DRUG FORMULATIONS AND CLINICAL METHODS

Development and Validation of a Reliable and Robust Method for the Analysis of Cannabinoids and Terpenes in Cannabis

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The requirements for an acceptable cannabis assay have changed dramatically over the years resulting in a large number of laboratories using a diverse array of analytical methodologies that have not been properly validated. Due to the lack of sufficiently validated methods, we conducted a single-laboratory validation study for the determination of cannabinoids and terpenes in a variety of commonly occurring cultivars. The procedure involves high-throughput homogenization to prepare sample extract, which is then profiled for cannabinoids and terpenes by HPLC-diode array detector and GC-flame ionization detector, respectively. Spike recovery studies for terpenes in the range of 0.03–1.5% were carried out with analytical standards, while recovery studies for Δ^9-tetrahydrocannabinolic acid, cannabidiolic acid, Δ^9-tetrahydrocannabivarinic acid, and cannabigerolic acid and their neutral counterparts in the range of 0.3–35% were carried out using cannabis extracts. In general, accuracy at all levels was within 5%, and RSDs were less than 3%. The interday and intraday repeatabilities of the procedure were evaluated with five different cultivars of varying chemotype, again resulting in acceptable RSDs. As an example of the application of this assay, it was used to illustrate the variability seen in cannabis coming from very advanced indoor cultivation operations.

The requirements for an acceptable cannabis assay have changed dramatically over the years. Historically the focus was the quantification of Δ^9-tetrahydrocannabinol (THC; 1), the main biologically active metabolite; however, intensive research over the past few decades has identified over 150 different cannabinoids (2). Pharmacological activities for a number of these, including Δ^9-tetrahydrocannabinolic acid (THCA), cannabidiol (CBD), cannabidiolic acid (CBDA), cannabigerol (CBG), cannabigerolic acid (CBGA), Δ^9-tetrahydrocannabivarin (THCV), Δ^9-tetrahydrocannabivarinic acid (THCVA), cannabidivarin (CBDV), and cannabidivarinic acid (CBDVA; 3), have been reported. Thus, quantification of these other cannabinoids is also important to understanding the pharmacological properties of cannabis. As of 2014 there have been over 545 constituents identified in cannabis (3), and apart from the cannabinoids, the 140 terpenoids (4) have garnered interest due to their organoleptic properties (4, 5), potential for chemically fingerprinting different cultivars (6–10), and putative synergistic interactions between the cannabinoids and the terpenoids (11, 12).

Of the 140 terpenoids identified in cannabis, our own qualitative GC/MS surveys of California landraces, as well as analytical results from other laboratories (13, 14), published studies on the terpene profiles of cannabis cultivars (7, 9, 10), and reviews of the biological activities of terpenes (11, 12) suggest there are approximately 17 that are the most common and can be used for examining the phenotypic and/or biological properties of cannabis cultivars. Publications documenting the chemotypic profiles of various cultivars (9, 10, 15–17), analytical results obtained within our laboratory, and analytical results posted by other cannabis testing laboratories (13, 14) also provide evidence that typical concentration ranges for the cannabinoids are from 0.1 to 40% of inflorescence dry weight and terpenoids range from 0.01 to 1.5% of inflorescence dry weight. Although there are certainly other classes of metabolites present in cannabis (2, 4), the above references as well as a survey of services offered by analytical laboratories that test cannabis (13, 14, 18) suggest much of the current focus lies with the cannabinoids and terpenoids. Thus, an economical, robust, and validated method for profiling all of these analytes over their observed ranges of concentration is needed.

As the number of states considering legalization of cannabis, medical or otherwise, is growing rapidly, the number of cannabis testing laboratories has also increased to keep up with demand; however, it is not always clear what standards they are held to, and this has resulted in a number of nonstandardized and nonvalidated methods being used (1, 19). While there may be a number of methods suitable for the analysis of cannabis, it is crucial for laboratories to perform rudimentary assay validation to demonstrate the assay is fit for its intended purpose. This is an absolute requirement for the confident use of any methodology, and the International Conference on Harmonization (ICH), United Nations Office on Drugs and Crime (UNDOC), and AOAC provide a number of guidance documents for this purpose (20–22). Based on the aforementioned considerations and the lack of a method that was validated for the analytes and concentration ranges of interest, we sought to develop an efficient and robust assay that covered the typical repertoire of analytes and working ranges mentioned above.

A number of publications have been released over the years (10, 15, 16, 23) that have presented validated assays for several analytes present in cannabis. These validations were suitable for the stated purposes of the studies; however, they

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have some limitations with respect to current goals. There are a number of methods for preparing cannabis and extracting the analytes (24); however, sonication is the most common and is the method recommended by both UNDOC and the American Herbal Pharmacopoeia (AHP) for the analysis of cannabis (1, 25). Methods in the AHP monograph, as well as those used by DeBacker et al. (15, 17) and Swift et al. (16) recommend drying and powdering the sample first; however, this would require a separate moisture determination to accurately assess the content of the initial inflorescence. This process would also alter the native content of the volatile terpenes, thus precluding their determination along with the cannabinoids from a single sample preparation. The above methods also recommend sonication for extraction of the analytes. This process can be less efficient with solid samples or large particle sizes, such as bulk plant material (26), and conditions optimized for single samples can result in loss of precision and accuracy when processing multiple samples at once (as is typical in most laboratories) due to variability in power across the bath that depends on sample depth, placement, and number of samples in the bath (26, 27). Using a probe for disruption minimizes variability, but also decreases throughput while increasing the possibility for cross-contamination by the probe (26). The shake flask method used by Fischedick et al. (10) avoids many of these issues, but it uses a relatively large volume of solvent (100 mL/1 g of sample) and requires multiple extractions. We decided to evaluate high throughput homogenization (HTH) of typical inflorescences as a single sample preparation method for the analysis of both terpenoids and cannabinoids. High-throughput homogenizers have been used for homogenizing microorganisms, plant tissues, and animal tissues and have been successful in relieving bottlenecks in a number of high-throughput screening strategies (26, 28).

The two most common instrumental methods for analysis of cannabinoids are GC-flame ionization detector (FID) and HPLC-UV (1, 24, 25); however, HPLC has been established as the method most suited for cannabinoid analysis of the native composition of the plant. The cannabinoids are biosynthesized as carboxylic acids; however, both heat, as applied from an ignition source or a GC injector, and time decarboxylate the carboxylic acids and convert them to their biologically active forms (15, 23, 24). Derivitization as the trimethylsilyl ethers prevents this decarboxylation (1, 24), but this requires extra sample processing steps. Furthermore, the conversion processes in the injector of a GC has been shown to be highly dependent on instrument configuration and is incomplete (29). The most accurate manner to assay the native composition of the inflorescence is to use a method that does not involve thermal stress, such as HPLC (15, 17, 23). DeBacker et al. (15) used HPLC to achieve separation of all the analytes of interest; however, it was a 36 min run and used mobile phases that involved buffer systems, which are more inconvenient to work with in typical settings. We sought to develop an HPLC method that used simple mobile phases, provided shorter run times, and adequately resolved all analytes of interest.

Although HPLC is the method of choice for cannabinoids, it is fairly well established that GC is the method of choice for small volatile organics such as the terpenoids (30, 31). Additionally, the large linear range of the FID makes it possible to cover the wide range of terpene concentrations (approximately 0.01 to 1.5%) with a single injection. A number of published methods (9, 10) document GC-FID methods that are very applicable to the analysis of terpenes in cannabis; however, since these methods also determined cannabinoid content, the instrument cycle times were over 1 h. We sought to develop a method that resolved all the analytes of interest with a shorter instrument cycle time.

When it comes to method validation, guidance states that methods must be validated for the analytes of interest at high, medium, and low concentrations that cover the desired working range (21). While the studies cited above were clearly sufficient for the stated purposes, they do not cover the desired analytes or concentration ranges of interest in the current study. For instance in the studies of Fischedick et al. (10) THCA was validated at concentrations of 19, 22, and 26% by weight and CBD at concentrations of 6, 7, and 8% by weight, and this does not cover the ranges or analytes mentioned above. DeBacker et al. (15) were able to validate all major analytes of interest by spiking cannabis extract into nettle; however, this covered concentration ranges, by weight, of 1.8–6.1% for THCA, 1.0–3.3% for CBDA, and 0.1–0.4% for CBGA. These levels reflect only a portion of the analytes and concentrations of interest in current research endeavors, as noted above.

A key contribution by DeBacker et al. (15) was to set a precedent for using cannabis extracts for validation studies. Ideally, the accuracy and precision of a method are assessed using certified reference materials (CRMs; 20–22); however, as noted by UNDOC (21), CRMs are rarely available for drugs of abuse. This is especially true in the United States where it is a legal impossibility for most laboratories to obtain cannabinoid standards for this process. This is even more problematic when concentrations of the analytes in the matrix can reach 40% by weight, as noted in the references above, and require large amounts of material. As stated in guidance from AOAC, sometimes an impure form of the analyte must be used until a pure form becomes available (22), and this is the current status of the cannabinoids in the United States. Due to legal restrictions on standards, the lack of official methods for comparison, and the lack of validation samples, we sought to expand the methods of DeBacker et al. (15) to more analytes and wider working ranges so any laboratory in the United States can validate their methodology for the analytes and ranges mentioned above.

Based on the previous discussion, this work sought to develop an efficient and relatively rapid method for determining both cannabinoids and terpenoids in cannabis samples from a single sample preparation, and to use spike recoveries to validate typical performance characteristics of the method (20–22). We sought to verify the specificity for the above-mentioned analytes, the applicability to the desired working ranges and different matrixes, and the reliability, expressed in terms of accuracy and reproducibility, by analyzing blanks spiked at three different concentrations covering the working range. We also sought to verify intermediate precision of the assay by analyzing replicates of five different authentic samples on the same day and on 5 consecutive days.

Experimental

Internal Standards, Extraction Solution, and Diluent

Denatured ethanol, nonane, 4-biphenyl carboxylic acid, and ibuprofen were obtained from Sigma- Aldrich (St. Louis, MO). The Certificates of Analyses for each lot were used determine the purity, and mass values were adjusted accordingly. The diluent was reagent grade ethanol. To prepare typical extraction solution with both 4-biphenyl carboxylic acid (BPCA) and nonane as the internal standard (ISTD), each was added to a volumetric flask that was brought up to volume with reagent grade ethanol and stirred for 3 h to give a solution that contained 0.1 mg/mL nonane and 2 mg/mL BPCA. The peak area of BPCA in new batches of extraction solution was verified to be within 2% of previous batches by diluting 1:6 and injecting into the HPLC system in triplicate. To prepare spike solution with both nonane and ibuprofen (IBU) as internal standards, each was added to a volumetric flask and it was brought up to volume with reagent grade ethanol to give a solution that contained 1.5 mg/mL nonane and 288 mg/mL IBU.

HTH Optimization

A 1600 Mini-G (OPS Diagnostics, Lebanon, NJ) was used for HTH. This process was optimized for solvent volume, sample mass, homogenization time, bead material, and bead volume. Optimized parameters were compared to the procedure used for the analysis of samples submitted to the University of Mississippi by the U.S. Drug Enforcement Administration (DEA) and recommended in the AHP monograph (1) as well as to the procedure recommended by UNDOC for the analysis of cannabis (25). Two cultivars, a Type I and Type II, were compared to verify equivalence. Sonication was then compared to the HTH procedure under typical sample loads and solvent usage with five separate cultivars.

The optimized extraction procedure involved pregrinding 5 to 7 g of bulk flower samples in a stainless steel coffee grinder. From this material, 1000 mg (±20 mg) was placed in a 50 mL BD Falcon polypropylene centrifuge tube (Amazon.com) with 1 mL 2.0 mm zirconia beads. To the tube was added 15.0 mL extraction solvent, via a solvent dispenser gravimetrically calibrated to deliver 15 mL ethanol, and the tubes were homogenized at 1500 rpm for 6 min. An aliquot was removed, placed in a 2.0 mL centrifuge tube, and centrifuged at 10 000 rpm for 5 min. An aliquot of the supernatant was removed and placed in a GC vial for terpene analysis. Another aliquot was placed in an HPLC vial and diluted 6-fold with diluent for quantification of the minor cannabinoids, while another was placed in a separate HPLC vial and diluted 96-fold with diluent for quantification of the major cannabinoids. It should be noted that pregrinding samples is only required for obtaining representative samples from large bulk sample masses. If the sample size is small enough, it can be placed directly in the Falcon tube for extraction.

