Application Note

Forensics

Analysis of Cannabinoids and their Metabolites in Human Urine Using the Agilent Chem Elut S Plate by LC/MS/MS

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Abstract

A robust, specific, and sensitive method was developed and validated for the quantitative analysis of tetrahydrocannabinol (THC), cannabidiol (CBD) and cannabinol (CBN), and the major metabolites of THC, 11-hydroxy-Δ^9-THC (THC-OH) and 11-nor-9-carboxy-Δ^9-THC (THC-COOH), in human urine. Human urine samples (150 µL) were digested using IMCSzyme, a genetically modified β-glucuronidase, extracted with the Agilent Chem Elut S 96-well plate, and then analyzed by LC/MS/MS. The method provided a dynamic linear range of 2 to 1,000 ng/mL with correlation coefficients of R^2 >0.99 for all five analytes. Method quantitation was verified with three spiking level quality control (QC) samples at 2, 50, and 1,000 ng/mL, with accuracy within 100 ±15%, and precision relative standard deviation (RSD) <15% in matrices. The method was demonstrated as a reliable solution for the emerging application of quantitation of THC and its metabolites, CBD, and CBN in human urine matrices.

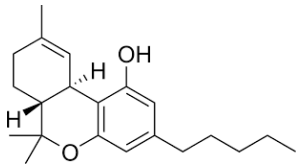
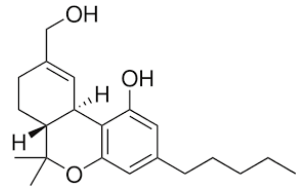
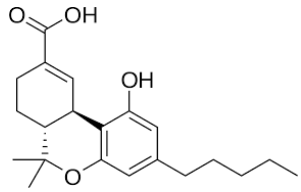
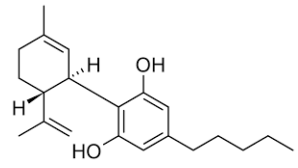
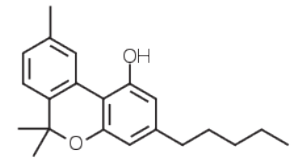
Introduction

The increasing use of marijuana both medicinally and recreationally is resulting in increased health risks, therefore there is a higher demand for fast and reliable monitoring in human biological matrices. Traditionally, cannabinoids and their metabolites, such as THC, CBD, CBN, and the major THC metabolites, THC-OH and THC-COOH, are measured in human urine to assess an individual's exposure to marijuana products.[1] Table 1 lists the five targets and their important properties.

Considering the high hydrophobicity of the targets, with log P above 4.5 (as shown in Table 1), these compounds are applicable for liquid–liquid extraction (LLE) and supported liquid extraction (SLE) methodology. SLE on a 96-well plate provides the advantage of high-throughput analysis and ease of adoption with automated sample preparation. The high-throughput sample processing based on a 96-well plate provides fast sample preparation and short processing time. This process improves the sample preparation productivity, but also prevents the target's degradation, which is usually caused by long processing times. The Agilent Chem Elut S 96-well plate also provides a great automatable option, which can further improve sample analysis speed and overall laboratory productivity. Compared to the traditionally used diatomaceous earth sorbent, the Chem Elut S synthetic sorbent improves sorbent batch-to-batch reproducibility and product performance consistency. The 96-well, 2 mL, square plate design offers large headspace for samples and eluent; a full skirt plate for easy automation platform compatibility; and fast and consistent elution.

Another critical consideration for cannabinoids and metabolites analysis, especially in urine, is that these compounds are highly prone to nonspecific binding during sample preparation. Therefore, it is important to minimize the contact of samples or standards with plastic container surfaces, including containers used to spike the standards in urine, as well as plastic 96-well collection plates. Instead, glass vials, plates with glass inserts, or glass-coated collection plates are highly recommended for this application.

Table 1. Molecules of interest.

Molecule	Structure	Chemical Formula	Log P
Tetrahydrocannabinol (THC)		$C_{21}H_{30}O_2$	5.94
11-Hydroxy-Δ^9-THC (THC-OH)		$C_{21}H_{30}O_3$	4.66
11-Nor-9-carboxy-Δ^9-THC (THC-COOH)		$C_{21}H_{28}O_4$	5.14
Cannabidiol (CBD)		$C_{21}H_{30}O_2$	6.33
Cannabinol (CBN)		$C_{21}H_{26}O_2$	6.41

Experimental

Reagents and chemicals

All reagents and solvents were LC/MS or HPLC grade. Methanol (MeOH), acetonitrile (ACN), and isopropanol (IPA) were from Honeywell (Muskegon, MI, USA). Hexane and ethyl acetate (EtOAc) were from J.T. Baker (Center Valley, PA, USA). Ammonium fluoride, ammonium formate, and all standards and internal standards (IS) were purchased from Sigma Aldrich (St. Louis, MO, USA).

Human urine (Mass Spect Gold) was purchased from Golden West Biological Inc. (Temecula, CA, USA). Genetically modified β-glucuronidase and buffer, IMCSzyme E1F kit, were from IMCS (Irmo, SC, USA).

Standards and solutions

Individual stock solutions of 1 mg/mL of THC, THC-OH, THC-COOH, CBD, and CBN were used to prepare a combined spiking solution of 20 µg/mL in MeOH. This combined standard solution was then used to prepare two spiking solutions, 2,000 ng/mL and 200 ng/mL, in MeOH. All three standard spiking solutions were used to fortify the calibration standards in urine. IS stock solutions of 100 µg/mL of THC-OH-D_3, THC-COOH-D_9, and THC-D_3, all in MeOH, were used to prepare IS spiking solution in IPA, at a concentration of 2,000 ng/mL for THC-D_3 and THC-COOH-D_9, and 200 ng/mL for THC-OH. All spiking solutions were prepared in an amber glass vial and stored at –20 °C.

