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Method validation for measurement of hair nicotine level in nonsmokers

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ABSTRACT: The development of strategies to address the growing worldwide burden of exposure to secondhand smoke (SHS) would be facilitated by sensitive and accurate methods for assessing SHS exposure. Hair provides a readily available matrix for assessing biomarkers of typical SHS exposure. We developed and applied an optimized analytical method using an isotope dilution gas chromatography–mass spectrometry (GC/MS) for hair nicotine measurement. The utility of this optimized method is illustrated by presenting data on SHS exposure of women and children from 31 countries. Using this isotope dilution method with spiked samples (3.3 ng/mg), we found that the greatest hair nicotine extraction efficiency was obtained with a 60 min shaking time. In the field study ($n = 2400$), a positive association was evident between hair nicotine concentrations from nonsmokers and higher numbers of cigarettes smoked per day in a household. Copyright © 2008 John Wiley & Sons, Ltd.

Keywords: nicotine; hair; secondhand smoke; children; gas chromatography–mass spectrometry

Introduction

In the presence of smokers, nonsmokers inhale secondhand smoke (SHS), the combination of side-stream smoke released from the cigarette's burning and mainstream smoke exhaled by the active smoker (Guerin *et al*., 1992). Exposure to SHS is a worldwide public health problem (IARC, 1986; NRC, 1986; US DHHS, 1986; Warren *et al*., 2006; Wipfli *et al*., 2008). Evaluating the magnitude of SHS exposure and identifying its sources are fundamental steps needed to reduce exposure and to target prevention strategies. SHS exposure is assessed by diverse methods, including questionnaires (Al-Delaimy *et al*., 2000; Delfino *et al*., 1993; Gaffney *et al*., 2003; Klepeis, 1999), air nicotine monitoring (Hammond and Leaderer, 1987; Leaderer, 1990; Navas-Acien *et al*., 2006; Repace *et al*., 2006; Wipfli *et al*., 2008), and biomonitoring of nicotine or cotinine in such biofluids as serum, urine or saliva (Benowitz, 1999; Coultas *et al*., 1987; Seccareccia *et al*., 2003; Pirkle *et al*., 2006). The most commonly used biomarker for SHS exposure is nicotine or its metabolite, cotinine, in human body fluids such as urine, saliva and serum (Pichini *et al*., 2000; Simoni *et al*., 2006; Thaqi *et al*., 2005).

The utility of nicotine or cotinine concentrations in body fluids is limited, however, by the relatively short half-lives of these biomarkers. The half-life of nicotine in body fluids is approximately 2–3 h, and that of cotinine is 1–2 days (Benowitz, 1996; Jaakkola and Jaakkola, 1997). Therefore, levels of these markers in the body fluids are indicative of recent, rather than long-term, exposure.

Longer-term biomarkers of SHS exposure have a potential utility for documenting usual patterns of exposure and for investigating health risks of chronic exposure to SHS (IARC, 1986; NRC, 1986; US DHHS, 1986; Warren *et al*., 2006). Several studies indicate that hair nicotine concentration is a useful biomarker of longer term smoke exposure (Al-Delaimy *et al*., 2002; Eliopoulos *et al*., 1996; Nafstad *et al*., 1995; Pichini *et al*., 1997b). With a rate of hair growth of about 1.1 cm/month (Uematsu, 1993), a small amount of hair (2–3 cm) from the scalp can potentially represent exposure to SHS over 2–3 months. In addition, hair nicotine has shown better ability to discriminate exposure status when compared with urinary cotinine (Al-Delaimy *et al*., 2002; Nafstad *et al*., 1995). Al-delaimy *et al*. reported that hair nicotine concentrations in a sample of children were more strongly associated with categories of smoking within a household (no smokers, smoking only outside the house, and smoking inside the house) than urinary cotinine concentrations (Al-Delaimy *et al*., 2002).

Hair nicotine concentrations have been assessed using various chromatographic techniques, including high-performance liquid chromatography (HPLC) with an ultraviolet detector (Pichini *et al*., 1997a), an electrochemical detector (Mahoney and Al-Delaimy, 2001), and mass spectrometry (MS, MS/MS) (Chetiyanukornkul *et al*., 2004; Kronstrand *et al*., 2004; Pirkle *et al*., 2006); and gas chromatography (GC) with a nitrogen–phosphorus detector or mass spectrometry (Kintz, 1992; Zahlsen and Nilsen, 1994; Torano

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Abbreviations used: GC/MS, gas chromatography–mass spectrometry; LOD, limit of detection; SHS, secondhand smoke.

