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Article

# Simplified Analysis of 11-Hydroxy-Delta-9-Tetrahydrocannabinol and 11-Carboxy-Delta-9-Tetrahydrocannabinol in Human Meconium: Method Development and Validation

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**We describe the development of a sensitive analytical method for the analysis of 11-hydroxy-delta-9-tetrahydrocannabinol (11-OH-THC) and 11-carboxy-delta-9-tetrahydrocannabinol (THCC) in meconium using a gas chromatography–mass spectrometry (GC/MS) platform. The method was validated according to protocols, which included assessment of accuracy, precision, robustness, stability in meconium and in-process stability, interference and sensitivity and specificity. The method consists of a solid phase extraction with alkaline hydrolysis and derivatization of the analytes with N, O-Bis(trimethylsilyl)trifluoroacteamide, followed by GC/MS analysis using selected ion monitoring. The method uses deuterated internal standards for both analytes. Calibration curves had $r^2$ values >0.998, and extraction efficiency was determined to be 84.7% for THCC and 78.6% for 11-OH-THC. The detection limit for both analytes was 5 ng/g. This confirmatory method was successfully applied to 183 meconium samples that had screened positive by enzyme-linked immunosorbent assay, and 67.2% were confirmed for THCC, and 2.2% were confirmed positive for 11-OH-THC. The mean (SD) and median (range) THCC ($n$ = 123) concentrations detected were 55.0 ng/g (±59.0) and 33.75 ng/g (5–265 ng/g), while the mean and median (range) for 11-OH-THC ($n$ = 4) concentrations were 8.25 ng/g (±4.71) and 6.5 ng/g (5–15 ng/g).**

## Introduction

Several methods have been described for monitoring prenatal exposure to marijuana through analysis of delta-9-tetrahydrocannabinol (THC) and its metabolites in blood, urine, hair, placenta, amniotic fluid, gastric aspirate (1), in umbilical cord tissue (2, 3), and meconium (1–7). Meconium, the black tarry substance that begins to collect in the intestine around the 12–16th week of gestation, has emerged as the preferred matrix for monitoring gestational marijuana use by the mother (1). Meconium can be effectively collected for about 5 days after birth to determine prenatal exposure of the newborn to drugs (1, 4). It is a complex matrix consisting of water, epithelial cells, lanugo, mucus, amniotic fluid, bile acids, salts, cholesterol, sugars, lipids, various organ secretions, and compounds ingested by the mother during the last few months of the pregnancy (4).

The relative merits of meconium and umbilical cord tissue for the determination of fetal exposure to drugs including cannabis has been assessed (2), and it was shown that there was 90.7% agreement between the meconium and tissue sample. There are advantages and disadvantages for both matrices that need to be taken into consideration. To test the umbilical cord it needs to be collected at birth, so prior suspicion of the drug use must be indicated in order to secure the sample, whereas meconium can be collected several days after the birth, so if a need for testing is identified after the birth, samples can still be procured (2). However, since the umbilical cord sample is collected at birth it can be tested immediately and results are available more quickly than in meconium, since it may take some days to collect a sufficient sample size. Additionally, the chemical properties of the drug may affect the concentrations of the analytes present in the meconium and cord samples causing different results between the two (2). Methods for the analysis of umbilical cord tissue can be costly and typically use more advanced instrumentation such as liquid chromatography–time of flight mass spectrometry (LC-TOF-MS), which may not be available in all laboratories (3). Some LC-TOF-MS methods may have a lower cutoff limit of 1 ng/g.

The value of monitoring for marijuana use during pregnancy is debated due to contradictory research on the effects of *in utero* exposure to the drug. Studies agree that marijuana is one of the most commonly used drugs during pregnancy and is the most common illicit drug used (8). It has been shown that THC can rapidly cross the placenta, but the exact effects on the fetus are undetermined (8–11). Fetal excretion of THC is delayed leading to a more prolonged exposure profile (8). It has been difficult to reach firm conclusions about the effects of marijuana use during pregnancy due to the number of potentially confounding factors involved, including polysubstance abuse, and socioeconomic factors such as income, social and family support, nutrition, and maternal health including prenatal care, stress, sexually transmitted diseases (STD), and domestic violence (8, 9). Some studies agree that there is little to no effect on the gestational length or presence of malformations in the newborn associated with the mother's use of marijuana during the pregnancy (8, 9, 12). One study suggests that there is, however, a possible detrimental effect on growth and length of gestation due to marijuana use during pregnancy, however that report relied only on maternal self-report of drug use which was not confirmed through toxicological testing (10). There are publications that suggest some effect on intelligence and cognitive functioning in neonates resulting from *in utero* marijuana exposure, but these results have again been inconsistent and may be a function of both genetics and the external environment (8, 9, 11). The exact effects of the drug on the brain and their persistence are not fully understood, but there is believed to be a negative effect on development of systems for mood, cognition and reward and goal

