



# CONFIRMATION OF CANNABINOID IN FORENSIC TOXICOLOGY CASEWORK BY ISOMER-SELECTIVE UPLC-MS/MS

Waters™

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## INTRODUCTION

In forensic toxicology, verifying use of  $\Delta^9$ -tetrahydrocannabinol ( $\Delta^9$ -THC) by urine confirmation testing has been traditionally limited to analysis of the major  $\Delta^9$ -THC excreted metabolite, 11-nor-9-carboxy- $\Delta^9$ -tetrahydrocannabinol ( $\Delta^9$ -cTHC). Legalization of hemp, however, has led to widespread production and sale of psychoactive cannabinoid (CBD) derivatives including  $\Delta^8$ -THC and  $\Delta^{10}$ -THC isomers.

We report an isomer-selective definitive method for the quantitative confirmation of  $\Delta^8$ -THC,  $\Delta^9$ -THC,  $\Delta^{10}$ -THC,  $\Delta^8$ -cTHC,  $\Delta^9$ -cTHC and CBD (Figure 1) by liquid chromatography/tandem mass spectrometry (UPLC-MS/MS).

Method validation along with method performance comparison to a prior  $\Delta^9$ -cTHC confirmation method is reported. Casework studies also include isomer metabolite prevalence and relative metabolism rates.

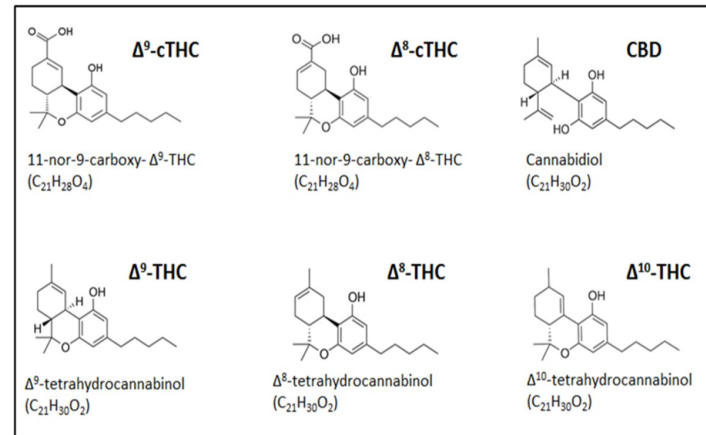


Figure 1. Analyte structures and elemental composition.

## METHODS

### Isomer-Selective Confirmation Method

**Chemicals:** Certified reference materials for  $\Delta^8$ -THC,  $\Delta^9$ -cTHC,  $\Delta^9$ -cTHC-glucuronide,  $\Delta^9$ -THC-D3,  $\Delta^9$ -cTHC-D3,  $\Delta^8$ -THC,  $\Delta^{10}$ -THC, CBD and CBD-D3 were from Millipore Sigma, and  $\Delta^8$ -cTHC from Cayman Chemical. IMCSzyme  $\beta$ -glucuronidase and buffer from Integrated Micro Chromatography Systems.

**Reagents:** Reference standard and quality control (QC) solutions of analytes (supplied at 0.1 mg/mL in methanol) were used to prepare multi-analyte calibrator and QC solutions at (10,000 ng/mL in methanol). The multi-analyte calibrator solution was diluted in analyte-negative urine:methanol (60:40) to prepare working calibrators at 10 (LLOQ), 25, 100, 250, 500 and 1000 (ULOQ) ng/mL concentrations. Working QC samples (15, 40, 400, 800 ng/mL), along with an LOD control (4 ng/mL) were similarly prepared with the multi-analyte QC solution. Internal standard solutions of  $\Delta^9$ -THC-D3,  $\Delta^9$ -cTHC-D3 and CBD-D3 in methanol (supplied at 0.1 mg/mL) were combined to prepare a stock multi-internal standard solution (10,000 ng/mL in methanol). Working multi-analyte internal standard solution (250 ng/mL) was prepared by dilution of the multi-internal standard stock solution with methanol. Inactive hydrolysis buffer reagent was prepared by a 1 in 5 dilution of rapid hydrolysis buffer in deionized water. Active hydrolysis reagent was then prepared by a 3 in 20 dilution of the IMCSzyme  $\beta$ -glucuronidase reagent in inactive hydrolysis buffer. A  $\Delta^8$ -cTHC-glucuronide stock (0.1 mg/mL in methanol) was diluted 10-fold in analyte-negative urine:methanol (60:40) to prepare a hydrolysis control.

