CUSTOMER APPLICATION NOTE 73413

Quantitating cannabinoids in edible chocolates using heated ultrasonic-assisted extraction

Complete **CANNABIS TESTING SOLUTIONS** for the Canadian market

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Goal

To develop and test a reliable quantitation of cannabinoids in chocolate using heated, ultrasonic-assisted extraction followed by cold stabilization and analysis using ultra-high-pressure liquid chromatography (UHPLC) coupled to either a diode array detector (DAD) or a mass spectrometer.

Introduction

With the recent legalization of recreational *Cannabis sativa L* (cannabis) use in Canada, routes of administration have diversified beyond typical inhalation. In Canada, 9.1% of respondents reported edible use as their primary method of cannabis consumption[1], and now that the production and sale of cannabis edibles has been legalized, this market is expected to surpass oils and tinctures. Underscoring this trend was a 2018 online survey of Canadians over 18, where 45.8% of respondents said they were willing to try edible cannabis products.[2] More specifically, respondents expressed interest in cannabis-based bakery and ready-made products (including candies); 46.1% and 26.6% respectively said they would consider buying these products.[2] These results were

consistent with a different survey of Canadians that indicated that the most commonly purchased cannabis edibles are baked goods (e.g., brownies and cookies) and candies (e.g., chocolate, hard candy, and gummies).[3]

The phytocannabinoids (i.e., the dominant pharmacologically-active constituents) in cannabis are a class of plant secondary metabolites that act on CB1 and/or CB2 receptors in the endocannabinoid system.[4] CB1 receptors have been linked to neurodegenerative disorders including multiple sclerosis and Huntington's Disease,[5] while CB2 receptors are involved in inflammatory processes.[6,7] *Cannabis sativa L* (cannabis) and its derivative products can contain an array of pharmacologically active secondary metabolites (Figure 1a) beyond the familiar phytocannabinoids Δ9-tetrahydrocannabinol (THC) and cannabidiol (CBD). Each phytocannabinoid is produced from acidic metabolic precursors (except CBN, which is a degradation product of THC and CBD) that have no documented effect on the endocannabinoid system.[8] The minor phytocannabinoids cannabigerol (CBG), cannabichromene (CBC), cannabinol (CBN), cannabidivarin (CBDV), and tetrahydrocannabivarin (THCV), each display a level of affinity towards CB1 and CB2 receptors, although, much less is known about their pharmacological effects.[9]

Following the inhalation of cannabis smoke or vapor, psychoactive effects are experienced within 3 minutes, with peak THC concentrations in plasma occurring within 3–10 minutes.[10] Contrastingly, following oral ingestion, effects are experienced 40–60 minutes after dosing, with peak THC concentrations occurring 1–5 hours after ingestion.[11,12] Thus, cannabis intoxication differs in its mechanism of action between the two routes, resulting in a different user experience. Regulations on a standard dose of THC in edibles have generally been set at 10 mg per serving in Canada. Dose control is essential for edible products, not only because of the delayed onset of intoxication, but also due to the potential for accidental consumption and cannabis poisoning. These problems have been exacerbated by quality control issues in consumer markets where edibles are legal. Determining the phytocannabinoid content of "THC free" products is also critical, since mislabeled products could result in accidental impairment; few products that claimed to contain a 1:1 ratio of THC to CBD actually contained this ratio.[13] Such inconsistencies in reported phytocannabinoid content represent a liability for producers and, more importantly, a major consumer safety and regulatory issue that needs to be addressed.

Dealing with such label claim issues requires the development of robust and accurate analytical methodologies to quantitate phytocannabinoids in edible matrices. However, edible matrices are complicated compared to cannabis flowers and extracts (where many excellent analytical methods have been published,[14-16] with many differing markedly in their macromolecular content, making a standard method for phytocannabinoid extraction and quantitation unlikely.[17] For example, a cannabis brownie may be comprised of 28.1% total fat, 49.3% carbohydrates, and 6.33% protein, while gummy bears are typically comprised of 77% carbohydrates, 6.9% protein, and 0% total fat.[18] Currently, few published methods exist for the extraction of major and minor phytocannabinoids from edible matrices. Previous studies have demonstrated the recovery of THC, CBD, and CBN from cannabis edibles, but failed to quantitate minor phytocannabinoids or any acidic forms.[19-23] These omissions are noteworthy given that minor phytocannabinoids likely have synergistic effects to the major phytocannabinoids, and their concentrations may impact user intoxication, experience, and metabolism.[9]

