FEB 05, 2024

DOI:
dx.doi.org/10.17504/protocols.io.bp2l6927dlqe/v1

Protocol Citation: Geraldine Magnin 2024. Procedure to analyze cannabinoids in bovine plasma using solid phase extraction & UPLC MS/MS. **protocols.io** https://dx.doi.org/10.17504/protocols.io.bp2l6927dlqe/v1

Procedure to analyze cannabinoids in bovine plasma using solid phase extraction & UPLC MS/MS

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DISCLAIMER

Reference to any commercial materials, equipment, or process does not in any way constitute approval, endorsement, or recommendation by the Food and Drug Administration.

ABSTRACT

This procedure described the quantification of 21 phytocannabinoids in 100uL bovine plasma. 200uL of acetonitrile is added to 100uL plasma extract, cleaned up by centrifugation and a 96-well plate solid phase extraction and analyzed by UPLC-MS/MS.Matrix-matched (not in solvent) calibration curve is used to quantify 21 cannabinoids at concentrations ranging 1-100 ng/mL (ppb). Internal Standards were applied for quantification some of the analytes.

Method validation/evaluation/verification:

The earlier version of the method (including in-house validation data) was published: https://pubmed.ncbi.nlm.nih.gov/35939416/

Then the method was extended (by Geraldine Magnin - lead) regarding the list of analytes (to include 21 cannabinoids in its 2nd version) and evaluated through Blinded Method Test (BMT) by Vet-LIRN. The BMT report summarizing method performance evaluation data is available upon request.

MANUSCRIPT CITATION:
Rapid quantification of cannabinoids in beef tissues and bodily fluids using direct-delivery electrospray ionization mass spectrometry. Shubhashis Chakrabarty, Eric M Serum, Thomas M Winders, Bryan Neville, Michael D Kleinhenz, Geraldine Magnin, Johann F Coetzee, Carl R Dahlen, Kendall C Swanson, David J Smith. Food Addit Contam Part A Chem Anal Control Expo Risk Assess. 2022 Oct;39(10):1705-1717. doi: 10.1080/19440049.2022.2107711. Epub 2022 Aug 8.https://pubmed.ncbi.nlm.nih.gov/35939416/

Protocol status: Working
We use this protocol and it's working

Created: Dec 01, 2022

Last Modified: Feb 05, 2024

PROTOCOL integer ID: 73400

Keywords: cannabinoids, animal diagnostic specimens, plasma, blood, serum, specimens, tissues, hemp, marijuana

Funders Acknowledgement:
FDA Vet-LIRN's
Grant ID: U18 FD006915-01

MATERIALS

Cannabinoids abbreviations

Table 1. List of cannabinoids and their abbreviation.

CBC	Cannabichromene
CBCA	Cannabichromenic acid
CBD	Cannabidiol
CBDA	Cannabidiolic acid
CBDV	Cannabidivarin
CBDVA	Cannabidivarinic acid
CBG	Cannabigerol
CBGA	Cannabigerolic acid
CBL	Cannabicyclol Tetrahydrocannabivarin
CBLA	Cannabicyclolic acid
CBN	Cannabinol
8-THC	D8-Tetrahydrocannabinol
9-THC	D9-Tetrahydrocannabinol
THCA-A	D9-Tetrahydrocannabinolic acid A
THC-acid	(±)-11-Nor-9-Carboxy-D9-tetrahydrocannabinol
THC-acid glu	Δ9-11-Nor-9-Carboxy-D9-tetrahydrocannabinol glucuronide
THC-glu	Δ9-Tetrahydrocannabinol glucuronide
THC-OH	11-Hydroxy-D9-tetrahydrocannabinol
THCP	Tetrahydrocannabiphorol
THCV	Tetrahydrocannabivarin
CBC-d9	Cannabichromene-d9
CBD-d3	Cannabidiol-d3
9-THC-d3	D9-Tetrahydrocannabinol-d3
THCA-A-d3	Δ9-Tetrahydrocannabinolic acid A-d3
THC-acid-d9	(±)-11-nor-9-Carboxy-D9-Tetrahydrocannabinol-d9
THC-acid-glu-d3	Δ9-11-Nor-9-Carboxy-D9-tetrahydrocannabinol

	glucuronide-d3
THC-OH-d3	11-Hydroxy-D9-Tetrahydrocannabinol-d3

Other abbrevations

NEG CTRL: Negative control
IS CTRL: Internal standard control
ACN: Acetonitrile
MeOH: Methanol
UPLC: Ultra high-pressure liquid chromatography
MRM: Multiple reaction monitoring
QC: Quality controls

Specimen

Plasma used for the validation was purchased through VWR International (P/N TDS-SBPUC35); the anticoagulant used is sodium citrate. Upon receiving, the plasma was stored in 50-mL aliquots at -80 °C. Plasma samples should be stored at -80 °C (to preserve the glucuronide metabolites which are not as stable as other cannabinoids) and let thawed on the bench for 20 min before analysis. The plasma is spun down at 4,500 g for 5 minutes before use.

