Matrix Effects and Matrix Affects: The Impact of Different Sample Matrices on Sample Preparation and Chromatographic Analysis

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Biological matrices differ significantly in their composition and in the nature and relative abundance of common components such as phospholipids. These differences can have substantial effects on LC-MS/MS methods. In addition to typical considerations such as removal of chromatographic interferences, minimising ion suppression or disrupting protein binding, more subtle differences between matrices often require matrix-specific modifications that are not always obvious. This manuscript details the extraction and analysis of THC, THC-OH and THC-COOH from plasma, oral fluid (OF), whole blood and urine using a novel solid-phase extraction sorbent in a µElution format, followed by direct analysis by UHPLC-MS/MS. Matrix-appropriate sample pretreatment, SPE protocol refinement and chromatographic column choice were all investigated and optimised to ensure consistent analyte recoveries and to minimise the specific matrix effects arising from the diverse matrices analysed. In addition to the discussion of matrix specific modifications, the final method for urinary cannabinoids is compared to a pre-existing validated forensic toxicology method in the analysis of a set of authentic urine samples.

Introduction

Scientists must consider many different variables when developing UHPLC-MS/MS methods. The analytes of interest represent only one of these variables. The sample matrix that contains these analytes presents its own set of unique challenges and these challenges can vary as the matrix varies. Further complicating the method development task is that failure to understand and remove the interferences present in different sample matrices can result in unpredictable matrix effects and chromatographic complications. For example, isobaric -compounds must be removed or separated chromatographically. Moreover, the method needs to be robust (with minimal matrix interferences) to ensure accurate and reproducible quantification. In biological matrices, phospholipids are one of the most common causes of matrix effects such as ion suppression, which can lead to poor reproducibility and compromised sensitivity [1]. If analysts incorporate techniques to remove phospholipids along with other interfering components such as salts and proteins from the sample, the impact of matrix differences can be minimised. Ideally this would result in a single sorbent and similar protocols being used for the analysis of a given set of compounds present in disparate matrices. To evaluate this possibility, the analysis of THC and its metabolites in urine, plasma, whole blood and oral fluid, was studied. Each sample matrix was pre-treated to achieve optimal release and recovery of these cannabinoids. A newly developed SPE sorbent, specifically designed to remove phospholipids while maintaining the ability to selectively purify analytes of interest, was used to remove matrix interferences . The sorbent relies on a hybrid (having both hydrophilic and lipophilic retention characteristics) reversed-phase mechanism to retain and isolate both polar and non-polar analytes. The water wettable nature of this sorbent enables the elimination of conditioning and equilibration steps, resulting in simplified SPE protocols. In addition, a novel functionality has been introduced which also enables the removal of phospholipids.

This manuscript details optimisations in sample pretreatment and sample preparation procedures for tetrahydrocannabinol (THC) and two of its main metabolites in urine, whole blood, plasma, and oral fluid. In addition to more obvious, matrix specific strategies, the use of chromatographic column screening as tool to minimise matrix effects is also investigated. Finally, authentic urine samples are used to compare the presented urine method to a previously validated method from a forensic toxicology laboratory.

Experimental

Reagents and material

All standards and stable isotope labeled internal standards including Tetrahydrocannabinol (Δ9-THC, THC), 11-nor-9-Carboxy-Δ9-THC (THC-COOH), 11-nor-9-Hydroxy-Δ9-THC (THC-OH), Δ9-THC -D3(THC-D3), 11-Carboxy-Δ^9-THC-D_3 (THC-COOH-D3), 11-Hydroxy-Δ^9-THC-D_3 (THC-OH-D3) were purchased from Cerilliant (Round Rock, TX, USA). β-Glucuronidase from E. Coli K 12 was purchased from Roche Life Science (Indianapolis, IN). Stock standards of THC, THC-OH and THC-COOH (100 µg/mL) were prepared in 40% methanol. A working internal standard (IS) solution of 1 µg/mL (for urine samples) or 100 ng/mL (for plasma, whole blood and oral fluid samples) THC-D3, THC-OH-D3 and THC-COOH-D3 was also prepared in 40% methanol. Working solutions to prepare

Table 1. Quality control results from extracted matrix samples. (N=6 for each compound at all three levels, A: Urine sample extraction; B: Plasma sample extraction; C: Whole blood sample extraction and D: Oral fluid sample extraction)

