

**Simultaneous Quantification of Amphetamine and Methamphetamine  
in Meconium Using ISOLUTE® HM-N Supported Liquid Extraction  
Columns and GC/MS.**

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## **Abstract**

A procedure for the rapid extraction and quantification of amphetamine and methamphetamine from meconium using ISOLUTE HM-N supported liquid extraction columns and gas chromatography – mass spectrometry (GC/MS) is described. Due to the matrix complexity of meconium samples, extraction and sample preparation prior to instrumental analysis can prove difficult and time consuming. The present study introduces a novel sample preparation technique for the simultaneous quantification of amphetamine and methamphetamine in meconium using GC/MS. Extraction of both analytes was achieved using ISOLUTE HM-N supported liquid extraction columns containing a modified form of diatomaceous earth. Limits of detection for both analytes were 30 ng/g and the lower limit of quantitation (LLOQ) was 75 ng/g. Linearity was achieved over the range 75 to 3000 ng/g. The methodology showed excellent intra run precision with % CV values ranging from 2-8% for both analytes. Inter run precision experiments produced % CV values between 7-10% for both analytes. The reported methodology proved suitable for the accurate quantification of amphetamine and methamphetamine in meconium samples and greatly reduced sample preparation time normally required for traditional solid phase extraction. Development and validation of such analytical methodologies will prove beneficial for the identification of prenatal substance abuse which is an ongoing concern across socioeconomic lines.

**Keywords:** Meconium; amphetamine; methamphetamine; GC/MS; supported liquid extraction

## **Introduction**

Prenatal substance abuse is an ongoing concern due to the characteristic physical and mental developmental problems that result from drug abuse during pregnancy. Various neonatal health and developmental problems are thought to be directly related to fetal exposure to drugs, alcohol, chemical agents, or other xenobiotics (1-4). Due to the increasing abuse and synthesis of the amphetamine like stimulants, there is a desire among analytical chemists for sensitive methodologies capable of detecting low levels of these drugs in meconium. Such methodologies would aid in further understanding the effects of fetal exposure on newborns. Clefting, cardiac anomalies, and fetal growth reduction deficits have all been seen in infants exposed to amphetamines during pregnancy (3). Animal studies involving prenatal exposure to amphetamines have allowed for the same observations and methamphetamine has been shown to cross the placenta within thirty seconds of intraperitoneal injection (5). Methamphetamine is the most widely abused amphetamine and animal studies observed increased maternal and offspring mortality, retinal eye defects, cleft palate, rib malformations, decreased rate of physical growth, and delayed motor development associated with prenatal methamphetamine exposure (6-9). Although peak concentrations are lower on the fetal side, slower elimination of the amphetamines means that the fetus is subject to prolonged exposure which can significantly impact neonatal health and development (5). In the first large scale investigation into the prevalence of methamphetamine use during pregnancy in areas of the United States where methamphetamine is a notable concern, it was found that 5.2 % of the 1632 subject mothers used methamphetamine at some point during their

pregnancy (10). The authors concluded that the methamphetamine exposed group was 3.5 times more likely to be small for gestational age than the unexposed group (11).

Meconium has become the specimen of choice for the detection of prenatal exposure to several drugs of abuse (12, 13). There are several reasons for this, including the relatively simple and non-invasive procedure used to collect meconium samples, making it more successful than urine collection (14). Meconium analysis also extends the window of drug detection to approximately the last 20 weeks of gestation as well as extending the window for specimen collection, as it is not fully evacuated until 125 hours post natally (15-17). Due to the complexity of the meconium matrix, analysis can prove difficult as sample preparation may require additional laborious steps in order to efficiently extract the desired analytes from the non-homogenous sample (18). Ostrea et al. (19) employed a two stage extraction procedure for the detection of illicit drugs and other xenobiotics in newborn infants. Such procedures involve an initial liquid extraction from the meconium after which the organic layer is evaporated and reconstituted in phosphate buffer in preparation for solid-phase extraction. Conventional SPE columns require multi-step conditioning and subsequent aspiration before the sample can be introduced onto the column. Most SPE extraction procedures involve 2-3 sequential washes before analytes are eluted with a suitable solvent made fresh daily. The combination of a two stage extraction involving a multi wash SPE procedure with the need to prepare elution solvents daily can prove very laborious in high throughput laboratories. ElSohly et al (20) achieved limits of detection of 50 ng/g for amphetamine and methamphetamine employing a multi-step liquid extraction procedure and GC/MS. Additional sample cleanup was achieved by incorporating a back extraction for the purpose of eliminating

neutral molecules present in the matrix. Although such extraction procedures have allowed the selective determination of amphetamines in meconium they can prove time costly and significantly affect sample turnaround time. The aim of the present study was to develop a novel methodology for the preparation of meconium samples which would allow for the rapid and simultaneous quantification of amphetamine and methamphetamine in meconium. In this report, we describe the first application of supported liquid extraction columns for the preparation of meconium specimens prior to analysis and quantification by GC/MS. ISOLUTE HM-N supported liquid extraction columns require no column conditioning and once the sample is introduced onto the column, elution is achieved with two washes of ethyl acetate.

