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Article

Accelerated Solvent Extraction for Gas Chromatographic Analysis of Nicotine and Cotinine in Meconium Samples

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Adverse effects associated with smoking during pregnancy are well documented. Although self-report surveys on drug consumption during pregnancy have been improved with new interviewing techniques, underreporting is still a concern. Therefore, a series of biological markers and specimens to diagnose fetal exposure to tobacco have been studied. In the present study, an analytical method was developed to detect nicotine and cotinine (the main nicotine metabolite) in meconium samples. Accelerated solvent extraction (ASE) followed by solid-phase extraction (SPE) were used as sample preparation techniques. The analytes were detected by gas-chromatography with nitrogen-phosphorus detection. The limits of detection were 3.0 and 30 ng/g for cotinine and nicotine, respectively. The method showed good linearity ($r^2 > 0.98$) in the concentration range studied (LOQ–500 ng/g). The intraday precision, given by the RSD of the method, was less than 15% for cotinine and nicotine. The method proved to be fast, practical, and sensitive. Smaller volumes of organic solvents are necessary compared to other chromatographic methods published in the scientific literature. This is the first report in which ASE was used as sample preparation technique in methods to detect xenobiotics in meconium.

Introduction

Tobacco is one of the main drugs consumed worldwide, and its use by women of childbearing age has played a major concern among experts and society in general. Although the dose and frequency that could affect the child before birth are unknown, information about the harmful effects of fetal exposure to tobacco constituents is widely disseminated (1). In fact, many addicted women continue tobacco use during pregnancy despite known adverse consequences on neonatal growth and development (2). Nicotine, carbon monoxide, and other toxic constituents of tobacco smoke can cause direct adverse effects on oxygen supply to the fetus, structure and function of the umbilical cord and placenta, fetal heart rate, and fetal breathing (3). Adverse effects associated with smoking during pregnancy include ectopic pregnancy, spontaneous abortion, prematurity, low birth weight, fetal growth restriction, preterm delivery, orofacial clefts, sudden infant death syndrome, craniosynostosis, clubfoot, childhood respiratory disease, attention deficit disorder, and some childhood cancers (4–9).

As a first attempt to identify smoking during pregnancy, maternal self-reports are widely used. However, maternal reports regarding smoking are sometimes unreliable. Many women underreport their smoking habits during pregnancy because of social pressure, guilt, or embarrassment (5, 7). Therefore, an accurate identification of children exposed in utero to tobacco is sorely needed to better assess the source and the proportion of effects. Clinical treatment and follow-up performed in children exposed could be more suitable if an efficacious identification of fetal exposure was conducted (10).

Identifying nicotine biomarkers in biological specimens provides an alternative method to self-reported gestational tobacco use. In recent years, toxicological analysis of meconium (the first neonatal feces) has shown particular promise in the evaluation of fetal exposure to psychoactive substances. The determination of nicotine and its biotransformation products, such as cotinine, in meconium have been proposed as possible biological markers for the assessment of long-term fetal tobacco exposure (11, 12). In spite of the many methods that have already been published for illicit and therapeutic drugs, few procedures exist for nicotine and metabolite analysis of meconium samples, perhaps because of its complex composition and analytical challenges (13).

The chromatographic methods for identification of nicotine biomarkers in meconium samples are generally based on time-consuming and laborious procedures involving homogenization of the samples with solvents and followed by centrifugation, prior to the solid-phase extraction procedure (SPE) for purification of analytes, with or without a hydrolysis step (6, 13, 14).

Accelerated solvent extraction (ASE), a relatively new technique, has gained considerable interest in several areas, especially in the analysis of substances in complex matrices, such as environmental samples (e.g., soil, sediment, and sewage sludge), different kinds of food (15), and some biological samples, such as feces (16). ASE combines the temperature and pressure increase with organic solvents to improve the efficiency of the extraction process compared with conventional techniques.

Samples (solid or semi-solid) are placed in an extraction cell, made of stainless steel, which in turn is subjected to the passage of pressurized solvent while the system is heated. The design of the extractor, capable of withstanding high pressures, allows the extraction temperature to be raised above the boiling point of the solvent used. The increased temperature accelerates the extraction kinetics, and high pressure keeps the solvent in liquid form during the process. Under these conditions, the solvent has properties that can improve the extraction process, such as low viscosity, high diffusion coefficients, and high solvent strength. When the extraction is complete, compressed nitrogen moves all of the solvent from the cell to the vial for analysis. The equipment provides precise control of temperature and pressure, allowing equality in the extraction of cells in different sequence analysis. The technique also combines automated extraction and filtration in a single process and generally uses less solvent than conventional extraction techniques (16, 17).

