Application Note

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# Efficient Quantitative Analysis of THC and Metabolites in Human Plasma 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 LC-MS/MS analysis of plasma. This Application Note describes plasma sample preparation and LC-MS/MS analysis of tetrahydrocannabinol ($\Delta^9$-THC or THC) and its major metabolites, 11-hydroxy-$\Delta^9$-THC (THC-OH) and 11-nor-9–carboxy-$\Delta^9$-THC (THC-COOH) from plasma using in-well protein precipitation (PPT) followed by PPL removal using Agilent Captiva EMR—Lipid 1 mL cartridge pass-through cleanup. Captiva EMR—Lipid cartridges produced cleaner eluents, with removal of over 99 % of unwanted PPLs from the plasma matrix, and over 90 % recovery of target analytes, with RSDs <10 %. Analysis of THC, THC-OH, and THC-COOH at 1 ng/mL yielded ideal peak shapes with good signal-to-noise ratio (S/N). Response from 0.5–100 ng/mL was linear with an $R^2$ >0.99. Limits of quantitation (LOQs) of 1.0 ng/g or lower in plasma were obtained. Results were consistent over 3 days of experiments.

## Introduction

Efficient sample preparation prior to LC-MS/MS analysis is an important consideration for routine sample analysis in forensic labs. Sample preparation is used to reduce system contamination and improve data integrity, method selectivity, and analytical sensitivity. Two of the major interferences found in plasma 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]. Proteins can be removed easily by a simple sample preparation method such as protein precipitation (PPT), but PPLs are difficult to effectively remove.

Common sample preparation techniques used in forensic labs include 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. However, of these techniques, PPT is most widely accepted. Using PPT, proteins are easily removed by adding an organic crash solvent, such as acetonitrile (ACN) or methanol (MeOH), to biological samples in a prescribed ratio. As the proteins denature, they form precipitates that can be removed by filtration or centrifugation. PPLs are not removed by PPT because they are soluble in organic crash solvents, and remain in the sample after filtration or centrifugation.

Cannabinoids are among the most common target analytes in forensic labs in support of casework. Fast and accurate confirmation and quantification of $\Delta^9$-THC (THC) and its primary metabolites 11-hydroxy-$\Delta^9$-THC (THC-OH) and 11-nor-9-$\Delta^9$-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 that reduces sample preparation steps, including off-line PPT, centrifugation, transfer, and dilution while allowing efficient protein and PPL removal and satisfactory recovery for target analytes, is highly desirable. This Application Note describes an approach that uses Agilent Captiva EMR—Lipid to remove PPLs after PPT, without analyte loss, in a simple pass-through cleanup step. The resulting extract is cleaner, reducing potential ion suppression, and column and mass spectrometer contamination.

Extraction of THC, THC-OH, and THC-COOH from plasma was performed using in-well PPT followed by PPL removal using the Captiva EMR—Lipid 1 mL cartridge. Subsequent quantitative analysis was performed using the Agilent 6490 Triple Quadrupole LC/MS system. Efficiency of PPL removal by EMR—Lipid cartridge cleanup was evaluated. Inter-day (days = 3) accuracy, precision, and recovery for THC and its metabolites were also determined.

For analysis of whole blood samples, the Agilent Application Note *Efficient Quantitative Analysis of THC and its Metabolites in Whole Blood Using Captiva EMR—Lipid and LC-MS/MS* is available[2].

## Experimental

### Reagents and Chemicals

$\Delta^9$-THC, 11-hydroxy-$\Delta^9$-THC, 11-nor-9-$\Delta^9$-carboxy-THC, $\Delta^9$-THC-d3, 11-hydroxy-$\Delta^9$-THC-d3, and 11-nor-9-carboxy-$\Delta^9$ -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, THC-OH, and THC-COOH was made at 10 µg/mL in methanol. The deuterated internal standard (IS) working solution of THC-d3, THC-OH-d3, and THC-COOH-d9 was made at 10 µg/mL in methanol.

### 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 plasma, 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 plasma. A 5 µL aliquot of 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 ZORBAX Rapid Resolution High Definition (RRHD) Bonus RP 2.1 × 50 mm 1.8 µm column | 857768-901 |
| Agilent 1290 Infinity Series Thermostatted Column Compartment | G1316C |
| Agilent 1290 Infinity Autosampler | G4226A |
| 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 to an Agilent 6490 Triple Quadrupole mass spectrometry system was used. Tables 2 and 3 provide the LC and MS conditions. The sample eluent after EMR—Lipid cartridge cleanup was injected directly without further dilution. The online dilution feature of the Agilent 1290 Infinity autosampler was used prior to injection, where 10 µL of diluent (water) was aspirated prior to 5 µL of sample, and the entire volume was injected into the LC system. The advantage of using the online dilution rather than sample dilution in-vial is that the sample remains in 100 % organic. This protects the analytes from degradation.