**Sample preparation:** Samples for analysis were prepared by adding 20  $\mu$ L of calibrators, controls or case specimens in 96-well plate positions (Waters, 2-mL square well plates), followed by additions of 20  $\mu$ L of working multi-internal standard solution and 20  $\mu$ L of active hydrolysis buffer. After mixing, the plates are incubated at room temperature for one hour, followed by addition of 150  $\mu$ L of 70% methanol containing 0.1% formic acid. Testing with and without enzymatic hydrolysis was performed for the hydrolysis control and for a subset of case samples by employing a second analysis sample containing 20  $\mu$ L of inactive hydrolysis buffer in place of the active hydrolysis reagent.

**UPLC-MS/MS analysis:** A Waters ACQUITY UPLC I-Class (FTN) system was interfaced with a Xevo® TQD tandem mass spectrometer detector. Chromatography was performed using a Waters CORTECS UPLC C18+ column (1.6  $\mu$ m, 2.1 x 50 mm) with a column temp. of 30° C. Mobile phase A was 0.1% formic acid in water and mobile phase B was LC-MS grade acetonitrile; flow rate was 0.4 mL/min. A series of step gradients were used as follows: 55% B (0-1.9 min), 67% B (1.9-4.4 min), 95% B (4.4-4.6 min) and 55% B (4.6-5.5 min). Injection volume was 10  $\mu$ L. Mass spectrometer conditions were: source temperature 150°C, capillary voltage 2.5 kV, nitrogen was used as the desolvation gas (at 800 L/h, 500°C) and as cone gas (at 10 L/h). Multiple reaction monitoring (MRM) conditions for analytes and internal standards are shown in Table 1.

### Prior Confirmation Method

The prior confirmation method was targeted for only  $\Delta^9$ -cTHC identification and quantitation. Reagents, calibrators and controls were prepared as described in the isomer-selective method with exclusion of the  $\Delta^8$ -THC,  $\Delta^{10}$ -THC,  $\Delta^8$ -cTHC and CBD reference material. UPLC-MS/MS analysis differs only in the use of a Waters BEH C18 1.7  $\mu$ m (2.1 x 100 mm) analytical column and the following mobile phase gradient: 50% B (0-0.8 min), 50-75% B (0.8-2.0 min), 75-95% B (2.0-2.5 min), 95-50% B (2.5-2.7 min) and 55% B (2.7-3.5 min).

## RESULTS & DISCUSSION

### CHROMATOGRAPHIC SELECTIVITY

Multiple column chemistries were evaluated to achieve rapid chromatographic resolution, especially for closely eluting isobaric-analytes (Figure 2).

Optimum resolution with minimum run time was obtained on solid-core C18+ particle column (Waters CORTECS, 50 mm, 1.6  $\mu$ m particle size). Step gradients, with near isocratic conditions at the beginning, and later in the 5.5 min chromatographic method, resolved the early eluting metabolite isomers as well as the later eluting CBD and THC isomers.

### PRECISION & ACCURACY

Within and between run method precision and accuracy was determined by replicate (n=11) analysis of QC pools. Statistical analysis of precision is displayed in Table 2 and shows an LOD percent relative standard deviation of 6-17% which was within the <20% validation criteria-limit. Low and high QCs were within the 15% quantitative control precision criteria.

Assay bias (Table 3) was within the <15% bias criteria for all control pools including LOD. Carryover was assessed for all analytes by replicate analysis (n=7) of an analyte-negative urine pool that followed the analysis of an ULOQ calibrator. Carryover was less than 0.03% for all analytes and met criteria.

Matrix interference was evaluated in 7 analyte negative donor urine specimens and met criteria for all analytes, based on an analyte response less than 50% of LOD control response. Interference was also tested in analyte-negative urine fortified with cannabidiol as well as 102 other drugs and metabolites routinely included in the definitive drug testing panels performed at the National Toxicology Center (Current Protocols e644, Volume 3 2023 doi: 10.1002/cpz1.644).