	Accuracy and Precision								
N=6	THC-OH			THC-COOH			THC		
QC Level (ng/mL)	Mean (ng/mL)	%Acc.	%RSD	Mean (ng/mL)	%Acc.	%RSD	Mean (ng/mL)	%Acc.	%RSD
A: Urine sample extraction									
0.750	0.661	88.6	1.7%	0.763	101	1.4%	0.722	96.3	0.4%
7.50	6.73	89.3	1.3%	7.37	98.3	1.3%	7.15	95.3	1.2%
75.0	73.1	97.9	1.8%	73.6	98.2	0.7%	75.5	101	0.8%
Mean		91.9	1.6%		99.1	1.1%		97.4	0.8%
B: Plasma sample extraction									
0.375	0.336	89.6	9.4%	0.365	97.3	6.2%	0.403	107	3.5%
1.75	1.62	92.8	5.0%	1.77	101	4.8%	1.82	104	1.9%
7.50	7.35	98.0	2.7%	7.65	102	1.9%	7.62	102	1.9%
37.5	37.3	99.5	4.1%	39.6	106	1.7%	38.1	102	2.4%
Mean		95.0	5.3%		101	3.7%		104	2.4%
C: Whole blood sample extraction									
0.375	0.330	97.9	0.6%	0.397	105	8.1%	0.408	108	3.0%
2.00	1.92	96.0	3.7%	1.89	94.7	2.3%	2.01	100	3.7%
7.50	7.50	100	2.7%	7.34	98.9	2.8%	7.42	98.9	1.4%
20.0	19.9	99.3	3.2%	20.0	100	2.1%	19.6	97.8	1.2%
37.5	36.2	96.5	2.2%	38.0	101	3.0%	35.3	94.2	0.7%
Mean		98.0	2.5%		100	3.7%		99.9	2.0%
D: Oral fluid sample extraction									
0.375	0.362	96.6	8.3%	0.352	93.8	7.1%	0.394	105	5.7%
1.75	1.77	101	3.4%	1.65	94.3	2.7%	1.69	96.6	3.2%
7.50	7.57	101	2.7%	6.94	92.5	3.9%	7.12	94.9	2.4%
37.5	36.9	98.3	1.9%	37.8	101	1.4%	36.34	96.9	0.8%
Mean		100	2.7%		95.9	2.7%		96.1	2.1%

individual calibrators and quality control standards were prepared daily in 40% methanol. Optima grade acetonitrile (ACN), methanol (MeOH), 2-isopropanol (IPA) and acetic acid (glacial) were obtained from Fisher Chemical (Fair Lawn, NJ). Formic acid (FA - 88% A.C.S. grade), and potassium phosphate monobasic, monohydrate and potassium phosphate diabasic were purchased from J.T. Baker (Phillipsburg, NJ). Sodium hydroxide (NaOH - anhydrous pellets, minimum 98%) and phosphoric acid (85% wt. H_2O) were purchased from Sigma-Aldrich (St. Louis, MO). All chemicals were of HPLC-grade unless stated otherwise.

Oasis PRiME HLB µElution Plates and vacuum manifold were from Waters (Milford, MA). Rat plasma and rat whole blood were purchased from Bioreclamation IVT (Westbury, NY). Urine and oral fluid were collected from healthy female adult volunteers. Quanitisal saliva collection devices were kindly donated by Immunalysis Corporation (Pomona, CA).

Sample pretreatment

Urine samples: 80 µL of each working calibrator or QC standard solution (prepared daily) was added to 1920 µL of human urine to make calibration curves and QC samples. Calibration ranges were from 0.1-100 ng/mL for THC-COOH and THC-OH and 0.2-100 ng/mL for THC. Quality control samples were prepared at 0.75, 7.5 and 75 ng/mL. Glucuronide hydrolysis: 40 µL of working IS solution was added to 2 mL of human urine sample in a glass vial, followed by 2.4 mL 0.1 M potassium phosphate buffer (pH 6.8) containing 10 µL β-Glucuronidase . Vials were capped, vortex mixed, and incubated at 37°C in a water bath for 16 hours. After allowing samples to cool down to room temperature, 150 µL of 10 M NaOH was added, vortex mixed and hydrolysed in a dry heating block for 30 min at 70°C. Once the samples had cooled, 850 µL of glacial acetic acid was added and the samples were vortex mixed. Solid-phase extraction was performed using Waters' Oasis PRiME HLB µElution Plates: 500 µL pretreated sample (equivalent to 180 µL urine) was directly applied to the plate without conditioning or equilibration. All wells of the SPE plate were then washed with 2 x 300 µL aliquots of 25:75 methanol/water. The samples were then eluted with 2 x 25 µL aliquots of 60:40 ACN/IPA and diluted with 50 µL of water. 5 µL was injected onto the UHPLC/MS/MS system.

Plasma samples: 100 µL of each working calibrator or QC standard solution and 100 µL IS solution were added to 1800 µL of plasma to make calibration curves and QC samples. Calibrator concentrations ranged from 0.1-100 ng/mL for all analytes. Quality control samples were prepared at 0.375, 1.75, 7.5 and 37.5 ng/mL, in plasma. 200 µL of 0.1% FA in ACN was added to 100 µL plasma in a micro centrifuge tube. Then the mixture was vortexed for 5 seconds and centrifuged for 5 min at 7000 relative centrifugal force (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 without conditioning or equilibration. All wells were then washed with 2 x 250 µL aliquots of 25:75 MeOH/H_2O. 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. 5 µL was injected onto the UPLC-MS/MS system.

Blood samples: 100 µL of each working calibrator or QC standard solution and 100 µL IS solution were added to 1800 µL of rat whole blood to prepare calibration curves and QC samples. Calibrator concentrations ranged from 0.05-100 ng/mL for all analytes. Quality control samples were prepared at 0.375, 2, 7.5, 20 and 37.5 ng/mL. 100 µL of spiked whole blood was added to 25 µL of a solution of 0.1 M zinc sulphfate/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% FA in ACN. The entire sample was vortexed for 10 seconds and centrifuged for 5 min at 7000 rcf. The supernatant was then diluted with 800 µL water. The entire pretreated sample was directly loaded on to the Oasis PRiME HLB µElution Plate in 2 aliquots without conditioning or equilibration. All wells were then washed with 2 x 250 µL aliquots of 25:75 MeOH/H_2O. All the wells were then eluted with 2 x 25 µL aliquots of 90:10 ACN/IPA and diluted with 50 µL of water. 5 µL was injected onto the UPLC-MS/MS system.