Sample preparation equipment and consumables

- Agilent Chem Elut S 96-well plate, 400 µL (part number 5610-2004)
- Agilent positive pressure manifold 96 processor (PPM-96) (part number 5191-4116)
- Plate, 96-well, with 1.2 mL glass inserts and plate matt
- Vortexer, VWR
- Evaporator, CentriVap Complete, Labconco
- Centrifuge 5424 R, Eppendorf
- Pipettes
- ViaFlo 96 Liquid Handler (Integra Biosciences, USA)

Instrument method

An Agilent 1290 Infinity II LC system coupled to an Agilent 6490 triple quadrupole LC/MS system was used. LC and MS conditions are listed in Tables 2 and 3. The dynamic multiple reaction monitoring (dMRM) parameters for data acquisition are listed in Table 4.

Table 2. Method LC conditions.

Parameter	Value
Column	Agilent ZORBAX Eclipse Plus C18, 2.1 × 100 mm, 1.8 µm column (p/n 959758-902) Agilent ZORBAX Eclipse Plus C18 guard, 2.1 × 5 mm, 1.8 µm (p/n 821725-901)
Flow Rate	0.4 mL/min
Column Temperature	40 °C
Injection Volume	5 µL
Mobile Phase	**A:** 10 mM ammonium formate, 0.5 mM ammonium fluoride in water, 0.125% FA **B:** 10 mM ammonium formate, 0.5 mM ammonium fluoride in 95:5 ACN/water, 0.125% FA
Needle Wash	1:1:1:1 ACN/MeOH/IPA/water, 0.2% formic acid
Gradient	**Time (min)** **%B** **Flow (mL/min)** 0.0 65 0.4 1.0 65 0.4 4.5 80 0.4 4.6 95 0.5
Stop Time	7 min
Post Time	2 min

Table 3. Method MS conditions.

Parameter	Value
Ionization Mode	Electrospray ionization (ESI)
Gas Temperature	220 °C
Gas Flow	18 L/min
Nebulizer	22 psi
Sheath Gas Heater	400 °C
Capillary Voltage	3,500 V (both positive and negative)
Nozzle Voltage	0 V (both positive and negative)
iFunnel Parameters	High-pressure RF: 120 V (positive), 110 V (negative)
	Low-pressure RF: 60 V (positive), 60 V (negative)
Polarity	Positive

Table 4. dMRM conditions for target analytes.

Analyte	Precursor Ion (*m/z*)	Product Ion (*m/z*)	Collision Energy (V)	Retention Time (min)
THC-OH	331.23	313.2	11	3.46
		193.1	27	
THC-OH D_3 (IS)	334.25	316.3	19	3.16
THC-COOH	345.21	327.3	15	3.56
		299.2	15	
THC-COOH D_9 (IS)	354.27	336.2	15	3.56
CBD	315.23	193	21	5.31
		41	80	
CBN	311.2	223	21	6.05
		43.1	37	
THC	315.23	193.1	27	6.3
		123.1	31	
THC D_3 (IS)	318.25	196.1	28	6.3

Calibration standards and quality control samples preparation

Cannabinoids and metabolites are highly prone to nonspecific binding with container surfaces, especially plastic surfaces, which results in significant analyte loss, and poor reproducibility and stability. This nonspecific binding can be more significant when analytes are in high-aqueous, but relatively simple and clean matrix, such as pure water or urine. In addition, the more hydrophobic the compound is, the more significant the nonspecific binding that may occur. THC is the most hydrophobic compound in the targets, and therefore experienced the most significant loss caused by nonspecific binding. Figure 1 shows the chromatograms of identical standard solutions prepared in water (A, B) and MeOH (C, D), in a glass vial (red) and a plastic vial (black). The comparison of results confirmed the previously mentioned effects caused by nonspecific binding between container surface and targets. Therefore, plastic vials and collection plates should be avoided, while glass vials and collection plates, with glass inserts or glass coating, should be used instead. In addition, 5% IPA was pre-added to a urine blank before standard spiking, which also helped prevent nonspecific binding loss. It also should be added as early after urine sample was collected.

Table 5 shows the calibration curve standards spiking details. The combined standard (STD) spiking solutions include: STD spiking I for 20 µg/mL, STD spiking II for 2,000 ng/mL, and STD spiking III for 200 ng/mL.

Table 5. Calibration curve STDs and QCs spiking in human urine.

Calibration STD or QC ID	Concentration in Urine (ng/mL)	STD Spiking Solution	STD Spiking Volume (µL)	Urine Volume (µL)	IPA Pre-addition (µL)
STD 1	2	STD spiking III	10	990	50
STD 2	5	STD spiking III	25	975	50
STD 3	10	STD spiking II	5	995	50
STD 4	50	STD spiking II	25	975	50
STD 5	100	STD spiking I	5	995	50
STD 6	500	STD spiking I	25	975	50
STD 7	800	STD spiking I	40	960	50
STD 8	1,000	STD spiking I	50	950	50
QC Low	2	STD spiking III	10	990	50
QC Mid	50	STD spiking II	25	975	50
QC High	1,000	STD spiking I	50	950	50