Given the high consumption of chocolate-based edibles[3] and their potential to be accidentally ingested by non-cannabis users, this matrix was identified as a critical need for the cannabis industry. Underscoring this was a call for methods issued by AOAC International in 2017, where they requested the quantitation of phytocannabinoids in milk, dark, and white chocolates. Recently, the extraction of phytocannabinoids from chocolate by cryomilling samples, followed by a QuEChERS extraction was reported.[23] While this method has merit, the chosen detection method of thin-layer chromatography coupled to DESI-mass spectrometry prevented the detection of minor or acidic cannabinoids, and DESI-mass spectrometry is not a widely implemented technique, so the uptake of the reported method is likely to be limited. Here we report the development of a robust analytical method to quantitate phytocannabinoids in chocolate using heated, ultrasonic-assisted extraction followed by cold stabilization and analysis using ultra-high-pressure liquid chromatography (UHPLC) coupled to either a diode array detector (DAD) or a Thermo Scientific™ Q Exactive™ Hybrid Quadrupole-Orbitrap™ mass spectrometer detector.

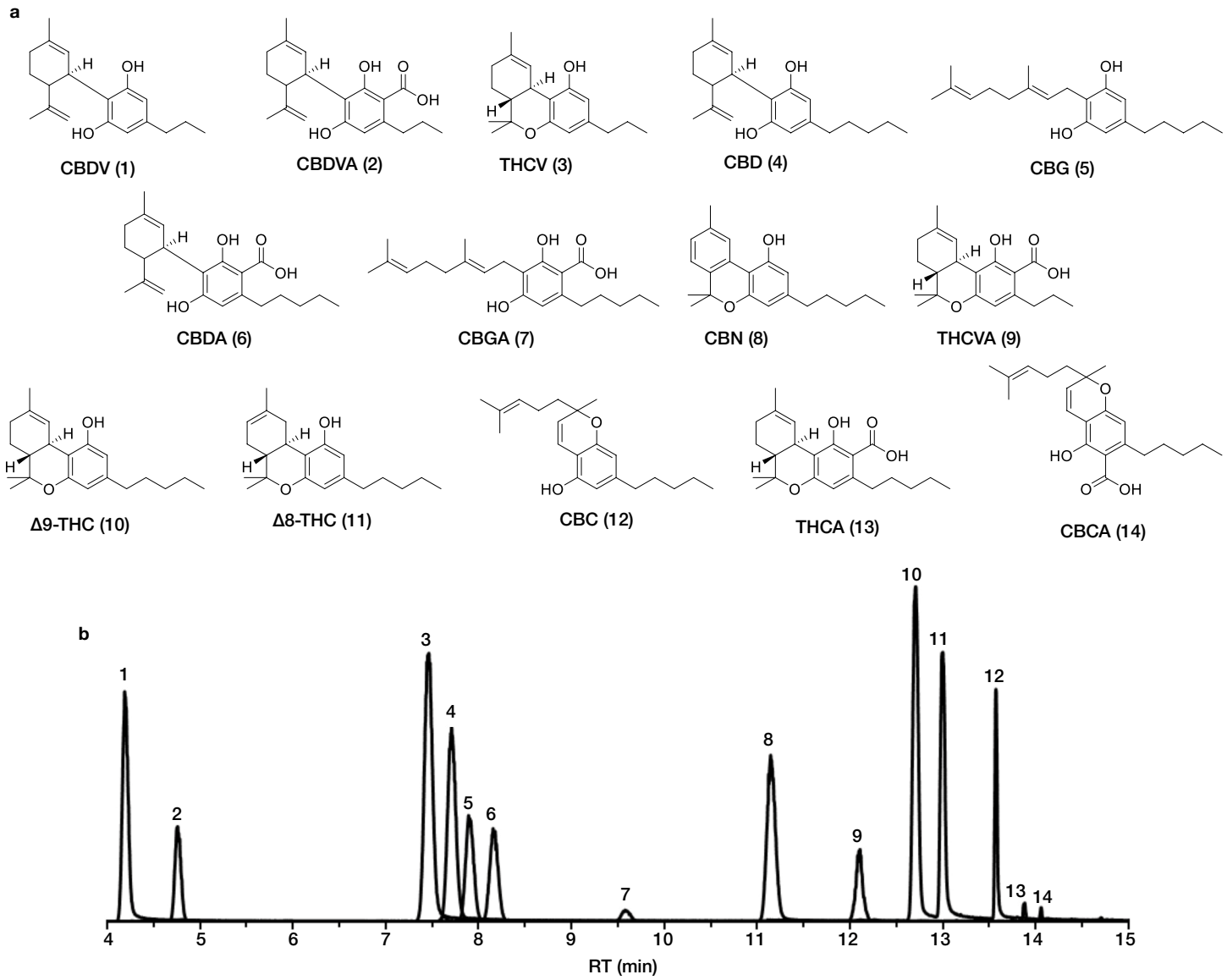

Figure 1. (a) Chemical structures of the 14 cannabinoids examined in this study; (b) chromatogram of a standard containing all 14 cannabinoids at 50 µg/mL

Experimental

Apparatus

- Micropipettes, Eppendorf Research® plus, 20 µL, 200 µL, 1000 µL, and 5000 µL (Eppendorf, Hamburg, Germany)
- Analytical balance, VWR-164AC 160 g (VWR International, Radnor, PA)
- Top loading balance, VWR-4502AC 4500 g (VWR International, Radnor, PA)
- Centrifuge 5804 R (Eppendorf, Hamburg, Germany)
- Polypropylene centrifuge tubes, 15 mL (Corning Inc., Corning, NY)
- Microcentrifuge tubes, 1.5 mL (Eppendorf, Hamburg, Germany)
- Heated sonicator, Digital-Pro 6L Professional Ultrasonic Cleaner
