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Liquid chromatography–tandem mass spectrometry for fatty acid ethyl esters in meconium: Assessment of prenatal exposure to alcohol in two European cohorts☆

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ABSTRACT

Fatty acid ethyl esters (FAEEs) in meconium emerged as a reliable, direct biological marker for establishing fetal exposure to ethanol. We developed an LC–MS/MS method for ethyl laurate, ethyl myristate, ethyl palmitate, ethyl palmitoleate, ethyl stearate, ethyl oleate, ethyl linoleate, ethyl linolenate, and ethyl arachidonate using ethyl heptadecanoate as the internal standard. The analytes were extracted from meconium with hexane, followed by solid-phase extraction with aminopropyl-silica columns. Chromatography was performed on a C_8 reversed-phase column using water/isopropanol/acetonitrile (20:40:40, v/v/v) as a mobile phase. A triple quadrupole mass spectrometer that monitored the transitions in multiple reaction-monitoring mode was used for the detection of the analytes. Limits of quantification (LOQs) varied between 0.12 and 0.20 nmol/g. Calibration curves were linear from LOQs to 50 nmol/g for all analytes, with a minimum $r^2 > 0.99$. At three concentrations spanning the linear dynamic range, mean recoveries ranged between 53.6 and 86.7% for the different analytes. The validated method was applied to analysis of meconium in newborns of two European cities. The two cohorts presented with different prevalence of gestational ethanol consumption during pregnancy.

1. Introduction

Consumption of ethanol during pregnancy is the leading preventable cause of neurodevelopmental delay in North America. Maternal alcohol use during pregnancy can cause Fetal Alcohol Spectrum Disorder (FASD), which is estimated to affect 1% of all North American live births [1]. FASD is an umbrella term used to describe a wide range of potentially lifelong effects that include physical, mental, behavior, and learning disabilities [2]. In order to diagnose the disorder and institute early intervention, preferably before the development of secondary disabilities, early detection of *in utero* exposure is of utmost importance [3].

In recent years, fatty acid ethyl esters (FAEEs) found in neonatal matrices such as meconium and neonatal hair emerged as a reliable, direct biological markers for the assessment of gestational alcohol exposure [4–8].

Although ethanol is the most widely consumed teratogen, most of the research on the prevalence of Fetal Alcohol Spectrum Disorder has come from North America.

In Europe, despite abundant alcohol consumption, the issue of maternal drinking during pregnancy is largely ignored.

For the first time in Europe, the "Meconium Project" was initiated in order to estimate the prevalence of drug use by pregnant women and the effects of chronic illicit drug exposure on the fetus and infant. This was achieved by meconium analysis, maternal structured interview and clinical observations. The first mother–infant dyads cohort was recruited in Barcelona, Spain, and a high prevalence of opiates (8.7%), cocaine (4.4%) and cannabis (5.3%) in meconium specimens was reported [9,10]. The assessment of fetal exposure to alcohol was the next step of the project, together with the addition of a second cohort of mother–infant dyads from Reggio Emilia, Italy.

Published methods for the analysis of FAEEs in meconium are mainly based on gas chromatographic separation of the compounds, coupled either with flame ionization detection (FID) or mass spectrometry [8,4,11,13]. In this latter case, chemical

