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Detection of in-utero ethanol exposure via EtG and EtS analysis in umbilical cord and placenta

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Table of Contents **Pages**

Abstract: Alcohol exposure during pregnancy constitutes one of the leading preventable causes of birth defects, mental retardation and neurodevelopmental disorders in exposed children. According to the 2016 National Survey on Drug Use and Health, alcohol was the second most prevalent substance (8.3%) after tobacco (10%), being this alcohol prevalence higher among 26-44 years old pregnant women than in the 18-25 years old group (9.1 vs 6.5%) (Center for Behavioral Health Statistics and Quality, 2017). The ethanol marker ethyl glucuronide (EtG) has proven to be a specific long-term marker of ethanol in-utero exposure in meconium; however, currently there are scarce or no data about EtG and ethyl sulfate (EtS) in umbilical cord and placenta. These tissues are alternative matrices to meconium that offer critical advantages; as they are always available at birth, their collection is noninvasive and easy, and they are considered waste products. We developed a method for the determination of EtG and EtS in 0.1 g of umbilical cord and placenta, achieving a limit of quantification of 5 ng/g in umbilical cord and 10 ng/g in placenta. Tissues were homogenized in methanol and the analytes of interest were extracted using weak anion exchange solid phase extraction and analyzed by liquid chromatography tandem mass spectrometry (LC-MSMS), in negative mode, monitoring 2 transitions per analyte. The methods were applied to 59 authentic umbilical cord and placenta samples from newborns whose meconium samples were positive for EtG (EtG > 5 ng/g). EtG and/or EtS were detected in 25 umbilical cord samples with ranges of 4.4-528.5 ng/g and 4.3-39 ng/g, respectively, and in 8 placenta samples with ranges of 26.5-266.5 and 11-24.3 ng/g, respectively. EtG and EtS showed a homogenous distribution throughout umbilical cord tissue (n=5). To date, this is the first method to investigate both minor metabolites of ethanol in term umbilical cord and placenta

samples for prenatal ethanol exposure.

1. Introduction

Although drinking alcohol while pregnant has long been regarded as harmful to a developing fetus, according to the Centers for Disease Control and Prevention (CDC), about 10% of pregnant women in US drink alcohol, while a third of that population participates in binge drinking (Tan et al., 2015). Alcohol consumption during pregnancy may happen due to preconceived notions among the population that a minor amount of alcohol during the course of pregnancy can be harmless. However, there is no amount of ethanol deemed safe for consumption during pregnancy (CDC, 2004), which ideally should lead to abstinence from alcohol in this population.

Alcohol exposure places a fetus at risk of developing conditions such as fetal alcohol syndrome and other alcohol related neurodevelopmental disorders which manifest as cognitive impairments (Denkins et al., 2000). Also, this exposure may result in a range of birth defects and subsequent neurodevelopmental problems, including distinctive craniofacial dysmorphology, compromised growth, and cognitive and social impairments (Vaiano et al., 2016). According to a study by Burd, Blair, & Dropps, (2012), due to the reduced fetal metabolic capacity and the reabsorption of ethanol from contaminated amniotic fluid, fetal blood alcohol concentration (BAC) levels can reach those of the mother within 1-2 hours of maternal ingestion. Thus, the effects experienced at these blood alcohol levels can affect the fetus with the same intensity as a grown adult, and because the fetus is in a vulnerable stage of development, such effects have been seen to hinder and negatively affect this crucial developmental period. As a result, the consumption of alcohol over the duration of a pregnancy is seen as an act that directly affects and compromises the well-being of a developing fetus.

While the possibility of birth defects and cognitive impairments is in and of itself detrimental enough, the risk of more life-threatening conditions can manifest from alcohol use during pregnancy. While the connection is still unclear, it has been seen that there is a possible link between the use of alcohol during pregnancy and an increased risk of stillbirth, miscarriage and sudden infant death syndrome (SIDS) (Bailey & Sokol, 2011). Though it is true that causality has not been established, it is wise to err on the side of safety considering the possible risks. In such cases, determining the cause of the above-mentioned outcomes can be imperative in deciding to take medical and/or legal interventions for the act of drinking and its consequences. Thus, it is crucial to be able to objectively determine whether or not there was fetal ethanol exposure and if it could have possibly contributed to such an outcome.

Several matrices and biomarkers have been investigated to determine fetal exposure to ethanol, in combination with the self-reported values of alcohol consumption over the duration of pregnancy. The use of biomarkers is superior to that of self-reported questionnaires alone, due to their lack of sensitivity and reliability (Vaiano et al., 2016). Various analytes studied include the traditional ethanol biomarkers such as the fatty acid ethyl esters (FAEEs), serum carbohydrate deficient aminotransferase and the transaminases aspartate transaminase (AST) and alanine transaminase (ALT), in addition to minor ethanol metabolites ethyl glucuronide (EtG) and ethyl sulfate (EtS). The traditional biomarkers lack the sensitivity and specificity that EtG and EtS afford, thus making them less desirable (Høiseth, Morini, Polettini, Christophersen, & Mørland, 2009). The traditional biomarkers are affected by factors outside of ethanol consumption that compromise the specificity of the data obtained from their analysis. If the origin of

elevated concentration of these biomarkers may be ambiguous, inconclusive data is obtained, and therefore, the goal of identifying in-utero exposure to ethanol is not fulfilled. Additionally, fatty acid ethyl esters have been seen to lack stability in biological samples as meconium (Himes et al. 2015), thus further compromising the data obtained.

EtG and EtS, which are minor metabolites of ethanol, have demonstrated to be specific and long-term biomarkers of ethanol use in different types of biological samples, such as urine, hair and meconium **(**Politi, Morini, Leone, & Polettini, 2006**)**. Meconium, the first feces of a newborn, has been used to detect fetal exposure to ethanol during pregnancy because it is an accumulation of the waste products produced during the second and mainly the third trimesters, therefore it may account for the exposure in that period of time. Several papers have established EtG cutoffs to detect ethanol exposure during pregnancy, based upon evaluation of newborns that were not exposed to ethanol, and the extent of agreement with FAEE concentrations and/or maternal self-reports (Pichini et al., 2012; Bakdash et al., 2010; Himes et al., 2015; Goecke et al., 2014; Morini et al., 2010a). These cutoffs are necessary because baseline EtG and EtS concentrations in meconium may be present due to ethanol exposure from sources other than drinking alcoholic beverages (Morini et al., 2010a; Bakdash et al., 2010). Meconium, however, can be difficult to collect, and its limited sample size can sometimes present a problem when it comes to confirmatory testing. In cases of stress on the fetus, meconium can be passed before birth rendering it inaccessible and thus unusable for analysis (Montgomery et al., 2005).

