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Article

Solid Phase Extraction Approach to the Analysis of Cannabinoids and their Metabolites in Urine by LC-MS/MS

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Abstract: Cannabis is the most frequently consumed illegal substance worldwide. More recently, an increasing number of legal cannabis products low in psychoactive Δ9-tetrahydrocannabinol (THC) but high in non-intoxicating cannabidiol (CBD) are being more widely consumed. While the detection and quantification of THC and its metabolites in urine matrices is an important forensic-toxicological task, additional detection of CBD is also important, for example, when examining the plausibility of consumer statements. This report describes the SPE method validation for the quantitative determination of cannabinoids and their metabolites in urine. The obtained limit of quantification (LOQ) was 0.01 ng mL^{-1} for all investigated analytes, while the average recoveries as well as linearity for 11-OH-THC, CBD, CBN, and THC met the AOAC requirement, except the THC-COOH.

Keywords: cannabinoids, urine, SPE, validation, LC-MS/MS.

1. Introduction

Cannabis sativa L. is a cosmopolitan species that is widely distributed around the world but it is indigenous to eastern Asia. This name includes different botanical forms: *Cannabis sativa* var. *sativa* and *Cannabis sativa* var. *indica*. *Cannabis sativa* L. is well known for its characteristic of producing a unique class of compounds, i.e., C 21 terpenophenolics – (phyto)cannabinoids [1]. According to the latest data, 144 cannabinoids have been detected to date. Their possible classification is tabulated (Table 1).

Table 1. Cannabinoids classification into subclasses.

Δ9-THC[1]	Δ8-THC	CBD[3]	CBN[5]	cannabielsoin	miscellaneous type
CBG[2]	cannabinodiol	CBL[4]	CBC[6]	cannabitriol	

[1]- tetrahydrocannabinol; [2]- Cannabigerol; [3]- Cannabidiol; [4]- Cannabicyclol; [5]- Cannabinol; [6]- Cannabichromene

The use of products based on *Cannabis sativa* L has increased in recent years, using them for the treatment of anorexia, pain, multiple sclerosis, emesis, neurodegenerative disorders, cancer,

cardiovascular disorders, epilepsy, inflammation, and other diseases [1]. In many countries, the use of *Cannabis* is allowed in many countries in the world. In Switzerland, hemp plants containing less than 1% THC and 3-20% CBD are being sold for smoking as a tobacco replacement and combined with other ingredients in daily products or with no need for specific declared use, while in the US and Canada, the use of plants with THC concentration ≤0.3% is also allowed. In this context, the commercialization of CBD-rich cannabis products with low THC content and variable CBD concentration is proliferating. Moreover, cannabis farmers have been working to create new cannabis varieties rich in CBD. Varieties expressing up to 25% total CBD and less than 1% total THC (0.3–0.7%) within the floral tissue have recently been farmed. CBD-rich products can either be sold as dried plant material to be inhaled using conventional methods (e.g., joints, bowls, and vaporizers) or as concentrated CBD extracts intended for oral (e.g., oils, tinctures), pulmonary (e.g., vaporizers or vape pens) or topical (e.g., cream) consumption [2].

The Association of Official Analytical Collaboration (AOAC) describes the minimum recommended performance requirements within the Standard Method Performance Requirements (SMPRs) for Quantitation of Cannabinoids in Cannabis Concentrates [3]. The SMPRs established the method performance characteristics for the determination of THC, THCA, CBDA, CBN, and CBD such as limit of quantification (LOQ; %, w/w) ≤0.3; analytical range (%, w/w) ≤0.3–ca. 100 and for CBN ≤0.3–ca. 50. The recovery, depending on the range must be 95-105% (≤0.3–1%, w/w), 97–103% (>1–10 %, w/w), and for the range >10–ca%, w/w from 98 to 102%. For the mentioned ranges RSDr, % should be ≤5, ≤4, and ≤2, respectively with the RSD_R, % of ≤7, ≤5, and ≤3. Method validation, including studies of precision, accuracy, linearity, the limit of detection (LOD), and quantification (LOQ) of the LC-MS/MS method was performed according to the FDA recommendations [4]. The calculation for the accuracy was done within-run and between runs: ± 15% of nominal concentrations; except ± 20% at LLOQ (lower limit of quantitation) and for the precision: ± 15% RSD, except ± 20% RSD at LLOQ.