## **Experimental**

### **Specimens**

Meconium specimens which had previously screened negative for amphetamines using a 50 ng/g cutoff at AIT laboratories (Indianapolis, IN) were collected and used as blanks. The suitability of blank meconium specimens for constructing calibration curves was

investigated by preparing spiked meconium samples at varying concentrations (n=30) and quantifying them using a calibration curve made up in negative meconium and a calibration curve made up in negative blood. Both calibration curves consisted of six points at concentrations of 25, 50, 100, 250, 500 and 1000 ng/mL. Results obtained using the meconium calibration curve showed excellent correlation with those obtained using the calibration curve made up in negative blood. All method validation experiments were performed using calibration curves prepared in blank meconium.

### **Chemicals**

Amphetamine, methamphetamine, amphetamine-d<sub>6</sub>, and methamphetamine-d<sub>9</sub> standards (1mg/mL in methanol) were obtained from Cerilliant (Round Rock, TX). ISOLUTE<sup>®</sup> HM-N supported liquid-liquid extraction columns were purchased from Biotage (Charlottesville, VA). All solvents were HPLC grade and obtained from Fisher Scientific (Pittsburgh PA).

### **Procedures**

*Extraction procedure:* Meconium samples were accurately weighed and then diluted by a factor of 3 (w/v) with 50:50 methanol/water to assist with the sonication procedure. Samples were shaken and sonicated for 10-15 minutes. Following sonication, 1 mL of the meconium samples was added to appropriately labeled culture tubes. Amphetamine and methamphetamine standards and deuterated internal standards were added to samples

which were then block vortexed for 15 minutes. Samples were centrifuged for 15 minutes at 3500 rpm after which the supernatants were transferred to appropriately labeled culture tubes (12x75). To improve sample recovery, each sample was made basic by the addition of 500  $\mu\text{L}$  of 2.0M NaOH. Samples were loaded into 5mL ISOLUTE HM-N supported liquid extraction columns and left to sit for 10 minutes. Analytes were initially eluted with 5 mL of ethyl acetate and after a 3 minute waiting period a second elution step was performed with 3 mL of ethyl acetate. 200  $\mu\text{L}$  of 1% HCl was added to all eluates to ensure formation of the hydrochloride salts in order to reduce the possibility of analyte loss during evaporation steps. Samples were dried down under a gentle stream of nitrogen.

### **Derivatization**

Following evaporation of the eluates, extracted amphetamines were derivatized with 50  $\mu\text{L}$  of 4-Carboxyhexafluorobutyryl chloride in 50  $\mu\text{L}$  of ethyl acetate at 60°C for 20 minutes. Samples were dried down under nitrogen, reconstituted in 40  $\mu\text{L}$  of ethyl acetate and transferred to GC vials for analysis.

### **GC/MS analysis**

All analyses were performed on an Agilent 6890 GC interfaced with an Agilent 5973 mass selective detector operating in the EI mode using selected ion monitoring (SIM). The GC was operated in the pulsed split mode and employed a HP-5MS column (15 m x 0.25-mm i.d., 0.25- $\mu\text{m}$  film thickness). The oven temperature was programmed as

follows: 120°C, held for 0.5 min, then ramped to 260°C at a rate of 35°C/min, where it was held for 0.5 min for a total run time of 4.5 min. Injector and detector temperatures were held at 175°C and 280°C respectively. Retention times and monitored ions for each analyte and internal standard are shown in Table 1.

