Identification of an Unknown Constituent in Hemp-Derived Extract Using Reversed-Phase Orthogonal Methodology

by Catharine E. Layton, Shawn C. Helmueller and Andrew J. Aubin*
Waters Corporation, 34 Maple St. Milford, MA, 01757, USA
**Corresponding author: Catharine_Layton@Waters.com*

The analysis of Cannabis sativa L. extracts can pose significant challenges due to complexities derived from extraction efficiency, cultivar genetic influences, and environmental factors such as weather and growing conditions. With over 400 constituents in the cannabis plant as a whole, potentially synergistic bioactive relationships are actively being investigated. The 'entourage effect' is an enhanced effect derived from a combination of two or more bioactive compounds. When referencing this phenomenon in the discussion of cannabis, this term usually is applied to cannabis strains selectively bred for target ratios of the most popular synergistic cannabinoids; such as cannabidiol (CBD) and (–)-*trans*-Δ⁹-tetrahydrocannabinol (Δ9 THC), although more recently, it has been applied to low Δ9 THC cannabis varieties classified as hemp. The goal of this manuscript is to demonstrate an approach for identification of an unknown constituent, present in a significant amount, in hemp-derived extract by employing orthogonal, reversed phase separation techniques on a fast, highly efficient Ultra-High Performance Liquid Chromatography (UHPLC) platform.

Introduction

Cannabis is a complex plant with over 400 chemical entities of which more than 60 are cannabinoid compounds [1]. Even though cannabis has been used and cultivated by mankind for at least 6000 years [2], our current knowledge regarding its pharmacological properties is based on studies which have taken place only since the end of the nineteenth century. Cannabis has significant potential for enlarging the library of naturally occurring bioactive metabolites. To date, several phytochemicals have been described in cannabis derived extract [3] including essential mono, poly and saturated fatty acids, cannabinoids, terpenes, plant sterols, vitamin E and chlorophyll. Many of these compounds are capable of optimising health and wellness alone, however the interplay of these active and inactive compounds as synergists can produce an enhanced physiological effect including inhibition of side effects, improved absorption, bacterial defence, and the ability to impact multiple molecular targets [4,5].The 'entourage effect' is a term introduced in cannabinoid science in 1998 to represent the novel endogenous synergistic cannabinoid molecular regulation route [6]. More recently, a similar phenomenon referred to by some as the 'hemptourage effect', has been applied to low THC cannabis varieties classified as hemp [7,8].

Cannabinoids accumulate in the secretory cavity of the glandular trichomes, which are mainly found in female flowers and in most aerial portions of the plant. They have also been detected in low quantity in other parts of the plants including the seeds [9] roots [10] and pollen [11]. Adding to the complexity, the concentration of phytocannabinoids depends on the age, variety, growth conditions, nutrition, humidity, light intensity, harvest time and storage conditions of the plant [12]. Many cannabinoids are present as non-enzymatically decarboxylated acids, which are converted into their corresponding neutral form after harvest and upon heating [13].

Cannabis testing laboratories provide important information regarding chemical composition of the cannabis plant for the same purpose that quality assurance laboratories provide purity results to ensure the safety of pharmaceutical products. In the absence of federal guidance, states have led cannabis testing laboratories to develop proprietary chromatographic methods in order to meet state testing specifications for raw materials, extracts, and/or infused products such as beverages, edibles and topicals. Within the past few years, studies including those performed by the US Food and Drug Administration, have reported the occurrence of inaccurate potency reporting for a significant number of infused cannabis products [14]. Factors which influence inaccurate results may be attributed to inadequate sampling procedures, inconsistent manufacturing techniques, or inaccurate testing methods.

In an attempt to capitalise on the need for improved testing methods, some analytical instrumentation manufacturers are actively promoting 'turn-key' chromatographic testing solutions. This one-size-fits-all standardised chromatographic separation may work well for the separation of pure cannabinoid reference standards however may not be adequate for product variability inherent during routine sample testing. Laboratories that purchase these solutions are left with the potential challenge of re-developing these 'turn-key' chromatographic methods in order to separate, quantify and identify compounds of interest within a particular batch or formulation [15]. Adding to this challenge, several of the important cannabinoids are isobars (identical in chemical composition and nominal mass) and are therefore distinguishable only by high-resolution MS/MS analysis (Table 1).

A multi-levelled approach to understanding, identifying, quantifying, and controlling bioactive compounds should include orthogonal or fundamentally complementary chromatographic methodology, as is often employed in pharmaceutical testing [17].