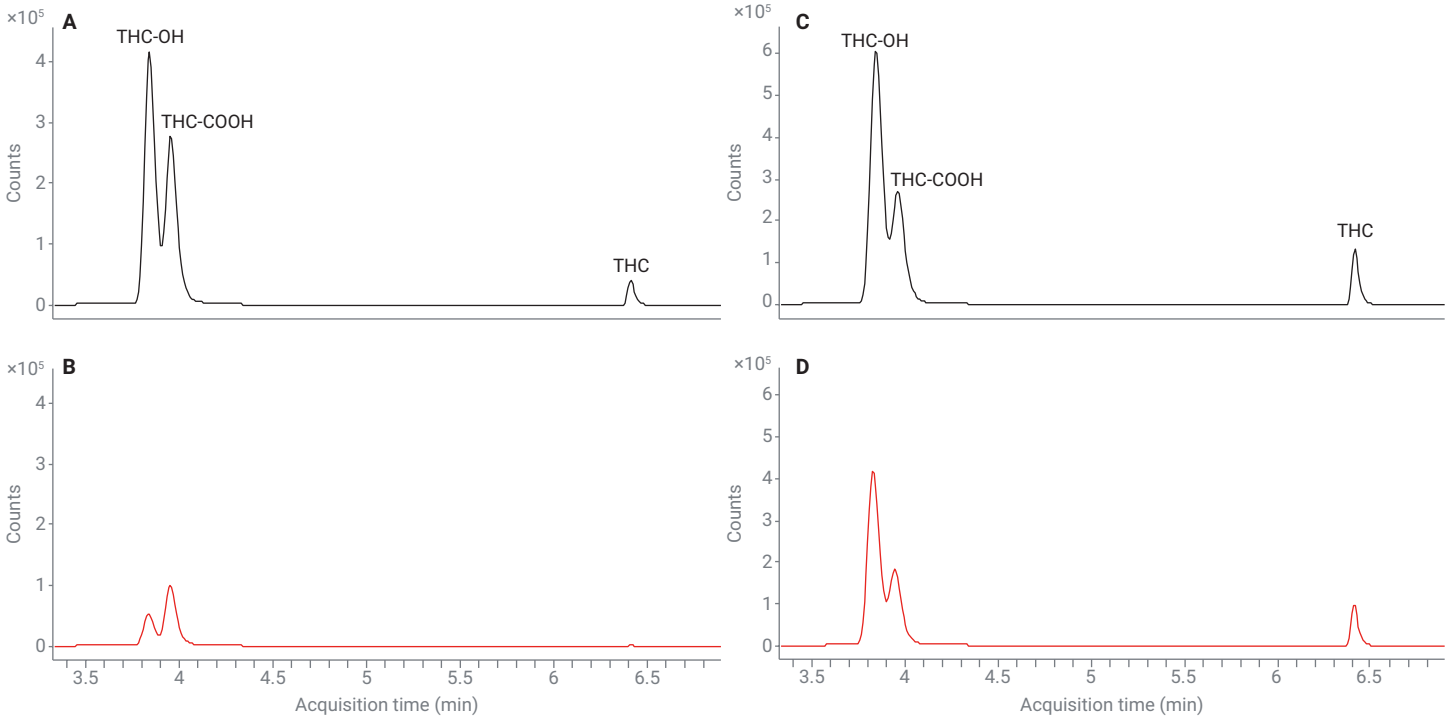


Figure 1. Nonspecific binding test for 50 ppb neat standards in water, prepared in (A) a glass vial and (B) a plastic vial, and for 50 ppb neat standards in MeOH, prepared in (C) a glass vial and (D) a plastic vial.

Sample digestion

Urine hydrolysis, either by enzyme or chemical hydrolysis, has been highly recommended to eliminate conjugated drug glucuronides before urine analysis. In comparison to traditionally used chemical hydrolysis, the enzymatic hydrolysis procedure provides mild digestion conditions, and therefore prevents drugs degradation and analysis accuracy. Therefore, enzyme digestion has been adopted widely in forensic labs, and was also used in this study. The IMCSzyme used in this study is a genetically modified β-glucuronidase, providing an immediate hydrolysis of THC-COOH glucuronide without incubation.[2] Prior to digestion, depending on the number of samples for digestion, a bulk master mix solution was made by mixing the IMCSzyme E1F enzyme and E1F buffer at the ratio of 2:3. Pipette mixing was used to mix the solution two to three times, and a vortex should not be used. An aliquot of 85 µL of master mix solution was then added to 160 µL of urine sample with IPA pre-addition.

Sample preparation

The sample preparation procedure is described step-by-step in Figure 2. Specifically, a plate with glass inserts or glass coating (1.2 mL or above) was used for sample aliquoting, IS addition, enzyme digestion, and post-acidification. Samples were then transferred to a Chem Elut S 96-well, 400 µL plate. After 5 to 10 minutes equilibration, samples were then eluted with four replicates of 450 µL 9:1 hexane/EtOAc. For the first two elutions, the 450 µL elution solvent was initially added to the preparation plate to rinse the well surface, and then transferred to the Chem Elut S plate for sample elution. For the third and fourth elution, the solvent was added to the Chem Elut S plate for direct elution. As the collection plate with glass inserts well volume (1.2 mL) was not enough to collect the entire eluent, a short mid-drying step was used after the second elution. The eluent volume was brought down to free the well capacity for further eluent collection. After the third and fourth elution, the collected sample eluent was dried completely.

