Application Note

Forensic Analysis

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Efficient Quantitative Analysis of THC and its Metabolites in Whole Blood Using Agilent Captiva EMR—Lipid and LC-MS/MS

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Abstract

Efficient extraction, cleanup, and analysis of complex biological samples are extremely beneficial to the forensic laboratory. Phospholipids (PPLs) have been identified as a major cause of matrix effects in the LC-MS/MS analysis of tetrahydrocannabinol (THC) and its metabolites in whole blood. This Application Note describes the extraction and LC-MS/MS analysis of Δ^9-THC (THC) and its major metabolites, 11-hydroxy-Δ^9-THC (THC-OH) and 11-nor-9-carboxy-Δ^9-THC (THC-COOH) from whole blood using in-well PPT followed by PPL removal using Agilent Captiva EMR—Lipid in a pass-through 1 mL cartridge. Captiva EMR—Lipid produced cleaner eluents with removal of over 97 % of the unwanted PPLs from whole blood matrix, and over 92 % recoveries for target analytes. Analysis of THC, THC-OH, and THC-COOH at 1 ng/mL yielded ideal peak shapes with good signal-to-noise (S/N). Response from 0.5 to 100 ng/mL was linear, with an R^2 >0.99. Limits of quantitation of 1.0 ng/g or lower were obtained, with RSD <11.5 %. Results were consistent over three days of experiments.

Introduction

Efficient sample preparation prior to LC-MS/MS analysis is an important consideration for forensic labs. Sample preparation is used to reduce system contamination, and improve data integrity, method selectivity, analytical sensitivity, and reliability. Two of the major interferences found in whole blood are proteins and phospholipids (PPLs). PPLs have been identified as a major cause of matrix effects in LC-MS/MS bioanalyses due to competitive ionization on the surface of droplets formed during electrospray ionization (ESI).[1]

Common forensic sample preparation techniques include protein precipitation (PPT), solid phase extraction (SPE), liquid-liquid extraction (LLE), and supported liquid extraction (SLE). Each technique has advantages and disadvantages in terms of speed, cost, and quality of the data generated. For example, PPT, LLE, and SLE do not remove PPLs, and SPE is more time-consuming and complicated to perform.[2] However, of these techniques, PPT is most widely accepted. Using PPT, proteins are easily and efficiently removed by adding an organic crash solvent, such as ACN or MeOH, to biological samples in a prescribed ratio. As the proteins denature, they form a precipitate that can be removed by filtration or centrifugation. However, PPLs are not removed by PPT because they are soluble in organic crash solvents.

Cannabinoids are among the most common target analytes in forensic labs in support of casework. Fast and accurate confirmation and quantification of Δ^9-THC (THC) and its primary metabolites 11-hydroxy-Δ^9-THC (THC-OH) and 11-nor-9-Δ^9-carboxy-THC (THC-COOH) in biological samples are essential. Nevertheless, THC and its metabolites can be prone to nonspecific binding during sample preparation.

A sample preparation method for whole blood that reduces sample preparation steps, including off-line PPT, centrifugation, transfer, and dilution, while allowing streamlined in-well PPT and PPL removal, is highly desirable. This Application Note describes an approach that relies on Agilent Captiva EMR—Lipid 1 mL cartridges to remove interferences, particularly PPLs, without analyte loss, in a simple pass-through format. The resulting extract is cleaner, reducing potential ion suppression, and column and mass spectrometer contamination.

Extraction of THC, THC-OH, and THC-COOH from whole blood was performed using in-well PPT followed by PPL removal using the Captiva EMR—Lipid cartridge. Subsequent quantitative analysis was performed using the Agilent 6490 Triple Quadrupole LC/MS system. The extent of PPL removal was evaluated. Inter-day (days = 3) accuracy, precision, and recovery for THC and its metabolites were also determined.

