QUANTITATIVE ANALYSIS OF THC AND RELATED CANNABINOIDS IN MULTIPLE MATRICES USING A NOVEL SOLID PHASE EXTRACTION SORBENT COUPLED WITH UPLC/MS/MS FOR CLINICAL RESEARCH AND TOXICOLOGY APPLICATIONS

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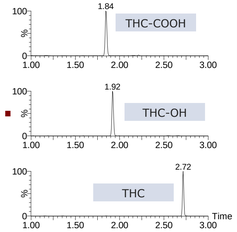
Introduction

Cannabis continues to be a highly abused recreational drug. In addition, the increasing number of states legalizing it for medical use, combined with the trend towards legalization for recreational purposes means than analytical methods for the quantification of Δ-9-tetrahydrocannabinol (THC), its metabolites and related cannabinoids continue to be necessary. Among drugs of abuse, natural cannabinoids present some unique analytical challenges. Excreted THC and related compounds are highly glucuronidated, requiring efficient deconjugation before analysis. In addition, the highly hydrophobic nature of natural cannabinoids makes them exceptionally susceptible to loss via non-specific binding, meaning that care must be taken with sample handling and processing of prepared extracts. Finally, matrix effects can be a challenge to control for these compounds, and can vary significantly in different biological matrices.

This work uses a novel reversed-phase (RP) solid phase extraction (SPE) sorbent, Oasis PRiME HLB, which has been developed to enable simpler and faster SPE protocols, while at the same time generating cleaner extracts than other sample preparation methods. 3 step load-wash-elute SPE protocols, eliminating conditioning and equilibration, were successfully employed to extract THC, THC-OH and THC-COOH from multiple matrices, including plasma, oral fluid (OF), whole blood and urine. This method details the extraction and analysis of these compounds using this novel RP SPE in a µElution format, followed by direct analysis by UPLC/MS/MS. Specific modifications to SPE protocols or chromatography have been detailed to optimize the method for the various matrices.

CHROMATOGRAPHY

The analysis was performed with a Waters ACQUITY I-Class UPLC combined with Xevo TQ-S MS instrument, with A BEH or CORTECS C18 2.1*100 mm UPLC column. The injection volume is 5 µL. The mobile phase gradient was started at 50% B (0.1% FA in ACN), rising to 95% in 3 min, followed by a 30 sec. hold. All compounds eluted within 3 minutes and all peak widths were under 3 seconds at 5% of baseline. All peaks were symmetrical with symmetries between 0.95-1.15.



Mass spectral parameters for all analytes and internal standards

Analyte	MRM transitions (m/z)	Con (V)	Col eV
THC-OH	331.3>313.1 331.3>193.1	40 40	18 30
THC-OH -d3	334.3>316.1	40	18
THC-COOH	345.3>327.3 345.3>299.3	50 50	20 25
THC-COOH-d3	348.3>330.3	50	20
THC	315.1>193.2 315.1>135.1	40 40	25 25
THC-d3	318.1>196.2	40	25

Figure 1. Chromatography and MS condition of THC-OH, THC-COOH and THC from an extracted urine sample on BEH C18 column

SAMPLE PREPARATION

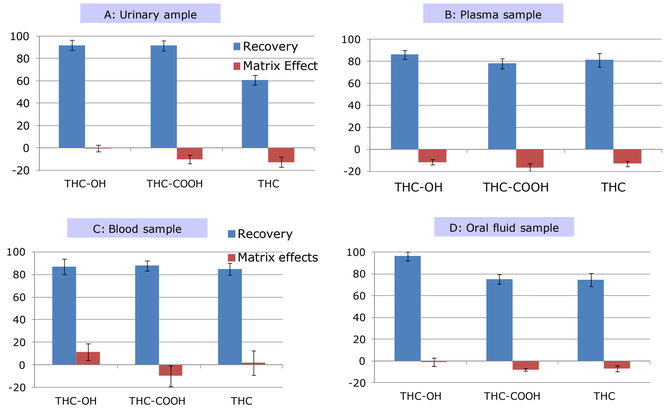
Urinary sample: Glucuronide hydrolysis: 40 µL of internal standard was added to 2 mL urine in a glass vial, then 2.4 mL 0.1M potassium phosphate buffer (pH 6.8) containing 10 µL β-Glucoronidase was added. Vials were capped, vortex mixed, and incubated at 37°C water bath for 16 hours. Following enzymatic hydrolysis, 150 µL of 10M NaOH was added, vortex mixed and samples heated for 30 min at 70 °C. Once the samples had cooled, samples were neutralized with 850 µL glacial acetic acid. 500 µL pretreated sample was directly applied to the Oasis PRiME HLB µElution plate. All wells of the SPE plate were then washed with 2 x 300 µL aliquots of 25% methanol. The samples were then eluted with 2 x 25 µL aliquots of 60:40 ACN:IPA and diluted with 50 µL of water. **The hydrolysis ensures the target drugs are fully deconjugated.**

