Chiral Analysis of Cannabinoids Using High-Pressure Liquid Chromatography

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Abstract

As synthesis of THC from hemp-derived cannabidiol (CBD) rises in popularity due to the 2018 U.S. Farm Bill, more rigorous testing methods of cannabis products may be necessary, including, but not limited to, chiral analysis, to ensure unregulated conversion products are not illegally entering the commercial market. Conversion of CBD to delta-9-tetrahydrocannabinol (Δ^9-THC) occurs under acidic conditions, giving rise to an unnatural (+)-Δ^9-THC enantiomer that does not originate in cannabis plants. This study focused on the development of a method to investigate the chiral separation of cannabinoids (namely Δ^{9-}-THC) using high pressure liquid chromatography (HPLC) equipped with a Daicel CHIRALPAK® ID amylose-based chiral column. Method optimization was first performed, and then the optimized method was employed on various cannabinoid isolates to determine the enantiomeric ratios of Δ^9-THC in the samples. This research indicates that HPLC chiral analysis could act as a viable means of determining whether cannabis concentrates originate from natural or synthetic sources.

Introduction

Cannabinoids are chemicals found in *Cannabis sativa L.* (Cannabis). Some of the most common phytocannabinoids include tetrahydrocannabinol (THC), cannabidiol (CBD), cannabidaverin (CBDV), cannabichromene (CBC), and cannabinol (CBN).[1] These neutral cannabinoids are formed via decarboxylation of their corresponding, most naturally abundant, acid precursor molecules (THCA, CBDA, CBDV, etc.), via heat or light exposure.[2] The most naturally abundant psychoactive phytocannabinoid is (-)-Δ^9-*trans*-THC.[3] Overall, the most published literature exists for (-)-Δ^9-*trans*-THC, given that it is responsible for the psychoactive effect of the plant.[1] With increasing popularity of cannabis and its growing legalization in many regions, arises the need for analytical methods to characterize cannabis and its derivatives.

Cannabinoids exhibit many pharmacokinetic activities, like other pharmacological compounds.[4] Like other pharmaceuticals, the stereochemistry of cannabinoids affects their pharmacological activity, which can thereby potentially impact the potency and/or toxicity of the compounds. It is well documented that, like other living organisms, cannabis tends to biosynthetically produce secondary metabolites, such as terpenes and cannabinoids, as single enantiomer compounds, due to downstream requirements for transformation or uptake.[5] This begs the question of whether lesser naturally occurring enantiomeric forms, likely arising from synthetic cannabis products, may have altered effects on cannabis (CB) receptors. This is particularly crucial when we consider that two enantiomers may interact differently in anisotropic/chiral media, such as certain receptors in the human body. These effects may be harmless, but other times may be disastrous (e.g. thalidomide).[6]

While there is little to no evidence of the effects of naturally occurring cannabinoids on CB receptors, there is some evidence that there are varying potency effects among enantiomeric

forms in synthetic cannabinoids**.** For instance, researchers have determined that the (-)-enantiomer of the synthetic cannabinoid dexanabinol (HU-210) is a highly potent active cannabinoid (similar to THC), whereas its (+)-enantiomeric counterpart (HU-211) is reported to completely lack the psychoactive properties characteristic of naturally derived THC.[3] This highlights the importance of separating the positional and stereoisomers of cannabinoids, prompting further investigation into the pharmacological effects of cannabinoids on the human body.[3,4] To analyze stereochemistry and positional isomers in particular, chiral analysis is a powerful tool.

Chiral analysis refers to the characterization of enantiomers in chiral compounds. An enantiomeric compound is a compound with two possible configurations that are nonsuperimposable mirror images of one another. Chiral analysis is typically achieved with some form of liquid chromatography (such as high-pressure liquid chromatography (HPLC)), and some form of chiral stationary phase (CSP), commonly referred to as a chiral column.[7] Following this analysis, the ratio of enantiomers can be observed within the analyte. HPLC is used to separate compounds based on the interaction between the mobile phase and the stationary phase. CSPs manipulate HPLC, based on the principle that enantiomers of the same compound can only be distinguished by the direction in which they rotate polarized light, biological activity, and their interaction with other chiral molecules.[7]