Materials

UPLC column: Eclipse Plus C18, Agilent Technologies (Santa Clara, CA) 100 x 2.1 mm, 1.8 m.
1.5-mL microcentrifuge tubes
Oasis PRIME HLB 96-well µElution Plate, 3 mg Sorbent per well, 1/pk (Waters, P/N 186008052)
Cap-mat 96 well 7 mm round plug pre-slit silicone/PTFE, 5/pk (Waters, P/N 186006332)
Pipette, single channel, variable, 0.5-5 mL (Eppendorf North America or equivalent)
Pipet, multichannel 25-300 mL

Equipment

Vortex mixer
Refrigerated microcentrifuge
96-well Sample Collection Plate, 2mL Square well 50/pkg (Waters, P/N 186002482)
Cap-mat 96 well 7 mm round plug pre-slit silicone/PTFE, 5/pkg (Waters, P/N 186006332)
Reservoir, White, 50mL, Non-Sterile, Fisher Scientific (Hampton, NH), P/N 50143700.
Positive Pressure Manifold Spacer, Waters Co. 9Milford MA), P/N 186007987.
UPLC system, Waters Acquity H equipped with a degasser, a column heater, a refrigerated autosampler, a quaternary pump. The UPLC is interfaced with a Waters triple quadrupole

spectrometer Xevo TQ-S.

Chemicals/Reagents

Methanol, LC-MS grade

Acetonitrile, LC-MS grade

Formic acid, LC-MS grade

Ultrapure 18W water is obtained in-house with a Millipore Synergy UV-R system.

Methanol-water (25:75)

Acetonitrile-Methanol (90:10)

Water with 0.2% formic acid

Acetonitrile-0.1% formic acid

Reference Standards

All standards are purchased in solutions from the following suppliers (Table 2)

Table 2. List of cannabinoids standards, suppliers and parts numbers

	A	B	C	D	E
	Cannabinoids standards	P/N	Supplier	Concentration (ug/mL)	Solvent
	CBC	C-143	Cerilliant Co	1,000	MeOH
	CBCA	30879	Cayman Chemical	1,000	ACN
	CBD	C-045	Cerilliant Co	1,000	MeOH
	CBDA	18090	Cayman Chemical	1,000	ACN
	CBDV	C-140	Cerilliant Co	1,000	MeOH
	CBDVA	C-152	Cerilliant Co	1,000	ACN
	CBG	C-141	Cerilliant Co	1,000	MeOH
	CBGA	20019	Cayman Chemical	1,000	ACN
	CBL	22036	Cayman Chemical	1,000	ACN
	CBLA	C-171	Cerilliant Co	500	ACN
	CBN	C-046	Cerilliant Co	1,000	MeOH
	8-THC	T-032	Cerilliant Co	1,000	MeOH

	A	B	C	D	E
	9-THC	T-005	Cerilliant Co	1,000	MeOH
	THCA-A	ISO60175	Cayman Chemical	1,000	ACN
	THC-11-acid	T-018	Cerilliant Co	100	MeOH
	THC-11-acid glu	T-038	Cerilliant Co	100	MeOH
	THC-glu	S16	ElSohly	10	MeOH
	THC-11-OH	H-026	Cerilliant Co	100	MeOH
	THCP	30171	Cayman Chemical	1,000	ACN
	THCV	T-094	Cerilliant Co	1,0000	MeOH
	Internal standards				
	CBC-d9	21294	Cayman Chemicals	100	
	CBD-d3	C-084	Cerilliant Co	100	MeOH
	9-THC-d3	T-003	Cerilliant Co	100	MeOH
	THCA-A-d3	T-145	Cerilliant Co	100	ACN
	THC-11-acid-d9	T-007	Cerilliant Co	100	MeOH
	THC-11-acid-glu-d3	T-080	Cerilliant Co	100	MeOH
	THC-11-OH-d3	H-041	Cerilliant Co	100	MeOH

BEFORE START INSTRUCTIONS

Bovine plasma. Upon receiving, plasma samples should be stored at -80 °C (to preserve the glucuronide metabolites which are not as stable as other cannabinoids). Before use, the plasma is thawed on the bench for 20 min mixed well with a vortex mixer and spun down at 4,500 g for 5 minutes.