Method Validation

The method was validated with respect to selectivity, linearity, accuracy (recovery and percentage relative bias), and repeatability precision (RSD_r). Guidelines from AOAC INTERNATIONAL were followed (22); however, guidelines from the ICH (20) and UNDOC (21) are all similar. Selectivity was verified by determining retention times of standards. Linearity was verified by using both the correlation coefficients and the residuals of the fitted calibration curves to verify goodness of fit. Validation samples were prepared by spiking blank matrix with known amounts of terpene standards or cannabinoid extracts. Accuracy (recovery) and RSD_r were determined by extracting the validation samples and comparing the results to those obtained from extraction solution (no matrix present) that had been spiked with equivalent amounts of standards. This was done at three different concentrations (low, medium, and high) covering the working range in replicate ($n = 5$). Accuracy is reported as recovery, and acceptable limits are concentration dependent and given by AOAC (22). RSD_r is determined from the RSD of the absolute measurements, and acceptable values are concentration-dependent and given by AOAC and Equation 3 in the *Calculations and Reporting* section below (22). Trueness, or bias under the reported conditions, is the difference between the accepted value and the average reported value (22). In this case, the trueness can be estimated as a byproduct of the recovery determinations since the true value of the validation samples is known from gravimetrically preparing them with standards or extracts. Trueness is expressed in terms of relative bias.

GC-FID Assay for the Terpenes

The terpenes were separated on a PerkinElmer (Waltham, MA) Clarus 680 GC instrument fitted with an FID detector, Elite 5MS column, and Restek Corp. (Bellefonte, PA) Precision SkyLiner. The injector temperature was set at 230°C, a 1.5 µL injection volume was used, and the split flow was set at 20:1. The carrier gas was hydrogen set at a flow rate of 1.3 mL/min, and the oven temperature program was a 3.5 min hold at 60°C, a ramp to 155°C at 3.5°C/min, and a ramp to 300°C at 30°C/min.

Terpene standards were obtained from Sigma-Aldrich, and the Certificates of Analyses were used to correct mass values. Calibration curves were prepared gravimetrically in diluent solution at concentrations of 1.000, 0.815, 0.655, 0.495, 0.335, 0.175, and 0.015 mg/mL and an internal standard (nonane) concentration of 0.1 mg/mL. The instrument was calibrated according to the manufacturer's procedures. The calibration curves were obtained in triplicate on separate days. In addition to requiring correlation coefficients greater than 0.99, the residuals were evaluated to verify the quality of the fit. As is typical with standard curve fitting procedures, the residuals should show a random distribution with a mean close to zero (2, 32). For laboratories that do not have statistical software, the residuals can be evaluated by calculating the difference of the experimental points from the fitted line and plotting these differences as a function of concentration.

HPLC-Diode Array Detector (DAD) Assay of the Cannabinoids

The assay was run on a 1290 HPLC system equipped with a G4212A DAD, G1316C temperature-controlled column compartment, G4226A autosampler, and G4204A quaternary pump. Separation of the cannabinoids was achieved on a Poroshell 120 EC-C18 column (2.7 µm, 150 × 2.1 mm id,

Part No. 693775-902) with a Poroshell 120 EC-C18 guard column (2.7 µm, 5 × 2.1 mm id, Part No. 821725-911) in place (Agilent Technologies, Santa Clara, CA). Instrument control, data acquisition, and integration were achieved with OpenLab CDS ChemStation Rev C.01.06[61] software (Agilent Technologies). The HPLC method used a 1.5 µL injection volume for all calibration standards, check standards, and sample analyses. Full spectra were recorded from 200 to 400 nm, and 214 nm was used for quantification of all analytes.

Mobile phases consisted of 0.1% formic acid (Sigma-Aldrich Part No. 56302-50ML-GL) in HPLC grade water (Sigma-Aldrich Chromasolv® Part No. 270733-4L) on the A side and 0.1% formic acid in HPLC grade acetonitrile (Sigma-Aldrich Chromasolv Part No. 34851-4L) on the B side. The flow rate was 0.5 mL/min, and the assay began with an 8 min isocratic hold at 66% B, followed by a linear gradient to 95% B over 4 min; 95% B was maintained for 1 min, then the column was re-equilibrated at 66% B for 4 min before the next injection. The total run time for the method was 17 min.

Cannabinoid standards for THCA, CBDA, THC, CBD, CBG, cannabichromene (CBC), Δ-8 THC, and cannabinol (CBN) were obtained from Restek Corp. as 1.0 mg/mL solutions in methanol. Calibration solutions for the acidic and neutral forms were prepared separately. Admittedly, the following procedure is generally not recommended for handling analytical standards, but due to legal issues dictating how these standards are supplied it is the most practical manner to combine multiple cannabinoid standards into a single solution with an internal standard. To prepare the calibration solutions, 1000 µL of each was placed in a small amber vial and the solvent was evaporated under a gentle flow of argon, after which the vial was placed under gentle vacuum until the theoretical weight (1 ± 0.1 mg) was obtained. The residues were dissolved in a total of 4000 µL diluent to give a stock cannabinoid solution of 0.250 mg/mL with 0.2 mg/mL IBU as the ISTD. The stock solutions of the neutral and acidic moieties were then diluted to concentrations of 0.250, 0.125, 0.063, 0.031, and 0.016 mg/mL.

Each set of calibration curves was obtained in triplicate on separate days, and the calibrations for the acids and neutrals were merged into a single instrumental method within the ChemStation software. The single raw data set was processed to obtain both ISTD calibration curves that referenced IBU as the ISTD and external standard (ESTD) calibration curves. In addition to requiring correlation coefficients greater than 0.99, the residuals were evaluated to verify the quality of the fit. As is typical with standard curve fitting procedures, the residuals should show a random distribution with a mean close to zero (2, 32). For laboratories that do not have statistical software, the residuals can be evaluated by calculating the difference of the experimental points from the fitted line and plotting these differences as a function of concentration. This is a critical process for the cannabinoid calibration curves since the "true" values of the validation samples are determined empirically from the curves, and this helps ensure they are not biased by nonlinearity of the calibration curves.

Guidance states that an impure specimen can serve as the reference standard if CRMs are not available, and there are cases where the complete characterization of products of natural origin is not possible (22). This is the case for CBGA, THCVA, and CBDVA, as well as the neutral counterparts of the propyl analogs, which are not easily obtained due to legal restrictions. For tentative identification of retention times for these analytes, cultivars purported to be rich in them were obtained and analyzed by GC/MS. The major peaks for these analytes had retention times that were similar to those published with authentic materials (1, 33) and had good correlation with mass spectral libraries. The same extracts were analyzed in both heated and unheated forms, and clear correlations could be seen between GC/MS and HPLC-UV peak areas and peak ratios, and elution orders were similar to those published with authentic materials (1, 34). Based on known similarities of spectral properties and molar absorption coefficients (35), these analytes were then quantified by referencing known calibration curves. CBGA and CBDVA referenced CBDA, THCVA referenced THCA, CBDV referenced CBD, and THCV referenced THC. These analytes are labeled with TI to stress the fact this was a tentative identification. During the preparation of this manuscript, standards for CBDV, THCV, and CBGA became available (Cerilliant, Round Rock, TX) and were used to verify the retention times and calibrate the instruments.

Terpene standards were also injected to verify there were no interferences from these analytes.

LOQ

Due to the impossibility of obtaining true blanks for all of the analytes, the LOQs were estimated from the calibration curves of the analytes and then verified experimentally as described in ICH Q2B (20).

Matrix Blanks

Since it is not possible to obtain cannabis that is devoid of terpenes and cannabinoids, blank matrixes were prepared by homogenizing cannabis samples in a stainless steel coffee grinder followed by repeated sonication of the bulk plant material in pentane and filtration. This material was then placed under vacuum overnight to remove traces of solvent. Extraction and analysis according to the current procedure indicated the complete absence of terpenes and residual amounts of the cannabinoids. The residual cannabinoid background concentrations (THCA 0.009, THC 0.001, CBDA 0.004, and CBGA 0.044%) were subtracted from those obtained in the spike recovery experiments.

Terpene Validation Samples

Although guidance recommends spiking each analytical standard into a blank matrix (20–22), 17 terpenes at high, medium, and low concentrations in replicate (n = 5) would require over 250 separate evaluations. To expedite this process, three separate solutions containing all the terpene analytical standards were made up gravimetrically in volumetric flasks at concentrations of 5 mg/mL in extraction solution.

To prepare the validation samples, each of these concentrated solutions were then used to volumetrically spike all the terpenes simultaneously into a conical vial containing approximately 1000 mg blank cannabis at target levels of 0.025, 0.175, and 1.500% by weight in the blank matrix. For instance, spiking 996 mg blank matrix with 50 µL introduced 0.25 mg of each terpene, spiking 970 mg with 350 µL introduced 1.75 mg of

Table 1. Terpene spikes, recovery, repeatability precision, and relative bias

	Analyte introduced, mg	True Wt%/ 1000 mg	RSD_r, %	Acceptable RSD_r, %	Accuracy (recovery), %	Recovery limits, %	Relative bias, %
Terpenoid				Low (n = 5)			
α-Pinene	0.25	0.025	0.35	3.5	102	85–110	2.16
Camphene	0.25	0.025	0.38	3.5	101	85–110	5.20
β-Pinene	0.25	0.025	0.52	3.5	103	85–110	2.56
Myrcene	0.25	0.025	0.73	3.5	104	85–110	5.52
α-Phellandrene	0.25	0.025	0.62	3.5	98	85–110	–2.16
Carene	0.25	0.025	0.45	3.5	101	85–110	1.04
α-Terpinene	0.25	0.025	0.82	3.5	96	85–110	–11.84
Limonene	0.25	0.025	0.60	3.5	101	85–110	1.36
β-Ocimene	0.25	0.025	0.52	3.5	100	85–110	0.48
γ-Terpinene	0.25	0.025	0.67	3.5	102	85–110	–2.32
Terpinolene	0.25	0.025	0.42	3.5	96	85–110	–4.00
Linalool	0.25	0.025	0.45	3.5	103	85–110	1.84
Fenchol	0.25	0.025	1.06	3.5	102	85–110	–1.76
α-Terpineol	0.25	0.025	1.16	3.5	101	85–110	0.80
β-Caryophyllene	0.25	0.025	0.33	3.5	105	85–110	0.72
α-Humulene	0.25	0.025	0.59	3.5	102	85–110	2.16
Caryophyllene oxide	0.25	0.025	0.49	3.5	102	85–110	–6.56
Terpenoid				Medium (n = 5)			
α-Pinene	1.75	0.175	0.06	2.6	100	90–108	2.26
Camphene	1.75	0.175	0.15	2.6	100	90–108	0.53
β-Pinene	1.75	0.175	0.20	2.6	101	90–108	1.84
Myrcene	1.75	0.175	0.32	2.6	101	90–108	3.54
α-Phellandrene	1.75	0.175	0.92	2.6	98	90–108	1.99
Carene	1.75	0.175	0.28	2.6	101	90–108	3.25
α-Terpinene	1.75	0.175	3.02	2.6	94	90–108	–5.49
Limonene	1.75	0.175	0.32	2.6	101	90–108	3.73
β-Ocimene	1.75	0.175	0.35	2.6	101	90–108	4.19
γ-Terpinene	1.75	0.175	0.34	2.6	101	90–108	4.65
Terpinolene	1.75	0.175	1.21	2.6	97	90–108	1.19
Linalool	1.75	0.175	0.60	2.6	103	90–108	1.58
Fenchol	1.75	0.175	0.58	2.6	103	90–108	1.25
α-Terpineol	1.75	0.175	0.66	2.6	104	90–108	2.11
β-Caryophyllene	1.75	0.175	0.53	2.6	103	90–108	2.33
α-Humulene	1.75	0.175	0.59	2.6	103	90–108	1.99
Caryophyllene oxide	1.75	0.175	0.59	2.6	103	90–108	2.02
Terpenoid				High (n = 5)			
α-Pinene	15	1.500	0.18	1.9	100	92–105	–2.71
Camphene	15	1.500	0.45	1.9	100	92–105	1.21
β-Pinene	15	1.500	0.47	1.9	101	92–105	–3.21
Myrcene	15	1.500	0.51	1.9	101	92–105	–1.88
α-Phellandrene	15	1.500	0.41	1.9	99	92–105	1.44
Carene	15	1.500	0.35	1.9	101	92–105	–4.60
α-Terpinene	15	1.500	0.29	1.9	98	92–105	–3.57
Limonene	15	1.500	0.40	1.9	101	92–105	–2.21
β-Ocimene	15	1.500	0.28	1.9	100	92–105	–3.47
γ-Terpinene	15	1.500	0.39	1.9	100	92–105	–2.13
Terpinolene	15	1.500	0.21	1.9	99	92–105	–5.31
Linalool	15	1.500	0.33	1.9	101	92–105	–4.88
Fenchol	15	1.500	0.46	1.9	101	92–105	–3.81
α-Terpineol	15	1.500	0.54	1.9	102	92–105	–1.60
β-Caryophyllene	15	1.500	0.53	1.9	101	92–105	–5.13
α-Humulene	15	1.500	0.41	1.9	101	92–105	–2.75
Caryophyllene oxide	15	1.500	0.42	1.9	100	92–105	–6.49

each terpene, and spiking 745 mg with 3 mL introduced 15 mg of each terpene. In this manner, the exact masses introduced (Table 1) can be converted to wt%/1000 mg of total mass (matrix plus total terpenes). These values are also shown in Table 1 and are the known true values, in wt%, for the terpene validation samples.