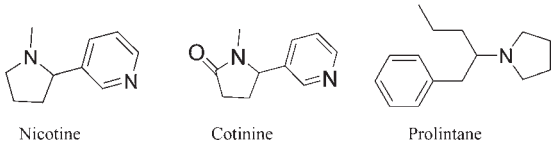

Figure 1. Chemical structures of nicotine, cotinine, and prolintane (internal standard).

The aim of the current study was to develop a gas chromatographic (GC) method for the determination of nicotine and cotinine in meconium samples, using accelerated solvent extraction as sample preparation technique. As far as we know, this is the first study in which accelerated solvent extraction was used in a method to detect xenobiotics in meconium samples.

Materials and Methods

Reagents and materials

Standard solutions of nicotine and cotinine (1.0 mg/mL in methanol) were obtained from Cerilliant (Round Rock, TX). The internal standard, prolintane, was prepared at the initial concentration of 1.0 mg/mL by dissolving in methanol the powder obtained from Boehringer Ingelheim (Ingelheim, Germany). Their chemical structures are shown in Figure 1. All other reagents were obtained from Merck (Darmstadt, Germany). Diatomaceous earth, used as inert material to fill the ASE cell extraction, was purchased from Dionex (Sunnyvale, CA). SPE cartridges (Bond-Elut Certify 3 mL/130 mg) were obtained from Varian (Harbor City, CA).

Preparation of standard solutions

Working solutions of the nicotine, cotinine and prolintane at concentrations of 1.0 and 10 μg/mL were prepared in methanol in volumetric glassware. Stock solutions were stored at −20°C when not in use.

Instrumentation

Accelerated solvent extraction equipment (ASE® 100 Accelerated Solvent Extractor) was obtained from Dionex. GC analyses for nicotine and cotinine were performed using a GC equipped with a nitrogen-phosphorus detector (GC–NPD) (model Agilent 6890N, Palo Alto, CA) and autosampler (model Agilent 7683). Chromatographic separation was achieved on an HP-5 fused-silica capillary column (30 m × 0.25 mm × 0.1-μm film thickness). The injections were made in splitless mode. Nitrogen was used as carrier gas in a constant flow of 1.2 mL/min. The injector and detector temperatures were 200°C and 250°C, respectively. The oven temperature was initially held at 100°C for 1 min and programmed to increase by 10°C/min to 200°C held for 4 min. The chromatographic total run time was 15 min. The injection volume was 1.0 μL.

Meconium samples

Meconium samples were collected from neonates born in University Hospital, São Paulo (HU-USP). The material was collected from diapers and pooled into one plastic container per infant. Samples were immediately stored at −0°C until analysis. This study was approved by the College of Pharmaceutical Sciences Ethics Committee, University of São Paulo. Informed consent was obtained from the mother in each case (Ethics Protocol Approval no. CEP 723/07).

