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Detection and quantification of fatty acid ethyl esters in meconium by headspace-solid-phase microextraction and gas chromatography–mass spectrometry

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ABSTRACT

Meconium fatty acid ethyl esters (FAEEs) are currently used as biomarkers to detect heavy prenatal alcohol exposure. We introduce a novel technique to quantify FAEEs in meconium using headspace-solid-phase microextraction (HS-SPME) coupled with gas chromatography–mass spectrometry (GC–MS). This method improves on previous approaches by decreasing sample preparation time, eliminating the need for organic solvents, and reducing the required sample size. Using 50 mg of meconium, the detection limits of FAEEs ranged from 0.05 to 0.16 nmol/g and had good reproducibility making it ideal for routine analysis of clinical samples.

1. Introduction

Fatty acid ethyl esters (FAEEs), non-oxidative metabolites of ethanol, are currently used as biomarkers for direct ethanol consumption in different biological matrices including hair [1], blood [2], and skin surface lipids [3]. Elevated FAEEs in meconium, the infant's first bowel movement, has also been developed as a biomarker for the detection of heavy prenatal ethanol exposure [4,5]. Using a cumulative sum of four different FAEEs in meconium (ethyl palmitate, ethyl linolate, ethyl oleate, and ethyl stearate), a positive cut-off of 2 nmol FAEEs per g meconium was established with 100% sensitivity and 98.4% specificity [4]. Having an objective biomarker for prenatal alcohol exposure is essential since confirmation of exposure is essential for the diagnosis of fetal alcohol spectrum disorder (FASD), a disorder characterized by prenatal and postnatal growth restriction, facial dysmorphology, and neurocognitive and behavioural dysfunction. An objective biomarker has the potential to identify many more neonates at risk of FASD compared to using maternal self-report of alcohol consumption during pregnancy, as maternal self-report is unreliable and underestimates exposure [6]. The ability of elevated FAEEs in meconium to predict deficits associated with FASD such as growth restriction [7,8], decreased executive function [8], and poorer neurodevelopmental outcome [9] have recently been documented.

Analytical methods currently used to analyze FAEEs in meconium are modifications of liquid–liquid and solid-phase extraction steps developed by Bernhardt et al. [10]. The FAEEs are then quantified by gas chromatography (GC) coupled with either flame ionization detection or mass spectrometry (MS) [4,11–14]. The disadvantages of these methods are that the two extraction steps involve hexane, an organic solvent. Inhalation of hexane can lead to dizziness, nausea, and headache, or even peripheral neuropathy. Other disadvantages are that the extraction steps are time-consuming and labour intensive, and that analysis requires a large amount of meconium (~1 g).

In other matrices, including hair and skin surface lipids, methods utilizing headspace-solid-phase microextraction (HS-SPME) to quantify FAEEs have been validated [1,3]. The advantages of HS-SPME compared to other methods include eliminating the need for organic solvents, automating the extraction to minimize sample preparation time, and obtaining clean extracts [15]. In the present study, we coupled HS-SPME with GC–MS to create a novel method to quantify FAEEs in meconium that has simple sample processing and requires a small amount of sample. Furthermore, by using deuterated internal standards of each FAEEs, accuracy is improved.

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This new method has good sensitivity and reproducibility, making it a suitable method for routine analysis of clinical samples.

2. Experimental

2.1. Chemicals

Ethyl palmitate, ethyl oleate, ethyl stearate, ethyl linolate, and their free carboxylic acids were obtained from Sigma–Aldrich Co. (St. Louis, MO) in addition to anhydrous ethanol-d_6 and thionyl chloride. HPLC-grade hexane was acquired from Caledon Laboratories Ltd. (Georgetown, ON).

2.2. Meconium

Meconium samples were submitted to the Motherisk Laboratory at the Hospital for Sick Children, Toronto, Canada for analysis of FAEEs. Blank meconium samples were obtained from this pool and defined as having each of the four FAEEs (ethyl palmitate, ethyl linolate, ethyl oleate, and ethyl stearate) below the limit of detection (LOD) using a previously established method [4].