To determine accuracy (recovery), the same volumes of terpene spikes added to the blank matrixes to make the validation samples were added to empty vials (no matrix or beads), and a volume of extraction solvent was then added to each vial (both empty vials and validation samples) such that the total volume (extraction volume plus spike volume) equaled 15 mL. Processing and analyzing the validation samples as described and comparing the results to the pure standards in the vial sets with no matrix present provided the percentage recovery ($n = 5$ at each concentration level). The absolute wt% determinations at each level were used to calculate RSD_r. To estimate trueness, reported as relative bias, the average values for the validation samples were compared to the known values, which had been determined gravimetrically and are listed in Table 1. All values were compared and reported as wt%, which can be done for the empty vials by simply assuming 1000 mg of matrix to convert the raw result to wt%.

Cannabinoid Validation Samples

Obtaining reference standards for the cannabinoids in quantities required for spike recovery studies is not possible in the United Sates due to both legal and financial considerations, as mentioned above. Guidance (22) states that an impure specimen can serve as the reference standard if CRMs are not available, and since this is the case with the cannabinoids, we used a method introduced by DeBacker et al. (15) and prepared our own validation samples by using concentrated forms of cannabinoid-containing extracts to spike analytes into a blank matrix. When using this procedure it is critical that the linearity and the goodness of fit of calibration curves are properly assessed since the "true" values of these concentrated spikes are determined empirically from the calibration curves, and nonlinearity of the curve can result in different "true" values that depend on the concentration measured.

The pentane extracts generated from preparing the blanks were concentrated and provided as oils that were rich in THCA and CBGA, CBDA, or THCVA and THC. These oils were then heated at 240°C for 15 min to decarboxylate the acidic forms

Table 2. Cannabinoid spikes, recovery, repeatability precision, and relative bias

Cannabinoid	Extract concn, Wt%	Extract added, mg	Analyte added, mg	True Wt%/ 1000 mg	RSD_r, %	Acceptable RSD_r, %	Recovery, %	Recovery limits, %	Relative bias, %
					Low				
THCA	67.2	3.0	2.0	0.20	4.1	5.1	104.57	90–108	11.80
CGBA(TI)	5.1	27.0	1.4	0.14	2.8	5.4	100.33	90–108	–4.00
CBDA	73.1	3.5	2.5	0.25	0.6	4.9	98.57	90–108	0.31
THCVA(TI)	9.1	24.0	2.2	0.22	1.4	5.0	101.87	90–108	–4.45
THC	17.0	24.0	4.1	0.41	1.2	4.6	103.77	90–108	2.15
CBG	3.8	49.5	1.9	0.19	0.5	5.1	102.36	90–108	0.74
CBD	52.1	4.5	2.3	0.23	0.6	5.0	102.13	90–108	0.35
THCV(TI)	11.1	27.0	3.0	0.30	0.3	4.8	101.18	90–108	–2.53
					Medium				
THCA	67.2	140.0	94	9.42	0.68	2.9	100.98	95–102	2.33
CGBA(TI)	5.1	500.0	25	2.53	1.94	3.5	101.74	95–102	0.58
CBDA	73.1	140.0	102	10.24	0.28	2.8	100.12	95–102	–1.46
THCVA(TI)	9.1	510.0	46	4.64	1.42	3.2	100.87	95–102	0.44
THC	17.0	510.0	87	8.66	0.73	2.9	102.09	95–102	6.85
CBG	3.8	720.0	27	2.74	0.40	3.4	102.77	95–102	6.93
CBD	52.1	310.0	161	16.13	0.42	2.6	100.71	95–102	–0.30
THCV(TI)	11.1	540.0	60	6.00	0.40	3.0	103.39	95–102	–1.60
					High				
THCA	67.2	500.0	336	33.61	0.50	2.4	100.50	95–102	2.63
CGBA(TI)	5.1	1170.0	59	5.91	1.34	3.1	101.59	95–102	4.28
CBDA	73.1	440.0	322	32.19	0.71	2.4	100.74	95–102	0.15
THCVA(TI)	9.1	1800.0	164	16.36	0.49	2.6	102.44	95–102	0.50
THC	17.0	1800.0	306	30.58	0.89	2.4	100.00	95–102	1.71
CBG	3.8	1590.0	60	6.04	0.65	3.0	101.76	95–102	5.17
CBD	52.1	669.0	348	34.83	0.49	2.3	101.25	95–102	–0.62
THCV(TI)	11.1	1920.0	214	21.35	0.67	2.5	105.21	95–102	–1.98

and provide three more extracts that were rich in CBG, CBD, or THCV. Since there was not a clearly dominant CBGA cultivar available for extraction, evaluation of CBGA was carried out with the same extract used for evaluating THCA. To determine the "true" values of the analytes in these extracts, they were each evaluated at different regions of the calibration curves by dissolving different masses of the extracts in extraction solution in volumetric flasks. Since this uses unknown extracts it is an empirical procedure, and sometimes several solutions need to be made up to find three that fall within the linear calibration range. Good agreement between these determinations is evidence of good linearity and the averages of these determinations were taken as the "true" values for each of the analytes and are shown in Table 2.

To prepare the validation samples, the concentrates were used to gravimetrically spike blank matrix with low, medium, and high levels of the cannabinoids. The exact masses added were dictated by the quantities of extracts that were available, the concentrations of analytes present, and their purity profiles. For instance, spiking 1000 mg blank matrix with 3 mg THCA concentrate introduced 2.1 mg THCA, spiking 1000 mg of blank matrix with 131 mg concentrate introduced 95 mg THCA, and spiking 1000 mg blank matrix with 462 mg concentrate introduced 336 mg THCA. Absolute mass values were then converted to wt%/1000 mg of total mass (matrix plus concentrate), and these values are also shown in Table 2. These values are taken as the "true" values, in wt%, for the cannabinoid validation samples.

To determine accuracy (recovery), the same masses of extracts that were added to the blank matrixes to make the validation samples were added to empty vials (no matrix or beads), and a 15 mL extraction solvent was then added to each vial (both empty vials and validation samples). Processing and analyzing the validation samples as described and comparing the results to the neat extracts in the vial sets with no matrix present provided the percentage recovery. The absolute wt% determinations at each level were used to calculate RSD_r. To estimate trueness, reported as relative bias, the average values for the validation samples were compared to the known values, which had been estimated gravimetrically and are listed in Table 2. All values are compared and reported as wt%, which can be done for the empty vials by simply assuming 1000 mg of sample to convert the raw result to wt%.

Authentic Samples for Evaluating Scope and Precision

In order to evaluate the scope and obtain a representative value for RSD_r as well as intermediate precision, different authentic samples containing different cannabinoid and terpene profiles were evaluated on 5 separate days as well as five times on the same day. Since this number of replicates required at least 10 g sample, approximately 15 g flowers were homogenized in a stainless steel coffee grinder to ensure a homogenous bulk sample for testing over the course of the study. This bulk material was stored at –20°C between analyses. The means of the analytes present should not differ significantly, and the acceptable RSD_r values for the analytes are given by Equation 3 below (22).

Application of the Method

As an example of the utility of this method, plants from a typical California production operation were sampled at the cultivation site to illustrate the variability that occurs with this agricultural crop in typical uncontrolled and unregulated production environments. One-third of the total population was randomly chosen across the growing area for sampling. Samples of flowers were taken from each plant at various vertical locations within the plant canopy. The samples were allowed to dry to approximately 10% moisture at ambient temperature, and then trimmed to mimic separate medical cannabis flowers that can be delivered to dispensaries as part of the same lot of material. Flowers were stored at –20°C in sealed plastic containers until analyses.

Calculations and Reporting

All instrumental assays for terpenes and cannabinoids at a single dilution ratio were configured to use the associated software packages (ChemStation C.01.06[61] on the Agilent 1290 and TotalChrom 6.3.2 on the Clarus 680) to calculate wt% values based on ISTD calibration curves, which was nonane (0.1 mg/mL) for the terpenes and IBU (0.2 mg/mL) for the cannabinoids. For analysis of both major and minor cannabinoids, which required two dilution factors, the intermediate values were obtained from ESTD calibration curves and reported as Raw wt%. In order to correct for recovery in the sample preparation and dilution processes, the final wt% for each dilution factor, y, was calculated according to the following equation (vide infra):

$$Final\ wt\%_{DF_y} = Raw\ wt\%/(1 + \%\Delta) \quad (1)$$

where:

$$\%\Delta = \frac{(A_{BPCA})_{Sample} - (A_{BPCA})_{Known}}{(A_{BPCA})_{Known}} \quad (2)$$

$(A_{BPCA})_{Known}$ is the experimentally determined peak area of BPCA at dilution factor y and $(A_{BPCA})_{Sample}$ is peak area of BPCA found in the sample at dilution factor y.

Acceptable RSD_r values were calculated according to the equation:

$$RSD(r) = 2C^{-0.1505} \quad (3)$$

where C is the analyte concentration expressed as a mass fraction. Guidance states the predicted relative standard deviation (PRSD) should be $C^{-0.1505}$, and acceptable values are typically ½ to 2 times this value (22), so Equation 3 is taken as the upper limit of acceptability.

Results and Discussion

GC-FID Analysis of the Terpenes

The GC method provided sufficient resolution of all terpenes of interest in 35 min, and Table 3 provides the relative retention times. The relative retention times of a number of other terpenes reported to be found in cannabis are also given to demonstrate selectivity, but since these analytes were not of interest, due to reasons mentioned in the introduction, the instrument was not calibrated for them.

Each calibration curve was obtained in triplicate. Table 3 shows the average injection RSDs over the entire range, correlation coefficients, and the averages of the residuals (reported as the percentage difference between the experimental points and the fitted line). A complete discussion of the analysis is outside the scope of this article; however, correlation coefficients greater than 0.99 and residuals with a random distribution and a mean close to zero are indicative of an acceptable fit of the data (2, 32).

This table also provides the retention times of each of the terpenes with the analytical HPLC method used for the cannabinoids to demonstrate selectivity for these components, which can show up in the HPLC method when present in very high concentrations.