Therefore, selecting a CSP compatible with the conditions of the chromatographic system to which it is equipped is crucial for optimal baseline separation of enantiomers. Among numerous CSPs that have been developed thus far, phenylcarbamates of cellulose and amylose have exhibited wide applicability to a broad spectrum of compounds.[7] In recent decades, immobilized polysaccharide-based CSPs have been advantageous for chiral separations due to

significant improvement regarding the number of compatible eluent/injection solvents (due to immobilization), enantioselectivity, loading capacity requirements, and column lifespans.[7]

Enantiomeric ratios, determined from chiral analysis, can lend evidence as to whether a cannabis product is plant-derived or synthesized. For instance, natural Δ^9-*cis*-THC exists as a scalemic mixture (non-racemic/not a 1:1 mixture of enantiomers), with 80-90% enantiomeric purity (ee).[8] CBC is unusual among cannabinoids, as it has been reported to present as either a racemate or a scalemate, when derived from natural cannabis sources.[8,9] Additionally, as previously established, cannabis tends to predominantly biosynthesize compounds in a single enantiomer form.

Therefore, the known natural enantiomeric ratios of various cannabinoids can allow for the determination of whether a cannabis product is synthetic or plant-derived upon chiral analysis. This can be highly advantageous, as many businesses claim that their cannabis products are 100% plant-derived, despite these claims potentially being highly disputable with the rise of legal hemp in the U.S., due to the 2018 Farm Bill. Following the 2018 U.S. Farm Bill, many cannabis products on the market are being derived and synthesized from hemp derived materials. For instance, CBD can be prepared by extraction from hemp.[10] Many other cannabinoids, such as Δ^8-THC, are then subsequently converted from hemp-derived CBD, under acid-catalyzed conditions.[10]

Many byproducts can result from organic synthesis due to residual organic solvents. Additionally, major impurities and/or unknown analogues have been detected in cannabis products, including an unknown compound, for which the name “cannabidibutol” was recently adopted.[10] Researchers have detected several impurities in Δ-8-THC, such as CBDV, 5”hydroxy-CBD/THC, and cannabidihexol.[11] This is a huge area of concern, as impurities in

cannabis products have the potential to cause unknown, potentially adverse health effects for consumers, particularly if stereochemistry is not controlled during a chemical synthesis. This prompts the investigation of impurities in cannabis products, via rigorous chemical analyses, including chiral analysis. With more widespread use of chiral analysis of cannabis compounds, chiral analysis could also be highly useful for many applications, including seized drugs in forensics, as it allows for a more detailed sample profile.[4] Chiral analysis may also eventually become a widely accepted practice in cannabis regulatory testing to regulate synthetic/conversion products in the cannabis industry.

Researchers have investigated impurities in cannabis products through chiral analysis. However, some isomers of cannabis compounds have proven difficult to separate from a mixture. In particular, the separation of the positional isomers of Δ^8-THC and Δ^9-THC in a mixture have posed some difficulties for researchers, due to their highly similar chemical structures.[12] Furthermore, many potency analyses focus on quantifying THC and CBD, which prompts further investigation of other cannabinoids. The focus of this study will be to add to this body of research via analysis of the enantiomeric ratios of Δ^9-THC in chemically converted and "natural" or mother liquor (MoLo) cannabis extracts. We anticipate that chemically converted extracts will most likely contain (+)-Δ^9-THC, given that the (-) enantiomer is predominantly produced in plant-derived cannabis.

Methods

Two standards, one consisting of a Δ-9-THC racemic mixture (Cayman Chemical, 1 mg mL^{-1}), and the other consisting of (+)-Δ-9-THC (Cayman Chemical, 1 mg mL^{-1}), were diluted in a ~1:4 ratio with HPLC-grade MeOH, in separate glass dram vials. These solutions were then syringe-filtered via 0.45 μm micropore membranes, into HPLC vials, to prepare the final

standard solutions. The standard solutions were run on an Agilent 1260 Infinity II HPLC equipped with an amylose tris (3-chlorophenylcarbamate) CHIRALPAK® ID (4.6 mm I.D. x 250 mm, 5 µm silica gel) analytical column and a diode array detector (DAD) (reference wavelength of 210 nm) under numerous conditions, until optimal separation was achieved.

