Solid-Phase Extraction and Analysis of THC and Carboxy-THC from Whole Blood Using a Novel Fluorinated Solid-Phase Extraction Sorbent and Fast Liquid Chromatography–Tandem Mass Spectrometry

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Abstract

In this study, solid-phase extraction (SPE) is described using a novel fluorinated [heptadecafluorotetrahydrodecyl (C10H4F17)] phase to isolate THC and its primary metabolite carboxy-THC from whole blood samples. SPE was performed in hydrophobic mode after samples of whole blood were precipitated with acetonitrile. After applying the sample to the SPE column in aqueous phosphate buffer (pH 7), the sorbent was washed with deionized water and phosphate buffer (pH 7) and dried. The SPE column was eluted with a solvent consisting of ethyl acetate/hexanes (50:50) containing 2% acetic acid. The eluate was collected, evaporated to dryness, and dissolved in mobile phase (50 μL) for analysis by fast liquid chromatography–tandem mass spectrometry in positive/negative multiple reaction monitoring mode. Chromatography was performed in gradient mode employing a C18 column and a mobile phase consisting of acetonitrile (containing 0.1% formic acid) and 0.1% aqueous formic acid. The total run time for each analysis was less than 5 min. The limits of detection/quantification for this method were determined to be 0.1 and 0.25 ng/mL, respectively. The method was found to be linear from 0.25 to 50 ng/mL (r² ≥ 0.995). Recoveries of the individual cannabinoids were found to be greater than 85%. In this report, results of authentic samples analyzed for THC and carboxy-THC are reported using this new methodology.

Introduction

For most forensic laboratories providing toxicological analyses, the confirmation and quantification of Δ9-tetrahydrocannabinol (THC) and its primary metabolite in whole blood are high on the list of requests from enforcement agencies, such as district attorneys and/or police departments (city, county, or state levels). The ability to efficiently extract and quickly analyze samples of blood submitted by such agencies is seen as a definite benefit to the laboratory in question. In this new procedure, the efficient extraction of the two cannabinoids via a novel fluorinated solid-phase extraction (SPE) sorbent coupled with fast analysis using tandem mass spectrometry coupled to liquid chromatography (LC–MS–MS) offers a new direction for laboratories to embark upon in terms of forensic toxicological analysis, especially in the area of drugs and driving cases.

It has been reported recently by Farrell et al. (1) that amongst forensic toxicology laboratories, cannabis is one of the most frequently encountered drugs. With regard to the reported analyses of THC and Δ9-tetrahydrocannabinol carboxylic acid (carboxy-THC) (Figures 1 and 2), previous methods have employed the use of liquid–liquid extraction (2), and SPE (both polymeric and silica-based sorbents) (3,4). These extractions have been followed in the main with gas chromatography (GC)–MS analysis (5–7) in which the extracts have been derivatized prior to chromatographic separation. With the advent of affordable tandem MS (coupled to LC), methods...
in which the need for derivatization has been eliminated have been published (8–10), thus saving valuable time for the analytical process. We extend this process further by applying fast LC to further enhance the productivity by reducing the time taken for each analytical run.

In our procedure, we describe the use of a fluorinated sorbent for the extraction of THC and its primary metabolite from whole blood after acetonitrile precipitation. Fluorous SPE has been in use within the pharmaceutical industry for some time, and reports discussing its employment for the isolation and purification of pharmaceuticals have entered the literature since 2000 (11,12) but not in the field of analytical toxicology. It was with this in mind that we approached the idea of employing a fluorinated SPE sorbent (Fluoro-C10) to efficiently extract these popularly observed compounds (THC and carboxy-THC) and analyze them with fast LC–MS–MS in both positive and negative multiple reaction monitoring (MRM) mode.

This SPE method coupled with fast LC–MS–MS provides a simple, sensitive, and reproducible quantitative method for the analysis of THC and its primary metabolite in whole blood. This procedure should be of great assistance to those analysts actively involved with the LC–MS–MS analysis of these drugs in biological matrices.