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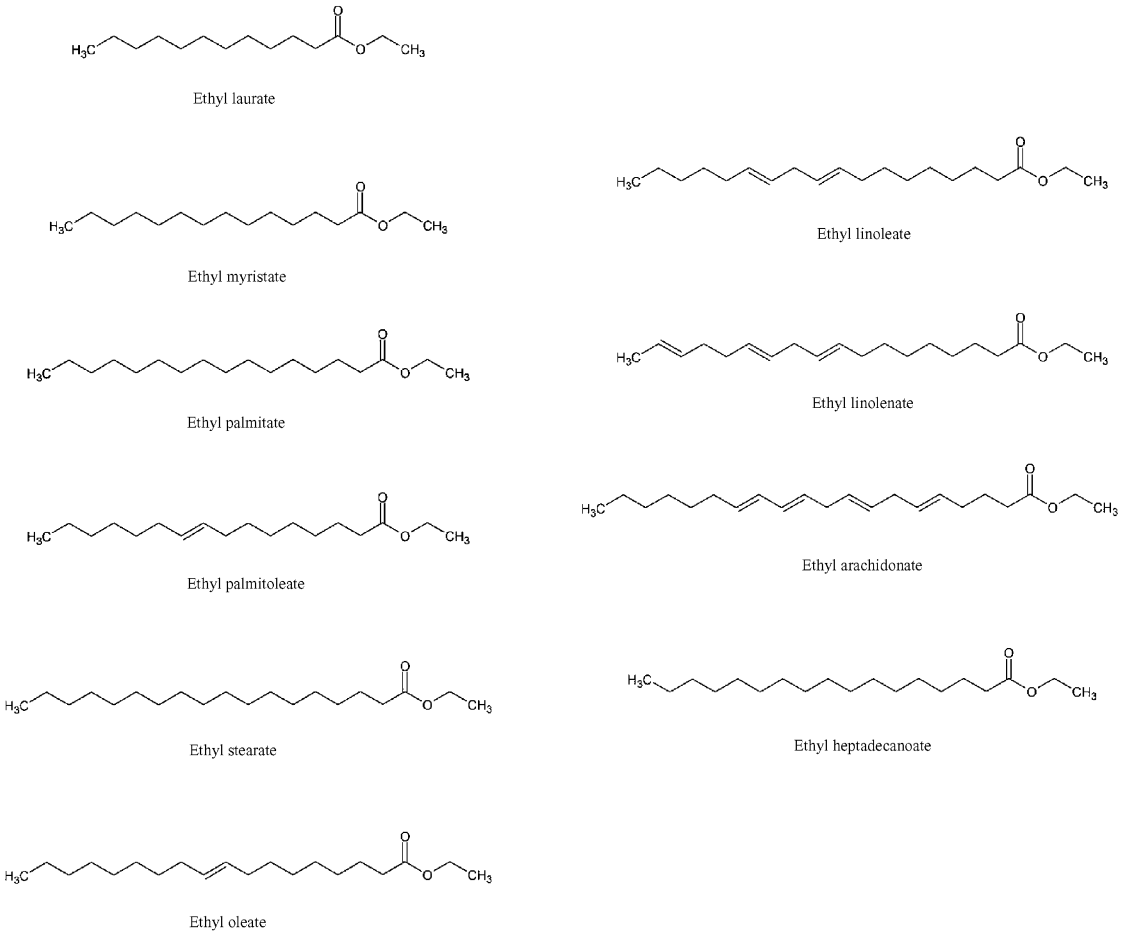

Fig. 1. Molecular structures of nine fatty acid ethyl esters.

ionization has been applied for improved detection and because electron impact ionization of these compounds yielded identical fragments for the various FAEEs [8,13].

New ionization techniques have made liquid chromatography–mass spectrometry (LC–MS) and LC–tandem MS (LC–MS/MS) extremely effective for the specific determination of different analytes in complex biological matrices. Within the framework of the "Meconium Project" LC–MS methodologies have been developed and validated for the detection of several drugs of abuse and metabolites in meconium [14–17].

We describe here an LC–MS/MS assay for the determination of the fatty acid ethyl esters ethyl laurate, ethyl myristate, ethyl palmitate, ethyl palmitoleate, ethyl stearate, ethyl oleate, ethyl linoleate, ethyl linolenate and ethyl arachidonate (Fig. 1) in meconium and its application to assess fetal exposure to alcohol in the above-reported cohorts.

2. Experimental

2.1. Chemicals

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 linolenate (E18:3), ethyl arachidonate (E20:4) esters were obtained from Sigma–Aldrich (Milan, Italy). The internal standard (IS) ethyl heptadecanoate ester (E17:0), 10 mg/L in hexane, was obtained from Chebios (Rome, Italy). Aminopropyl-silica solid-phase extraction (SPE) columns (100 mg sorbent amount, 1 mL volume, 40 μm particle size) were purchased from Varian (Turin, Italy). Ultrapure water and all other reagents of analytical grade were obtained from Carlo Erba (Milan, Italy).

2.2. Instrumentation

LC–MS/MS analyses were performed using a Waters Alliance HPLC system interfaced to a Micromass Quattro micro API triple quadrupole mass spectrometer equipped with electrospray ionization (ESI) probe (Waters, Etten-Leur, The Netherlands).

Chromatographic separation was achieved using an Eclipse XDB-C8 column (150 × 4.6 mm, 5 μm, Agilent Technologies, Palo Alto, CA) with water:isopropanol:acetonitrile (20:40:40, v/v/v) as the mobile phase at a flow of 0.7 mL/min. All chromatographic solvents were degassed with helium before use. The column temperature was set at 30 °C.