Dilution linearity was verified by dilution and testing of control samples with analyte concentrations up to 10,000 ng/mL, using up to a 10-fold pre-analysis dilution of the urine with an analyte-negative urine:methanol (60:40) solution.

### ISOMER-SPECIFIC CALIBRATION

Peak integration and calibration performance using a linear regression (1/x weighting) model was also evaluated over 11 analytical runs. Representative analysis for  $\Delta^8$ -THC,  $\Delta^9$ -THC and  $\Delta^{10}$ -THC (Figure 3) and for  $\Delta^8$ -cTHC,  $\Delta^9$ -cTHC, and CBD (Figure 4) show peak integration with smoothing on the left panels, plus calibration regression data (x) points and line with quality control results graphically displayed (o) in the right panels. Analyte-specific calibration was performed for each isomer, and the R<sup>2</sup> for linear regression analysis exceeded 0.99 for all analytes across the 11 analytical runs.

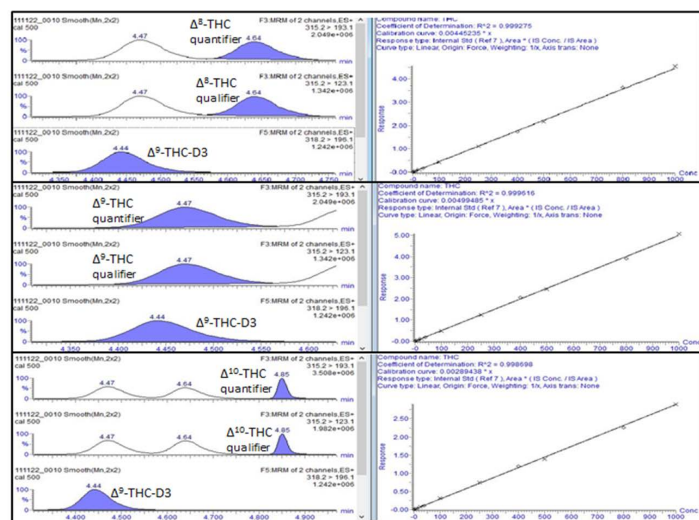


Figure 3. Integration and regression analysis of THC isomer calibration and QC data.

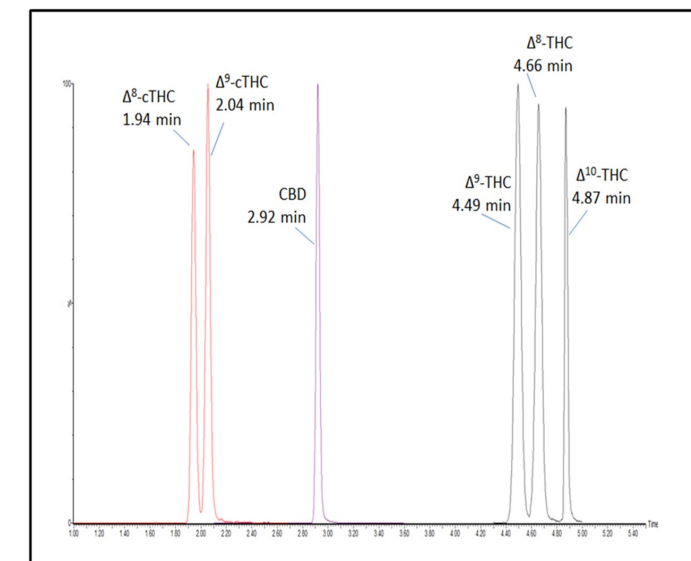


Figure 2. Chromatographic resolution of analytes. Representative analysis of a calibrator prepared in urine at 50 ng/mL.

| Analytes           | Precision, %CV |          |          |           |           |
|--------------------|----------------|----------|----------|-----------|-----------|
|                    | LOD 4ng/mL     | 15 ng/mL | 40 ng/mL | 400 ng/mL | 800 ng/mL |
| $\Delta^8$ -THC    | 15.3           | 12.8     | 9.6      | 4.6       | 4.0       |
| $\Delta^9$ -THC    | 9.3            | 7.1      | 5.4      | 3.4       | 3.7       |
| $\Delta^{10}$ -THC | 17.3           | 14.5     | 10.0     | 9.1       | 9.2       |
| $\Delta^8$ -cTHC   | 13.8           | 10.1     | 5.0      | 6.9       | 2.1       |
| $\Delta^9$ -cTHC   | 14.2           | 12.0     | 4.8      | 5.3       | 3.1       |
| CBD                | 6.1            | 5.1      | 4.0      | 3.6       | 2.1       |