The literature references several methods of extracting cannabinoids from urine. Namely. Gerace et al. [2] primarily raised the pH of urine by adding sodium hydroxide, and after being incubated at 55 °C and adjusting the pH to 6.5-7.5 by adding glacial acetic acid mixture was transferred into a new tube and diluted with a mixture methanol/acetonitrile. After centrifugation, an aliquot was injected into the UHPLC-MS^n system. Frei et al. [5], used solid phase extraction (SPE), with a C18 cartridge for the extraction of cannabinoids from urine. C18 was activated with methanol and acetic acid, and the elution of analytes was performed with acetonitrile. After that, the derivatization and analysis by gas chromatography with tandem mass spectrometry (GC-MS/MS) were performed. The microextraction by packed sorbent (MEPS) was used by Rosendo and colleagues [6], in the analysis of cannabinoids in urine. After extraction, GC-MS/MS was used for the qualitative and quantitative analysis. Korać et al. [7], used the modified QuEChERS extraction method for the determination of the THC, CBN, CBD, and THC-COOH in urine by GC-MS.

The increased use of medicines based on this plant has led to a more detailed control of the quantification of cannabinoids in different products. Also, forensic laboratories have activated cannabinoid analyses because of the possible abuse of marijuana and hemp. A variety of analytical techniques have been developed for the quantification and qualification of cannabinoids and other compounds in plant and biological samples. Advances in analytical methods have also resulted in the detection of various compounds from sample extracts. The purpose of this work is to explore the possibility of cannabinoids such as CBD, CBN, THC and its metabolites THC-COOH and 11-Hydroxy-Δ^9-tetrahydrocannabinol – 11-OH-THC, extraction from spiking urine samples by Solid Phase Extraction (SPE) technique followed by the liquid chromatography-tandem mass spectrometry (LC-MS/MS). The delta9-THC-D3 was used as an internal standard (IS).

2. Materials and Methods

Chemical, reagents, and solutions

The delta 9-Tetrahydrocannabinol (delta9-THC), solution 0.1 mg mL^{-1}, LGC standard, LGCAMP1088.03-02, Lot 1187310, Cannabinol (CBN), 1.0 mg mL^{-1} in acetonitrile, LGC standard LGC-AMP0806, Lot 38229000 (1 mL), Cannabidiol solution (CBD) 1000 μg mL^{-1} in methanol, LGC standard, DRE-Al0946000ME-1000, Lot I2-H511581ME (1 mL), (±)-11-Hydroxy-Δ9-THC (THC-OH), 100 μg mL^{-1} in methanol, LGC standard, LGCAMP1088.03-02, Lot 1187310 (1 mL), (±) trans-11-nor-9-Carboxy-Δ9-THC (THC-COOH), 100 μg mL^{-1} in methanol, LGC standard, LGCAMP1088.05-02, Lot 1049664 (1 mL), delta9-THC-D3 100 μg mL^{-1} in methanol, 1 mg, CAY-19332-lMG were used. All stock standard solutions were stored at – 18 ºC. A working standard solution was prepared by measuring 50 μL of each stock solution into a 5 mL volumetric flask and diluting with methanol. The obtained concentration of the working standard was 1 μg mL^{-1}.

Methanol and acetonitrile were parched from Loba Chemie, Mumbai, India. SPE column: MAX SPE Cartridge, 3 mL, Biocomma, China, NH_4OH was perched by Sigma Aldrich, USA.

Acquisition parameters

Liquid chromatography–triple quadrupole mass spectrometry analysis was done following chromatograph conditions given in Tables 2 and 3.

Table 2. Agilent 1290 Infinity II UHPLC parameters.

Parameter	**Value**	
Column	Agilent Zorbax Eclipse Plus C18 4.6 × 50 mm, 1.8 μm	
Column temperature	35 °C	
Injection volume	10μL	
Mobile phase	A) 0.1% formic acid in Water B) 0.1% formic acid in Acetonitrile	
Flow rate	0.3 mL min^{-1}	
Gradient	Time (min)	%B
	0	30
	5	90
	10	95
	14	95
	15	30
Stop time	15 minutes	
Post time	5 min	

Table 3. Agilent Triple Quadrupole 6460 and Agilent Jet Stream source parameters.

