Tecan AC Extraction Plate™

Sample preparation with the AC Extraction Plate: extraction of urinary THC metabolites for LC-MSMS quantification

Introduction

The consumption of cannabis, or marijuana, is illegal in many countries and is also considered harmful to health. Δ9-tetrahydrocannabinol (THC), the main active compound in marijuana, is metabolized to Δ9-(11-OH)-tetrahydrocannabinol (11-OH-THC) and 11-nor-Δ9-carboxy-tetrahydrocannabinol (THC-COOH). In many countries, routine workplace drug testing – for example for airline pilots, government workers or medical staff – involves screening for the free THC-COOH metabolite obtained by hydrolyzing THC-COOH glucuronide. Similarly, former drug abusers are regularly tested for abstinence compliance. Liquid chromatography coupled with tandem mass spectrometry (LC-MSMS) provides an easy and cost-effective means for fast, sensitive and precise analysis of THC and its metabolites. Acidic compounds such as THC-COOH are often run in negative ion mode, but can also be analyzed using positive electrospray ionization.

Prior to analysis by LC-MSMS, a sample clean-up step is essential to remove matrix components such as proteins, lipids, carbohydrates and salts which may interfere with ionization and analyte detection. This application note describes a novel sample preparation method for the extraction of THC-COOH from urine for subsequent LC-MSMS analysis, based on Tecan Immobilized Coating Extraction (TICE™) technology. The main focus is the creation of a sample preparation workflow which is perfectly suited to automation. Tecan's AC Extraction Plate with TICE technology enables a straightforward workflow that is reproducible and cost efficient. Compared to most common sample preparation methods, extraction using the AC Extraction Plate involves fewer steps which are more easily implemented into automation. The final eluate can be directly injected into an LC-MSMS system for quantitative analysis.

The AC Extraction Plate

The centerpiece of the newly developed sample preparation method is the AC Extraction Plate, a 0.6 ml, 96-well, deep well plate. Each well contains the proprietary TICE coating, which acts as an extraction phase for relatively non-polar small molecules. In a highly aqueous environment, analytes such as THC and its metabolites are strongly absorbed by the TICE coating, while proteins, phospholipids, carbohydrates and salts remain in the solution. After rinsing the well with a wash solution to remove any residual matrix, the analyte is eluted from the stationary phase with an appropriate eluent containing a high percentage of organic solvent. The eluate can be directly injected into an LC-MSMS system for analysis. Procedural variations are mitigated by the addition of an internal standard at the beginning of the sample preparation process. For this application, stable isotope-labeled D_3-THC-COOH was used as the internal standard.

The workflow

The AC Extraction Plate well is filled with an extraction mix containing the internal standard, and an aliquot of the urine sample is added. After horizontal mixing on a shaker, the diluted urine is removed, leaving the analyte(s) of interest retained in the well's TICE coating. A wash solution is added, the plate is shaken again, and the wash solution is discarded. After the addition of an elution solvent, the analyte is eluted from the coating by shaking the plate. The eluate is then transferred to either an HPLC vial or an uncoated 96-well plate, and loaded into an LC autosampler for sample injection. The general process is illustrated in Figure 1.

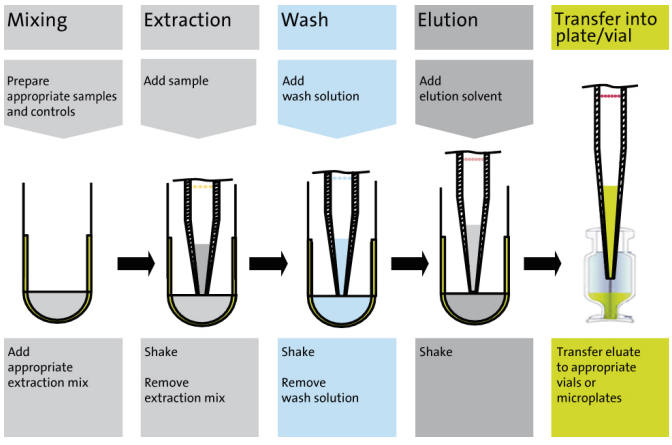

Figure 1 Typical AC Extraction Plate sample preparation workflow.

Materials and methods

Instruments and laboratory equipment

- MixMate® shaker for 96-well plates (Eppendorf)
- LC-MSMS system: UltiMate® 3000 HPLC (Dionex) coupled to a 3200 QTRAP® tandem quadrupole mass spectrometer equipped with an electrospray ionization source (AB Sciex)

Determination of appropriate organic/aqueous solvent mixes for the three sample preparation steps (a DEEM approach)

A Direct Extraction-Elution Method (DEEM) was performed using a spiked analyte solution to determine the absorption/desorption behavior relative to organic content. The ratio of organic to aqueous solvent was varied by 20 µl in each column of the AC Extraction Plate while maintaining a constant total well volume of 200 µl. After shaking the plate for 10 min at 1,200 rpm to enable absorption of the analyte by the coating, the amount of unabsorbed analyte remaining in the supernatant was determined and plotted against the percentage of organic content used. The resulting DEEM curves indicated suitable operating ranges for the extraction, wash and elution steps (Figure 2).