Table 2. Statistical analysis of precision in 11 analyses of LOD and QC.

| Analytes           | Mean QC Concentration (% Target Bias) |                    |                     |                   |                   |  |
|--------------------|---------------------------------------|--------------------|---------------------|-------------------|-------------------|--|
|                    | LOD 4 ng/mL                           | 15 ng/mL           | 40 ng/mL            | 400 ng/mL         | 800 ng/mL         |  |
| $\Delta^8$ -THC    | 3.9 ng/mL (-1.6%)                     | 14.5 ng/mL (3.4%)  | 37.7 ng/mL (5.7%)   | 390 ng/mL (-2.6%) | 761 ng/mL (-4.9%) |  |
| $\Delta^9$ -THC    | 3.7 ng/mL (-5.7%)                     | 13.9 ng/mL (-7.3%) | 37.9 ng/mL (-5.2%)  | 402 ng/mL (0.5%)  | 767 ng/mL (-4.1%) |  |
| $\Delta^{10}$ -THC | 3.8 ng/mL (-6.8%)                     | 14.3 ng/mL (-4.4%) | 35.9 ng/mL (-10.2%) | 394 ng/mL (-1.5%) | 752 ng/mL (-6.0%) |  |
| $\Delta^8$ -cTHC   | 3.9 ng/mL (-3.4%)                     | 13.9 ng/mL (2.3%)  | 38.5 ng/mL (-3.8%)  | 402 ng/mL (0.5%)  | 769 ng/mL (-3.9%) |  |
| $\Delta^9$ -cTHC   | 4.1 ng/mL (1.6%)                      | 13.6 ng/mL (-9.6%) | 38.7 ng/mL (-3.2%)  | 405 ng/mL (1.2%)  | 778 ng/mL (-2.7%) |  |
| CBD                | 3.6 ng/mL (-8.9%)                     | 13.3 ng/mL (11.5%) | 36.4 ng/mL (-9.0%)  | 398 ng/mL (-0.6%) | 752 ng/mL (-6.1%) |  |

Table 3. Mean recovery and percent bias data for QCs (n=11).

### MATRIX EFFECTS

Matrix effect was determined in 12 analyte-negative urine matrices by comparison with response of a non-matrix control. Figure 5 displays the percent matrix effect for analytes and internal standard across the urine matrices at concentrations of 200 ng/mL and 500 ng/mL. At 200 ng/mL (left panel), the early eluting metabolites and their internal standards demonstrated ion suppression, with means of -18% ( $\Delta^8$ -cTHC), -28% ( $\Delta^9$ -cTHC) and -20% ( $\Delta^9$ -cTHC-D3). The THC isomers showed ion enhancement, with means of 25% ( $\Delta^8$ -THC), 24% ( $\Delta^{10}$ -THC) and 32% ( $\Delta^9$ -THC). Ion enhancement (27%) was also determined for their internal standard ( $\Delta^9$ -THC-D3). CBD and CBD-D3 both averaged 18% ion enhancement. In the right panel data is displayed the same study performed at standard concentrations of 500 ng/mL. Compared with the lower concentration study, the metabolites and their internal standard all averaged similar, but less ion suppression of -12% ( $\Delta^8$ -cTHC), -9% ( $\Delta^9$ -cTHC), and -9% ( $\Delta^9$ -cTHC-D3), further demonstrating the matrix normalization power of stable isotope internal standardization. CBD and CBD-D3 again showed a comparable ion enhancement (28%) but greater ion enhancement for both at the higher concentration. Except for the ion suppression of  $\Delta^{10}$ -THC (-11%), the pattern of ion enhancement was parallel for  $\Delta^8$ -THC (18%),  $\Delta^9$ -THC (19%), and their internal standard  $\Delta^9$ -THC-D3 (21%). Matrix effects criteria were met for all analytes.

