%	3%	81%	3%	LC
Etofenprox	59%	15	55	8	GC
Etoxazole	76%	2%	69%	2%	LC
Fenoxycarb	85%	6%	84%	2%	LC
Fenpyroximate(E)	74%	3%	59%	2%	LC
Fipronil*	94%	5%	86%	2%	GC
Flonicamid	86%	11%	88%	3%	LC
Fludioxonil	78%	5%	61%	12%	GC
Hexythiazox	72%	3%	63%	4%	LC

Cleanup:	Verde		PSA/C18/GCB		
	% Rec	% RSD	% Rec	% RSD	Analysis
Imazalil (Enilconazole)	49%	5%	66%	2%	LC
Imidacloprid	88%	4%	86%	2%	LC
Kresoxim methyl	84%	13%	79%	7%	LC
Malathion	84%	5%	73%	6%	LC
Metalaxyl	88%	4%	87%	2%	LC
Methiocarb (Mercaptodimethur)	88%	6%	82%	2%	LC
Methomyl	89%	5%	88%	2%	LC
MGK-264	81%	2%	75%	5%	GC
Myclobutanil	87%	3%	88%	2%	LC
Oxamyl	89%	4%	95%	2%	LC
Paclobutrazol	77%	3%	85%	0.5%	LC
Permethrin	54%	2%	58%	6%	GC
Phosmet (Imidan)	90%	5%	79%	7%	LC
Piperonyl butoxide	81%	5%	73%	2%	LC
Prallethrin	72%	10%	67%	7%	LC
Propiconazole	73%	7%	79%	7%	LC
Propoxur	89%	5%	87%	1%	LC
Pyrethrin	71%	6%	67%	29%	LC
Pyridaben	68%	5%	62%	1%	LC
Spinosyn A	42%	10%	42%	2%	LC
Spinosyn D	42%	9%	35%	19%	LC
Spirotetramat	75%	1%	76%	1%	LC
Spiromesifen*	80%	5%	61%	12%	GC
Spiroxamine	26%	1%	27%	3%	LC
Tebuconazole	67%	4%	80%	1%	LC
Thiacloprid	87%	5%	86%	1%	LC
Thiamethoxam	87%	5%	86%	3%	LC
Trifloxystrobin	85%	5%	79%	2%	LC

**See reference 4.*

due to hydrophobic retention on the GCB; which has a higher surface area than the carbon used in the Supel™ QuE Verde mix.

Several pesticides exhibited poor recoveries after both cleanup techniques:

- **bifenazate**: Recovery was around 50% after both cleanups. Bifenazate is susceptible to oxidation to bifenazate-diazine[8], which may have occurred to some degree during the extraction and cleanup process.
- **daminozide:** Very low recovery after both cleanups. This compound is a carboxylic acid, and is thus retained by PSA (present in both cleanups).
- **dichlorvos:** Matrix interference prevented analysis of this pesticide in the PSA/C18/GCB extracts. In the Supel™ QuE Verde cleaned extracts, the peak could be detected, but recoveries were low and variable. The low recovery using Verde is most likely due to retention on the Z-Sep+ portion of the sorbent. This same behavior has been observed in the past with this compound when using zirconia sorbents by both the author and others.[9]
- **etofenprox:** This is a very hydrophobic pesticide (log p= 7.1) and may exhibit poor extraction efficiency and/or retention by the C18 and Z-Sep+ portions of the cleanup sorbents (although less so on the later).
- **imazalil:** This is a relatively polar pesticide, which can be retained by PSA (present in both cleanups). Recovery issues have been observed by others with this compound when using Supel™ QuE Verde for cleanup as well as other zirconia containing sorbent mixtures.[9,10]
- **spinosyn A & D:** Lower recoveries of these large, macrocyclic lactones have been observed when using C18, carbon and zirconia containing sorbents.[11] In the case of zirconia, the use of citrate buffering in the QuEChERS extraction has been observed to increase recovery, possibly by displacement of the analytes from the zirconia.[9]
- **spiroxamine:** Recovery was very low, and about the same level after both cleanups. This could indicate an issue with extraction efficiency.