MS/MS characterization of the compounds under investigation was achieved using the triple quadrupole and electrospray ionization in positive ionization mode. The, analytes, dissolved in mobile phase at a concentration of 10 mg/L, were infused through an integrated syringe pump into the ESI probe at a rate of 10 μL/min for tuning the mass spectrometer and optimizing the acquisition parameters. The following conditions, optimized during method development to obtain the best assay sensitivity and specificity, were used: capillary voltage at 4.5 kV, source temperature at 120 °C,

Table 1
MRM (*m*/*z*) transitions for quantification of fatty acid ethyl esters (FAEEs)

FAEE	MRM transition (*m*/*z*)	Cone voltage (V)	Collision energy (eV)	Retention time (min)
Ethyl laurate	229 → 201	20	10	6.72
Ethyl linolenate	307 → 261	15	15	8.09
Ethyl myristate	257 → 229	15	10	9.25
Ethyl arachidonate	333 → 287	20	15	9.36
Ethyl palmitoleate	283 → 237	20	10	9.80
Ethyl linoleate	309 → 245	20	15	10.41
Ethyl palmitate	285 → 257	20	15	13.27
Ethyl oleate	311 → 265	20	10	14.04
Ethyl stearate	313 → 285	20	15	19.55
Ethyl heptadecanoate (IS)	299 → 271	20	10	16.02

desolvation temperature at 400 °C. Cone gas flow and desolvation gas flow were set at 50 L/h and 400 L/h, respectively. The collision gas was argon at a collision cell pressure of 0.25 Pa.

For multiple reaction monitoring (MRM) of the individual compounds and IS, the pseudomolecular ions $(M+H)^+$ were selected in the first quadrupole, and the collision energy was adjusted to optimize the signal for the most abundant product ions. A quantification and a confirmation transition were chosen for each compound. Quantification transitions are reported in Table 1.

2.3. Preparation of calibrators and quality control samples

Stock calibration solutions containing analytes at concentrations of 1 g/L were prepared in hexane. Working solutions at concentrations 10 and 1 mg/L were prepared by dilution of the stock calibrators with hexane, and stored at −20 °C until analysis.

Using the working solutions, calibrators were prepared in nmol/g concentrations in meconium, in agreement with international literature, which reports FAEEs amount in these units.

Calibrators containing analyte concentrations ranging from the limit of quantification (LOQ ranging from 0.12 to 0.20 depending from different FAEE), to 50.0 nmol/g (in ng/g: ethyl laurate from 45.6 to 11420 ng/g; ethyl myristate from 46.1 to 12,820 ng/g; ethyl palmitate from 51.2 to 14,225 ng/g; ethyl palmitoleate from 42.3 to 14,125 ng/g; ethyl stearate from 50 to 15,625 ng/g; ethyl oleate from 43.4 to 15,525 ng/g; ethyl linoleate from 49.3 to 15,425 ng/g; ethyl linolenate from 49.0 to 15,325 ng/g; ethyl arachidonate from 40.2 to 16,625 ng/g) were prepared daily for each analytical batch. For this purpose, suitable amounts of working solutions in hexane were added to 1 g of pre-checked drug-free meconium. Quality control (QC) samples of 45.0 nmol/g (high control), 9.0 nmol/g (medium control), 0.45 nmol/g (low control) were prepared for each analyte in drug-free meconium and stored at −20 °C. They were included in each analytical batch to check calibration, recovery, precision, and the stability of samples under storage conditions.

2.4. Sample preparation

Similar to previously reported methods [4,6], one gram of meconium was transferred into 15-mL screw-capped glass tubes and 25 μL of IS (60 mg/L), 250 μL distilled water and 500 μL acetone were added. The tubes were vortexed for 1 min, followed by the addition of 5 mL hexane. The tubes were placed in a horizontal shaker for 10 min. After centrifugation at 1350 g for 10 min, the organic layer was transferred to new tubes and the solvent was evaporated to dryness at 30 °C under a nitrogen stream. The dried samples were then resuspended in 500 μL hexane, vortexed for 15 s and applied onto aminopropyl-silica solid-phase extraction (SPE) columns, which had been preconditioned with 1 mL hexane. The analytes were eluted with 2 mL hexane. The eluent was evaporated to dryness under a stream of nitrogen and redissolved in 100 μL mobile phase. A 50 μL volume was injected into LC column.

2.5. Validation procedures

The method was validated following the accepted criteria for bioanalytical method validation [18,19]. We evaluated selectivity, carry over, matrix effect, extraction efficiency, linearity, limit of detection (LOD) and LOQ, precision, and stability as previously reported [20,21] and introduced some additional parameters which were also evaluated.

Since it is known that certain FAEEs can be present in low amounts in meconium of neonates not exposed to alcohol [12], selectivity experiments were carried out with 10 meconium samples previously analyzed at the Hospital for Sick Children, Toronto, and reported to be negative for any FAEEs. Furthermore, aliquots of these samples were pooled, analyzed to verify the absence of any FAEEs under investigation, and used as blank meconium in the validation procedures.