Table 4 provides the multiple reaction monitoring (MRM) acquisition parameters. To evaluate PPL removal by Captiva EMR—Lipid, 11 PPL compounds were monitored using the MRM transitions 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,<br>Draw 5 µL from sample with default speed,<br>Wash needle as specified in the method |
| Mobile phase | A) 5 mM Ammonium formate in water, 0.1 % FA<br>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<br>0.0 65<br>0.1 65<br>4.0 95<br>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 V |
| Delta electron multiplier voltage (EMV) | 200 V |
| 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 | 327.3 (12) | 299.1 (20) | 2.26 |
| THC-COOH-d9 | 354.27 | 336.2 | 12 | 2.26 |

**Table 5.** MRM acquisition parameters for PPL compounds.

| Precursor ion (*m/z*) | Product ion (*m/z*) | Collision energy (eV) |
|---|---|---|
| 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 qualitative and quantitative data analysis. Inter-day (days = 3) accuracy, precision, and recovery of the method were determined.

### Sample Preparation Procedure

1. Add 500 µL of ACN (1 % FA) to an Agilent Captiva EMR—Lipid 1 mL cartridge.
2. Add 100 µL of human plasma.
3. Thoroughly mix, in-well.
4. Pull a vacuum of 1.5–3 psi.
5. Add 200 µL of 1:4 $H_2O$:ACN.
6. Pull the vacuum until the entire volume has passed through the cartridge, then increase the pressure to 11–13 psi to pull the remaining solvent through.
7. Evaporate under $N_2$ at 45 °C, then reconstitute in 100 µL MeOH (0.1 % FA).
8. Inject 5 µL + 10 µL of water for dilution directly into the LC system.

**Note:** For analysis of whole blood samples, the Agilent Application Note *Efficient Quantitative Analysis of THC and its Metabolites in Whole Blood Using Captiva EMR—Lipid and LC-MS/MS* is available[2].

Because PPT by MeOH forms finer precipitates than ACN does, ACN is recommended to maximize PPT and avoid gelation prior to Captiva EMR—Lipid treatment. A ratio ranging from 1:3 to 1:5 (sample/solvent) is recommended. Sample is added after the crash solvent. Acid (formic acid) helps break up proteins, and reduces protein binding.

Preferably, active in-well mixing is done using wide-bore pipette tips. 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

### Efficient Lipid Matrix Removal

The EMR—Lipid approach is simple and universally applicable to reducing matrix effects and improving analyte recoveries. The EMR—Lipid sorbent selectively traps lipids by size exclusion and hydrophobic interaction (Figure 1). Unbranched hydrocarbon chains (lipids) enter the sorbent, but bulky analytes do not. Lipid chains that enter the sorbent are then trapped by hydrophobic interactions. Lipid removal is greater than 99 % for PPLs, with high analyte recovery.

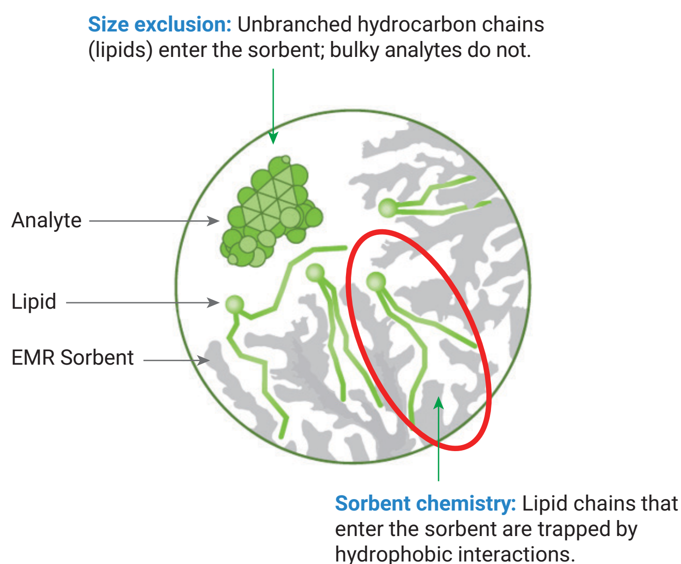



**Figure 1.** EMR-Lipid mechanism of action: size exclusion and sorbent chemistry.

PPLs are major constituents of cell membranes, and are abundant in plasma. PPLs consist of a hydrophilic head group composed of phosphate and choline units, and a hydrophobic tail made up of long alkyl chains. EMR—Lipid retains aliphatic compounds with a long carbon chain such as PPLs, free fatty acids, and triglycerides. EMR—Lipid does not interact with compounds with branched chains, short carbon chains, or functional groups such as carboxylic acids, phospho-amines, amines, amides, carbonyls, or hydroxyls.