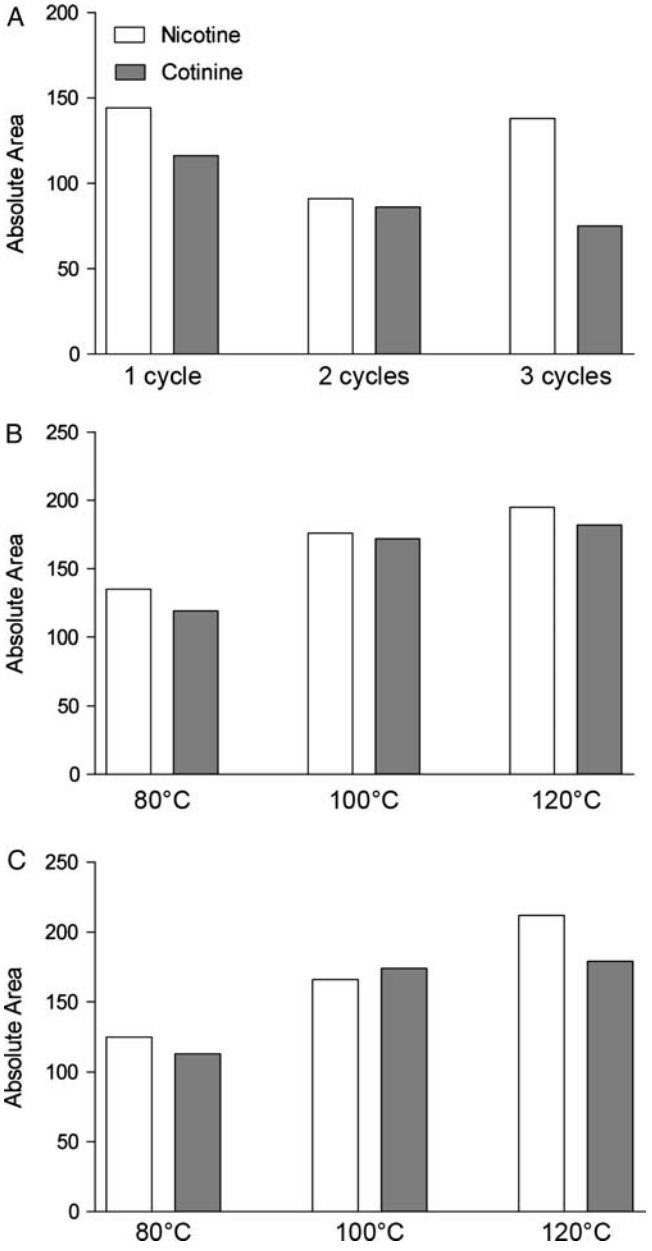

Figure 2. Optimization of ASE technique. All results are expressed as average of absolute area. Influence of the number of cycles in ASE extraction efficiency (A); influence of the temperature on the extraction yield using solvent phosphate buffer 0.1 M (pH 6.0) (B); and influence of the temperature on the extraction yield using solvent hexane/acetone (1:1) (C).

Preparation of meconium samples

Meconium samples (500 ± 10 mg) were combined with 10 μL of standard solution prolintane at a concentration of 10 μg/mL.

Afterwards, approximately 2.4 g of diatomaceous earth was used to fill the 10-mL cell extraction that was further attached to the equipment. The procedure of ASE is initiated by passing the solvent (0.1 M phosphate buffer, pH 6.0) through the sample cell under controlled pressure (1500 psi) and temperature (120°C). The conditions of extraction were as follows: 5 min to warm up, 3 min of static cycle, and 1 min of purging. The collection tube was placed in the exit of ASE system and about 10 mL of the aqueous extract was collected. The extract was submitted to a subsequent SPE procedure. Bond-Elut Certify cartridge was conditioned with 3 mL methanol and 3 mL of 0.1 M phosphate buffer (pH 6.0). The extract obtained from ASE was loaded and allowed to flow under vacuum (one drop/s). The cartridge was washed out with 2 × 3 mL of deionized water, followed by 3 mL of 0.1 M HCl, and 3 × 3 mL of methanol. The analytes were eluted with 2 mL of freshly prepared dichloromethane/isopropanol/ammonium hydroxide (12:3:0.3). The eluate was evaporated under a flow of N_2 at 40°C. The residue was reconstituted by dissolving in 50 μL methanol and transferred to a glass autosampler vials to be injected into the GC–NPD.

Optimization of the ASE procedure

The optimization of the ASE procedure was performed taking into consideration the influence of the temperature, quantity of static cycle and the best solvent on the extraction yield. Method optimization was carried out on meconium samples spiked with a known concentration of 100 ng/g of nicotine and cotinine. The efficiency of extraction was assessed by the absolute average chromatographic peak area produced by each analyte in triplicate. The following parameters were studied: temperature (80, 100, and 120°C); number of static cycles (1, 2 and 3) with each one lasting for 3 min; and choice of solvent (hexane/acetone, 1:1, v/v and 0.1 M phosphate buffer, pH 6.0).

Validation of the method

The validation of the method was performed by establishing limits of detection (LOD) and quantification (LOQ), linearity, intra- and interassay precision, and recovery values of the analytes.

LOD and LOQ

The LOD and LOQ were determined by the empirical method that consisted of analyzing a series of meconium samples containing decreasing amounts of the nicotine and cotinine. The LOD was the lowest concentration that presented an RSD that did not exceed 20%, and the LOQ the lowest concentration that presented an RSD that did not exceed 15% in six replicates. LOD and LOQ should still satisfy the predetermined acceptance criteria of qualification (retention time within 2% compared with standards analyzed in the same batch). LOQ should fall within ±20% of the expected value (nominal concentration).