As an alternative matrix for detection of in-utero exposure to ethanol, umbilical cord has seldom been investigated. Only one study conducted in 2012 determined a

method for the detection of two ethanol biomarkers, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanol (POPE) and EtG, but did not look at EtS (Jones, Jones, Plate, & Lewis, 2012). The inclusion of EtS can provide greater evidence of ethanol consumption as opposed to EtG alone, as they are both direct metabolites of ethanol. Because the cord tissue provides the connection through which the fetus is nourished, it is reasonable that whatever compounds are detected in this tissue will have been passed to the fetus as well. The umbilical cord contains 3 vessels – 2 arteries and 1 vein – which help to circulate nutrients and oxygen from the mother to the growing fetus (Bosselmann & Mielke, 2015). These vessels feed into the hepatic portal vein and inferior vena cava of the fetus (Ellis, 2005), sending these nutrients directly into the bloodstream for circulation and partially bypassing first pass metabolism. Obtaining umbilical cord tissue is not invasive nor does it require extra work, as the umbilical cord is always severed after birth, making it a convenient sample for analysis. Because the cord tissue is available upon birth, there is also no wait time, as is the case with meconium (Palmer, Wood, & Krasowski, 2017), and analytical data can be provided sooner to determine the necessary course of action.

An additional matrix for investigation is the placenta, the organ connecting the fetus to the uterine wall of the mother and allowing nutrient and oxygen exchange between mother and baby (Burton & Jauniaux, 2015). Amongst its many functions, because the placenta is responsible for the transport of nutrients to the fetus, it is hypothesized that substances present in the placenta will be present in the fetus as well. Previous research regarding EtG and EtS in placenta has been conducted, with successful results in the detection of both analytes. Morini et al. (2011) presented a fully validated method applied to authentic placenta samples and paired fetal tissues, while Swortwood

et al. (2018) presented a fully validated method applied to authentic placenta samples and paired fetal liver. Both articles investigated samples obtained from pregnancies voluntarily terminated around 8-20 weeks of gestation, indicating ethanol exposure in the first and early second trimester.

The goal of the present study was to develop and validate analytical methods for the determination of EtG and EtS in umbilical cord and placenta samples. These methods were applied to authentic term umbilical cord and placental samples from newborns whose paired meconium samples tested positive for EtG. Such methods and data will allow for the determination and will improve the interpretation of ethanol exposure during pregnancy, to aid individuals in the clinical and forensic scientific fields in understanding the newborns' outcomes and the medical, legal and social consequences of this exposure. By finding appropriate biomarkers and matrices and utilizing them properly in order to detect in-utero alcohol exposure as early as possible, the risk of developmental damage and long-term impairments can be minimized, therefore improving the livelihood of the affected children.

2. Materials and methods

2.1. Reagents and materials

EtG and EtS were purchased from Cerilliant (Round Rock, TX, USA), as well as the deuterated analogs, EtG-d_5 and EtS-d_5. Reagent grade formic acid, and liquid chromatography mass spectrometry (LCMS) grade acetonitrile, isopropanol, formic acid and methanol were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Reagent grade dichloromethane, ammonium hydroxide and methanol were purchased

from Pharmco-Aaper (Brookfield, CT, USA). Strata-X-AW 33 µm polymeric weak anion exchange cartridges of 3 mL/200 mg were purchased from Phenomenex (Torrance, CA, USA). Sarstedt Inc 10 mL sc tubes 16x100 mm and 350 µL fused insert vials were acquired from Thermo Fisher Scientific. Filter vials nano/Filter vial® 0.2 µm PVDF with red non-slit cap were from Thomson Instrument Company (Oceanside, CA, USA).

2.2. Method optimization

In order to obtain the most efficient method while maximizing sensitivity and specificity, mass spectrometry, chromatography, homogenization and extraction methods were optimized. Mass spectrometry for EtG and EtS was first optimized by determining which transitions to monitor by direct injection (no column) of 2 µL of individual solutions at 0.1 µg/mL. Using the molecular weight and the electrospray ion source in negative mode, the precursor ion was obtained and a list of fragment ions with various intensities were compiled. The two fragments with the highest intensities were used to produce the target transitions. The parameters (entry and exit quadrupole voltages and collision energies) to provide the greatest intensity for these transitions were also optimized and recorded for the methods to be developed. Further optimization was performed by varying the probe voltage and flow rates of nebulizing, drying and desolvation line gases to obtain results with the greatest intensities.

Chromatographic optimization was performed by utilizing methods proposed by other authors (Pragst et al., 2010; Himes, Concheiro, Scheidweiler, & Huestis, 2014; Vaiano et al., 2016) with slight modifications to mobile phase compositions, chromatographic columns, gradients and flow rates. The goal was to produce a gradient in which both analytes, EtG and EtS, were retained for enough time while providing

adequate baseline resolution of peaks. Peak shifting was minimized by including a re-equilibration period after the gradient.

Mechanical homogenization (tissue blender) of the umbilical cord and placenta tissue was optimized by utilizing various solvents including methanol and water with additives to prevent agglutination of the homogenate, such as formic acid and ammonium hydroxide.

Multiple extraction methods for umbilical cord and placenta matrices were investigated and modified to determine the most efficient and successful method (Himes et al., 2014; Sanvisens et al., 2016). Extraction of umbilical cord and placenta samples was complicated by the fact that EtG and EtS appear to be governed by different mechanisms, making their retention and elution complex. Various cartridge chemistries were applied including strong and weak anion exchange, carbon packing and aminopropyl cartridges. Because of the different properties of EtG and EtS, various loading conditions and elution as a 1 or 2 step process were also tested.

2.3. Preparation of calibration and quality control samples

Calibrators were prepared at 5, 25, 50, 100 and 500 ng/g of umbilical cord, and 10, 25, 50, 100 and 500 ng/g of placenta using working solutions containing both EtG and EtS at 0.01, 0.1 and 1 µg/mL each, prepared from serial dilutions of 1 mg/mL stock solutions of EtG and EtS. Internal standard (IStd) EtG-d_5 and EtS-d_5 mixture at 1 µg/mL was prepared in methanol. All solutions were stored in amber vials at -20°C.

2.4. Sample homogenization

Umbilical cord and placenta samples were cut into ~ 2-mm sized portions and weighed to 100 mg (± 5 mg) in a 10-mL Sarsted tubes. Samples were spiked with 25 µL

of the IStd working solution at 1 µg/mL. Scissors and tweezers used for cutting the samples were rinsed with water and methanol and dried before moving onto samples from different donors to avoid cross contamination. Two mL of methanol were added and samples were vortexed and homogenized using a tissue blender Omni International Mixer/Homogenizer (Kennesaw, GA, USA) until there were no longer any visible chunks of the tissues. Trapped chunks of the tissue were manually removed using a wooden toothpick and the blender blades were rinsed between samples by immersing the blades in a 50-mL falcon tube filled halfway with water and turning on the power, then wiping down the instrument. The same process was then repeated using a falcon tube filled with methanol and wiped down before moving onto the next sample. Homogenates were then centrifuged at 7,830 rpm at 4˚C for 10 minutes using an Eppendorf 5430 centrifuge (Hauppauge, NY, USA). The supernatant was collected (~ 2 mL) and submitted to solid phase extraction.