Though the analytes shown in Figure 2, THC, THC-OH, and THC-COOH, do contain an unbranched carbon chain, the chain is not long enough to become trapped by hydrophobic interactions with the sorbent. In addition, the bulky ring component of the analytes inhibits their retention by the sorbent.

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 spiked plasma at 1 ng/mL THC, THC-OH, and THC-COOH (Figure 3) shows the chromatogram obtained using in-well PPT followed by EMR—Lipid cartridge cleanup. Even at the 1 ng/mL level, ideal peak shape due to reduced matrix effect and interferences resulted in good separation and signal-to-noise (S/N) for accurate integration. When performing forensic analysis to establish impairment, accurate detection and quantification at 5 ng/mL is typically desired.

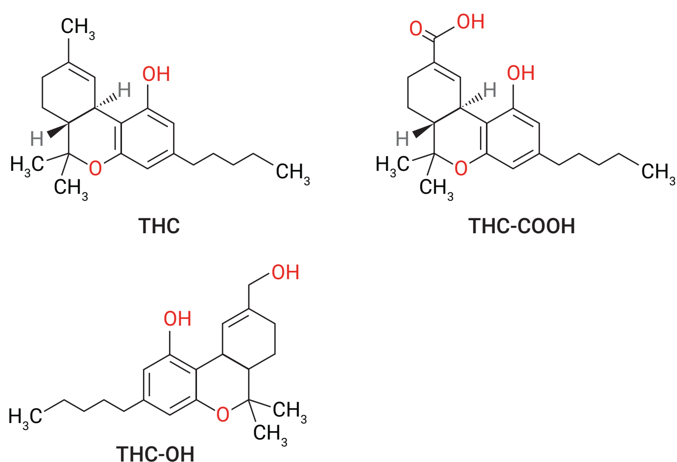



**Figure 2.** Common THC-related analyte structures.

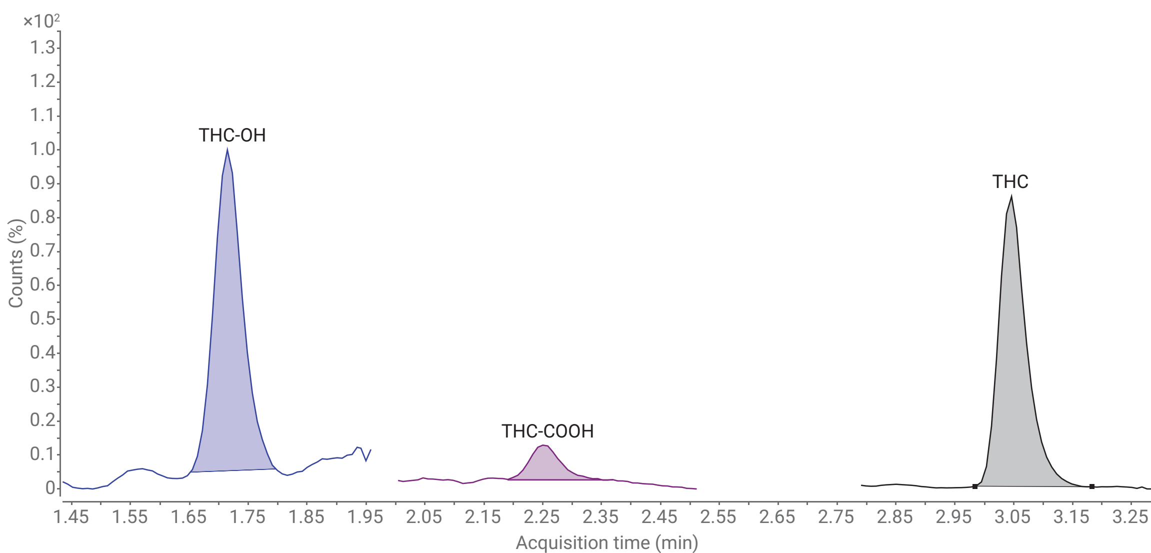



**Figure 3.** MRM chromatograms of plasma spiked at 1 ng/mL.

## PPL Removal

PPLs are the main constituents of cell membranes and the main class of compounds that cause significant matrix effect[3,4]. Glycerophophocholines and lysophosphatidylcholines represent 70 % and 10 % of the total plasma PPLs, respectively[5], and are the major source of matrix effects. To determine the effectiveness of PPL removal from plasma using Captiva EMR—Lipid, 11 naturally occurring PPL compounds were monitored.