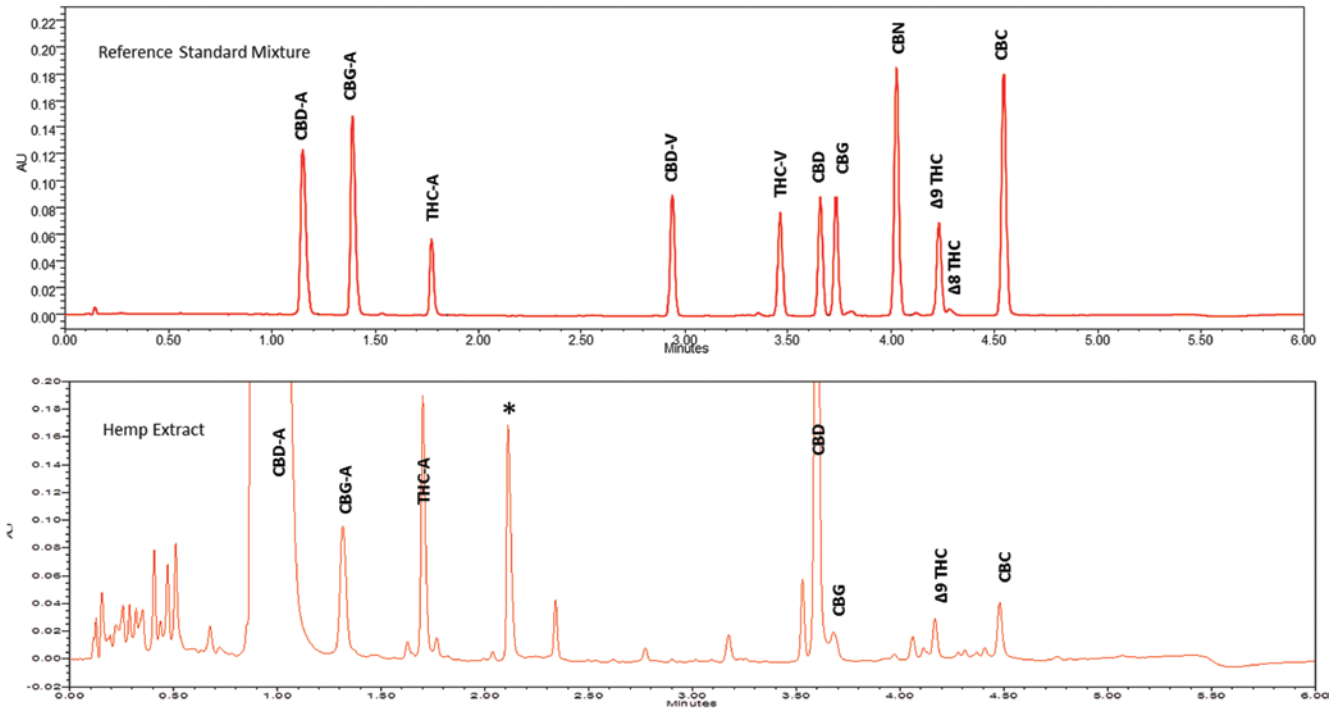

Figure 1: Overlay of cannabinoid reference standard mixture and hemp extract separated using mobile phase at pH 10. The unknown constituent is marked by an asterisk.

Chromatographic orthogonality is the use of multiple separation mechanisms in order to gain additional analyte information. It is recognised that many cannabis laboratories may not have access to high-end scientific instrumentation therefore a simple approach to chromatographic orthogonality is most useful. Orthogonality was accomplished in this study using basic and acidic pH mobile phases on the same chromatographic platform (i.e. column and instrument) to identify an unknown constituent in cannabis extract.

Experimental

Hemp Extract Preparation

At a partnering cannabis testing laboratory, hemp extract was generated from a 20 lb feed stock of Vermont grown hemp (*Cannabis sativa L.*) with seeds and stems removed. The buds and leaves were ground, homogenised, and divided into five 4 lb bags. A sample was analysed from each bag with the average total cannabinoid content determined as 5.04 wt%. Six extractions were performed at the 5 L scale via a solvent-free Bio-Botanical Extraction System (SFE-BBES) (Waters, Milford, MA. USA).

Table 1: Empirical formula of major cannabinoids and monoisotopic mass [16].