2.5. Solid Phase Extraction (SPE)

Extraction of EtG and EtS in umbilical cord and placenta tissues was performed using Strata-X-AW weak anion exchange 3 mL/200 mg cartridges conditioned with 2 mL of methanol, followed by 2 mL of 1% formic acid in water. The supernatant from the sample homogenates were loaded into the cartridge and allowed to flow via gravity. Cartridges were washed with 2 mL of water and then dried under vacuum (~ 15-20 Hg) for 15 minutes. EtG samples were eluted first via gravity with 2 mL of 5% formic acid in methanol and light vacuum was applied to collect the entire sample. Cartridges were washed with 2 mL of methanol and then briefly dried using vacuum. EtS samples were then eluted via gravity using 5% ammonium hydroxide in acetonitrile and light vacuum

was applied once more to collect the entire sample. Fresh elution solvents were prepared prior to each extraction to maintain the integrity of its properties.

Both eluates were evaporated under a stream of N_2 gas in a Biotage TurboVap LV (Charlotte, NC, USA) at 50˚C. Umbilical extracts were reconstituted in 100 µL of 0.1% formic acid in water, vortexed for a minimum of 15 seconds, centrifuged at 7,830 rpm at 4˚C for 10 minutes using an Eppendorf 5430 centrifuge, and 60 µL were transferred to the injection vials. In the case of the placenta extracts, samples eluted using 5% formic acid in methanol (EtG eluate) contained blood and particles that could not be removed by centrifugation. Because of that, these extracts were reconstituted using 200 µL and filtered using nano filter vials from Thomson Instrument Company. All analyses were carried out injecting 20 µL of sample.

2.6. Instrumentation

Samples were analyzed on a liquid chromatography tandem mass spectrometry LC-MS 8050 instrument from Shimadzu (Columbia, MD, USA). The Nexera HPLC system consisted of a binary LC-20AD XR pump, online degassing unit DGU-20A 3R, cooled autosampler SIL-20A XR and column oven CTO-20AC. The chromatographic column employed was a Kinetex polar C18 column (2.1 mm x 100 mm, 2.6 µm particle size, 100 Å pore size) with a KrudKatcher ULTRA HPLC In-Line Filter guard column (0.5 µm x 0.004 in) from Phenomenex (Torrence, CA, USA). Mobile phase A consisted of 0.1% formic acid in ultra-high purity (UHP) water, and mobile phase B consisted of 0.1% formic acid in LC-MS grade acetonitrile. The column oven was operated at 30˚C and the flow rate was set to 0.3 mL/min. The following gradient adapted from a previously published method in meconium (Himes et al., 2014) was utilized for analysis:

at time 0 minutes gradient is increased from 0% B to 95% B until 3.6 minutes and held at 95% until 7.5 minutes. From 7.5 minutes to 7.6 minutes, the gradient is deceased from 95% B to 0% B and held at 0% until 11 minutes.

The mass spectrometer employed was a triple quadrupole mass spectrometer using an electrospray ionization source operating in negative mode (ESI-). Both methods employed the following source parameters: ionization voltage at -3 kV, nebulizing gas flow at 2 L/min, heating gas flow at 15 L/min, drying gas flow at 5 L/min, interface temperature at 300˚C, heating block temperature at 400˚C and desolvation line temperature at 250˚C. The compound transitions were monitored using the multiple reaction monitoring (MRM) mode. The two transitions for each compound and their deuterated analogs are listed in table 1.

Table 1: MRM transitions monitored and energy conditions for EtG, EtS, and their deuterated analogs.

Compound	Precursor ion	Transition 1	Q1	CE	Q2	Transition 2	Q1	CE	Q2
EtG	220.8	75.2	11	15	14	84.9	15	17	21
EtS	125.1	97.05	13	20	17	80	13	31	14
EtG-d_5	226	75.1	15	17	13	84.9	16	16	23
EtS-d_5	130	98.05	13	18	18	79.95	13	32	14

2.7. Method validation

Guidelines for method validation were obtained from procedures presented by the Scientific Working Group for Forensic Toxicology (Scientific Working Group for Forensic Toxicology (SWGTOX), 2013). Parameters under investigation were linearity, limit of detection (LOD), limit of quantification (LOQ), intra- and inter-day accuracy and precision using quality control (QC) samples, endogenous and exogenous interferences,

carryover, autosampler stability, extraction efficiency, matrix effect and overall process efficiency.

Determination of linearity for the calibration model was investigated over 5 different days using at least 5 non-zero calibrators. Each calibration curve was prepared using a different donor source of either umbilical cord or placenta. The calibration model was evaluated using a least-squares residual model incorporating different weighting factors (non-weighted, $1/x$ and $1/x^2$) to produce the simplest yet most accurate calibration model. Linearity was considered acceptable if the individual residuals were within a ± 20% range of the indicated concentration, and the coefficient of determination was ≥ 0.99. The LOD and LOQ were chosen to be at the same concentration as the lowest non-zero calibrator, and were examined in triplicate over 3 different days using a different donor source for each day. The accuracy and precision of the LOD/LOQ samples were required to be within ± 20% of the established concentration in order to pass.

Inter and intra-day precision and accuracy were assessed using QC samples at 15 ng/g (low QC) and 300 ng/g (high QC). Accuracy and precision were evaluated with triplicate samples at each concentration over 5 different days. Accuracy was determined by measuring the bias via percent error of QC samples, and was determined to be acceptable if results were within a ± 20% range of the indicated concentration. Precision was assessed by measuring the coefficient of variation (CV) between the samples and was considered acceptable if below 20%. The formulas utilized to study these parameters are summarized in Table 2.

Table 2: Formulas for Validation Parameters.

Parameter Studied	Formula Utilized
Accuracy	(Mean Concentration/Nominal Concentration) * 100%
Bias	[(Mean Concentration-Nominal Concentration)/Nominal Concentration)] * 100%
Imprecision (Coefficient of Variation)	(Standard Deviation/Mean Concentration) * 100%

To determine carryover, blank samples from the calibration model, spiked only with IStd, were reinjected directly after the highest calibrator, on 5 different days. Carryover was considered absent if the concentration calculated from the signal at the point of interest was below that of the LOD. Autosampler stability was evaluated by reinjecting 6 QC samples (3 low and 3 high) after 48 hours in the autosampler at 10˚C. Samples were considered stable in the autosampler if the concentrations calculated were within ± 20% of their initial concentration.

Extraction efficiency (EE), matrix effect (ME) and process efficiency (PE) were determined via a series of neats and samples fortified before and after extractions at high QC (HQC) and low QC (LQC) levels. To evaluate, a set of 6 neats was produced by fortifying empty Sarsted tubes with the correct volumes of working solutions and IStd necessary to obtain either 15 ng/g or 300 ng/g. A second set of samples was produced by fortifying 6 blank umbilical cord or placenta samples from different donors at the LQC level, and 6 at the HQC level, and extracting via SPE. These were the “before extraction” samples. A third set of samples was produced by extracting 12 blank umbilical cord or placenta samples from different donors without fortification. After SPE, the samples were fortified in the same manner as the neats and “before extraction” samples – 6 at the LQC

level and 6 at the HQC level. This set was the "after extraction" samples. Samples were evaporated to dryness following the same protocol discussed previously and reconstituted in either 100 µL or 200 µL of 0.1% formic acid in water depending on the procedure protocol. Samples from the neat set and the "after extraction" set were compared to evaluate ME. EE was examined by comparing samples from the "before extraction" and "after extraction" set, and overall PE was evaluated by comparing the neat set with the "before extraction" set. The formulas utilized for studying ME, EE and PE are summarized in Table 3, using mean peak areas for calculations.