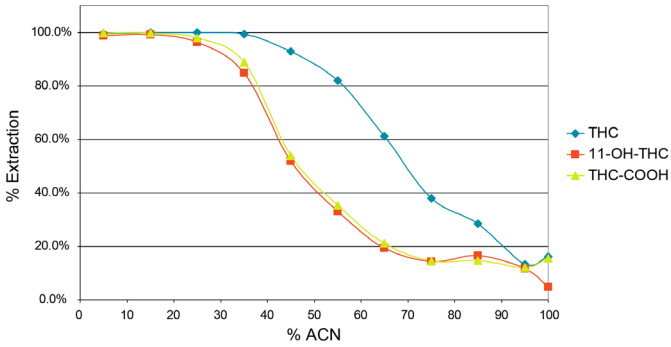

Figure 2 DEEM curves for THC, 11-OH-THC and THC-COOH.

The following solutions were chosen for the sample preparation steps:

- Modifier buffer: H_2O/CH_3CN (75/25; v/v) + 0.2 % HCOOH
- Wash solution: H_2O/CH_3CN (75/25; v/v) + 0.2 % HCOOH
- Elution solvent: H_2O/CH_3CN (15/85; v/v) + 0.2 % HCOOH

For the extraction step, 100 µl of the modifier solution was mixed with 10 µl of internal standard in MeOH in each well. A urine sample (90 µl) was added to each well, making a total volume of 200 µl, with an organic content of 17.5 %. The wash step was performed using 25 % organic solvent, while the elution step was carried out with an organic content of 85 %.

All solvents used were LC-MS Chromasolv® grade (Sigma Aldrich, USA). The internal standard D_3-THC-COOH was obtained from Cerilliant, USA.

Urine calibrators

Seven calibrators containing defined concentrations of THC-COOH in urine were extracted. The concentrations chosen cover the forensically relevant range.

Samples (urine matrix)	THC-COOH (ng/ml)
Calibration Level 1	2.5
Calibration Level 2	5
Calibration Level 3	7.5
Calibration Level 4	10
Calibration Level 5	20
Calibration Level 6	50
Calibration Level 7	100

Table 1 Concentrations of THC-COOH in urine calibration samples.

Sample preparation and extraction procedure

The modifier buffer was mixed with internal standard solution at a ratio of 10:1 (v/v) within each well of the AC Extraction Plate. This mixture represents the extraction mix used for the first step of the actual sample preparation procedure.

Calibrators and samples were treated as outlined in Table 2, using manual single-channel, 8-channel or multi-dispense pipettes.

Step	Procedure
1	110 µl of extraction mix was generated in each well of the AC Extraction Plate
2	90 µl of urine calibrator or urine sample was added
3	The AC Extraction Plate was shaken horizontally for 10 min at 1,200 rpm*
4	The supernatant was completely removed from each well and 200 µl of wash solution was added
5	The AC Extraction Plate was shaken horizontally for 2 min at 1,200 rpm*
6	The wash solution was completely removed and 200 µl of elution solvent was added
7	The AC Extraction Plate was shaken horizontally for 5 min at 1,200 rpm*
8	190 µl of each eluate was transferred from the AC Extraction Plate to a non-coated Axygen autosampler plate fitted with a pierceable cover (to minimize evaporation of solvent) and placed in a cooled autosampler (10 °C) for immediate analysis by LC-MSMS

* Tested with MixMate; optimum rpm settings may vary with type and orbit of plate shaker.

Table 2 Detailed AC Extraction Plate sample preparation workflow for THC-COOH extraction and analysis by LC-MSMS.

LC-MSMS parameters

LC

Column: Kinetex™ C8 2.6 µm, 50 x 2.1 mm,100 Å (Phenomenex)
Column temperature: 40 °C

Eluent A: 0.1 % HCOOH
Eluent B: acetonitrile with 0.1 % HCOOH

Injection volume: 40 µl
Initial flow rate: 0.3 ml/min

LC set-up and flow rates:

1. Pump 1; 300 µl/min of 100 % B, for injection onto the trapping column
2. Pump 2; 500 µl/min of 100 % A, for the addition of water via a T-union during injection onto the trapping column (Polar RP)
3. Pump 3; 300 µl/min, gradient from 30-97.5 % B over 6 min, for backflushing the analytical column and chromatographic separation
4. Total HPLC cycle time: 12.0 min

MS/MS parameters

Electrospray ionization

Positive mode

- CUR 25
- CAD 5
- IS 5500
- TEM 650
- GS1 60
- GS2 40

Mass transitions

	Analyte	D_3-analyte (internal standard)
THC	315 > 193	318 > 196
11-OH-THC	331 > 313	334 > 316
THC-COOH	345 > 327	348 > 330

Table 3 The mass transitions used for LC-MSMS analysis.