Cannabinoid	Empirical Formula	Monoisotopic Mass
Δ9 THC CBD CBC Δ8 THC	$C_{21}H_{30}O_2$	314
THC-A CBD-A	$C_{22}H_{30}O_4$	358
THC-V CBD-V	$C_{19}H_{26}O_2$	286
CBG-A CBG	$C_{22}H_{32}O_4$ $C_{21}H_{32}O_2$	360 316
CBN	$C_{21}H_{32}O_2$	310

Table 2: SFE – BBES extraction and collection conditions

Extraction	Condition
Flow Rate	170 g/min
Pressure	344 bar
Temperature	50°C
Time	210 nm
Collection	**Condition**
CS1 Pressure	158 bar
CS1 Temperature	45°C
CS2 Pressure	75 bar
CS2 Temperature	40°C
CS3 Pressure	53 bar
CS3 Temperature	35°C

The cyclone separator 1 (CS 1) extracts were combined and homogenised. Ethanol was used to clean CS 1 post extraction. Approximately 85 g raw extract were added to 1.2 L of the ethanol wash, and the solution stored at -20°C until analysis and purification. Plant waxes were removed from the homogenised solution by vacuum filtration, and pigments were removed through a patent pending clarification strategy (Table 2).

Orthogonal Reversed-Phase Chromatography

Reversed-phase separations were performed using an ACQUITY H-Class UPLC System (Waters, Milford, MA. USA) equipped with a PDA (UV) detector at 228 nm, with a 4.8 nm resolution, and a 3D data λ range at 200-400 nm. The UHPLC was also equipped with a single quadrupole QDa (MS) detector with a programmed ESI (-) mass scan range of 100-600 Da, cone voltage at 15 V, capillary temperature of 500°C and capillary voltage of 0.80 kV. An accessory fraction collector (Waters Fraction Manager – Analytical (WFM-A)) (Waters, Milford, MA. USA) was added for automated collection of the peak of interest. Separations were achieved using an ACQUITY CSH C_{18}, 130Å, 1.7µm, 2.1 mm x 50 mm column (Waters, Milford, MA. USA) at a temperature of 30°C and a flow rate of 1.0 mL/min. All data was collected and processed by Empower® 3 Chromatography Data Software.

A 'turn-key' chromatographic separation of the major cannabinoid reference standards (Cerilliant, Round Rock, TX. USA) cannabidivarian (CBD-V, C-140), tetrahydrocannabivarian (THC-V), cannabigerol (CBG, C-141), Δ^9 –trans-tetrahydrocannabinol (Δ^9-THC), Δ^8 –trans-tetrahydrocannabinol (Δ^8-THC, T-032), cannabinol (CBN), Δ^9 –trans-tetrahydrocannabinolic acid (THC-A), cannabidiol (CBD), cannabidiolic acid (CBD-A), cannabigerolic acid (CBG-A) and cannabichromene (CBC) prepared at 1.0 mg/mL in methanol was performed at pH 10 using 10mM ammonium bicarbonate (mobile phase A) and acetonitrile (mobile phase B).

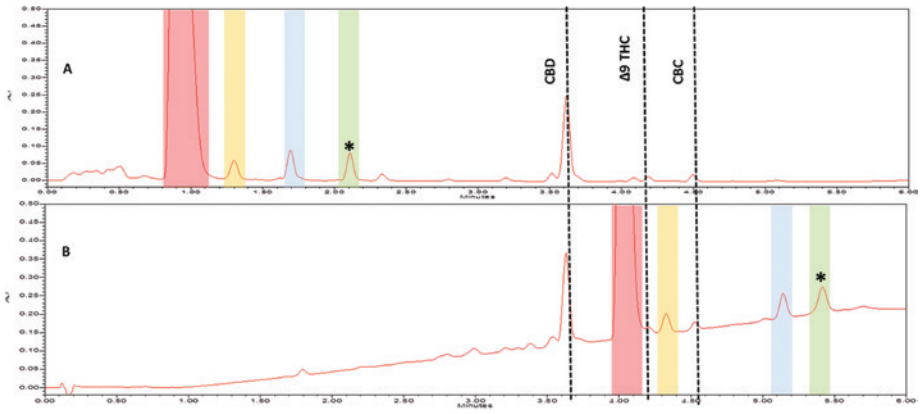

Figure 2: Elution order of biosynthetic cannabinoid precursor forms in hemp extract as a result of mobile phase pH. Separation at (A) pH 10 and (B) pH 3. Cannabinoid acids are tracked in colour; CBD-A (red), CBG-A (yellow) and THC-A (blue). The unknown constituent (green) is marked with an asterisk.

