Monograph: Issue 3

Cayman Novel Psychoactive Substances Metabolism Monograph

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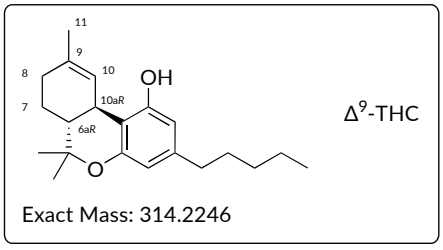
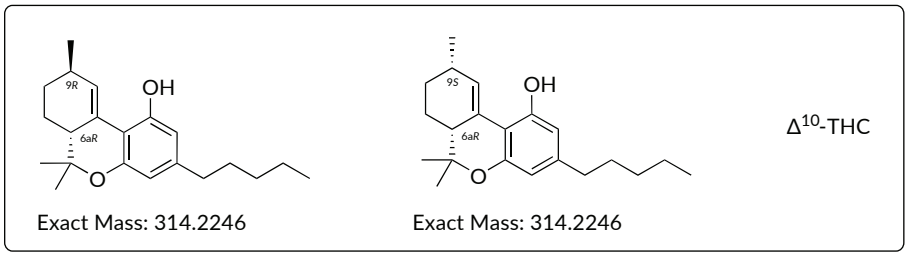
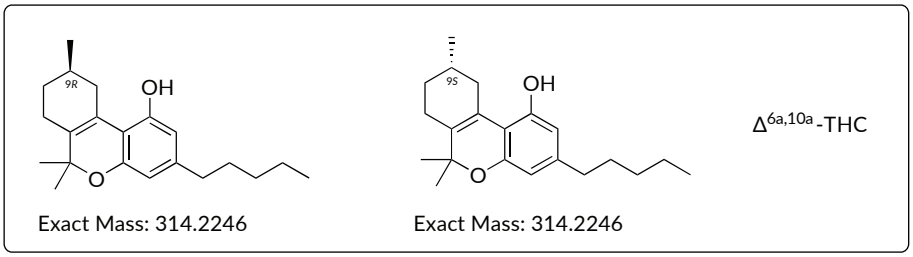
Δ^9-THC and Semi-Synthetic Cannabinoids Δ^{10}-THC and $\Delta^{6a,10a}$-THC

Item Nos. ISO60157, 33011, 33012, 33013, and 33014







The goal of this monograph is to identify the probable metabolites of the newly emerging semi-synthetic cannabinoids Δ^{10}-THC and $\Delta^{6a,10a}$-THC using human liver microsomes (HLMs) and high-resolution mass spectrometry. The metabolism of Δ^9-THC with HLMs was also investigated to provide forensic toxicologists a comparative understanding of the key fragments needed for identification.

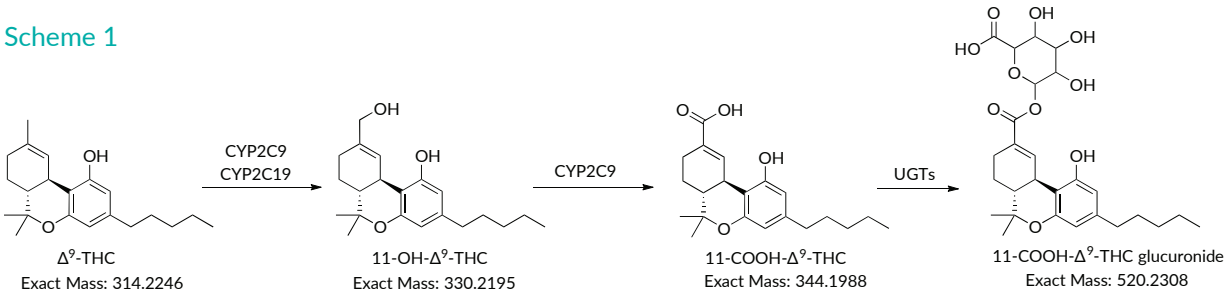
Introduction

The metabolism of the psychoactive constituents of *Cannabis sativa* has been of interest for decades. Δ^9-Tetrahydrocannabinol (Δ^9-THC) is the major contributor to the psychoactivity associated with smoking or ingesting *Cannabis*. Thus, the metabolites of this constituent have been detected, isolated, and studied dating back to initial work by Wall.[1] With the increasing legalization of recreational *Cannabis* use, coupled with the resulting increase in the use of e-cigarette/vaping devices, semi-synthetic cannabinoids such as Δ^{10}-THC and $\Delta^{6a,10a}$-THC have become more prevalent. These structural isomers are not naturally occurring in *Cannabis* but can be formed during the manufacturing process intended to make cannabidiol (CBD), Δ^9-THC, and other naturally occurring phytocannabinoids. These components are also specifically being targeted and marketed in said vaping products. Thus, studies on the metabolites of such compounds have become of increasing interest.[2,3] In this study, *in vitro* metabolism with human liver microsomes (HLMs) was compared for established, naturally occurring metabolites of Δ^9-THC, to the semi-synthetic cannabinoids (6a*R*,9*R*)-Δ^{10}-THC, (6a*R*,9*S*)-Δ^{10}-THC, 9(*R*)-$\Delta^{6a,10a}$-THC, and 9(*S*)-$\Delta^{6a,10a}$-THC. Mass spectral data were collected using UHPLC and ESI ionization with SIM-MS/MS scanning. *In vitro* metabolites from naturally occurring Δ^9-THC were compared to those formed from the semi-synthetic cannabinoids Δ^{10}-THC and $\Delta^{6a,10a}$-THC.

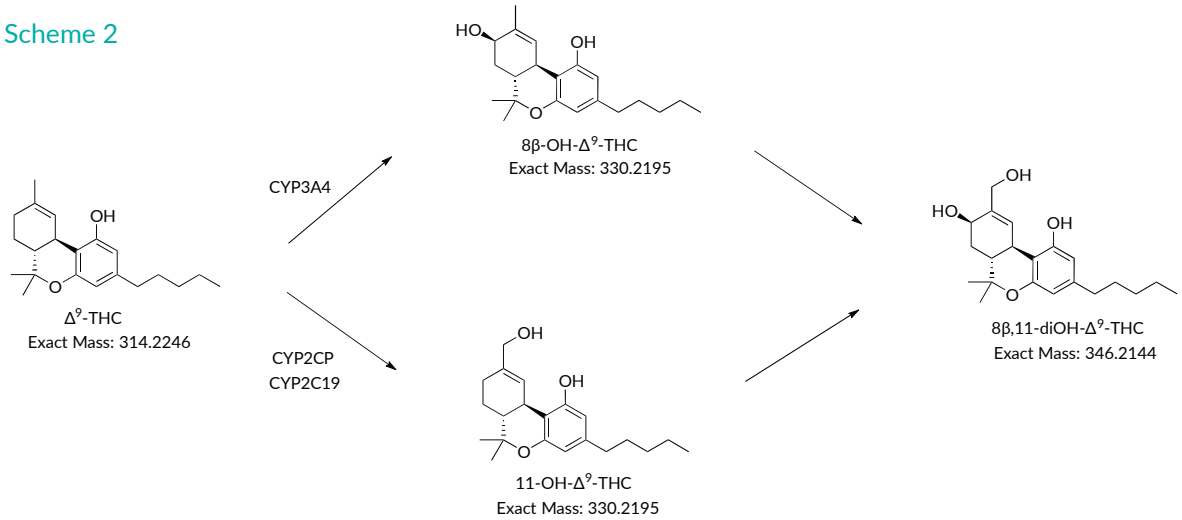
Metabolism of Δ^9-THC

The metabolism of Δ^9-THC has been studied extensively, mostly *via in vivo* (blood, urine, tissue) methodologies.[4] The primary metabolites detected (**Schemes 1 and 2**) have consistently been 11-hydroxy-Δ^9-tetrahydrocannabinol (11-OH-Δ^9-THC), 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol (11-COOH-Δ^9-THC), 8α-hydroxy-Δ^9-tetrahydrocannabinol (8α-OH-Δ^9-THC), and 8β-hydroxy-Δ^9-tetrahydrocannabinol (8β-OH-Δ^9-THC). Many other minor metabolites were also detected in trace amounts, and recent literature indicates that experiments using pooled HLMs generate other monohydroxylated metabolites in even higher abundance than 11-OH-Δ^9-THC.[5]

Scheme 1



Scheme 2



Major **(Scheme 1)** and minor **(Scheme 2)** pathways of Δ^9-THC metabolism.