Linearity

The study of linearity was established by the analyses of meconium samples spiked in triplicate at the following concentrations: 5, 10, 50, 100, 300, and 500 ng/g (for cotinine) and 40, 100, 200, 300, 400, and 500 ng/g (for nicotine). The relationship between peak-area ratios (analyte/IS) and concentrations of the analytes in the samples was determined by linear regression.

Table I
Confidence Parameters of the Validated Method for the Determination of the Cotinine and Nicotine in Meconium

	Cotinine	Nicotine
LOD	3 ng/g	30 ng/g
LOQ	5 ng/g	40 ng/g
Intraassay precision (RSD %)		
CQ1*	10.2	11.9
CQ2	7.3	12.9
CQ3	4.7	7.7
Interassay precision (RSD %)		
CQ1	20.1	13.0
CQ2	19.4	18.9
CQ3	6.4	4.3
Accuracy (%)		
CQ1	95.1	96.0
CQ2	88.7	93.1
CQ3	92.5	96.8
Recovery (%)		
CQ1	65	72
CQ2	76	85
CQ3	83	82

* CQ1 = 20 ng/g cotinine and 120 ng/g nicotine; CQ2 = 250 ng/g cotinine and 250 ng/g nicotine; and CQ3 = 400 ng/g cotinine and 400 ng/g nicotine.

Intra- and interassay precision

Imprecision, defined as the relative standard deviation (RSD), was determined by intra- and interday repetitions. They were performed by analyzing meconium samples fortified with nicotine and cotinine at low, medium, and high concentrations on three different days. The following concentrations were used in the assays: cotinine (20, 250, and 400 ng/g) and nicotine (120, 250, and 400 ng/g). Six replicate analyses were performed at each concentration.

Accuracy

The accuracy of the method was evaluated by analyzing, in triplicate, meconium samples spiked with nicotine and cotinine at low, medium and high concentrations (the same concentrations used in the precision test). The experimental concentrations, quantified using the standard calibration curves, were then expressed as a percentage [(mean measured concentration/nominal concentration) × 100].

Recovery

The extraction efficiency of nicotine and cotinine was evaluated through the recovery studies that were performed by preparing two sets of samples of each concentration. One of them (set A), consisting of three concentrations (the same concentrations used in the precision study: low, medium, and high), was extracted using the method described in the Preparation of meconium samples section. The analyses were performed six times for each concentration. The other one (set B) also consisted of six replicates of each concentration (low, medium, and high). However, standard solutions of the analytes were spiked to the extract immediately before drying under nitrogen stream. To both sets (A and B), internal standard was added