- Vortex mixer, Fisherbrand™ Digital
- HPLC autosampler vials, amber, 2 mL (Chromatographic Specialties, Brockville, ON, Canada)
- Pasteur pipets, Fisherbrand™ disposable borosilicate glass
- Vacuum concentrator, Thermo Scientific™ Savant™ SPD121P-115 SpeedVac™

Equivalent apparatus may be substituted.

Chemicals and solvents

- Acetonitrile, HPLC grade, Fisher Scientific
- Methanol, HPLC grade, Fisher Scientific
- Water, HPLC grade, Fisher Scientific
- Formic acid, Reagent grade, ≥95%

Cannabinoids

All sourced from Cerilliant Corp. (Round Rock, TX)

- Cannabinchromene (CBC), 1.0 mg/mL in methanol
- Cannabinchromenic acid (CBCA), 1.0 mg/mL in acetonitrile
- Cannabidiol (CBD), 1.0 mg/mL in methanol
- Cannabidiolic acid (CBDA), 1.0 mg/mL in acetonitrile
- Cannabidivarin (CBDV), 1.0 mg/mL in methanol
- Cannabidivarinic acid (CBDA), 1.0 mg/mL in acetonitrile
- Cannabigerol (CBG), 1.0 mg/mL in methanol
- Cannabigerolic acid (CBGA), 1.0 mg/mL in acetonitrile
- Cannabinol (CBN), 1.0 mg/mL in methanol
- Δ8-tetrahydrocannabinol (Δ8-THC), 1.0 mg/mL in methanol
- Δ9-tetrahydrocannabinol (Δ9-THC), 1.0 mg/mL in methanol
- Δ9-tetrahydrocannabinolic acid (THCA), 1.0 mg/mL in acetonitrile
- Tetrahydrocannabivarin (THCV), 1.0 mg/mL in methanol
- Tetrahydrocannabivarinic acid (THCVA), 1.0 mg/mL in acetonitrile

Equivalent chemicals may be substituted.

Preparation of solutions and calibration standards

Mobile phase A

Mobile phase A (0.1% formic acid in 85:15 water:acetonitrile, v/v) was prepared by diluting 1 mL formic acid in 850 mL HPLC-grade water and 150 mL HPLC-grade acetonitrile and mixed thoroughly. Solvents were added gravimetrically.

Mobile phase B

Mobile phase B (0.05% formic acid in 64:36 methanol: acetonitrile, v/v) was prepared by diluting 0.5 mL formic acid in 640 mL HPLC-grade methanol and 360 mL HPLC-grade acetonitrile and mixed thoroughly. Solvents were added gravimetrically.