2.3. Preparation of stock solutions and internal standards

Stock solutions (1 mg/ml) of the four ethyl esters were prepared by dissolving each of the four FAEEs in hexane. A mixture of the four FAEEs was prepared by adding 4 μl of each FAEEs stock to 3.984 ml hexane for a final concentration of 1.0 μg/ml. The solution was vortexed and was further diluted to 50 ng/ml by diluting 200 μl of the 1 μg/ml standard solution into 3.8 ml of hexane. The 1.0 μg/ml and 50 ng/ml standard solutions were stored at −20 °C.

Deuterated standards (D_5-ethyl esters) of each of the four FAEEs were used as suitable internal standards. The D_5-ethyl esters have suitable mass spectrometric properties as they have similar recoveries as the FAEEs and do not have interfering peaks [1]. Deuterated standards for each of the four FAEEs were prepared according to a previously published method [1]. Briefly, 10 mg of each free fatty acid was added to 50 μl ethanol-d_6 in a separate 7 ml round bottom culture tube. The tubes were placed on dry ice to cool the mixture to −78 °C and subsequently 10 μl of thionyl chloride was added. The tubes were capped and heated to 40 °C for 2 h. The mixture was placed under a stream of nitrogen at 10 psi to remove excessive thionyl chloride and ethanol-d_6. To the remaining residue, 1 ml hexane was added, vortexed and evaporated again under nitrogen to remove any traces of reagents. This step was repeated two times. The residues were dissolved in hexane to obtain a concentration of 2 mg/ml and stored at −20 °C. The purity of each D_5-ethyl ester was determined by GC–MS analysis by direct injection. A stock solution of the four D_5-ethyl esters was prepared by adding 20 μl of each of D_5-ethyl palmitate, D_5-ethyl linolate, D_5-ethyl oleate, and D_5-ethyl stearate at 2 mg/ml to 3.92 ml hexane to give a final concentration of 10 μg/ml. The mixture was vortexed and further diluted to 1 μg/ml by adding 400 μl of the of 10 μg/ml solution to 3.60 ml of hexane.

2.4. Sample preparation and solid-phase microextraction

After optimisation, the following procedure was used: to a glass culture tube, 25 μl of the internal standard solution containing each of the four D_5-ethyl esters in hexane (1 μg/ml of each) was added. The hexane was completely evaporated at 40 °C under a nitrogen stream. In order to avoid losses of internal standard by evaporation, the nitrogen stream was stopped immediately after the solvent was removed. Subsequently, 50 mg of meconium and 750 μl of phosphate buffer (0.1 M, pH 7.6) was added and vortexed for 1 min. The mixture was transferred to a 10 ml headspace SPME vial using a glass pipette. The glass culture tube was then further rinsed with 250 μl of phosphate buffer and again transferred to the SPME vial for a total volume of 1 ml. The vials were capped with a steel screw cap with PTFE/Silicone septa and placed into the vial rack of the autosampler.

For HS-SPME, the following conditions were used: preheating 5 min at 90 °C and 250 rpm agitation, headspace adsorption 40 min at 90 °C and 250 rpm agitation, desorption 12 min at 260 °C. The agitator on time was 15 s and the off time was 10 s.

2.5. Gas chromatography–mass spectrometry

A gas chromatograph with a mass selective detector GC/MS-QP2010 (Shimadzu, Columbia, MD, USA) coupled with a AOC-5000 autosampler (Shimadzu, Columbia, MD, USA) was used for analysis. All steps of the HS-SPME experiments including preheating of the sample, sample agitation, headspace adsorption, and desorption in the GC injection port were programmed and automatically carried out by the autosampler. The SPME experiments used a 65 μM Polydimethylsiloxane–divinylbenzene fiber (PDMS/DVB) (Supelco, Bellefonte, PA). The PDMS/DVB fiber was previously found to be optimal for HS-SPME of FAEEs by Pragst et al. [1].