Oral fluid samples: 200 µL of each working

Table 2. THC-COOH concentrations from the forensic toxicology validated method and the Oasis PRiME HLB method

Sample Number	Urine THC Concentration (ng/mL) Original Oasis MAX Method	Urine THC Concentration (ng/mL) PRiME HLB Method	Mean Concentration (ng/mL)	ABS %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%

BQL: Below Quantifiable Limit

calibrator or QC standard solution was added to 1800 µL of oral fluid to prepare calibration curves and QC samples. Calibrator concentrations ranged from 0.05-100 ng/mL for all analytes. Quality control samples were prepared at 0.375, 1.75, 7.5 and 37.5 ng/mL in oral fluid. Oral fluid samples were collected with Quantisal collection device from Immunalysis Corporation according to the manufacturer's directions. The collection applicator was saturated with oral fluid, and then placed in a collection vial, which contained 3.0 mL of sample stabilisation buffer. Per manufacturers 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 collection kit was stored in a refrigerator overnight to simulate the transit time of the sample and to allow for complete equilibration between the sample in the pad and the stabilisation buffer mix in the collection vial. 500 µL aliquots of buffer stabilised oral fluid samples (equivalent to 100 µL oral fluid) were pre-treated by adding 200 µL 4% H_3PO_4 and 10 µL of working IS mixture. The entire pre-treated sample (total of 710 µL) was directly loaded on to the Oasis PRiME HLB µElution Plate without conditioning or equilibration, followed by washing with 2 x 250 µL 5% NH_4OH in 25:75 MeOH/H_2O. All the wells were then eluted with 2 x 25 µL 90:10 ACN/MeOH and diluted with 50 µL of water. 5 µL was injected onto the UPLC-MS/MS system.

Calculation for analyte recovery and matrix effect

Analyte recovery was calculated according to the method of Matuszewski [15] using the following equation:

$$\%Recovery = \frac{(Area\ A)}{(Area\ B)} \times 100\%$$

Where A equals the peak area of a specific analyte (which has been pre-spiked into the matrix) in an extracted sample and B equals the peak area of the same analyte in an extracted blank matrix sample in which the compounds were added post-extraction.

Matrix effects were calculated according to the following equation:

$$\text{Matrix Effects} = \frac{\text{Peak area in the presence of matrix}}{\text{Peak area in the absence of matrix}} - 1 \times 100\%$$

The peak area in the presence of matrix refers to the peak area of a specific analyte in an extracted matrix sample in which the compounds were added post-extraction. The peak area in the absence of matrix refers to peak area of an analyte in a neat solvent solution.

Chromatographic Analysis

Analysis was performed using an ACQUITY I Class UPLC combined with Xevo TQ-S triple quadrupole MS instrument supplied by Waters Corporation (Milford, MA). The autosampler and column compartment temperatures were set at 10°C and 40°C, respectively. Depending upon the matrix, Waters BEH C_{18}, 1.7 µm, 2.1*100 mm or CORTECS C_{18}, 1.6 µm, 2.1*100 mm Columns were used for separation. An HSS (high strength silica) C_{18} UPLC® 1.8 µm, 2.1*100 mm column was tested during the oral fluid extraction to optimise matrix effects. The injection volume was 5 µL. The mobile phases consisted of solvent A: Milli-Q water containing 0.1% formic acid and solvent B: acetonitrile containing 0.1% formic acid. The flow rate was 0.6 mL/min. THC and metabolites were chromatographically separated using the following gradient: initial hold at 50% solvent B followed by a linear ramp to 95% solvent B in 3 min. The proportion of solvent B was held at 95% for 30 seconds after which it was lowered to 50% in 0.1 min and held at this level for 0.4 min. The entire cycle time was 5 min. The MS was operated in positive ESI mode with the following conditions: Capillary Voltage at 2.0 kV; Desolvation Gas at 1000 L/hr; Cone Gas at 150 L/hr; Desolvation Temperature at 500°C, and Source Temperature at 150°C. The Cone voltage was optimised for each analyte. The MRMs for THC-OH were 331.3>313.1 (primary MRM with cone voltage at 40V and collision energy at 18eV) and 331.3>193.1 (confirmatory MRM with cone voltage (CV) at 40V and collision energy at 30eV) with its IS at 334.3>316.1. The MRMs for THC-COOH were 345.3>327.3 (primary with 50V and 20eV) and 345.3>299.3 (confirmatory with

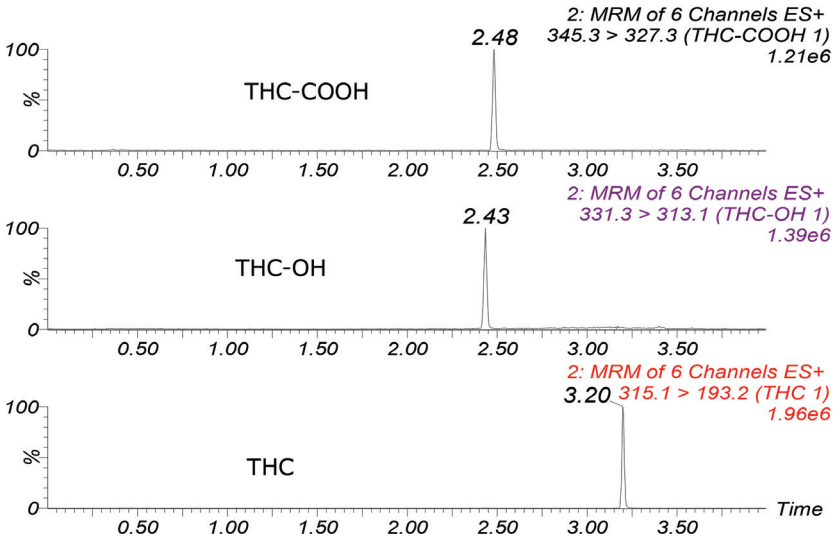

Figure 1. Chromatography of THC-OH, THC-COOH and THC from an extracted urine sample on the ACQUITY UPLC BEH C_{18} Column, 1.8 µm; 2.1 x 50 mm. The concentrations are 2 ng/mL for all compounds