Parameter	**Value**
Drying gas temperature	300 °C
Drying gas flow	10 L min^{-1}
Sheath gas temperature	350 °C
Sheath gas flow	11 L min^{-1}
Nebulizer pressure	40 psi
Capillary voltage	3500 V (+)
Nozzle voltage	500 V (+)
Cycle time	500 ms

Q1 scan of the mass spectra was recorded to select the most abundant mass to charge ratio (m/z) ion using continuous infusion of each cannabinoid directly into the MS using a syringe pump at a flow rate of 0.1 mL min^{-1}. In this study, the proton adduct [H+] of the molecular ion was chosen as the precursor ion for all analytes. Then, an enhanced product ion scan was conducted to obtain the product mass spectra of the precursor ion. The first transition, which corresponds to the most abundant production was used for identification and quantification, while the second one for confirmation purposes [8]. To obtain maximum sensitivity for the identification and quantification of the analytes, collision energy (CE), cell Acc energy (CA), and fragmentation energy (Frag.) were performed for each analyte using 1 μg mL^{-1} solution of individual compounds in methanol. Finally, the presence of precursor and product ions was investigated using multiple reaction monitoring (MRM) experiments with a cycle time of 500 ms. The optimized LC-MS/MS parameters are summarized in Table 4.

Table 4. Transitions for cannabinoids detection in dMRM mode.

Compound	Precursor *(m/z)*	Product *(m/z)*	Frag. *(V)*	CE *(V)*	CA *(V)*	RT *(min)*	Polarity
Cannabinol	311.2	223	110	24	5	5.99	Positive
Cannabinol	311.2	178	110	76	5	5.99	Positive
Cannabidiol	315.1	193.1	110	24	5	5.54	Positive
Cannabidiol	315.1	123.1	110	36	5	5.54	Positive
(±)-11-Hydroxy-Δ9-THC	331	313	110	12	5	4.99	Positive
(±)-11-Hydroxy-Δ9-THC	331	175	110	22	5	4.99	Positive
(±)-11-nor-9-Carboxy-Δ9-THC	345	327	110	15	5	5.08	Positive
(±)-11-nor-9-Carboxy-Δ9-THC	345	299	110	15	5	5.08	Positive
delta 9-Tetrahydrocannabinol	315.23	193.4	110	24	5	6.25	Positive
delta 9-Tetrahydrocannabinol	315.23	123.3	110	36	5	6.25	Positive
(-)-delta 9-THC-D3	318.25	123	97	36	5	6.25	Positive
(-)-delta 9-THC-D3	318.25	77	97	72	5	6.25	Positive

Sample preparation

Spiking of the blank urine samples as follows: 1 mL of blank urine sample was spiked with 50 μL of IS (conc. 1 μg mL^{-1}), and working standard solution 10, 25. 50 and 100 μL (conc. 1 μg mL^{-1}), resulting in final concentrations of 10, 25, 50 and 100 ng mL^{-1}. The 100 μL of NH_4OH was added to 1 mL of sample and it was shaken on vortex for 15 s, and heated for 90 min at 80 °C. The sample prepared in this way is purified through an SPE column. SPE anion exchange columns were activated with 1 mL of methanol and 1 mL of water. After adding the sample, the cartridge was washed with 1 mL of 5% methanol. Eluting of analytes was done with 1 mL methanol and injected into the LC-MS/MS.

Validation parameters

The validation parameters were done in accordance with the propositions of the AOAC [3].

Calibration curve

The linearity in which cannabinoids and their metabolites were determined was checked at four calibration levels ranging from 0.01 to 0.1 μg mL^{-1} (procedural calibration), spiking sample at the beginning.

Limit of detection (LOD) and limit of quantification (LOQ)

The LOQ was determined experimentally by enriching blank water samples so that the final concentration of cannabinoids and metabolites was 0.01 μg mL^{-1}.

The LOD was determined using MassHunter software, based on a signal-to-noise ratio = 5. The LOD was calculated based on the ratio of the peak area to the standard deviation of the noise in the chromatogram for the lowest spiked sample concentration.

The LOD represents the lowest concentration that can be determined by a given method but is not quantified with satisfactory reliability.

The LOQ represents the smallest concentration that can be determined with satisfactory accuracy and precision by a given method.

Recovery

The recovery was determined by enriching blank samples in four replicates at two concentration levels. In both methods, enrichment of samples was at the levels of 0.025 and 0.1 μg L^{-1}.

Reproducibility of the method

The reproducibility of the methods was tested by preparing one sample in five repetitions at the same concentration level. The obtained results were statistically processed using Microsoft Excel 2013 and the obtained %RSD value was compared with the criterion using the Horowitz equation. The obtained value of RSDr, RSD_R, (%) is compared with the calculated RSD (%), i.e. the theoretical relative standard deviation (AOAC Peer-Verified Methods Program Manual on Policies and Procedures):

$$RSD_R < 2^{(1-0.5logC)}$$

$$RSD_r < 2^{(1-0.5logC)} * 0.67$$

Where are:

RSD_R – Relative standard deviation of interlaboratory reproducibility

RSDr - Relative standard deviation of repeatability

3. Results and Discussion

Validation parameters

Total ion chromatogram (TIC), of the spiking urine samples at the concentration level of 0.025 μg mL^{-1} obtained using the SPE method are shown in Figure 1.