The method for GC–MS separation and detection of FAEEs was adapted from Pragst et al. [1]. A FactorFour Capillary Column (30 m × 0.25 mm × 0.25 μm, Varian, CA, USA) was used for separation of the four FAEEs. Helium (1.0 ml/min) was used as the carrier gas. Splitless injection mode was used and the injection temperature was 260 °C. The oven temperature was programmed as follows: 2 min at 70 °C, then 20 °C/min up to 300 °C, then 1 min at 300 °C. The mass spectrometer was scanned from m/z 80 to 350 on a 0.5 s cycle. The ion source temperature and interface temperatures were 230 and 310 °C, respectively. The retention times for ethyl palmitate, ethyl linolate, ethyl oleate, and ethyl stearate were 11.39, 12.22, 12.23, and 12.32 min, respectively. The retention times for the deuterated internal standards of ethyl palmitate, ethyl linolate, ethyl oleate, and ethyl stearate were 11.37, 12.20, 12.21, and 12.30 min, respectively. The peak areas of the molecular ions (m/z) for ethyl palmitate, ethyl linolate, ethyl oleate, and ethyl stearate (284, 308, 310, and 312, respectively), as a ratio to the corresponding peak areas of the molecular ions (m/z) for D_5-ethyl palmitate, D_5-ethyl linolate, D_5-ethyl oleate, and D_5-ethyl stearate (289, 313, 315, and 317, respectively) were used to quantify each FAEEs.

3. Results

3.1. Optimisation of experimental parameters

Optimisation, calibration, and validation of this method were carried out using blank meconium samples spiked with each of the four FAEEs (25 μl of the 1.0 μg/ml standard solution) and the internal standard solution containing the four D_5-ethyl esters (25 μl of the 1.0 μg/ml standard solution) as described in the above paragraph. Each experiment was done with $n = 2$ at each parameter. All experiments were repeated on a separate occasion using a different source of blank meconium.

The effects of adsorption time on peak area during HS-SPME were measured from 15 to 60 min in 5-min intervals. For all four esters, the extraction equilibrium between the liquid phase and the fiber was obtained after 40 min. Thus, 40 min was selected as the routine adsorption time since it provided the largest peak area. At all time intervals, the ratio of FAEEs to d-FAEEs (deuterated-FAEEs) was consistent with a variability of less than 7%.

The effect of sample weight on peak area during HS-SPME was determined using 0, 25, 50, 100, and 200 mg of meconium. The results were calculated from the peak areas obtained from the

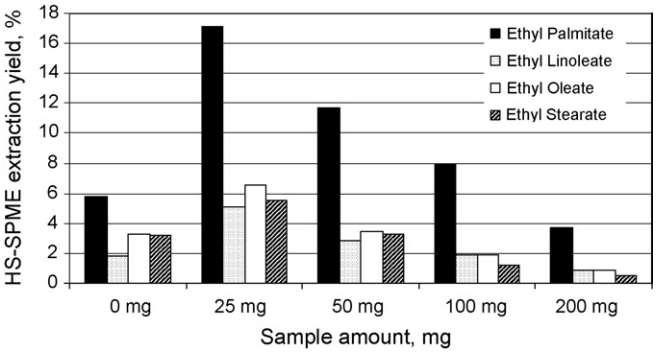

Fig. 1. Effect of sample amount on the FAEEs extraction yields during HS-SPME. To 0, 25, 50, 100, or 200 mg of meconium, 1 ml phosphate buffer pH 7.6 and 25 ng of each of the four FAEEs were added. Extraction yield was calculated by comparing the peak area of the molecular ion after HS-SPME compared to direct injection of the same concentration. The extraction yield % is the average of 2 duplicates.