Conclusions

In the analysis of a majority of the pesticide list required by the state Oregon for cannabis, several recommendations can be made:

- QuEChERS extraction and cleanup can be used; and both LC-MS/MS and GC-MS/MS will be required for analysis.
- Cleanup using Supel™ QuE Verde can be substituted for PSA/C18/GCB. Both cleanups will reduce the green color of the extracts; however Verde was found to produce a slightly lower GC/MS background. Cannabinioids were co-extracted with the pesticides. Significant levels were still present after both cleanups, although slightly less after Verde. Pesticide recovery using Verde was found to be better overall, especially for several pesticides.
- Compared to C18, the Ascentis® RP-Amide column provided less overlap between the elution ranges of the targeted pesticides and the co-extracted cannabinoids. This separation would allow a switch to waste on the LC-MS/MS system after elution of the last pesticide, which would in turn prevent some of the cannabinoids from entering the MS.
- In the LC-MS/MS analysis, a high percentage of aqueous was necessary in the starting mobile phase conditions to increase retention of the more polar pesticides. As a result, injecting extracts in 100% organic resulted in distorted peak shapes for early eluting peaks. Switching from a 2.7 µm Ascentis® Express RP-Amide to a 3 µm Ascentis® RP-Amide (fully porous particle) improved these peak shapes.
- A guard column prior to the Ascentis® RP-Amide will further improve peak shapes when injecting 100% organic, and is recommended to prolong the life of the analytical column.

References

1. Oregon Health Authority, Oregon Medical Marijuana Program, **www.oregon.gov/oha**, accessed 6/7/17.
2. Exhibit A, Table 3. Pesticide Analytes and their action levels. *Oregon Administrative Rules 333-007-0400*; Oregon/gov/oha, effective 5/31/2017.
3. Chapter 6. Testing Laboratories. *Section 5719 Residual Pesticides;* Bureau of Cannabis Controls Text of Regulations, CA Code of Regulations, Title 16, Div. 42, pp 107-110.
4. Stenerson, K.; Halpenny, M. *Analysis of Pesticide Residues in Cannabis Using QuEChERS Extraction and Cleanup Followed by GC-MS/MS Analysis;* Supelco Reporter 34.1: 17-19.
5. Kowalski, J.; Dahl, J.H.; Rigdon, A.; Cochran, J.; Laine, D.; Fagras, G., Evaluation of Modified QuEChERS for Pesticide Analysis in Cannabis. *LCGC North America (supplement)* **2017,** *May,* 8-22.
6. Kinsella, B.; Telepchak, T.J.; Mackowsky, D.; Duncan, D.A.; Fanning, T., Pesticide and Mycotoxin Analysis: Mastering the Complexity of the Cannabis Matrix. *LCGC North America (supplement)* **2017,** *May,* 33-38.
7. Herrmann, S.S.; Andersen, G.; Poulsen, M.E., *Determination of pesticide residues in maize for livestock feed by GC-MS/MS and LC-MS/MS (QuEChERS method);* Validation Report 18; European Union Reference Laboratory, EURL for Cereals and Feeding stuff, National Food Institute Technical University of Denmark, December 2014.
8. *Analysis of Bifenazate (sum) by the QuEChERS Method using LC-MS/MS;* EURL-SRM Analytical Method Report, ver. 1, updated 3/17/2017.
9. Morris, B.D.; Schriner, R. B., Development of an automated column solid-phase extraction cleanup of QuEChERS extracts, using a zirconia-based sorbent, for pesticide residue analyses by LC-MS/MS. *J. Agric. Food Chem.* **2015**, *63*, 5107-5119.
10. Han, J.; Sapozhnikova, Y.; Matarria, J., Evaluation of a new carbon/zirconia-based sorbent for the cleanup of food extracts in multiclass analysis of pesticides and environmental contaminants. *J. Sep Science* **2016**, *39*, 4592-4602.
11. Han, L.; Sapozhnikova, Y.; Lehotay, S.J., Streamlined sample cleanup using combined dispersive solid-phase extraction and in-vial filtration for analysis of pesticides and environmental pollutants in shrimp. *Anal Chimica Acta* **2014**, *827*, 40-46.

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Heavy Metal Analysis of *Cannabis sativa* by ICP-MS and the Need for Proper Sample Homogenization

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Abstract

A step-by-step protocol for the analysis of various heavy metals in *Cannabis sativa* plant material by ICP-MS was developed. Cannabis is known to accumulate metals in various parts of the plant, such as seeds, leaves or stems, to a different extent. As a consequence, several protocols for the homogenization of cannabis buds were developed in order to prepare reproducible samples and analysis results. For comparison, cannabis buds were separated into seeds, stems, and leaves and the plant parts were subjected to ICP-MS.