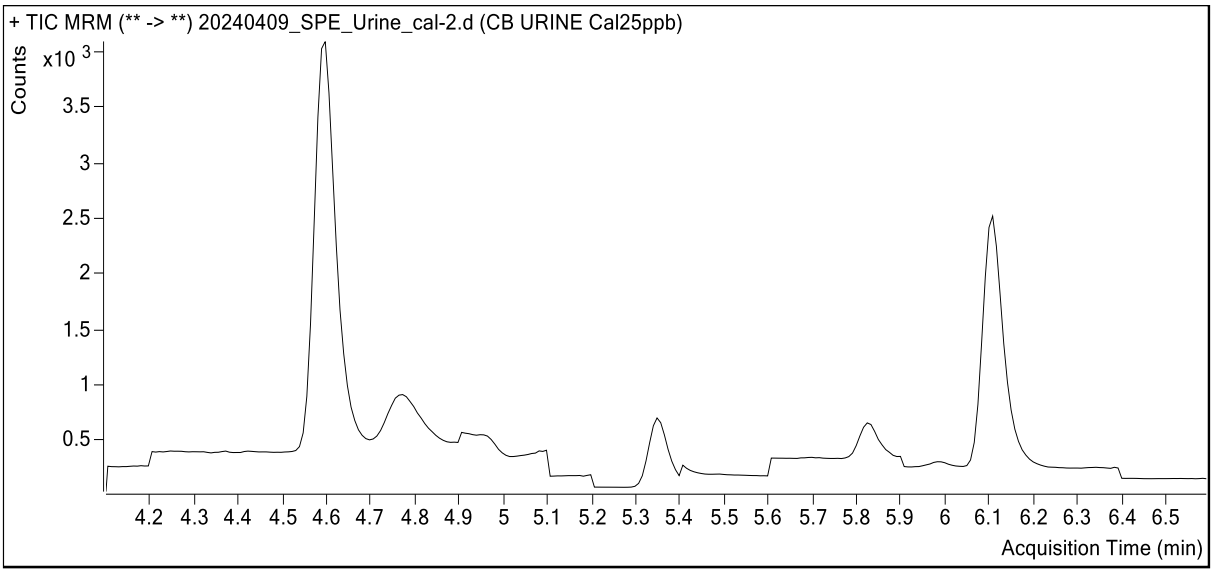

Figure 1. TIC chromatogram of the enriched urine sample (C= 0.025 μg mL^{-1}).

The calibration curve of the method for determining cannabinoids and their metabolites was tested for the concentrations of 0.01, 0.025, 0.05, and 0.1 μg mL^{-1} and is shown in Figures 2 and 3.