Table 3. Terpenoid retention times and calibration curve analysis

Analyte	GC RRT, min[a]	HPLC RRT, min	R^2	Mean residuals, %	Avg. RSD, %
α-Pinene	1.180	4.768	0.999	–1.10	0.56
Camphene	1.271	3.732	0.999	–1.10	0.72
Sabinene	1.406				
β-Pinene	1.428	3.871	0.999	–1.00	0.72
Myrcene	1.484	3.153	0.999	–0.93	1.04
α-Phellandrene	1.601	3.738	0.999	–1.26	0.90
Careen	1.619	4.200	0.999	–1.00	0.93
α-Terpinene	1.671	3.531	0.999	0.57	0.88
Cymene	1.738				
Limonene	1.754	3.713	0.999	–1.09	0.91
Cineole	1.789				
trans-Ocimene	1.866	3.041	0.999	–0.48	1.34
γ-Terpinene	1.957	3.603	0.999	–1.16	1.06
Terpinolene	2.157	3.568	0.999	–1.08	1.21
Linalool	2.262	0.854	0.999	0.77	2.08
Fenchol	2.415	0.382	0.999	0.31	2.15
Borneol	2.751				
α-Terpineol	3.018	0.833	0.999	0.04	2.10
Nerol	3.114				
Neral	3.205				
Geraniol	3.299				
Geranial	3.407				
β-Caryophyllene	4.818	8.439	0.999	0.36	2.24
trans-Nerolidol	4.886				
cis-Nerolidol	5.061				
α-Humulene	5.096	8.248	0.999	0.33	2.28
Caryophyllene oxide	6.041	2.594	0.998	0.09	2.48

[a] RRT = Relative retention time.

HPLC-DAD Analysis of the Cannabinoids

The HPLC method provided sufficient resolution of the major cannabinoids of interest in 17 min (Figure 1), and the relative retention times are provided in Table 4. The upper chromatogram is an overlay of the neutral cannabinoid standards and the acidic cannabinoid standards. Baseline resolution of CBG/CBD and THCA/CBC is lost at the highest calibration level but there is still good linearity, and RSDs are within acceptable limits at all concentration levels.

As mentioned previously, authentic standards for CBGA, THCVA, THCV, CBDVA, and CBDV are not easily obtained. Rather than wait for the legal climate to change, we sought tentative identifications by MS to provide a method for laboratories to quantify these important analytes. A full discussion of our survey of CBGA-rich and propyl-rich cultivars is outside the scope of this publication; however, we worked closely with cultivators to obtain a number of cultivars with these purported analytes present in high levels of 7 to 20%, which greatly facilitates their identification. Since our laboratory (like many others) does not have the resources for an LC/MS system to directly correlate with LC-UV, we analyzed extracts of these cultivars by GC/MS and tentatively identified the major analytes by their mass spectra and similarities of retention times to those derived from authentic material (33).

We then compared the chromatograms from GC/MS to those from HPLC-UV, and there were very clear correlations between the major peak areas as well as their ratios. This was especially true of cultivars containing THCA, CBDA, THCVA, and CBDVA where proposed biosynthetic pathways (36) suggest the relative ratios of THCA:CBDA and THCVA:CBDVA should be nearly equivalent. The THCA:CBDA ratios were determined from authentic standards, and there was good correlation with the ratios from the tentatively identified THCVA:CBDVA peaks. Both heated and unheated extracts were analyzed by GC/MS and HPLC-UV to identify the retention times of both the acidic and decarboxylated neutral species by HPLC, and again clear correlations could be seen between peak areas as well as their appearance and disappearance from decarboxylation. This is especially evident with CBGA and CBG, since there was good correlation between the disappearance of the CBGA peak (TI) and appearance of the CBG peak (identified from authentic standards). The central chromatogram in Figure 1 is an overlay of an unheated (blue) and heated (red) extract containing CBDVA and THCVA (color figure is available online at http://aoac.publisher.ingentaconnect.com/content/aoac/jaoac). The disappearance of the peaks attributed to CBDVA and THCVA and the appearance of the peaks attributed to CBDV and THCV can be seen. The retention times and elution orders of the decarboxylated species compared favorably with those obtained from authentic materials (34). The lower chromatogram in Figure 1 is a cultivar containing both CBDA and CBGA. These were identified by GC/MS (as well as authentic standard for

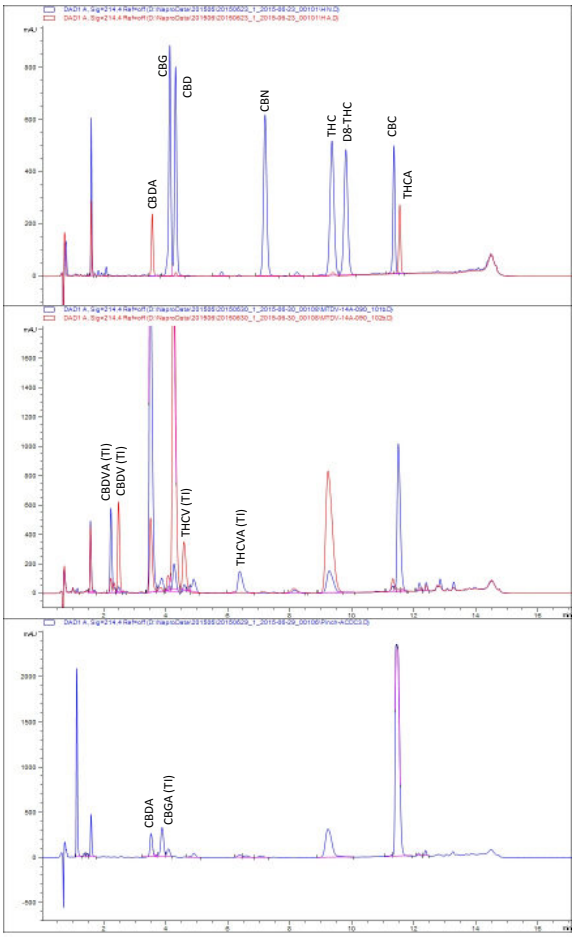

Figure 1. (Top) Acidic and neutral cannabinoid standards. (Middle) Heated and unheated extracts containing THCV(A) (TI) and CBDV(A) (TI). (Bottom) Extract containing CBDA and CBGA (TI).

CBDA by HPLC), and when this extract was heated both peaks disappeared and gave rise to peaks identified as their neutral counterparts by authentic standards. Based on these studies, we tentatively identified the retention times of CBGA, THCVA, CBDVA, THCV, and CBDV analytes, and these analytes are labeled with TI. CBCA is missing from our analyses due to the lack of either a readily available standard or a cultivar known to be rich in CBCA. Based on other analyses (23) CBCA likely elutes after THCA near the end of the run, so a slight extension of the run may be required. As mentioned above, tentatively identified analytes were quantified by referencing the calibration curves of analytical standards with similar spectral properties (35). While this is certainly not the ideal situation, it provides a path forward until analytical standards for these other important analytes become readily available.

Each calibration curve obtained with analytical standards was produced in triplicate, and Table 4 shows the average injection RSDs over the entire range, correlation coefficients, and the averages of the residuals (reported as the percentage difference between the experimental points and the fitted line). The data set from the calibration runs could be used to generate both ISTD and ESTD calibration curves (by simply selecting or deselecting the option before reprocessing results), and the parameters are shown for both. Correlation coefficients greater than 0.99 and

Table 4. Cannabinoid retention times and calibration curve analysis

		ISTD			ESTD		
Analyte	HPLC RRT, min	R^2	Mean residuals, %	Avg. RSD, %	R^2	Mean residuals, %	Avg. RSD, %
BPCA	0.570						
Ibuprofen	1.000						
CBDVA (TI)	1.418						
CBDV (TI)	1.575						
CBDA	2.249	1.000	0.38	1.01	1.000	0.08	0.90
CBGA (TI)	2.420						
CBG	2.609	1.000	–0.09	0.39	1.000	–0.62	0.28
CBD	2.727	1.000	0.07	0.47	1.000	–0.45	0.33
THCV (TI)	2.919						
THCVA (TI)	4.064						
CBN	4.560	1.000	0.37	0.41	1.000	–0.15	0.42
THC	5.931	1.000	–0.33	0.38	1.000	–0.86	0.36
D8-THC	6.216	1.000	–0.23	0.61	1.000	–0.76	0.55
CBC	7.201	1.000	0.13	0.50	1.000	–0.39	0.33
THCA	7.320	1.000	0.26	0.52	1.000	–0.04	0.43

residuals with a random distribution and a mean close to zero are indicative of an acceptable fit of the data (2, 32).

LOQ

Due to the impossibility of obtaining authentic blanks for all the analytes, the LOQs were estimated from the calibration curves. ICH Q2B guidance states that the LOQ can be estimated from the calibration curve of an analyte using the formula LOQ = 10 (σ/m), where σ is the SD of the y-intercept and m is the slope (20). This was done for each analyte, and LOQ was generally found to be in the range of 0.005–0.008 mg/mL. This was then tested experimentally by diluting standards to 0.008 and 0.004 mg/mL and verifying the measured values were within 20% of the known values. All the analytes satisfied the requirement at the 0.008 mg/mL level and this was taken as the LOQ for all of the analytes. Given the standard sample mass, extraction volumes, and dilution ratios used for these assays, this corresponds to 0.012% for the terpenoids and 0.07% for the cannabinoids at the 1:6 dilution. Although this was not verified for every analyte, Tables 5–9 for the intermediate precision studies show that analytes found near these levels displayed acceptable RSDs as given by Equation 3, suggesting these LOQs are adequately defined.

ISTDs in the Cannabinoid Assay

The uses and benefits of ISTDs in analytical assays and sample preparation are well established (22, 37–39). The use of nonane as the ISTD for terpene analysis is analytically rigorous and compensates for variability in recovery and peak area in typical fashion. The use of both IBU and BPCA in the cannabinoid assay, however, requires some discussion.

The method of using two different ISTDs for different purposes was the result of several factors encountered during method development and sample analysis: (*1*) the availability, stability, cost, and supplied form of cannabinoid analytical standards can make it impractical for many laboratories to prepare multiple calibration solutions with different concentrations of ISTDs; (*2*) the linear range of the cannabinoid assay was approximately 1.5 orders of magnitude (0.016–0.250 mg/mL); however, analyte concentrations can span over 2 orders of magnitude (0.1–40% by weight), and this requires separate dilution ratios for both the minor and major cannabinoids to remain in the linear range of the calibration curve; and (*3*) when testing unknown samples (especially water hash and extracts), the cannabinoid concentrations are unpredictable and can be even higher (as high as 80% by weight). Since the sample had already been processed, the only way to bring the properly stored extraction slurry (4°C for no longer than 24 h) into the linear range for reanalysis was to use a larger dilution factor.

When an assay and calibration curves are developed with an ISTD present, the concentration of the ISTD in the final aliquot for analysis must remain fixed at the level used to generate the calibration curves. If it is anticipated a sample is "typical" and only the major cannabinoid profile is desired (2.5–36% by weight with the given mass and solvent ratios), only a single 96-fold dilution is needed. In this case, spiking the sample with 1 mL of ISTD spike solution, adding 14 mL of diluent, extracting the sample, and then diluting 96-fold provides an aliquot for analysis with a theoretical 0.2 mg/mL IBU concentration for analysis with the HPLC method that utilizes the ISTD curve.

While the use of IBU as the ISTD in the aforementioned manner is analytically rigorous and is used by our laboratory at times, we typically use the method described herein where BPCA is used as the ISTD. In this case the surrogate is added to the sample at a fixed concentration via the extraction solution, and variation in analyte recovery due to sample preparation and/or dilution is estimated by the recovery of the surrogate, which can then be used as a correction factor for the raw result. The instrument parameters are set to use the ESTD calibration curves, and a correction factor given by Equation 1 is applied to the raw result. This procedure is also analytically rigorous (22, 39), and while it does not benefit generation of the calibration curves as with the first method, careful method development to ensure linearity and injection precision mitigates this issue. This procedure has the added benefits that it does not rely on the precision and/or accuracy of ISTD spikes by different laboratory technicians, and it can be applied to any dilution ratio needed by comparing the experimental peak area of the surrogate to the known peak area of the surrogate determined at each concentration.

In this specific case, the extraction solution with BPCA at 2.0 mg/mL is diluted 6-fold (0.333 mg/mL) and 96-fold (0.021 mg/mL) in replicate, and the average peak areas are determined. The correction for recovery is then given by Equation 1 for each of those dilution factors. If a processed sample is found to be outside the linear range of the calibration curve, a correction factor can quickly be determined at a different dilution ratio, and the properly stored extract rediluted for analysis. This process is much faster than preparing new calibration solutions of expensive cannabinoid standards with ISTDs at the appropriate concentrations and recalibrating the instrument. Typically, the reporting methods and appropriate correction factors for several dilution factors are stored in the ChemStation software and simply applied to the individual sample dilutions as needed.