Experimental

Reagents and materials
THC, THC-d3, carboxy-THC, and carboxy-THC-d3 were supplied as liquid standards from Cerilliant (Round Rock, TX) as 1 mg/mL solutions in the case of THC and 0.1 mg/mL solutions for THC-d3, carboxy-THC, and carboxy-THC-d3. Acetonitrile, acetic acid (glacial), ethyl acetate, hexanes (containing 60–66% n-hexane), and methanol were obtained from Fisher Scientific (Pittsburgh, PA). Phosphate buffer (0.1 M, pH 7) was purchased already prepared from Fisher Scientific. Formic acid was obtained from Acros Chemicals (via Fisher Scientific). All chemicals were of ACS-grade unless stated otherwise. SPE Fluoro-C10 columns (6 mL, 200 mg) were a kind donation from UCT Inc., (Bristol, PA). SPE was performed using Varian Vac Elut SPS 24 vacuum manifold (Walnut Grove, CA).

Formic acid was made up as a 0.1% (v/v) solution by the addition of 1 mL of the acid to 900 mL of deionized (DI) water and diluting to 1 L. Acetonitrile containing 0.1% formic acid (v/v) was made up by adding 1 mL of formic acid to 900 mL of acetonitrile and diluting to 1 L with the same.

Chromatographic analysis
Analysis was performed using an API 3200 Q-Traps instrument supplied by Applied Biosystems (Foster City, CA). The chromatographic system consisted of a Shimadzu CMB 20 A controller, two Shimadzu LC 20 AD pumps including degasser, a Shimadzu SIL 20 AC autosampler, and a Shimadzu CTO AC autosampler unit set to keep the samples at 10°C (Kyoto, Japan). The instrument was fitted with an Imtakt US-C18 column (50 × 2 mm, 5 µm) from Silvertone Sciences (Philadelphia, PA) and was attached to a Union US-C18 guard column (5 × 2 mm), which was obtained from the same supplier. The LC column oven was maintained at 40°C throughout the analyses.

Retention times for THC, THC-d3, carboxy-THC, and carboxy-THC-d3 are shown in Table I. The instrument was readied for re-injection after 4.5 min. The MS was performed on an API 3200 QTRAP using both positive/negative MRM modes. Positive ion mode was employed for THC/THC-d3 under the following conditions: curtain gas 15 lb psi, collision gas medium, ion spray voltage 5000 V, temperature 650°C, ion source gas (a): 50 psi, ion source gas (b): 50 psi. Negative mode was employed for carboxy-THC and carboxy-THC-d3 under the following conditions: curtain gas 15 psi, collision gas medium, ion spray voltage −4500 V, temperature 650°C, ion source gas (a): 50 psi, ion source gas (b): 50 psi.

The following transitions were monitored as follows with quantification ions displayed first: m/z 343.1 → 299.3 and 245.3 for carboxy-THC, and m/z 346.1 → 302.3 and 248.3 for carboxy-THC-d3. The following transitions were monitored as follows with quantification ions displayed first: m/z 315.2 → 193.2 and 123.1 for THC, and m/z 318.2 → 196.2 and 123.1 for THC-d3. The values for declustering potential (DP), collision energy (CE), entrance potential (EP), and collision cell exit potential (CXP) are given for each compound in Table I. Data was collected using Analyst Software (Version 1.4.2).

11-Hydroxy-THC was not subject to analysis by this procedure as it was found that it elutes close to carboxy-THC in the fast LC–MS–MS program. Positive MRM mode is required to maximize sensitivity for 11-hydroxy-THC, whereas negative MRM mode is required for carboxy-THC. The software (Analyst 1.4.2) does not allow for efficient switching from negative to positive modes to capture data from both compounds, thus only carboxy-THC and THC were considered as part of the analytical project.

Retention times for THC, THC-d3, carboxy-THC, and carboxy-THC-d3 were 2.57, 2.55, 2.07, and 2.06 min, respectively.
Calibrators and controls
A solution of THC was prepared at a concentration of 1 μg/mL by the dilution of 10 μL of stock solution with methanol to 10 mL in a volumetric flask. Solutions of THC-d₃, carboxy-THC, and carboxy-THC-d₃ were prepared similarly by the dilution of 100 μL of the individual stock solutions to 10 mL with methanol using individual volumetric flasks.