Blank meconium samples spiked with 100, 150 and 200 nmol/g of the investigated FAEEs were prepared as over-the-curve samples, to be tested for calibration curve fitting, recovery, and precision once they were diluted 10-fold.

Autosampler stability was assessed by placing processed QC samples in an autosampler at room temperature for 24 h.

A cross validation between our developed method and a standardized assay was performed by re-analyzing with LC–MS/MS 46 different meconium samples analyzed already, 12 months prior by GC-FID at the Division of Clinical Pharmacology/Toxicology, Hospital for Sick Children, Toronto.

2.6. Method application

The LC–MS/MS method was applied for the analysis of the meconium samples collected for the "Meconium Project" [14]. The two cohorts included neonates born between October 2002 and February 2004 at the Hospital del Mar, Barcelona and neonates born between January and March 2008 at of the Neonatal Intensive Care Unit of Arcispedale Santa Maria Nuova, Reggio Emilia, Italy.

The study was approved by the Institutional Ethical Committees and conducted in accordance with the Declaration of Helsinki. Signed informed consent was obtained from the mothers of the newborns. All the newborns were clinically examined at delivery and individual samples of meconium, collected within the first 24 h, were immediately stored in separate aliquots at −20 °C until analysis.

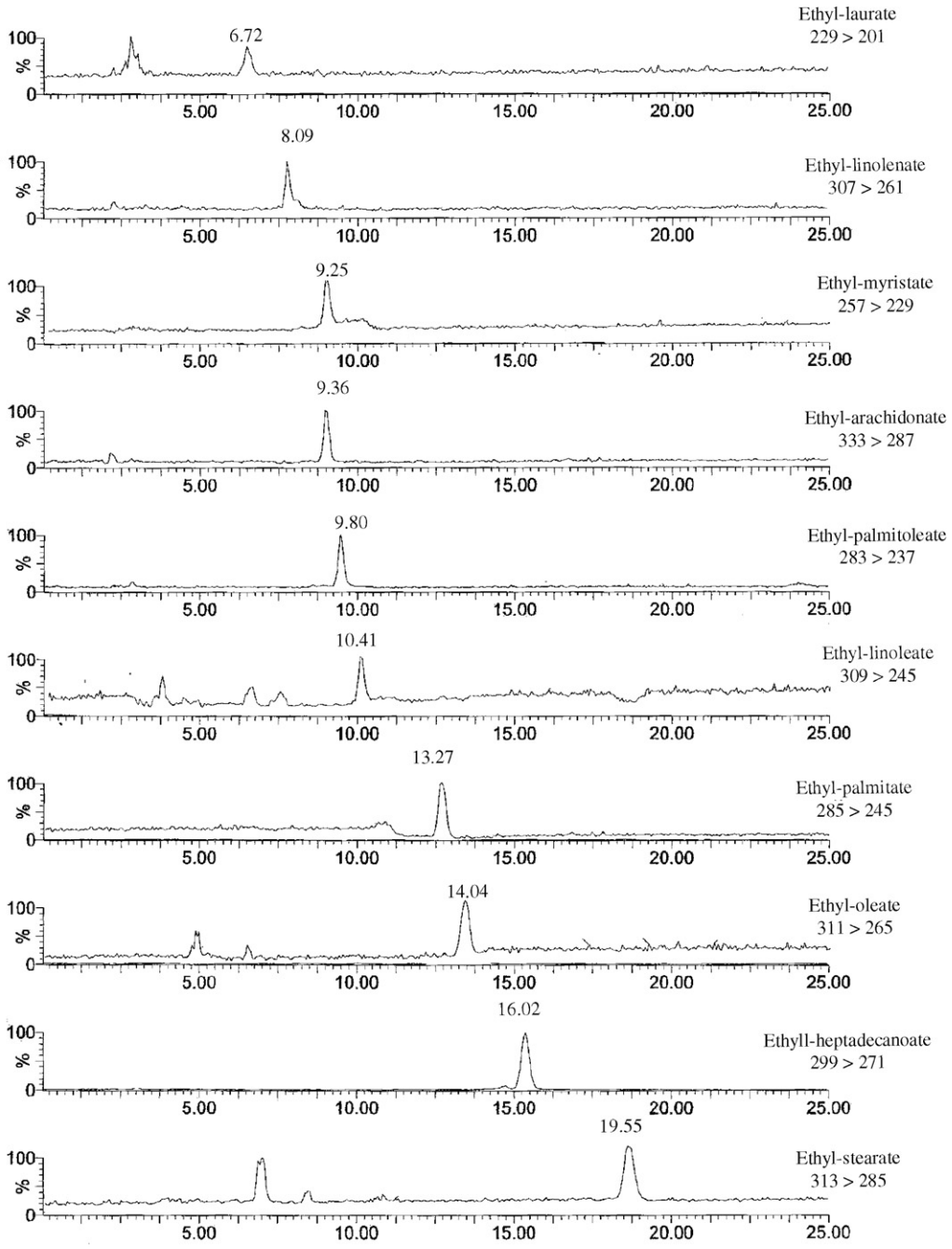

Fig. 2. MRM chromatogram of an extracted sample containing 0.20 nmol/g ethyl laurate, 0.31 nmol/g ethyl myristate, 0.26 nmol/g ethyl palmitate, 0.96 nmol/g ethyl palmitoleate, 0.39 nmol/g ethyl stearate, 0.44 nmol/g ethyl oleate, 0.16 nmol/g ethyl linoleate, 0.21 nmol/g ethyl linolenate and 0.35 nmol/g ethyl arachidonate.

3. Results and discussion

3.1. Chromatography and validation results

A representative MRM chromatogram of an extracted meconium sample is shown in Fig. 2. Separation of the compounds and IS was completed in 21 min, a run time significantly shorter than those obtained by other authors for the nine FAEEs [8,12].

When analyte concentration in samples was initially higher than the calibration curve range, samples were diluted in mobile phase as reported above- and re-injected (over-the-curve samples). No carryover was observed in the over-the-curve samples, nor when blank meconium samples were injected after the highest point of the calibration curve. No additional peaks due to endogenous substances that could have interfered were observed at the retention times of the analytes under investigation (Fig. 3). Similarly, none of the most common drugs of abuse or medications, carried through the entire procedure, interfered with the assay and quantification of the low QC samples.

No significant ion suppression (less than 10% analytical signal suppression due to matrix effect) occurred during chromatographic runs.Absolute analytical extraction recoveries (mean ± S.D.) obtained after the extraction procedure for the three different QC samples ranged between 55.4 and 95.6% for the different FAEEs (Table 2). The extraction efficiencies observed for the individual FAEE species were similar to those reported previously [22].

