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# Quantification of fatty acid ethyl esters (FAEE) and ethyl glucuronide (EtG) in meconium for detection of alcohol abuse during pregnancy: Correlation study between both biomarkers

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ABSTRACT

This article presents results from 47 meconium samples, which were analyzed for fatty acid ethyl esters (FAEE) and ethyl glucuronide (EtG) for detection of gestational alcohol consumption. A validated microwave assisted extraction (MAE) method in combination with GC–MS developed in the Institute of Forensic Science (Santiago de Compostela) was used for FAEE and the cumulative concentration of ethyl myristate, ethyl palmitate and ethyl stearate with a cut-off of 600 ng/g was applied for interpretation. A simple method for identification and quantification of EtG has been evaluated by ultrasonication followed solid phase extraction (SPE). Successful validation parameters were obtained for both biochemical markers of alcohol intake. FAEE and EtG concentrations in meconium ranged between values lower than LOD and 32,892 ng/g or 218 ng/g respectively. We have analyzed FAEE and EtG in the same meconium aliquot, enabling comparison of the efficiency of gestational ethanol exposure detection. Certain agreement between the two biomarkers was found as they are both a very specific alcohol markers, making it a useful analysis for confirmation.



## 1. Introduction

Heavy drinking constitutes a significant problem in our societies as alcohol is the most popular legal drug used in our times. Its chronic consumption is a known cause of many medical conditions due to the toxic effects of ethanol or its metabolites. In addition, chronic heavy drinking during pregnancy often leads to the appearance of the fetal alcohol syndrome (FAS), the most well known consequence of FASD (fetal alcohol spectrum disorders). It is characterized by facial dysmorphology, reduced growth, neurological deficit, mental disorders, development disabilities and mental retardation [1]. Most ingested ethanol (90–98%) is oxidized to acetaldehyde in the liver, with only a small percentage undergoing non-oxidative metabolism yielding fatty acid ethyl esters (FAEE) or ethyl glucuronide (EtG), whose windows of detection are longer than ethanol [2]. Because of these reasons, the availability of reliable biological markers of ethanol consumption becomes an extremely important issue in both clinical and forensic settings to prove recent use of ethanol and/or chronic heavy drinking. It includes markers of alcohol related toxic effects, such as mean corpuscular volume (MCV) and gamma-glutamyltransferase (gGT); indirect markers, such as carbohydrate-deficient transferrin (CDT) and 5-hydroxytryptophol (5-HTOL); and direct markers, including blood ethanol itself, as well as alcohol derivatives such as fatty acid ethyl esters (FAEE), ethyl glucuronide (EtG), ethyl sulfate (EtS), and phosphatidylethanol (PEth) [3].

The FAEE group consists of more than 20 different compounds from which ethyl laurate (E12:0), ethyl myristate (E14:0), ethyl palmitate (E16:0), ethyl palmitoleate (E16:1), ethyl stearate(E18:0), ethyl oleate (E18:1), ethyl linoleate (E18:2), ethyl linoleneate (E18:3), ethyl arachidonate (E20:4), and ethyl docosahexanoate (E22:6) were included in different combinations in meconium analysis in different studies [4–10]. FAEE are formed by an enzymetic esterification of ethanol with free endogenous fatty acids, triglycerides, lipoproteins and phospholipids by two enzymes, FAEE synthase and acyl-CoA/ethanol O-acyl-transferase

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(AEAT) [1]. They have a marked lipophilic character and are stable at neutral pH. An important finding for the evaluation of gestational ethanol exposure is the fact that FAEE do not cross the placenta into the fetal circulation but, since they can be detected in fetal matrices, must be produced in the fetus itself from the ethanol which crosses the placenta.

EtG (ethyl glucuronide) is a minor, stable, nonvolatile, water-soluble and direct phase II metabolite of ethanol formed after conjugation with glucuronic acid via UDP-glucuronosyl transferases. The specificity and sensitivity of EtG exceed those of all other known ethanol markers, being detectable only if alcohol has been consumed [11,12].

Meconium is the first stool passed by the newborn within 72 h after birth. It is a dark viscous material and contains 60–80% water. It begins forming at approximately 12–13 weeks of gestation but serves as a reservoir of fetal chemical exposure only two to at maximum three months before birth considering that the amount at the beginning is very low and its exponential growth with time. That is why it provides a long time window for detection of chronic exposure to alcohol. Meconium is an optimum sample because of ease of collection and its analysis results in high sensitivity and specificity [10].

In the present study, FAEE and EtG were determined in 47 meconium specimens. FAEE were measured by microwave assisted extraction in combination with GC–MS according to a previously described validated procedure for E14, E16 and E18 [1]. A new LC–MS–MS method was developed and validated for quantification of EtG from meconium. It is investigated by comparing whether the two markers lead to similar results and whether the combined use enables improved interpretation with respect to gestational ethanol exposure.