For analysis of plasma samples, the Agilent Application Note *Efficient Quantitative Analysis of THC and Metabolites in Human Plasma Using Captiva EMR—Lipid by LC-MS/MS* is available.[3]

Experimental

Reagents and Chemicals

Δ^9-THC, 11-hydroxy-Δ^9-THC, 11-nor-Δ^9-carboxy-THC, Δ^9-THC-d3, 11-hydroxy-Δ^9-THC-d3, and 11-nor-9-carboxy-Δ^9-THC-d9 were purchased from Sigma-Aldrich (St Louis, MO, USA). LC-MS/MS grade ammonium formate was also purchased from Sigma-Aldrich. All solvents were LC grade or higher, and were from Burdick and Jackson (Muskegon, MI, USA).

Solutions

A combined standard working solution of THC and its metabolites, THC-OH and THC-COOH, was made at 10 µg/mL in methanol. The deuterated THC-d3, THC-OH-d3, and THC-COOH-d9 were combined in a working solution at 10 µg/mL in methanol, and used as internal standard (IS).

Calibration Standards and Quality Control Samples

Prespiked quality control (QC) samples were fortified with standard working solution to the appropriate concentrations in replicates of seven. The QC samples were low QC (LQC), middle QC (MQC), and high QC (HQC) corresponding to 1, 10, and 50 ng/mL levels in whole blood, respectively. The deuterated solution mix (IS) was spiked at 50 ng/mL at each QC level.

Blank matrix after cleanup by Captiva EMR—Lipid was post-spiked with a working solution of THC and its metabolites, corresponding to 1, 10, and 50 ng/mL concentrations in whole blood. A 5 µL aliquot of a 1.0 µg/mL IS solution was also added.

Matrix-matched calibration curves were prepared with the standard working solution. Blank matrix after Captiva EMR—Lipid was post-spiked to correspond to 0.5, 1, 5, 10, 50, and 100 ng/mL in extract. Five microliters of IS at 1.0 µg/mL was added to each calibration level.

Equipment and Instrumentation

Table 1 provides the list of the equipment and instrumentation used to perform the analysis.

Table 1. Equipment and instrumentation used for sample preparation and analysis.

Component	Part number
Sample Preparation	
Agilent Captiva EMR—Lipid, 1 mL cartridge	5190-1002
Agilent Vac Elut SPS 24 Manifold with collection rack for 12 × 75 mm test tubes	12234041
Eppendorf pipettes and repeater pipettor (VWR, NJ, USA)	
Liquid Chromatography System	
Agilent 1290 Infinity LC System	G4204A
Agilent 1290 Infinity Series Thermostatted Column Compartment	G1316C
Agilent 1290 Infinity Autosampler	G4226A
Agilent ZORBAX Rapid Resolution High Definition (RRHD) Bonus RP 2.1 × 50 mm 1.8 µm column	857768-901
Agilent 1290 Infinity Inline filter, 0.3 µm	5067-6189
Vial Inserts 400 µL glass, flat bottom, deactivated	5183-2086
MS analyzed vial kit. 2 mL amber screw top vials with write-on spot, blue screw caps, and PTFE/silicone septa	5190-2280
Mass Spectrometry System	
Agilent 6490 Triple Quadrupole LC/MS System	
Agilent MassHunter Software	

LC-MS/MS Analysis

An Agilent 1290 Infinity LC System coupled with an Agilent 6490 Triple Quadrupole mass spectrometry system was used for LC-MS/MS analysis. Tables 2 and 3 provide the LC and MS conditions. The samples were not diluted prior to placement into the autosampler. The dilution capabilities of the 1290 Infinity autosampler were used prior to injection, where 10 µL of diluent (water) was aspirated prior to 5 µL of sample. The entire volume was injected into the LC system. The advantage of using the online dilution feature of the autosampler compared to diluting the sample in the autosampler vial is that the sample remains in 100 % organic, where most compounds are more stable.

Table 4 provides triple quadrupole multiple reaction monitoring (MRM) acquisition parameters, including precursor, qualifier and quantifier ions, collision energies (CE), and retention times. To evaluate PPL removal by Captiva EMR—Lipid, 11 PPL MRM transitions were monitored, as shown in Table 5.

Table 2. LC conditions.