Known metabolism of Δ^{10}-THC and $\Delta^{6a,10a}$-THC is limited by the absence of *in vitro* and *in vivo* studies. Comparison of the *in vitro* metabolism of these semi-synthetic cannabinoids to Δ^{9}-THC as a control was the aim of this study. Synthetic reference standards were used for comparison to experimental data: Δ^{9}-THC, 11-OH-Δ^{9}-THC, 11-COOH-Δ^{9}-THC, 8α-OH-Δ^{9}-THC, 8β-OH-Δ^{9}-THC, (6a*R*,9*R*)-Δ^{10}-THC, (6a*R*,9*S*)-Δ^{10}-THC, 9(*R*)-$\Delta^{6a,10a}$-THC, 9(*S*)-$\Delta^{6a,10a}$-THC, (6a*R*,9*R*)-11-OH-Δ^{10}-THC, (6a*R*,9*R*)-11-COOH-Δ^{10}-THC, 9(*R*)-11-COOH-$\Delta^{6a,10a}$-THC (**Appendix Table 1**).

Δ^{9}-THC was incubated at 37°C in the presence of NADPH regeneration system, pooled HLMs, and phosphate buffer (pH 7.4) for 60 minutes. Upon quenching with acetonitrile and centrifugation, samples were injected into the UHPLC-MS/MS with selected ion monitoring (SIM) instrumental setup for data collection. Multiple time points (5, 10, 20, 40, and 60 minutes) were analyzed but with very few observable differences, so time was not considered a factor in this metabolism study.

Several previously identified metabolites, *via in vivo* studies, of the control Δ^{9}-THC include those shown in **Figure 1**.

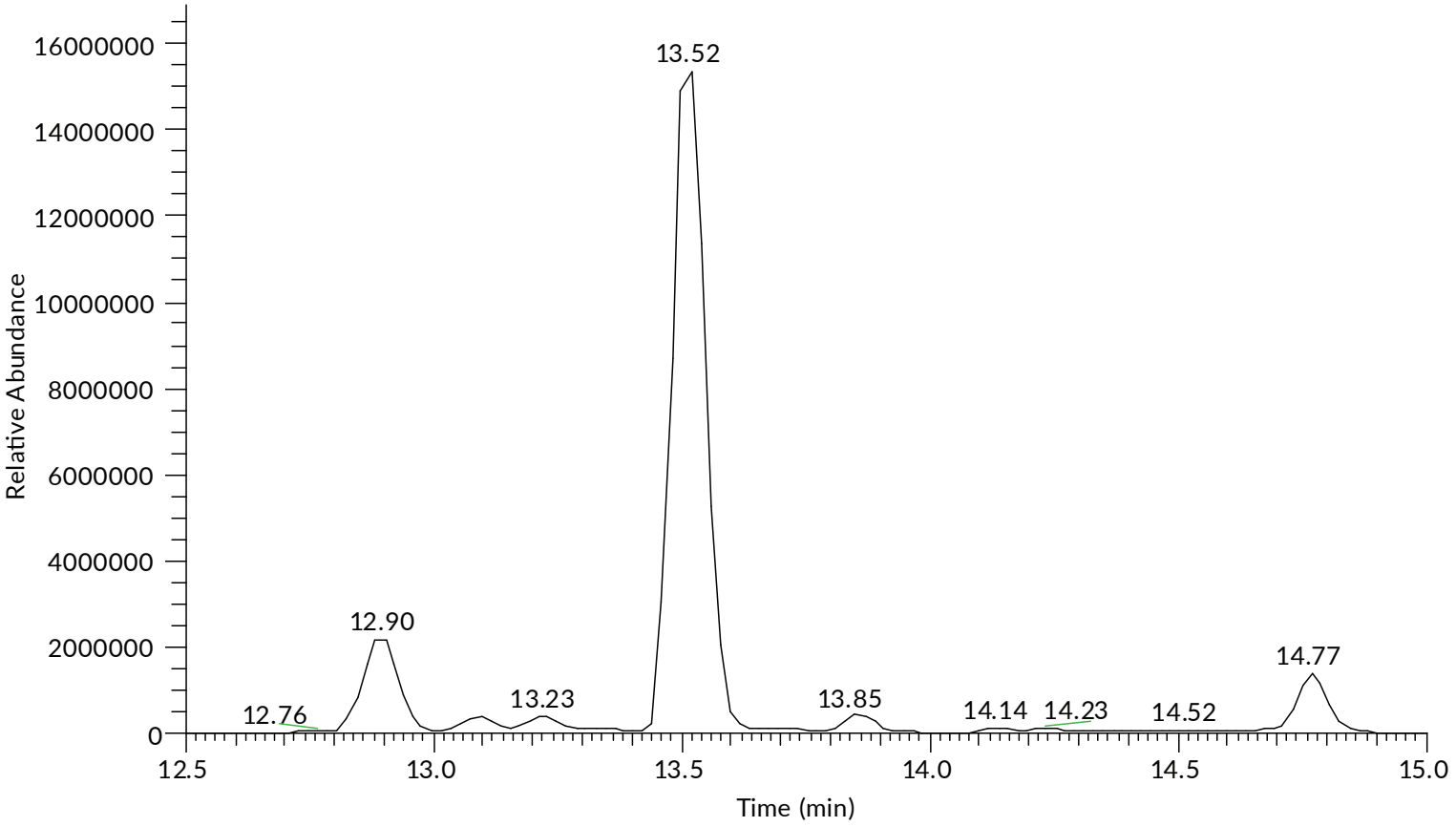


Figure 1. Representative ion chromatogram from Δ^{9}-THC incubation with pooled HLMs.

Data were analyzed for the SIM detection of three main masses of interest: 331 (monohydroxylation), 347 (dihydroxylation), and 345 (carboxylation). The data showed no appreciable indication of the presence of dihydroxylated or carboxylated products (*i.e.*, 11-COOH-Δ^9-THC), as was also observed with the other metabolism studies reported in the next section. Thus, the analysis focused on the monohydroxylated products. As is the case for previous *in vivo* studies, this work shows what can be reasonably determined to be hydroxylation somewhere in the cyclohexene ring moiety or on the pentyl chain. The key difference in the MS/MS spectra between ring hydroxylation and chain hydroxylation is exemplified by the fragments, as shown in **Figure 2**. In both cases, a major resorcinol-type fragment is observed. In the case of hydroxylation at carbon-11 or within the ring system, a fragment of *m/z* 193 is observed. However, in the case of hydroxylation along the pentyl chain, further fragmentation occurs, resulting in elimination of the hydroxyl group to afford a fragment of *m/z* 191. This is consistent with the *in vitro* and *in vivo* metabolism studies of 9(*R*)-hexahydrocannabinol and 9(*S*)-hexahydrocannabinol (HHC).[7]

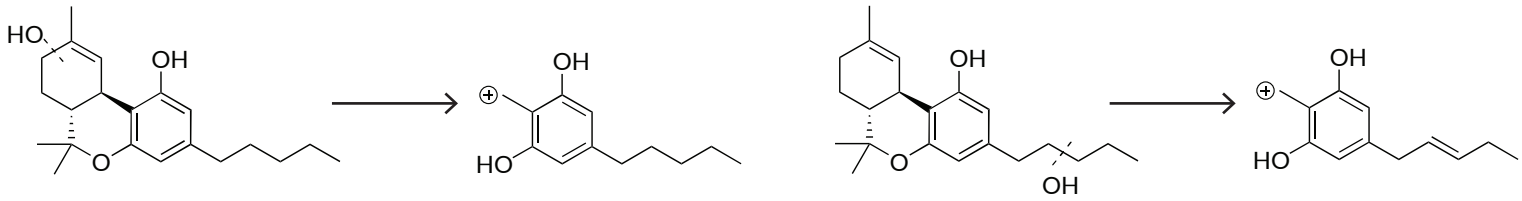


Figure 2. Key resorcinol-type fragments distinguishing ring and chain hydroxylation of THC.