Preparation of solutions

1 **Standards stock mixture.** From the commercially available solutions, a stock solution containing the mixture of analytes at [M] 10 µg/mL in ACN is prepared and stored at -20 °C. In a 4-mL glass vial,

1.1 add 20 µL of CBC, CBCA, CBD, CBD-7-acid, CBDA, CBDV, CBDVA, CBG, CBGA, CBLA, CBN, 8-THC, 9-THC, THCA-A, THCP, THCV each at [M] 10000 µg/mL

1.2 add 200 µL of THC-11-acid, and THC-11-OH at [M] 100 µg/mL

1.3 add 40 µL of CBL at [M] 500 µg/mL

1.4 add 1240 µL of ACN

1.5 The stock standard solution is aliquoted in 0.5-mL portions stored at -20 °C.

Note

THC-11-acid-glu and THC-glu are added to the working solution on the day of the analysis because they are not stable at -20 °C (they should be stored at -80 °C).

2 **Internal standards mixture**. A mixture of internal standards is prepared at [M] 10 µg/mL in ACN and stored at -20 °C. In a 4-mL glass vial.

2.1 add 200 µL of each IS (THC-11-acid-glu-d_3, THC-11-OH-d_3, THC-11-acid-d_9, 9-THC-d_3, CBD-d_3, CBC-d_9, THCA-A-d_3)at [M] 100 µg/mL

2.2 add 600 µL of ACN.

2.3 The stock IS solution is aliquoted in 0.5-mL portions stored at -20 °C.

3 **Standards mixture**. Each day of the analysis, fresh standard solutions are prepared in ACN-0.1% formic acid, at the following concentrations: 0.5; 1; 2.5; 5; 10; 25; 50 and 100 ng/mL. In a 1.5-mL polypropylene microcentrifuge tube, mix the following:

3.1 For 1,000 ng/mL:

- 100 µL of cannabinoids working solution at [M] 10 µg/mL in ACN
- 10 µL of THC-11-acid-glu at [M] 100 µg/mL
- 100 µL of THC-glu at [M] 10 µg/mL
- 790 µL of ACN

3.2 For 100 ng/mL

- 100 µL of cannabinoids working solution at 1,000 ng/mL in ACN
- 900 µL of ACN

3.3 For 50 ng/mL

- 50 µL of cannabinoids working solution at 1,000 ng/mL in ACN
- 950 µL of ACN

3.4 For 25 ng/mL

- 25 µL of cannabinoids working solution at 1,000 ng/mL in ACN.
- 975 µL of ACN.

3.5 For 10 ng/mL

- 100 µL of cannabinoids working solution at 100 ng/mL in ACN.
- 900 µL of ACN.

3.6 For 5 ng/mL:

- 100 µL of cannabinoids working solution at 50 ng/mL in ACN.
- 900 µL of ACN.

3.7 For 2.5 ng/mL

- 100 µL of cannabinoids working solution at 25 ng/mL in ACN.
- 900 µL of ACN.

3.8 For 1.0 ng/mL

- 100 µL of cannabinoids working solution at 10 ng/mL in ACN
- 900 µL of ACN

4 **Working solution of internal standards mixture.** An IS working solution is prepared at 100 ng/mL in ACN-0.1% formic acid as follow:

4.1 In a 15-mL polypropylene tube,

4.2 add 100 µL of IS mixture at [M] 10 ng/mL

4.3 add 9.9 mL of ACN-0.1 % formic acid.

5 **Working quality control standards solutions**

5.1 For 950 ng/mL
- 475 µL of cannabinoids working solution at 1,000 ng/mL in ACN
- 25 µL of ACN

5.2 For 475 ng/mL
- 100 µL of cannabinoids working solution at 950 ng/mL in ACN
- 100 µL of ACN

5.3 For 47.5 ng/mL
- 50 µL of cannabinoids working solution at [M] 475 µg/mL in ACN

- 450 µL of ACN

Preparation of QCs

6 Quality controls are prepared each day of the analysis in negative control bovine plasma containing a mixture of the cannabinoids at the following concentrations: 4.75, 47.5, 95 ng/mL. In 1.5-mL microcentrifuge tubes:

6.1 QC3 (95 ng/mL)- Add:
- 10 µL of mix 950 ng/mL
- 990 µL of NEG CTRL bovine plasma

6.2 QC2 (47.5 ng/mL)- Add:
- 10 µL of mix 475 ng/mL
- 990 µL of NEG CTRL bovine plasm

6.3 QC1 (4.75 ng/mL)- Add:
- 10 µL of mix 47.5 ng/mL
- 990 µL of NEG CTRL bovine plasma

Protein precipitation

7 **Negative control**: In a 1.5-mL microcentrifuge tube, mix:

7.1 100 µL of NEG CTRL bovine plasma

7.2 100 µL of ACN

7.3 100 µL of acetonitrile-0.1% formic acid

8 **IS control:** In a 1.5-mL microcentrifuge tube, mix:

8.1 100 µL of NEG CTRL bovine plasma

8.2 100 µL of ACN

8.3 100 µL of IS mixture at 100 ng/mL in ACN-formic acid 0.1%

9 **Standard:** In a 1.5-mL microcentrifuge tube, mix:

9.1 100 µL of NEG CTRL bovine plasma

9.2 100 µL of working standard in ACN

9.3 100 µL of IS working mixture at 100 ng/mL in ACN-formic acid 0.1%.

10 **Samples and QCs**: In a 1.5-mL microcentrifuge tube, mix:

10.1 100 µL of plasma (samples or QCs)

10.2 100 µL of ACN

10.3 100 µL of internal standard mixture at 100 ng/mL in ACN-0.1% formic acid

11 Vortex each mixture for 5 seconds and centrifuge for 5 minutes at 13,000 g. The supernatant is then transferred to another 1.5-mL microcentrifuge tube and diluted with 0.4 mL of water before clean-up.

Solid-phase extraction

12 Fill out the plate template below before added each solution (NEG CTRL, IS CTRL, standards, QCs or sample to the wells.

	A	B	C	D	E	F	G	H	I	J	K	L
	NEG	IS	1.0	2.5	5	10	25	50	100	QC1	QC2	QC3

13 Stack the HLB µElution plate on top of a waste collection plate

14 Load each diluted supernatant into a well using positive pressure with nitrogen.

15 Wash each well twice with 250 µL of MeOH-water (25:75).

16 Stack the HLB µElution plate on top of a clean collection plate.

17 Elute the cannabinoids with 2 x 25 µL aliquots of ACN-MeOH (90:10).

18 Add 50 µL of water with 0.2% formic acid in each well before analysis.

19 Stack a cap mat on top of the plate.

20 Mix gently.

E. Analytical parameters

21 The analysis is performed with a system from Waters Corporation (Milford, MA) including an Acquity H UPLC and a TQ-S triple quadrupole mass spectrometer. The software used to control the UPLC and the mass spectrometer is MassLynx 4.2.

22 **Chromatographic separation**

22.1 UPLC column: Eclipse Plus C18 from Agilent Technologies (Santa Clara, CA) 100 x 2.1 mm, 1.8 µ.

22.2 Column temperature: 55 °C
Autosampler compartment: 8 °C
Flow rate: 0.5 mL/min
Injection volume: 5 µL

Table 1. Gradient used for the chromatographic separation of cannabinoids

	A	B	C
	Time (min)	Acetonitrile %	Aqueous formic acid 0.1%

A	B	C
0.00	40	60
6.50	14	86
6.51	0	100
7.50	0	100
7.51	40	60
10.00	40	60

The total run time is 10 minutes.

23 Mass spectrometer parameters

Data acquisition is performed by Electrospray Ionization (ESI) in positive (ES+) and negative mode (ES-). The source parameters are described in Table 2.

Table 2. Source parameters

A	B
Temperature	
Source	150 °C
Desolvation	550 °C
Gas Flow	
Desolvation	1,000 L/hour
Cone	150 L/hour
Nebulizer	7.0 bar
Voltages	
Capillary	3.0 kV

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Multiple reaction monitoring (MRM) mode is used to detect quantify each cannabinoid. The precursor ion, the products ions, the quantifier ion (bold), the qualifiers ions, the cone voltage, the collision energy (CE) as well as the ionization mode (ES+ or ES-) are indicated in Table 5.

Table 5. MRM parameters for each cannabinoid. (Quantifiers product ions are in bold; other product ions are used as qualifiers). Note: The dwell time is adjusted automatically in order to have 20 data points across each peak.