To demonstrate the use of this correction, a CO_2 wax was analyzed for THCA by dissolving the wax in a suitable volume of extraction solution in a volumetric flask so as to not require any dilution for analysis ($n = 2$), and the average was taken as the "true" value. The wax was then reanalyzed by dissolving 267 mg in 10 mL of extraction solution and diluting 96-fold in the typical manner, as well as intentionally adding 20% more and 20% less of the extract ($n = 5$ for each). The process was then repeated by dissolving 168 mg in 100 mL of extraction solution and diluting 6-fold in the typical manner, as well as introducing the same intentional dilution errors ($n = 5$ for each). Table 10 shows the

Table 5. ACDC analyzed on 5 different days and five times on the same day

Replicate	α-Pinene	β-Pinene	Myrcene	Limonene	Linalool	α-Terpineol	β-Caryophyllene	α-Humulene	THCA	CBDA	CBGA (TI)	CBD
D1, %	0.384	0.173	1.173	0.079	0.020	0.017	0.273	0.087	0.49	15.232	0.68	0.489
D2, %	0.326	0.153	1.036	0.072	0.020	0.017	0.259	0.082	0.50	15.460	0.69	0.472
D3, %	0.298	0.144	0.975	0.068	0.021	0.017	0.256	0.082	0.50	15.32	0.69	0.48
D4, %	0.293	0.142	0.962	0.068	0.021	0.016	0.253	0.081	0.49	15.23	0.67	0.48
D5a, %	0.266	0.134	0.907	0.065	0.022	0.017	0.249	0.080	0.49	15.232	0.68	0.489
D5b, %	0.256	0.131	0.886	0.064	0.022	0.017	0.253	0.081	0.50	15.460	0.69	0.472
D5c, %	0.249	0.128	0.859	0.063	0.022	0.016	0.248	0.079	0.50	15.32	0.69	0.48
D5d, %	0.247	0.128	0.860	0.063	0.023	0.017	0.254	0.081	0.49	15.23	0.67	0.48
D5e, %	0.239	0.124	0.835	0.062	0.022	0.017	0.253	0.080	0.49	15.21	0.65	0.51
Interday avg., %	0.313	0.149	1.011	0.070	0.021	0.017	0.258	0.082	0.49	15.29	0.67	0.49
RSD, %	14.3	9.8	10.1	7.4	4.1	1.9	3.6	3.4	0.9	0.7	2.5	3.2
Intraday avg., %	0.251	0.129	0.870	0.063	0.022	0.017	0.251	0.080	0.49	15.29	0.67	0.49
RSD, %	4.0	3.0	3.2	2.2	0.9	1.0	1.1	1.3	0.9	0.7	2.5	3.2
Acceptable RSD_r, %	4.7	5.3	4.0	5.9	7.1	7.4	4.9	5.8	4.4	2.7	4.2	4.4

Table 6. Master Kush analyzed on 5 different days and five times on the same day

Replicate	α-Pinene	β-Pinene	Myrcene	Limonene	Linalool	α-Terpineol	β-Caryophyllene	α-Humulene	THCA	CBGA(TI)	THC
D1, %	0.038	0.073	0.197	0.473	0.154	0.049	0.051	0.433	19.82	0.66	0.50
D2, %	0.032	0.063	0.166	0.408	0.139	0.045	0.046	0.390	18.44	0.64	0.49
D3, %	0.033	0.065	0.168	0.421	0.151	0.049	0.051	0.427	20.12	0.70	0.52
D4, %	0.031	0.061	0.155	0.391	0.142	0.046	0.048	0.396	18.99	0.66	0.52
D5a, %	0.030	0.059	0.149	0.382	0.145	0.047	0.049	0.405	19.37	0.65	0.56
D5b, %	0.029	0.059	0.147	0.377	0.145	0.047	0.049	0.405	19.33	0.63	0.56
D5c, %	0.030	0.060	0.148	0.382	0.147	0.047	0.050	0.416	19.58	0.65	0.57
D5d, %	0.029	0.058	0.143	0.370	0.143	0.046	0.049	0.405	19.01	0.68	0.52
D5e, %	0.029	0.059	0.144	0.375	0.148	0.047	0.050	0.417	19.65	0.64	0.58
Interday average, %	0.033	0.064	0.167	0.415	0.146	0.047	0.049	0.410	19.35	0.66	0.52
RSD, %	10.0	8.3	11.1	8.6	4.3	4.1	4.0	4.6	3.4	3.4	5.7
Intraday average, %	0.030	0.059	0.146	0.377	0.145	0.047	0.049	0.409	19.39	0.65	0.56
RSD, %	1.4	1.1	1.8	1.3	1.2	0.9	1.5	1.6	1.3	2.8	4.2
Acceptable RSD_r, %	6.7	6.0	5.2	4.6	5.3	6.3	6.3	4.6	2.6	4.2	4.4

Table 7. Pincher's Creek analyzed on 5 different days and five times on the same day

Replicate	α-Pinene	β-Pinene	Myrcene	Limonene	β-Ocimene	Linalool	Fenchol	α-Terpineol	β-Caryophyllene	α-Humulene	Caryophyllene oxide	THCA	CBGA (TI)	THCVA (TI)	THC	CBG
D1, %	0.086	0.059	0.428	0.148	0.229	0.068	0.024	0.025	0.269	0.099	0.019	18.41	2.07	0.151	1.06	0.13
D2, %	0.077	0.054	0.380	0.136	0.208	0.065	0.024	0.024	0.258	0.094	0.020	18.47	2.06	0.156	1.06	0.13
D3, %	0.073	0.052	0.363	0.132	0.201	0.067	0.024	0.025	0.262	0.096	0.020	18.42	2.08	0.16	1.10	0.13
D4, %	0.070	0.050	0.343	0.125	0.191	0.065	0.024	0.024	0.253	0.093	0.020	18.14	2.03	0.15	1.08	0.13
D5a, %	0.068	0.049	0.334	0.124	0.188	0.066	0.024	0.024	0.257	0.094	0.022	18.23	2.06	0.151	1.13	0.13
D5b, %	0.067	0.049	0.326	0.122	0.185	0.065	0.024	0.024	0.254	0.094	0.021	18.15	2.06	0.151	1.18	0.13
D5c, %	0.066	0.048	0.317	0.119	0.181	0.065	0.024	0.024	0.252	0.093	0.020	18.06	2.04	0.15	1.13	0.13
D5d, %	0.066	0.047	0.313	0.118	0.178	0.064	0.023	0.024	0.249	0.092	0.021	18.42	2.05	0.15	1.13	0.13
D5e, %	0.066	0.048	0.314	0.119	0.180	0.065	0.024	0.024	0.253	0.093	0.022	18.56	2.08	0.16	1.15	0.13
Interday average, %	0.075	0.053	0.370	0.133	0.204	0.066	0.024	0.024	0.260	0.095	0.020	18.33	2.06	0.15	1.09	0.13
RSD, %	9.5	7.0	10.1	7.2	8.1	2.1	1.6	2.1	2.4	2.4	6.9	0.8	1.0	2.3	2.4	1.0
Intraday average, %	0.067	0.048	0.321	0.120	0.182	0.065	0.024	0.024	0.253	0.093	0.021	18.28	2.06	0.15	1.14	0.13
RSD, %	1.4	1.8	2.8	2.3	2.2	1.1	1.3	0.6	1.1	1.1	3.1	1.1	0.7	1.4	1.9	1.5
Acceptable RSD_r, %	5.9	6.2	4.6	5.4	5.1	6.0	7.0	7.0	4.9	5.7	7.2	2.6	3.6	5.3	3.9	5.4

Table 8. A THCVA hybrid analyzed on 5 different days and five times on the same day

Replicate	α-Pinene	β-Pinene	Myrcene	Limonene	β-Ocimene	Linalool	Fenchol	α-Terpineol	β-Caryophyllene	α-Humulene	THCA	CBDA	CBGA (TI)	THCVA (TI)	CBDVA (TI)	THC	CBD	CBG	THCV (TI)	CBDV (TI)
D1, %	0.077	0.029	0.359	0.109	0.227	0.066	0.019	0.019	0.261	0.225	3.68	10.58	0.54	0.81	1.39	0.48	0.28	0.09	0.07	0.04
D2, %	0.062	0.024	0.298	0.093	0.196	0.060	0.017	0.017	0.237	0.206	3.44	9.67	0.50	0.78	1.29	0.45	0.26	0.08	0.06	0.04
D3, %	0.058	0.023	0.278	0.089	0.184	0.061	0.017	0.018	0.234	0.203	3.46	9.70	0.51	0.79	1.30	0.44	0.27	0.08	0.07	0.04
D4, %	0.057	0.023	0.276	0.089	0.185	0.062	0.018	0.018	0.239	0.207	3.51	9.46	0.50	0.80	1.31	0.45	0.27	0.08	0.07	0.04
D5a, %	0.052	0.021	0.255	0.084	0.173	0.059	0.017	0.017	0.227	0.198	3.51	9.79	0.49	0.78	1.29	0.47	0.29	0.08	0.07	0.04
D5b, %	0.052	0.021	0.256	0.084	0.175	0.061	0.017	0.018	0.232	0.202	3.52	9.78	0.50	0.75	1.31	0.48	0.29	0.08	0.07	0.04
D5c, %	0.051	0.021	0.252	0.083	0.173	0.059	0.017	0.017	0.227	0.197	3.43	9.60	0.48	0.77	1.27	0.45	0.28	0.08	0.07	0.04
D5d, %	0.050	0.021	0.246	0.082	0.171	0.060	0.017	0.018	0.232	0.201	3.41	9.79	0.48	0.78	1.29	0.46	0.29	0.08	0.07	0.04
D5e, %	0.050	0.021	0.246	0.082	0.174	0.063	0.018	0.018	0.238	0.207	3.51	10.03	0.50	0.77	1.34	0.48	0.30	0.08	0.07	0.04
Interday average, %	0.061	0.024	0.293	0.093	0.193	0.062	0.017	0.018	0.240	0.208	3.52	9.84	0.51	0.79	1.31	0.46	0.27	0.08	0.07	0.04
RSD, %	15.4	11.9	13.5	10.3	10.6	4.6	5.0	4.4	5.2	4.9	2.6	4.4	3.7	1.6	3.2	3.7	3.4	4.0	2.4	5.3
Intraday average, %	0.051	0.021	0.251	0.083	0.173	0.060	0.017	0.018	0.231	0.201	3.48	9.80	0.49	0.77	1.30	0.47	0.29	0.08	0.07	0.04
RSD, %	2.3	0.9	2.1	1.2	0.9	2.5	2.8	1.8	1.9	1.8	1.5	1.6	2.0	1.4	2.0	3.2	2.2	2.1	2.4	1.7
Acceptable RSD_n, %	6.1	7.0	4.8	5.7	5.1	6.1	7.3	7.3	4.9	5.1	3.3	2.8	4.4	4.1	3.8	4.5	4.9	5.8	6.0	6.5