The major product analyzed at retention time (R.T.) 13.52 minutes produced an MS/MS spectrum, with SIM at 331 *m/z*, that is in good agreement with the spectrum of the standard 11-OH-Δ^9-THC (**Figure 3**). This corroborates the known data that 11-OH-Δ^9-THC is a major metabolite produced in living organs and tissues. A key fragment ion of *m/z* 193 is observed here, providing evidence for monohydroxylation on the ring rather than on the tail.

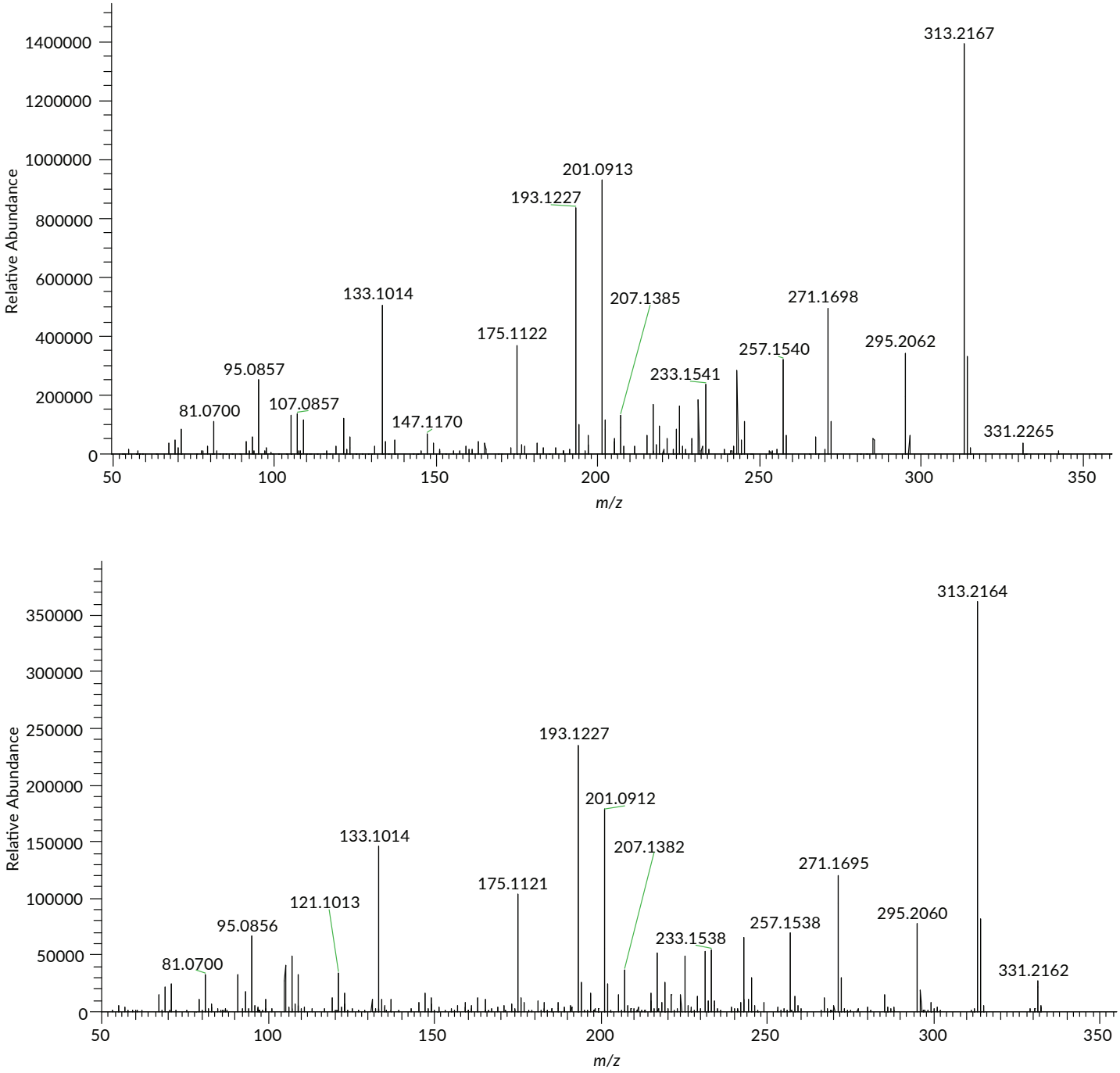


Figure 3. Mass spectrum **(top)** of 11-OH-Δ⁹-THC reference standard and that **(bottom)** of the major metabolic product at R.T. 13.52 minutes.

Figure 4 displays possible structures for three other main fragment ions of interest. The *m/z* 313 ion is likely due to simple cleavage of the hydroxyl moiety from the 11-position. The *m/z* 271, 201, and 133 fragments are likely due to additional aliphatic fragmentation, resulting in double bond movement due to conjugation.