Methods 1, 2, 3, 4, and 5 were prepared, each with varying mobile phases and a constant flow rate of 2 mL/min. The respective conditions for each method are depicted, as follows, in Figure 1:

Method 1
- **0-50 min:** 50:50 (ACN / H_2O)
- **50-55 min:** 50:50 -> 90:10 (ACN / H_2O)
- **55-60 min:** 90:10 (ACN / H_2O)

Method 2
- **0-15 min:** 50:49:1 (ACN / H_2O / 0.1% formic acid in H_2O)
- **15-20 min:** 50:49:1 -> 90:9:1 (ACN / H_2O / 0.1% formic acid in H_2O)
- **20-25 min:** 90:9:1 (ACN / H_2O / 0.1% formic acid in H_2O)

Method 3
- **0-30 min:** 30:69:1 → 50:49:1 (ACN / H_2O / 0.1% formic acid in H_2O)
- **30-35 min:** 50:49:1 → 90:9:1 (ACN / H_2O / 0.1% formic acid in H_2O)

Method 4
- **0-30 min:** 30:69:1 → 60:39:1 (ACN / H_2O / 0.1% formic acid in H_2O)
- **30-35 min:** 60:39:1 → 90:9:1 (ACN / H_2O / 0.1% formic acid in H_2O)

Method 5
- **0-30 min:** 50:49:1 (MeOH / H_2O / 0.1% formic acid in H_2O)
- **30-35 min:** 50:49:1 → 90:9:1 (MeOH / H_2O / 0.1% formic acid in H_2O)

*Figure SEQ Figure * ARABIC 1: Method Development Conditions*

Once a method enabling optimal separation of the standards was achieved, 12 cannabis distillates were subsequently tested under the same conditions to test the efficacy of the optimal method. Among the 12 samples, 3 were known to be plant-extracted mother liquor (MoLo), and 4 were known to be synthetic. The 5 remaining distillates were of unknown origin. Prior to HPLC analysis, each extract was diluted using HPLC-grade MeOH (~1 mg/mL) into glass dram vials. ~0.2 mL aliquots of the diluted extracts were subsequently syringe-filtered into respective HPLC vials containing an additional ~0.8 mL of HPLC-grade MeOH, via a 0.45 µm micropore membrane.

Supplemental reference nuclear magnetic resonance (NMR) spectroscopy was also utilized to determine the presence of cannabinoids apart from Δ^9-THC, including synthetic components (i.e. Δ^8-THC and Δ^8-iso-THC). CBD, CBN, and CBG isolate samples were also prepared and analyzed utilizing the optimal chiral separation method, to assess their presence within samples. Δ^8-THC and cis-Δ^9-THC standards were prepared and analyzed in the same fashion as the *trans*-Δ^9-THC standards for further confirmation.

Figure 2 depicts an example chromatogram, exhibiting separation between the (-) and (+) enantiomers of Δ-9-THC, under the optimal conditions.

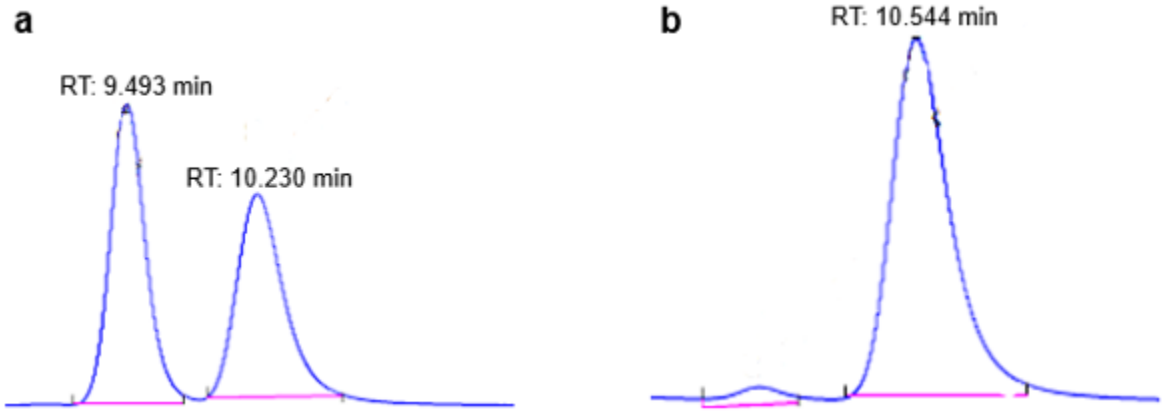

*Figure 2: Enantiomeric separation of Δ^9-trans-THC enantiomers utilizing most optimal chiral separation method. a) Separation of (+) and (-) enantiomers of Δ^9-trans-*THC b) (+)-Δ^9-*trans*-THC

Following the preliminary analysis of the 12 cannabis distillates, 100 distillate samples of unknown origin, from Cambium Analytica, were obtained and analyzed using the optimal method. These samples were prepared using the same procedure utilized for the 12 cannabis distillates. 4 control samples containing the (-) enantiomer of Δ^9-THC were additionally analyzed.