Performance parameters

All experimental data was obtained with the AC Extraction Plate using the chemicals and workflow as described above.

1. Linearity

Linear regression analysis over seven different calibration levels demonstrated good linearity for THC-COOH between 2.5 and 100 ng/ml, showing a correlation coefficient (r value) >0.998 using a weighting of 1/x (Figure 3).

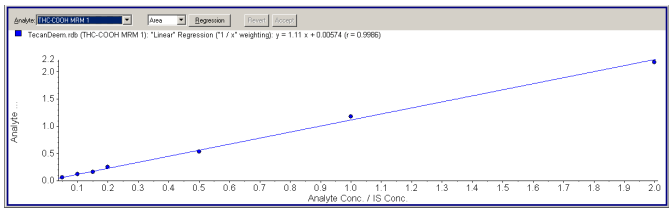

Figure 3 Seven point calibration curve for THC-COOH at concentrations between 2.5 and 100 ng/ml in urine.

2. Lower limit of quantitation (LLOQ)

The LLOQ was determined at 2.5 ng/ml THC-COOH (Figure 4).

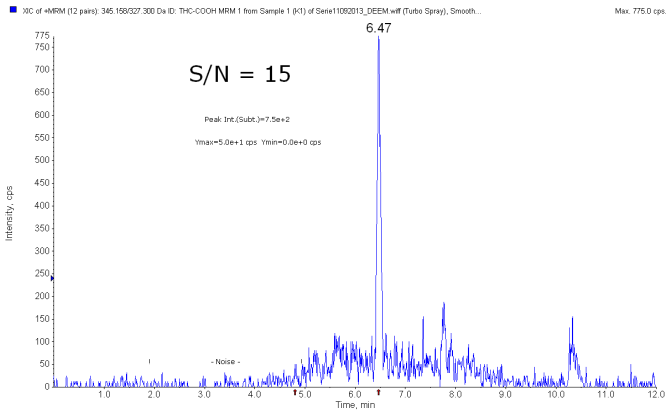

Figure 4 Lowest urine calibration sample containing 2.5 ng/ml THC-COOH: S/N > 10.

3. Reproducibility

The peak areas of the D_3-THC-COOH internal standard were determined to establish the reproducibility of the extraction and analysis. A low CV of 5.6 % was obtained (Figure 5).

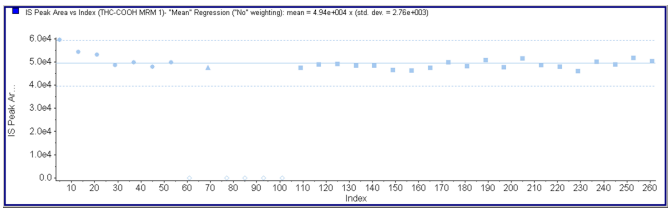

Figure 5 D_3-THC-COOH internal standard peak areas. Mean and 20 % deviation of peak areas are shown; empty spheres represent the double blanks.

4. Direct comparison with a 'dilute and shoot' approach

Identical urine calibration samples, blanks and samples were analyzed using the AC Extraction Plate protocol, as well as by diluting 1:1 with water for a 'dilute and shoot' approach.

The 'dilute and shoot' approach shows a matrix effect, with significantly lower internal standard peak areas observed for the samples compared to the calibrators (Figure 6).

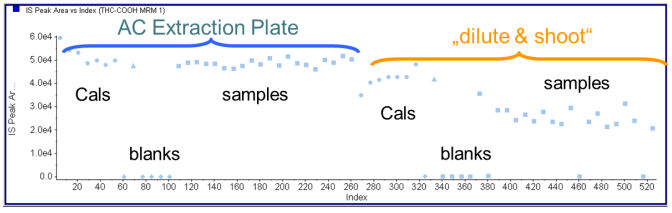

Figure 6 Comparison of the internal standard peak areas of the same samples after preparation with the AC Extraction Plate (left) and the 'dilute and shoot' approach (right); spheres = calibrators, squares = samples, diamonds = blanks.

Conclusion

The metabolite THC-COOH was successfully determined from urine matrix by a combination of AC Extraction Plate for sample preparation followed by LC-MSMS quantification. The range of THC-COOH calibrator concentrations covered the forensically relevant range. The complete procedure can be successfully performed manually or automated on a liquid handling platform such as the Freedom EVO®. The procedure may be adapted to further THC metabolites or other small molecules.

The AC Extraction Plate featuring TICE technology is a fast and easy-to-automate sample preparation method requiring minimal sample pretreatment. No time consuming or rather costly to automate procedures – such as protein precipitation with centrifugation, filtration or evaporation – are needed thus accelerating and simplifying the entire workflow. This sample preparation method can easily be automated.

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