Starting mobile phase

Starting mobile phase (60% mobile phase B, 40% mobile phase A) was prepared by adding 9 mL of mobile phase B and 6 mL of mobile phase A to a 15 mL centrifuge tube and mixed thoroughly.

Cannabinoid standard mix

Cannabinoid standard mix (50 µg/mL) was prepared by transferring 50 µL of each of the fourteen cannabinoid standards *(vide supra)* to a 2 mL amber HPLC vial along with 300 µL of the starting mobile phase and mixing thoroughly. This resulted in a stock solution where each of the cannabinoid standards was present at a concentration of 50 µg/mL.

Calibration standards

A calibration curve was prepared at 0.05 µg/mL, 0.1 µg/mL, 0.25 µg/mL, 0.5 µg/mL, 1 µg/mL, 2.5 µg/mL, 5 µg/mL, 10 µg/mL, and 25 µg/mL by diluting the 50 µg/mL cannabinoid standard mix with the appropriate volume of the starting mobile phase.

Preparation of chocolate extracts

Dark chocolate, milk chocolate, and white chocolate chips were purchased at local grocery stores and used as received. Five hundred milligrams of chocolate were accurately weighed using an analytical balance into a 15 mL centrifuge tube and 10.00 mL ACN was added. Samples were then manually mixed before sonication at 50 °C for 10 min, with additional manual mixing at 5 min and at the end of sonication. Extracts were placed in a freezer at -20 °C for 2 h. Afterwards, extracts were centrifuged at 3000 × g for 5 min. A 1 mL aliquot of each extract was transferred to a 1.5 mL microcentrifuge tube and centrifuged at 13,000 × g for a further 5 min. Extracts were diluted 100-fold, using the starting mobile phase as the diluent, into 2 mL amber glass vials and analyzed using by UHPLC-MS without further work-up.

Instrumental analysis

Liquid chromatography separations were carried out on the Thermo Scientific™ Vanquish™ Flex UHPLC system consisting of the following:

- Vanquish Binary Pump F (P/N VF-P10-A)
- Vanquish Split Sampler FT (P/N VF-A10-A) with 25 µL sample loop (P/N 6850.1911)
- Vanquish DAD FG (P/N VF-D11-A) with 5 µL flow cell (P/N 6083.0520)
- Column oven

A Thermo Scientific™ Accucore™ C18, 150 × 4.6 mm, 2.6 µm column (P/N 17126-154630) was used. The UHPLC conditions are listed in Table 1. The MS analysis was performed on a Q Exactive Hybrid Quadrupole-Orbitrap mass spectrometer with a HESI-II heated electrospray ionization source in positive mode. The MS and acquisition parameters are listed in Tables 2 and 3, respectively.

Table 1. UHPLC conditions for the separation of phytocannabinoids

Parameter	Value	
Column/pre-heater temperature	50 °C	
Flow rate	1.5 mL/min	
Injection volume	5 µL	
Gradient	Time (min)	%B
	0.0	60
	0.5	60
	8.5	65
	11.2	70
	13.0	95
	14.0	98
	16.0	98
	16.1	60
	18.0	60

Table 2. MS and source parameters

Parameter	Value
Sheath/aux gas	40/15 Arb units
Sweep gas	1 Arb units
Spray voltage	3.50 V
Capillary temperature	320 °C
S-Lens RF	50.0
Aux gas heater	350 °C
Acquisition mode	Full MS/dd-MS^2 (Top 5) (Table 3)

Table 3. Acquisition parameters for the full MS /dd-MS^2 (Top 5) scans

MS parameter	Full MS	dd-MS^2 (Top 5)
Resolution	70,000	17,500
AGC target	1e6	4e3
Maximum IT (ms)	250	50
Scan range (*m/z*)	200–400	2.0
Loop count	-	5
NCE	-	17.5, 35.0, 52.5
Dynamic exclusion (s)	-	2.0

Data processing

Data were acquired and processed with the Thermo Scientific™ TraceFinder™ (version 4.1) software package and Thermo Scientific™ FreeStyle™ (version 1.3) application. Data reduction and statistical calculations were performed using Microsoft® Excel®. The ICIS detection algorithm was used to integrate all data, using 5-point Savitzky-Golay peak smoothing and ±5 ppm mass accuracy tolerance for the precursor ion. All data was matched against a high-resolution accurate mass spectral library constructed using phytocannabinoid standards. Calibration curves were calculated as quadratic equations using 1/× weighting. Unless noted, data are reported as the mean ± the standard error of the mean (SEM).