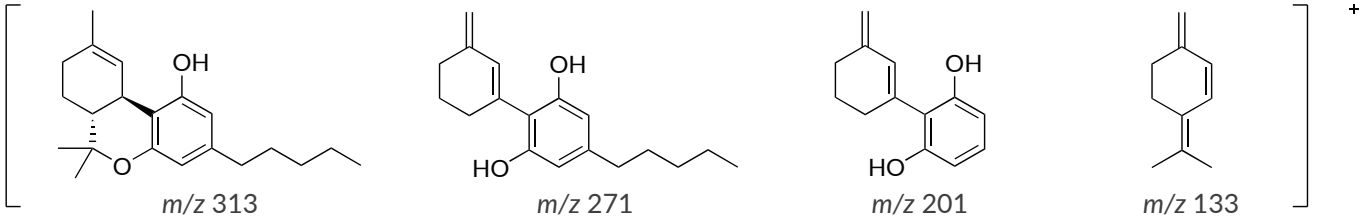
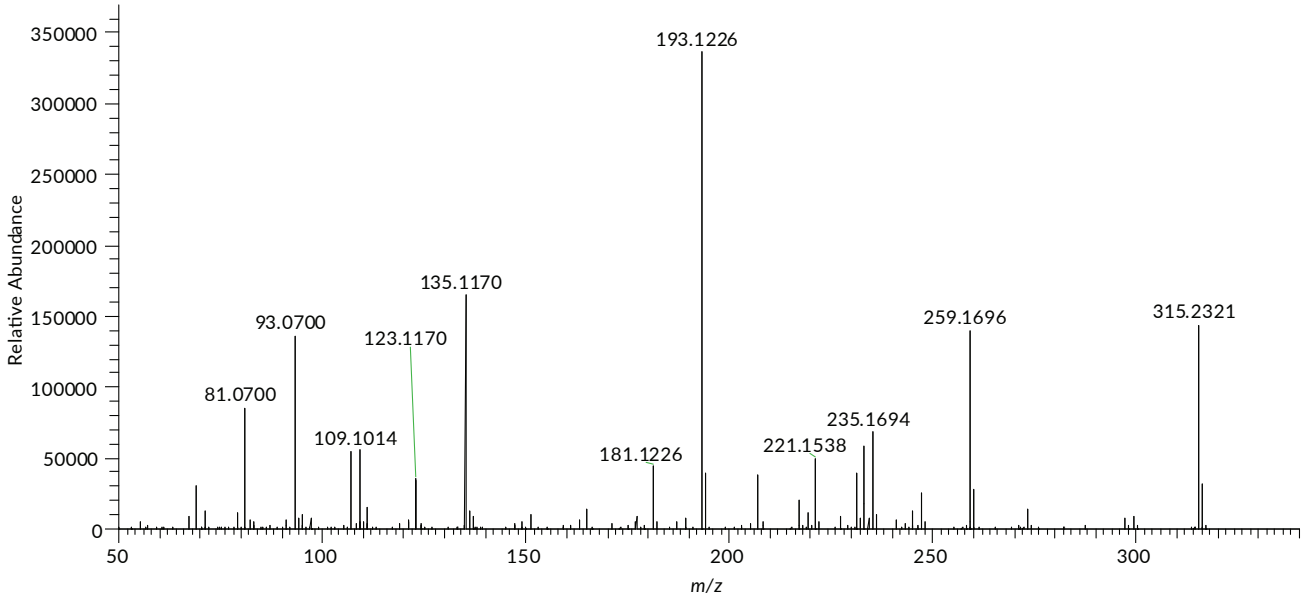
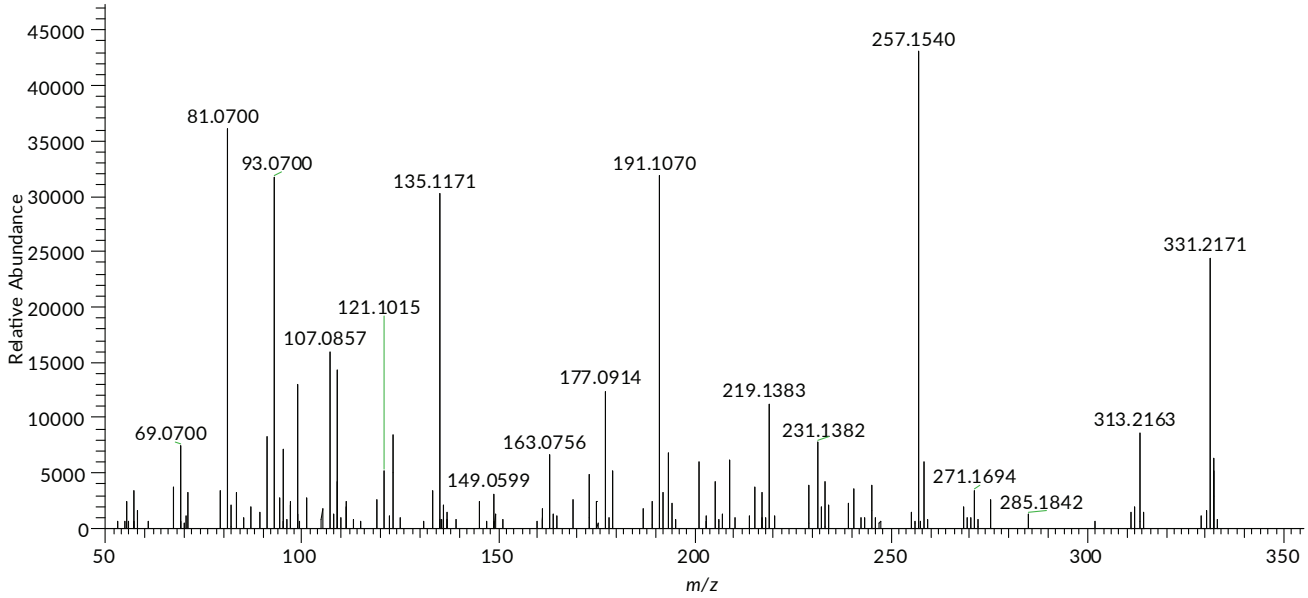


Figure 4. Proposed fragment ions in the mass spectrum of 11-OH-Δ⁹-THC.

The material eluting at 12.90 minutes produced a mass spectrum (**Figure 5, middle**) that may be indicative of pentyl chain hydroxylation. Pentyl chain metabolism has been reported in prior *in vivo* studies.[6] Of key note is the presence of the fragment ion of *m/z* 191, which is a major ion in the spectrum. The *m/z* 135 ion is also present in significant abundance in all parent (non-metabolite) THC standards (*i.e.*, Δ^9-, Δ^{10}-, and $\Delta^{6a,10a}$-THC) but is not present in the metabolite reference standards, which show an *m/z* 133 ion as in **Figure 5**. Other significant peaks include *m/z* 331 (molecular ion), 257, 135, and 81. The *m/z* 81 ion is present in greater abundance in the parent reference standard Δ^9-THC but is only a minor fragment in the hydroxylated and carboxylated reference standards (*e.g.*, 11-OH-Δ^9-THC).[1]





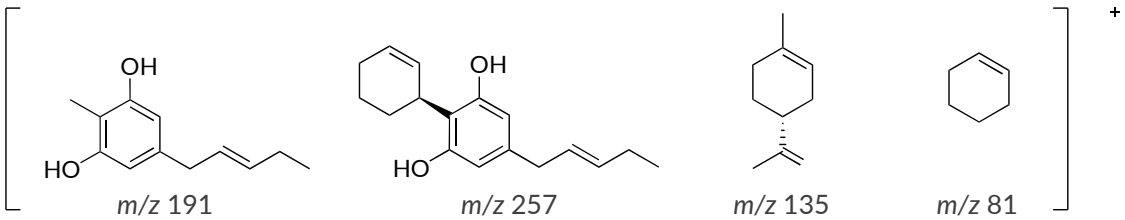


Figure 5. Mass spectrum **(top)** of parent Δ^9-THC, mass spectrum of peak at R.T. 12.90 minutes **(middle)**, and plausible fragment ions **(bottom)**.

Further evidence, seen in the mass spectrum of parent Δ^9-THC (**Figure 5, top**) is the *m/z* 259 ion. An analogous ion is observed in the metabolite with *m/z* 257 (**Figure 5, middle**), wherein the loss of two mass units indicates a unit of unsaturation, likely from elimination of the hydroxyl group along the pentyl chain. Comparison of these spectra, coupled with the observation that the major metabolite detected gives a spectral match to 11-OH-Δ^9-THC, supports the hypothesis that the major metabolites formed in this *in vitro* system are likely monohydroxylation in or around the cyclohexene ring moiety (in this case, at the 11 position) as well as monohydroxylation somewhere along the pentyl chain. The resulting mass spectral data alone cannot precisely determine the exact location where hydroxylation occurs on the pentyl chain. Matching retention times to reference standards would be necessary.

Analysis of the third most prevalent product at R.T. 14.77 minutes was inconclusive. The spectrum (**Figure 6**) displays a quite simple output with the molecular ion *m/z* 331 and the common *m/z* 193 ion, indicative of hydroxylation not occurring along the pentyl chain, being the most abundant. Mass spectra of the trace products did not appear to match any of the reference standards included in this study.