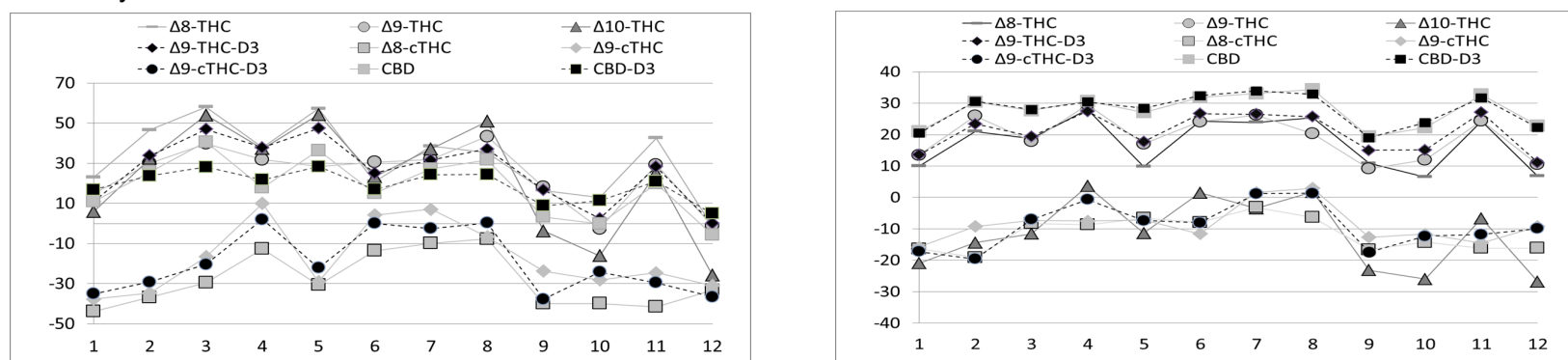


Figure 5. Matrix effects on analytes and internal standards at 200 ng/mL (left panel) and 500 ng/mL (right panel) in 12 urine matrices.

### ISOMER PREVALENCE IN CASEWORK

Prevalence of cannabinoid isomers was evaluated in 220 de-identified case specimens using isomer-selective analysis. All cases were confirmed positive for CBD and/or  $\Delta^9$ -cTHC and/or  $\Delta^8$ -cTHC. Table 4 shows the relative prevalence of these three analytes in the 220 case samples.

The least frequent finding was CBD with a 10% prevalence based on parent drug testing. The majority of samples (99%) were positive for  $\Delta^9$ -cTHC at an average concentration of 766 ng/mL and with a maximum concentration of 8880 ng/mL.

A 14% prevalence of  $\Delta^8$ -cTHC cases (30 cases) was determined with a mean concentration of 530 ng/mL and a maximum concentration of 4300 ng/mL.

It is interesting to note that the percent prevalence of  $\Delta^8$ -THC use, based on toxicology casework findings, matched closely with the percent prevalence of  $\Delta^8$ -THC in e-liquids used in vaping as determined in a recent New York State study (J Anal Toxicol 2022;46:743). The parent drugs,  $\Delta^8$ -THC,  $\Delta^9$ -THC, and  $\Delta^{10}$ -THC, were not detected in any of the case samples.

Figure 6 compares the concentration of  $\Delta^8$ -cTHC and  $\Delta^9$ -cTHC isomers in the 30 cases with positive  $\Delta^8$ -cTHC findings. In 27 cases, there were detectable-to-high concentrations of  $\Delta^9$ -cTHC. The relative isomer concentration is annotated for each case and shows a wide variation in  $\Delta^8$ -cTHC and  $\Delta^9$ -cTHC excretion by users of the cannabinoid isomers. Co-excretion of  $\Delta^9$ -THC metabolite was found in 90% of  $\Delta^8$ -cTHC users, indicates a high rate of concomitant use of  $\Delta^8$ -THC and  $\Delta^9$ -THC which is consistent with knowledge that CBD-sourced derivatives contain both  $\Delta^8$ -THC and  $\Delta^9$ -THC in varying ratios.