50 V and 25 eV) with its IS at 348.3>330.3. The MRMs for THC were 315.1>193.2 (primary, 40V and 25eV) and 315.1>135.1 (confirmatory, 40V and 25 eV) with its IS at 318.1>196.2. Data were acquired and analysed using MassLynx Software (V4.1). Quantification was performed using TargetLynx.

Results and discussion:

Chromatography

Figure 1 shows the chromatography of the three cannabinoids from an extracted urine sample at 2 ng/mL, using a BEH C_{18} column. All compounds eluted within 3 minutes and were characterised by peak widths under 3 seconds at 5% of baseline. All peaks were symmetrical with symmetries between 0.95-1.15. For oral fluid samples, a CORTECS C_{18} column was used to minimise matrix effects as discussed in the matrix effect study section. With the same separation conditions, all compounds eluted in 3 minutes. Peak shape was excellent with peak widths under 1.8 seconds at 5% of baseline.

Recovery and Matrix Effects

In these studies, absolute % matrix effects were used rather than matrix factor or IS normalised calculations. While IS normalised matrix factors could be used, for many methods there isn't an IS for all compounds. Measurement of % matrix effects gives one an overall, and 'representative' sense of the cleanliness and specificity of the clean-up. In addition, lower % matrix effects also help one feel more confident in the robustness of the method, in that minimising matrix effects minimises variability between samples and reduces the risk of unexpected, sample specific suppression/enhancement.

Urine samples: Figure 2A demonstrates that THC-OH and THC-COOH were reproducibly recovered at 90%. While THC recovery was somewhat lower (60%), it too was consistent. The RSDs for recovery of all analytes were under 8%. Matrix effects were minimal, at less than 15% for all compounds. Both hydrolysis steps were necessary to maximise recovery and to ensure that the target drugs were fully deconjugated. Abraham et al [14] found that both an enzymatic and alkaline hydrolysis were necessary for THC and related compounds. Excreted cannabinoids in urine tend to be highly conjugated, requiring glucuronide hydrolysis in order to analyse the free metabolites. While THC-COOH can be effectively hydrolysed using alkaline hydrolysis procedures, THC and THC-OH glucuronide conjugates require enzymatic hydrolysis procedures to fully deconjugate these molecules [14]. To that end, a hybrid hydrolysis procedure, employing enzymatic hydrolysis followed by alkaline hydrolysis was employed. 25% MeOH/H2O was used in the wash step to ensure target analytes were retained on the sorbent while the less hydrophobic, high concentration interferences were washed away. Oasis PRiME HLB also provided better recovery, variability and matrix effects than LLE, with a more simplified procedure .[2].

Plasma samples: Extraction recoveries in plasma (Figure 2B) were ~80%, and were consistent with all RSDs under 6%. The simple load, wash, elute SPE method resulted in absolute matrix effects that were less than 20% for all compounds with standard deviations <3%. Protein precipitation pre-treatment was necessary to disrupt binding between THCs and endogenous plasma proteins, enabling high analyte recovery. The use of 90/10 ACN/MeOH as the elution solution provides optimal solubility and elution of THC compounds while leaving the majority of endogenous phospholipids on the sorbent, thus decreasing matrix effects. The differences in recoveries between plasma and urine samples may be due to a number of factors. First, the plasma sample preparation procedure requires a protein precipitation step to disrupt protein binding of the cannabinoids to plasma proteins. If this step is less than 100% efficient in disrupting protein binding, extraction efficiencies may be compromised. Secondly, a different elution solvent was used with the plasma samples (90:10 ACN:MeOH) vs. the urine samples (60:40 ACN:IPA) in order to minimise the amount of phospholipids in the final eluate. The different composition of the elution solvent could easily result in the different extraction efficiencies seen between the two matrices.

Blood samples: The average THC extraction recovery in blood (Figure 2C) was greater than 85% with average RSDs within 5-7%, demonstrating the high reproducibility of Oasis PRiME HLB. The slightly higher recovery relative to plasma samples may be a result of the substitution of IPA for MeOH in the elution solvent. Matrix effects were again minimal, at less than 15% for all compounds. In this experiment, the 90/10 ACN/IPA elution solution removes a similar % of phospholipids as 90/10 ACN/MeOH, but results in more consistent recoveries. Average %RSD for recovery is 6% using 90/10 ACN/IPA in blood samples. Oasis PRiME HLB provided comparable recovery, variability and matrix effects to mixed-mode SPE, with a more simplified procedure than previously published [3].