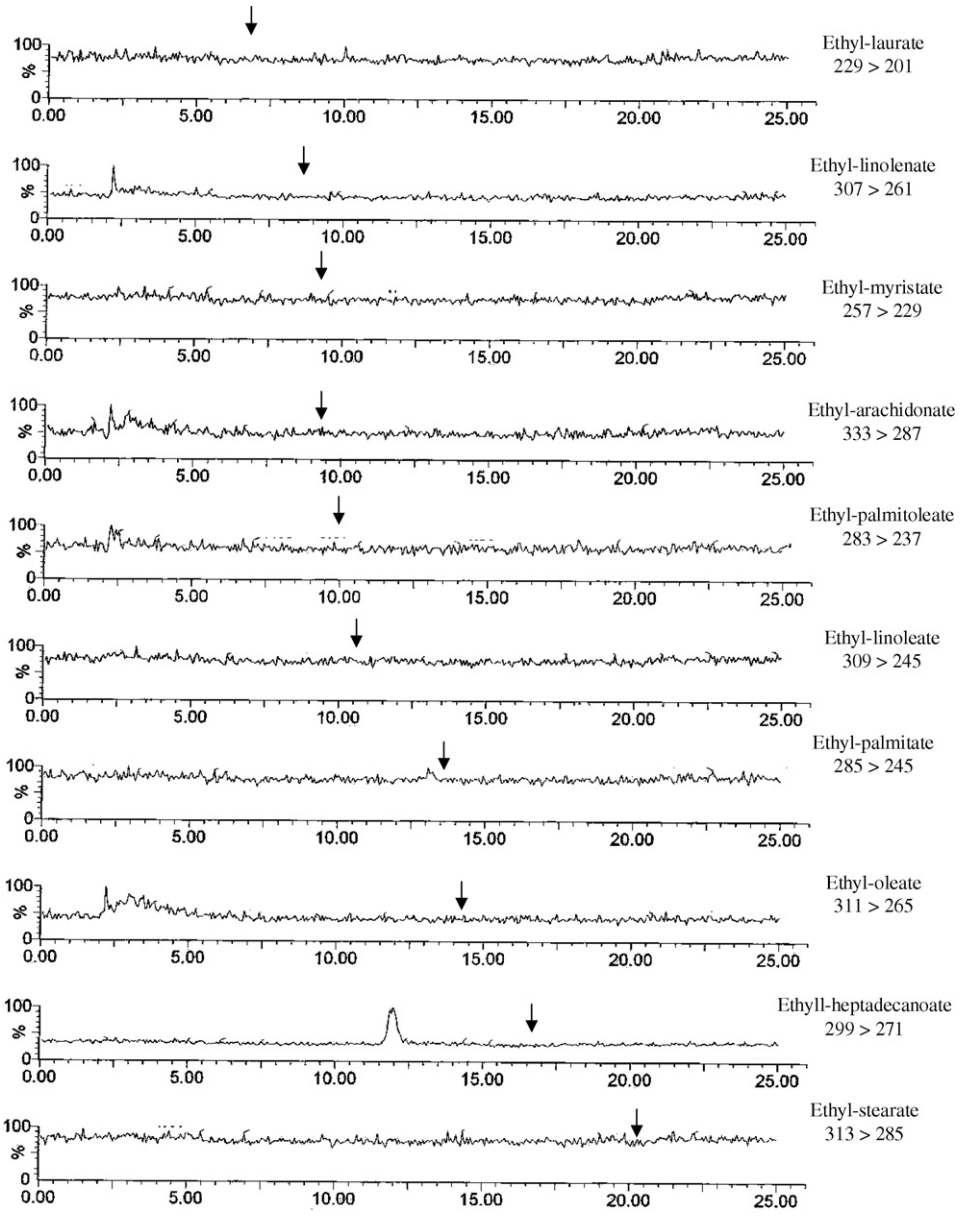

Fig. 3. MRM chromatogram of blank meconium sample.

Linear calibration curves showed determination coefficients (r^2) higher than 0.99 in all cases. LOD and LOQ values, calculated from S.D. of the mean noise level over the retention time window of each analyte in blank meconium samples, were similar or in some case better than those previously obtained [8,12,13] (Table 3).

The results obtained for intra-assay and inter-assay precision and accuracy met the internationally established acceptance criteria [18,19] (Table 2). Once diluted, over-the-curve samples fitted the calibration curve, and when tested for precision and recovery gave values always better than 10% relative standard deviation (R.S.D.) and error.

We observed less than 10% degradation from the initial concentration after three freeze/thaw cycles and for autosampler stability (QC samples re-injected after 24 h and compared to freshly prepared and injected samples). Similar results (differences < 10%) were obtained in the case of mid-term stability test for real samples, assuring the feasibility of stored samples analysis.

The comparison of the quantitative results obtained for the 46 meconium samples previously analyzed by GC-FID the at Division of Clinical Pharmacology/Toxicology, Hospital for Sick Children, and analyzed 12 months later by LC–MS/MS in our laboratory, showed a correlation coefficient (r) of 0.984 ($y = 0.89x + 0.7$, relative %error = 11%; where y = LC–MS/MS values and x = GC-FID values). This also confirmed the relative stability of meconium samples stored at −20 °C.

3.2. Method application