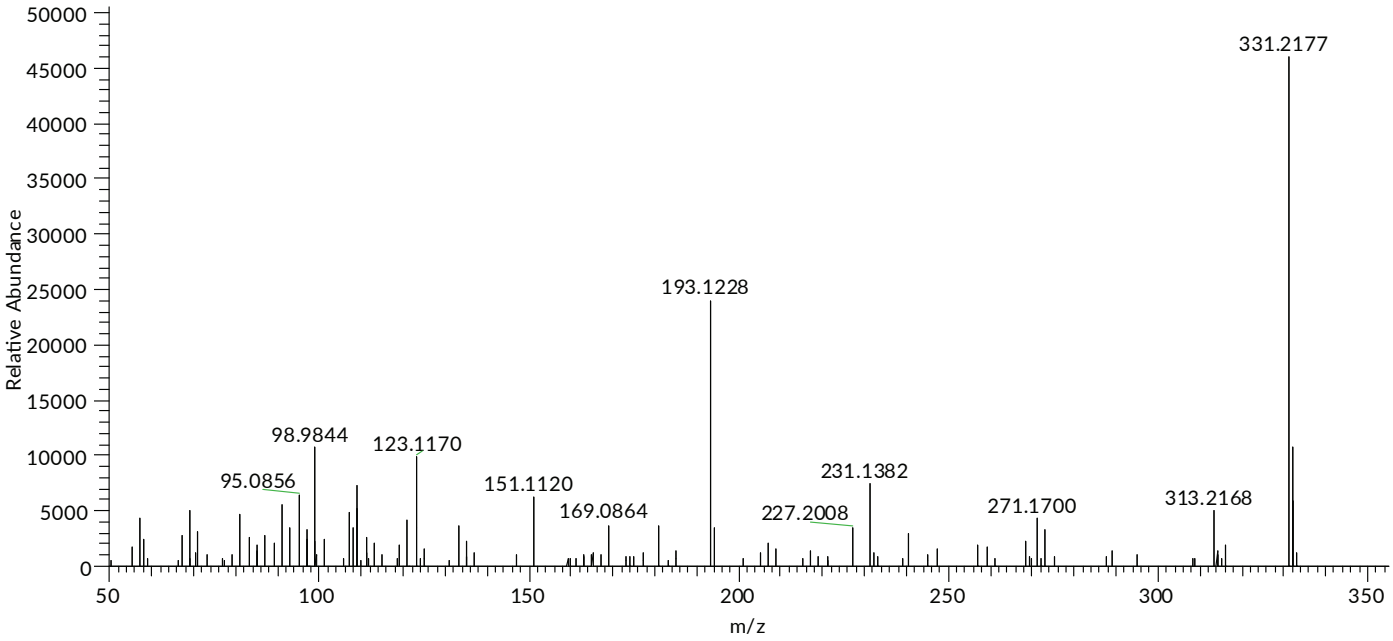


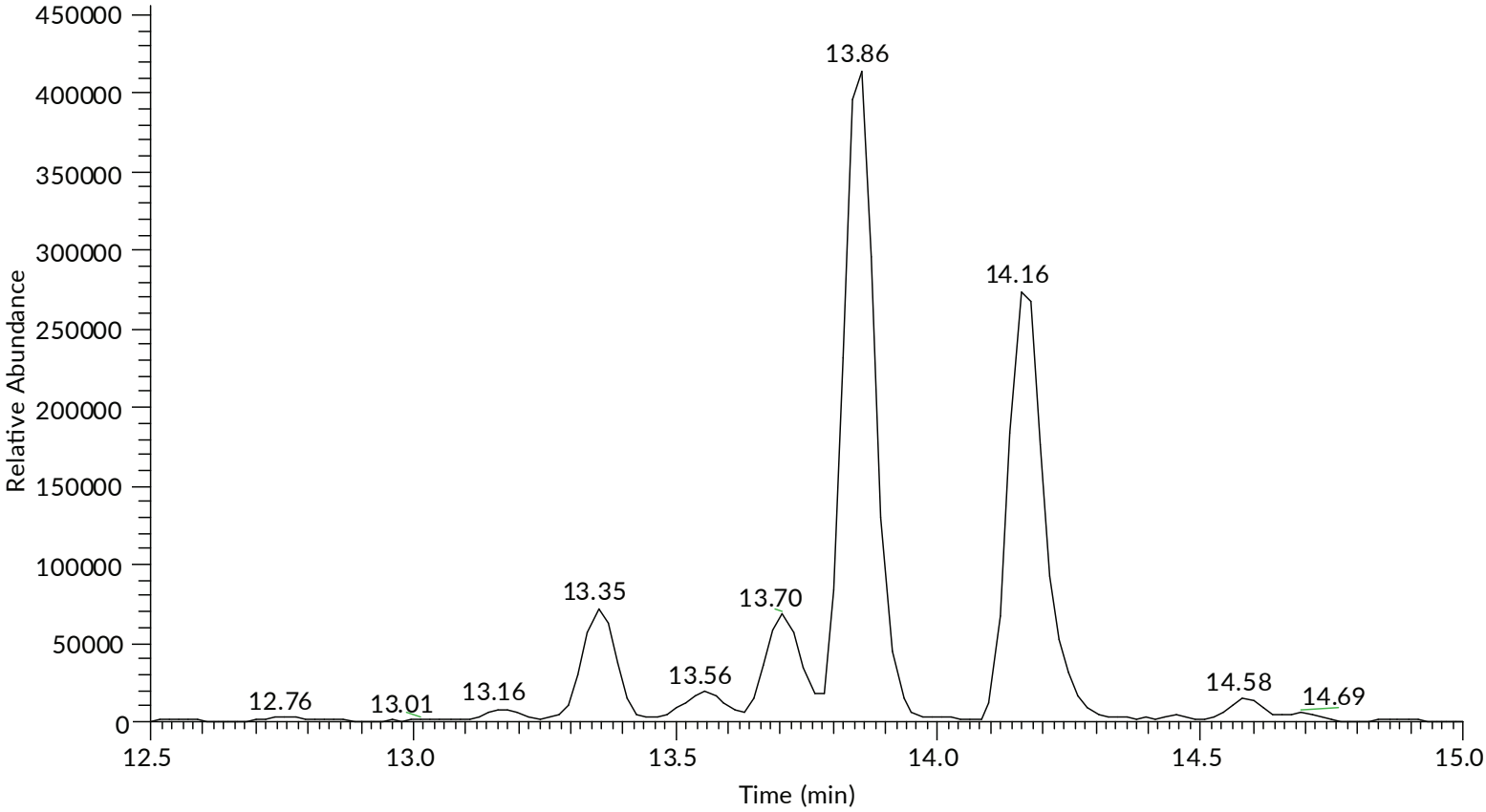
Figure 6. Mass spectrum **(top)** of the peak at R.T. 14.77 minutes and a generic structure of hydroxylation within the ring moiety **(bottom)**.

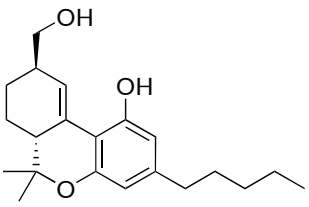
Metabolism of Semi-Synthetic Cannabinoids

(6a*R*,9*R*)-Δ^{10}-THC, (6a*R*,9*S*)-Δ^{10}-THC, 9(*R*)-$\Delta^{6a,10a}$-THC, and 9(*S*)-$\Delta^{6a,10a}$-THC were incubated and analyzed under the same conditions as for Δ^9-THC.

(6a*R*,9*R*)-Δ^{10}-THC

The *in vitro* experiment on (6a*R*,9*R*)-Δ^{10}-THC produced two major and two minor products (**Figure 7**).





Exact Mass 330.2195

Figure 7. Ion chromatogram **(top)** from (6a*R*,9*R*)-Δ^{10}-THC metabolism experiment and structure **(bottom)** of presumed major metabolite.

The product metabolite at R.T. 14.16 minutes differs in R.T. to the (6a*R*,9*R*)-11-OH-Δ^{10}-THC reference standard (Item No. 38231) and the mass spectrum lacks the fragment ion of *m/z* 301 observed in the standard (**Figure 8, top)**. However, it does share other spectral similarities indicative of hydroxylation within the ring system. Key similarities include the large signals at *m/z* 331 (molecular ion), which clearly indicates the addition of a hydroxyl species, and at *m/z* 193, which, as discussed, suggests hydroxylation within the ring system (as opposed to anywhere on the pentyl chain). The strong molecular ion is of most interest as this implies hydroxylation further away from the double bond. Alternatively, hydroxylation in an allylic position would show a predominant *m/z* 313 ion, resulting from loss of the hydroxyl group that is stabilized by the adjacent double bond.