Parameter	Value
Column	Agilent ZORBAX Rapid Resolution High Definition (RRHD) Bonus RP 2.1 × 50 mm 1.8 µm column
Flow rate	0.5 mL/min
Colum temperature	50 °C
Autosampler temperature	5 °C
Injection volume	5 µL
Injector program	Draw 10 µL from location "P2-F1" with default speed Draw 5 µL from sample with default speed Wash needle as specified in the method
Mobile phase	A) 5 mM Ammonium Formate in Water, 0.1 % FA B) 5 mM Ammonium Formate in MeOH, 0.1 % FA
Needle wash	ACN:MeOH:IPA:H_2O, 0.2 % FA (1:1:1:1)
Gradient	Time (min) %B 0.0 65 0.1 65 4.0 95 5.0 95
Stop time	5.10 minutes
Post time	1.5 minutes

Table 3. MS conditions.

Parameter	Value
Ionization mode	ESI
Gas temperature	120 °C
Gas flow	20 L/min
Nebulizer	50 psi
Sheath gas heater	325 °C
Capillary voltage	3,500 V
Vcharging	300
Delta electron multiplier voltage (EMV)	200
Polarity	Positive

Table 4. MRM acquisition parameters for THC compounds.

Compound	Precursor ion	Quantifier ion (CE)	Qualifier ion (CE)	Retention time (min)
THC-OH	331.23	313.2 (12)	193.1 (24)	1.70
THC-OH-d3	334.25	316.3 (12)		1.70
THC	315.23	193.2 (24)	123.0 (44)	3.05
THC-d3	318.25	196.1 (28)	28	3.04
THC-COOH	345.21	299.1 (20)	327.3 (12)	2.26
THC-COOH-d9	354.27	336.2	12	2.26

Table 5. MRM acquisition parameters for 11 PPL compounds.

Precursor ion (*m/z*)	Product ion (*m/z*)	Collision energy
808	184	30
806	184	30
786	184	30
784	184	30
760	184	30
758	184	30
704	184	30
524	184	30
522	184	30
520	184	30
496	184	30

Agilent MassHunter Software was used for instrument control, and for qualitative and quantitative data analysis. Inter-day (days = 3) accuracy, precision, and recovery from the method for THC and its metabolites were determined.

Sample Preparation Procedure

1. Add 500 µL of COLD* 15:85 MeOH:ACN to an Agilent Captiva EMR—Lipid 1 mL cartridge.
2. Add 100 µL of human whole blood sample.
3. Thoroughly mix in-well using a disposable glass pipette, or allow 5–7 minutes for passive mixing.
4. Pull a vacuum of 3.5–4 psi.
5. Add 200 µL of COLD 1:4 H_2O:ACN
6. Pull the vacuum until the entire volume is through the cartridge, then increase to 11–13 psi to pull the remaining solvent through.
7. Evaporate, then reconstitute in 100 µL MeOH (0.1 % FA).
8. Inject 5 µL + 10 µL water for dilution directly into the LC system.

* Cold 15:85 MeOH:ACN was stored in a –20 °C freezer and placed in a frozen container while in use.

Note: For analysis of plasma samples, the Agilent Application Note *Efficient Quantitative Analysis of THC and Metabolites in Human Plasma Using Captiva EMR—Lipid by LC-MS/MS* is available.[3]

A ratio from 1:3 to 1:5 (sample/solvent) is common and recommended for complete protein precipitation. The use of cold MeOH/ACN solvent is a convenient approach to cause hemolysis or a rupturing (lysis) of red blood cells. This releases their contents (cytoplasm) into the surrounding blood plasma, forming a powdery precipitant, as shown in Figure 1.

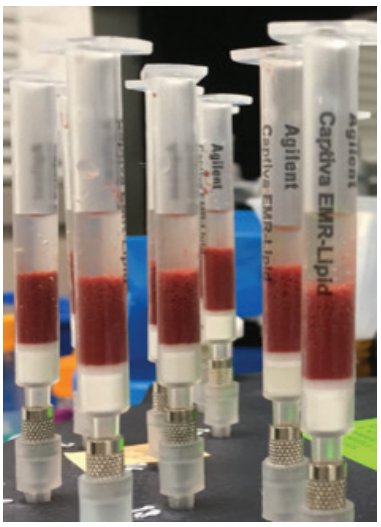


Figure 1. Whole blood after active mixing forms a powdery precipitant prior to vacuum.

