Supelco®
Analytical Products

Application Note

Determination of Δ⁹-tetrahydrocannabinol (THC), cannabidiol (CBD) and their metabolites in serum by automated protein precipitation and clean-up by dispersive in-tip HybridSPE® coupled with LC-MS/MS

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Introduction

Δ⁹-tetrahydrocannabinol (THC) and cannabidiol (CBD) are the two major pharmacologically active compounds present in cannabis. THC is the psychoactive component. There has been growing use of THC as sedative, antiemetic, and antiepileptic, etc..[1,2] CBD doesn't have psychotropic effect. But it has been increasingly used for treating medical conditions such as Lennox-Gastaut and Dravert syndrome.[3,4] The major metabolites of THC are 11-hydroxy-Δ⁹-THC and 11-nor-9-carboxy-Δ⁹-THC. The major metabolites of CBD are 7-hydroxy cannabidiol and 7-carboxy cannabidiol. Chemical structures of these compounds are presented in **Figure 1**.

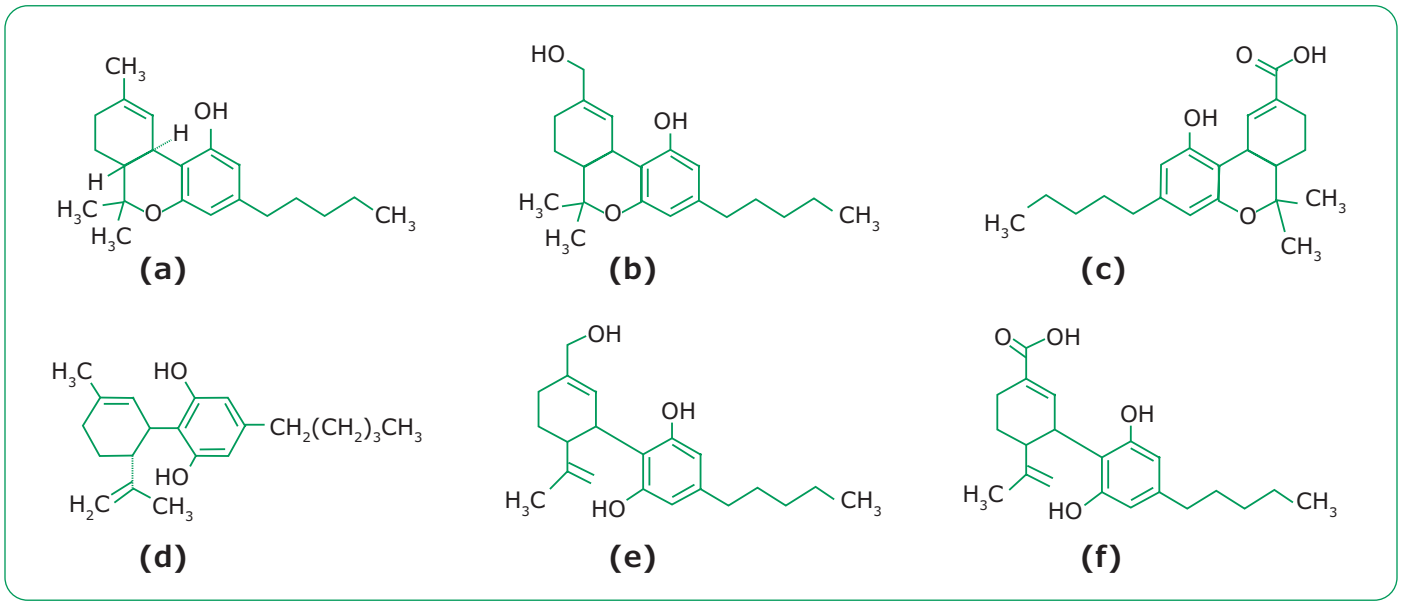

Figure 1. Chemical Structures of (a) Δ⁹-THC, (b) 11-Hydroxy-Δ⁹-THC, (c) 11-nor-9-Carboxy-Δ⁹-THC, (d) Cannabidiol, (e) 7-Hydroxy cannabidiol, (f) 7-Carboxy cannabidiol.

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To facilitate the research activities of clinical trials and medical uses of THC and CBD, analytical methods have been developed to monitor their concentrations in a variety of matrixes such as plasma, whole blood, urine, and oral fluids.[5,6] The major challenges for analytical method development were low recovery and matrix effect.