Analysis was performed with and without beta glucuronidase treatment in 13 de-identified case samples containing both  $\Delta^8$ -cTHC and  $\Delta^9$ -cTHC to determine relative percent of the glucuronidated forms of excreted  $\Delta^8$ -cTHC and  $\Delta^9$ -cTHC. Figure 7 shows the percent glucuronidation varying significantly between donors for both  $\Delta^8$ -cTHC and  $\Delta^9$ -cTHC. The study also showed, however, that the percent glucuronidation was similar for  $\Delta^8$ -cTHC and  $\Delta^9$ -cTHC within individual donors.

Regression analysis comparing intra-individual percent glucuronidation revealed a correlation coefficient of 0.940 between the metabolite isomers with a regression slope of 1.03 and intercept of 7.6 ng/mL. These findings suggest that UDP-glucuronide transferase enzyme specificity is similar for the major excretion metabolites in  $\Delta^8$ -THC and  $\Delta^9$ -THC users. Further studies are needed to determine whether relative isomer clearance rates are similar and whether the ratio of urinary  $\Delta^8$ -cTHC and  $\Delta^9$ -cTHC can be used to predict the ratio  $\Delta^8$ -THC and  $\Delta^9$ -THC in the administered product, as this information may be useful in identifying a common production lot or derivative product source.

### PRIOR METHOD COMPARISON & INTERFERENCE

Performance of the isomer-selective method was compared with the prior confirmation method for quantitation  $\Delta^9$ -cTHC in 39 de-identified case specimens. Three of the case specimens tested by the non-selective method showed chromatographic interference that did not allow conclusive identification or quantitation of  $\Delta^9$ -cTHC. Figure 8 shows the correlation of  $\Delta^9$ -cTHC concentration in the 36 remaining cases. Regression analysis slope, intercept and correlation coefficient provide evidenced of unbiased agreement between the methods.  $\Delta^8$ -cTHC was not detected in the 36 specimens, based upon the isomer-selective method results.

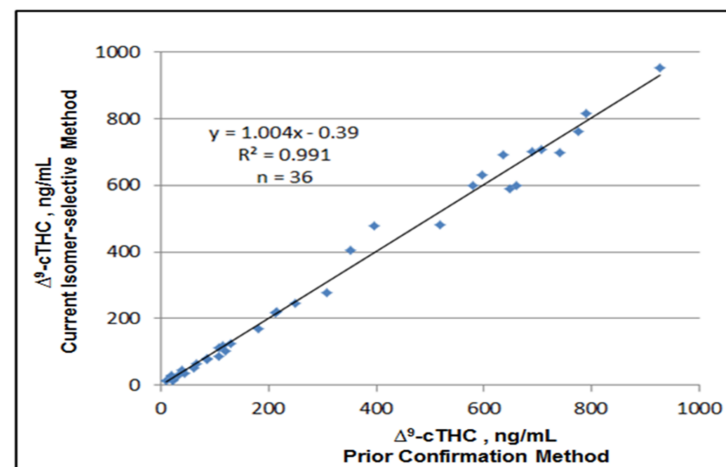


Figure 8. Comparison of  $\Delta^9$ -cTHC concentration in 36 positive case samples co-analyzed by the isomer-selective and prior confirmation methods.

Further studies were performed with the three case samples demonstrating interference by the prior confirmatory method. Isobaric interference by  $\Delta^8$ -cTHC in testing for  $\Delta^9$ -cTHC by the prior confirmation method is shown in Figure 9. Panels A and B display MRM chromatograms with normal peak symmetry for quantifier and qualifier ions in a representative  $\Delta^9$ -cTHC 500 ng/mL calibrator (Panel A) and in a case sample without detectable  $\Delta^8$ -cTHC (Panel B). Panel C shows the results for analysis of a combined reference standard containing both  $\Delta^8$ -cTHC and  $\Delta^9$ -cTHC. Both quantifier (top) and qualifier (middle) MRM chromatograms show a frontal peak interference by isobaric  $\Delta^8$ -cTHC which is partially co-eluting with  $\Delta^9$ -cTHC. The same pattern of interference is observed in three case samples (Panels D-F) with the partially co-eluting interference peak demonstrating a retention times consistent with the  $\Delta^8$ -cTHC and  $\Delta^9$ -cTHC reference sample analysis in Panel C. The progressive increase in  $\Delta^8$ -cTHC interference observed in panels D through F is consistent with the progressively higher relative concentration ratio of  $\Delta^8$ -cTHC to  $\Delta^9$ -cTHC as determined by additional isomer-selective method testing. The findings confirm  $\Delta^8$ -cTHC interference with  $\Delta^9$ -cTHC in confirmation testing by the prior method and emphasize the need for isomer-selective testing.