Preferably, active in-well mixing is done using wide-bore pipette tips, or by another mixing device. The vacuum initiates flow through the Captiva EMR—Lipid cartridge. A controlled flow rate of one drop per 3–5 seconds is recommended for optimal lipid removal. After sample elution off the cartridge, higher vacuum is applied to maximize sample recovery.

Results and Discussion

Unwanted Lipid Matrix Removal

The EMR—Lipid approach is simple, and universally applicable to reducing matrix effects and improving analyte recoveries for the analysis of polar, midpolar, and nonpolar target analytes. EMR—Lipid uses a unique sorbent chemistry that, when activated by water, causes the EMR—Lipid sorbent to selectively trap lipids by size exclusion and hydrophobic interaction (Figure 2). Unbranched hydrocarbon chains on lipids enter the sorbent, but bulky analytes do not. Lipid chains that enter the sorbent are then trapped by hydrophobic interactions. PPLs are major constituents of cell membranes, and are abundant in whole blood. PPLs consist of a hydrophilic head group composed of phosphate and choline units, and a hydrophobic tail made up of long alkyl chains.

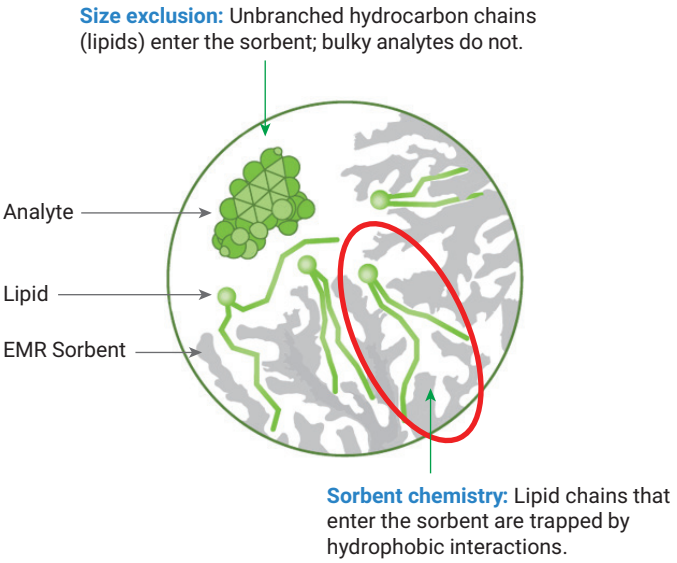


Figure 2. EMR-Lipid mechanism of action: size exclusion and sorbent chemistry.

Though the analytes shown in Figure 3, THC, THC-OH, and THC-COOH, do contain a straight carbon chain, the chain is not long enough to form stable hydrophobic interactions with the EMR sorbent. In addition, the bulky ring component of the analytes inhibits their retention by the sorbent.

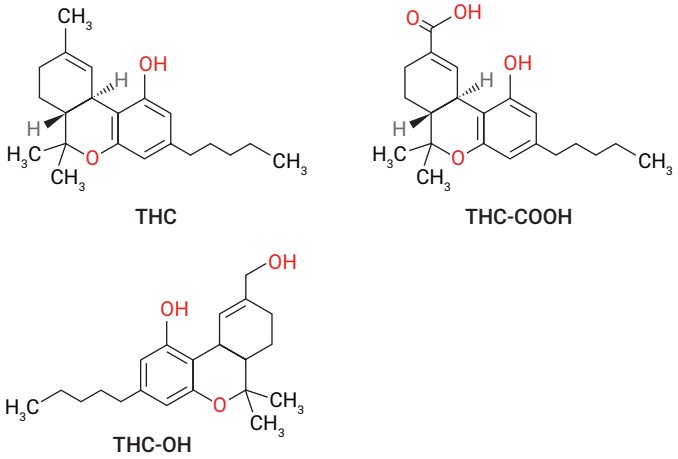


Figure 3. THC and metabolite structures.

The EMR—Lipid technology is available in 96-well plate or 1-mL cartridge formats, and contains a solvent retention frit for in-well PPT for applications requiring high-throughput. This unique design minimizes clogging.