HybridSPE® utilizes the proprietary zirconia atoms functionally bonded to the HybridSPE® stationary phase to retain phospholipids via a highly selective Lewis acid-base interaction between zirconia atoms and the phosphonate moiety consistent with all phospholipids. HybridSPE® DPX tips are available with 30 mg and 50 mg adsorbents (**Figure 2**). The HybridSPE® DPX 30 mg tips are designed for use with biological sample volumes between 30-100 µL. While the HybridSPE® DPX 50 mg tips are designed for use with biological sample volumes between 100-300 µL. Biological plasma or serum samples are first subjected to protein precipitation via the addition and mixing with acidified acetonitrile. The resulting supernatant is extracted using the HybridSPE® DPX tip which acts as a chemical filter that specifically targets the removal of endogenous sample phospholipids. The resulting eluent is ready for immediate LC/MS or LC/MS/MS analysis.

In this application, a method was developed to quantify THC and CBD and their metabolites in serum by proteins precipitation and clean-up by HybridSPE® DPX tips.

Figure 2. HybridSPE® DPX tips for Hamilton®

Method

Protein Precipitation

Load serum, 300 µL, spiked with analytes (1-100 ng/mL) and internal standards (20 or 50 ng/mL) into wells of a 2 mL 96 well plate. Precipitate proteins by combining the serum samples with a precipitating agent (0.9 mL of acetonitrile with 1% formic acid, -20 °C). Facilitate precipitation by agitating (1200 rpm) for 3 min. Let the solution settle for 5 min. Transfer 400 µL of the supernatant into new wells for cleanup.

HybridSPE®

HybridSPE® DPX Tips for Hamilton® (50 mg bed, tip volume 1 mL) (52978-U)		
	Solution	**Aspirate/Dispense**
Condition:	Acetonitrile with 0.5% citric acid	Cycles = 1 Volume = 800 µL
Clean-up: (removal of phospholipids)	Settled protein precipitated serum	Cycles = 2 Volume = 500 µL
Wash:	Not applicable	

Hamilton® STARlet was programmed to automatically perform protein precipitation and HybridSPE® cleanup. The automatic process was completed in less than 15 min for 8 samples. And the process could be scaled up to process 96 samples simultaneously using a 96 tip header.

LC-MS/MS Analysis

Analysis of samples using the Shimadzu 8030 MS coupled with Shimadzu LC-30AD. The LC separation parameters are listed in **Table 1.** The MS/MS detection parameters are listed in **Table 2** and **Table 3.**

Table 1. Analytical Conditions for Shimadzu 8030 MS coupled with Shimadzu LC-30AD

Column:	Ascentis® Express Phenyl-Hexyl column 10 cm x 2.1 mm, 2.7 µm (53336-U)				
Mobile phase A:	LC-MS grade water with 0.1% formic acid				
Mobile phase B:	LC-MS grade acetonitrile with 0.1% formic acid				
Column Temp:	25 °C				
Inj. Vol:	5 µL				
Flow Rate:	0.35 mL/min				
Gradient:					
Time (min)	0	0.5	3.5	5	6.5
B%	40	40	70	95	95
Detector:	MS, ESI(+), Scheduled MRM				

Table 2. Analytes and the MS-MS detection parameters

Peak	Analytes	Ret. Time	m/z	Product	Dwell	Q1 Pre Bias(V)	CE	Q3 Pre Bias(V)
1	7-Carboxy cannabidiol-D3 (IS)	4.534	348.1	330.2	100	-30	-17	-25
2	7-Carboxy cannabidiol	4.539	345.1	327.2	100	-30	-16	-25
3	7-Hydroxy cannabidiol-D3 (IS)	4.581	334.2	316.1	100	-30	-13	-24
4	7-Hydroxy cannabidiol	4.585	331.3	313.1	100	-30	-13	-24
5	11-Hydroxy- Δ^9-THC -D3 (IS)	5.032	334.2	316.1	100	-30	-16	-24
6	11-Hydroxy- Δ^9-THC	5.038	331.4	313.2	100	-30	-16	-23
7	11-nor-9-Carboxy- Δ^9-THC -D3 (IS)	5.070	348.3	330.1	100	-40	-19	-25
8	11-nor-9-Carboxy- Δ^9-THC	5.075	345.3	327.1	100	-30	-18	-17
9	Cannabidiol-D3 (IS)	5.467	318.4	196.1	100	-30	-22	-14
10	Cannabidiol	5.471	315.4	193.0	100	-30	-24	-24
11	Δ^9-THC -D3 (IS)	5.812	318.4	196.1	100	-30	-24	-14
12	Δ^9-THC	5.818	315.4	193.1	100	-30	-25	-23