From the initial 1151 meconium samples collected for the "Meconium Project" in Barcelona, the 98 meconium samples positive for any of the principal drugs of abuse (opiates, cocaine and cannabis), plus an additional 376 randomly selected negative samples were investigated. The application of LC–MS/MS for neonatal screening of *in utero* exposure to alcohol was facilitated by the fact that LC–MS/MS was the methodology applied in the project

Table 2
Absolute recovery ($n = 4$), intra-day ($n = 5$) and inter-day ($n = 25$ precision and accuracy for fatty acid ethyl esters (FAEEs)

FAEE	Concentration (nmol/g)	Absolute recovery (%mean ± S.D.)	Intra-assay precision (CV, %)	Inter-assay precision (CV, %)	Intra-assay accuracy (%Error)	Inter-assay accuracy (%Error)
	0.45	59.7 ± 1.4	9.1	8.9	9.9	4.3
Ethyl laurate	9.0	66.7 ± 5.8	12.0	10.5	7.6	7.7
	45.0	68.4 ± 2.4	7.8	9.2	15.7	14.4
	0.45	64.7 ± 5.6	11.9	15.0	11.1	13.5
Ethyl linolenate	9.0	74.8 ± 5.1	14.5	14.4	10.6	10.7
	45.0	67.7 ± 2.6	7.3	9.1	13.9	16.3
	0.45	61.6 ± 5.0	8.7	9.8	8.3	14.1
Ethyl myristate	9.0	58.0 ± 3.1	10.5	7.6	5.6	12.1
	45.0	60.0 ± 9.5	6.6	11.0	6.3	9.0
	0.45	98.3 ± 6.6	6.0	6.6	11.7	10.4
Ethyl arachidonate	9.0	95.6 ± 4.3	6.3	6.5	5.1	6.2
	45.0	93.4 ± 1.8	9.7	11.4	9.4	11.4
	0.45	80.2 ± 2.2	9.9	5.1	6.9	4.2
Ethyl palmitoleate	9.0	86.7 ± 9.2	6.4	3.8	5.6	9.2
	45.0	84.2 ± 1.9	6.9	3.1	2.1	9.7
	0.45	85.8 ± 9.3	2.8	9.9	9.4	6.6
Ethyl linoleate	9.0	86.6 ± 2.0	7.1	10.2	10.4	10.4
	45.0	82.6 ± 1.4	4.7	5.2	3.4	9.8
	0.45	54.8 ± 6.3	10.9	4.4	11.3	6.2
Ethyl palmitate	9.0	53.6 ± 2.2	9.5	9.1	8.3	10.1
	45.0	55.4 ± 2.4	9.1	10.1	10.3	9.4
	0.45	66.4 ± 2.4	6.4	6.1	8.4	8.4
Ethyl oleate	9.0	69.7 ± 7.2	9.6	5.6	7.7	7.6
	45.0	66.6 ± 0.5	10.1	10.1	11.5	12.5
	0.45	64.2 ± 3.9	10.1	5.2	8.6	6.4
Ethyl stearate	9.0	63.1 ± 3.5	6.1	7.1	9.8	10.3
	45.0	66.4 ± 4.3	7.4	8.1	5.8	7.2