HS-SPME conditions and compared to direct injection of pure standards in hexane at the same concentration. The highest % extraction yield with HS-SPME was obtained with the lowest amount of meconium, 25 mg (5.1–17.1%) (Fig. 1). Increasing sample amount has previously been shown to decrease HS-SPME extraction efficiency in other matrices [16,17]. A sample amount of 50 mg was chosen as the optimal weight since it had only a slightly lower extraction efficiency (2.9–11.7%) compared to 25 mg (Fig. 1) but had a much better efficiency than 100 mg (1.2–8.0%). The percent recoveries using 50 mg of meconium were 11.7% for ethyl palmitate, 2.9% for ethyl linolate, 3.5% for ethyl oleate, and 3.3% for ethyl stearate (Fig. 1). The amount of recovery using HS-SPME represents the amount of FAEEs present during equilibrium in the headspace. The range of recoveries in this experiment is consistent with other SPME methods that have recoveries ranging from 0.1 to more than 60% [1].

Differences in the composition of individual meconium samples influence HS-SPME recovery as peak areas for the D_5-ethyl esters differed between meconium samples. The effect of the matrix on HS-SPME recovery of FAEE is consistent with recovery of FAEEs from hair [1]. For example, differences in the amount of lipid in each sample will influence the movement of FAEEs into the headspace. To account for matrix effects, replications of method development experiments were repeated in different meconium samples (see Section 3.1). There was no difference in retention time or peak shape of the individual FAEEs or the D_5-ethyl esters when comparing HS-SPME extractions from meconium with solvent only (phosphate buffer).

3.2. Calibration curve, limits of detection and quantification

For preparation of the calibration curve, 50 mg of meconium was spiked with 100, 200, 400, 600, 1000, and 2000 ng/g of the four FAEEs in addition to 500 ng/g of the four d-FAEEs. By using the average of the molecular weights for the four FAEEs, 1 nmol/g is approximately 303.5 ng/g. These concentrations were chosen as they represent the range of FAEEs typically found in meconium samples [4]. For production of the calibration curve, the data were plotted with the ratio of the peak area of each FAEEs to d-FAEEs against the amount of FAEEs spiked into the sample. A line of best fit was added to the data points and an equation was generated. The calibration curves for each of the four FAEEs were linear within this range and had a $R^2 > 0.99$. Typical curve equations and R^2 values are given in Table 1.

The LOD and limit of quantitation (LOQ) were determined by spiking blank meconium with repeatedly lower concentrations of each FAEEs. The LOD was the concentration that gave a signal to noise ratio of >3. The LOD for the four FAEEs ranged from 0.05 to 0.16 nmol/g (Table 1). The LOQ was the concentration that gave a signal to noise ratio of >10 and a coefficient of variation below 20% between runs. The LOQ of the four FAEEs ranged from 0.13 to 0.32 nmol/g (Table 1).

3.3. Reproducibility

The method reproducibility was examined by determining the intraday and interday precisions. The intraday precision was evaluated by determining the peak area ratios of each FAEEs to its deuterated standard in five spiked replicates at a low and high concentration and determining the coefficient of variation (CV). The intraday precision showed CVs ranging from 2.40 to 9.01% at 1 ng/mg and 5.64 to 13.76% at 0.10 ng/mg for the different FAEEs (Table 2). The interday variation was similarly calculated but using values from six runs performed on different days. The interday precision showed CVs ranging from 2.73 to 11.27% at 1 ng/mg and 11.49 to 13.94% at 0.10 ng/mg. The CV for a quality control positive sample run during routine analysis with clinical meconium samples was 5.57% ($n = 11$).

The potential for carryover of FAEEs to the subsequent sample in the batch was investigated by running a blank meconium sample with IS after the highest point in the calibration curve. There were no peaks above baseline at the corresponding retention times for each of the four FAEEs after the highest calibration curve point. Furthermore, the potential for carryover of the IS and other

Table 2
The reproducibility of the analysis of FAEEs in meconium by HS-SPME.