Chromatographic Performance

The MRM chromatogram of prespiked whole blood at 1 ng/mL THC, THC-OH, and THC-COOH (Figure 4) shows the chromatographic performance that can be obtained using the EMR—Lipid protocol. Even at the 1 ng/mL level, ideal peak shape due to reduced matrix effect and interferences was obtained, resulting in good separation and signal-to-noise (S/N) for accurate integration. When performing forensic analysis to establish impairment, accurate detection and quantification to 5 ng/mL is typically desired.

Phospholipid Removal

Direct analysis of THC and other cannabinoids in crude acetonitrile extracts (PPT only) of whole blood by LC-ESI-MS/MS is subject to pronounced ion suppression from coeluting PPLs. The interferences are mainly caused by the lysophosphatidylcholine and lysophosphatidylethanolamine classes of PPLs.[4,5]

To determine the effectiveness of PPL removal from whole blood using Captiva EMR—Lipid, 11 naturally occurring PPLs were monitored. Specifically, the phosphatidylcholine product ion fragment at *m/z* 184 was used to monitor the PPLs in whole blood extract after protein precipitation and Captiva EMR—Lipid removal.

Figure 5 shows that 97 % of the PPLs were eliminated from the extracted whole blood samples, some of which would have coeluted with the target analyte. The high abundance of PPLs shown in Figure 5 (black trace; PPT with cold MeOH:ACN,15:85 only) subjects the detector to potential saturation, and could impact the quality of quantification. In addition, a high abundance of PPLs can contaminate a MS system over time.

Quantitative Performance

Calibration curve linearity for THC and metabolites was evaluated. Figure 6 shows that good linearity of response was observed at the six concentration levels tested (0.5–100 ng/mL, n = 5). The average coefficient of determination (R^2) for each curve was greater than 0.99, with linearity from 0.5–100 ng/mL in whole blood, regression fit for linear and 1/x weighting.

Analytical sensitivity was excellent, with limits of quantitation (LOQs) of 1.0 ng/g or lower in whole blood for the compounds tested. Method LOQs were based on %RSD ≤15 and S/N ≥10. Method reproducibility was determined by spiking the standards into whole blood at 1, 10, and 50 ng/mL in replicates of seven. The table in Figure 7 shows that RSDs ranged from 2.4 to 11.5 % and were acceptable for the matrix.