Calibrators were prepared by the addition of 0.25, 1, 2, 5, 10, and 50 ng of THC, and carboxy-THC to 1-mL samples of drug-free whole blood. To the calibrators were added 25 ng of the deuterated internal standards (THC-d₃, carboxy-THC-d₃). Control samples were prepared by the addition of 4 ng and 15 ng of THC and carboxy-THC to 1 mL of the drug-free whole blood along with 25 ng of the internal standards. All determinations were performed in duplicate (i.e., each point on the calibration curve was analyzed twice as were the control samples) and the mean values employed. In order to eliminate the possibility of carboxy-THC-d₃/THC-d₃ contributions to the THC/carboxy-THC chromatography, two samples of drug-free whole blood spiked only with the deuterated internal standards were analyzed.

The dynamic range of the analysis was set to cover the range of carboxy-THC concentrations (0.2–50 ng/mL) observed in cases submitted to the Massachusetts State Police Crime Laboratory. For samples that exceed 50 ng/mL, an aliquot would be taken of the original sample and diluted with an appropriate volume of drug-free whole blood. This would be re-extracted and analyzed.

Sample pretreatment
In this project, samples of whole blood (1 mL) from previously analyzed cases were treated in exactly the same fashion as calibrators and controls with respect to the addition of internal standards. The analysis of the case samples was performed in duplicate.

Two milliliters of cold acetonitrile was added (dropwise) to each sample (1 mL) while it was vortex mixing. Following addition of the acetonitrile, the sample was further vortex mixed for approximately 1 min. Each sample was centrifuged at 3000 rpm for 10 min to separate blood precipitants from the acetonitrile.

Following centrifugation, the acetonitrile phase was transferred into individual clean glass tubes (non-silanized). The solvent was evaporated to approximately 200 μL using an evaporator set at 35°C and employing nitrogen as the drying gas. To each tube was added 5 mL of 0.1 M phosphate buffer (pH 7), and the samples were vortex mixed for approximately 0.5 min.

SPE
Fluorinated SPE columns (F-SPE) were conditioned by the sequential addition of 1 × 3 mL of methanol, 1 × 3 mL of DI water, and 1 × 1 mL of 0.1 M phosphate buffer (pH 7). Each liquid was allowed to percolate through the sorbent using gravity without allowing the sorbent to dry out in between addition of the liquids.

After the phosphate buffer had passed through the F-SPE column, each sample was loaded onto an individually marked F-SPE tube and allowed to flow through the sorbent using gravitational flow. The columns were then washed with 1 × 3 mL of DI water followed by 1 × 3 mL of 0.1 M phosphate buffer (pH 7) and dried under full vacuum for 10 min.

Each F-SPE column was eluted by the addition of 1 × 3 mL of a hexanes/ethyl acetate solvent mixture (50:50, v/v) containing 2% acetic acid. The elution solvent was allowed to flow through the sorbent with the aid of gravity and collected in silanized glass tubes for evaporation. Silanized glass tubes were employed at this stage to eliminate the potential of adsorption of the cannabinoids onto the surface of the glass tubes.
The eluent from each F-SPE column was evaporated to dryness using a gentle stream of nitrogen at 35°C after which the samples were dissolved in mobile phase (50 μL) for analysis by LC–MS–MS. Representative chromatograms are shown in Figures 3–6.

Recovery studies

To determine the recovery values across the dynamic range of the analysis, the results of the F-SPE extractions of the blood (as duplicate analyses) were compared to those values obtained from unextracted standards at corresponding concentrations. The unextracted standards were prepared by evaporation of methanolic solutions of THC/carboxy-THC [and THC-d₅/carboxy-THC-d₅ (25 ng)] over the range of calibrators. The dried residues were dissolved in mobile phase (50 μL) prior to analysis by fast LC–MS–MS.

Matrix effects

Studies into the matrix effects were performed according to procedures described by Matuszewski et al. (13). Essentially, the analytes were added to the matrix before and after F-SPE extractions, and a measure of matrix effects was ascertained. The difference in MS response between pre- and post-addition of analytes to the extracted matrix is a measure of how much the matrix contributes to the signal response and is distinct from a recovery study (where the difference in analyte concentration pre- and post-SPE (i.e., neat, unextracted samples) is measured).