driven behaviors (11). No major teratogenic effects or psychotic symptoms have been reported in the newborn associated with prenatal exposure to marijuana (8, 9). A related consideration is the effect of the marijuana usage on maternal health. If the mother's health is negatively impacted, this might lead to corresponding negative impacts on the fetus's health which may be the actual cause of some inconstancies between studies (9). Other studies suggest a possible link between prenatal exposure and early onset of marijuana use in the child (8, 9). In addressing some of these conflicting concerns regarding maternal drug use and its effects *in utero* or in the neonate, there is a need for a reliable and sensitive objective method for demonstrating gestational marijuana use through analysis of meconium.

Because of the complex nature of the meconium matrix, extraction methods often require multiple steps, the use of costly specialized extraction columns (13, 14), high resolution multidimensional chromatographic techniques (14, 15) and long analytical run times (7) to maximize sensitivity and avoid interference. We describe the development and validation of a robust, cost-effective extraction and instrumental analysis method with a 13.5-min run time, using traditional single column gas chromatography–mass spectrometry (GC/MS) for the analysis of 11-hydroxy-delta-9-tetrahydrocannabinol (11-OH-THC) and 11-carboxy-delta-9-tetrahydrocannabinol (THCC) in meconium.

## Reagents and Materials

Cannabinoid spiking standards (THCC and 11-OH-THC) and deuterated internal standard (11-OH-THC-d3 and THCC-d3) were obtained from Cerilliant (Round Rock, TX). Strata-X-C 33u Polymeric Strong Cation (Part No: 8BSO29-UBL, Phenomenex Torrance, CA) was used. Then, 0.1 M phosphate buffer (pH 7) was prepared from monobasic and dibasic sodium phosphate (Sigma-Aldrich, St Louis, MO) and pH adjustments made with 10 M NaOH (Fisher Scientific Waltham, MA). Acetonitrile, methanol, concentrated ammonium hydroxide, concentrated hydrochloric acid, and methylene chloride were obtained from Fisher Scientific, and isopropyl alcohol was obtained from OmniSolv (Charlotte, NC). N, O-Bis(trimethylsilyl) trifluoroacteamide (BSTFA) was purchased from Sigma-Aldrich. Drug-free meconium for calibration and quality control was obtained by pooling discarded meconium samples that had tested negative for cannabinoids by a cannabinoid enzyme-linked immunosorbent assay (ELISA, Immunalysis, Pomona, CA) and an existing validated GC/MS method before use. The existing method was less sensitive, less robust and more time consuming than the current method, and had a low confirmation rate of 21% for THCC and 0% for 11-OH-THC versus the cannabinoid ELISA immunoassay. Sample extracts were analyzed by single column GC/MS (Agilent 7890A/5975C with a7693 liquid autosampler). The column was a ZB50 (Phenomenex, part no. 7HG-G004-11).

## Methods

### *Sample preparation, hydrolysis and precipitation*

Approximately 0.25 g of meconium is placed in a 13 × 100 mm disposable test tube, tared and weighed. An equivalent weight of water is added, and the sample is vortex mixed for ~1 min or until the meconium goes into solution. Internal standard spiking solution (50 μL, 0.2 ng/μL) containing 11-OH-THC-d3 and THCC-d3 is added, and the solution mixed. Phosphate buffer (pH 7, 0.5 mL) is added to each tube. Then, 12 M KOH (25 μL) is added to each tube and vortex mixed. Samples are incubated uncapped at 60 ± 5°C in a calibrated heating block or a water bath for 15 min to hydrolyze conjugated metabolites. Following hydrolysis, concentrated HCl (100 μL) is added and the samples mixed. Acetonitrile (1 mL) is added to each tube and mixed well, then centrifuged for 5 min at 2500 rpm. The supernatant is poured off into clean, labeled 13 × 100 mm test tubes, and phosphate buffer (pH 7, 1 mL) is added. Samples are mixed and then centrifuged at 3500 rpm for 5 min.