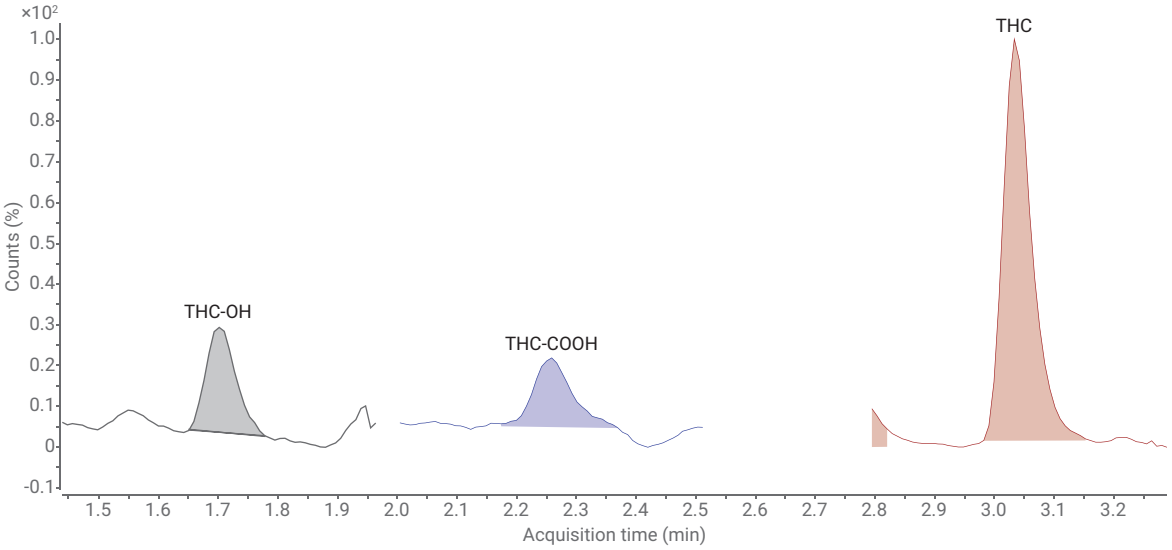


Figure 4. MRM chromatograms of whole blood prespiked at 1 ng/mL

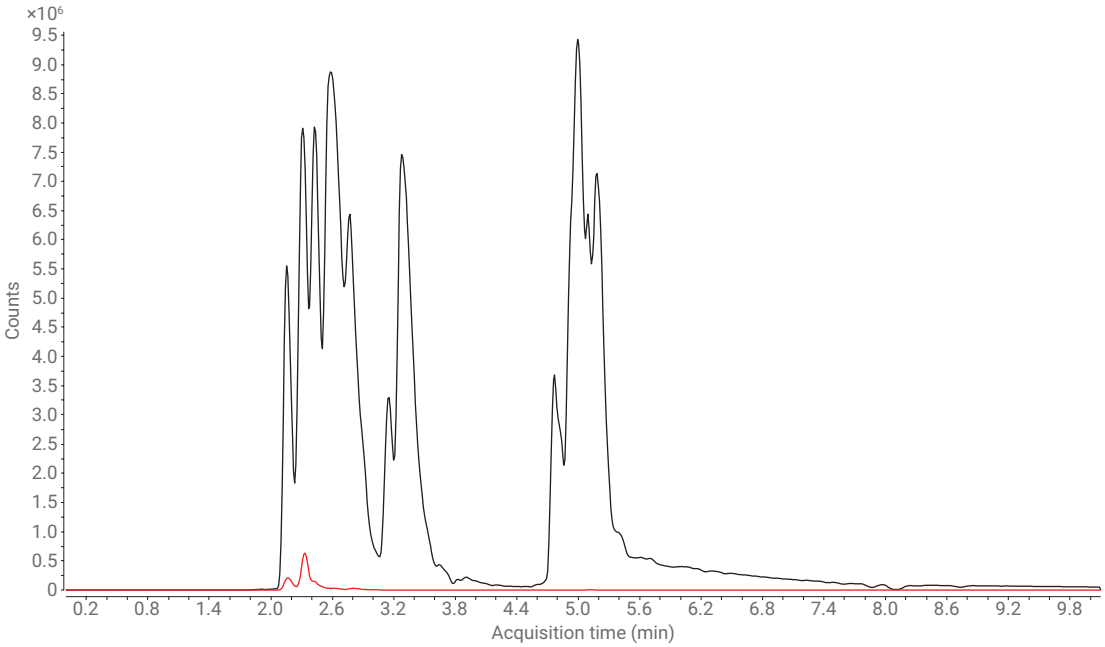


Figure 5. MRM chromatograms of 11 PPLs monitored at product ion *m/z* 184 with (red trace) and without (black trace) Agilent Captiva EMR—Lipid removal.

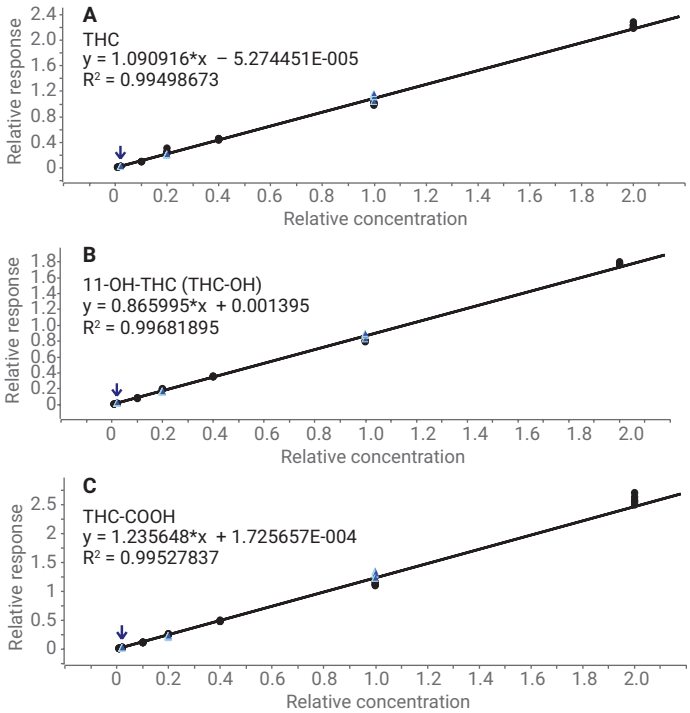
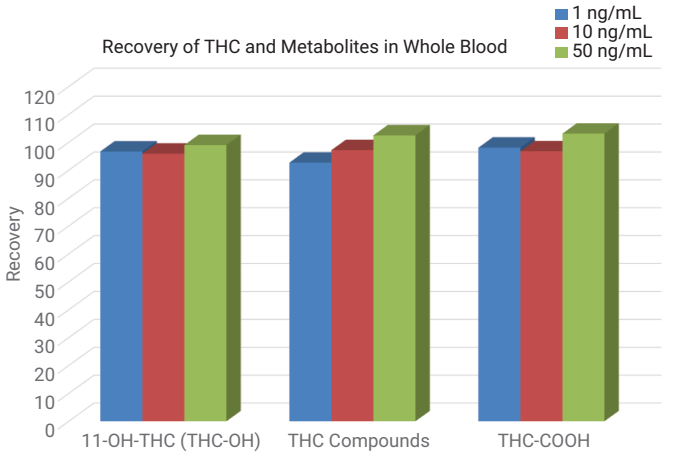