A separation of the hemp extract was performed at pH 10 using the 'turn-key' method with 10mM ammonium bicarbonate (mobile phase A) and acetonitrile (mobile phase B). As an orthogonal approach, a separation at pH 3 was performed using 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B). For efficiency purposes, separation conditions were the same for both pHs starting at 36% mobile phase B with a 0.5 minute hold. A linear ramp was made over 5 minutes to a maximum of 84% mobile phase B. The separation was followed by a rapid equilibration back to starting conditions.

The unknown constituent of interest was collected with the WFM-A using the separation at pH 10 and evaporated to dryness. After reconstitution of the collected fraction in 200 µL of 50:50 methanol / water (v/v), the solution was injected to confirm that the retention time of the collected peak matched the unknown constituent retention time in the extract. The fraction was heated at 120°C for one hour in a heat block to induce decarboxylation.

Results and Discussion

The separation of neutral cannabinoid reference standards CBD, CBG, CBN, Δ9 THC, Δ8 THC and CBC, as well as biosynthetic precursor forms CBD-A, CBG-A and THC-A, and variants CBD-V and THC-V, was achieved using a potential 'turn-key' reversed-phase method at pH 10 (Figure 1). In raw hemp extract, a peak of significant area overlaid with the retention time of CBD-A, as expected in unheated, selectively bred high CBD hemp. Cannabinoids CBG-A, THC-A, CBD, CBG, Δ9 THC and CBC were also present in the extract in lower quantities by detection at 228 nm. An unknown constituent eluted at 2.1 minutes and exhibited an area response at the detection wavelength comparable to THC-A (Figure 1). The unknown constituent's retention time and PDA-UV profile did not correspond to any of the cannabinoid reference standards, with a distinct lambda max at 246 nm, while the QDa-MS *m/z* of 357.2 Da was identical to isobaric cannabinoids CBD-A and THC-A (data not shown).

By reversed-phase, the elution order of analytes which can be ionised is influenced by mobile phase pH, while the retention of neutral compounds is not affected [18].

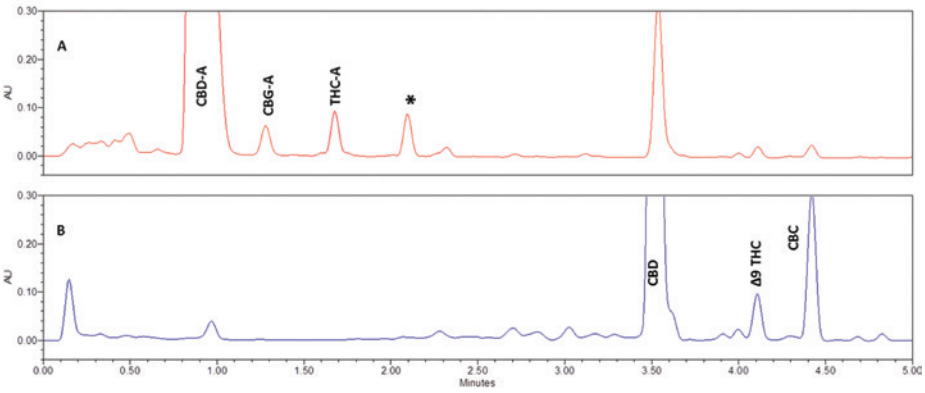

Figure 3: UPLC separation in basic mobile phase of raw hemp extract (A) before decarboxylation and (B) after decarboxylation. The unknown peak of interest is marked with an asterisk.

Acidic cannabinoids; CBD-A, THC-A, and CBG-A showed greater retention in acidic mobile phase (pH 3) than when separated under basic conditions (pH 10), while the retention of the neutral cannabinoids remained the same (Figure 2). The elution order of the unknown constituent was altered by the change in pH, therefore it was determined that the unknown constituent was susceptible to ionisation.

When the extract was heated to induce decarboxylation, the acidic cannabinoids CBD-A, THC-A, CBG-A, and the unknown constituent, were no longer present in the chromatography. There was an increase in the abundance of the neutral cannabinoids, as expected from the conversion of the acid form to the neutral form induced by the decarboxylation process (Figure 3) [19].