### *Solid phase extraction*

Strata-X-C SPE columns are conditioned with methanol (2 mL), and the samples are applied to the columns under vacuum. The columns are washed with 2 × 1 mL of 50:50 water–methanol solution and dried. The analytes are eluted from the columns with 2 mL of the mixed elution solvent (780:200:20 methylene chloride–isopropyl alcohol–concentrated ammonium hydroxide) and evaporated to dryness.

### *Derivatization*

Once dry, BSTFA + 1% TMS (50 μL) is added to each tube, capped securely, vortex mixed and incubated at 70 ± 5°C for 30 min. The derivatized extracts are transferred to autosampler vials with microinserts and placed on the GC–MS autosampler.

### *Calibration and control*

The calibration curve was a linear curve with a lower limit of quantitation of 5 ng/g and an upper linear range of 150 ng/g (points included 5, 20, 50, 100 and 150). The controls for the hydrolysis and extraction were commercially available urine controls containing 11-carboxy-delta-9-tetrahydrocannabinol glucuronide (THCCG) from ElSohly Laboratories Inc. (Oxford, MS), which also served as hydrolysis controls, because no commercially available meconium THCCG controls were available. The use of urine hydrolysis controls for THCCG was selected for ease of use, and assessment of the suitability of this material was established during validation. Replicates ($n = 6$) of the ElSohly urine THCCG control (18.5 ng/mL) were prepared alongside an equivalent number of replicates of drug-free meconium samples spiked with 18.5 ng/mL of THCCG after dilution with water, per the sample preparation procedure described above. The urine and meconium samples were subjected to the alkaline hydrolysis procedure according as described above. The recovery of the THCC from this procedure was evaluated by analysis using the analytical method described, and the theoretical concentration of the spiked meconium was calculated and compared with the actual resultant concentration and both were found to be within 10% of target.

The high control (18.5 ng/mL) was run undiluted while the lower control (9.5 ng/mL) was a 2-fold dilution of the high control using deionized water.

### *GC/MS analysis*

After extraction, 2 μL was injected in splitless mode on the Agilent 7890A/5975C GC–MS using a 7693 liquid autosampler. The GC–MS had a single 30 mm × 0.25 mm × 0.25 μm, Phenomenex ZB 50 column installed. The mass spectrometer was equipped with an electron ionization source and utilized selective ion monitoring. The analytical method entailed use of a temperature-programmed analysis consisting of an initial oven temperature of 180°C held for 0.75 min; the temperature then increased by 10°C/min to 300°C, then ramped up at 40°C/min to 340°C. The total runtime is ~13.5 min long. The septum purge flow was 32 mL/min, and the injection used a pulse pressure of 30 psi until 0.75 min. The inlet temperature was held at 280°C for the entire run. The ions ($m/z$) monitored for the THCC-d3 were 374 (quantitation), 491 and 476 (qualifier ions); for the THCC analyte 371 (quantitation), 472 and 488 (qualifier ions); for the 11-OH-THC-d3 374 (quantitation), 477, 462 (qualifier ions) and for the 11-OH analyte 371 (quantitative), 474 and 459 (qualifier ions).

### *Data analysis and review*

To determine the acceptability of a run several factors were considered. The calibrators within the curve were required to be within 20% of their nominal values. The curves themselves were required to have a correlation coefficient ($r^2$) value of ≥0.98. The internal standard responses were required to be within ±50% of the mean response for the calibrators. The ion ratios must be within ±20% of the mean ratio determined from the calibrators. The controls run with the samples are required to quantify within ±20% of the established mean. If the run failed to meet any of those criteria, it was deemed unacceptable and was rejected.

### *Method validation*

Before validation started two pre-validation experiments, stock standard verification and determination of reporting limit were performed to determine the validity of the materials used for the calibrators and controls and to define the reportable limits of the assay. The validation experiments included assessing the stability of the analyte in the meconium matrix, specificity and sensitivity of the method, the extraction efficiency and matrix effect, carry-over between samples, interfering substances and robustness of the method.