Gravimetric analysis

Extracts (n = 3/condition) for each type of chocolate were prepared as above with the amount of time at -20 °C varied between 1, 2, and 4 h. Control extracts were kept at ambient conditions for the same time points. After cold stabilization, samples were centrifuged at 13,000 × g for 5 min. The resulting supernatant was evaporated to dryness using N_2. Dryness was evaluated by repeated weighing of samples until there was no more than a 0.25 percent difference between successive readings. The amount of residual material remaining after drying the supernatant was expressed relative to the original mass of the chocolate.

Single-laboratory validation with fortified samples

Extracts were fortified with the fourteen cannabinoid standards at 0.008% (low), 0.4% (mid), or 4% (high; w/w) after the final dilution to facilitate method validation using commercially available analytical standards. The absolute area response of all analytes in a blank sample analyzed immediately after a calibrator were required to be ≤0.1% of the absolute area of the same analytes in the high calibrator when carryover was evaluated. Recovery was determined by fortifying blank samples at low, mid, and high concentrations, with five replicates at each level in the chocolate three matrices. Repeatability (RSD_r) was determined for each matrix at the mid concentration and intermediate precision (single analyst, RSD_R) was calculated using three separate days with five replicates at the mid concentration for each matrix. The method detection limit (MDL), defined as the minimum concentration of analyte that can be reported with 99% confidence that a measured concentration is distinguishable from a blank sample,[24] was found by fortifying a total of seven replicates of each matrix across three days of validation at the concentration, with two analysts contributing to the preparation of extracts. The MDLs were calculated with the following equation:

$$MDL = t \times S_s$$

Where *t* is 3.134, the Student's *t*-value for a single-tailed 99th percentile t statistic and standard deviation estimate with 6 degrees of freedom, and S_s is the standard deviation of the seven fortified replicate samples for each matrix. The method reporting limit (MRL),[24] defined as the lowest concentration level reported for a given test method (which must be equal to or greater than the MDL), was determined from seven replicates in each matrix prepared on a single day by a single analyst. The Half Range for the Prediction Interval of Results (HR_{PIR}) is calculated from this data:

$$HR_{PIR} = 3.963 \times s$$

Where 3.963 is a constant for the seven extraction replicates and s is the standard deviation. This value is used to calculate the upper and lower Prediction Interval of Results (PIR):

$$PIR = (x \pm HR_{PIR}) / (\text{Fortified Concentration}) \times 100\%$$

If the upper PIR is less than 150% and the lower PIR is greater than 50%, the MRL is verified.

Results and discussion

Chromatography

The final chromatographic method was able to satisfactorily resolve all 14 cannabinoids contained within the standard mix (Figure 1b). CBDV was the first cannabinoid to elute, at 4.20 min. The final cannabinoid to elute was CBCA just under 10 minutes later at 14.06 min. The neutral form cannabinoids exhibit higher relative intensities than their acidic form counterparts, which was to be expected as the ESI system was run in positive ionization mode. In particular, the acidic cannabinoids CBGA (9.58 min), THCA (13.88 min), and CBCA (14.06 min) demonstrated much lower relative signal intensities than the other cannabinoids, but this did not present a problem in quantitating these compounds. The ability to quantitate acidic cannabinoids is highly advantageous, as it provides a better look at the potency of a cannabis-infused chocolate, since these acidic precursors can easily be decarboxylated to form their neutral cannabinoid counterparts.[8]

Cold stabilization

The extraction of cannabinoids from chocolate to acetonitrile also brings along waxes present in the chocolate (tellingly, solvent extraction has been used in the quantitation of fatty acids in chocolate[23]). These fats can have severely detrimental effects on the performance of analytical instruments and drastically reduce operational lifetime. In addition, coextracted fats could also represent a significant source of matrix interferences, reducing the accuracy of quantitation. Cold stabilization (also called winterization) was proposed as a solution to both these problems. At colder temperatures, fats and oils crystalize and precipitate out of the extraction solvent, allowing for their removal from the extracted cannabinoids after centrifugation. The cannabinoid analytes are contained within the supernatant and can be aliquoted without issue. For the purposes of this study, -20 °C was selected as the cold stabilization temperature due to the ubiquity of freezers with this temperature setting.