Introduction

Cannabis is a plant genus that consists of three different species (*Cannabis sativa, indica* and *ruderalis*) and all of these are known to accumulate heavy metals in different parts of the plant (roots, leaves, seeds etc.). Due to this ability cannabis has been used for the remediation of contaminated soil (phytoremediation and phytoextraction).[1,2,3,4] On the other hand this inclination can hinder the use of cannabis in the food or medical industry. Therefore, all plant materials used in either food or pharma products, should be tested for their heavy metal content.

As of July 2020, 24 US states and Canada issued regulations for the testing of heavy metal content in cannabis, and all of them provided limits for arsenic, cadmium, lead and mercury metals (referred to as the "big four"). In addition, several states have set limits for one or more of these metals: chromium, barium, silver, selenium, antimony, copper, nickel and zinc.

Dried cannabis plant material is a very inhomogeneous matter and consists of leaves, buds including resin, stems of various thickness and seeds. All these plant parts accumulate heavy metals to a different extent. As it was shown in numerous studies, the heavy metal uptake depends on both the plant part and the element[1,5,6] In addition, uptake is influenced by external factors such as fertilization and liming,[1] ultimately causing an uneven distribution of metals throughout the plant. Hence, if the focus of studies is on the overall heavy metal content of cannabis, the material needs to be thoroughly homogenized before sample analysis. The recommended process for this sample breakdown is grinding. Various milling techniques exist, each differing in their technical complexity, such as mortar and pestle, rolling pin, knife mill, cutting mill, rotor mill, and ball mill.

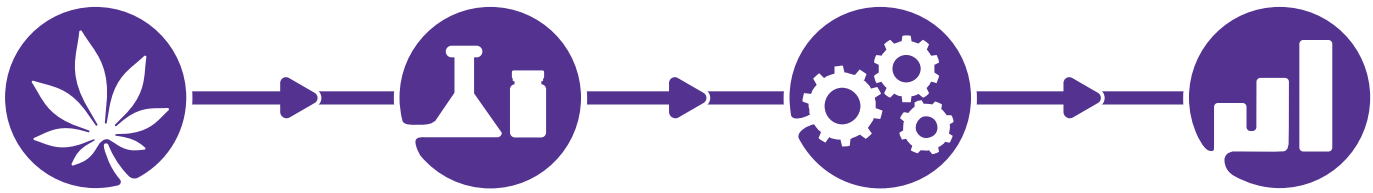
Experimental

In this paper, a four-step workflow was applied for the process of sample homogenization, standardization, sample digestion, and analysis.

The cannabis resins which have a sticky, smearing appearance make it necessary to always freeze samples prior to milling.

Freezing can either be accomplished at -20 °C in a freezer, by making use of dry ice (-78 °C) or by utilizing liquid nitrogen (-196 °C) as a cooling agent.

The setup of any grinder must be performed according to the target analytes. For the analysis of the big



four heavy metals, irrespective of the type of grinder chosen, stainless steel tools can be used. In contrast, if the abundance of additional metals such as Cr or Ni in a sample is of interest, knife mills with titanium blades or mills with grinding tools made of ZrO_2 or PTFE need to be selected. As these tools are normally smaller in size, the milling process is more time consuming.

The seeds of the cannabis species are the plant part that is most potent and is investigated in this homogenization study.

Four different grinding methods were applied for the milling of three *Cannabis sativa* hemp varieties. ICP-MS was used to determine the heavy metal content of the samples and based on the results the homogenization efficiency of all approaches was compared. In a second set of experiments, one hemp variety was separated into seeds, leaves, and stems, followed by ICP-MS analysis to identify possible variations of heavy metal concentrations in the different plant parts.

Sample Homogenization / Milling

Three different *Cannabis sativa* varieties: "Finola", "Felina" and "Santhica", were purchased from a drug store. All of them were qualified as industrial hemp per German regulation and were sold as "hemp flowers". The samples were obtained as 25 g batches of dried buds.