Table 9. TrainWreck analyzed on 5 different days and five times on the same day

Replicate	α-Pinene	β-Pinene	Myrcene	α-Phellandrene	Carene	α-Terpinene	Limonene	β-Ocimene	γ-Terpinene	Terpinolene	Linalool	α-Terpineol	β-Caryophyllene	α-Humulene	THCA	CBGA (TI)	THC	CBG
D1, %	0.062	0.103	0.342	0.049	0.042	0.034	0.141	0.305	0.020	0.931	0.035	0.034	0.173	0.048	16.97	1.20	1.15	0.14
D2, %	0.046	0.082	0.265	0.039	0.034	0.028	0.117	0.253	0.018	0.794	0.033	0.033	0.163	0.045	16.55	1.16	1.19	0.14
D3, %	0.041	0.076	0.240	0.036	0.032	0.025	0.108	0.232	0.018	0.713	0.033	0.033	0.160	0.045	16.72	1.18	1.16	0.14
D4, %	0.040	0.074	0.233	0.034	0.031	0.022	0.106	0.230	0.018	0.672	0.033	0.033	0.161	0.045	16.96	1.19	1.20	0.14
D5a, %	0.036	0.069	0.214	0.032	0.030	0.021	0.100	0.216	0.016	0.653	0.033	0.033	0.161	0.045	17.46	1.21	1.26	0.15
D5b, %	0.035	0.067	0.206	0.031	0.029	0.020	0.096	0.209	0.016	0.636	0.032	0.033	0.157	0.044	16.84	1.19	1.24	0.14
D5c, %	0.034	0.066	0.201	0.030	0.028	0.020	0.095	0.206	0.016	0.632	0.033	0.033	0.158	0.044	17.11	1.19	1.22	0.14
D5d, %	0.033	0.063	0.192	0.029	0.027	0.019	0.091	0.199	0.015	0.611	0.032	0.032	0.156	0.044	16.76	1.17	1.20	0.14
D5e, %	0.033	0.063	0.188	0.028	0.027	0.018	0.090	0.196	0.015	0.597	0.032	0.032	0.155	0.043	16.89	1.18	1.21	0.14
Interday average, %	0.045	0.081	0.259	0.038	0.034	0.026	0.114	0.247	0.018	0.753	0.033	0.033	0.163	0.045	16.93	1.19	1.19	0.14
RSD, %	22.2	16.4	19.3	18.2	13.8	21.1	14.0	14.1	7.8	15.1	2.2	1.7	3.2	3.0	2.0	1.6	3.6	1.8
Intraday average, %	0.034	0.065	0.200	0.030	0.028	0.020	0.094	0.205	0.016	0.626	0.033	0.033	0.157	0.044	17.01	1.19	1.23	0.14
RSD, %	4.3	4.0	5.2	4.7	3.9	5.1	4.2	3.9	3.3	3.5	1.3	1.1	1.5	1.5	1.6	1.3	1.9	1.8
Acceptable RSD_n, %	6.4	5.8	4.9	6.5	6.6	6.9	5.5	4.9	7.3	4.2	6.6	6.6	5.2	6.3	2.6	3.9	3.9	5.3

Table 10. Dilution error correction with BPCA

	Raw, %		Corrected, %		Relative error, %		RSD, %	
	Average	±	Average	±	Raw	Corrected	Raw	Corrected
"True"	73.8	1.56	73.8	1.56	0.0	0.0	2.1	2.1
96-fold	68.3	0.69	73.6	0.24	–7.4	–0.2	1.0	0.3
6-fold	72.2	0.61	75.7	0.29	–2.2	2.6	0.8	0.4
96+20%	83.28	1.02	73.88	0.25	12.8	0.1	1.2	0.3
6+20%	84.15	0.93	77.40	0.14	14.0	4.9	1.1	0.2
96–20%	56.77	1.06	73.14	0.38	–23.1	–0.9	1.9	0.5
6–20%	59.26	1.07	73.54	0.38	–19.7	–0.4	1.8	0.5

results for both corrected and uncorrected values. In all cases the corrected values are much closer to the true value, RSDs are reduced by half, and the relative error is also greatly reduced. While gross errors such as an error of 20% in sample volumes still show some deviation from the true value, they are still reasonable, and typical dilution errors are easily compensated for as shown by the first set of dilutions. This also demonstrates the ability of a single BPCA concentration to compensate for recovery at both a 6-fold (169 mg in 100 mL) and 96-fold (267 mg in 10 mL) dilution.

To demonstrate the applicability to typical sample processing, a sample of flowers was homogenized in a stainless steel coffee grinder, and two different technicians each analyzed five separate samples in replicate (n = 5) using a different pipet technique for diluting each sample. The techniques were standard with (*1*) prewetting the tip, (*2*) reverse pipet, (*3*) standard with rinsing the pipet tip in the diluent, (*4*) multichannel pipet, (*5*) and serial dilution (*5*). They were specifically instructed to show "less care," so this was not an evaluation of the various pipetting techniques. In this case, the "true" value was not known since it was being determined by the analyses. The average values are represented as the lines in Figure 2. For the uncorrected wt% values the overall average was lower, and there was a clear systematic error related to the technician, with technician A always generating lower values than technician B. The corrected wt% values, however, were all very similar regardless of the pipet technique used or the technician. The RSDs are shown in the lower chart, and on average the RSDs using the correction factor were half of those not using the correction factor. This suggests that using BPCA as a surrogate to correct for recovery was quite applicable to the typical workflow in the laboratory.

If the samples generally have known analyte concentrations and ranges, the linear range of the assay, volumes of spike solution with IBU, and dilution ratios can be tailored to use a single ISTD (IBU) in the typical fashion with the ISTD calibration curves. Verifying a wider linear range for a single calibration curve would simplify the situation; however, a linear calibration range (as determined via analysis of both correlation coefficients and residuals) on an HPLC-UV assay over 2 orders of magnitude (for concentrations of THCA from 0.1 to approximately 40%) can be difficult to attain. Likewise, once cannabinoid standards are made available in pure solid forms, it will become easier to prepare multiple calibration solutions with different ISTDs and concentrations. However, for a laboratory processing a large amount of unknowns, having a second method that used a surrogate (BPCA) to correct for recovery at any dilution factor provided a pragmatic solution that minimized variability and systematized the process while still accommodating the wide range of analyte concentrations. There are some well known limitations to using a single surrogate at a single concentration to approximate the behavior of a number of analytes with a wide

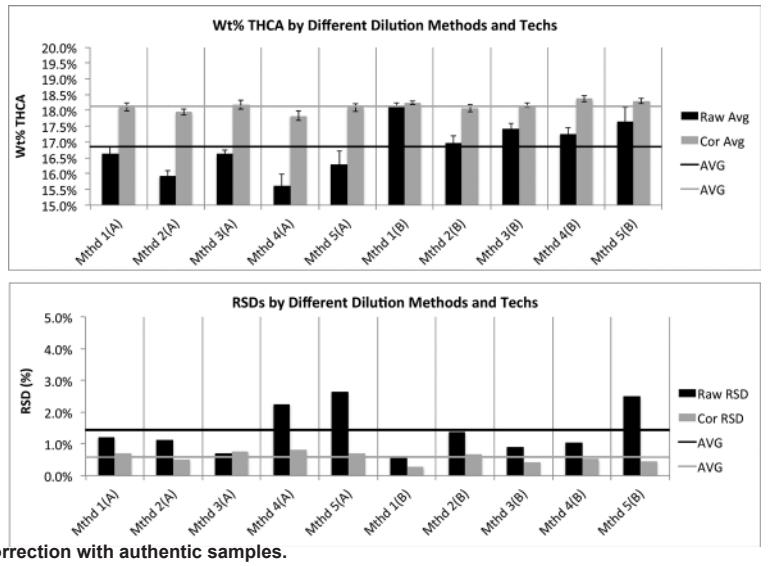

Figure 2. Total recovery correction with authentic samples.

range of concentrations (39); however, method development and validation suggest this process performs adequately for this assay.

Optimization of the HTH Parameters

For reasons mentioned earlier, we sought to utilize HTH for sample preparation. We sought to optimize the process with respect to bead type and bead volume, extraction time, extraction solvent volume, and sample mass by maximizing the amounts of analytes extracted and as well as minimizing the RSDs. Enough flower material to be considered compositionally equivalent for that study was reduced to a homogeneous mixture in a stainless steel coffee grinder, and each condition was evaluated in triplicate. Every analyte was evaluated individually; however, they closely mirrored the total amounts so only totals are discussed in the interest of brevity. Individual analytes will be addressed in the *Spike Recovery* and *Intermediate Precision* study sections below.

Table 11 shows the concentrations of analytes extracted and the RSDs for different volumes and types of beads. The parameters appeared to have no statistically significant effect on the total amounts of analytes extracted, and the results were essentially identical. Due to the fact that 1 mL of 2.0 mm zirconia beads gave the lowest RSDs for both the cannabinoids and the terpenes, and due to the lower cost and disposable nature of these beads, they were chosen for all subsequent experiments.

Table 11 shows the concentrations of analytes extracted and the RSDs for the five different extraction times that were evaluated. The parameters appeared to have no statistically significant effect on the total amounts of analytes extracted; however, a slight trend suggested longer times resulted in lower concentrations and higher RSD for the terpenes, so 6 min was chosen as the time for all subsequent experiments.

Table 11 shows the concentrations of analytes extracted and the RSDs for three different extraction volumes that were evaluated. Smaller volumes were not evaluated since 1000 mg of plant material in 10 mL of solvent provided a thicker slurry that was difficult to work with. Again, the parameters appeared to have no statistically significant effect on the total amounts of analytes extracted. While the RSD was slightly higher for the terpenes in 15 mL of solvent it was still quite low, and this volume was chosen for all subsequent processing to minimize cost and waste.

Table 11 shows the concentrations of analytes extracted and the RSDs for four different sample masses that were evaluated. While the absolute amounts extracted all fell within the error bars, the RSDs showed a clear dependence on sample size for both the cannabinoids and the terpenes. This was not completely unexpected as the bulk homogenized plant material was not a fine powder, and there were still pieces of stem and leaf present as well as aggregates of plant matter due to the sticky nature of the samples. These factors had a much greater influence on the homogeneity of small sample sizes and affected precision in a similar manner. A sample size of 1000 ± 20 mg was used for subsequent analyses.

Table 11. Optimization of HTH parameters

Variable	Total cannabinoids, Wt%	±, %	RSD, %	Total terpenoids, Wt%	±, %	RSD, %
Bead size and volume						
5/32 stainless (1 mL)	20.27	0.15	0.8	1.470	0.000	0.0
2.8 mm stainless (1 mL)	20.69	0.29	1.4	1.483	0.031	2.1
3.0 mm zirconium (1 mL)	20.41	0.50	2.4	1.450	0.000	0.0
2.0 mm zirconium (1 mL)	20.44	0.15	0.7	1.440	0.010	0.7
5/32 stainless (2 mL)	20.83	0.52	2.5	1.415	0.007	0.5
2.8 mm stainless (2 mL)	20.65	0.29	1.4	1.420	0.035	2.4
3.0 mm zirconium (2 mL)	20.43	0.36	1.8	1.430	0.026	1.9
2.0 mm zirconium (2 mL)	21.15	0.57	2.7	1.400	0.020	1.4
Extraction time, min						
2	21.72	0.10	0.4	1.557	0.006	0.4
4	21.72	0.13	0.6	1.530	0.010	0.7
6	22.00	0.25	1.1	1.543	0.006	0.4
8	22.13	0.42	1.9	1.533	0.006	0.4
10	22.05	0.20	0.9	1.490	0.026	1.8
Solvent volume, mL						
15	24.24	0.45	1.9	1.880	0.046	2.4
20	24.45	0.58	2.4	1.940	0.026	1.4
25	24.13	0.33	1.4	1.920	0.052	2.7
Sample mass, mg						
250	25.83	0.69	2.7	1.929	0.064	3.3
500	25.26	0.47	1.9	1.919	0.056	2.9
750	25.66	0.24	0.9	2.007	0.025	1.3
1000	25.28	0.17	0.7	2.016	0.011	0.5

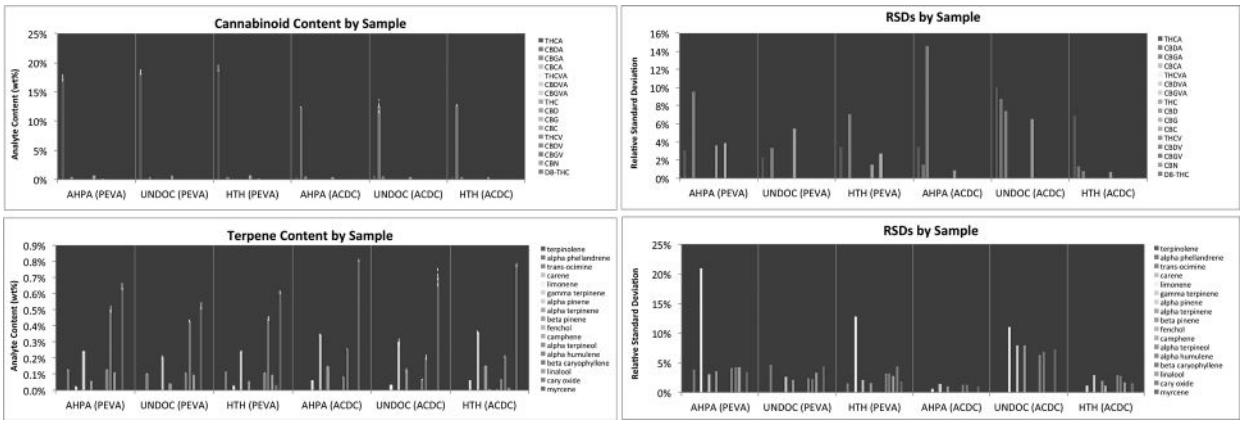

Figure 3. Comparison of HTH to AHPA and UNDOC sonication procedures.