Plasma sample: 200 µL 0.1% FA in ACN was added to 100 µL spiked plasma to precipitate out the protein in a micro centrifuge tube. Then the mixture was vortexed for 5 seconds and centrifuged for 5 min at 7000 rcf. The supernatant was then diluted with 400 µL water prior to loading. The entire pre-treated sample was directly loaded on to the Oasis PRiME HLB µElution plate. All wells were then washed with 2 x 250 µL aliquots of 25:75 methanol:water. All the wells were then eluted with 2 x 25 µL aliquots of **90:10 ACN:MeOH** and diluted with 50 µL of water prior to analysis. **The use of 90:10 ACN:MeOH ensures a maximum removal of phospholipids in the elution thus resulting in a cleaner extract.**

Blood sample:100 µL spiked whole blood was added to 25 µL of a solution of 0.1 M zinc sulfate/ammonium acetate, and the mixture was vortexed for 5 seconds to lyse the cells. All samples were then precipitated by adding 375 µL 0.1% formic acid in ACN. The entire sample was vortexed for 10 seconds and centrifuged for 5 min at 7000 rcf. The supernatant was diluted with 800 µL water prior to loading. The entire pretreated sample was directly loaded on to the Oasis PRiME HLB µElution plate in 2 aliquots. All wells were then washed with 2 x 250 µL aliquots of 25:75 methanol:water. All the wells were then eluted with 2 x 25 µL aliquots of **90:10 ACN:IPA** and diluted with 50 µL of water. **The use of 90:10 ACN:IPA ensures a consistent recovery and a maximum removal of phospholipids in the elution**

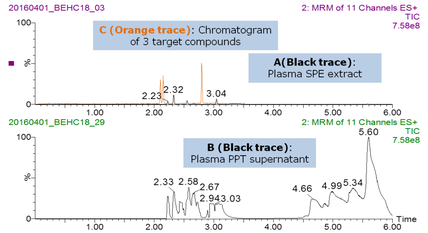
Oral fluid sample: Oral fluid samples were collected with Quantisal collection device from Immunalysis. The collection applicator was saturated with oral fluid (spiked), and then placed in a collection vial, which contained 3.0 mL of sample stabilization buffer. Per Quantisal instruction, this was claimed to be the equivalent of collecting 1.0±0.1 mL of sample. **1 mL acetonitrile was then added to the collection vial to help improve extraction.** The kit was stored in a refrigerator overnight. 500 µL aliquots of buffer stabilized oral fluid samples were pre-treated by adding 200 µL 4% H_3PO_4 and 10 µL of working IS mixture. The entire pre-treated sample was directly loaded on to the Oasis PRiME HLB µElution plate, followed by washing with **2 x 250 µL 5% NH4OH in 25:75 methanol:water.** All the wells were then eluted with 2 x 25 µL 90:10 ACN:MeOH and diluted with 50 µL of water. **While other matrices used the BEH C18 column, for oral fluid, the CORTECS column helped minimize ion suppression that was not seen in other matrices. In addition, the addition of 5% strong ammonia to the wash solution helped to minimize this suppression, resulting in the near complete elimination of matrix effects.**

RESULTS—ANALYTE RECOVERY AND MATRIX EFFECTS



PHOSPHOLIPIDS REMOVAL

As the main constituent of cell membranes, phospholipids are the main source of matrix effects in LC–MS bioanalysis. [2] The figure below shows chromatograms of combined phospholipid traces from the novel SPE sorbent extract (A) and an identical sample subject to protein precipitation (B). Compared with protein precipitation (PPT) sample, This novel sorbent removes over 99% of phospholipids, resulting in a much cleaner extraction. The chromatography of the three target compounds is also shown (C), demonstrating the potential interference of phospholipids if they were not removed during the extraction.