The experiments for the analysis of homogenization efficiencies of milling and grinding techniques were performed by applying four different methods, described below:

- Rolling pin (RP)
- Mortar and pestle (MP)
- Knife mill (KM)
- (Cryo) Ball mill (CM)

All four procedures are very different in terms of their speed, throughput, and ability to provide homogeneous samples in a reproducible way. In addition, using the knife mill can lead to an increase in sample temperature and hence in a change of sample composition. This needs to be considered, if samples are meant to be subjected not only to ICP-MS, but also to HPLC or GC analysis (e.g., for the determination of cannabinoid or terpene content). The detailed proceedings for each of the milling techniques applied are described in the following sections.

Rolling Pin

Approximately 10 g of dried buds were weighed into an airtight zip bag and cooled in a freezer at -20 °C for one hour. Subsequently the bag was placed on a hard support and the sample was ground using a wooden household rolling pin. After three to five minutes no further sample breakdown was observed and the process was stopped. Stems in the sample needed to be broken into shorter pieces of approximately 10 mm length by hand. An image of the final sample revealed rather large pieces of stem segments and undamaged seeds in an overall inhomogeneous material (**Figure 1**).

Figure 1. Hemp Buds "Finola" After Three Minutes Grinding Using a Wooden Rolling Pin.

Mortar and pestle

Approximately 10 g of aliquoted, dried buds were weighed into an airtight zip bag and cooled in a freezer at -20 °C for one hour. Then one or two buds were withdrawn from the bag, placed in a china mortar and ground for five minutes utilizing a porcelain pestle. The resulting coarse powder still contained large pieces of rigid stems, which needed to be broken by hand in order to obtain shorter pieces of approximately 10 mm length. The entire process was repeated, until a 10 g sample had been prepared. Rather large pieces of stem material were visible in the otherwise homogeneous, final sample.

Knife mill

For knife milling 18 g of hemp buds were placed in a mill equipped with titanium blades and ground for 30 seconds at 4,000 rpm (one cycle) or 10,000 rpm (two cycles), respectively. The 30 sec/4000 rpm method resulted in a sample that looked similar to that obtained with the rolling pin. In contrast, the 30 sec/10,000 rpm milling process generated a coarse powder comparable to ground coffee beans, with no stem segments or seeds visible.

(Cryo) Ball mill

One hemp bud (approx. 2.5 g) was manually broken into pieces and placed into a 50 mL stainless steel milling beaker. A 25 mm stainless steel milling ball was added and the sealed beaker was mounted to a cryo ball mill equipped with a liquid nitrogen filling system. The grinding parameters were as follows: Pre-cooling at 5 Hz, two cycles of 90 s at 30 Hz and 30 s at 5 Hz (for intermediate cooling). This process resulted in a very fine powder with a particle size of <100 µm (**Figure 2**).

Figure 2. Hemp Buds "Finola" after Grinding in a Cryo Ball Mill. Resulting Particle Size <100 µm.

For a second set of experiments, the hemp variety "Santhica" was manually separated into seeds, leaves, and stems to identify possible variations of heavy metal concentrations in the different plant parts (**Figure 3**).

These samples were directly subjected to digestion (without a preceding grinding step) and then analyzed by ICP-MS.

Figure 3. *Cannabis sativa* "Santhica" Separated into Leaves (top), Stems (middle) and Seeds (bottom).

Digestion of samples

All hemp samples were digested as follows: 50 (±1) mg of ground sample was weighed into a microwave vial and after addition of 3 mL nitric acid (65%), 1 mL hydrogen peroxide (30%) and a metal spiking solution, the sample was digested. After completion of digestion, the solution was quantitatively transferred into a 50 mL polypropylene tube, and 50 µL of indium internal standard solution was added. The final solution was then brought up to 50 mL final volume with ultrapure water. For detailed microwave digestion conditions please contact the author.

Preparation of standard solutions

In order to compensate for sample matrix effects, a standard addition approach utilizing various Certified Reference Material Heavy Metal Mix *Trace*CERT® standard solutions was applied for the preparation of all calibration curves. The final calibration curve covered a range from 1-20 µg/g total of heavy metals. The curve comprised of four data points; three standard addition solutions plus one unspiked sample solution. Alternatively, standard solutions were also prepared by using ICP Certipur® single element standards and ICP *Trace*CERT® single element standards (data not shown). For accuracy reasons, the composition of the addition solutions was adjusted to the heavy metal concentration in each of the three samples.

ICP-MS analysis

Analysis of samples, standards, and spikes was done by ICP-MS. For detailed conditions kindly contact the author.

The analysis was performed in the sequence: Blank, sample 1 – x, additions.