Table 3: Formulas for matrix effect, extraction efficiency and process efficiency.

Parameter Studied	Formula Utilized
Matrix Effect	$[(Mean_{After}-Mean_{Neat})/ Mean_{Neat}] * 100\%$
Extraction Efficiency	$(Mean_{Before}/Mean_{After}) * 100\%$
Process Efficiency	$(Mean_{Before}/Mean_{Neat}) * 100\%$

Homogenate stability was also tested using one LQC and one HQC sample. Cord samples were fortified with appropriate volumes of analyte and IStd working solutions and homogenized following the previously mentioned procedure. After centrifugation, sample homogenates were collected and placed in the refrigerator at 4˚C overnight and extracted the following day. These samples were compared to QC samples that were homogenized, extracted and analyzed in the same day to determine sample stability. This stability component was added in order to assess the ability to split sample preparation and sample extraction into 2 days for the analysis of authentic samples in order to maximize efficiency.

Endogenous and exogenous interferences were assessed to determine method specificity. Endogenous interferences were determined by extracting blank umbilical cord or placenta samples and analyzing with the addition of IStd. Using a total of 10 different blank umbilical cord sources and 7 different blank placenta sources, endogenous interferences were assessed by reviewing the blank samples. Exogenous interferences were determined by analysis of neat samples containing common drugs of abuse, including cocaine, cannabis, various amphetamines and opioids, at a concentration equal to that of the highest calibrator (500 ng/g). Interferences were considered absent if the concentration calculated from the signal at the points of interest was below that of the LOD.

2.8. Identification criteria

In order to confirm the presence and identity of substances in a sample, identification criteria must be adhered to. In order to confirm the success of the sample extraction and overall method, the IStd must be present in all of the samples that it was added to. Moving forward, the substance detected must be within a 0.2-minute range of the average retention time of the substance in the calibrators. Two transitions (quantifier and qualifier, indicated in table 1) must also be detected for each compound, and must be present in a specific ratio to one another. This ratio of the qualifier to quantifier transitions must be within 20% of the average determined for the calibrators. Only after all of these standards have been met can a compound be confirmed present.

2.9. Authentic umbilical cord and placenta specimens

Human blank umbilical cord samples were purchased from Lee BioSolutions (Maryland Heights, MO, USA), and were stored at -20°C. Authentic umbilical cord and

placenta samples (blank and positive cases) were collected during a study to investigate alternative matrices to detect in-utero drug exposure (Concheiro et al., 2017). Umbilical cord and placenta samples were collected at delivery in polypropylene containers and stored at -20°C until analysis, and meconium samples were collected from newborn diapers up to 3 days after delivery in polypropylene containers and also stored at -20°C until analysis. The participants were pregnant women who delivered at the University Hospitals of Santiago de Compostela and Vigo, Spain, from January 2012 to December 2015. The participants were informed about the study both in writing and orally before the delivery, and they gave written consent. The subjects were not paid for their participation. The study was approved by the Ethics Committee of the University of Santiago de Compostela, Spain.

EtG meconium analysis was performed at the Toxicology Service of the University of Santiago de Compostela (Spain) following their routine analysis procedure. Briefly, 0.5 g of meconium were ultrasonicated in acetonitrile, and the supernatant was submitted to aminopropyl solid phase extraction cartridges (Sep-Pack NH_2 cartridges, Waters, Milfrod, MA). The analysis was performed by LC-MSMS (Quattro Micro™ API ESCI triple quadrupole, Waters), in ESI-, using 2 transitions per compound. The chromatographic separation was performed using a Hypercarb 100x2.1 mm column (Thermo Fisher Scientific). EtG linearity in meconium was from 5 to 500 ng/g.

3. Results

3.1. Umbilical cord and placenta method validation results

For EtG and EtS in umbilical cord, the method calibration was achieved via a linear, non-forced model. EtG was determined to behave superiorly with an inversely weighted model, while an inverse square model was best suited for EtS. In umbilical cord the LOQ and LOD for EtG and EtS were both 5 ng/g, and the linear range was 5-500 ng/g. Linearity for the method met the criteria for acceptability by demonstrating a coefficient of determination greater than 0.99 and maintaining residuals within a 20% range for both EtG and EtS. No endogenous or exogenous interferences were detected for the method.

For EtG, the inter-day imprecision was between 4.65-11.18% and the intra-day imprecision was between 1.63-11.81%, based on a 5-day analysis of low and high QC samples and LOQ samples. Inter-day accuracy was between 90-95.78% with a bias measurement between -10 -4.22%, assessed via the same QC and LOQ samples, and intra-day accuracy was between 82.67- 102% with a bias measurement between -17.33-2%.

For EtS, the inter-day imprecision was between 1.85-6.81% and the intra-day imprecision was between 1.09-9.08%, based on a 5-day analysis of low and high QC samples and LOQ samples. Inter-day accuracy was between 87.2-100% with a bias measurement between -12.8-0%, assessed via the same QC and LOQ samples, and intra-day accuracy was between 83.61- 102.89% with a bias measurement between -16.39-2.89%. These results are presented in Tables 4 and 5.

There was no evidence of carryover detected for either EtG or EtS, as calculated results for the reinjected blank were below the LOD/LOQ for the method. Auto-sampler stability results demonstrate there was no indication of analyte instability for either EtG or EtS after 24 hours and 48 hours in the auto-sampler at 10˚C. Results after 24 and 48 hours demonstrate a percent difference below 6% and accuracy within ± 13% of the target concentration.

ME, EE and PE were also evaluated at LQC and HQC concentrations for EtG and EtS, as well as the respective deuterated analogs. ME for LQC samples demonstrated a 75.08% ion suppression for EtG with a CV of 10.12% for the after-fortification sample set (n=6), and a 71.54% ion suppression for EtG-d_5 with a CV of 3.91 for the after-fortification sample set (n=6). EtS had a 19.67% ion suppression with a CV of 33.75% for the after-fortification sample set (n=6) and a 28.82% ion suppression for EtS-d_5 with a CV of 16.45% for the after-fortification sample set (n=6). ME for the HQC samples demonstrated a 58.77% ion suppression for EtG with a CV of 6% for the after-fortification sample set (n=6), and a 59.05% ion suppression for EtG-d_5 with a CV of 2.46% for the after-fortification sample set (n=6). EtS had a 9.84% ion suppression with a CV of 8.21% for the after-fortification sample set (n=6), and a 23.08% ion suppression for EtS-d_5 with a CV of 4.31% for the after-fortification sample set (n=6). For EtG and EtS respectively, at the LQC concentration, EE was 74.21% and 83.96%, and PE was 18.49% and 67.44%, and at the HQC concentration, EE was 68.99% and 79.28%, and PE was 28.44% and 71.48%. For EtG-d_5 and EtS-d_5 respectively, at the LQC concentration, EE was 75.71% and 85.89%, and PE was 21.57% and 61.14%, and at the HQC concentration, EE was 71.95% and 81.1%, and PE was 29.47% and 62.38%.