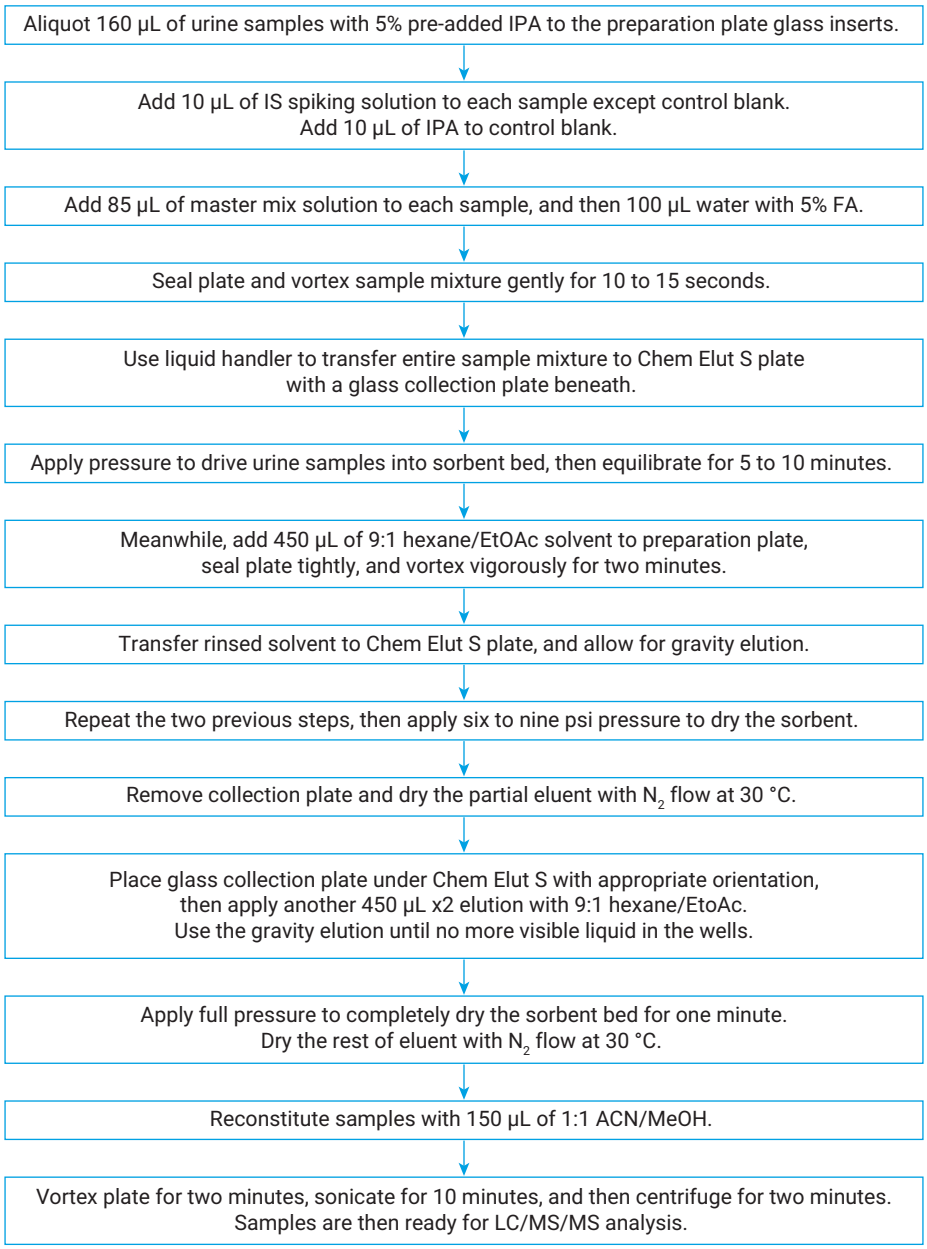


Figure 2. Sample preparation procedure for cannabinoids analysis in human urine, using the Agilent Chem Elut S 400 µL, 96-well plate.

Analyte absolute recovery and matrix effect

Analyte absolute recoveries were evaluated based on analyte peak area comparison between prespiked QCs and matrix-matched QCs at the spiking level of 50 ng/mL in urine. In many forensic testing labs, a level of 50 ng/mL targeted drugs in urine is an important threshold for THC screening and quantitation. Therefore, this level was used as the major spiking level for method verification. Matrix evaluation was based on analyte peak area comparison between matrix-matched QCs and neat standards at an equivalent concentration of 50 ng/mL. In addition, the THC-COOH glucuronide digestion recovery and reproducibility was also assessed at the spiking level of 50 ng/mL in urine.

Method verification

The developed method was verified through accuracy and precision (A&P) runs. Two sets of calibration standards, six replicates of three levels of QC samples, and matrix blanks were prepared appropriately. Two sets of calibration standards were run at the beginning and end of the sequence, bracketing the three levels of QC samples from low to high level. For method selectivity validation, four urine matrix lots were evaluated for matrix blank and LLOQ accuracy and precision. The combined accuracy and precision were validated at the spiking level of 50 ng/mL free THC-COOH and THC-COOH glucuronide.

Results and discussion

Analytes nonspecific binding

As mentioned previously, the targeted analytes in this study experienced significant nonspecific binding with the container surface, especially the plastic surface. As a result, it is important to use a glass container for sample collection; standard spiking; sample pre-preparation in a plate before extraction; and sample collection after extraction. The results showed that the use of the Chem Elut S plate is acceptable, even though the plate is plastic. First, samples only encountered the plastic surface for a short time during sample loading. Second, a highly hydrophobic solvent was used for multiple rinses/elution, which can recover analytes that have bonded with the plastic surface.

Besides the use of a glass container/plate, the addition of IPA was another important strategy to prevent nonspecific binding. Figure 3 shows the effect of IPA pre-addition to urine samples on the recovery and reproducibility. These results demonstrate that improved recovery and reproducibility were observed for all analytes with IPA pre-addition. The improvement effect is more significant for more hydrophobic analytes, such as CBD, CBN, and THC, which exhibited as the improved recoveries, and the reduced RSDs from >20% to <10%. The IPA addition was conducted in two steps: direct addition of the 5% of IPA to the urine matrix, before standard spiking. If possible, this addition should be added as early as possible after collection of the urine sample. After sample aliquoting into the preparation plate wells, 10 µL of IS spiking solution in IPA was added to each sample. As a result, the total IPA in the final volume was approximately 10%. Although the addition of IPA is helpful for preventing nonspecific binding, it is not recommended to use >15% of total IPA addition. First, >15% of IPA in urine could compromise the enzyme digestion efficiency. Second, >15% IPA could result in a high risk of breakthrough during sample loading and elution on the Chem Elut S plate.