Oral fluid samples: Extraction recoveries in oral fluid (Figure 2D) were > 75% with all % RSDs within 6%. Matrix effects were negligible, at less than 10% for all compounds. During sample pretreatment, 1mL ACN was added to the collection device to improve extraction efficiency. This was particularly important for THC (the most hydrophobic of the panel) whose recovery increased from 65% to 100%. The SPE wash step required optimisation to eliminate suppression from the oral fluid matrix. The addition of 5% strong ammonia to the wash solution minimised suppression, resulting in the near complete elimination of matrix effects. This modification may help remove

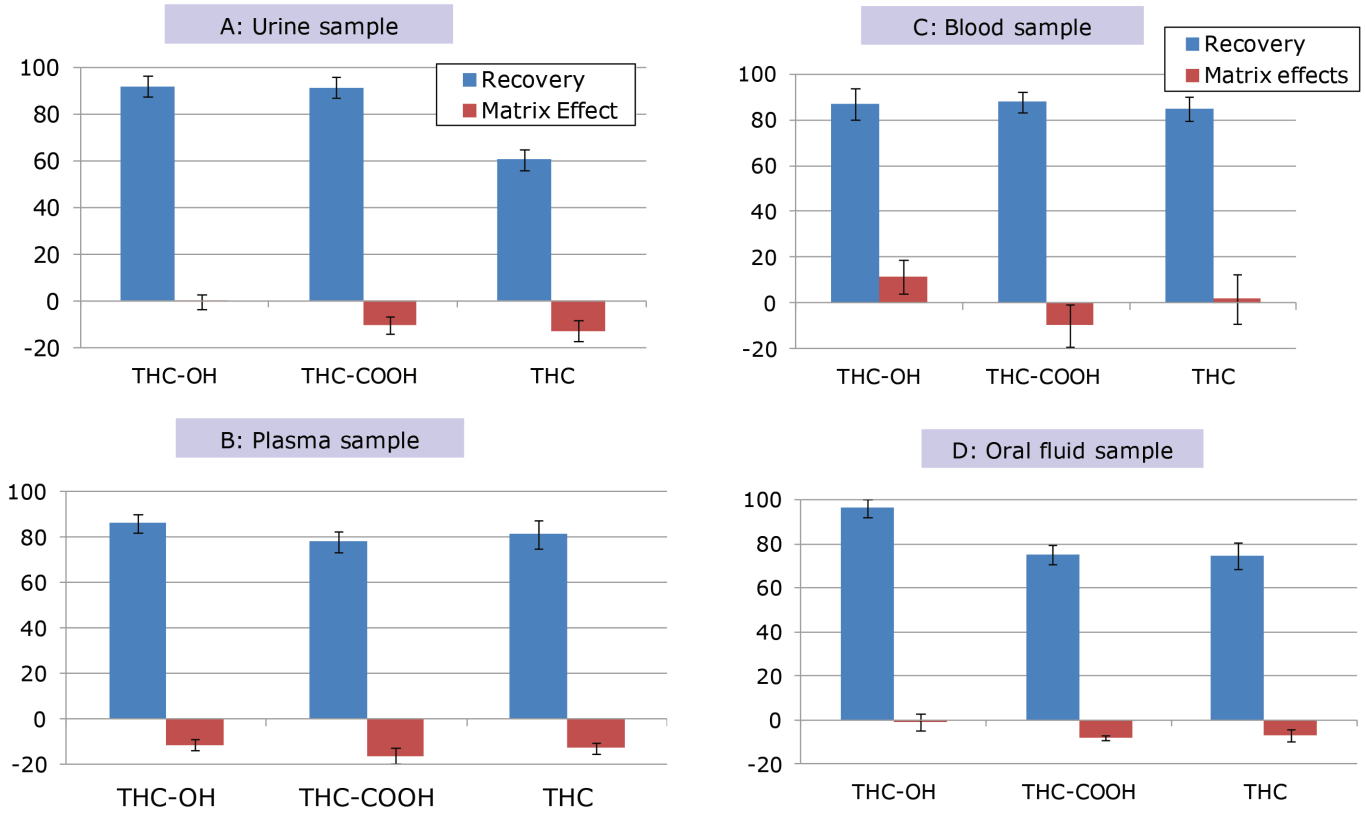

Figure 2. Recovery and matrix effects of THC-OH, THC-COOH, and THC after extraction using the Oasis PRiME µElution Plate. A: Urine sample extraction; B: Plasma sample extraction; C: Whole blood sample extraction and D: Oral fluid sample extraction

either a natural component of oral fluid or a component of the stabilisation buffer not present in the other matrices. While other matrices used the BEH C_{18} column, the CORTECS Column helped minimise ion suppression from oral fluid that was not seen in other matrices. Once again, either the oral fluid or stabilisation buffer may contain constituents that interfere with the anayltes of interest. Using a column with a different selectivity may chromatographically separate these components resulting in the observed elimination of ion suppression.

Phospholipid Removal for blood and plasma samples

As the main constituent of cell membranes, phospholipids are a primary source of matrix effects in LC–MS bioanalysis. They can be found in all biological matrices in significant concentrations especially in whole blood and plasma [4]. The matrix effects caused by phospholipids are one of the greatest challenges in bioanalytical method development and validation. One of the unique attributes of the Oasis PRiME HLB sorbent is its ability to remove these endogenous phospholipids. Figure 3 shows chromatograms of combined phospholipid traces (in black colour) from an Oasis PRiME HLB extract (A) and an identical sample prepared by protein precipitation (B). Compared with the protein precipitation (PPT) sample preparation, Oasis PRiME HLB extraction removes over 99% of phospholipids, resulting in a much cleaner eluate. This can translate to reduced matrix effects, longer column lifetimes, and less mass spectrometer source maintenance. The chromatography of the three target compounds is also shown in orange (C), demonstrating the potential interference (co-elution) of phospholipids if they were not removed during the extraction.