Figure 6. Calibration curves. A) THC; B) THC-OH; C) THC-COOH. Range 0.5–100 ng/mL in whole blood, n = 5.



Compound	1 ng/mL		10 ng/mL		50 ng/mL	
	Recovery	%RSD	Recovery	%RSD	Recovery	%RSD
THC-OH	96.7	11.5	95.9	3.5	99.0	2.4
THC	92.7	6.2	97.2	2.8	102.5	3.5
THC-COOH	98.1	9.2	96.8	3.7	103.1	3.6

Figure 7. Method %RSD of THC and its metabolites in whole blood (Day 1.)

Active mixing for PPT at step 3 allowed optimum recovery of 92.7 % to 103.1 % for THC and its metabolites, THC-OH and THC-COOH, with RSDs of less than 11.5 %, and calibration curve R^2 of 0.99. Passive PPT yielded recoveries of 91 % to 105 %, with RSDs of less than 10 %, but calibration curve R^2 of 0.98. Satisfactory recovery was achieved due to the unique PPL removal mechanism of Captiva EMR—Lipid. Other techniques often cannot distinguish between PPLs and hydrophobic compounds such as THC (Log P, 7.6).

Over the course of 3 days, inter-day method recovery and precision remained consistently good at 93.4 % to 109.2 %, with RSDs less than 11.5 % at 1, 10, and 50 ng/mL.

Conclusion

This Application Note presents a simple and rapid workflow to prepare whole blood samples for LC-MS/MS forensic analysis of THC and its metabolites. Extraction of THC and two of its major metabolites (THC-OH and THC-COOH) from whole blood was performed using in-well PPT followed by PPL removal using an Agilent Captiva EMR—Lipid 1 mL cartridge. Captiva EMR—Lipid efficiently removed 97 % of the unwanted PPLs from the whole blood matrix, with excellent recovery of target analytes. The sample extract was cleaner than using PPT alone, thereby reducing the potential for ion suppression, LC-MS/MS system contamination, and downtime. In-well PPT had the benefit of less sample handling and transfer.

Analysis of THC, THC-OH, and THC-COOH at 1 ng/mL, which is lower than the level needed to establish impairment, yielded ideal peak shapes and good S/N. Response for THC and its metabolites over seven concentration levels (0.5–100 ng/mL) was linear, with an R^2 greater than 0.99. LOQs of 1.0 ng/g or lower were obtained, with RSDs less than 11.5 %. Recoveries were exceptional, at 92 % or higher, for THC and its metabolites at the levels tested. Results were consistent when repeating the analysis over 3 days.

Captiva EMR—Lipid methodology can readily be incorporated into existing workflows, and does not require additional sample preparation devices or glassware. In either the 96-well plate or 1 mL cartridge formats, Captiva EMR—Lipid is compatible with automation, enabling high-throughput applications. The frit design provides easy and efficient elution of samples without clogging.

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