Gravimetric analysis

As depicted by Figure 2, there was a smaller amount of residual material present in the supernatant after evaporation in samples that had been cold stabilized at -20 °C for one, two, or four hours when compared to a sample that had been held at ambient room temperature (~21 °C) for the same amount of time. This effect was much more pronounced for the two- and four-hour time points than the one-hour time point. Two hours was ultimately selected as the time allotted to cold stabilization in this study, as it provided a balance between effective sample

clean-up and allowing for high sample throughput within a working day. There is a greater overall level of residual material in white chocolate than in milk chocolate, which in turn has a greater amount of residual material than dark chocolate. This is not unexpected when the differences in listed fat content provided by the labels of the three matrices are considered: white chocolate and milk chocolate contain more fat than dark chocolate. There is a slight decrease in the amount of residual material between time points at ambient room temperature, and this is likely a result of some small amount of fats and waxes precipitating out of solution even at these temperatures. However, it is clear that cold stabilization is far more effective at removing residual material from the solvent.

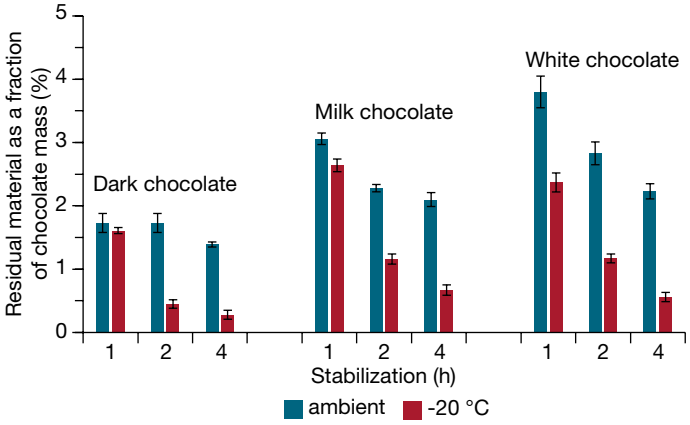

Figure 2. Mass of residual material in supernatant expressed as a percentage of the original mass of chocolate, with and without cold stabilization at -20 °C

DAD amenability

While the majority of data presented here was produced by an Orbitrap MS, all 14 analytes are resolved to the extent that the method is fully amenable to use with a diode array detector. All samples were run through the diode array detector mounted on the Vanquish Flex UHPLC after separation but before injection into the mass spectrometer. While cannabinoids at low concentrations may be difficult to see using a diode array detector when the sample injection volume is the 5 µL common to the rest of this study, the system and method had no issue with sample injection volumes up to 50 µL, greatly increasing the signal intensity and accounting for the differences in sensitivity between detectors. Figure 3 demonstrates this, providing an example chromatogram of a 1 µg/mL cannabinoid standard after a 50 µL injection detected by a diode array detector.

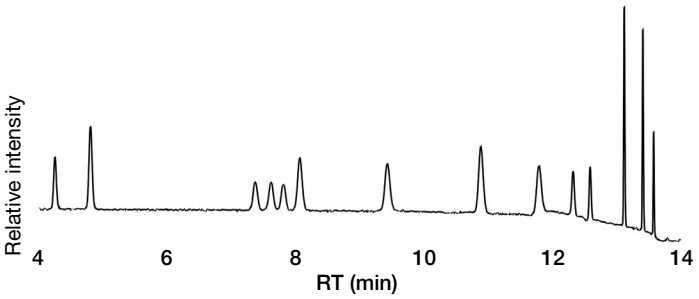

Figure 3. DAD data (230 nm) at 50 µL (vs. 5 µL for MS) demonstrating the ability of the chromatographic method to accommodate for the sensitivity differences between the reported MS-based detection and DAD detectors

Method validation

For the purposes of method validation, extracts were fortified at low, medium, and high levels (0.008%, 0.4%, and 4% weight of cannabinoid per weight of chocolate) as part of the final dilution. It was necessary to fortify at this point in the extraction due to the prohibitively high cost of infusing chocolates with the cannabinoids of interest at levels representative of the actual concentration range of cannabinoids in cannabis edibles.

Carryover was determined to be ≤0.1% for all analytes, ensuring that there was no concern of this interfering with measured responses.