The resulting calibration curves for As, Cd, Hg, and Pb revealed excellent linearity over the entire calibration range, with r^2 values of > 0.9995 for all.

Addition solution for the determination of recovery rates

The recovery rates for the big four heavy metals are listed in **Table 1**. All recoveries were in the range of ±10%.

Table 1. Recovery rates for three hemp varieties (RP and MP grinding were applied to each sample) using a CRM Heavy Metal Mix *Trace*CERT® standard solution I, II or III (see details in products section).

	Cannabis Finola		Cannabis Santhica		Cannabis Felina	
Element	RP Mix I [%]	MP Mix I [%]	RP Mix II [%]	MP Mix II [%]	RP Mix III [%]	MP Mix III [%]
As	104	102	98	99	104	98
Cd	98	97	99	94	102	100
Hg	90	91	108	110	100	97
Pb	94	95	96	102	98	99

Results & discussion

Seed milling method investigation

The heavy metal content of the three hemp varieties that were subjected to the different milling processes are listed in **Table 2**.

The mercury content of all samples was below the limit of detection, and only one sample, out of the 14 samples displayed a cadmium level above the detection limit (0.1 µg/g). For arsenic the results were similar, with five samples containing As close to the LOD (0.1 µg/g). The findings for lead were a bit different, and the detected concentrations ranged from 0.3 to 1.0 µg/g. It is noteworthy, that these values do in part exceed the limits of various US states and Canada by a factor of 2-3 (depending on the intended use). The analysis of the "big four" elements was reproducible, and except for one deviation (Pb content of hemp variety "Felina" ground with MP) the milling technique did not affect the detected heavy metal concentrations.

Cryo milling was performed utilizing stainless steel equipment and resulted in the detection of elevated levels of chromium in all cryo-ground samples. Though nickel is also a content of stainless-steel alloys, increased amounts (compared to RP and MP milling) were only found in the "Finola" sample. This result can be attributed to the difference in grinding time. Some US states (as of now MI, MD, MO, NY) issued regulations, which make the analysis of chromium in cannabis necessary. In this case, it is essential to utilize a cryo milling approach and zirconia or PTFE grinding equipment in order to avoid sample contamination. In contrast to cryo milling, knife milling was performed (highlighted in **Table 2**) using titanium blades and therefore did not affect the Cr (and Ni) content of cannabis samples.

Plant part analysis

The heavy metal content of stems, seeds, and leaves of the hemp variety "Santhica" and the respective recovery rates are listed in **Table 3**. All results but the lead content of seeds are in line with the data shown in the previous section. This finding corresponds to results published in various publications, that also reported the Pb concentration in seeds being lower than in other plant parts such as leaves, stems, flowers, or roots.[5,6]

Table 2. Heavy Metal Content of three Hemp Varieties Determined by ICP-MS. Four different Grinding Procedures were Applied. Roman Figures Indicate the Use of a Specific CRM Heavy Metal Mix *Trace*CERT® Standard Solution (III to VIII) for the Preparation of Respective Addition Solutions.

	Cannabis Finola				Cannabis Santhica					Cannabis Felina				
Element	RP [µg/g]	MP [µg/g]	CM-VII [µg/g]	CM-VIII [µg/g]	RP [µg/g]	MP [µg/g]	KM [µg/g]	CM-V [µg/g]	CM-VI [µg/g]	RP [µg/g]	MP [µg/g]	KM [µg/g]	CM-III [µg/g]	CM-IV [µg/g]
As	0.1	0.1	0.1	0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	0.1	< 0.1	< 0.1	< 0.1	< 0.1
Cd	< 0.1	< 0.1	< 0.1	<0.1	< 0.1	0.1	<0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
Cr	0.3	0.4	**12.0**	**12.0**	0.2	0.3	0.3	3.6	2.5	0.3	0.5	0.6	3.7	4.3
Hg	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
Ni	0.8	1.1	**2.0**	**2.1**	0.4	1.7	0.4	0.7	0.6	0.5	0.6	0.6	0.7	0.7
Pb	0.6	0.7	0.7	0.7	0.3	0.5	0.5	0.4	0.4	0.5	1.0	0.5	0.5	0.5

Table 3. Heavy Metal Content of "Santhica" Stems, Seeds, and Leaves as Determined by ICP-MS (Duplicates) and Respective Recovery Rates (RR) using CRM Heavy Metal Mix *Trace*CERT® Standard Solution II. No Grinding was Performed prior to Digestion.