Table 3. MS Source parameters

Source parameter	
Nebulizing gas flow (L/min)	3
DL temperature (°C)	250
Heat block temperature (°C)	400
Drying gas flow (L/min)	15

Results and discussion

Acetonitrile was selected to precipitate protein in serum samples. Formic acid, 0.1%, was included to improve the analyte recovery by disruption of the protein-analyte interactions. To facilitate the separation of precipitated protein from supernatant, acetonitrile was kept at -20 °C prior to combining of acetonitrile with serum. After agitation and settling of the serum-acetonitrile mixture, a clear supernatant was obtained and 400 µL of the supernatant was transferred to new wells and subjected to HybridSPE® cleanup to remove phospholipids. To prevent retention of acidic analytes by HybridSPE®, 0.5% citric acid was added into acetonitrile to condition the HybridSPE® tips. Citric acid is a stronger Lewis acid than formic acid and inhibits the retention of chelating compounds. But it is not a strong enough Lewis base to inhibit phospholipids from retaining on the HybridSPE® phase. Hamilton® STARlet was programed to perform the automatic sample preparation. The eluent from the HybridSPE® cleanup was ready for LC-MS/MS analysis.

Separation of THC, CBD and their metabolites, 7-carboxy cannabidiol, 7-hydroxy cannabidiol, 11-hydroxy- Δ^9-THC, 11-nor-9-carboxy- Δ^9-THC, was demonstrated in **Figure 3**. It appears from the chromatogram that there are no significant interferences to quantify each analyte.

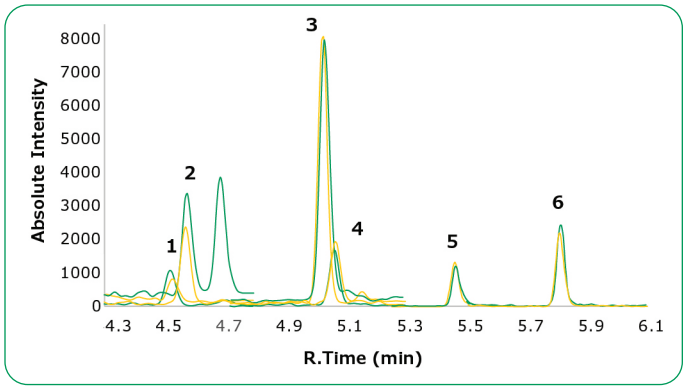

Figure 3. Chromatogram of sample of 25 ng/mL showing the elution of (1) 7-carboxy cannabidiol, (2) 7-hydroxy cannabidiol, (3) 11-hydroxy- Δ^9-THC, (4) 11-nor-9-carboxy- Δ^9-THC, (5) CBD and (6) Δ^9-THC.

Calibration

Quantification of individual analytes was done using deuterated analogs as the internal standards (**Table 2**). To simplify the procedure to prepare calibration standards, acetonitrile/water (75/25, v/v) with 0.75% formic acid was used as the matrix. The calibration range was from 1 ng/mL to 100 ng/mL. The concentrations of internal standards were 50 ng/mL for 7-carboxy cannabidiol-D3 and 11-nor-9-carboxy- Δ^9-THC-D3, and 25 ng/mL for other analytes. Linear regression was used for all calibration curves and correlation coefficients were all 0.99 or higher. **Figure 4** shows a sample calibration curve for the analyte 11-nor-9-carboxy- Δ^9-THC.