Figure 4 shows that 99 % of PPLs were eliminated from the extracted plasma samples, some of which would have coeluted with the target analytes[6]. The high abundance of PPLs shown in Figure 4 (black trace; PPT with ACN, 1 % FA 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.

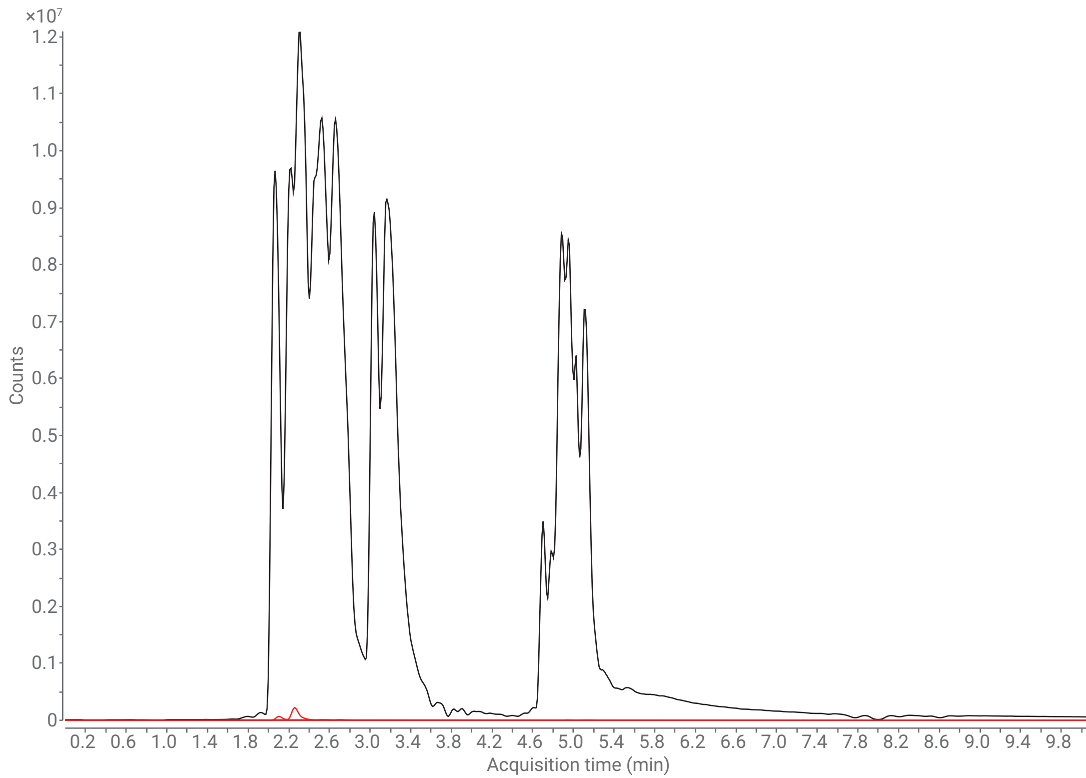



**Figure 4.** MRM chromatograms of 11 PPLs monitored at product ion *m/z* 184 with (red trace) and without (black trace) Agilent Captiva EMR—Lipid removal.

## Quantitative Performance

Calibration curve linearity for THC and its metabolites was evaluated. Figure 5 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, regression fit for linear and 1/x weighting.