	Stems			Seeds			Leaves		
Element	#1 [µg/g]	#2 [µg/g]	RR [%]	#1 [µg/g]	#2 [µg/g]	RR [%]	#1 [µg/g]	#2 [µg/g]	RR [%]
As	< 0.1	< 0.1	99	< 0.1	< 0.1	96	< 0.1	< 0.1	99
Cd	< 0.1	<0.1	100	< 0.1	< 0.1	98	< 0.1	<0.1	102
Hg	< 0.1	< 0.1	96	< 0.1	< 0.1	103	< 0.1	< 0.1	100
Pb	0.4	0.4	97	< 0.1	< 0.1	101	0.4	0.4	99

Conclusion

This work demonstrates a comprehensive ICP-MS workflow, using the standard addition calibration method, for the determination of heavy metals in *Cannabis sativa hemp* variety plant materials. Critical elements in the process include homogenization of samples and use of accurate traceable Certified Reference Material mixes, that are tailored to state specific regulations for heavy metals in cannabis. Reproducible samples were prepared by grinding cannabis with different mill types and techniques. Samples were then digested utilizing a specific digestion protocol, optimized to provide clear digestion solutions. The resulting solutions were subjected to ICP-MS analysis. Calibration data was obtained by the preparation and analysis of standard addition solutions obtained by diluting various different heavy metal CRM mixes containing arsenic, cadmium, lead and mercury. The final results were consistent for all samples and revealed an As, Cd, and Hg concentration of <0.1 to 0.1 µg/g. The detected lead content of the three cannabis varieties ranged from 0.3 to 1.0 µg/g.

Literature

1. M. Griga, M. Bjelkova (2013) Flax (Linum usitatissimum L.) and Hemp (Cannabis sativa L.) as Fibre Crops for Phytoextraction of Heavy Metals: Biological, Agro-technological and Economical Point of View. In: D.K. Gupta (ed) Plant-Based Remediation Processes. Springer, Berlin.
2. P. Linger, A. Ostwald, J. Haensler, Biol. Plant. 49 (2005) 567–576.
3. R. Ahmad, Z. Tehsin, S.T. Malik, S.A. Asad, M. Shahzad, M. Bilal, M.M. Shah, S.A. Khan, Clean Soil Air Water 44 (2016) 195-201.
4. G. Shi, C. Liu, M. Cai, Y. Ma, Q. Cai, Appl. Biochem. Biotechnol. 168 (2012) 163-173.
5. V. Angelova, R. Ivanova, V. Delibaltova, K. Ivanov, Ind. Crops Prod. 19 (2004) 197–205.
6. L.O. Eboh, B.E. Thomas, Pak. J. Nutr. 4 (2005) 349–351.