	A	B	C	D	E	F	G	H
	Cannabinoids	RT (min)	Precursor (m/z) (Cone, V)	Product (m/z)(CE, V)			Mode	IS
	THC-11-acid-glu	1.00	521.5 (44)	299.4 (32)	327.4 (24)	345.4 (14)	ES+	THC-11-acid-glu-d3
	THC-11-acid-glu-d3	1.00	524.5 (2)	348.3 (14)			ES+	n/a
	CBD-7-acid	1.08	524.5 (74)	119.1 (26)	193.3 (28)	299.2 (18)	ES+	THC-11-acid-d9
	THC-glu	1.48	491.3 (2)	123.0 (52)	193.1 (36)	315.1 (16)	ES+	THC-11-acid-glu-d3
	CBVDA	2.01	329.3 (2)	217.1 (24)	283.1 (20)	311.2 (20)	ES-	THC-11-acid-d9
	THC-11-acid	2.18	345.4 (60)	193.2 (28)	299.3 (20)	327.3 (14)	ES+	THC-11-acid-d9)
	THC-11-acid-d9	2.14	354.5 (28)	196.6 (26)			ES+	n/a
	THC-11-acid-d9	2.14	352.5 (68)	308.4 (20)			ES-	n/a
	THC-11-OH	2.17	331.4 (36)	193.2 (26)	201.2 (24)	313.4 (14)	ES+	THC-11-OH-d3
	THC-11-OH-d3	2.17	334.4 (18)	196.3 (26)			ES+	n/a
	CBDV	2.24	287.2 (66)	135.1 (18)	165.1 (26)	231.1 (18)	ES+	CBD-d3
	CBDA	2.91	357.4 (4)	179.2 (26)	245.4 (28)	339.4 (20)	ES-	THC-acid-d9
	CBGA	3.07	359.5 (32)	191.3 (34)	315.5 (20)	341.4 (20)	ES-	THC-acid-d9
	THCV	3.25	287.5 (40)	135.2 (16)	165.2 (20)	231.2 (16)	ES+	CBD-d3
	CBG	3.28	317.4 (50)	123.1 (30)	193.0 (18)		ES+	CBD-d3
	CBD	3.36	315.2 (14)	135.0 (20)	193.0 (22)	259.1 (20)	ES+	CBD-d3
	CBD-d3	3.36	318.5 (34)	196.2 (24)			ES+	n/a
	CBN	4.19	311.4 (72)	208.2 (28)	223.2 (20)	241.3 (18)	ES+	CBD-d3
	9-THC	4.78	315.4 (46)	123.1 (34)	135.2 (20)	193.2 (22)	ES+	9-THC-d3
	9-THC-d3	5.78	318.5 (36)	196.2 (24)			ES+	n/a
	8-THC	4.89	315.4 (24)	123.1 (30)	135.2 (18)	193.2 (22)	ES+	9-THC-d3

	A	B	C	D	E	F	G	H
	CBL	5.25	315.4 (42)	123.0 (280	165.0 (26)	235.1 (18)	ES+	CBC-d9
	THCA-A	5.42	359.4 (30)	219.2 (32)	261.3 (24)	341.3 (16)	ES+	THCA-A-d3
	THCA-A	5.42	357.4 (8)	191.2 (34)	245.4 (30)	313.4 (24)	ES-	THCA-A-d3
	THCA-A-d3	5.43	362.3 (26)	264.1 (26)			ES+	n/a
	THCA-A-d3	5.43	360.3 (76)	316.6 (28)			ES-	n/a
	CBC		315.4 (16)	123.1 (30)	193.2 (18)	259.3 (16)	ES+	CBC-d9
	CBC-d9	5.38	324.4 (40)	268.3 (14)			ES+	n/a
	CBLA	5.83	359.3 (52)	177.0 (36)	219.0 (32)	261.0 (26)	ES-	THCA-A-d3
	CBCA	5.99	357.4 (70)	179.2 (24)	313.4 (22)	339.4 (22)	ES-	THCA-A-d3
	THCP	6.43	343.4 (48)	123.0 (22)	135.1 (22)	221.1 (22)	ES+	CBC-d9

Table 6: Linear range and limit of quantitation

	A	B	C
	Cannabinoid	LOQ ng/mL	Linear range (ng/mL)
	THC-11-acid glu	1.0	1-100
	THC-11-glu	1.0	1-100
	CBD-7-acid	1.0	1-100
	CBDVA	2.5	2.5-100
	THC-acid	1.0	1-100
	THC-11-OH	1.0	1-100
	CBDV	1.0	1-100
	CBDA	1.0	1-100
	CBGA	2.5	2.5-100
	THCV	1.0	1-100
	CBG	1.0	1-100
	CBD	1.0	1-100
	CBN	1.0	1-100
	9-THC	1.0	1-100
	8-THC	1.0	1-100

	A	B	C
	CBL	1.0	1-100
	CBC	1.0	1-100
	THCA-A	1.0	1-100
	CBCA	2.5	2.5-100
	CBLA	1.0	1-100
	THCP	1.0	1-100