Figure 4 shows the profile of phospholipids remaining in various eluates from plasma extracted with this novel sorbent. A total of 18 different elution solvents were evaluated to compare the amount of phospholipids remaining, including different percentages of MeOH in water, ACN in water, and ACN/MeOH combinations. The figure shows that ACN does not elute as many phospholipids as MeOH. Overall, >80% ACN in water or >75/25 ACN/MeOH are optimal elution solvents for eliminating phospholipids using this novel sorbent. In the plasma and whole blood sample extractions, elution solutions were 90/10 ACN/MeOH or ACN/IPA to remove the maximum amount of phospholipids. IPA yielded more consistent recovery and similar phospholipid removal when used for whole blood extraction. However, it was not evaluated along with ACN and MeOH during the initial assessment of phospholipid removal.

Quantitative Results

Calibration and quality control samples were prepared as previously described in the materials and method section for all matrix samples. Calibration ranges were from 0.05/0.2-100 ng/mL for THC and its metabolites. Quality control samples were prepared at low, medium, and high concentrations as appropriate for the calibration ranges.

Urine samples: All compounds had linear responses over the full calibration range with R^2 values of ≥0.99 with 1/x weighting. Lower limits of quantification (LLOQ) were 0.1 ng/mL for THC-COOH and THC-OH and 0.2 ng/mL for THC. Quality control samples were accurate and precise. All results were within 15% of expected values and %RSDs were under 2% (N=6). This data can be seen in Table 1A. The excellent accuracy and precision demonstrate the consistency and robustness of the method.

Plasma samples: All compounds had linear responses over the entire calibration range with R^2 values of ≥0.99 with 1/x weighting

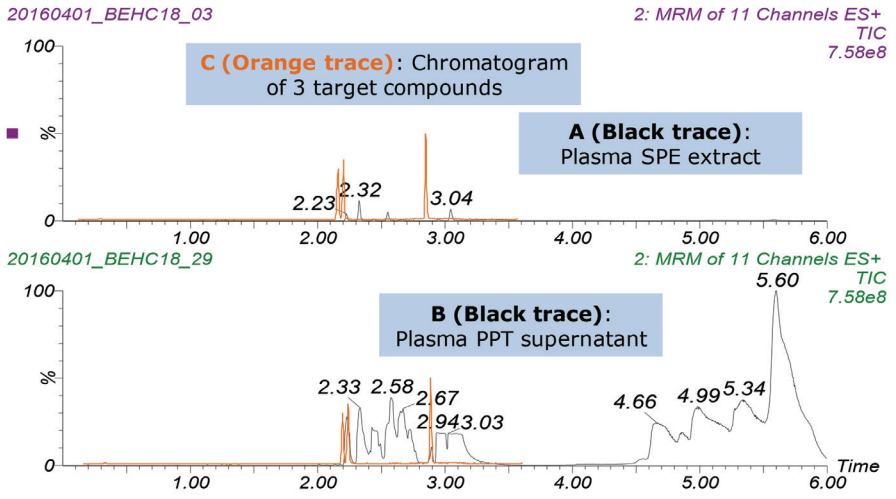

Figure 3. Chromatography of phospholipids remaining in the Oasis PRiME HLB final eluate, after extraction, vs. plasma protein precipitation. Scales are linked. An overlaid chromatogram shows THC-OH, THC-COOH and THC (orange trace) in relation to the phospholipid traces (black). A: Phospholipids remaining in a plasma sample after SPE extraction; B: Phospholipids remaining in a plasma sample after PPT; C: Chromatogram of 3 target compounds

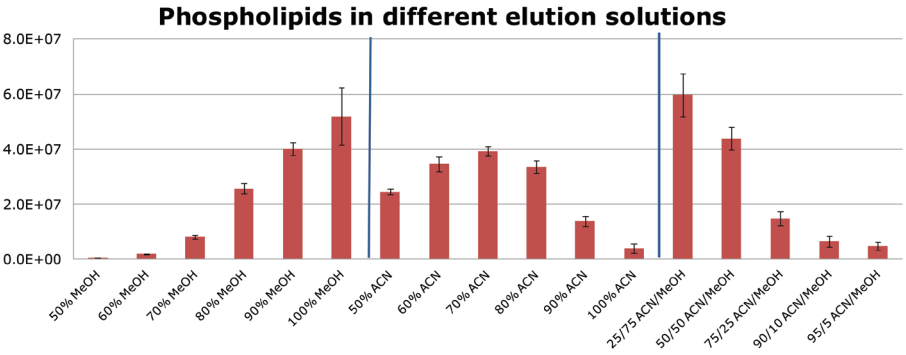

Figure 4. Phospholipid profile: Sum of 11 phospholipids in final eluate using different elution solution compositions. (The 11 phospholipids MRMs are: 496.4>184.4, 520.4>184.4, 522.4>184.4, 524.4>184.4, 704.4>184.4, 758.4>184.4, 760.4>184.4, 784.4>184.4, 786.4>184.4, 806.4>184.4, 808.4>184.4).

and average deviations <5%. Lower limits of quantification (LLOQ) were 0.1 ng/mL for all compounds. Quality control samples prepared at 0.375, 1.75, 7.5 and 37.5 ng/mL were accurate and precise. All results were within 10% of expected values with average RSDs between 2-5% (N=6). This data can be seen in Table 1B and it demonstrates that the method is linear, accurate and precise over a calibration range that includes the entire scope of expected values of samples. The method was also proved to be both selective and sensitive enough to routinely measure THC in plasma well below 2-3 ng/mL cut off level [6]. This was exemplified by the excellent accuracy and precision at the 0.375 ng/mL QC sample level, where calculated concentrations of all six replicates were within an average of 10% of expected.