Accuracy

Accuracy was expressed as the average percent recovery of five replicate samples at a given fortification level compared to the known concentration at that fortification level. For the purposes of this study, recoveries were determined accurate if they varied by less than or equal to ±20% at the low and medium levels and by less than or equal to ±10% at the high level. The accuracy of these spike recoveries is given in Table 4 for dark chocolate, Table 5 for milk chocolate, and Table 6 for white chocolate. For the most part, recoveries fall within these bounds with a few minor exceptions and two notable exceptions. In the dark chocolate matrix (Table 4) for example, all compounds fall within the prescribed region except for CBG and CBN, which both fall short of the minimum acceptable recovery level by a few percent. In milk chocolate (Table 5) it is apparent that cannabinoids were recovered high at the low fortification level; while the recoveries are still within 20% of the true value (with the exception of THCVA, which displayed an 123% recovery), it is clear that this set of data is biased high. Similarly, recoveries are biased low at the low fortification level in white chocolate (Table 6) with five cannabinoids (CBDV, CBDVA, CBDA, CBGA, and THCA) falling short of the requisite recovery levels. Overall,

however, these recoveries indicate that this method can accurately report cannabinoid concentrations in chocolate matrices, allowing for this method to be employed for routine testing.

Repeatability and intermediate precision

Repeatability, expressed here as % Relative Standard Deviation (RSD_r), was determined using peak area across a set of five replicate samples prepared at the mid fortification level. The calculated RSD_r for each compound is given by Tables 4, 5, and 6 for the dark, milk, and white chocolate matrices, respectively. In all three matrices, repeatability is quite good, with an average RSD_r across all compounds of 6.27% in dark chocolate, 6.62% in milk chocolate, and 3.87% in white chocolate. Similarly, intermediate precision was assessed as the % Relative Standard Deviation (RSD_R) of 15 total replicates over the course of three days (five replicates per matrix per day). As might be expected, RSD_R was greater than RSD_r. Across all 14 analytes in a given matrix, the average RSD_R was found to be 12.1% in dark chocolate, 7.99% in milk chocolate, and 9.45% in white chocolate. While the variance in RSD_r between replicates

Table 4. Method validation summary for cannabinoids in a dark chocolate matrix (data produced by an Orbitrap MS)

		Recovery			Conducted at mid level		Method limits (% w/w)	
ID	Analyte	0.008% w/w	0.4% w/w	4% w/w	% RSD_r	% RSD_R	MDL	MRL
1	CBDV	93.5	96.1	94.0	6.52	15.9	0.005	0.008
2	CBDVA	90.0	92.4	97.8	6.05	15.4	0.003	0.008
3	THCV	102	97.6	97.3	5.69	13.2	0.004	0.008
4	CBD	101	97.4	103	6.29	11.4	0.004	0.008
5	CBG	101	92.8	87.3	6.40	14.9	0.005	0.008
6	CBDA	99.3	91.3	95.8	6.79	12.2	0.003	0.008
7	CBGA	102	92.5	93.9	3.64	9.80	0.003	0.008
8	CBN	113	93.7	89.3	7.63	12.1	0.004	0.008
9	THCVA	104	93.3	91.6	7.66	10.4	0.004	0.008
10	Δ9-THC	101	94.8	91.3	5.96	9.14	0.005	0.008
11	Δ8-THC	96.8	97.6	92.6	6.51	17.6	0.004	0.008
12	CBC	103	94.8	90.4	5.94	10.3	0.004	0.008
13	THCA	92.3	88.7	92.1	6.15	8.64	0.003	0.008
14	CBCA	107	85.4	98.1	6.57	7.63	0.003	0.008

Table 5. Method validation summary for cannabinoids in a milk chocolate matrix (data produced by an Orbitrap MS)