## CONCLUSIONS

1. Prior confirmation method for  $\Delta^9$ -cTHC demonstrates limited test scope and potential for CBD-sourced derivative interference
2. Development, validation and application of an isomer-selective confirmation method was achieved with applicability to high-volume confirmation testing.
3. Optimization of chromatographic separation was found to be essential for isomer-selective analysis, especially for the closely eluting isobaric parent drugs and metabolite isomers.
4. A 14% prevalence for the  $\Delta^8$ -cTHC isomer was determined in toxicology casework, indicating significant concomitant use of  $\Delta^8$ -THC and  $\Delta^9$ -THC containing products.
5. Percent glucuronidation was similar for  $\Delta^8$ -cTHC and  $\Delta^9$ -cTHC within individual donors, therefore the relative metabolite concentration may reflect the ratio of isomers in the administered drug preparation.
6. Toxicology laboratories should remain vigilant for additional cannabinoid derivatives that may require further expansion in the scope and selectivity of confirmation testing for psychoactive cannabinoid use.

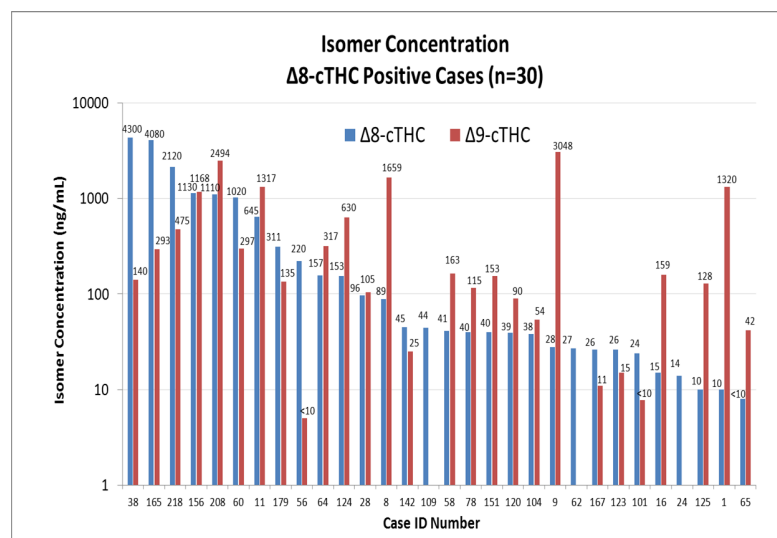


Figure 6. Relative concentration of THC metabolite isomers in  $\Delta^8$ -cTHC positive cases.

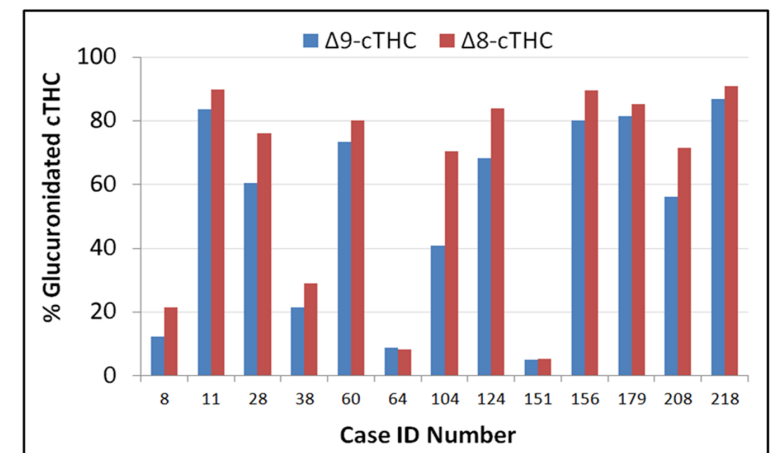


Figure 7. Comparison of glucuronidation of  $\Delta^8$ -cTHC and  $\Delta^9$ -cTHC within and between users of  $\Delta^8$ -THC and  $\Delta^9$ -THC containing products.