	Intraday CV		Interday CV	
	1000 ng/g	100 ng/g	1000 ng/g	100 ng/g
Ethyl palmitate	2.40	6.08	9.38	12.16
Ethyl linolate	9.01	5.64	11.27	13.94
Ethyl oleate	5.54	13.76	2.73	12.64
Ethyl stearate	2.84	9.63	5.67	11.49

The intraday precision was evaluated by determining the peak area ratios of each FAEEs to its deuterated standard in five spiked replicates at a low and high concentration. The interday variation was similarly calculated but using values from six runs performed on different days.

Table 1
Calibration curves, LOD, and LOQ in the analysis of FAEEs in meconium by HS-SPME.

FAEEs	(*Asample/Astandard*) = *a* (amount of FAEE) + *b*			LOD (nmol/g)	LOQ (nmol/g)
	a	*b*	R^2		
Ethyl palmitate	0.0286	0.01	0.9979	0.05	0.14
Ethyl linolate	0.0426	−0.0888	0.9933	0.05	0.32
Ethyl oleate	0.0225	0.0183	0.9936	0.16	0.32
Ethyl stearate	0.0268	0.0615	0.9959	0.05	0.13

For preparation of the calibration curve, 50 mg of meconium was spiked with 100, 200, 400, 600, 1000, and 2000 ng/g of the four FAEEs in addition to 500 ng/g of the four d-FAEEs. The data were plotted with the ratio of the peak area of each FAEEs to d-FAEEs ($A^{sample}/A^{standard}$) against the amount of FAEEs. The LOD is the FAEEs concentration with a signal to noise ratio of >3 and the LOQ is the FAEEs concentration with a signal to noise ratio of >10 and a coefficient of variation below 20% between runs.

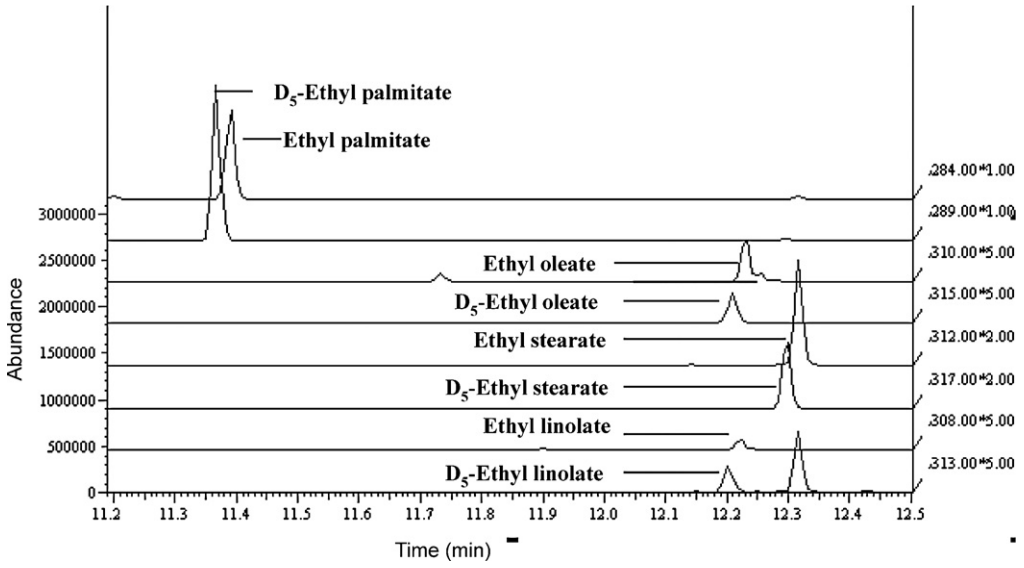

Fig. 2. GC–MS chromatogram obtained from a meconium sample with cumulative FAEEs above 2 nmol/g using the optimised HS-SPME procedure as described in Sections 2.4 and 2.5. The (*m*/*z*) value is given to the right and the abundance is multiplied by the given factor. Measured concentrations: ethyl palmitate 1.81 nmol/g, ethyl linolate 0.42 nmol/g, ethyl oleate 2.62 nmol/g, ethyl stearate 3.03 nmol/g.