Featured products

Description	Cat. No.
Certified Reference Materials (CRMs)	
Single element standards for ICP Certipur® 1000 mg/L	
Arsenic	**1.70303**
Cadmium	**1.70309**
Indium	**1.70324**
Lead	**1.70328**
Mercury	**1.70333**
Single element standards for ICP TraceCERT® 1000 mg/L	
Arsenic	**01969**
Cadmium	**36379**
Indium	**00734**
Lead	**41318**
Mercury	**28941**
State specific heavy metals CRM mixes TraceCERT®, 100 mL each	
Heavy metal mix I Arkansas, (As 20, Cd 20, Hg 10, Pb 50) mg/L	**94846**
Heavy metal mix II California, (As 15, Cd 5, Hg 30, Pb 5) mg/L	**94794**
Heavy metal mix III Colorado, (As 40, Cd 40, Hg 20, Pb 100) mg/L	**94772**
Heavy metal mix IV Nevada and Washington, (As 200, Cd 82, Hg 40, Pb 120) mg/L	**95094**
Heavy metal mix V Connecticut and New Mexico, (As 14, Cd 9, Hg 29, Pb 29) mg/L	**95117**
Heavy metal mix VI Vermont, (As 100, Cd 41, Hg 20, Pb 100) mg/L	**04295**
Heavy metal mix VII Pennsylvania, (As 15, Cd 3, Hg 5, Pb 10) mg/L	**03056**
Heavy metal mix VIII New Hampshire, (As 5, Cd 3, Hg 9, Pb 9) mg/L	**95562**
Heavy metal mix IX (As, Cd, Hg, Pb all 100 mg/L each)	**89471**
Heavy metal mix according to USP <561> articles of botanical origin, (As 20, Cd 5, Hg 10, Pb 50) mg/L	**18208**
Metal mix I for cannabis testing (As, Hg, Cd, Pb, Cr, Ba, Ag, Se, Sb, Cu, Ni, Zn, all 100 mg/L each)	**91539**
Solvents, reagents and acids	
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Description	Cat. No.
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IKA® MF10 basic microfine grinder drive, 230 V	**Z645176**
IKA® MF 10.1 cutting-grinding head	**Z645249**
IKA® MF 10.2 impact grinding head	**Z645257**
IIKA® M 20 universal mill, 115 V	**Z645133**
IKA® M 20 universal mill, 230 V	**Z645141**
IKA® A11 basic analytical mill	**Z341789**
Agate mortar and pestle	**Z409111**
Porcelain mortar and pestle	**Z247499**
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Centrifuge tubes polypropylene 50 mL	**T2193**
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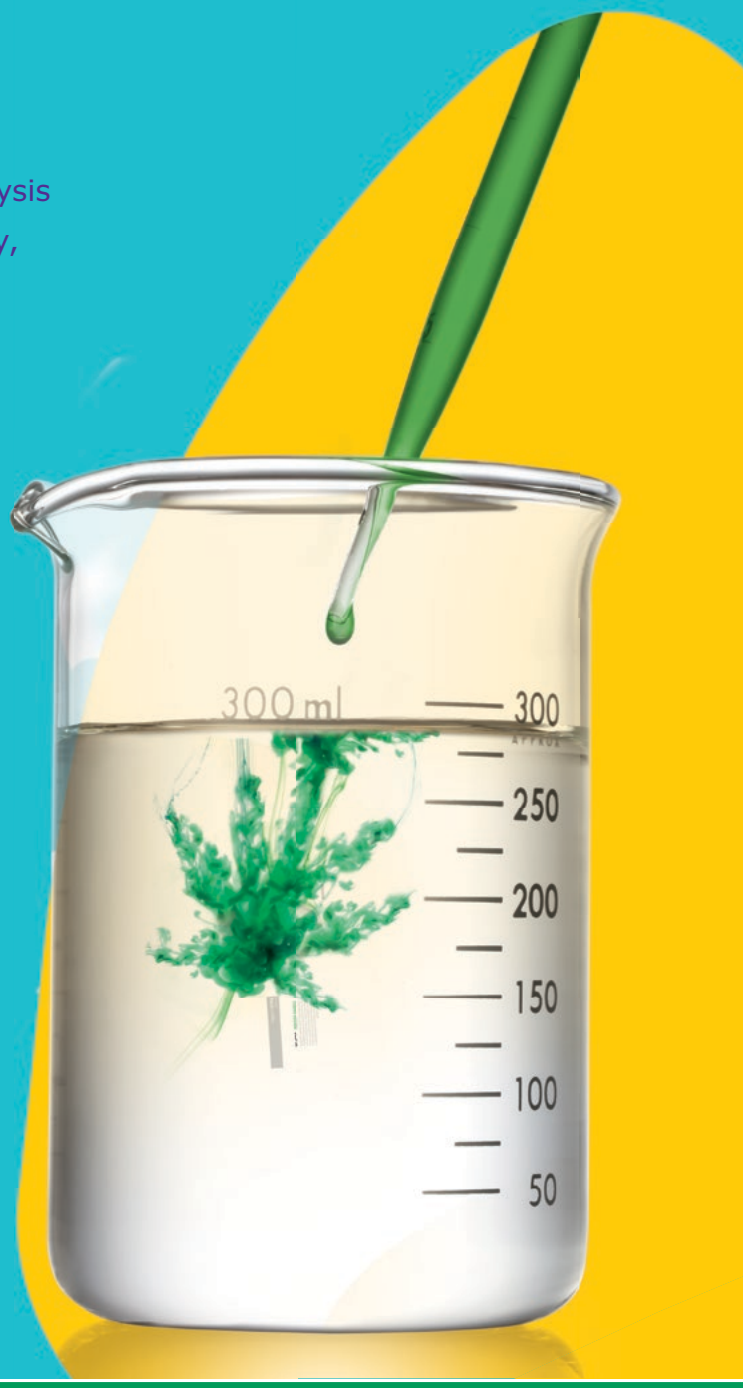

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