Table 4: Accuracy and imprecision results for EtG in umbilical cord.

		Conc (ng/g)	Day 1	Day 2	Day 3	Day 4	Day 5
Imprecision (%)	**Intra-day (n=3)**	**5**	9.8	2.33	10.87	10	11.81
		15	2.13	9.39	10.14	7.83	2.13
		300	3.83	6.1	1.63	3.94	1.9
	Inter-day (n=5)	**5**	11.18				
		15	7.91				
		300	4.65				
Accuracy (%)	**Intra-day (n=3)**	**5**	102	86	101.33	100	88
		15	94	88.67	94.44	90.22	82.67
		300	89.27	90.52	88.49	96.36	88.93
	Inter-day (n=5)	**5**	95.78				
		15	90				
		300	90.71				

Table 5: Accuracy and imprecision results for EtS in umbilical cord.

		Conc (ng/g)	Day 1	Day 2	Day 3	Day 4	Day 5
Imprecision (%)	**Intra-day (n=3)**	**5**	2.28	1.15	1.15	1.17	2.34
		15	6.4	8.2	9.08	3.34	1.09
		300	7.19	5.9	2.95	1.59	1.98
	Inter-day (n=5)	**5**	1.85				
		15	6.81				
		300	4.9				
Accuracy (%)	**Intra-day (n=3)**	**5**	101.33	100.67	100.67	98.67	98.67
		15	99.33	94.22	102.89	94.22	93.11
		300	90.81	83.61	85.18	88.98	87.42
	Inter-day (n=5)	**5**	100				
		15	96.75				
		300	87.2				

For EtG and EtS in placenta, the method calibration was achieved via a non-forced model. EtG was determined to behave superiorly with a linear, inversely squared weighted model, while a non-weighted quadratic model was best suited for EtS. In placenta the LOQ and LOD for EtG and EtS were both 10 ng/g, and the linear range was

10-500 ng/g. Linearity for the method met the criteria for acceptability by demonstrating a coefficient of determination greater than 0.99 and maintaining residuals within a 20% range for both EtG and EtS. No endogenous or exogenous interferences were detected for the method.

For EtG, the inter-day imprecision was between 5.1 - 9.68% and the intra-day imprecision was between 0.98 - 11.3%, based on a 5-day analysis of low and high QC samples and 3-day analysis of LOQ samples. Inter-day accuracy was between 91.76 – 103.2% with a bias measurement between -8.24 – 3.20%, assessed via the same QC and LOQ samples, and intra-day accuracy was between 88.28 – 112.44% with a bias measurement between -11.72 – 12.44%.

For EtS, the inter-day imprecision was between 5.54 – 7.87% and the intra-day imprecision was between 0.58 – 6.97%, based on a 5-day analysis of low and high QC samples and 3-day analysis of LOQ samples. Inter-day accuracy was between 88.78 – 100.58% with a bias measurement between -11.22 – 0.58%, assessed via the same QC and LOQ samples, and intra-day accuracy was between 83.08 – 108.67% with a bias measurement between -16.92 – 8.67%. These results are summarized in Tables 6 and 7.

Accuracy and imprecision data for days 1 and 2 for EtG and day 1 for EtS are not available due to sample volume of the extract. After evaporation and reconstitution of the sample eluate, samples were centrifuged and a portion was transferred to the analysis vial or filter vial. Due to the limited sample size, multiple injections of the same sample were not possible to study the accuracy and imprecision on that day.

There was no evidence of carryover detected for either EtG or EtS, as calculated results for the reinjected blank were below the LOD/LOQ for the method. Auto-sampler

stability results demonstrated there was no indication of analyte instability for either EtG or EtS after 48 hours in the auto-sampler at 10˚C. Results after 48 hours demonstrate a percent difference below 7% and accuracy within ±7%.

ME, EE and PE were also evaluated at low QC and high QC concentrations for EtG and EtS, as well as the respective deuterated analogs. ME for LQC samples demonstrated a 67.73% ion suppression for EtG with a CV of 10.57% (n=6) for the after-fortification sample set, and a 65.65% ion suppression for EtG-d_5 with a CV of 6.34% for the after-fortification sample set (n=6). EtS has a 5.32% ion suppression for EtS with a CV of 15.97% (n=6) for the after-fortification sample set, and a 0.57% ion suppression for EtS-d_5 with a CV of 8.67% for the after-fortification sample set (n=6). ME for the HQC samples demonstrated a 69.92% ion suppression for EtG with a CV of 7.15% for the after-fortification sample set (n=6), and a 67.1% ion suppression for EtG-d_5 with a CV of 7.1% for the after-fortification sample set (n=6). EtS had a 15.23% ion suppression with a CV of 7.81% for the after-fortification sample set (n=6), and an 8.8% ion suppression for EtS-d_5 with a CV of 5% for the after-fortification sample set (n=6). For EtG and EtS respectively at the LQC concentration, the EE of the process was 81.33% and 92.69%, and the PE was 26.25% and 97.62%, and at the HQC the EE of the process was 75% and 83.43%, and the overall PE was 22.56% and 70.73%. For EtG-d_5 and EtS-d_5 respectively at the LQC concentration, the EE of the process was 77.56% and 86.82%, and the PE was 26.64% and 87.31%, and at the HQC the EE of the process was 76.21% and 83.71%, and the overall PE was 25.08% and 76.34%.

Table 6: Accuracy and imprecision results for EtG in placenta.

		Conc (ng/g)	Day 1	Day 2	Day 3	Day 4	Day 5
Imprecision (%)	**Intra-day (n=3)**	**5**	-----	-----	9.66	7.66	0.98
		15	6.74	11.3	7.82	9.37	8.27
		300	3.65	3.53	4.22	4.62	4.53
	Inter-day (n=5)	**5 (n=3)**	6.18				
		15	9.68				
		300	5.11				
Accuracy (%)	**Intra-day (n=3)**	**5**	-----	-----	102	102	102
		15	112.44	106.67	99.56	102.89	94.44
		300	97.39	88.28	90.1	93.49	89.53
	Inter-day (n=5)	**5 (n=3)**	102				
		15	103.2				
		300	91.76				

Table 7: Accuracy and imprecision results for EtS in placenta.