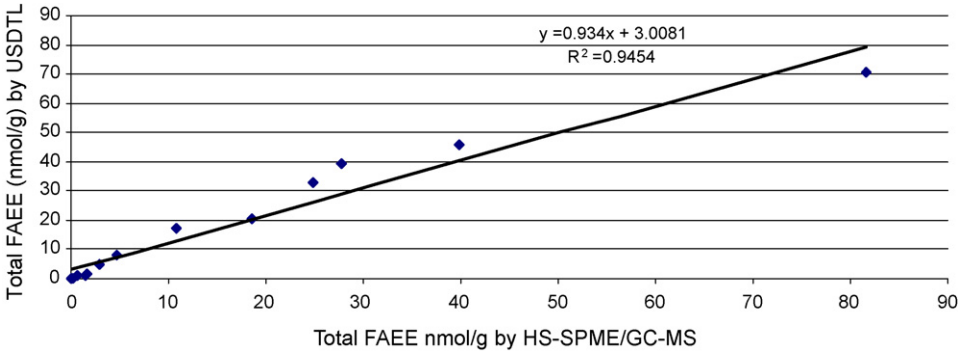

Fig. 3. Regression analysis comparing cumulative concentrations of four FAEEs found in 14 meconium samples. Both the HS-SPME with GC–MS and the USDTL methods had complete agreement in the classification of samples as either positive or negative.

components found in meconium was determined by running only phosphate buffer under the SPME and GC–MS conditions. There were no significant peaks above baseline indicating that there was no significant carryover.

3.4. Comparison of SPME to solid-phase extraction methods

For method comparison and validation, meconium samples were analyzed by HS-SPME with GC–MS. The FAEEs were quantified by back calculating the amount of each individual FAEEs from a calibration curve using least squares regression. An example of a positive sample obtained from the HS-SPME method is given in Fig. 2. The same samples were sent to the US Drug Testing Laboratory (Des Plaines, IL, USA) for independent analysis by a previously established method. Samples contained a range of FAEEs levels above and below the positive cut-off (<LOQ to 89.6 nmol/g). In total, there were six negative samples (<2 nmol/g) and eight positive samples (>2 nmol/g) analyzed by the two methods. There was complete agreement on the classification of the meconium samples above or below the cut-off. There was also good correlation between the two methods (R^2 = 0.945) (Fig. 3). Table 3 shows typical results for ten clinical samples analyzed by HS-SPME.

Table 3
Concentrations of FAEEs in clinical meconium samples (nmol/g) analyzed by HS-SPME and GC–MS.

Sample #	Ethyl palmitate	Ethyl linoleate	Ethyl oleate	Ethyl stearate	Total FAEEs	Classification
1	8.93	Below LOD	17.60	1.26	27.79	Positive
2	3.57	1.99	11.66	1.16	18.38	Positive
3	Below LOD	Below LOD	Below LOD	Below LOD	Below LOD	Negative
4	Below LOD	Below LOD	Below LOD	Below LOD	Below LOD	Negative
5	0.68	0.43	1.60	0.24	2.95	Positive
6	Below LOD	Below LOD	Below LOD	Below LOD	Below LOD	Negative
7	0.25	Below LOD	0.38	Below LOD	0.63	Negative
8	0.90	Below LOD	3.73	Below LOD	4.63	Positive
9	1.14	1.48	4.77	0.22	7.61	Positive
10	0.40	Below LOD	0.94	0.15	1.49	Negative

4. Discussion

Previous methods to extract and quantify FAEEs in meconium required lengthy extraction steps, involved the use of organic solvents, and required around 1 g of meconium. Combining HS-SPME with GC–MS allows automation of the extraction of FAEEs from meconium by the SPME fiber, thus eliminating the need for organic solvents and decreasing sample preparation time. A deuterated standard for each of the measured FAEEs enables a high accuracy of the concentrations. This novel method for extracting and quantifying FAEEs in meconium uses only one extraction step that is fully automated. Other matrices including blood and hair have been analyzed for drug or drug metabolites without the use of any additional extraction steps [16,18].