Since none of the above variables were found to significantly influence the results, it was assumed the extraction was optimized and efficient. To verify this, the bead beating procedure was compared to accepted sonication procedures, one recommended by the AHP and used by the University of Mississippi to process samples submitted by the DEA (1), and the other recommended by UNDOC for the analysis of cannabis (25).

The AHP procedure involved macerating a 100 mg sample in 3 mL of methanol–chloroform (9 + 1, v/v) for 1 h followed by sonication for 5 min and filtration into a vial for analysis. The UNDOC procedure involved 200 mg of cannabis with 20 mL of ethanol for 20 min. Slight modifications to the recommended procedures had to be used for direct comparison. The cannabis could not be dried and powdered since this would affect the terpene profiles, and we increased the sample mass tested to 500 mg to reduce RSDs with fresh inflorescences (as demonstrated above) but retained the mass-to-solvent ratios recommended by each method. Due to interfering peaks with the chloroform–methanol extraction, BPCA correction or recovery could not be applied, so raw results were reported for the AHPA method.

This was carried out in triplicate for a Type I and Type II cultivar with slightly different terpene profiles. Figure 3 shows bar charts of the average wt% as well as the RSDs for each analyte. There were no significant differences between the methods and the profiles were nearly identical, with the exception of slightly lower RSDs for the HTH method. The larger RSDs (>5%) were for analytes that were present at low levels (limonene was not detected with the UNDOC method due to the larger solvent volume); however, they all exhibited the same general behavior. Demonstrating equivalence to recommended methodologies indicates the HTH extraction was complete.

The robustness of the procedure with respect to cultivar and throughput was also evaluated. This is an important step since there is a very wide range of morphologically different flowers that very often contain distinct analyte profiles. While it is not possible to do this for every different cultivar, five cultivars were chosen to cover varying flower morphologies, from loose to compact, and a variety of cannabinoids and terpenes. These five cultivars were extracted by both HTH and sonication procedures under typical workflow conditions in the laboratory (i.e., multiple samples being processed at the same time and reduced solvent volumes), and the results were compared.

The results for processing samples by HTH and sonication procedures in replicate (n = 5) are shown in Figure 4. It can be seen that the relative "fingerprints" of each cultivar are identical for both methods, indicating that all the analytes were extracted equally; however, total extraction for both cannabinoids and terpenes was significantly higher with bead beating than with sonication, almost double for some cultivars. The RSDs were also significantly lower with bead beating than with sonication, and average bead beating RSDs across all cultivars was less than 2% for both cannabinoids and terpenes, opposed to 21.2 and 16.5%, respectively, for sonication. It is also seen that the RSDs for all of the analytes in each sample were of similar magnitude, again indicating similar behavior of the analytes within each method and cultivar.

It is important to acknowledge that some sonication parameters in the literature for the extraction of cannabinoids typically use much larger solvent to sample mass ratios, which undoubtedly improves extraction efficiency. However, these larger ratios not only increase the amount of solvent needed for analysis but also reduce the concentrations of many terpenes in the resulting extract to levels that result in unacceptable detection limits, so parameters that were optimized for HTH were used. The higher RSDs noted with sonication may also have been due to different locations of sample tubes within the sonication bath, as well as differing flower morphologies. The HTH mitigates all of these issues, and this is a critical feature of an efficient sample processing method.

Terpene Spike Recovery

Table 1 shows the mass of each terpene standard introduced, the true wt% of each terpene in 1000 mg of matrix, the accuracy (recovery), RSD_r, and trueness (% relative bias) for each of the terpenes at low, medium, and high concentrations. The acceptable limit for RSD_r is given by Equation 3. The acceptable values for recovery at each of the concentration ranges are also listed and are given by AOAC (22). In all cases, the precision and recovery met accepted limits. The relative bias is also reported, which is the difference between the average measured value and the known value determined by gravimetric addition of the standards. In most cases bias closely follows recovery, indicating good linearity of the calibration curves, preparation of the spikes, and that the assay should give results close to the true values.

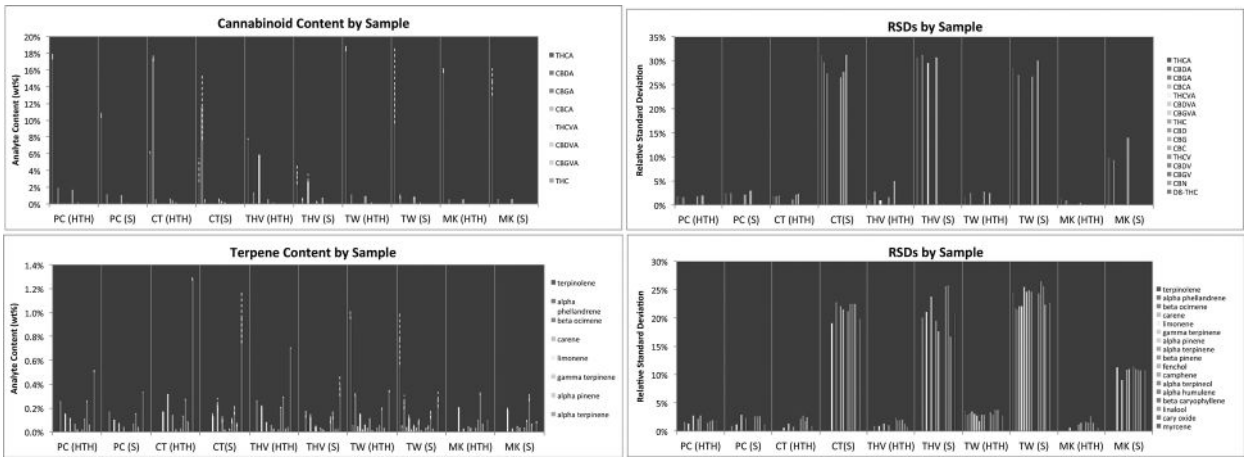

Figure 4. Comparison of HTH and sonication with five different cultivars.

A clear outlier with respect to relative bias was α-terpinene. Despite acceptable recovery values there was significant negate bias compared to the others, indicating the measured value is less than the value anticipated based on the mass spiked into the matrix. Note that all three terpinenes (α-phellandrene, α-terpinene, and terpinolene) exhibited lower recoveries and bias. This is a real effect and has been found to occur in extracts kept in clear vials loaded in the GC autosampler, as was the case for these spike recovery studies. This does not occur with standards kept in clear vials on top of the gas chromatograph for a short period (<24 h), and the rate of decomposition in extracts is reduced dramatically when amber vials are used.

All three of the terpinenes exhibited identical behavior; however, only terpinolene is discussed in detail due to the prevalence of terpinolene dominant cultivars. Figure 5 shows the levels of terpinolene in an extract of TrainWreck at three time points, 0, 3, and 18 h, in both clear and amber vials loaded in the gas chromatograph autosampler tray. An obvious decrease in the level of terpinolene can be seen in the clear vial. To explore this further, terpene standards were added to diluent in a clear vial, and they were added to the same volume of extract from a nonterpinolene containing cultivar in both clear and amber vials. The charts show that the standards were stable, but when they were diluted into extract in a clear vial there was a steady decrease in concentration. This decrease was not seen when the mixture was placed in an amber vial. Given the prevalence of terpinolene dominant cultivars, such as TrainWreck and Jack the Ripper, it is critical that extracts are placed in amber vials for analysis to obtain accurate results for the terpene profiles.

Cannabinoid Spike Recovery

Table 2 shows the concentration of the desired analyte in the extract, the mass of extract added to the blank matrix, the mass of analyte introduced via the extract, and the conversion of mg of analyte to wt%/1000 mg of matrix. The table also lists the accuracy (recovery), RSD_r, and trueness (% relative bias) for each of the cannabinoids at low, medium, and high concentrations. The acceptable limit for RSD_r is also listed, and this is given by Equation 3. The acceptable values for recovery at each of the concentration ranges are also listed and are given by AOAC (22). The exact levels of analytes introduced were dictated by the amount of extract available to do the experiments as well as the purity profiles of the extracts.

The precision was acceptable for all of the cannabinoids, although it was relatively higher for THCA at the low concentration range. This was undoubtedly due to the variable residual amounts of THCA present in the blank matrix, and an average background value was subtracted out of each matrix spike. Note that CBGA also exhibited a higher RSD, and this analyte was also present in the blank matrix at a higher level. All analytes met accepted recovery limits with the exception of THCV, which was slightly over the acceptable value. This was most likely due to the fact that this extract contained an even larger amount of CBD, which overloaded the column and resulted in some loss of baseline resolution of those two analytes. The relative bias was also quite good, suggesting this assay can provide results that are reasonably close to true values.

A few comments are necessary on this method of spike recovery and validation. While rigorous methods for recovery and/or bias use analytical standards or CRMs, this is not an option for most laboratories due to legal restrictions. Obtaining 400 mg of analytically pure THCA to carry out spike recovery studies with 1000 mg of matrix is neither legally nor financially feasible for laboratories within the United States. There are also no accepted methodologies to which to compare, nor are authentic reference standards with the analytes in the matrix at known values available. The Emerald Test is an excellent first step towards verifying laboratory instrument calibration (40); however, this test is on an aliquot of methanol containing 0.1–1.0 mg/mL Δ^9-THC, which only covers one order of magnitude and says little about the laboratories' sample processing techniques. Having some manner to estimate the bias is useful since nonlinear calibration curves can provide satisfactory results when determining percentage recovery, but those values may not say much about the deviation from the true value. Similarly, a systematic error in sample processing, such as the pipetting error seen with technician A in Figure 2, can also provide satisfactory results for recovery yet still exhibit bias from the true value. Using extracts to spike cannabinoid analytes into a matrix is a procedure introduced by DeBacker et al. (15) and is in line with guidance from a number of organizations that recognize the difficulties with obtaining CRMs for drugs of abuse and state there are times an impure specimen of the may serve as reference material temporarily until a purer form

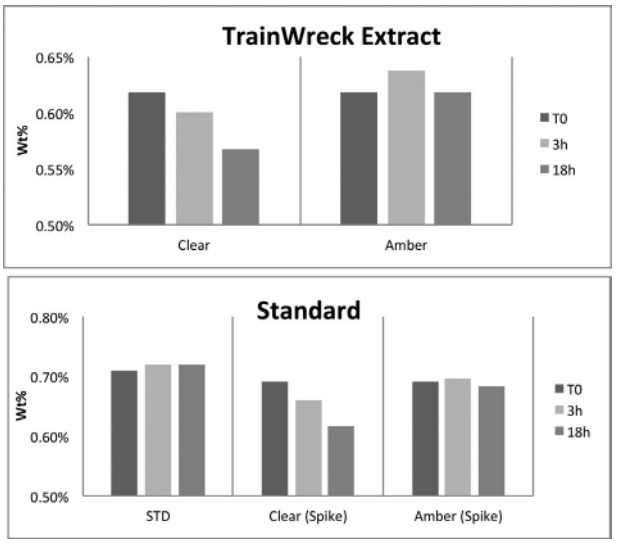

Figure 5. Decomposition of terpinolene in clear vials.

becomes available (21, 22). This study extended that procedure to a more efficient sample preparation method and a wider range of analytes and concentrations. Careful analysis of calibration curves, careful assessment of extracts to determine the "true" concentrations, and careful spike recovery studies provide preliminary methods for laboratories to validate their assays until more rigorous options become available.

These studies are best performed with oils or shatters since waxes and water hash can display enough heterogeneity on the 3–4 mg scale to give higher RSDs at the low spike levels. Note that in some cases the mass of the blank matrix plus the extract was over 1000 mg. The plant material must be added to provide putative adsorption sites that may affect recovery and possible cellular interferences; however, the extra mass had no effect on the final result since conversion of the raw result from mg/mL to wt% for both the spike (no matrix) and the matrix spike (matrix present) assumes 1000 mg of sample. In other words, the raw quantitative results in mg/mL were independent of blank matrix mass introduced if there were no matrix effects present or if those effects were minimized by the ISTD. The concentration of the specific analyte depends only on the mass of that analyte introduced via the extract and the volume of extraction solvent, and any possible change in volumes or analyte adsorption due to the extra mass introduced was accounted for by the ISTD. The spike recovery results in Table 5 show it made little difference if there was no matrix present or 1000 mg of matrix present, which highlights the critical nature of the ISTD in assays such as this.