The specificity and sensitivity of the method was determined using blinded specimens, and this experiment also shows the accuracy of the method. Blinded samples were prepared by spiking individual verified drug-free meconium samples with the target analytes at a range of concentrations (7.5–150 ng/g). These concentrations were chosen to show the method's effectiveness throughout the linear range starting at 150% of the lowest calibrator to the highest calibrator concentration. A total of 30 spiked positive specimens across the range of 6.5 ng/g (30% above cutoff) to the concentration of the highest calibrator (150 ng/mL) and 30 negative specimens (0–3.5 ng/g) were prepared and analyzed. The spiked specimens were individual drug-free meconium samples ($n = 30$), not pooled. A hydrolysis study was performed to determine the need for, and optimum conditions for, hydrolysis of cannabinoid conjugates in the meconium. The stability of the analyte within the matrix was tested by creating a pool of sample with a known concentration of analyte present and testing it on the day it was prepared. The samples were then stored under different conditions and run on subsequent days with the results compared back to the time zero run. Carry-over was tested by running a negative sample after the highest calibrator (150 ng/mL) on every validation run. Several common drugs ($n = 48$) were tested to determine if they would cause interference with the analysis, including over the counter drugs such as non-steroidal anti-inflammatory drugs, antihistamines, caffeine, as well as prescription medications including opiates, antidepressants, muscle relaxants and sleep aids. The extraction efficiency was determined by extracting samples of a known compound and comparing their response to those of un-extracted 'neat' samples with the analyte and internal standard added to methanol.

## Results

An assessment of the need for hydrolysis showed that in authentic patient samples, there was a significant increase in abundance of the target analytes following addition of base for hydrolysis of glucuronide or sulfate conjugates. Hydrolysis under the conditions described above was completed very rapidly (<15 min), and no additional increase in signal was obtained with extended incubation times.

After applying the method as described, calibration curves with $r^2$ values >0.998 were routinely obtained. Figure 1 shows an example of chromatography at the second calibrator (20 ng/g), and Figure 2 shows calibration curves for the THCC and 11-OH-THC. The method gave cleaner extracts than a previously used liquid/liquid extraction method, and hydrolysis improved recovery of the drug from the sample.

Precision at the cutoff was used to characterize the performance of the method at its reporting limit. This was done to prove the reliability of the method at the low concentrations because the cutoff value was determined administratively. Analytically, the LLOQ was determined to be 2.5 ng/g, but an administrative cutoff of 5 ng/g was used for consistency with other reports. At the cutoff (5 ng/g for both analytes), the coefficient of variation (% CV) was determined to be 4.30% for THCC and 3.10% for 11-OH-THC based on analysis of a series of 5 ng/g controls analyzed in two separate runs on two separate days ($n = 40$).

It was determined by serial analysis of spiked controls that the analytes in question were stable up to 14 days at room temperature and were stable up to 30 days under refrigerated conditions. The sensitivity and specificity of the extraction were tested and evaluated by analyzing 60 blind samples (30 positive and 30 negative) prepared as described above. The results were subsequently compared with the true pedigree of the samples to identify any false-positive or false-negative results. This method produced no false positives or false negatives giving it an apparent sensitivity and specificity of 100%.

No carry-over was observed below the upper limit of linearity (high calibrator) of 150 ng/g. No interferences with common therapeutic or other drugs of abuse were encountered in the interference study. Extraction efficiency was determined to be 84.7% for THCC and 78.6% for 11-OH-THC.

Robustness of the method was tested by having two different analysts extract 20 of the blind samples prepared for the