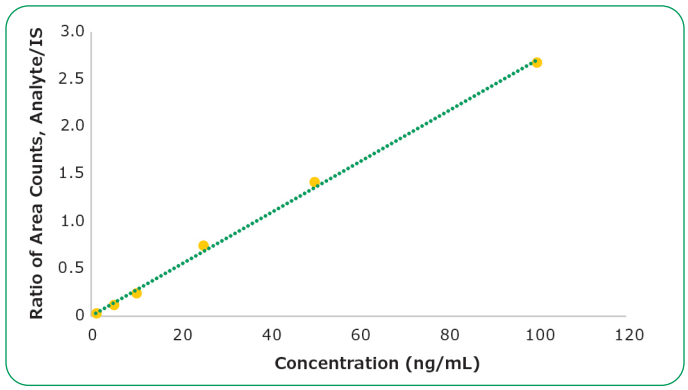

Figure 4. Sample calibration curve (11-nor-9-carboxy- Δ^9-THC). For this analyte, y = 0.027x + 0.005; R^2 = 0.9982.

Determination of spiked serum samples

Pooled human serum was spiked with working standards so that the concentrations of analytes in the serum samples ranged from 1 ng/mL to 100 ng/mL. The IS concentration in the serum samples were 25/50 ng/mL as described earlier. The spiked serum samples were shaken at 500 rpm for 15 min prior to automatic protein precipitation and HybridSPE® cleanup followed by LC-MS/MS analysis. **Figure 5** shows the chromatograms of 7-carboxy cannabidiol at different concentrations.

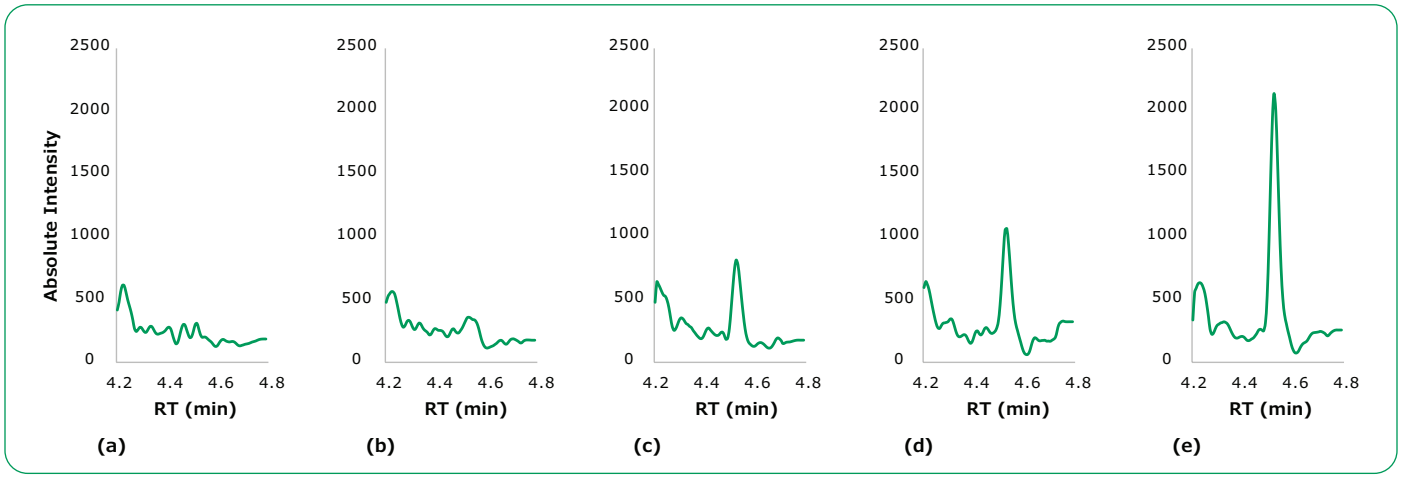

Figure 5. Chromatograms of 7-carboxy cannabidiol in serum samples at concentration of (a) 5, (b) 10, (c) 25, (d) 50 and (e) 100 ng/mL.

The accuracy of the developed method, which was calculated based on the percentage of the determined concentration versus spiked concentration, is shown in **Table 4**. The average accuracy values for 7-carboxy cannabidiol, 7-hydroxy cannabidiol, 11-hydroxy-Δ^9-THC, 11-nor-9-carboxy-Δ^9-THC, CBD, and THC at different concentration levels were 118%, 92%, 88%,122%, 98%, and 110%, respectively, which demonstrate the method was accurate.