		Conc (ng/g)	Day 1	Day 2	Day 3	Day 4	Day 5
Imprecision (%)	**Intra-day (n=3)**	**5**	-----	2.96	1.63	0.58	0.58
		15	6.97	1.15	2.28	5.5	6.46
		300	3.2	4.44	3.91	3.25	2.61
	Inter-day (n=5)	**5 (n=4)**	5.76				
		15	7.87				
		300	5.54				
Accuracy (%)	**Intra-day (n=3)**	**5**	-----	108.67	93.67	100.33	99.67
		15	88.89	100	102.89	100.89	108.67
		300	89.53	85.76	94.44	91.1	83.08
	Inter-day (n=5)	**5 (n=4)**	100.58				
		15	100.27				
		300	88.78				

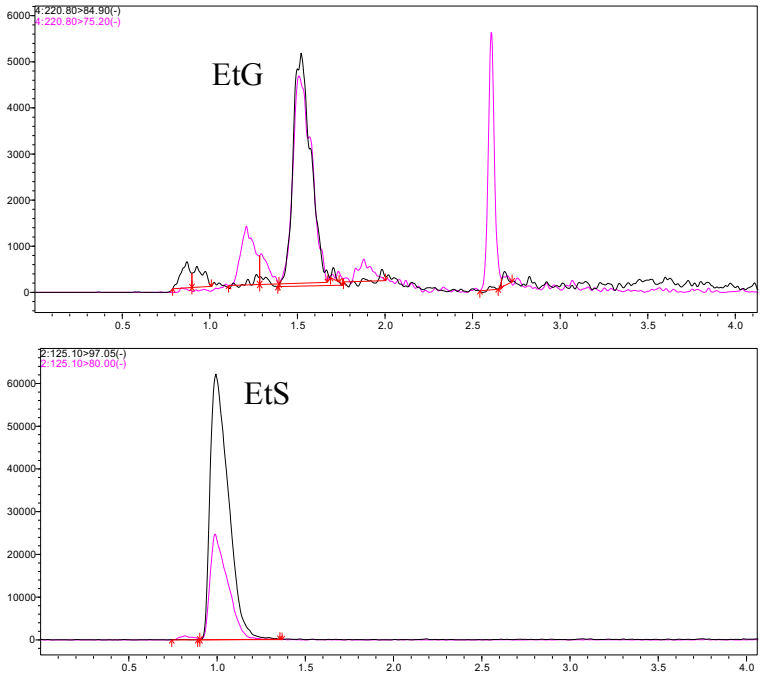
3.2. Authentic specimens' results

3.2.1. EtG and EtS in Umbilical Cord

EtG and/or EtS were detected in 25 umbilical cord samples out of 59. The average±SD EtG concentration detected was 144.91±204.63 ng/g (n=8) and the average±SD EtS concentration was 14.72±11.41 ng/g (n=22). Raw data indicates EtG has

a 13.6% detection rate and EtS has a 37.3% detection rate in umbilical cord. Both EtG and EtS were detected in 5 samples, with concentration ranges of 4.8-528.5 ng/g and 5.2-39 ng/g, respectively. EtG alone was detected in 3 additional samples analyzed (4.4-19.4 ng/g) and EtS alone was detected in 17 additional samples (4.3-38.8 ng/g).

Figure 1: MRM chromatogram of the authentic sample 2013_V31 positive for EtG at 46.4 ng/g and EtS at 22.3 ng/g. In black the quantifier transition, in pink the qualifier transition.



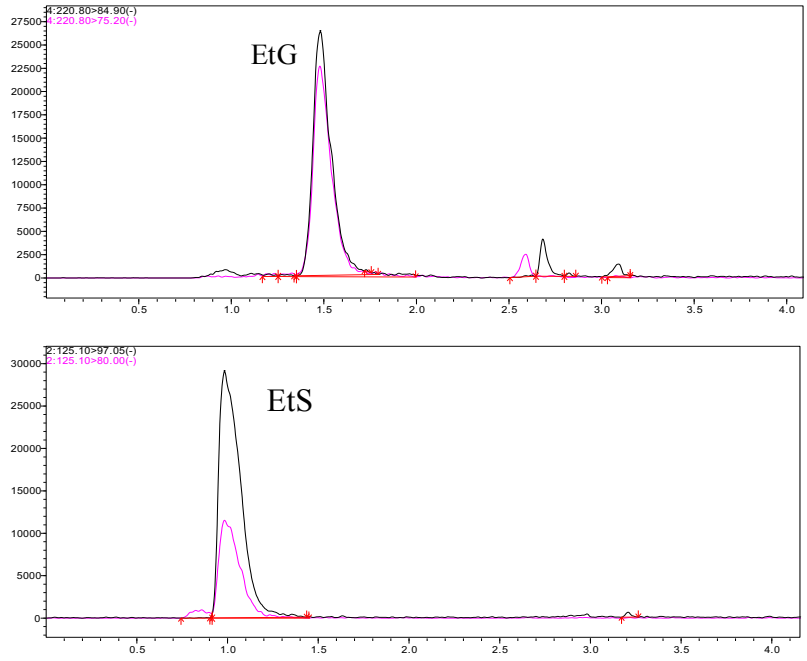
To investigate the distribution of EtG and EtS in umbilical cord, 5 authentic umbilical cord samples, where both EtG and EtS were present at a concentration significantly above the LOQ, were investigated. The range of EtG in the 5 samples the first time they were analyzed was 4.8-528.5 ng/g, and for EtS was 5.2-39 ng/g. The 5 samples of interest were segmented into 3 parts, the top, middle and the end portions of the cord sample. The range of EtG after segmental analysis was 4.9-544.8 ng/g and for

EtS was 5-38.9 ng/g. Both EtG and EtS showed a homogenous distribution across the umbilical cord tissue with a CV < 10.3% (n=5).

3.2.2. EtG and EtS in Placenta

EtG and/or EtS were detected in 8 placenta samples out of 59. The average±SD EtG concentration detected was 129.6±120 ng/g (n=5), while the average EtS concentration was 15.18±5.51 ng/g (n=6). Raw detection rates in placenta were almost the same for both EtG and EtS, at 8.5% and 10.2% respectively. EtG and EtS were both detected in 3 placenta samples with concentration ranges of 76.9 – 266.5 ng/g and 13.2 – 24.3 ng/g, respectively. An additional 2 samples contained EtG only (26.5 – 26.7 ng/g), and an additional 3 samples contained EtS alone (11 – 12 ng/g).

Figure 2: MRM chromatogram of the authentic sample S101 positive for EtG at 251.4 ng/g and EtS at 19.6 ng/g. In black the quantifier transition, in pink the qualifier transition.



3.2.3. Comparison of EtG and EtS in umbilical cord, placenta and meconium

As compared to the 59 meconium samples positive for EtG, 25 umbilical cord samples tested positive for EtG and/or EtS. These paired umbilical cord samples demonstrated a higher frequency of detection for EtS (n=22) than for EtG (n=8). Whereas EtG was detected above 5 ng/g in 59 meconium, 34 paired umbilical cord samples had no detectable EtG or EtS levels above the same cutoff. In the case of placenta, 8 samples tested positive for EtG and/or EtS above 10 ng/g. The frequency of detection of EtG (n=5) and EtS (n=6) in placenta was similar. The average±SD concentration for EtG in meconium was 611.86±2761.2 ng/g, while the average concentration in cord was 144.91±204.63 ng/g and in placenta 129.6±120 ng/g, which were within a similar range to one another as opposed to meconium. EtS in umbilical cord and placenta were also in a similar range, 14.72±11.41 ng/g and 15.18±5.51 ng/g respectively.