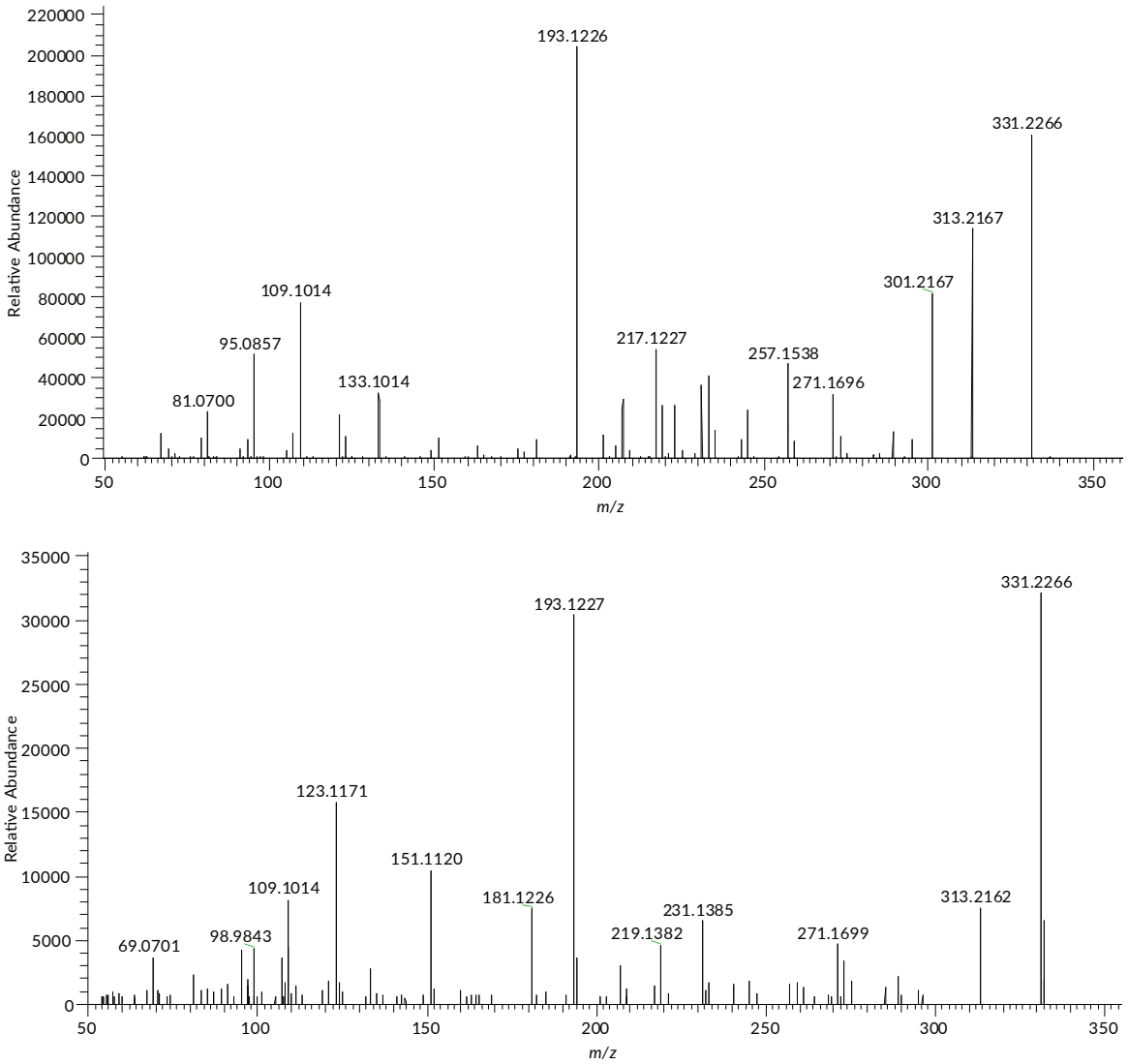
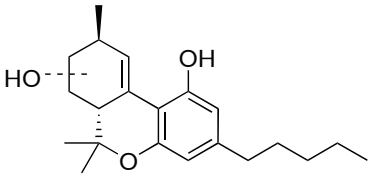


Figure 8. Mass spectra of the reference standard (6a*R*,9*R*)-11-OH-Δ[10]-THC **(top)** and of the metabolic product **(bottom)** at R.T. 14.16 minutes.

While hydroxylation/carboxylation frequently occurs at more reactive allylic positions, aliphatic oxidation can occur at less reactive positions in biological systems. As such, it is possible that hydroxylation at the 7 or 8 positions in the ring could occur (**Figure 9**), leading to products with similar identifying features like ion *m/z* 193 but potentially with quite different fragmentation pathways and retention times.



Exact Mass 330.2195

Figure 9. Generic structure of hydroxylation within the ring moiety of (6a*R*,9*R*)-Δ[10]-THC.

The signal at R.T. 13.86 minutes has a R.T. matching that of the (6a*R*,9*R*)-11-OH-Δ^{10}-THC reference standard. However, the mass spectrum appears as a mixture of products that coelute with the (6a*R*,9*R*)-11-OH-Δ^{10}-THC, This includes products with hydroxylation along the pentyl chain, as indicated by ions of *m/z* 191 and 257.

(6a*R*,9*S*)-Δ^{10}-THC

The *in vitro* experiment on (6a*R*,9*S*)-Δ^{10}-THC produced one major and two minor metabolites noted by the chromatogram (**Figure 10**). The mass spectra of the signals at R.T. 13.21 minutes and R.T. 14.13 minutes are almost identical to those observed at R.T. 13.86 minutes and R.T. 14.16 minutes, respectively, in the (6a*R*,9*R*)-Δ^{10}-THC (Item No. 33011) experiment (**Figure 7**).