Table 4. The method accuracy at different concentration levels

Concentration (ng/mL)	5	10	25	50	100
7-Carboxy cannabidiol	NA	NA	132	121	103
7-Hydroxy cannabidiol	87	90	114	84	84
11-Hydroxy-Δ^9-THC	77	82	111	83	87
11-nor-9-Carboxy-Δ^9-THC	NA	125	139	102	124
Cannabidiol	107	80	116	94	92
Δ^9-THC	110	113	131	94	104

NA: not available

The reproducibility of the method was estimated by analysis of four replicate samples at the concentration of 25 ng/mL. The method was reproducible with RSD% for all analytes at less than 20% except 7-carboxy cannabidiol. The high RSD% for 7-carboxy cannabidiol was due to the fact that the concentration of 25 ng/mL was close to its limit of quantitation (LOQ). The limit of detection (LOD) and LOQ were estimated by 3 and 10 times of the standard deviation of the analyte concentration determined from 6 samples at the concentration of 1 ng/mL. The LOD for all analytes are from 1 to 8 ng/mL. The LOQ for all analytes are from 2 to 26 ng/mL (**Table 5**). And calibration curves show linear ranges from LOQ up to 100 ng/mL, which was the highest concentration employed in the study.

Table 5. The reproducibility, LOD, LOQ and linear range of the method

	Reproducibility (RSD%)*	LOD (ng/mL)	LOQ (ng/mL)	Linear range (ng/mL)
7-Carboxy cannabidiol	28	8	26	26-100
7-Hydroxy cannabidiol	16	1	3	3-100
11-Hydroxy-Δ^9-THC	15	1	3	3-100
11-nor-9-Carboxy-Δ^9-THC	12	4	14	14-100
Cannabidiol	19	1	3	3-100
Δ^9-THC	13	1	2	2-100

*** Note:** n=4

Matrix and cleanup effects

The matrix effect was investigated by comparison of the absolute responses from the standards prepared in acetonitrile/water (75/25, v/v, with 0.75% formic acid) with the absolute responses from the standards prepared in the serum extract, which was the supernatant after protein precipitation. **Figure 6** presents the external calibration curves plotted with peak area vs. concentration in different matrixes. It is apparent that significant ion suppression was observed for 7-carboxy cannabidiol, 7-hydroxy cannabidiol, 11-hydroxy-Δ^9-THC, and 11-nor-9-carboxy-Δ^9-THC. Ionization of cannabidiol, CBD, seemed not affected by matrixes. Ion enhancement matrix effect was observed for Δ^9-THC.