In paired meconium samples (EtG > 5 ng/g), there was a 13.6% agreement in EtG detection in umbilical cord (n_{cord}=8, $n_{meconium}$=59). The meconium concentrations in the paired positive samples were 31.5-19,680 ng/g (n=8). Percent agreement was determined by comparing the amount of positive cord samples against the paired positive meconium samples to determine which pairs yielded two positive results in the respective matrices. In pairs where EtG was detected in meconium (EtG > 5 ng/g), there was a 37.3% agreement in EtS detection (n_{cord}=22, $n_{meconium}$=59), and the meconium concentrations in the paired positive samples were 5.62-19,680 ng/g (n=22). Finally, in pairs were EtG was detected in meconium (EtG > 5 ng/g), there was an 8.5% agreement in both EtG and EtS detection in umbilical cord (n_{cord}=5, $n_{meconium}$=59), and meconium concentrations in the paired positive samples were 31.5-19,680 ng/g (n=5).

Applying different EtG meconium cutoffs from various sources to detect in-utero ethanol exposure, we determined the percentage of umbilical cord samples that tested positive for EtG and/or EtS above the LOQ. The meconium EtG cutoffs employed were: 30 ng/g cutoff (Himes et al., 2015); 120 ng/g cutoff (Goecke et al., 2014); 274 ng/g cutoff (Bakdash et al., 2010); 333 ng/g cutoff (Morini et al., 2010b); and 444 ng/g cutoff (Pichini et al., 2012). These results are summarized in Table 8.

Table 8: Various rates of detection in umbilical cord based on meconium EtG cutoffs.

EtG meconium cutoff	N paired samples	% Agreement with umbilical cord samples (# of samples)			
		EtG positive	EtS positive	EtG and EtS positive	EtG or EtS positive
5 ng/g	59	13.6% (8)	37.3% (22)	8.5% (5)	42.4% (25)
30 ng/g	44	18.2% (8)	40.9% (18)	11.4% (5)	47.7% (21)
120 ng/g	18	22.2% (4)	44.4% (8)	16.7% (3)	50% (9)
274 ng/g	12	33.3% (4)	41.7% (5)	25% (3)	50% (6)
333 ng/g	10	40% (4)	50% (5)	30% (3)	60% (6)
444 ng/g	8	50% (4)	62.5% (5)	37.5% (3)	75% (6)

In paired meconium samples (EtG>5 ng/g), there was an 8.5% agreement in EtG detection in placenta ($n_{placenta}$=5, $n_{meconium}$=59). The meconium concentrations in the paired positive samples were 68.82-19,680 ng/g (n=5). In pairs where EtG was detected in meconium (EtG>5ng/g), there was a 10.2% agreement in EtS detection in placenta ($n_{placenta}$=6, $n_{meconium}$=59), and the meconium concentrations in the paired positive samples were 23.8-19,680 ng/g (n=6). Finally, in pairs were EtG was detected in meconium, there was a 5.1% agreement in both EtG and EtS detection in placenta ($n_{placenta}$=3, $n_{meconium}$=59), and meconium concentrations in the paired positive samples were 68.82-

19,680 ng/g (n=3). The remainder of the data utilizing various meconium EtG cutoffs is presented in Table 9.

Table 9: Various rates of detection in placenta based on meconium EtG cutoffs.

EtG meconium cutoff	N paired samples	% Agreement with placenta samples (# of samples)			
		EtG positive	EtS positive	EtG and EtS positive	EtG or EtS positive
5 ng/g	59	8.5% (5)	10.2% (6)	5.1% (3)	13.6% (8)
30 ng/g	44	11.4% (5)	11.4% (5)	6.8% (3)	15.9% (7)
120 ng/g	18	22.2% (4)	11.1% (2)	11.1% (2)	22.2% (4)
274 ng/g	12	33.3% (4)	16.7% (2)	16.7% (2)	33.3% (4)
333 ng/g	10	40% (4)	20% (2)	20% (2)	40% (4)
444 ng/g	8	50% (4)	25% (2)	25% (2)	50% (4)

3.2.4 Comparison of EtG and EtS in biological samples and self-reports

Among the 59 cases analyzed, one mother reported daily alcohol use during the pregnancy, 9 reported alcohol consumption on the weekends, 18 sporadic use (sometimes during pregnancy), 21 no use, and 10 did not answered. In the case of the daily user, the meconium EtG was the highest (19,680 ng/g) and EtG and EtS were detected in umbilical cord (397.5 and 32.1 ng/g respectively) and in placenta (251.4 ng/g and 19.6 ng/g).

In the cases where alcohol use was reported on the weekends (n=9), meconium EtG concentrations ranged from 11.6 to 490.8 ng/g. EtG and/or EtS were detected in 5 umbilical cord samples (EtG, 145.1 ng/g, n=1; EtS, 5.4-38.8 ng/g, n=5), and in 2 placenta samples (EtG, 76.9 ng/g, n=1; EtS, 11-13.2 ng/g, n=2). If sporadic alcohol use was declared (n=18), meconium EtG concentrations ranged from 5.2 to 370.3 ng/g. EtG and/or EtS were detected in 7 umbilical cord samples (EtG, 4.8-19.4 ng/g, n=2; EtS, 4.5-

26.3 ng/g, n=6), and in 2 placenta samples (EtG, 26.5 ng/g, n=1; EtS, 11 ng/g, n=1).

Although 21 mothers declared no alcohol use during pregnancy, EtG was detected in meconium at concentrations from 7.9 to 792.6 ng/g. Ten umbilical cord samples were positive for EtG and/or EtS (EtG, 13.2-46.4 ng/g, n=2; EtS, 4.3-22.3 ng/g, n=9), and 2 placenta samples (EtG, 26.7 ng/g, n=1; EtS, 12 ng/g, n=1). In the 10 cases where the mothers did not answer, EtG was detected in meconium from 19.7 to 8,549 ng/g. Two cases were positive for EtG and/or EtS in umbilical cord (EtG, 4.4-528.5 ng/g, n=2; EtS, 39 ng/g, n=1), and one placenta was positive for EtG (266.5 ng/g) and EtS (24.3 ng/g).

4. Discussion

We developed and validated two sensitive and specific analytical methods for the determination of EtG and EtS in both umbilical cord and placental tissues. From a single sample aliquot of 0.1 g, the samples were homogenized in a tissue blender, extracted by weak anion exchange solid phase extraction and analyzed by LC-MSMS. We were able to achieve an LOD/LOQ of 5 ng/g and a linear range of 5-500 ng/g for both EtG and EtS in cord tissue and an LOD/LOQ of 10 ng/g and linear range of 10-500 ng/g for both analytes in placenta. Most methods have been developed for use with meconium and have looked at both EtG and EtS with success (Himes et al., 2014; Morini et al., 2010a; Morini et al., 2010b); however, the literature in umbilical cord and placenta is scarce. Only one analytical paper has been published for the determination of EtG in umbilical cord (Jones et al., 2012) and two for the determination of EtG and EtS in placenta (Morini et al., 2011; Swortwood et al., 2018). All of these publications employed LC-MSMS as the analytical technique. Jones et al. reported a lower EtG LOQ of 3 ng/g than

in our method (5 ng/g), but that method required 1 g of umbilical cord (x10 the amount required in our method) and EtS was not included. Umbilical cord samples were homogenized with a bullet blender and extracted by strong anion exchange solid phase extraction (Jones et al., 2012). Morini et al. (2011) developed a method for the determination of EtG and EtS in 0.5 g of placenta achieving a LOQ of 5 ng/g. EtG and EtS were extracted by briefly vortexing the tissue in acetonitrile, and after 1:10 dilution in water, the samples were injected in the LC-MSMS instrument. Swortwood et al. (2018) homogenized 0.25 g of placenta in methanol in a bead mill, and half of the aliquot was employed to extract EtG and EtS by strong anion exchange solid phase extraction following a procedure previously described by these authors for meconium (Himes et al., 2014). The LOQ was 20 ng/g for EtG and 5 ng/g for EtS.