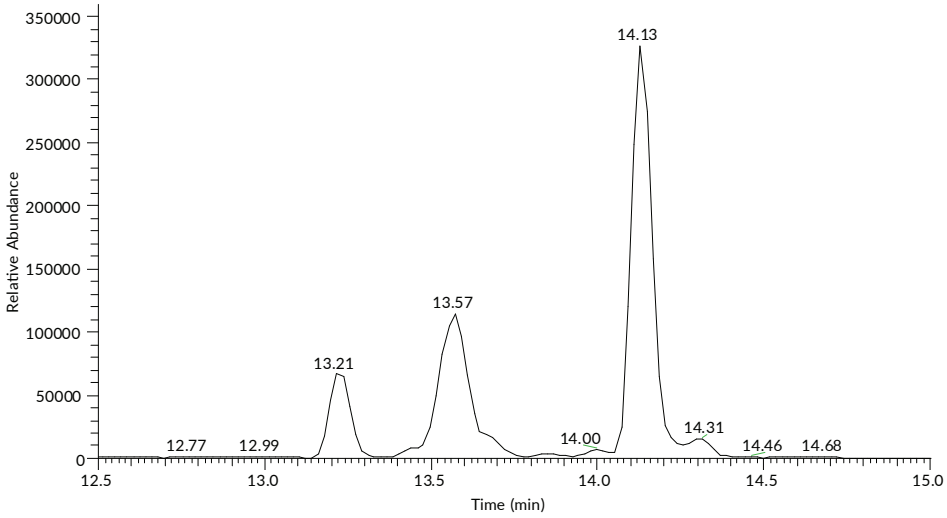
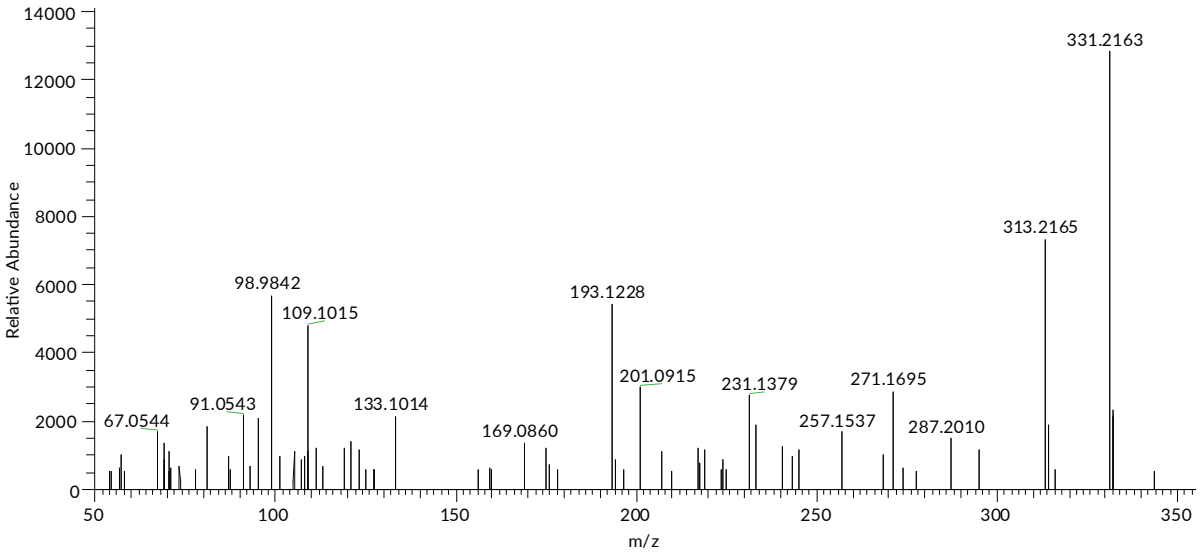
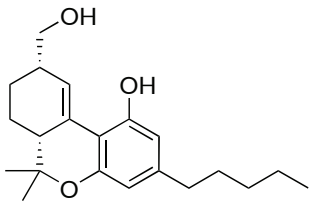


Figure 10. Ion chromatogram from (6a*R*,9*S*)-Δ^{10}-THC metabolism experiment.

The signal at R.T. 13.57 minutes offers a slightly different set of mass spectral data (**Figure 11**).





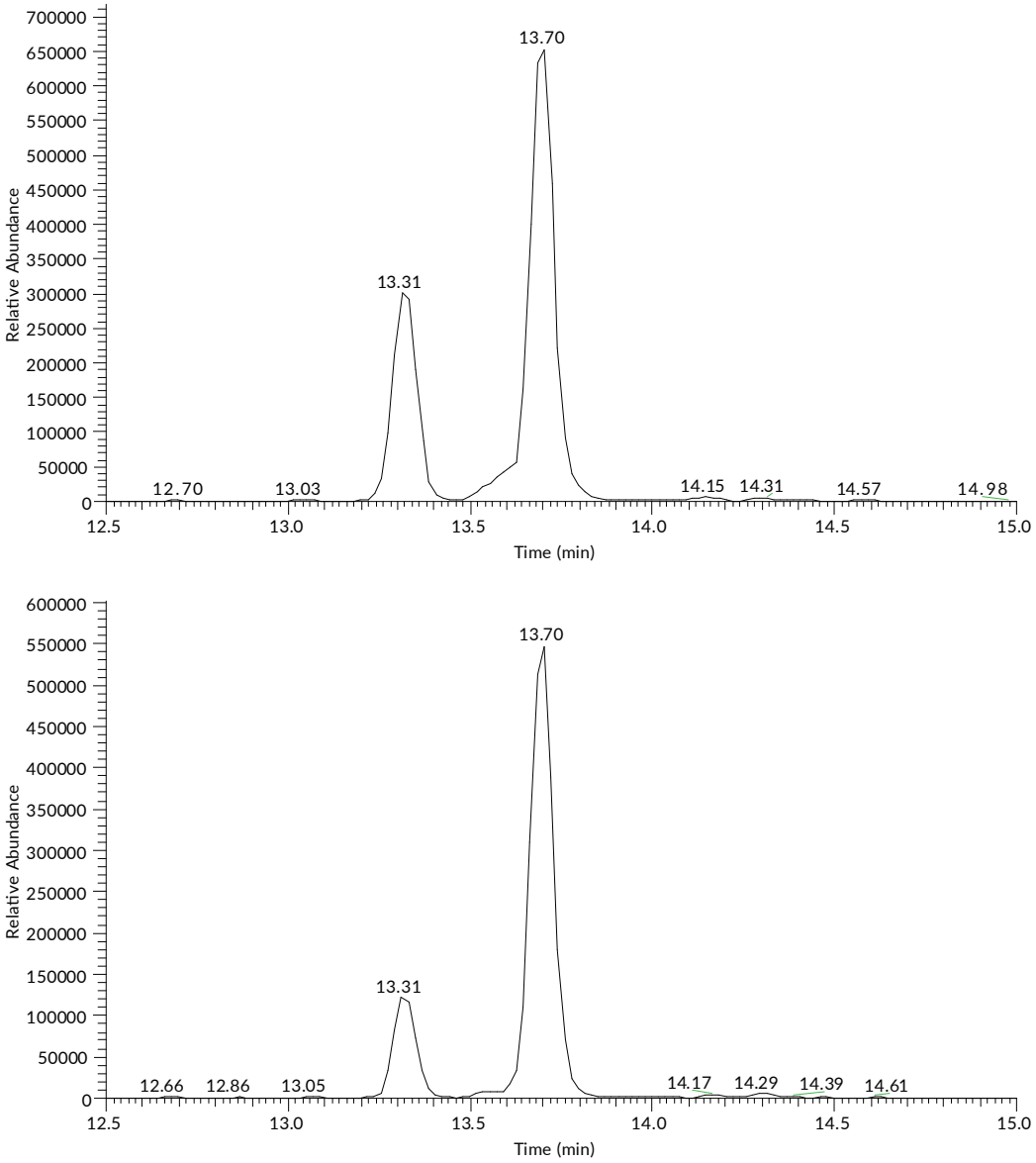
Exact Mass 330.2195

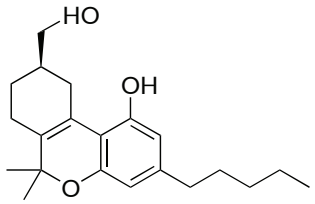
Figure 11. Mass spectrum **(top)** of the metabolic product at R.T. 13.57 minutes and the structure of (6a*R*,9*S*)-11-OH-Δ^{10}-THC **(bottom)**.

There is no reference standard for (6a*R*,9*S*)-11-OH-Δ^{10}-THC, but the mass spectrum in **Figure 11** shares some similarities with 11-OH-Δ^{9}-THC, markedly ions of *m/z* 133, 193, 201, 271, 295, and 313. The *m/z* 331 molecular ion has large abundance, which may indicate hydroxylation at the 11 position as it is no longer allylic like in 11-OH-Δ^{9}-THC, as discussed in the previous section. As with the (6a*R*,9*R*) diastereomer, hydroxylation at other positions around the ring cannot be discounted. Confirmation with reference standards by R.T. matching is required.

9(*R*)-Δ^{6a,10a}-THC & 9(*S*)-Δ^{6a,10a}-THC

Data from the *in vitro* metabolism of the enantiomers 9(*R*)-Δ^{6a,10a}-THC (Item No. 33013) and 9(*S*)-Δ^{6a,10a}-THC (Item No. 33014) are nearly identical to one another (**Figure 12**). Peak retention times match and the mass spectra associated with each respective signal are consistent with each other.