		Recovery			Conducted at mid level		Method limits (% w/w)	
ID	Analyte	0.008% w/w	0.4% w/w	4% w/w	% RSD_r	% RSD_R	MDL	MRL
1	CBDV	112	95.5	98.8	6.60	7.26	0.003	0.008
2	*CBDVA*	117	92.5	101	6.03	7.05	0.003	0.008
3	THCV	116	95.4	99.0	6.03	7.59	0.003	0.008
4	CBD	116	97.9	99.6	7.05	7.75	0.003	0.008
5	CBG	114	97.8	99.2	6.00	7.44	0.003	0.008
6	CBDA	115	93.8	99.6	5.51	7.27	0.003	0.008
7	CBGA	119	97.9	101	6.79	6.89	0.003	0.008
8	CBN	114	97.6	96.3	6.39	7.76	0.003	0.008
9	THCVA	123	97.3	98.4	5.90	7.44	0.004	0.008
10	Δ9-THC	114	102	97.6	5.97	7.07	0.003	0.008
11	Δ8-THC	115	101	95.0	5.96	7.82	0.003	0.008
12	CBC	109	97.6	97.0	5.63	8.64	0.003	0.008
13	*THCA*	118	98.0	97.7	9.50	11.5	0.003	0.008
14	CBCA	109	100	103	9.26	10.3	0.002	0.008

Table 6. Method validation summary for cannabinoids in a white chocolate matrix (data produced by an Orbitrap MS)

		Recovery			Conducted at mid level		Method limits (% w/w)	
ID	Analyte	0.008% w/w	0.4% w/w	4% w/w	% RSD_r	% RSD_R	MDL	MRL
1	CBDV	77.6	94.7	91.2	4.13	12.0	0.006	0.008
2	CBDVA	76.1	92.5	95.8	4.12	10.2	0.004	0.008
3	THCV	86.1	96.6	97.1	3.42	9.60	0.005	0.008
4	CBD	86.1	96.0	104	3.74	8.56	0.005	0.008
5	CBG	84.8	91.9	82.8	3.79	12.9	0.006	0.008
6	CBDA	77.4	91.5	95.3	4.17	10.9	0.005	0.008
7	CBGA	76.0	90.8	88.9	3.18	8.66	0.006	0.008
8	CBN	98.0	92.0	84.7	3.16	10.2	0.005	0.008
9	THCVA	85.0	89.8	83.4	3.49	14.4	0.006	0.008
10	Δ9-THC	86.9	94.9	90.5	4.44	7.08	0.006	0.008
11	Δ8-THC	84.0	97.4	92.1	3.80	16.1	0.006	0.008
12	CBC	86.5	93.8	92.2	5.09	7.59	0.005	0.008
13	THCA	78.0	87.5	89.3	3.55	4.73	0.005	0.008
14	CBCA	93.5	85.7	91.6	4.06	5.24	0.004	0.008

in the dark and milk chocolates are not statistically different, the RSD_r is statistically smaller in the white chocolate samples compared to the other two matrices (Student's *t*-test, α = 0.05). The reason for this difference is not known but is likely instrumental in origin. The average RSD_R was not statistically different between milk and white chocolate, but the RSD_R was larger in dark chocolate than the other matrices (Student's *t*-test, α = 0.05). For all compounds in all three matrices, both RSD_r and RSD_R yielded Horwitz Ratios (HorRat)[25] between 0.3 and 1.3, meaning that they pass the internal laboratory validation requirements. As a result, it can be readily shown that the developed method is suitably reliable for extensive quantitative purpose.

Method Detection Limit and Method Reporting Limit

The MDL and MRL were determined in a similar fashion, the primary difference being that the verification of the MDL was conducted across three separate days of analysis while the MRL verification was conducted on a single day. For this method, the MRL was selected as 0.008% w/w, the concentration at the low fortification level. Per our validation procedures, the calculated value of the MDL for a given compound in a given matrix must be lower than the selected MRL. This was true across the board, with MDLs ranging from 0.002 to 0.006% w/w (see Tables 4, 5, and 6 for the MDLs and MRLs for each compound in the dark, milk, and white chocolate matrices). Both MDLs and MRLs were verified for all compounds in all matrices.

Conclusion

The final developed method allows for the reliable quantitation of cannabinoids in chocolate matrices, filling a pressing need of regulatory bodies and cannabis producers. The developed method provides several advantages in addition to its quantitative capabilities, such as cold stabilization. This technique proved to be effective at removing co-extracted waxes from the supernatant extracts, thereby reducing both matrix interferences and wear on the analytical instrument. The method's compatibility with multiple types of detector, including the popular diode array detector, gives the method a greater applicability than other methods developed to solve the same problem.[23] The current method shows great potential for ensuring the accuracy of product labels in cannabis-infused chocolate products. In the future, adaption and expansion of the method to encompass other challenging matrices such as gummy candies will serve to increase the applicability and usefulness of this method, providing greater accuracy in edible product labeling and thereby reducing consumer risk.

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