Another advantage of this new method is that the required sample amount is only 50 mg of meconium, compared to the 1 g required by previous methods. By requiring a substantially lower amount of sample, this new method may allow analysis of samples that would otherwise be reported as "non-sufficient quantity" (NSQ). Furthermore, by requiring a small sample amount for FAEEs analysis, there would be adequate amount of meconium sample available to use for detection of other drugs of abuse such as cocaine, marijuana, and amphetamines. Analyses for multiple drugs of abuse are commonly requested by clients of the Motherisk Laboratory.

The small sample amount required by this method in addition to eliminating the need for organic solvents and decreasing sample preparation time make this method optimal for analysis of FAEEs in meconium. Furthermore, this method has good reproducibility and limits of quantification, making it ideal for routine analysis of clinical samples. Overall, this method provides a simple and reliable means to quantify FAEEs in meconium to determine if an infant has been exposed to risky amounts of alcohol *in utero*.

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References

[1] F. Pragst, V. Auwaerter, F. Sporkert, K. Spiegel, Forens. Sci. Int. 121 (2001) 76.
[2] M. Laposata, Clin. Chem. 43 (1997) 1527.
[3] F. Pragst, V. Auwaerter, B. Kiessling, C. Dyes, Forens. Sci. Int. 143 (2004) 77.
[4] D. Chan, B. Bar-Oz, B. Pellerin, C. Paciorek, J. Klein, B. Kapur, D. Farine, G. Koren, Therap. Drug Monitor. 25 (2003) 271.
[5] C.F. Bearer, L.M. Santiago, M.A. O'Riordan, K. Buck, S.C. Lee, L.T. Singer, J. Pediatr. 146 (2005) 824.
[6] J. Gareri, H. Lynn, M. Handley, C. Rao, G. Koren, Therap. Drug Monitor. 30 (2008) 239.
[7] C. Derauf, A.R. Katz, D.A. Frank, A. Grandinetti, D. Easa, J. Drug Issues 33 (2003) 1001.
[8] J.S. Noland, L.T. Singer, R.E. Arendt, S. Minnes, E.J. Short, C.F. Bearer, Alcohol.: Clin. Exp. Res. 27 (2003) 647.
[9] J. Peterson, H.L. Kirchner, W. Xue, S. Minnes, L.T. Singer, C.F. Bearer, J. Pediatr. 152 (2008) 788.
[10] T.G. Bernhardt, P.A. Cannistraro, D.A. Bird, K.M. Doyle, M. Laposata, J. Chromatogr. B: Biomed. Appl. 675 (1996) 189.
[11] C.F. Bearer, S. Lee, A.E. Salvator, S. Minnes, A. Swick, T. Yamashita, L.T. Singer, Alcohol.: Clin. Exper. Res. 23 (1999) 487.
[12] C.F. Bearer, J.L. Jacobson, S.W. Jacobson, D. Barr, J. Croxford, C.D. Molteno, D.L. Viljoen, A.S. Marais, L.M. Chiodo, A.S. Cwik, J. Pediatr. 143 (2003) 463.
[13] J. Klein, T. Karaskov, G. Koren, Therap. Drug Monitor. 21 (1999) 644.
[14] C.M. Moore, D. Lewis, Clin. Chim. Acta 312 (2001) 235.
[15] F. Pragst, Anal. Bioanal. Chem. 388 (2007) 1393.
[16] R.R. Boyle, S. McLean, S. Brandon, G.J. Pass, N.W. Davies, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 780 (2002) 397.
[17] F. Sporkert, F. Pragst, Forens. Sci. Int. 107 (2000) 129.
[18] A.M. Bermejo, P. Lopez, I. Alvarez, M.J. Tabernero, P. Fernández, Forens. Sci. Int. 156 (2006) 2.