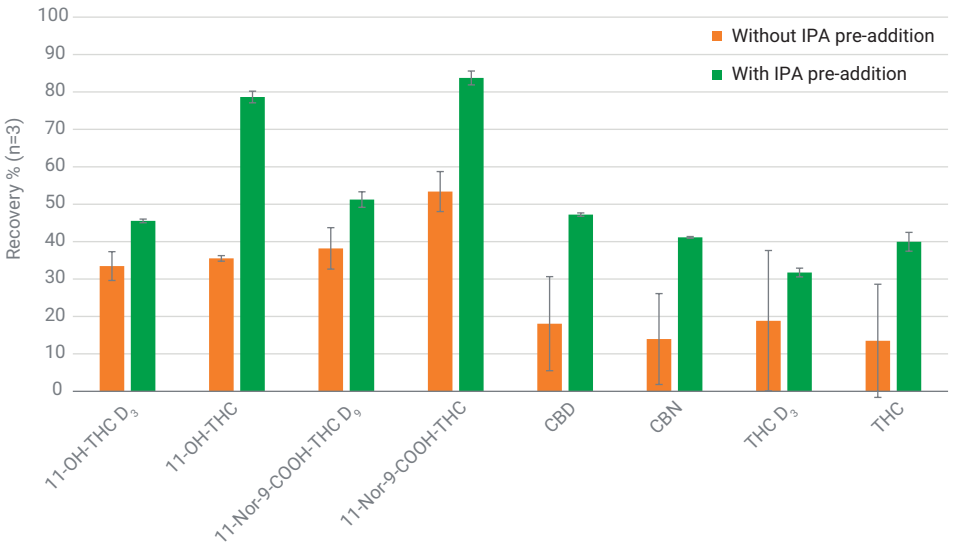


Figure 3. The impact of IPA pre-addition to urine (green), compared to without IPA pre-addition (orange).

The third critical strategy to prevent nonspecific loss was to use the elution solvent to rinse the preparation plate and then elute the sample on the Chem Elut S plate. Even with the use of a glass preparation plate, nonspecific binding loss still was significant. The use of extraction solvent to rinse the preparation plate became critical to recover the bonded analytes from the contact surface. Figure 4 shows the analyte recovery profiles, from no rinse to four rinses with 450 µL extraction solvent. The results showed that: 1) the rinsing step provided significant improvement on analytes recovery; 2) two rinses provided the best efficiency, with the highest recoveries for all analytes, and 3) additional rinsing did not further improve the recovery, but instead caused some additional loss. Therefore, two rinses with 450 µL of 9:1 hexane/EtOAc was used for the final method.

Other preventive strategies, including minimal sample vortexing; keeping urine samples frozen prior to use; and carrying out the entire procedure in succession, helped reduce the nonspecific binding, and maintain analyte stability during sample preparation.

Supported liquid extraction (SLE) on Chem Elut S plate

Once the nonspecific issues were resolved, the SLE method development on the Chem Elut S plate was straightforward. As shown in Table 1, all analytes in this study were hydrophobic compounds, therefore, a more hydrophobic solvent mixture of 9:1 hexane/EtOAc provided the best recoveries, where the 10% EtOAc solvent was used to improve the recoveries of THC-OH and THC-COOH. The multiple elutions were used to improve analyte recovery, especially for the highly hydrophobic analytes such as THC. Considering the ~10% IPA in the final sample mixture, the loading volume was reduced to 350 µL, which prevented the potential sample breakthrough caused by the bridging effect of 10% IPA.

For the sample pretreatment step after digestion but prior to extraction, the use of different dilution buffers was explored, including: A) an acidic buffer to neutralize the targets; B) NaCl solution for targets salting out; C) the ion pairing reagent dibutlyammonium acetate buffer; and D) a combined dilution buffer. Results showed that the use of the acidic dilution buffer provided best results for analyte recovery and reproducibility. Although stability was reported for THC and its metabolites in acidic sample conditions[1], the results do not show any impact of using acidic dilution buffer on the data quality. This can mostly be attributed to the expedited sample extraction procedure after the addition of acidic buffer on the 96-well Chem Elut S plate, which provided the efficient control for analyte degradation in acidic conditions.

Even with extra rinsing steps, and a mid-drying step due to the collection plate capacity, the SLE method on the Chem Elut S plate still provided significant advantages over the traditional LLE method. Notable advantages included: time saving and reduced labor from sample mixing and transferring; improved method reproducibility; and prevention of human errors due to fewer steps and manipulation.

For the batch processing on the 96-well Chem Elut S plate, the appropriate glass or glass-coated collection plates are critical for the success of the high-throughput sample preparation. There may be concerns over the relatively high cost of using a special collection plate, or the testing needs for only a small quantity of samples. However, the direct transfer of the 96-well plate-based method to the cartridge-based method is an alternative, where the corresponding Chem Elut S 3 mL cartridges (part number 5610-2006) and appropriate glass tubes can be used for preparing samples. However, sample processing involving different treatments of individual samples can slow down the entire sample processing, which may slightly increase the risk of analyte instability during sample preparation. Therefore, it is important to choose the most appropriate sample preparation method and Chem Elut S products for the application, based on the practical situation.