It is also noteworthy to mention that issues with the check valves/tubing resulted in instrument drift, upon troubleshooting these issues. Hence, delay of retention times (relative to the preliminary analysis of the 12 cannabis distillates) for analytes of interest was observed, upon

analysis of the 100 cannabis distillates. For instance, the (-) and (+) enantiomer, respectively, were exhibited at retention times of ~10.067 and ~10.860 minutes during the analysis of the 100 samples, as opposed to the retention times of ~9.493 and ~10.230 minutes observed in the prior analysis of the 12 cannabis distillates.

Results

A suitable method was developed for optimal separation of the Δ-9-THC enantiomers. The mobile phase that provided the most efficient separation consisted of 50.0% ACN; 49.0% H_2O; 1.0% 0.1% formic acid in H_2O (0-15 min.), and 90.0% ACN; 9.0% H_2O: 1.0% 0.1% formic acid in H_2O (20-25 min.), with enantiomeric separations completing in less than 11 minutes (method 2, depicted in Figure 2). Figure 2 depicts a side-by-side comparison of the chromatograms for the racemic mixture of Δ^9-THC and the single enantiomer ((+)-Δ^9-THC), under these conditions. The unnatural (+)-Δ^9-THC enantiomer exhibits a retention time of ~10.320 minutes, whereas the natural (-) enantiomer exhibits a retention time of ~9.493 minutes. Upon analysis of the 100 cannabis distillate samples, the retention times of the (-) and (+) enantiomer, respectively, shifted to ~10.067 and ~10.860 minutes.

Figure 3 depicts the results from the method tests for method tests 1, 3, 4, and 5 on the racemic Δ^9-THC standard. Evidently, method test 1 exhibited decent separation, but method 2 was determined to be the most optimal, with separation more closely matching the width of each peak. Method tests 3, 4, and 5 each had relatively poor separation, and each compound took ~26-36 minutes to resolve, with method 5 experiencing the latest retention times of each enantiomer, at 36.326 and 36.556 minutes, respectively.

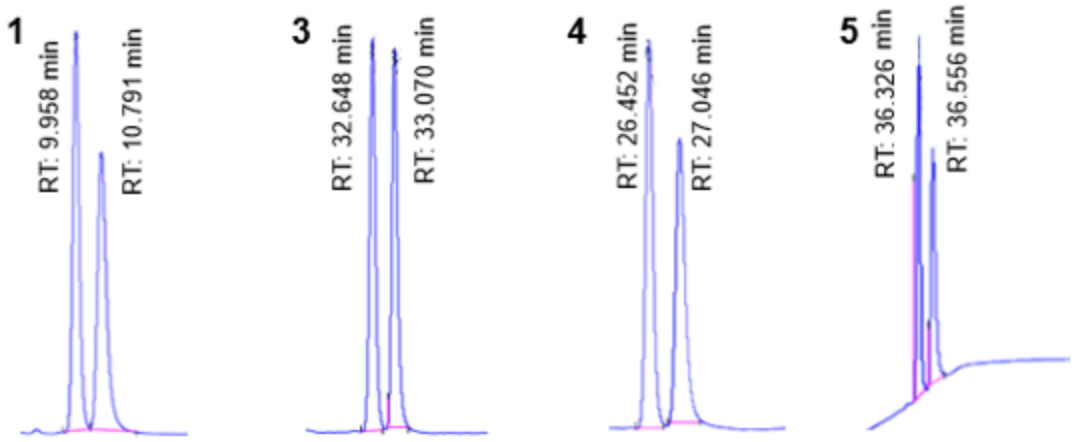

Figure 3: Methods 1, 3, 4, and 5 (respectively) and their ability to separate (+) and (-) enantiomers of Δ^9-trans-THC.