The unknown constituent was collected after separation at pH 10 via the WFM-A. The isolated fraction was subjected to heat to confirm that it, when present alone, was susceptible to decarboxylation. Two main peaks were observed after heat exposure (Figure 4). When separated at pH 10, the first eluting peak corresponded with the retention time of the unknown constituent, while an additional peak correlated to the retention time of the cannabinoid reference standard CBC. This information, including the vulnerability to ionisation, as shown when orthogonal chromatographic mobile phase pHs were employed, and isobaric similarity to CBD-A and THC-A, suggested the unknown constituent was a biosynthetic acid form of CBC, known as cannabichromenic acid (CBC-A).

To investigate the hypothesis, a certified CBC-A reference standard was obtained. The CBC-A reference standard showed a retention time and QDa-MS spectra that matched that of the unknown constituent. Since many cannabinoids are isobaric, the mass information was not enough alone to confirm the unknown constituent's identity. The unique PDA-UV spectral profile of the CBD-A reference standard provided the most conclusive result. The PDA-UV spectral profile of CBC-A reference standard was identical to that of the unknown constituent with a major lambda maximum at 246.4 nm, and minor maxima at 293.1 nm and 326.4 nm (Figure 5). Combined with the retention time and MS results, the unknown constituent in the hemp extract was positively identified as the non-decarboxylated acid form of CBC, known as CBC-A.

CBC, a non-psychoactive cannabinoid, has been reported to have a wide range of therapeutic properties with anti-depressant and anti-inflammatory activity. Some studies on mice have shown that the administration of

THC or CBD with CBC increases inflammatory modulation, suggesting a strong synergistic effect [20]. Studies in the 1970s suggested that CBC was the second most abundant cannabinoid in cannabis plants, although today that is no longer the case. This is due to selective breeding favouring plants high in THC and CBD, with varieties originating from the tropics with initially high concentrations of CBC having little resemblance to modern commercial hybrids [20].

Conclusion

The data demonstrates the utility of orthogonal methodology employed within a reversed-phase platform to determine the identity of an unknown constituent in Vermont hemp extract as CBC-A. In the case presented, the data obtained from the proposed 'turn-key' separation at a single pH, was not enough alone to draw unequivocal conclusions regarding the identity of the compound of interest. The data also reveals the utility of MS, PDA-UV, and a fraction collector such as the WFM-A, as important tools to distinguish isobaric constituents in C. *sativa* extract such as CBD-A, THC-A and CBC-A.

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References

1. Atakan, Zerrin. "Cannabis, a Complex Plant: Different Compounds and Different Effects on Individuals." Therapeutic Advances in Psychopharmacology 2(6): 241–254. 2012.

2. Li, H. "An Archaeological and Historical Account of Cannabis in China". Economic Botany 28: 437–448. 1973.

3. Gould, J. "The Cannabis Crop". Nature 525 S2–S3. 2015.

4. Wagner, H. "Synergy Research: Approaching a New Generation of Phytopharmaceuticals". Phytomedicine. 16 (2-3): 97–110. 2009.

5.Burnett, Malik. "Medical Marijuana: Much More Than Just THC and CBD". Medical Jane.

6. Mechoulam, R. and Ben-Shabat, S. "From gan-zi-gun-nu to anandamideand 2-arachidonoylglycerol: The Ongoing Story of Cannabis". Nat. Prod. Rep.16: 131–143. 1999.

7. Plus CBDiol. "What is CBD". www.pluscbdoil.com. Date accessed December 26, 2017.

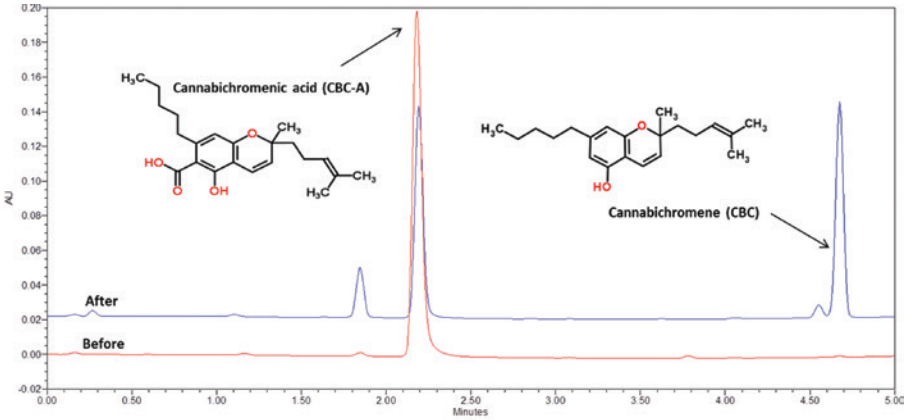

Figure 4: Overlay of the CBC-A reference standard before and after decarboxylation with partial conversion to the neutral form CBC. The chemical structure [16] is shown next to the respective peak.