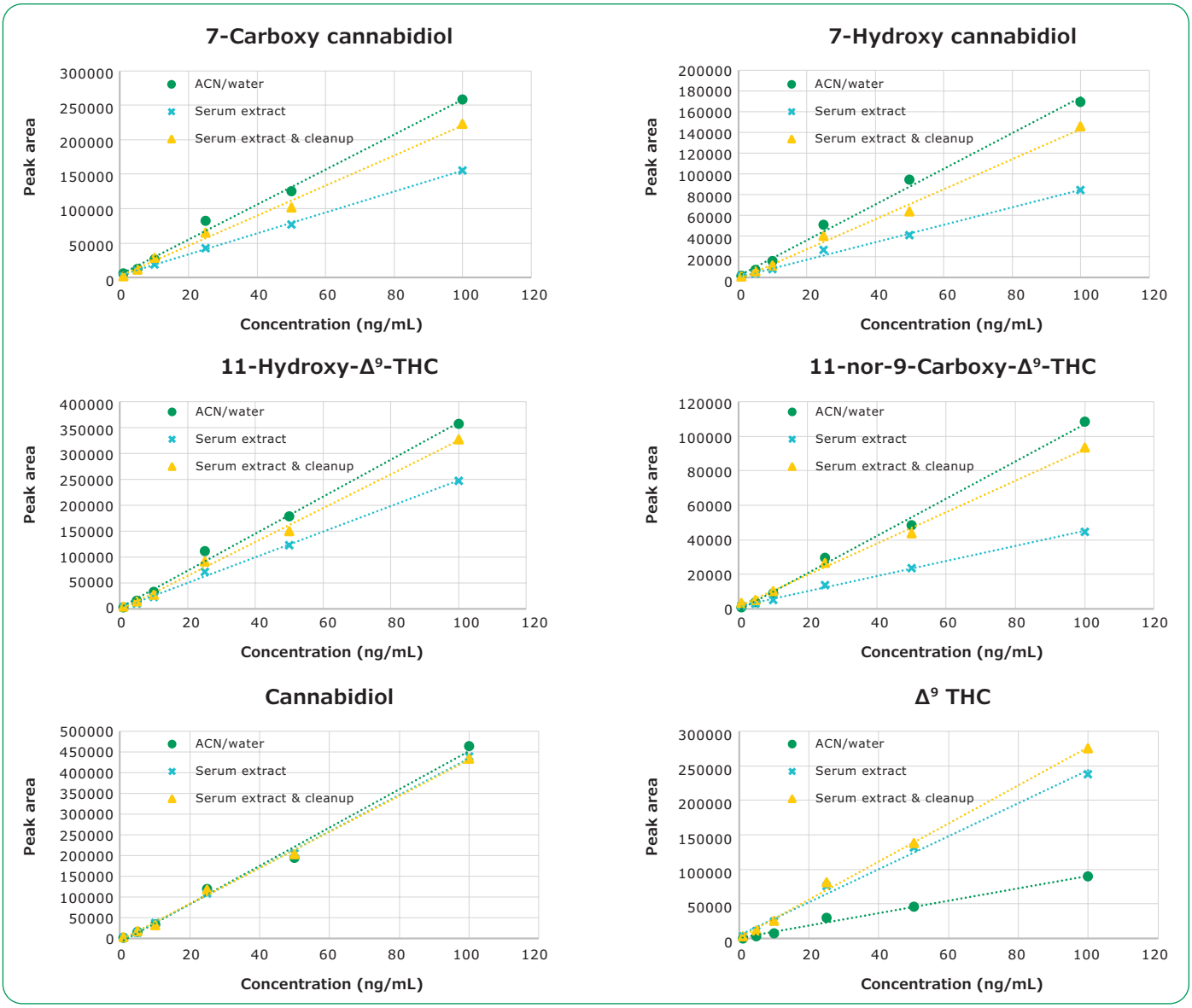

Figure 6. Calibration in different matrixes.

To estimate the cleanup effect of HybridSPE®, the supernatant after protein precipitation was subjected to loading and dispensing through HybridSPE® DPX tips twice. The eluent (serum extract & cleanup) was used as the matrix to prepare standards. The calibration curves were shown in **Figure 6**, and the estimated matrix effects were listed in **Table 6**. The percentage matrix effect for all the analytes were calculated using the following equation:

$$\textit{Matrix effect (\%)} = \frac{\textit{Calibration slope in serum extract or serum extract \& cleanup} - \textit{Calibration slope in acetonitrile/water}}{\textit{Calibration slope in acetonitrile/water}} \times 100\%$$

Table 6. Matrix effect (%)

	Serum extract & HybridSPE® cleanup	Serum extract
7-Carboxy cannabidiol	-14	-40
7-Hydroxy cannabidiol	-16	-51
11-Hydroxy-Δ⁹-THC	-9	-31
11-nor-9-Carboxy-Δ⁹-THC	-16	-59
Cannabidiol	-4	-6
Δ⁹-THC	166	207

Note: negative number means ion suppression. Positive number means ion enhancement.

Figure 6 and **Table 6** show that ion suppression for 7-carboxy cannabidiol, 7-hydroxy cannabidiol, 11-hydroxy-Δ⁹-THC, and 11-nor-9-carboxy-Δ⁹-THC was significantly minimized when standards were prepared in the eluent from HybridSPE®. The matrix effect (%) were about -9% to -16%. Ionization of CBD seemed not affected by HybridSPE® cleanup. Ion enhancement matrix effect was still observed for THC, but to a lesser extent.

Summary

A quantitative LC-MS/MS method was developed to quantify THC, CBD, and their metabolites in serum by coupling automated protein precipitation and HybridSPE® DPX tips cleanup. The method was optimized with addition of 1% formic acid in acetonitrile to precipitate protein and addition of 0.5% citric acid in acetonitrile to condition HybridSPE® DPX tips. Hamilton® STARlet liquid handler performed the automatic sample preparation, which was completed in about 13 min for 8 samples (the process could be scaled up to process 96 samples in 15 min). Significant matrix effect was diminished with the combination of the protein precipitation and HybridSPE® cleanup processes. Experimental results showed that only less than 16% matrix effect was observed after the sample preparation. With addition of internal standards, the quantitative LC/MS-MS method was demonstrated to be accurate (70%-130%), reproducible (RSD%<30%), and had a linear range of low ppb to 100 ppb.