Blood samples: Calibration ranges were from 0.1-100 ng/mL for THC-OH and 0.05-100 ng/mL for THC and THC-COOH. All compounds had linear responses over the full calibration range with R^2 values of ≥0.99 with 1/x weighting. Quality control samples at low, medium, and high concentrations, as appropriate for the calibration range, were accurate and precise. All results were within 10% of expected values with average RSDs between 2-4% (N=6). This data can be seen in Table 1C.

Oral fluid samples: All compounds had linear responses over the calibration range with R^2 values of ≥0.999 using 1/x weighting. Lower limits of quantification (LLOQ) were 0.1 ng/mL for THC-OH and THC-COOH and 0.05 ng/mL for THC. Quality control samples prepared at 0.375, 1.75, 7.5 and 37.5 ng/mL were accurate and precise. All QC values were within 10% of their target values, and most were within 5%. This data can be seen in Table 1D. This demonstrates that the method is linear, accurate and precise over a calibration range that includes the entire scope of expected values of samples. The method was also proved to be both selective and sensitive enough to routinely measure THC and its metabolites in oral fluid sample.

These extraction methods, with only minor protocol changes between them, have been shown to deliver consistently high extraction recoveries in all matrices tested. All compounds had linear responses over the entire calibration range with R^2 values of 0.99 or more for all four matrices. In each case, all FDA recommendations for accuracy, precision, linearity and analytical sensitivity were met for validated methods [7]. Calibration and quality control (QC) results indicate that the methods are linear, accurate and precise within 4 orders of magnitude. Research data shows that 2-5 ng/mL THCs (THC-COOH for urine) are an indicator of recent marijuana exposure (cut off concentration) [6, 8-9]. This method detects THC and its metabolites down to 0.05-0.2ng/mL in all four different matrices, well below the threshold value for recent marijuana exposure. This is also highlighted by the excellent accuracy and precision at the low QC (0.375-0.75 ng/mL see table 3) sample level, where calculated concentrations of all six replicates were within an average of 9% of expected.

The LLOQs for THC and its metabolites in different matrices varied from 0.05 to 0.2 ng/mL. Since LLOQs were defined as the lowest concentration at which accuracy was within 20% of the nominal value and %CVs were less than 20% these differing values were the concentrations at which these criteria were met in each matrix. Most likely, the differences were due to subtle differences in sample matrices or the slight differences in recoveries seen.

Matrix effect study

Matrix effects refer to the enhancement or suppression of the analyte response caused by coeluting endogenous matrix constituents. The impact is due to changes in ionisation or simply acting as isobaric interferences as initially recognised by Kebarle et al. in the early 1990s [10]. Even though there is currently no universal solution to solve the matrix effects problem [11], there are a couple of strategies analysts can apply to minimise it. In this study, we employed SPE clean-up to get rid of salts, proteins and even endogenous phospholipids in the analysis. LC column choice can also be a very effective way to eliminate matrix effects. Figures 5 show matrix effects from oral fluid samples for THC-COOH and THC using different columns. These include a high strength silica (HSS) C_{18} for maximum retention, a fully porous ethylene bridged hybrid BEH C_{18} and a solid core CORTECS C_{18} UPLC® Column

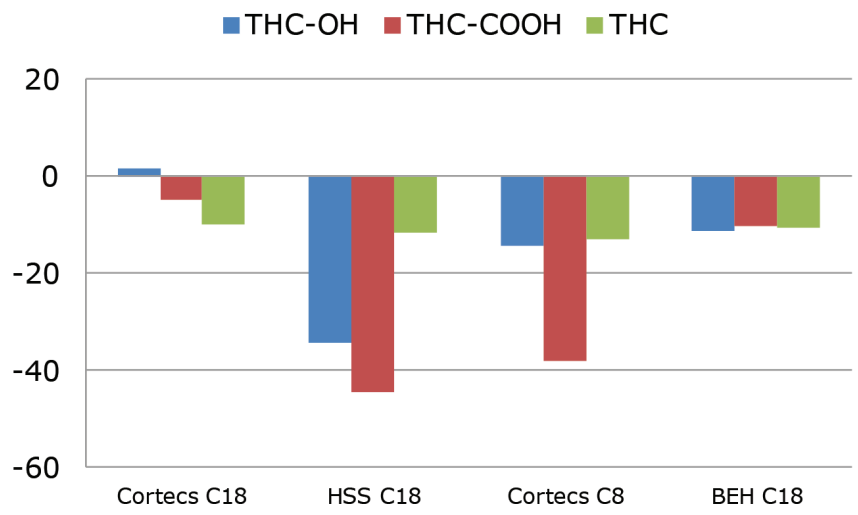

Figure 5. % Matrix effects observed in oral fluid extracts separated on different columns including a high strength silica HSS C_{18}, an ethylene bridged hybrid BEH C_{18} and a solid-core CORTECS C_{18} UPLC Column as well as a solid core CORTECS C_8 UPLC Column.