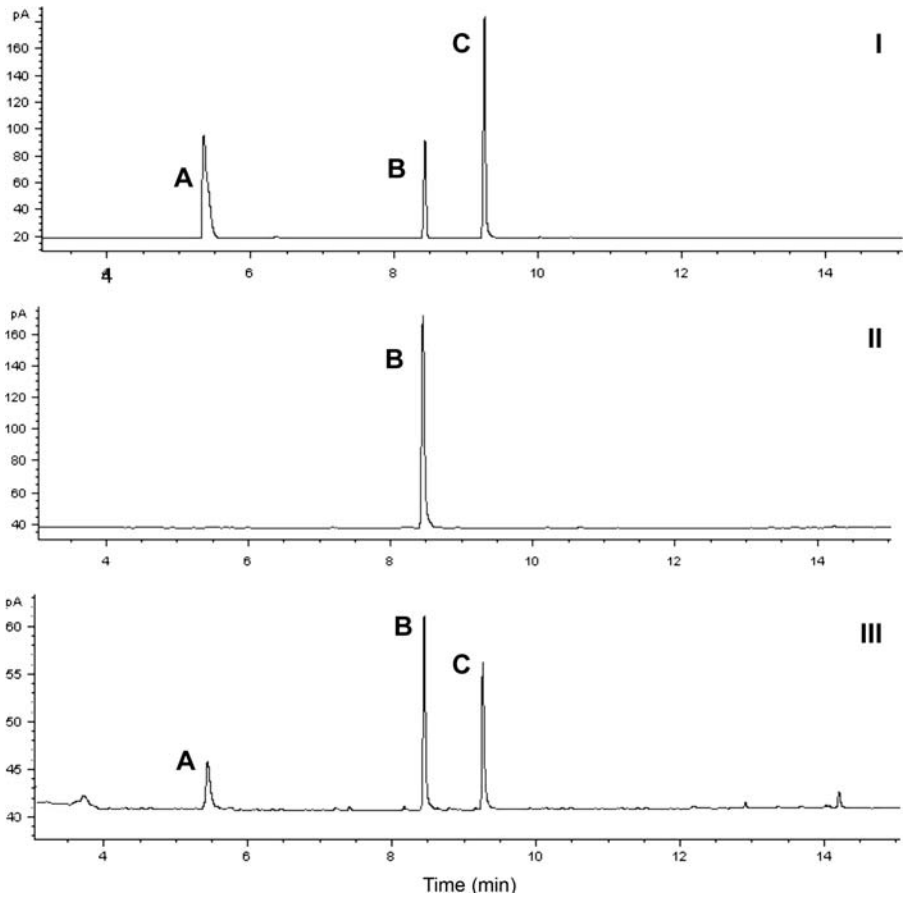

Figure 3. Chromatograms obtained with the ASE and GC–NPD analysis of a meconium samples. Sample spiked with nicotine (A) and cotinine (C) at concentration of 100 ng/g and the internal standard prolintane (B) (I); blank meconium sample (II); and positive sample containing 55.3 ng/g of nicotine and 61.6 ng/g of cotinine (III).

prior to the extraction of the matrix. The absolute recovery, expressed as a percentage, was evaluated by comparison of the mean response of extracted samples fortified before extraction and the response of the extracted blank matrix samples to which analytes had been added at the same concentration just before the drying step.

Results and Discussion

Sample preparation

In general, the detection of drugs and metabolites in meconium samples is extremely difficult because of the high concentration of endogenous compounds such as lipids, proteins, and salts and the low concentration of the analytes present in the specimen. Currently published methods to extract substances from meconium for chromatographic analysis are generally time-consuming and laborious, often involving at least two steps of sample preparation: homogenization of meconium in a liquid prior to SPE for purification of analytes (18).

In the last few years, few analytical methods were published in the scientific literature for the detection of nicotine biomarkers in meconium samples. In one of the first published methods, Boranowski et al. (14) emulsified 2.0 g of sample with 20 mL of phosphate buffer (pH 6.0) and carried out an extraction with chloroform. After the evaporation of the extract, the residue was dissolved in buffer, and an SPE procedure was used for purification of analytes (nicotine, cotinine, and caffeine), prior to liquid chromatographic (HPLC) analysis. Köhler et al. (6) used a modified method published by Dempsey et al. (19) to assess prenatal tobacco smoke exposure. In summary, the following procedure was conducted: meconium (0.5 g) was homogenized with 3 mL of methanol and alkaline-hydrolyzed under ultrasonic treatment for 30 min. Following centrifugation the supernatant was evaporated and reconstituted with buffer. Afterwards, the solution was submitted to an SPE step. The analytes (nicotine, cotinine, and *trans*-3′-hydroxycotinine) were detected by HPLC. Gray et al. (13) extracted nicotine biomarkers (nicotine, cotinine, *trans*-3′-hydroxycotinine, nornicotine, and norcotinine) from meconium by using acidified methanol homogenization. After centrifugation, the supernatant was evaporated and reconstituted with phosphate buffer for overnight enzymatic hydrolysis (18 h). An SPE procedure of the solution was still necessary for further detection of analytes by liquid chromatography–tandem mass spectrometry (LC–MS–MS). More recently, Marin et al. (20) detected nicotine and metabolites in paired umbilical cord tissue and meconium samples using LC–MS–MS. Preparation of samples also involved homogenization with organic solvents and clean-up of extracts with SPE.

In the present work, ASE was used for the pretreatment of meconium before the SPE step. The use of ASE technique instead of organic solvent homogenization provided a simpler and faster method because no evaporation step was necessary prior the SPE procedure. Also, no organic solvent (chloroform or methanol) was used for pretreatment of samples. Instead, buffer solution could be used, because ASE works with a filtration system that makes possible the direct use of the aqueous extract to the subsequent SPE procedure. Because hydrolysis is a lengthy and costly process, this procedure was not taken into consideration in the present method. In addition, in a study performed by Gray et al. (5), who analyzed 125 paired meconium samples (with and without hydrolysis), they identified only 1 additional positive specimen in the group of hydrolyzed samples. The authors also verified that nicotine, cotinine, and *trans*-3′-hydroxycotinine are the most prevalent and abundant biomarkers found in meconium of tobacco-exposed neonates. Unfortunately, *trans*-3′-hydroxycotinine standard was not commercially available in Brazil to be included in our study.