Material

Cat. No.	Material
53336-U	Ascentis® Express Phenyl-Hexyl, 2.7 µm HPLC Column, 2.7 µm particle size, L × I.D. 10 cm × 2.1 mm
1.00030	Acetonitrile gradient grade for liquid chromatography LiChrosolv® Reag. Ph Eur
1.15333	Water for chromatography (LC-MS Grade) LiChrosolv®
00940	Formic acid for LC-MS LiChropur™, 97.5-98.5% (T)
791725	Citric acid anhydrous, free-flowing, Redi-Dri™, ACS reagent, ≥99.5%
52978-U	HybridSPE® DPX tip
Z717266	Nunc™ 96 DeepWell™ plate, 2mL
C224	7-Carboxy cannabidiol-D3 100 µg/mL in methanol, certified reference material, ampule of 1 mL, Cerilliant®
C181	7-Carboxy cannabidiol 1 mg/mL in methanol, certified reference material, ampule of 1 mL, Cerilliant®
C223	7-Hydroxy cannabidiol-D3 100 µg/mL in methanol, certified reference material, ampule of 1 mL, Cerilliant®
C180	7-Hydroxy cannabidiol 1 mg/mL in methanol, certified reference material, ampule of 1 mL, Cerilliant®

Cat. No.	Material
H041	11-Hydroxy- Δ^9-THC-D3 100 µg/mL in methanol, certified reference material, ampule of 1 mL, Cerilliant®
H027	11-Hydroxy- Δ^9-THC 1 mg/mL in methanol, certified reference material, ampule of 1 mL, Cerilliant®
T004	11-nor-9-Carboxy-Δ^9-THC-D3 100 µg/mL in methanol, certified reference material, ampule of 1 mL, Cerilliant®
T010	11-nor-9-Carboxy- Δ^9-THC 1 mg/mL in methanol, certified reference material, ampule of 1 mL, Cerilliant®
C084	Cannabidiol-D3 100 µg/mL in methanol, certified reference material, ampule of 1 mL, Cerilliant®
C045	Cannabidiol 1 mg/mL in methanol, certified reference material, ampule of 1 mL, Cerilliant®
T003	Δ^9-THC-D3 100 µg/mL in methanol, certified reference material, ampule of 1 mL, Cerilliant®
T005	Δ^9-THC 1 mg/mL in methanol, certified reference material, ampule of 1 mL, Cerilliant®
29651-U	Certified Vial Kit, Low Adsorption (LA), 2 mL, pk of 100

References

1. Zuardi, AW, Guimaraes, FS, et al., Cannabis and Cannabinoids: Pharmacology, Toxicology, and Therapeutic Potential (Book). Choice Curr. Rev. Acad. Lib. 2003; Vol. 40: 359.
2. Karler, R, Turkanis, SA, The cannabinoids as potential antiepileptics. J. Clin. Pharmacol. 1981; Vol.21: 437S-448S.
3. Karler, R, Cely, W, Turkanis, SA, The anticonvulsant activity of cannabidiol and cannabinol. Life Sci. 1973; Vol. 13: 1527–1531.
4. Taylor L, Gidal B, Blakey G, Tayo B, Morrison G. A Phase I, Randomized, Double-Blind, Placebo-Controlled, Single Ascending Dose, Multiple Dose, and Food Effect Trial of the Safety, Tolerability and Pharmacokinetics of Highly Purified Cannabidiol in Healthy Subjects. CNS Drugs. 2018 Nov; 32(11):1053-1067.
5. Mohamed MK, Takyi-Williams J, Baudot B, Grobler A. Development and validation of a LC-HRMS method for the quantification of cannabinoids and their metabolites in human plasma. Eur J Pharm Sci. 2021 Apr 1; 159:105705.
6. Grauwiler SB, Scholer A, Drewe J. Development of a LC/MS/MS method for the analysis of cannabinoids in human EDTA-plasma and urine after small doses of Cannabis sativa extracts. J Chromatogr B Analyt Technol Biomed Life Sci. 2007 May 1; 850(1-2):515-22.

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