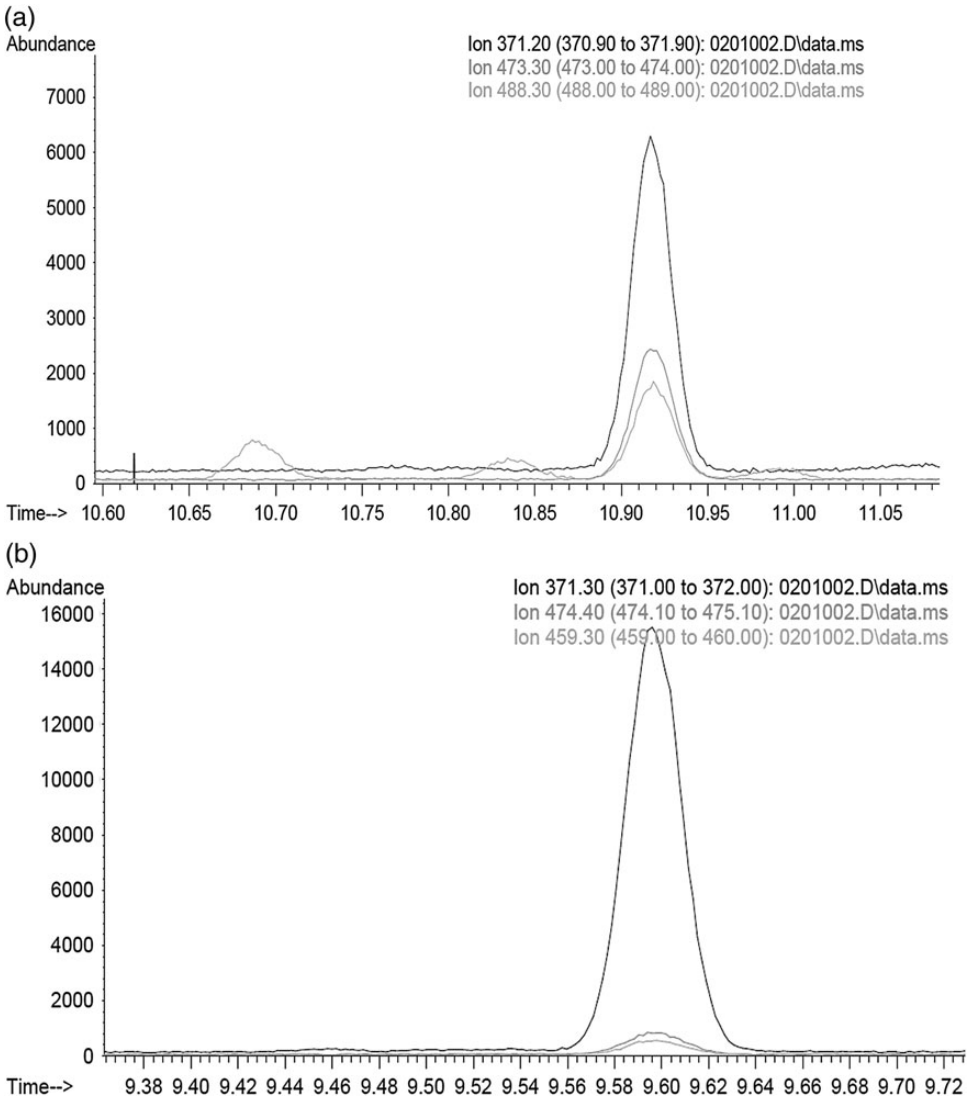


**Figure 1.** Examples of chromatography for (a) THCC and (b) 11-OH-THC.

sensitivity and specificity experiments. Both analysts obtained identical qualitative results with respect to the designated cutoff of 5 ng/g, and all quantitative results were within 15% of each other.

This method allows for more sensitive detection of the target analytes in comparison to Coles *et al.*, being able to detect 5 ng/g compared with 10 ng/g, and achieved better recovery for both analytes (16). While the efficiency for hydrolysis of the 11-OH-THC conjugates was not assessed, it was demonstrated to be very effective for hydrolysis of the THCC conjugate, which is the predominant species in this and other reported studies, and allowed for a shorter, more efficient incubation time without compromising the benefits of this qualitative procedure for disclosing THC exposure.

### *Analysis of patient samples*

Over a 4-month period, a total of 183 meconium samples were analyzed using the method described. The samples tested had previously screened positive by Cannabinoid ELISA (Immunalysis, Pomona, CA) at a cutoff of 20 ng/mL. Two samples (1%) were canceled for THCC analysis due to matrix interference issues that could not be resolved using this method. Further, 67.2% of the screen positive samples were confirmed positive for THCC ($n = 121$) using this method while only 2.18% ($n = 4$) were confirmed positive for 11-OH-THC. All cases that were positive for 11-OH-THC were also positive for THCC. The mean (SD) and median (range) THCC ($n = 123$) concentrations detected in the patient samples that confirmed were 55.0 ng/g ($\pm 59.0$) and 33.75 ng/g (5–265 ng/g), while the mean and median (range) concentrations for 11-OH-THC ($n = 4$) were 8.25 ng/g ($\pm 4.71$) and 6.5 ng/g (5–15 ng/g). The distribution of THCC concentrations is shown in Figure 3. Quantitative results were determined for research purposes only, and results for patient samples were reported qualitatively as positive or negative around the administrative cutoff of 5 ng/g.