## 2. Material and methods

### 2.1. Meconium samples and selection of cases

Meconium, collected from the diapers within the first 24–48 h of the newborn's life, was placed in a plastic tube, homogenized, and immediately stored at −20 °C. It was sent to the Laboratory of Toxicological Analysis of the Institute of Forensic Science (University of Santiago de Compostela) and the Laboratory of Forensic Toxicology of the Institute of Public Health, Section of Legal Medicine of the Catholic University (Rome) to measure the analytes concentration. The specimens were obtained from the neonatology wards of the Complejo Hospitalario Universitario of Santiago de Compostela (CHUS) with signed informed consent from all the newborns' parents. It also provided all the information on mother–infant dyads when possible. The age range of the pregnant women ranged from 25 to 43. All of the expectant mothers had a pregnancy controlled by the Galician Health Service. Only 4 women admitted the occasional consumption of alcohol from the total women included in the study.

Out of the 110 meconium samples collected, 47 were divided up to analyze FAEE and EtG because of an insufficient sample quantity and frozen at −20 °C until analysis. After analysis, the samples were stored at −20 °C for possible repetitions.

### 2.2. Standards and reagents

Ethyl myristate, ethyl palmitate, and ethyl stearate were purchased from Sigma–Aldrich (Madrid, Spain). Because the deuterated standards d5-ethyl myristate, d5-ethyl palmitate, and d5-ethyl stearate were not available commercially they were prepared in our laboratory by a published method [13]. All the reagents used for the manufacture of the deuterated FAEE were also obtained from Sigma–Aldrich.

Ehyl glucuronide and d5-ethyl glucuronide were purchased from Cerilliant. SPE Bond Elut $NH_2$ columns were purchased from Varian (Barcelona, Spain). All other reagents were obtained in analytical grade purity from Merck (Barcelona, Spain). Distilled water was obtained from a Milli-Q water system (Millipore, Bedford, MA, USA).

### 2.3. Sample preparation for FAEE determination in meconium

Ethyl myristate, ethyl palmitate and ethyl stearate were analyzed according to an optimized and validated method described previously by Cabarcos et al. [1]. The technique selected was MAE because of the good resolution of the chromatographic peaks and the high recoveries obtained. This extraction procedure has never used before to separate the compounds of meconium, except in the case of Illán et al. [14], who used it combined with head space-solid phase microextraction (HS-SPME) to extract the FAEE from skin. As a brief description of the procedure, 50 μl of a solution of the internal standards d5-ethyl myristate, d5-ethyl palmitate and d5-ethyl stearate (10 μg/mL in *n*-heptane), 1 mL $H_2O$ and 12 mL hexane were added to the vessels used for microwave assisted extraction with 500 mg of meconium. MAE temperature program was 2 min heating from room temperature to 90 °C, which was held for 10 min. After extraction, the vessels were cooled to room temperature before opening. After centrifugation for 5 min the extracts were transferred to tubes for preconcentration. They were evaporated to dryness with an inert gas stream ($N_2$) at 40 °C, resuspended in 40 μL hexane and vortex mixed for 5 min. A 2 μL volume was then injected for GC–MS analysis.

The conditions for GC–MS separation and detection were described previously [1]. Limits of detection and quantification were 50 and 100 ng/g for all analytes except ethyl stearate (LOD 100 ng/g and LLOQ 500 ng/g). Calibration curves were linear from the LLOQ to 5000 ng/g. A cumulative sum of 600 ng FAEE/g meconium (2 nmol/g) was considered positive, suggesting heavy prenatal ethanol exposure.

### 2.4. Sample preparation for EtG determination in meconium

In addition to FAEE testing in meconium and the purpose of discriminating between heavy maternal ethanol use, occasional use or no use at all, the determination of EtG in meconium was proposed as alternative biomarker. In 2008, for the first time, Morini et al. [15] validated a method based on LC-MS/MS with postcolumn addition of acetonitrile for the measurement of EtG in meconium. In the present paper, EtG was extracted from meconium by solid phase extraction. To 500 mg of meconium, 2 mL of acetonitrile and 50 ng/g d5-ethyl glucuronide was added. After ultrasonication for 15 minutes and centrifugation for 10 minutes, the mixture was applied to a Bond Elut $NH_2$ solid phase extraction (SPE) cartridges previously conditioned with 2 mL of methanol, followed by 2 mL of deionized water and 2 mL of acetonitrile. The SPE cartridges were then washed with 2 mL of n-hexane. Elution was done using 2 mL of water. The eluate was then evaporated to dryness under a stream of nitrogen at 40 °C. The residue was dissolved with 100 μL of mobile phase. Finally, 20 μL was injected into a liquid chromatography–tandem mass spectrometry (LC–MS/MS) using electrospray ionization in negative mode. Chromatograhic separation was achieved using a Synergi Polar column (150 mm × 2 mm; Phenomenex). A binary gradient consisting of (A) $H_2O$/formic acid and (B) methanol was used. Detection was performed in the multiple reaction monitoring (MRM) mode. The precursor ion (*m*/*z* 221) used was at *m*/*z* value corresponding to the molecular ion minus one proton. Two product ions (*m*/*z* 75, 85) can be obtained