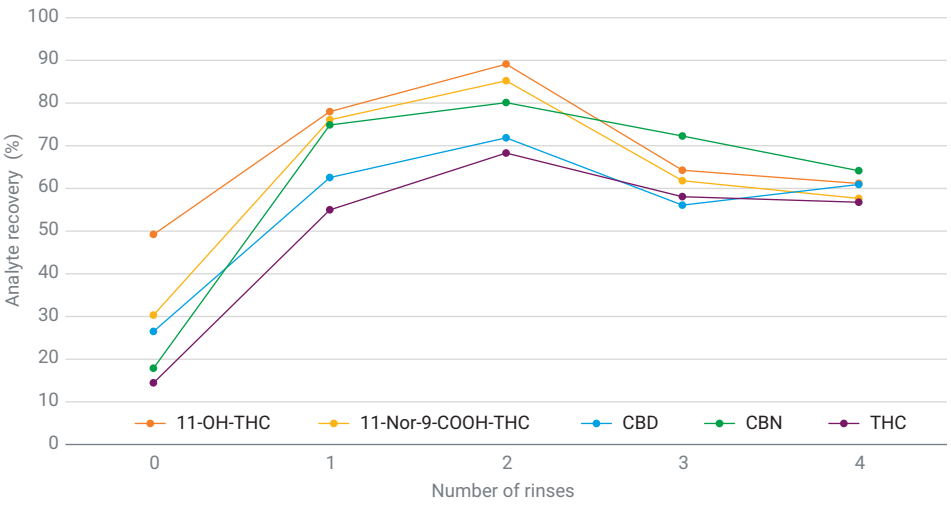


Figure 4. The impact of extraction solvent rinse of preparation plate on recovery of the analytes. The extraction solvent 9:1 hexane/EtOAc, 450 µL.

Method sensitivity and selectivity

As part of the method verification, the method sensitivity was assessed based on signal-to-noise ratio (S/N) at the lowest limit of quantitation (LLOQ) level. Method selectivity was assessed based on the comparison of the matrix blank contribution to the corresponding analyte peak area at LLOQ level, and LLOQ accuracy and precision in multiple urine matrix lots. Figure 5 shows the chromatograms of the matrix blank and the LLOQ of each analyte in human urine. At the defined LLOQ of 2 ng/mL, the S/N of the analytes was >10, and the contribution from the matrix blank was less than 20% of the analytes' responses at LLOQ level. Table 6 shows the accuracy and precision results when using four different urine matrix lots at the LLOQ spiking level of 2 ng/mL in urine. The results clearly demonstrated the method sensitivity and selectivity, confirming the quantitation method reliability.

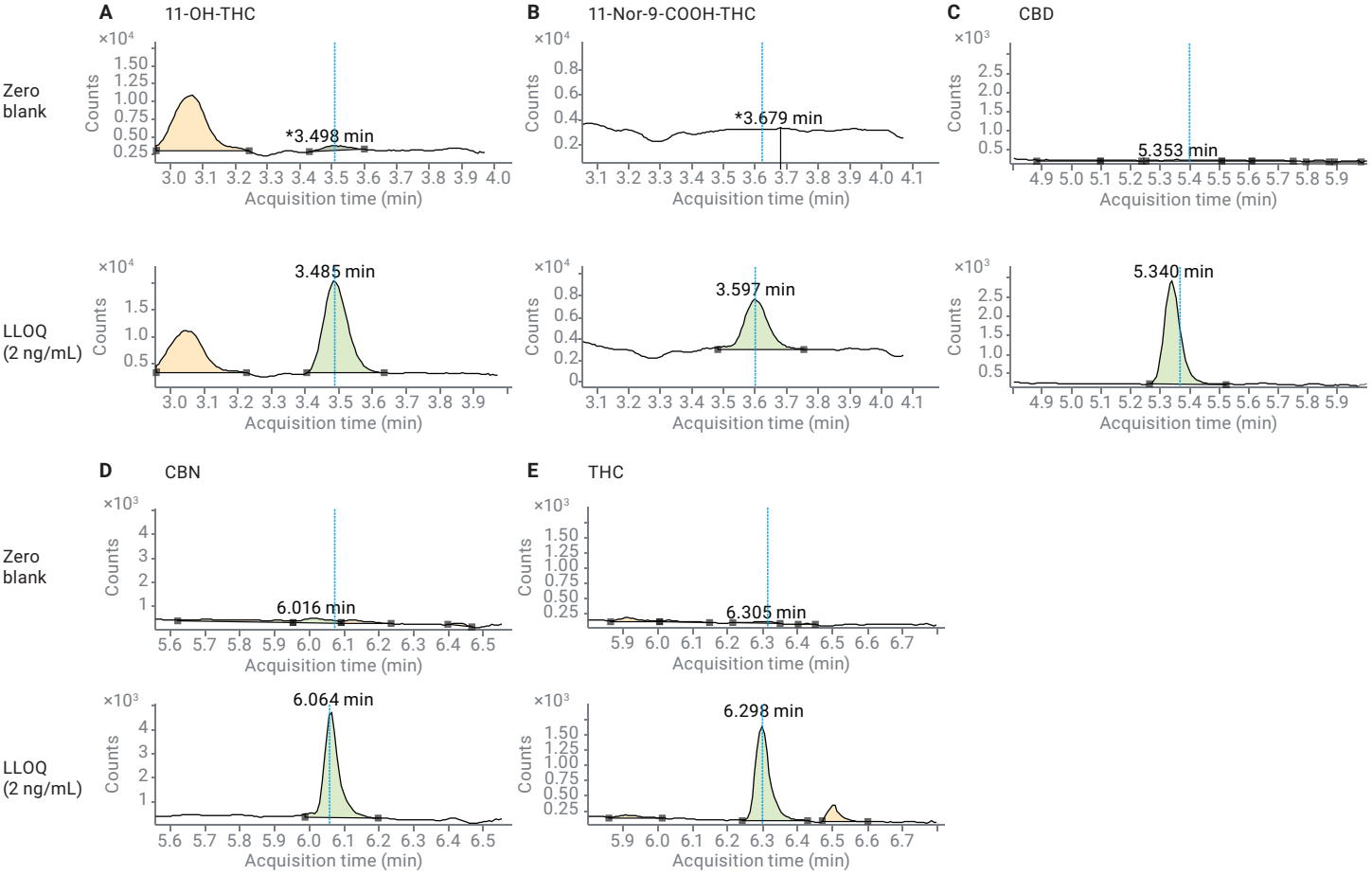


Figure 5. LC/MS/MS MRM chromatograms of human urine matrix blank (top) and 2 ng/mL LLOQ (bottom) for each analyte: (A) 11-H-THC; (B) 11-nor-COOH-THC; (C) CBD; (D) CBN; and (E) THC.