In this area of the study, samples of drug-free whole blood (1 mL) were extracted using the F-SPE procedure. After elution and evaporation, THC and carboxy-THC were added at concentrations corresponding to 4 and 15 ng/mL, respectively. To these solutions was also added 25 ng of THC-d₅/carboxy-THC-d₅. Following further evaporation, the samples were dissolved in mobile phase (50 μL) for analysis by fast LC–MS–MS. Another set of extractions was performed in which 4 and 15 ng/mL of THC/carboxy-THC (and 25 ng THC-d₅/carboxy-THC-d₅) was added to samples of drug-free whole blood. The samples were vortex mixed and extracted according to the F-SPE procedure. Following elution and evaporation, the samples were dissolved in mobile phase (50 μL) for analysis.

A comparison of the pre-F-SPE spiked samples to the post-F-SPE spiked samples provided the data for the matrix effects.

 Ion suppression

THC and carboxy-THC (concentration: 0.5 ng/mL of each) were infused into the tandem MS using the on-board syringe pump (controlled by Analyst 1.4.2 software) via a Hamilton syringe (model #1001TTL, volume = 1 mL) supplied by Fisher Scientific at a flow rate of 5 μL/min. At the same time as the THC/carboxy-THC solution was flowing into the MS, a 10-μL aliquot of the F-SPE extracted matrix [drug-free blood (1 mL) in mobile phase (50 μL)] was injected using the autosampler syringe on the Shimadzu LC using Analyst 1.4.2 software. The LC and MS were arranged so that samples from the LC were mixed into the flow of THC/carboxy-THC in a three-port T-section before the total flow entered the MS. Any suppression effects on the THC/carboxy-THC could be monitored at the MRMs for the noted cannabinoids.

Selectivity

In analyzing blood samples via F-SPE and LC–MS–MS, it is essential to ensure that the interfering effects of other drug compounds be eliminated. In this procedure, whole blood was spiked with 49 drugs at a concentration of...
100 ng/mL (bupropion, lidocaine, methadone, amitriptyline, nortriptyline, thioridazine, trazodone, mesoridazine, pethidine, diphenhydramine, phenyltoloxamine, imipramine, desipramine, benztropine, trimethoprim, diltiazem, haloperidol, strychnine, morphine, codeine, 6-acetylmorphine, oxycodone, oxymorphone, hydrocodone, noroxycodeone, hydromorphone, diazepam, nordiazepam, oxazepam, temazepam, alprazolam, α-hydroxyalprazolam, lorazepam, triazolam, α-hydroxytriazolam, flunitrazepam, 7-aminoflunitrazepam, chloridiazepoxide, midazolam, α-hydroxymidazolam, flurazepam, desalkylflurazepam, cocaine, ecgonine methyl ester, ecgonine ethyl ester, benzoylecgonine, cocaethylene, clonazepam, and 7-aminoclonazepam) and extracted according to the F-SPE method. It was observed that the interfering effect of these compounds was not found to be significant. A representative chromatogram of whole blood spiked with the noted compounds, extracted, and analyzed by LC–MS–MS using the chromatographic program/MRMs for THC, THC-d3, carboxy-THC, and carboxy-THC-d3 is shown in Figure 7, indicating the performance of the F-SPE method.

Although cannabinoids such as Δ⁸-tetrahydrocannabinol (Δ⁸-THC), cannabidiol, and 11-hydroxy-THC are structurally related to THC and carboxy-THC, these compounds were not investigated at this time. It is noted 11-hydroxy-THC has a molecular weight of 330.4 and is significantly different from THC (molecular weight = 314.4), and carboxy-THC (molecular weight = 344.4) could cause any interference. However, although cannabidiol, Δ⁸-THC, and THC do share a common molecular weight (314.4), the differences in the molecular structures (Figures 8 and 9) (14,15) of these compounds should permit chromatographic separation from THC and carboxy-THC, thus eliminating the potential for interference. The chromatographic separation of cannabidiol, Δ⁸-THC, THC, and carboxy-THC was not investigated as part of this study.