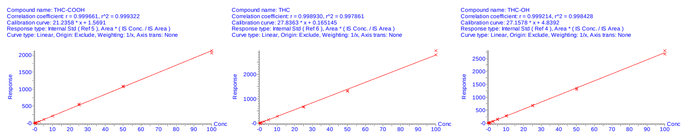
CASE SAMPLES

25 authentic urine samples were analyzed and compared to the validated method at Dominion Diagnostics. Table below details the results obtained by the two methods. 78% of the sample results are within 20% of each other, exceeding the FDA-GLP specification of 67% for incurred sample reanalysis.[1]

Sample Number	Urine THC Concentration (ng/mL) Reference Method	Urine THC Concentration (ng/mL) Novel RP sorbent Method	Mean Concentration (ng/mL)	% Bias
1	BQL*	BQL	N/A	N/A
2	BQL	BQL	N/A	N/A
3	7.90	6.70	7.30	-16%
4	14.3	12.0	13.2	-17%
5	14.6	13.4	14.0	-9%
6	15.5	15.0	15.3	-3%
7	21.9	16.4	19.2	-29%
8	22.8	19.8	21.3	-14%
9	23.1	21.4	22.3	-8%
10	26.5	25.1	25.8	-5%
11	35.2	31.5	33.4	-11%
12	37.6	31.4	34.5	-18%
13	42.2	31.4	36.8	-29%
14	101	92.4	96.6	-9%
15	101	94.4	97.8	-7%
16	104	84.5	94.3	-21%
17	105	82.1	93.7	-25%
18	134	112	123	-18%
19	199	154	176	-26%
20	264	239	251	-10%
21	312	297	304	-5%
22	328	297	312	-10%
23	384	409	396	6%
24	398	423	410	6%
25	458	445	451	-3%
			Agreement	78%

RESULTS - METHOD VALIDATION IN URINE

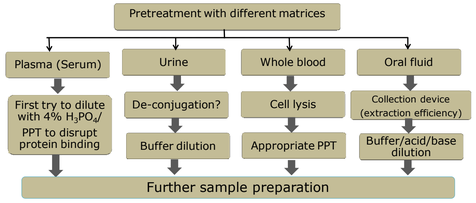
All compounds had linear response over the entire calibration range with R^2 values of 0.99 or more for all four matrices. Calibration and quality control (QC) results indicate that the method is linear, accurate and precise within 4 orders of magnitude. Figure below is the validation data for whole blood sample with excellent linearity range, correlation and accurate and precise QC data. Similar accurate validation results for other matrices can be found in Waters application notes [2, 3, 4]



	Accuracy and Precision								
N=6	THC-OH (0.1-100ng/mL)			THC-COOH (0.1-100ng/mL)			THC (0.05-100ng/mL)		
QC Level (ng/mL)	Mean (ng/mL)	%Acc.	%RSD	Mean (ng/mL)	% Acc.	%RSD	Mean (ng/mL)	% Acc.	%RSD
0.375	0.33	97.9	0.6%	0.40	105.8	8.1%	0.41	108.2	3.0%
2	1.92	96.0	3.7%	1.89	94.7	2.3%	2.01	100.5	3.7%
7.5	7.50	100.0	2.7%	7.34	98.9	2.8%	7.42	98.9	1.4%
20	19.87	99.3	3.2%	20.04	100.2	2.1%	19.56	97.8	1.2%
37.5	36.19	96.5	2.2%	37.95	101.2	3.0%	35.32	94.2	0.7%
Mean		**98**	**2%**		**100**	**4%**		**99.9**	**2%**

CONCLUSION

- **Optimized both sample pretreatment, SPE procedures, and chromatographic column choices for multiple matrices to ensure consistent analyte recoveries and minimum matrix effects.**
- **Introduced a novel SPE sorbent extraction with simpler procedures (eliminate condition and equilibration steps) and cleaner extracts (removes >99% phospholipids).**
- **Documented accurate analysis over a wide of calibration range compared to a fully validated reference method from an external laboratory.**



Sample pretreatment suggestions for different matrices

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