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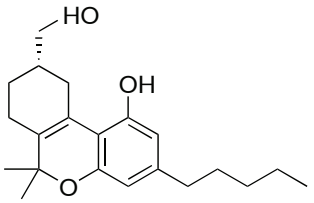

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Figure 12. Ion chromatogram from 9(*R*)-Δ^{6a,10a}-THC metabolism experiment with the corresponding presumed major metabolite **(top)** and those from 9(*S*)-Δ^{6a,10a}-THC metabolism experiment **(bottom)**.

The mass spectral results consistently display the likelihood of monohydroxylation around the cyclohexene ring for one of the main metabolite products (in this case corresponding to R.T. 13.70 minutes), as well as monohydroxylation along the pentyl chain (those corresponding to R.T. 13.31 minutes). Many familiar ion signals are present in the spectra that have been seen and discussed above (**Figure 13**) (*m/z* 135, 191, 257, 313, 331 in the case of chain hydroxylation and *m/z* 133, 193, 271, 313, 331 in the case of ring hydroxylation). Noteworthy is the similarity between the mass spectrum of the signal at 13.70 minutes and that of the (6a*R*,9*R*)-11-OH-Δ^{10}-THC reference standard (**Figure 8, top**), indicative of hydroxylation at the 11-position of 9(*R*)-Δ^{6a,10a}-THC and 9(*S*)-Δ^{6a,10a}-THC.

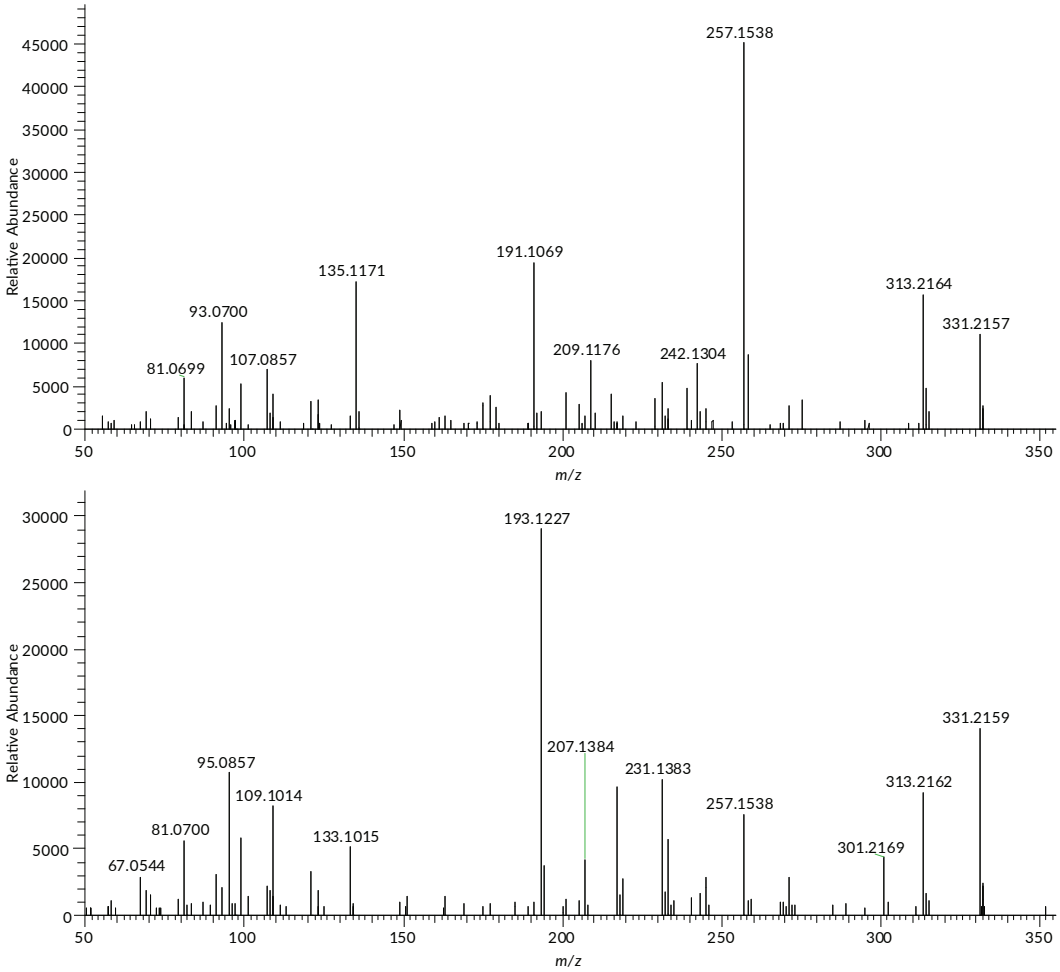

Figure 13. Mass spectra of the metabolic products at R.T. 13.31 minutes **(top)** and 13.70 minutes **(bottom)** from 9(*R*)-Δ^{6a,10a}-THC metabolism experiment.

Conclusion

HLM incubation of (6a*R*,9*R*)-Δ^{10}-THC, (6a*R*,9*S*)-Δ^{10}-THC, 9(*R*)-Δ^{6a,10a}-THC, 9(*S*)-Δ^{6a,10a}-THC resulted in identification of predominantly monohydroxylated metabolites. Similar *in vitro* metabolism was noted with the control experiment on Δ^{9}-THC. Putative monohydroxylated metabolites, such as (6a*R*,9*R*)-11-OH-Δ^{10}-THC (Item No. 41299), may serve as useful biomarkers for consumption of the semi-synthetic cannabinoids Δ^{10}-THC and Δ^{6a,10a}-THC. Fragmentation of the monohydroxylated metabolites reveals that hydroxylation is occurring within the THC ring system (exemplified by *m/z* 193) as well as along the pentyl chain (exemplified by *m/z* 191) of these semi-synthetic cannabinoids. Large molecular ions indicative of allylic hydroxylation (*m/z* 313) and hydroxylation occurring more distal to the olefin (*m/z* 331) demonstrate the hydroxylation at multiple points within the THC ring system. Additional *in vivo* studies are necessary to fully understand the metabolism of Δ^{10}-THC and Δ^{6a,10a}-THC. Synthesis of reference standards to support identification of these putative metabolites is also ongoing.

Appendix

Methodology and Instrumentation

Test compounds were formulated into 1 mM stock solutions in ethanol and then incubated at 37°C for one hour in pH 7.4 phosphate buffer with microsomes and cofactor. Mixtures were quenched with acetonitrile, centrifuged, and injected into instrumentation for mass spectral analysis.

Materials

Table 1. Reference standards used in this study.

Item No.	Product Name
ISO60157	Δ^9-THC
21667	11-OH-Δ^9-THC
20754	11-COOH-Δ^9-THC
39793	8α-OH-Δ^9-THC
39794	8β-OH-Δ^9-THC
33011	(6a*R*,9*R*)-Δ^{10}-THC
33012	(6a*R*,9*S*)-Δ^{10}-THC
33013	9(*R*)-$\Delta^{6a,10a}$-THC
33014	9(*S*)-$\Delta^{6a,10a}$-THC
38231	(6a*R*,9*R*)-11-OH-Δ^{10}-THC
38235	(6a*R*,9*R*)-11-COOH-Δ^{10}-THC
38237	9(*R*)-11-COOH-$\Delta^{6a,10a}$-THC
41299	(6a*R*,9*R*)-11-OH-Δ^{10}-THC (exempt preparation)

Table 2. Materials for *in vitro* metabolism experiments in this study.

Material	Supplier Information
Pooled Human Liver Microsomes	Sekisui XenoTech, H0610
NADPH Regeneration System	Promega, V9510

Table 3. Instrumentation for experiments in this study.

Instrument	Supplier Information
Dionex™ UltiMate™ 3000 UPLC system	Thermo Fisher Scientific
Q Exactive™ Plus Orbitrap MS System	Thermo Fisher Scientific
Ascentis Express C18 100 x 2.1 mm, 2.7 µm	Supelco, 53823-U
Xcalibur™ Software v. 4.2	Thermo Fisher Scientific

References

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