to assess prenatal exposure to opiates, cocaine and amphetamines as well [14,16]. In particular, LC–MS/MS with the same chromatographic column was used for detecting opiates, cocaine, and FAEEs in meconium, rendering the assay suitable for use as high-throughput application. In addition, 115 meconium samples (only one positive to cocaine) from neonates born between January and March 2008 at of the Neonatal Intensive Care Unit of Arcispedale Santa Maria Nuova, Reggio Emilia, Italy were also investigated. Both hospitals are located in an urban area with similar prevalence of immigrants (around 40%), although Hospital del Mar is located in an urban area with low socioeconomic status while Arcispedale Santa Maria Nuova in an urban area of medium socioeconomic status.

Nine different FAEEs were quantified in meconium specimens, but only seven of them were used as biomarkers of chronic maternal alcohol intake during pregnancy and fetal exposure to maternal consumption. Based on previous studies, laurate and myristate ethyl esters were excluded from the statistical analysis [8,13]. Nonetheless, these two FAEEs were quantified as well in order to verify if, as in case of North American populations, they were present non-specifically in meconium samples.

Based on the sum total of the seven selected FAEEs, we set a cut-off of 2 nmol/g to differentiate heavy maternal alcohol consumption during pregnancy from occasional use or no use, in agreement with previously reported studies [13,22].

Of the 474 meconium samples from the Barcelona cohort analyzed for FAEEs, 211 (44.5%) contained a total amount of the seven FAEEs above 2 nmol/g meconium (Table 4), demonstrating a high prevalence of heavy alcohol intake during pregnancy in this cohort. Of the remaining 263 samples with seven FAEEs below the cut-off, 221 had quanfiable amounts of single FAEEs. Of the 98 meconium samples that were positive for opiates, cocaine or cannabis, 53 had total FAEEs below and 45 above the established cut-off, showing similar rates of gestational alcohol consumption between drug abusing and non-abusing pregnant women.

Conversely, of the 115 meconium specimens from Reggio Emilia cohort, only 2 (1.7%) contained a total amount of the seven FAEEs

Table 3
Method calibration for FAEEs in meconium

FAEE	Calibration line slope[a]	Calibration line intercept[a]	Correlation coefficient (r^2)	Limit of detection (LOD) nmol/g (ng/g)	Limit of quantification (LOQ) nmol/g (ng/g)
Ethyl laurate	0.213 ± 0.04	0.034 ± 0.08	0.994 ± 0.004	0.07 (15.9)	0.20 (45.6)
Ethyl linolenate	0.419 ± 0.08	0.077 ± 0.03	0.991 ± 0.001	0.05 (15.3)	0.16 (29.3)
Ethyl myristate	0.766 ± 0.07	0.193 ± 0.11	0.993 ± 0.001	0.05 (12.8)	0.18 (46.1)
Ethyl arachidonate	0.669 ± 0.08	0.115 ± 0.09	0.995 ± 0.005	0.04 (13.3)	0.12 (40.2)
Ethyl palmitoleate	1.157 ± 0.08	0.445 ± 0.29	0.990 ± 0.001	0.04 (11.3)	0.15 (42.3)
Ethyl linoleate	0.492 ± 0.003	0.251 ± 0.11	0.994 ± 0.004	0.05 (15.3)	0.16 (49.3)
Ethyl palmitate	2.445 ± 0.140	0.975 ± 0.44	0.991 ± 0.002	0.05 (14.2)	0.18 (51.2)
Ethyl oleate	2.265 ± 0.005	0.033 ± 0.26	0.995 ± 0.001	0.04 (12.4)	0.14 (43.4)
Ethyl stearate	1.871 ± 0.147	0.377 ± 0.43	0.991 ± 0.001	0.05 (15.6)	0.16 (50.0)

[a] Mean and S.D. of five replicates.