It is feasible that other non-targeted metabolites in the meconium, or endogenous materials have cross-reactivity on the ELISA

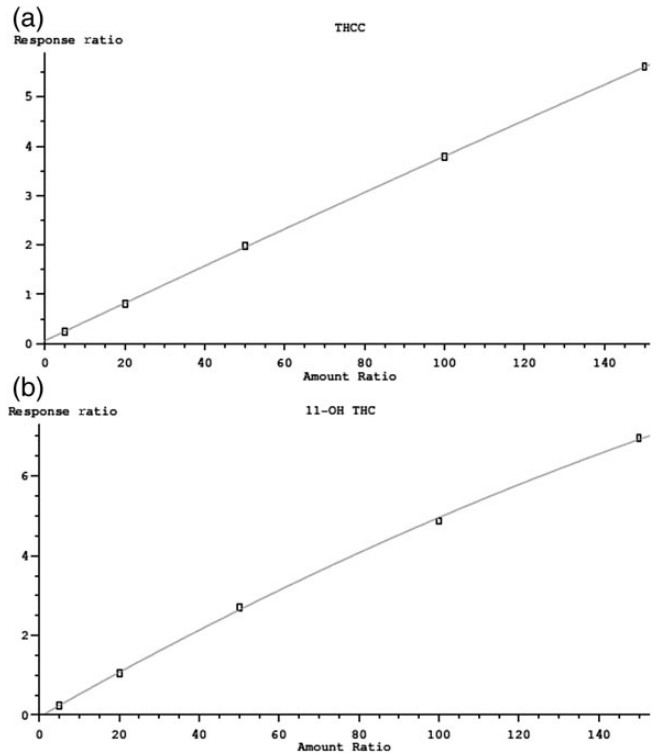


**Figure 2.** Examples of calibration curves for (a) THCC and (b) 11-OH-THC.

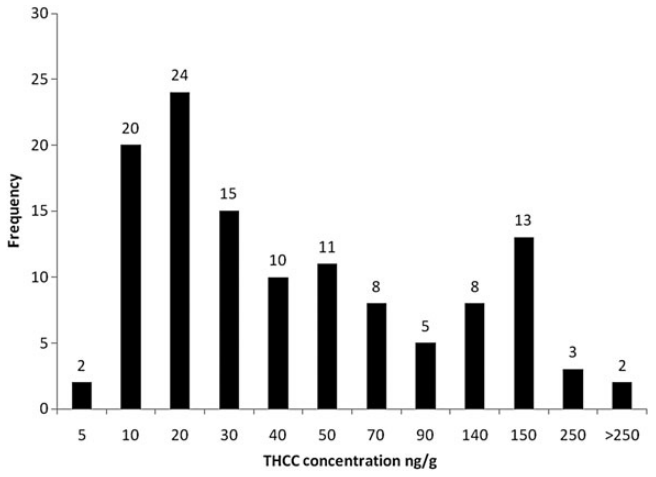


**Figure 3.** Distribution of total THCC concentrations in patient samples ($n = 121$).

resulting in screen positive results that would not confirm at the detection threshold for this assay. The results underline the importance of a confirmatory mass spectrometric analysis and should discourage any action based simply on an immunoassay result. The confirmation rate performance was a significant improvement over the prior method, where the confirmation rate was 21% for THCC.

Coles *et al.* (16) have reported higher rates cannabinoid positivity in populations tested for cannabinoids. The positivity rates, however, will depend on the nature of the population being tested and the reason for samples being collected and submitted for analysis. Coles *et al.* reported 6.5% of 246 patient samples testing positive for cannabinoids were positive for only 11-OH-THC, 19.1% positive for only THCC and 61% positive for both analytes. ElSohly and Feng (17) reported results of meconium analysis from newborns of mothers with known marijuana use ($n = 16$) and found 14 positive for THCC and 11-OH-THC, and two positive for 11-OH-THC alone. Results in our subjects revealed 2.18% positive for 11-OH-THC and 67.2% positive for both THCC and 11-OH-THC. There were no samples positive for 11-OH-THC only; however, the ELISA assay used in our laboratory has only a 5% cross reactivity with 11-OH-THC, so those samples positive for only this metabolite may go undetected if samples are first screened by ELISA.

## Conclusion

The developed method is viable for qualitative or quantitative analysis of THCC and 11-OH-THC in meconium as an indicator of marijuana use by the mother during pregnancy. The method is robust, time and cost effective and produces reliable results consistently as shown by the validation data. The method passed all criteria in the validation procedure demonstrating its ability to analyze samples over a variety of concentrations in this highly variable matrix.

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