8. Leafly. "Understanding the Hemptourage Effect and the Diversity of Compounds". www.leafly.com. Date accessed December 26, 2017.

9. Ross S. A., Mehmedic Z., Murphy T. P., ElSohly M. A. "GC-MS Analysis of the Total Δ9-THC Content of Both Drug- and Fiber-type Cannabis Seeds". J. Anal. Toxicol. 4 715–717. 2000.

10. Stout J. M., Boubakir Z., Ambrose S. J., Purves R. W., Page J. E. "The Hexanoyl-CoA Precursor for Cannabinoid Biosynthesis is Formed by an Acyl-activating Enzyme in Cannabis Sativa Trichomes". Plant J. 71 353–365. 2012.

11. Ross S. A., ElSohly M. A., Sultana G. N. N., Mehmedic Z., Hossain C. F., Chandra S. "Flavonoid Glycosides and Cannabinoids from the Pollen of Cannabis sativa L". Phytochem. Anal. 16 45–48. 2005.

12.Khan B., Warner P., Wang H." Antibacterial Properties of Hemp and Other Natural Fiber Plants: A Review". Bioresources 9 3642–3659. 2014.

13. Flores-Sanchez I. J., Verpoorte R. "Secondary Metabolism in Cannabis". Phytochem. Rev. 7, 615–639. 2008.

14 .Vandrey, R et.al. "Cannabinoid Dose and Label Accuracy in Edible Medical Cannabis Products". JAMA. 313(24):2491-2493. 2015.

15. Egerton, David. "Defining Cannabis Standards in a Green Market". American Laboratory. February 17, 2017.

16. Royal Society of Chemistry. http://www.chemspider.com . Date accessed December 22, 2017.

17. Argentine MD, Owens PK, Olsen BA. "Strategies for the Investigation and Control of Process-Related Impurities in Drug Substances". Advanced Drug Delivery. 59(1):12-28. Rev. January 10, 2007.

18. Layton, Catharine. "Beginners Guide to Preparative Chromatography". Waters Corporation. Library of Congress Number 2017933626. 2017.

19. Wang,Mei et.al. "Decarboxylation Study of Acidic Cannabinoids: A Novel Approach Using Ultra-High Performance Supercritical Fluid Chromatography/Photodiode Array Mass Spectrometry". Cannabis Cannabinoid Research. 1(1): 262–271. 2016.

20. Alchimia. "Cannabichromene (CBC); Therapeutic Potential". https://www.alchimiaweb.com. Date accessed December 22, 2017.

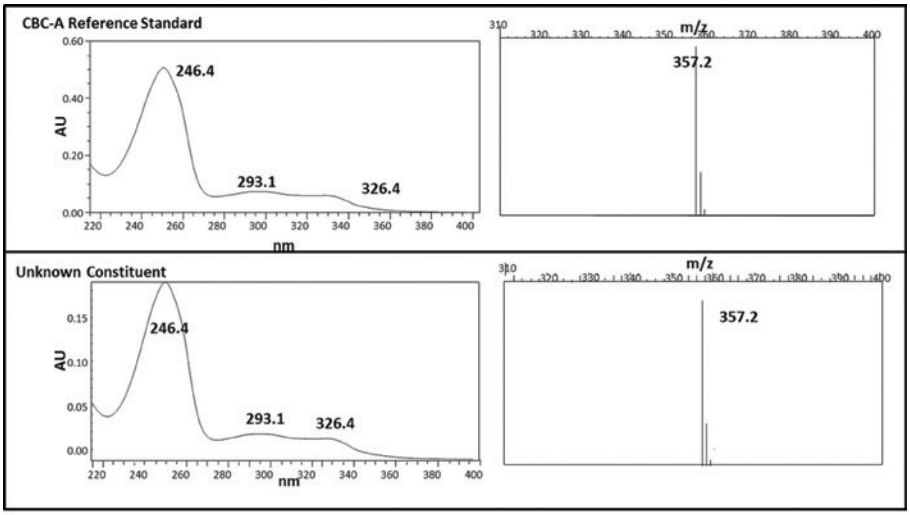

Figure 5: PDA-UV and QDa-MS spectra of (A) the unknown eluting at 2.1 mins in hemp extract and (B) CBC-A reference standard.