Optimization of the ASE procedure

For the optimization of the ASE procedure, the influence of the number of cycles in extraction efficiency was evaluated. The number of static cycles can be selected to improve the efficiency of the extraction, whereas a longer contact time of solvent with the sample could help in maintaining the balance of the extraction. In practice, it was observed that one cycle produced the highest efficiency of the extraction (Figure 2A).

Two solvent systems were evaluated in three different temperatures of extraction (80, 100, and 120°C). The choice of the mixture hexane/acetone (1:1) was based on the study of Curwin et al. (21), who used this solvent system to extract nicotine from wipes by means of the ASE technique. Phosphate buffer (0.1 M, pH 6.0) was also tested because it would be more practical to have an extract already dissolved in the same solvent to be used in the next step of sample preparation (SPE). Interestingly, although they have distinct physical-chemical properties, both solvents system showed to have similar efficiency in the extraction yield for the three tested temperatures (Figures 2B and 2C). Nevertheless, organic solvents have disadvantages when compared with aqueous solutions because they are more toxic to the analyst and hazardous to the environment. Therefore, phosphate buffer was chosen as solvent to be used in the ASE system. The temperature of 120°C provided the best result for extraction of the analytes in meconium.

Validation of the method

The confidence parameters of the validated method (LOD, LOQ, intra- and interassay precision, accuracy, and recovery) for the determination of nicotine and cotinine in meconium are shown in Table I.

Calibration curves were linear over the specified range (LOQ–500 ng/g). The linear regression equations and coefficients of correlation were as follows: nicotine $y = 0.0144x - 0.5509$; $r^2 = 0.9872$ and cotinine $y = 0.1400x + 5.4319$; $r^2 = 0.9901$, where y and x represent the relationship between the peak-area ratio (compound/internal standard) and the corresponding calibration concentrations, respectively.

The method showed good linearity over a broad concentration range (LOQ–500 ng/g). Considering that meconium is a complex matrix and the sample preparation consisted of two extraction techniques, the precision was considered acceptable over the studied concentration range (RSD < 15% for intraassays). The ASE procedure followed by SPE produced clean extracts for GC–NPD analysis with good recovery (average 77.2%).

Table II
Concentrations of Cotinine and Nicotine (ng/g) Detected in 16 Positive Meconium Samples Analyzed by the Developed Method

ID	Cotinine	Nicotine
1	90.7	329.3
2	41.8	131.8
3	50.2	221.5
4	61.6	55.3
5	35.3	266.6
6	38.0	n.d.*
7	46.8	n.d.
8	58.5	194.5
9	34.1	195.8
10	30.9	133.2
11	45.5	175.4
12	49.0	234.0
13	31.8	113.9
14	34.4	141.6
15	53.1	195.1
16	32.0	180.8

* n.d. = not detected

Method application

The developed method was applied to 16 meconium samples collected from neonates whose mothers admitted using tobacco during pregnancy. Samples were collected from neonates who were born in the University Hospital of São Paulo (HU-USP). Figure 3 shows GC–NPD chromatograms obtained with the practical use of this method to the analysis of meconium samples [a sample fortified with 100 ng/g of analytes (I), blank sample (II), and a positive sample containing 55.3 ng/g of nicotine and 61.6 ng/g of cotinine (III)]. Table II shows the results for 16 meconium samples analyzed by the ASE/SPE method.

Conclusions

A GC–NPD screening method for the determination of cotinine and nicotine in meconium samples was developed. Sample pretreatment was simplified using the accelerated solvent extraction technique compared to previous published chromatographic methods. Also, smaller volumes of organic solvents were necessary. Taking into consideration that meconium is a complex matrix the values of precision (RSD less than 15% for intraassay precision) are acceptable. The method can be readily used to evaluate tobacco exposure during pregnancy. ASE can be a useful technique to be employed in the analysis of complex biological samples.

Acknowledgments

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