Table 6. Accuracy and precision results for different urine matrix lots at the spiking level of LOQ (2 ng/mL in urine).

	11-OH-THC (n = 3)			11-Nor-9-COOH-THC (n = 3)			CBD (n = 3)			CBN (n = 3)			THC (n = 3)		
Urine Matrix Lot	Calculated Concentration (ng/mL)	Accuracy (%)	RSD (%)	Calculated Concentration (ng/mL)	Accuracy (%)	RSD (%)	Calculated Concentration (ng/mL)	Accuracy (%)	RSD (%)	Calculated Concentration (ng/mL)	Accuracy (%)	RSD (%)	Calculated Concentration (ng/mL)	Accuracy (%)	RSD (%)
1	2.05	102	0.7	2.15	107	3.8	2.32	116	1.9	2.25	113	11.1	1.91	96	6.7
2	2.23	111	8.1	2.13	107	4.7	2.18	109	6.4	1.71	85	6.4	2.01	101	18.7
3	2.16	108	7.2	2.05	102	1.1	2.39	120	1.8	2.27	114	5.0	2.17	108	5.9
4	2.06	103	9.9	2.03	102	4.8	2.34	117	6.8	1.99	99	11.0	1.81	90	9.0

Calibration curve linearity

Method linearity was demonstrated in the dynamic range of 2 to 1,000 ng/mL in matrix. The calibration curves were regressed using linear regression fit, with the weight of 1/x. The calibration curves for each analyte in human urine are shown in Figure 6. All curves showed excellent linearity over the calibration range, with R^2 >0.99.

Method instrument carryover was assessed by running two matrix blanks after the highest limit of quantitation (HLOQ) sample, which was 1,000 ng/mL. Results demonstrated that <20% of carryover contribution to the target response at LLOQ level for all the targets. This was due to the use of appropriate post-column washing after gradient, and the highly effective needle wash solution to wash the entire injection port.

Quantitation accuracy and precision

The optimized method using IMCSzyme digestion, followed by extraction on the Chem Elut S 96-well plate, was verified by A&P runs to collect the complete quantitation results. The results shown in Table 7 include the accuracy and precision RSD (%) for all analytes at three levels in human urine. The ISs used in this method include THC-OH D_3, THC-COOH D_9, and THC D_3. For THC-OH, THC-COOH and THC, the corresponding stable-labeled IS was used for quantitation. For CBD and CBN, THC-COOH D_9 was used. Quantitation results from the A&P run demonstrated excellent method accuracy and precision results, meeting the typical acceptance criteria for clinical testing, defined as an accuracy of 100 ±15% and RSD ≤15% for all spiking levels, except the LLOQ spike level. For the LLOQ spiking level, the acceptance criteria are defined as an accuracy of 100 ±20% and RSD ≤20%.

THC-COOH glucuronide digestion

THC-COOH glucuronide is the most common metabolite in urine. Therefore, it is important to use the appropriate digestion to convert THC-COOH glucuronide into free drug format for the accurate quantitation of THC-COOH. The THC-COOH glucuronide digestion efficiency was evaluated by spiking the THC-COOH glucuronide in urine, going through the digestion and extraction, and then quantitating for THC-COOH. For the recovery test, only THC-COOH glucuronide was spiked in urine at the level of 75.75 ng/mL, corresponding to THC-COOH level of 50 ng/mL (with the conversion factor of 0.66). After digestion and extraction, the pre-spiked samples were then compared to postspiked samples with 50 ng/mL of THC-COOH. The combined recovery for digestion and extraction was 81% with RSD of 1.6% for the replicates of six.