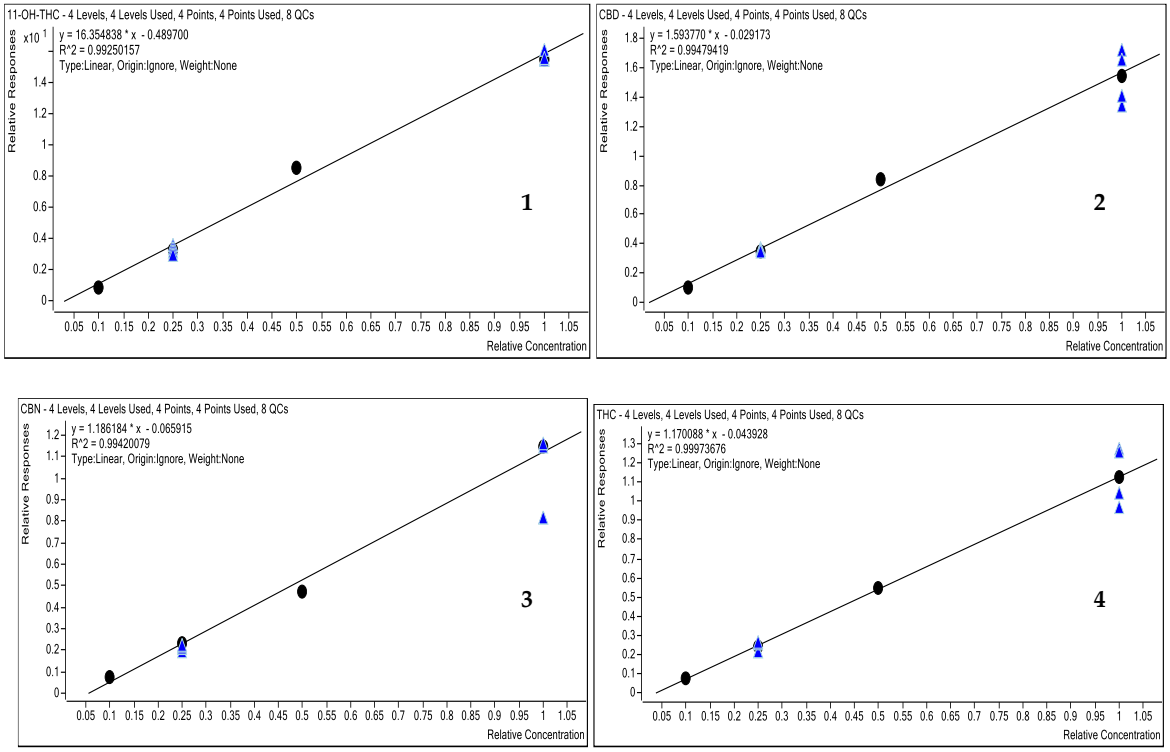

Figure 2. Calibration curve of 11-OH-THC (1), CBD (2), CBN (3) and THC (4).

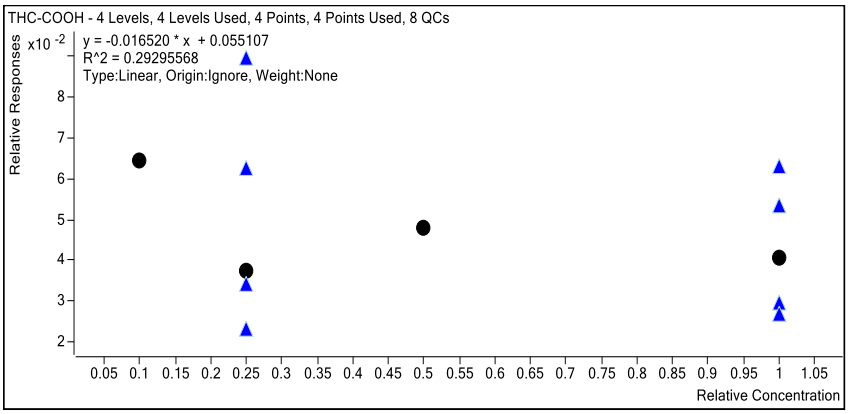

Figure 3. Calibration curve of THC-COOH.

The coefficient of correlation for 11-OH-THC, CBD, CBN, and THC met the linearity requirement for the given test range (R^2>0.99) for mass spectrometry. Linearity for THC-COOH does not exist ie R^2<0.99, which leads to the conclusion that the method of extraction and clean-up of the sample is not suitable for this analyte.

Recovery

The recovery (Re, %) was determined by enriching blank samples in four replicates at the levels of 0.025 and 0.1 μg mL^{-1}. The table (Table 5), shows recovery values (Rec) by levels and repetitions, their average values as well as the achieved recovery also the average relative standard deviation (%RSDr) are tabulated.

Table 5. Recovery, RSD.

Level μg/mL	11-OH-THC	Average Rec, RSD, %	CBN	Average Rec, RSD, %	CBD	Average Rec. RSD, %	THC	Average Rec, RSD, %
0.25	90.3		96.7		98.7		104.8	
0.25	86.1		97.7		94.3		106.7	
0.25	100.2		88.1		100.6		89.5	
0.25	95.5	**95.9±4.16**	92.1	**91.1±11.53**	97.8	**97.8±7.19**	89.1	**99.2±11.36**
1.0	101.3		102.3		109.5		112.4	
1.0	98.3		103.2		105.5		111.5	
1.0	97.7		74.5		86.3		86.7	
1.0	98.0		74.6		89.9		92.8	

4. Conclusions

We have developed and validated an LC-MS/MS method for the simultaneous determination of four cannabinoids in urine samples. The sample pre-treatment is very simple. Accuracy and precision fulfill the criteria of < 20% at a concentration equal to the LOQ and < 15% at higher concentrations for 11-OH-THC, CBD, CBN, and THC while the applied purification method through MAX SPE columns proved to be inappropriate for THC-COOH. The main advantages of the present method lie in its simple sample preparation, reliable results (recovery from 91.1 to 99.2%, and RSD 4.16 to 11.53%), and short analysis time for 11-OH-THC, CBD, CBN, and THC.

Conflicts of Interest: The authors declare no conflict of interest.

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