Results and Discussion

Recovery studies
It was found that for samples of blood spiked at 4 ng/mL and 15 ng/mL, concentrations of 3.8 ± 0.2 ng/mL and 15.2 ± 0.2 ng/mL (THC) and 4.2 ± 0.2 ng/mL and 15.4 ± 0.4 ng/mL (carboxy-THC), respectively, were obtained. These values were obtained from the calibration curves constructed from blood samples spiked over the dynamic range and extracted using the F-SPE method. These values were chosen as a measure of determining the accuracy/imprecision at the lower to mid-range of the calibration curve.

Recovery values of 85 ± 2% (THC) and 89 ± 2% (carboxy-THC) were obtained over the calibration range for this analysis (i.e., > 85% overall for both analytes).

This method was found to be linear (r² ≥ 0.995) over the dynamic range 0.25 ng/mL to 50 ng/mL for THC and carboxy-THC (Figures 10 and 11).

Imprecision of analysis
The intraday precision was assessed by analyzing the 4 ng/mL and 15 ng/mL controls 10 times each, resulting in an intraday precision of 4% (THC) and 6% (carboxy-THC). The interday precision was determined by analyzing the same two control levels over a period of five days. The control samples and calibration curves were prepared fresh on each of the five days. This resulted in an interday precision of 6% (THC) and 8% (carboxy-THC) (n = 10).

Limits of detection (LOD) and quantification (LOQ)
The LOD of a particular method can be defined as the level at which the signal-to-noise ratio for the particular analyte is ≥ 3:1. The limit of quantification (LOQ) for the method is the level at which the signal-to-noise ratio for a particular analyte is ≥ 10:1.

To determine the LOD of the method, drug-free whole blood samples (1 mL spiked with THC and carboxy-THC at decreasing concentrations below 1 ng/mL) were analyzed in duplicate employing the F-SPE/LC–MS–MS. This process was employed to assess the concentration at which the signal-to-noise ratio (3:1) just failed. This value was determined to be 0.1 ng/mL. In terms of LOQ, samples of whole blood (1 mL) were spiked with THC/carboxy-THC at levels below 1 ng/mL and extracted according to the F-SPE procedure until the analytes failed to meet the signal-to-noise ratio (10:1). This value was determined to be 0.25 ng/mL. Representative chromatograms for the LOD and LOQ are shown (Figures 5 and 6).

Matrix effects and ion suppression studies
It was found that the matrix effects for this F-SPE application were < 6% (i.e., over 94% of the matrix was removed). Matrix effects (whether they increase or decrease the MS signal response) may be derived from the transfer of extracted...
endogenous materials through the extraction process into the final analytical solution. By removing these endogenous materials as effectively as possible, the less impact they can have upon the final MS signal. This novel use of F-SPE has been shown to significantly reduce the matrix effects.

In the ion suppression study, THC, THC-\(d_3\), carboxy-THC, and carboxy-THC-\(d_3\) MRMs were monitored while the drug-free extracted matrix was injected into the system. This study is a measure of the suppression effect of the extracted matrix upon the signal response. In an ideal situation, the matrix is removed entirely by the extraction procedure, and no reduction in signal response is noted. The use of F-SPE in the extraction process was shown to reduce the effect of signal suppression by removing those interfering compounds. Chromatograms indicating the lack of ion suppression from the F-SPE extraction procedure are shown in Figure 12.

**Case Samples**

In this project, 10 samples from closed-out cases were subjected to analysis using F-SPE and fast LC–MS–MS. The values obtained from the original analyses ranged from 0.4 to 2.5 ng/mL (THC) and 1.9 to 40.0 ng/mL (carboxy-THC). When reanalyzed, the values of THC and carboxy-THC were found to be as shown in Table II.

The values obtained by this new methodology were found to be consistent with the original test values. The previously analyzed samples were received by the Massachusetts State Police Crime Laboratory in 2008. The samples were submitted to the laboratory already preserved with sodium fluoride. After the initial analysis (performed according to the validated method contained within the current standard operating procedure manual employing Clean Screen THC SPE columns purchased from UCT), these samples were analyzed by LC–MS–MS on the same API 3200 Q-Trap instrument employing identical tandem MS conditions. For case samples, an LOQ of 0.25 ng/mL and a linear range of 0.25–50 ng/mL, respectively, were employed.
interaction of THC/carboxy-THC and the F-SPE sorbent is not fully understood, it is thought by the authors to involve some degree of hydrophobicity and localized ion-exchange functionality.