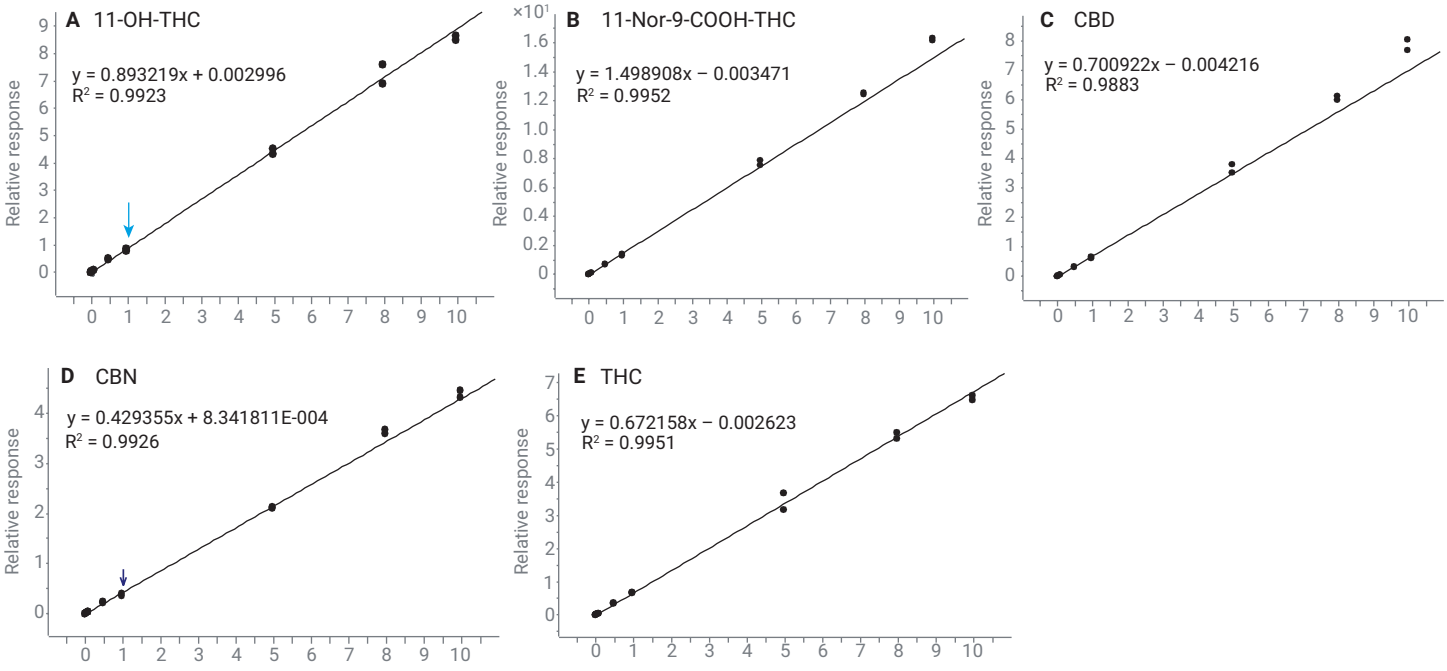


Figure 6. Calibration curves of analytes in human urine for the range of 2 to 1,000 ng/mL: (A) 11-OH-THC; (B) 11-nor-9-COOH-THC; (C) CBD; (D) CBN; and (E) THC.

Table 7. Method accuracy and precision results for THC and its metabolites, CBD, and CBN in human urine.

Target Analyte	Experiment	QC Low (2 ng/mL)			QC Mid (50 ng/mL)			QC High (1,000 ng/mL)		
		Calculated Concentration (ng/mL)	Accuracy (%)	RSD (%)	Calculated Concentration (ng/mL)	Accuracy (%)	RSD (%)	Calculated Concentration (ng/mL)	Accuracy (%)	RSD (%)
11-OH-THC	Day 1 (n = 6)	1.93	96	8.5	55.43	111	2.9	924.71	92	2.7
	Day 2 (n = 6)	1.97	99	5.3	55.02	110	1.0	931.90	93	2.5
	Day 3 (n = 6)	1.98	99	5.3	53.71	107	2.9	1,012.69	101	4.9
	Interday (n = 18)	1.96	98	6.2	54.72	109	2.7	956.43	96	5.5
11-Nor-9-COOH-THC	Day 1 (n = 6)	2.04	102	4.7	49.20	98	2.4	1,084.78	108	3.9
	Day 2 (n = 6)	1.87	93	3.7	50.18	100	4.4	1,085.18	109	4.0
	Day 3 (n = 6)	1.97	99	2.3	49.28	99	3.7	1,087.18	109	4.8
	Interday (n = 18)	1.96	98	5.1	49.55	99	3.5	1,085.71	109	4.0
CBD	Day 1 (n = 6)	2.09	105	4.3	50.42	101	3.5	1,110.14	111	2.3
	Day 2 (n = 6)	1.91	96	4.1	50.18	100	3.4	1,137.08	114	8.0
	Day 3 (n = 6)	2.14	107	7.3	50.84	102	6.3	1,066.11	107	10.3
	Interday (n = 18)	2.05	102	7.2	50.48	101	4.4	1,104.44	110	7.6
CBN	Day 1 (n = 6)	2.06	103	6.7	54.57	109	2.7	905.28	91	4.5
	Day 2 (n = 6)	2.12	106	3.1	55.09	110	2.8	1,028.07	103	7.4
	Day 3 (n = 6)	1.89	94	12.0	55.71	111	3.1	1,063.99	106	5.2
	Interday (n = 18)	2.02	101	8.8	55.12	110	2.8	999.11	100	9.0
THC	Day 1 (n = 6)	2.09	104	4.7	54.50	109	4.6	1,119.21	112	2.8
	Day 2 (n = 6)	2.17	108	8.6	53.06	106	4.6	1,107.75	111	5.9
	Day 3 (n = 6)	2.02	101	8.1	51.52	103	5.1	1,108.74	111	5.2
	Interday (n = 18)	2.09	105	7.5	53.11	106	5.1	1,111.90	111	4.5

The quantitation accuracy and precision were also verified. A mixed QC sample was spiked with 50 ng/mL of THC-COOH, and 75.75 ng/mL of THC-COOH glucuronide, and then quantitated against the regular THC-COOH calibration curve. The results gave an average calculated concentration of 93.25 ng/mL, with the accuracy of 93% and RSD of 2.6% for the replicates of six.

The results confirmed an acceptable enzyme digestion and SLE extraction efficiency, and accurate and precise quantitation abilities for both free drug and glucuronide formats.

Conclusion

A robust method using IMCSzyme digestion followed by the Agilent Chem Elut S plate extraction was established for fast and reliable analysis of THC, CBD, CBN, and THC major metabolites THC-OH and THC-COOH, in human urine using LC/MS/MS. Multiple strategies were applied for effective control of nonspecific binding loss and variations. The method provided excellent quantitation results for analyte accuracy and precision, calibration curve linearity, sensitivity and selectivity, efficient recovery and matrix removal, and a simplified workflow.

References

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