Table 1 (below) summarizes the presence of MoLo (mother liquor) and synthetic components (Δ^8-THC and Δ^8-iso-THC) within each sample, as well as the enantiomeric ratios of the (+) and (-) enantiomers of Δ^9-THC within each sample. As presented in Table 1, the MoLo extracts all contained CBG and CBC, and no Δ^8-THC or Δ^8-iso-THC. Furthermore, there was no presence of the non-natural (+)-Δ^9-THC enantiomer in these extracts. These same findings were determined for Distillate #5. The only other extract that had a 100% ratio of the natural (-)-$\mathbf{\Delta^9}$-THC enantiomer was Distillate #8. The remaining 7 extracts (distillates #4, 6, 7, and 9-12) all contained the unnatural (+)-Δ^9-THC enantiomer, with (-):(+) ratios of 5.6:1, 9.75:1, 7.21:1, 14.3:1, 8.86:1, 14.82:1, and 15.31:1, respectively.

Table 1. Δ^9-THC Enantiomeric Ratios in Samples, MoLo & Synthetic Components

MoLo Verification	MoLo Components		Synthetic Components		Δ^9-THC Enantiomeric Ratio (-):(+)
Sample #	CBG	CBC	Δ^8-THC	Δ^8-iso-THC	
Distillate 1	✓	✓	✗	✗	100:0
Distillate 2	✓	✓	✗	✗	100:0
Distillate 3	✓	✓	✗	✗	100:0
Distillate 4	✗	✗	✓	✓	5.6:1
Distillate 5	✓	✓	✗	✗	100:0
Distillate 6	✗	✗	✓	✓	9.75:1
Distillate 7	✗	✗	✓	✓	7.21:1
Distillate 8	✓	✗	✗	✗	100:0
Distillate 9	✗	✗	✓	✓	14.3:1
Distillate 10	✗	✗	✓	✓	8.86:1
Distillate 11	✗	✗	✓	✓	14.82:1
Distillate 12	✗	✗	✓	✓	15.31:1

**✓ = presence of component, ✗ = absence of component
MoLo, unknown origin, synthetic

Notably, the extracts containing the non-natural Δ^9-THC enantiomer contained no CBG or cannabichromene (CBC), and all contained Δ^8-THC and Δ^8-iso-THC. It is also notable to mention that distillates 1-3 were known to be extracted from plant matter, and distillates 9-12 were known to be synthetic conversion samples.

Upon analysis of the additional 100 cannabis samples, 6% of the 100 samples contained the unnatural (+) enantiomer of *trans*-Δ^9-THC. Among the samples containing the (+) enantiomer, the average relative percentage compared to the (-) enantiomer was 3.14%. The 4 control samples all exhibited an average retention time of 10.058 minutes, consistent with the

retention times of the (-)-*trans*-Δ^9-THC enantiomer. The presence of cannabinoids, a part from Δ^9-THC, was not assessed in the batch of 100 samples.

Discussion

Overall, the method employed was successful at separating the (+) and (-) enantiomers of Δ^9-THC. Our results exhibit that known synthetic conversion cannabis samples frequently contain (+)-Δ^9-THC, Δ^8- and Δ^8-iso-THC, and do not contain CBG or CBC. Conversely, cannabis samples known to be extracted from plant material do not contain (+)-Δ^9-THC, Δ^8- and Δ^8-iso-THC. As distillates 1-3 were known to be extracted from plant matter, and distillates 9-12 were known to be synthetic conversion samples, our anticipated hypothesis that known synthetic conversion samples would contain the unnatural enantiomer of Δ^9-THC, was supported. Furthermore, other synthetic components, such as Δ^8- and Δ^8-iso-THC were present in all known synthetic conversion samples and absent in cannabis samples known to be extracted from plant matter, further supporting our hypothesis. The presence of the (+) enantiomer of Δ^9-THC could be a result of diluting samples with foreign distillate and/or some type of cultivar naturally containing the (+) enantiomer.

Conclusions

Based on our findings, the method employed in this literature lends significant evidence as to whether a cannabis sample is plant-derived or synthetic. This method could be adjusted and further applied in the cannabis industry, as well as for forensic analysis of seized drug samples,

and other applications in the future. Future research could focus on the chiral separation of other components in cannabis, including, but not limited to, major chiral cannabinoids. Future research could also focus on investigating the pharmacological effects of chiral cannabinoids, as there is little to no literature that aims to determine potential varying effects among enantiomeric counterparts of chiral cannabinoids.

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