Conclusions

In this procedure where a novel F-SPE sorbent extraction methodology was coupled to fast LC–MS–MS, a highly efficient technique has been developed. The use of this process should greatly assist analysts in the field of drug-related driving cases to quickly resolve the issues involved in reporting values for both THC and its primary metabolite (carboxy-THC) in whole blood samples. Comparison of re-analyzed cases with their previous historical data has shown the effectiveness of this new, improved methodology.

In these days of acetonitrile shortages, this method reduces the needs and requirement for this compound within the SPE procedure, thus making this new methodology a very attractive prospect for laboratories engaged in the analysis of THC and carboxy-THC.

With an LOD/LOQ of 0.1 ng/mL and 0.25 ng/mL, respectively to analyze easily achievable, quantifying both drugs rapidly is within the grasp of those chemists charged with the analysis of these compounds in the legal system.

Table II. Case Sample Results

<table>
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<tr>
<th>Case #</th>
<th>THC/(ng/mL)</th>
<th>Carboxy-THC/(ng/mL)</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>1.4 (1.6)*</td>
<td>38.0 (40.0)</td>
</tr>
<tr>
<td>2</td>
<td>2.8 (2.5)</td>
<td>35.0 (37.0)</td>
</tr>
<tr>
<td>3</td>
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<td>5.5 (6.5)</td>
</tr>
<tr>
<td>4</td>
<td>0.6 (0.8)</td>
<td>7.5 (7.2)</td>
</tr>
<tr>
<td>5</td>
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<td>2.9 (3.2)</td>
</tr>
<tr>
<td>6</td>
<td>0.3 (0.6)</td>
<td>1.7 (1.9)</td>
</tr>
<tr>
<td>7</td>
<td>1.2 (1.1)</td>
<td>5.5 (5.3)</td>
</tr>
<tr>
<td>8</td>
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<td>1.9 (2.3)</td>
</tr>
<tr>
<td>9</td>
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<td>5.8 (5.3)</td>
</tr>
<tr>
<td>10</td>
<td>0.7 (0.8)</td>
<td>6.5 (6.6)</td>
</tr>
</tbody>
</table>

* Previous results shown in parentheses.

F₃C–(CF₂)₇–(CH₂)₂–

Figure 13. Structure of F-SPE material.

After the analyses were completed, the samples were stored under refrigeration at 4°C.

Tandem MS

This project was aimed at introducing a more efficient methodology to the forensic community involved in toxicological analyses of THC and carboxy-THC. Although carboxy-THC and its deuterated analogue can be detected using the tandem MS in positive MRM mode (9), it was found that sensitivity (i.e., greater signal-to-noise ratio) is increased by altering the MRM mode from positive to negative mode. As the carboxy-THC is eluted from the analytical LC column before the THC (Figure 4), the system was programmed to run in negative MRM mode and switched to positive mode after carboxy-THC was eluted to capture THC in the MS. 11-Hydroxy-THC elutes closely to carboxy-THC and is detected using positive MRM mode. The software controlling the tandem MS (Analyst 1.4.2) is unable to switch polarity between positive and negative MRM modes quickly enough to collect data for both compounds, thus 11-hydroxy-THC was not included in this project.

F-SPE

Heptadecafluorotetrahydrodecyl (C₁₀H₄F₁₇) (Figure 13) is a fluoro-substituted decyl sorbent. As noted earlier, F-SPE has been used in the area of pharmaceutical chemistry for isolating and purifying materials for several years, and this is the first reporting of such a compound in the area of forensic toxicology for performing a similar task, although from a more complex matrix.

This application finds itself operating in a hydrophobic mode. The C₁₀ moiety providing this type of interaction for sorbing of the molecules whereas the addition of the fluorine atoms is believed by the authors to lend an increase in polarity to the bonded silica. Although the full mechanism for the interaction of THC/carboxy-THC and the F-SPE sorbent is not

References

7. R.D. Scurlock, G.B. Ohlson, and D.K. Worthen. The detection of Δ⁴-tetrahydrocannabinol (THC) and 11-nor-9-carboxy-Δ⁴-tetra-