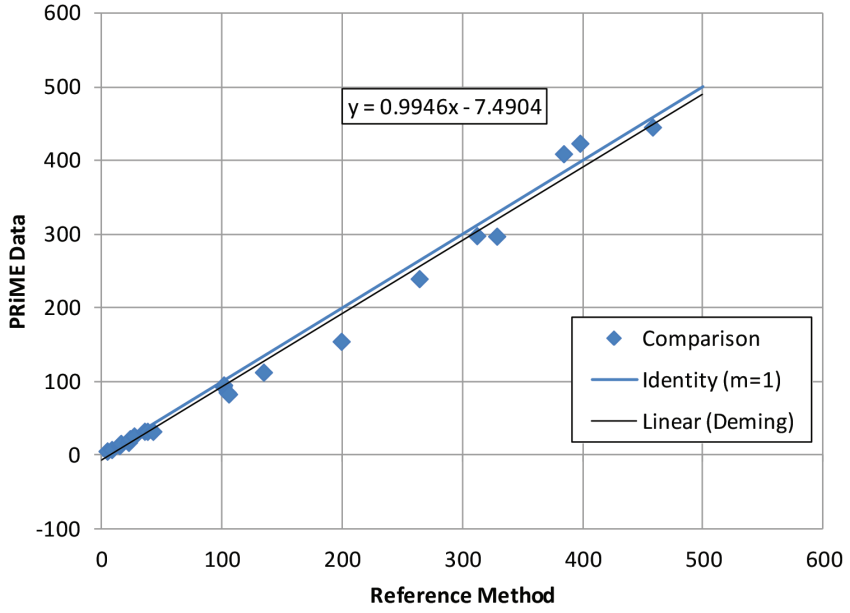

Figure 6. Correlation between the reference method and the Oasis PRiME HLB method for the analysis of THC-COOH in authentic urine samples. The blue line indicates the theoretical identity line of a perfect correlation. The black line is the plotted Deming regression with the equation listed on the chart.

as well as a superficially porous CORTECS C_8 (the least retentive) UPLC® Column. With the same base particle, CORTECS C_{18} showed reduced matrix effects and higher retention than CORTECS C_8. The use of the same ligand (C_{18}) with different base particles (CORTECS-solid-core, BEH-porous, and HSS-silica) resulted in different matrix effects and chromatography. CORTECS C_{18} was most effective in minimising matrix effects for all analytes while providing the best peak shape (efficiency) possibly due to the inherent advantages of superficially porous particles - chiefly reduced diffusion path and improved packing technology. [12]. This difference could also simply be a result of the differential selectivity seen with the Cortecs vs. BEH base particle. HSS C_{18} provided the highest reversed phase retention at similar C_{18} ligand density (all ~3 µmol/m^2). This indicates that not only different ligands, but different base particles have an effect not only on the separation as expected, but also on the observed matrix effects. Matrix interferences in the oral fluid sample were most effectively eliminated using the CORTECS C_{18} UPLC Column, in contrast to the BEH C_{18} column used for the three other matrices. The CORTECS column was not applied to the other three matrices as the matrix effects with BEH column were all within 20%, which is at acceptable level for a bioanalysis assay.

Method Comparison: Case Samples

In this research, 25 authentic urine samples were analysed by the in-house method and results were subsequently compared to the validated forensic toxicology method at Dominion Diagnostics Labs. (Authentic urine samples were obtained from Dominion Diagnostics, North Kingstown, RI). While the urine method previously described in this paper demonstrated excellent accuracy over a wide calibration range, a side by side comparison with a fully validated method from an external laboratory is a key component of method validation. The samples ranged in concentration from 6.70-458 ng/mL, covering nearly the entire linear range of the forensic toxicology method (5.00-500 ng/mL). A Deming regression (Figure 6) had a slope of 0.995 demonstrating parallelism between the two methods. The correlation (R) of 0.998 indicated an excellent correlation between the results obtained by the two laboratories. Table 2 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. [13]. Most results showed a slight negative bias not seen in the standards or QCs. Since the standards and QC samples were prepared in surrogate matrix (Surine), it is possible that the combination of different SPE methods and different chromatographic conditions differentially remove or chromatographically resolve an endogenous substance from the urine samples causing slight signal suppression during ionisation. Despite the fact that the samples were subject to different extraction procedures as well as different LC-MS/MS conditions, the results show excellent agreement and indicate that the simplified SPE methodology, which eliminates conditioning and equilibration, gives equivalent results for authentic urine samples.

Conclusions:

In this work, a novel SPE sorbent and a simple load, wash, elute protocol were applied to efficiently minimise matrix effects (from both phospholipids and other endogenous components) across four common, yet disparate, biological matrices. This research demonstrates the impact of sample pretreatment, SPE methodology, and chromatographic column choice on the robustness of an assay for THC and its metabolites in urine, whole blood, plasma

and oral fluid samples.

LLOQs of 0.1 ng/mL or 0.05 ng/mL were easily achieved for all analytes. Accuracy and precision of both standard curve and QC samples all fell well within the 15/20 guidelines recommended by the FDA. On average, accuracy of standard curve points was within 2-3% of expected. Accuracy of QC samples averaged 98%, while QC precision averaged 3%. Oasis PRiME HLB has been successfully used to achieve consistent recoveries with minimal matrix effects as well as accurate quantification over 4 orders of magnitude from wide variety of challenging matrix samples.

Finally, authentic incurred urine samples were analysed in separate laboratories using both the newly developed, simplified method and a fully validated forensic toxicology method. A strong correlation between the results demonstrated the consistency and validity of this new, improved methodology.

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