The present developed and validated methods were applied to 59 authentic umbilical cord and placenta samples, from newborns whose meconium tested positive for EtG (EtG > 5 ng/g). EtG was detected in 8 umbilical cord samples with concentration ranges of 4.4-528.5 ng/g. Jones et al. (2012) applied their method to 308 de-identified umbilical cord samples that were received by their laboratory for routine toxicological analysis. They detected EtG in 12 samples with a concentration range of 4-666 ng/g, similar to the concentrations determined in our study. We observed that EtS was detected in a total of 22 cases (4.3-39 ng/g), 5 cases along with EtG and 17 cases alone. Morini et al. (2010a, b) noted that the presence of EtS without EtG may indicate a false positive in meconium, as it is advisable to have both markers to confirm ethanol consumption. However, in this study the EtS only cases in umbilical cord were positive for EtG in meconium (5.63-792.6 ng/g), suggesting ethanol exposure during pregnancy. The

observed differences between EtG and EtS detection in umbilical cord samples could be explained due their different stability in biological samples. EtG is prone to bacterial decomposition, while EtS is not (Baranowski et al., 2008). Currently, there are no data available about the stability of EtG and EtS in this type of biological samples, therefore further research is necessary.

Regarding placenta, EtG was detected in 5 samples and EtS in 6 out of 59 cases, with concentration ranges of 26.5 – 266.5 ng/g and 11 – 24.3 ng/g, respectively. EtG and EtS were detected together in 3 cases, EtG only in 2 cases and EtS only in 3 cases. Morini et al. (2011) analyzed EtG and EtS in 35 placenta samples from fetuses voluntarily terminated at 12 weeks of gestation. Four samples were positive for both EtG and EtS and only 2 samples were positive for only EtS. The concentration ranges for EtG and EtS respectively were 122-1306.49 ng/g and 9.59-175.32 ng/g. Swortwood et al. (2018) applied their method for the determination of EtG and EtS in placenta of 48 authentic samples from pregnancies voluntarily terminated at 8-20 weeks. Ten samples were positive for both EtG and EtS, and an additional 6 samples were positive for EtG only and 7 for EtS only. The concentration range for EtG and EtS respectively was 34.3-1168 ng/g and 6.9-214 ng/g. Both studies reported higher maximum concentrations for EtG and EtS in placenta from terminated pregnancies by 8-20 weeks, than in our study from term placentas. Different alcohol consumption pattern among the mothers, or tissue differences due to the week of gestation, may explain these differences.

Currently, there are no data available comparing EtG and EtS in umbilical cord and other tissues. Regarding placenta, comparisons of placenta and other tissues, such as fetal and liver tissues, have been done previously (Morini et al., 2011; Swortwood et al.,

2018). Morini et al. (2011) analyzed 35 fetal tissue-placenta pairs at gestational week 12. Four cases tested positive for EtG and/or EtS in fetal tissue and placenta, and 2 placenta samples tested positive for EtS but negative in fetal tissue. Swortwood et al. (2018) compared 47 paired placenta and fetal liver samples at gestational week 8-20. Twenty-one were negative in both samples; 11 cases showed EtG and EtS in liver and placenta, and in 15 cases EtG and/or EtS were detected in only one matrix (12 only in placenta, and 3 only in liver).

In the present study, we compared for the first time EtG and EtS detection in matched umbilical cord, placenta and meconium samples. We observed higher concentrations of EtG in meconium compared to umbilical cord and placenta, while umbilical cord and placenta showed similar range of concentration for EtG and EtS. Out of 59 cases with positive meconium results (EtG > 5 ng/g), EtG and/or EtS were detected in 25 umbilical cord and in 8 placenta samples. Different distribution/accumulation of drugs and metabolites have been reported in umbilical cord, placenta and meconium, due to the tissues different composition, formation and dynamics, and chemical properties of the analytes (Concheiro & Huestis, 2018). Several studies showed a good agreement between meconium and umbilical cord for cocaine, amphetamines, opioids, benzodiazepines, cannabis and tobacco detection, but in general meconium had better sensitivity (Concheiro et al., 2017; Labardee et al., 2017; Colby et al., 2017; Kim et al., 2017; Marin et al., 2011). Comparisons with placenta are scarce. Previous studies from our group showed similar concentrations in placenta and umbilical cord for cocaine, opioids and methadone (Concheiro et al., 2010; Concheiro et al., 2017). Regarding tissue distribution, we investigated the distribution of EtG and EtS in the umbilical cord. As

already reported for other drugs of abuse (de Castro et al., 2011), we observed a homogeneous distribution of EtG and EtS throughout the umbilical cord tissue.

We compared the umbilical cord and placenta results with different cut-offs of EtG in meconium, which has been suggested in the literature (30, 120, 274, 333 and 444 ng/g). The 30 ng/g cutoff was determined by Himes et al. (2015), comparing the extent of agreement between positive meconium samples and maternal self-reports of ethanol consumption (Himes et al., 2015). Goecke et al. (2014) also determined their meconium EtG cutoff at 120 ng/g based on analysis of the statistically significant p-value when studying their results against self-reports. Bakdash et al. (2010) utilized FAEE concentrations >500 ng/g in conjunction with EtG concentrations to establish a meconium EtG cutoff at 274 ng/g. Morini et al. (2010b) utilized both EtG and EtS, as a confirmation, in order to determine a meconium cutoff. They did so by establishing the increase in positive samples as the cutoff value for the two target analytes was increased and then cross comparing those levels with FAEEs levels. Morini et al. (2010b) determined that when the cutoff for EtG was increased to 333 ng/g, all of the samples that tested positive were also positive for EtS at 1.5 ng/g or higher. Finally, Pichini et al. (2012) established the EtG cutoff at 444 ng/g by comparing meconium samples from teetotalers against meconium samples from births where mothers admitted to drink ethanol during pregnancy and meconium FAEEs were > 2 nmol/g. We found agreements up to 75% with umbilical cord and 50% with placenta, when the highest cutoff (Pichini et al., 2012) was applied.

5. Conclusion

We have successfully developed and validated two analytical methods for the

detection of maternal alcohol consumption and fetal alcohol exposure via EtG and EtS in umbilical cord and placenta. Our method proved to be sensitive and specific, needing only 0.1 g tissue to achieve 5 and 10 ng/g LOQ in umbilical cord and placenta, respectively.

Umbilical cord and placenta showed to be alternative matrices to meconium to detect ethanol in-utero exposure, showing umbilical cord a better agreement with meconium than placenta. We demonstrated a homogenous distribution of EtG and EtS throughout the umbilical cord tissue. Further research is guaranteed to investigate in more depth the window of detection of umbilical cord and placenta for EtG and EtS, and the amount of ethanol exposure that yields positive results in these biological samples.

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