### **Method Validation**

Aliquot volumes of amphetamine and methamphetamine working standards were added to 1 mL of drug free meconium following sonication with methanol:water to obtain samples in the concentration range of 10-2500 ng/mL. 25 µL of Amphetamine-d<sub>6</sub> and methamphetamine-d<sub>9</sub> internal standard solutions (10 µg/mL) were added to all samples resulting in a concentration of 250 ng/mL. Spiked samples were extracted and analyzed in order to experimentally determine the limit of detection (LOD), lower limit of quantitation (LLOQ) and the range of linearity, which was used to construct suitable calibration curves employed for the purpose of quantification (Table 2). In order to evaluate the reproducibility of the methodology, inter-run and intra-run precision was evaluated by analyzing 30 high quality control (HQC) standards at a concentration of 250 ng/mL and 30 low quality control (LQC) standards at a concentration of 50 ng/mL over a period of three days (Table 3). Precision at the lower limit of quantitation (25 ng/mL) was also evaluated by analyzing 10 replicate samples at this concentration (Table 3). Sample stability following a freeze and thaw cycle was also evaluated by analyzing 3 quality control standards at varying concentrations following a freeze and thaw cycle (Table 4). The methodology was deemed selective following the accurate quantitation of both

amphetamine and methamphetamine standards in the presence of possible exogenous interferants at varying concentrations (Table 5). Cocaine, propoxyphene, methylenedioxyamphetamine, paramethoxyamphetamine, and  $\Delta^9$ -tetrahydrocannabinol were chosen as possible exogenous interferants as they constitute the remainder of analytes currently analyzed by GC/MS at AIT laboratories. Meconium samples that had screened positive for amphetamines using a 50ng/g cutoff were analyzed in order to determine the suitability of the developed method for authentic samples.

## **Results and Discussion**

Due to the complex nature of meconium matrices, extraction of analytes for quantitative instrumental analysis can prove difficult and time consuming. ISOLUTE HM-N supported liquid extraction columns greatly reduce the sample preparation time for meconium testing. Due to the high protein and lipid composition of meconium, significant sample clean up, such as a preliminary liquid –liquid extraction, is often required before traditional solid phase extraction can be employed. This is to ensure that the high content of large molecules in the meconium matrix do not clog the adsorbent material of the SPE cartridge. This additional preparation step combined with the multiple washes required for solid phase extractions can result in time costly assays that significantly affect sample turnover time in high throughput toxicology laboratories. The present study introduces a rapid sample preparation technique for the quantitative analysis of amphetamine and methamphetamine in meconium samples using supported liquid extraction columns. ISOLUTE HM-N columns contain a modified form of

diatomaceous earth which is packed into high purity polypropylene columns. Supported liquid extractions using the ISOLUTE HM-N columns are analogous to traditional liquid-liquid extractions. Modified diatomaceous earth contained in the ISOLUTE HM-N columns has a high capacity for retaining aqueous samples. When an aqueous sample is applied, the sample spreads over the hydrophilic surface of the column packing resulting in adsorption of the aqueous phase. An efficient extraction then occurs when an immiscible organic solvent is applied to the column. Analytes are then eluted as the organic solvent passes through the column. ISOLUTE HM-N supported liquid extraction columns have been designed to deal with unusual or difficult matrices such as meconium which can often cause conventional SPE extraction columns to plug due to the turbid nature of the sonicated sample. The methodology significantly reduced sample preparation time as there was no need for a preliminary liquid-liquid extraction or a multi-step solid phase extraction. ISOLUTE HM-N supported liquid extraction columns allowed for the simple and rapid extraction of both analytes with 2 aliquots of ethyl acetate. Following extraction and derivatization, analytes were separated and quantified in less than 5 min using GC/MS (Figure 1). The limit of detection (LOD) which is defined as the lowest analyte concentration required to produce a signal-to-noise (S/N) ratio of 3, was 10 ng/mL for both amphetamine and methamphetamine. This instrumental LOD corresponds to an initial specimen concentration of 30 ng/g, due to the 3:1 (w/v) dilution prior to analysis. The lower limit of quantitation (LLOQ) which is defined as the lowest analyte concentration required to produce a signal-to-noise (S/N) ratio of 10 with an imprecision no greater than 20% was found to be 25 ng/mL. Again, this instrumentally determined value corresponds to an initial specimen concentration of 75 ng/g due to the