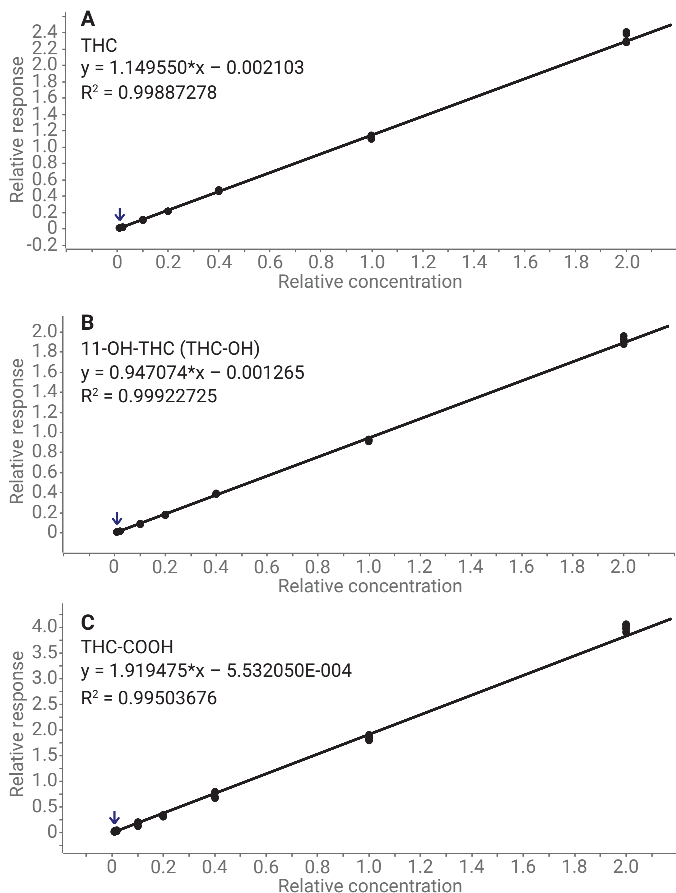
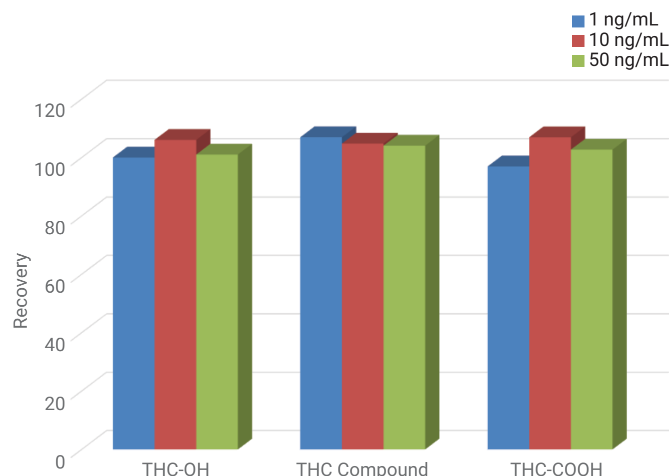



**Figure 5.** Calibration curves. A) THC; B) THC-OH; C) THC-COOH. Range 0.5–100 ng/mL in plasma, n = 5.

Analytical sensitivity was excellent, with LOQs of 1.0 ng/g or lower in plasma for the target compounds. Method LOQs were based on %RSD ≤10 and S/N ≥10.

Method reproducibility was determined by spiking the standards into plasma at 1, 10, and 50 ng/mL in replicates of seven. The table in Figure 6 shows that the %RSDs ranged from 1.2 to 7.6, and were acceptable. Recoveries of THC and its metabolites THC-OH, and THC-COOH were exceptional at 97 % to 107 % with RSDs of less than 7.6 % at 1, 10, and 50 ng/mL (Figure 6). 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 98.6 to 116.9 % with RSDs less than 10 % at 1, 10, and 50 ng/mL.




| Compound | 1 ng/mL | | 10 ng/mL | | 50 ng/mL | |
|---|---|---|---|---|---|---|
| | Recovery | %RSD | Recovery | %RSD | Recovery | %RSD |
| THC-OH | 100 | 7.6 | 106 | 1.4 | 101 | 1.4 |
| THC | 107 | 1.2 | 105 | 3.2 | 104 | 3.2 |
| THC-COOH | 97 | 5.6 | 107 | 4.2 | 103 | 4.2 |

**Figure 6.** Method recovery and precision (%RSD) of THC and metabolites in plasma (Day 1.)

## Conclusion

This Application Note presents a simple and rapid workflow to prepare plasma samples for the analysis of THC and its metabolites by LC-MS/MS for forensic studies. Extraction of target analytes from plasma was performed using in-well PPT followed by Agilent Captiva EMR—Lipid 1 mL cartridge cleanup. Captiva EMR—Lipid removed >99 % of the PPLs from the plasma matrix, with excellent recovery of target analytes. The sample extract was cleaner than that obtained using PPT alone, thereby reducing the matrix ion suppression, and improving analytical accuracy, precision, and reproducibility. The cleaner extract also reduced LC-MS/MS system contamination and possible downtime for maintenance. 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, 5 ng/mL, yielded ideal peak shapes and good S/N. Calibration curves in the range of 0.5–100 ng/mL in plasma were linear, with $R^2$ >0.99. LOQs of 1.0 ng/g or lower were obtained for the three analytes, with RSDs <10 %. Recoveries were exceptional at 90 % or higher. 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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