Table 4
Fatty acid ethyl ester concentrations as median and interquartile range in meconium samples from Barcelona cohort of the "Meconium Project"

FAEE	Concentration[a] (n = 474)	Concentration[a] when 7 total FAEEs were <2 nmol/g (n = 211)	Concentration[a] when 7 total FAEEs were >2 nmol/g (n = 263)
Ethyl laurate	0.0; 0.0–0.0 (0.0; 0.0–0.0)	0.0; 0.0–0.0 (0.0; 0.0–0.0)	0.0; 0.0–0.2 (0.0; 0.0–45.7)
Ethyl myristate	0.0; 0.0–0.5 (0.0; 0.0–141.0)	0.0; 0.0–0.0 (0.0; 0.0–0.0)	0.6; 0.0–4.0 (143.6; 0.0–1038.4)
Ethyl palmitate	0.2; 0.0–1.3 (45.5; 0.0–381.2)	0.0; 0.0–0.04 (0.0; 0.0–11.4)	1.5; 0.6–5.3 (435.3; 159.7–1496.9)
Ethyl palmitoleate	0.0; 0.0–1.5 (0.0; 0.0–429.4)	0.0; 0.0–0.0 (0.0; 0.0–0.0)	1.6; 0.3–6.1 (467.0; 86.2–1717.6)
Ethyl stearate	0.0; 0.0–0.3 (0.0; 0.0–84.4)	0.0; 0.0–0.0 (0.0; 0.0–0.0)	0.3; 0.0–0.8 (81.2; 0.0–260.6)
Ethyl oleate	0.4; 0.0–2.5 (123.4; 0.0–791.7)	0.0; 0.0–0.1 (0.0; 0.0–45.0)	3.0.; 1.11–9.6 (919.1; 344.3–2968.4)
Ethyl linoleate	0.2; 0.0–1.0 (55.5; 0.0–320.8)	0.0; 0.0–0.04 (0.0; 0.0–10.8)	1.1; 0.4–4.3 (348.6; 138.8–1315.7)
Ethyl linolenate	0.0; 0.0–0.0 (0.0; 0.0–0.0)	0.0; 0.0–0.0 (0.0; 0.0–0.0)	0.0; 0.0–0.0 (0.0; 0.0–0.0)
Ethyl arachidonate	0.0; 0.0–0.7 (0.0; 0.0–239.3)	0.0; 0.0–0.0 (0.0; 0.0–0.0)	0.8; 0.2–1.8 (279.3; 61.5–585.2)
9 Total FAEEs (nmol/g) (ng/g)	1.7; 0.0–10.6 (496.9; 0.0–3084.6)	0.0; 0.0–0.6 (0.0; 0.0–187.6)	12.8; 4.8–37.8 (4427.7; 1679.5–13107.3)
7 Total FAEEs excluding laurate and myristate (nmol/g) (ng/g)	1.5; 0.0–9.2 (438.0; 0.0–2712.7)	0.0; 0.0–0.6 (0.0; 0.0–187.6)	11.2; 4.4–28.4 (3889.9; 1523.3–9868.7)

[a] FAEEs expressed in nmol/g (ng/g value in parenthesis) meconium.

above 2 nmol/g meconium (data not shown). Interestingly, of the remaining 113 samples, only 10 had quantifiable amounts of single FAEEs, while in all the other samples the concentration of the seven FAEEs was always under the method LOQ.

The results of this study highlight once more the importance of assessing chronic fetal exposure to alcohol through the objective measurement of established biomarkers. The prevalence of gestational alcohol consumption in the Barcelona cohort (44.5%) is extremely high when compared to that of Reggio Emilia (1.7%) and to that of 3.4% in a population-based study in Ontario [12] or a 16.7% positive rate in a population-based study in Hawaii [13]. On one hand, it can be argued that such a high prevalence could be linked to the lower socioeconomic characteristics of this cohort. This is the only distinguishing feature of the studied population. On the other hand, it is known that a daily consumption of mainly wine and beer during principal meals is a common pattern in Mediterranean countries. It is worth mentioning that not a single case of fetal alcohol syndrome with characteristic facial features was found in our newborn cohort [23]. Nevertheless, since it is recognized that *in utero* exposure to alcohol is one of the principal diagnostic criteria (if not the principal itself) for fetal alcohol spectrum disorders, follow-up of exposed newborns has been ongoing to assess the possible presence of alcohol-related neurobehavioural problems [24].

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