3:1 (w/v) sample dilution. Although no dose – response relationship is currently available for amphetamines in meconium, achieved limits of detection are encouraging as self reported heavy prenatal use of methamphetamine has lead to high concentrations in meconium (200 – 1000 ng/g) (21). The methodology showed excellent inter-run and intra-run precision which was evaluated by analyzing 30 high quality control standards (250 ng/mL) and 30 low quality control standards (50 ng/mL) over three consecutive days (n=10/day). Intra-run precision experiments provided average coefficient of variance values of 2.06% and 7.17% for amphetamine HQC and LQC standards respectively, and 2.61% and 3.8% for methamphetamine HQC and LQC standards respectively. Inter-run precision was evaluated to assess the robustness of the methodology. Inter-run precision experiments provided coefficient of variance values of 7.34% and 9.68% for amphetamine HQC and LQC standards respectively, and 7.19% and 7.62% for methamphetamine HQC and LQC standards respectively. Accuracy, which is defined as the closeness of a measured value to the true or accepted value was excellent in all experiments (Tables 3-5). Precision of the analytical methodology was assessed at the lower limit of quantitation (LLOQ) to ensure reproducible results were obtainable at low concentrations of both analytes (Table 3). Replicates of spiked meconium samples (n=10) containing amphetamine and methamphetamine at a concentration of 25 ng/mL were analyzed and results showed excellent accuracy and precision for both analytes (Table 3). Amphetamine was quantified at a mean concentration of 25.61 ng/mL, with a coefficient of variance of 2.96%. Methamphetamine was quantified at an average concentration of 26.71 ng/mL, with a coefficient of variance of 3.14%. Sample stability was also evaluated to ensure that

sample storage protocols would not affect the quantification of either analyte. Blank meconium samples were spiked with amphetamine and methamphetamine to give samples at concentrations of 100, 250, and 500 ng/mL. Samples were frozen for two days after which they were thawed out, extracted and analyzed using the above methodologies. Quantification accuracy was excellent for both analytes at all three concentrations indicating that both amphetamine and methamphetamine remain at stable concentrations in the meconium matrix when stored at -20°C (Table 4). Selectivity, which is defined as the extent to which a method can determine particular analytes in mixtures or matrices without interferences from other components, was assessed by analyzing meconium samples which had been spiked with possible exogenous interferants. Negative meconium samples were spiked to achieve amphetamine and methamphetamine concentrations of 100 ng/mL (n=3). Samples were spiked with  $\Delta$ 9-tetrahydrocannabinol (THC), propoxyphene, methylenedioxymethamphetamine (MDMA), paramethoxyamphetamine (PMA), and cocaine at concentrations of 50, 100, and 250 ng/mL. Samples were analyzed and quantified to ensure selective identification and accurate quantification of both analytes was possible in the presence of possible exogenous interferants. Cocaine, propoxyphene, methylenedioxymethamphetamine, paramethoxyamphetamine, and  $\Delta$ 9-tetrahydrocannabinol were chosen as possible exogenous interferants as they constitute the remainder of analytes currently analyzed by GC/MS at AIT laboratories and accurate quantification of amphetamine and methamphetamine at all three interferant concentrations indicated that the presence of exogenous sample interferants will not affect the analysis of amphetamine and methamphetamine (Table 5). Following method validation, meconium specimens which

had previously screened positive for amphetamines using a cutoff of 50 ng/g were analyzed in order to assess the suitability of the developed method for the analysis of authentic samples. Both analytes were successfully detected and quantified in authentic meconium specimens. Such results are encouraging as they indicate that the established LOD and LOQ are low enough to allow the successful detection of amphetamine and methamphetamine at concentrations typically found in meconium specimens (Figure 2).

## **Conclusions**

Meconium is a complex biological matrix that can indicate prenatal exposure to drugs of abuse. Unfortunately, meconium specimens require extensive sample pre-treatment before they are suitable for instrumental analysis. Extensive sample preparation can prove detrimental to sample turn around time in high throughput toxicology laboratories. ISOLUTE HM-N supported liquid-liquid extraction columns provide an attractive sample pre-treatment technique for the extraction of amphetamine and methamphetamine from meconium specimens. ISOLUTE HM-N columns are designed to deal with difficult matrices such as sonicated meconium specimens which can sometimes cause traditional SPE columns to plug due to sample turbidity. Due to the ISOLUTE HM-N columns ability to retain aqueous samples, sonicated meconium samples can be directly loaded onto the column without the need for a preparative liquid-liquid extraction. ISOLUTE HM-N supported liquid extraction columns require no column conditioning and once the sample has been loaded onto the column, analytes are eluted with two washes of ethyl acetate.

Rapid and selective extraction of both analytes was possible with the columns and subsequent quantitation was achieved in less than 5.0 min using GC/MS. The proposed methodology could greatly reduce the sample preparation time for meconium testing which is becoming an extremely important tool across socioeconomic lines for the identification of prenatal exposure to drugs of abuse.

### **Acknowledgments**

This work was financially supported by AIT laboratories. The authors would also like to express their gratitude to Crystal Stines, Kevin Shanks and Marcie Keys of AIT laboratories for their ongoing support and assistance.

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