This is especially useful with authentic samples that have variable moisture content. In theory, a 1000 mg sample with 15% moisture would add 150 µL of water to the extraction solvent, which dilutes the analytes. Similarly, extraction solvent can be absorbed by the plant matter, which concentrates the analytes. In both cases, ISTDs compensate for these changes and provide more accurate and reliable results (22, 37, 39).

Intermediate Precision

Both intraday and interday precisions for the entire extraction and analysis process were determined by extracting five cultivars five times over the course of 5 days, and five times on a single day. These cultivars were chosen to obtain a broad representation of analyte profiles and flower morphologies. In order to start with a homogeneous sample, a 20 g sample of bulk flower was homogenized on the first day for extraction, and the remainder of the ground flower was stored at –20°C between extractions. The interday analyses were carried out on Days 1–5, while the intraday analyses were all carried out on Day 5 ($n = 5$).

Tables 5–9 show each of the individual determinations as well as the averages and RSDs. Days 1–5a were used to determine the interday average and precision and 5a–5e were used to determine the intraday average and precision. The acceptable values for RSD_r are concentration dependent and given by Equation 3 (22). As a general rule, both interday and intraday precisions of the cannabinoid assay were well within accepted limits for all analytes and were generally less than 3%. It is also seen that intraday precision was generally slightly better than interday precision. The most notable deviations were seen with CBDA in the THV hybrid and THCA/THC in Master Kush; however, they still met accepted limits. The outliers found in Master Kush are most likely related to sample morphology, as this flower was the most difficult to handle due to morphological characteristics of the sample. It should be noted that while the cannabinoids are stable for short periods when stored below room temperature, decomposition of the acidic species was seen at room temperature within 24 h. While a chilled sample tray mitigates this issue, as a general rule samples are not processed if the analysis cannot be completed within 24 h.

Approximately half of the monoterpenes failed to meet acceptable interday precision limits, but they passed intraday precision criteria. This was a consequence of the sampling methodology, where large amounts of flowers were preground and stored at –20°C to ensure a more homogenous sample for testing over the course of the study. After grinding there was a rapid decrease of the many of the monoterpenes once flowers were homogenized in a grinder and the trichomes were ruptured. This loss occurs even when stored at –20°C. This can be seen in the table for the individual daily measurements, and this decrease was most significant on Day 1 after the initial homogenization. It should be noted that if Day 1 is removed from the analysis, the average values are higher and the RSDs are lower and satisfy Equation 3. This is due to the fact the rate of monoterpenes loss was the greatest during the first 24 h as seen in the tables. Note the cannabinoids and the less volatile terpenols and sesquiterpenes had comparable results for both intraday and interday analyses. This strongly suggests that once large sample sizes are homogenized they must be extracted the same day to obtain representative assay results for the monoterpenes.

This is a critical observation as typical sampling procedures for both agricultural crops and cannabis (25, 41) require an initial sample size that is compositionally representative of the crop. This large sample mass is subsequently homogenized so a small mass amenable to analysis can be extracted and still be considered representative of the whole. Once the initial sample is homogenized, it should be extracted immediately for accurate results.

Plant Variability

Agricultural crops inherently have a large amount of natural variation resulting from differences in environmental

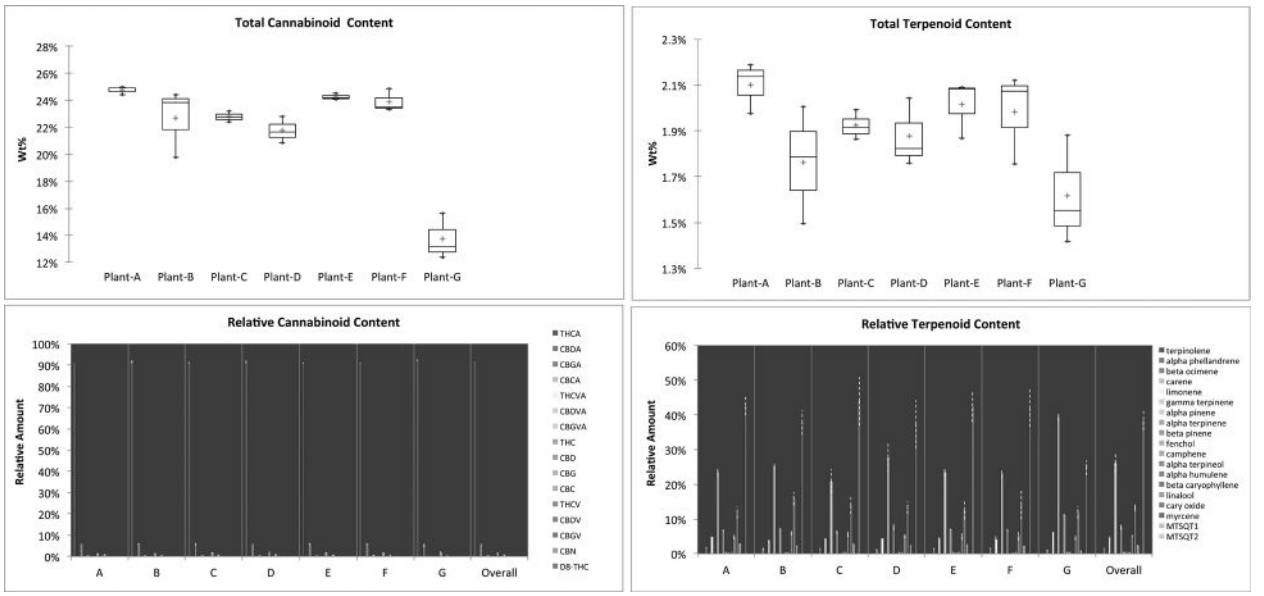

Figure 6. Heterogeneity of cannabinoid and terpenoid content in different flowers from the same lot.

conditions, genetic background, developmental stage, farming practices, and seasonal changes (42), which result in differences in organoleptic profile, appearance, nutrient composition, shelf life, and crop yield.

The method described herein has been used not only to determine the chemotype of cannabis preparations destined for human consumption but also to gain a better understanding of the sources of variability associated with the production of cannabis in a typical indoor production facility. Discussion of applying this method to look at the causes of variation is beyond the scope of this article; however, the relatively higher throughput can be used to look at analyte content across a typical cultivation site. Cannabis is a highly variable plant, and cannabinoid content in finished flowers is extremely inhomogeneous (41, 43). The variability in raw materials is the principal reason medical producers, such as GW Pharmaceuticals, produce standardized extracts to guarantee product uniformity (43). However, finished flowers remain popular products in the United States, and variation in this product is an important concept for dispensaries, patients, and legislators to understand when interpreting test results and enacting regulations.

A typical indoor California cultivation site was sampled as described in *Experimental* section. Boxplots are a typical way to graphically represent descriptive statistics and are used for the discussion on total content, while bar charts are used to display the individual analyte profiles. Exact methods of cultivation and/or product preparation are irrelevant since this represents typical finished flowers distributed as a "lot" of material (vide infra) at California dispensaries.

Figure 6 shows the boxplots of total cannabinoid content for seven plants that were each sampled at high, medium, and low heights within the canopy. The total cannabinoid content across plants ranged from 12 to 25% (w/w) of the dried plant material. This is a relative variation of approximately 100% for plants from the same production lot. Plant G is obviously an outlier, so after discarding this data the range is from 19.8 to 24.9%, which is still relative variation of 25% for flowers from the same lot of material. The boxplots show the intraplant variability of total cannabinoids is generally lower than the interplant variability; however, this does not hold true for every individual plant, and it can be seen the variation in Plant B is nearly the same as interplant variation. The relative cannabinoid profiles are also shown and they are identical for each plant, indicating the variation in cannabinoid content is due to variation in overall cannabinoid production.

The figure also shows the boxplots of the total terpene content by plant. The range of total terpenes in the plants was 1.4–2.2% (w/w). The relative variation in terpene content across these plants was approximately 90%. Variation in terpene content was generally much higher than the variation in cannabinoid content. In this case, the total content of Plant G fell within the range of other plants; however, the relative terpenoid profiles show there was a down regulation of myrcene and an up regulation of pinene. If all of these plants were combined into a single lot (as discussed below), inflorescences from Plant G could potentially have different properties than those from the rest of the lot. This is an extreme example, and in this specific case these plants exhibited morphological traits that would allow the cultivators to remove them from processing; however, even discarding this outlier the rest of the "typical" lot displays significant inhomogeneity.

There are no clear definitions as to what constitutes a lot of medical cannabis, but it has been suggested that a lot is no more than approximately 2 kg (based on average yield from an indoor facility using tables) and can be distinguished by plant count (no more than 20), or defined by those of identical genetic background in the space receiving similar nutritional and environmental influences (41). While definitions will undoubtedly continue to evolve, the above samples were chosen from a lot that conforms to these suggestions, and these results demonstrate the potential for large differences in secondary metabolite levels in plants that constitute the same lot of material.

This variation in analyte content is important to understand when interpreting test results from different analytical laboratories for the same lot of material at a given dispensary. Even laboratories using identical validated testing

methodologies can obtain disparate results unless they are testing the exact same flower, which is never the case. In many cases, the laboratory results are only as good as the sampling procedures. Unless specific sampling protocols are followed, collated data sets that display the "lot-to-lot" variation in different cultivars (41) may simply be observing plant-to-plant variation within a lot.

The boxplots illustrate the fact that a representative value for a lot of medical cannabis must come from a large sample size that is compositionally representative and reduced to a size amenable to testing; thus, homogenizing the sample followed by immediate extraction is required. However, real information about variation is lost when bulking samples in this manner, and plants such as "Plant G" get hidden in the average values. For true statistical power to look at variation across a single crop, a high throughput method such as this is needed. It may be more suitable to report a range of analyte values for a lot of material rather than a single value, which is a more realistic representation of the content of pharmacologically active compounds. Rather than bulking smaller samples and homogenizing them, reporting a range would require the separate analysis of many smaller samples to report an average value and a range. Similarly, when looking at causes of variation in any crop due to environment and/or gene-environment (GxE) interactions, a large number of samples need to be analyzed to determine statistical significance. Using properly validated analytical methods ensures laboratories are observing actual crop variation rather than deficiencies in analytical methodologies.

Conclusions

Presented herein is an optimized and validated method for the analysis of both terpenes and cannabinoids in cannabis from a single sample preparation. This method is only minimally affected by sample morphology and has been found to be fairly robust from a process standpoint. A single sample extraction procedure, which requires minimal sample handling and solvent usage, provides extract that can be analyzed for both terpenes and cannabinoids by GC-FID and HPLC-DAD, respectively. The single GC assay for the terpenes requires 36 min, and the two HPLC assays for the major and minor cannabinoids take 18 min each, so a complete profile of a sample is obtained in approximately 36 min on the two separate instruments from a single sample preparation.

The recovery, repeatability, and trueness of the method were found to be acceptable for all terpenes and cannabinoids analyzed, and this was demonstrated with spike recoveries at analyte levels that reflect those found in most samples on the market today. Both intraday and interday precisions of the complete extraction and analysis procedure were demonstrated on five different cultivars containing different cannabinoid contents, terpene profiles, and flower morphologies. In most cases, the RSD_r values were lower than the acceptable PRSDs. While interday precision was not satisfactory for the volatile monoterpenes, this proved to be an artifact of the experimental design and highlighted the need for timely analysis of samples once trichomes are ruptured. The throughput, accuracy, and precision of this assay make it amenable to assessing the development and environmental variation of this crop. This is important given the inherent variability of all agricultural crops and must be taken into consideration when evaluating the effects that cultivation, production, and processing techniques can have on chemotype.

While the methods applied to the analysis of the terpenoids are rigorous, those developed for the analysis of the cannabinoids required some decidedly nonideal procedures. However, this is a function of the current legal environment, and rather than wait for it to change, this provides pragmatic methods for typical laboratories involved in the analysis of cannabis to validate their methods through careful analysis of calibration curves and spike recoveries.

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