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and van Kan, 2003). Immunoassay techniques, such as enzyme-linked immunosorbent assay, fluorescence immunoassay and radioimmunoassay, have also been used (Eliopoulos *et al*., 1994, 1996; Klein and Koren, 1999; Florescu *et al*., 2007). Chromatographic methods have been chosen more often because of their high sensitivity and specificity to detect trace levels of nicotine or cotinine in saliva, urine and serum of smokers and nonsmokers exposed to SHS (Crooks and Byrd, 1999; Jacob and Byrd, 1999). In general, immunoassay techniques have a comparable sensitivity to chromatographic methods but some techniques, e.g. radioimmunoassay, have a relatively low specificity due to potential cross-reaction between anti-cotinine antibody and not only cotinine but other nicotine metabolites (Benowitz, 1996). Among chromatographic methods with various detection systems, gas or liquid chromatography with mass spectrometry is most commonly used due to its advantage of simultaneous measurement of nicotine and its metabolites with deuterium labels used as internal standards (Crooks and Byrd, 1999; Jacob and Byrd, 1999). Despite the potential usefulness of assessing SHS exposure using hair nicotine and analysis by chromatography with mass spectrometry, few studies have been reported on an isotope dilution GC/MS method for measuring nicotine in hair. In addition, few studies have reported experimental data justifying important sample preparation and extraction details for GC/MS.

The purpose of this paper is to describe optimized analytical extraction parameters with isotope dilution GC/MS techniques for hair nicotine measurement and to demonstrate the feasibility of our optimized method by analyzing hair samples from a study of nonsmoking women and children in 31 countries. Optimized extraction parameters include: sample shaking time; extraction solvent selection; and the impact of repeated extractions. In addition, estimates of measurement bias and accuracy estimates are presented.

Experimental

The optimized analytical method for hair nicotine analysis using an isotope dilution gas chromatography–mass spectrometry was adapted with modification using a method reported by Kintz (1992) and Zahlsen and Nilsen (1994). Hair samples from a non-smoking, non-SHS-exposed individual were collected for method optimization experiments. Multiple samples of hair 3 cm in length, starting from the scalp, were cut and weighed into 30 mg aliquots.

Determination of Method Optimization Parameters

We optimized extraction parameters for hair nicotine measurement with a GC/MS by evaluating the effects of sample shaking time during liquid–liquid extraction, extraction solvents and impact repeated extraction.

Sample shaking time. To evaluate liquid–liquid extraction efficiency by shaking time extraction, fifteen 30 mg hair samples (three samples per group) were spiked to a concentration of 3.3 ng/mg and placed in a shaker for 10, 30, 60, 90 and 120 min. Following shaking, the samples were processed according to the extraction process described below.

Extraction solvent. We compared the extraction efficiency of two solvents, dichloromethane and diethyl ether. These two solvents were selected because they have similar polarities to each other as well as to nicotine. Diethyl ether was the solvent used in the method developed by Kintz (1992).

Impact of repeated extraction. We also compared amount of nicotine extracted from smokers' (15–20 cigarettes per day) hair samples ($n = 6$) and spiked (3.3 ng/mg) nonsmokers' hair samples ($n = 3$) by single, double and triple liquid–liquid extraction to validate extraction recovery. We calculated nicotine extraction recovery by calculating the ratio of nicotine obtained by single and/or double extraction to the sum of the amounts of nicotine obtained by all three extractions for smokers' hair samples. Our recoveries were further validated by comparing the absolute amount of nicotine obtained by single and double extraction from the non-smokers' hair samples spiked with a known mass of nicotine.

Hair Nicotine Extraction Method

Thirty milligrams of hair were transferred to a polypropylene centrifuge tube (SARSTEDT, no. 62.554.205) for washing. Remaining portions of the hair samples were stored for subsequent analyses. Since we were only interested in the measurement of nicotine accumulated in hair by inhalation, systemic transport, and subsequent incorporation of nicotine into the growing hair, samples were washed using 3 mL of dichloromethane by sonication (Aquasonic, Model 250HT) for 30 min to remove nicotine adhering to the surface of the hair. When the hair strands were completely dry after the washing step, 1.5 mL of 1 M NaOH and 70 ng of internal standard (Nicotinine-d3, Supelco) were added to the sample preparation tube. Tubes were then capped to reduce evaporation. Samples were incubated at 50°C for 24 h, then cooled to room temperature. After incubation, 3.5 mL solvent (either diethyl ether or dichloromethane) was added to the tubes, the tubes were shaken using a horizontal shaker (IKA, KS260 basic) for various shaking times at 200 mot/min, and finally centrifuged at 2000***g*** for 10 min, as described above. The organic phase in each tube was then transferred to a clean polypropylene evaporation tube. To prevent volatilization of nicotine, 17.5 μL octanol was added to each tube. The diethyl ether or dichloromethane hair extraction process was repeated a second or third time to evaluate extraction efficiency. The organic layer from combined extractions was evaporated completely in a water bath at 50°C for approximately 20 min. Finally, 52.5 μL of methanol was added to the remaining solution of octanol and nicotine, bringing the volume to 70 μL. The final solution was transferred to an insert mounted in an autosampler vial. During method optimization processes, these steps were completed using both dichloromethane and diethyl ether to compare extraction efficiency of the two solvents.

Instrumentation

Nicotine analysis was conducted using a GC/MS (GC-17/MS-QP5000, Shimadzu) in selected ion monitoring (SIM) and splitless modes. The nicotine was separated by a capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness, Rtx-624, Restek, Bellefonte, PA, USA). The GC oven temperature was maintained at 70°C for 1 min and then ramped up to 280°C at a rate of 25°C/min; it was then maintained for 1 min. Temperatures of injection port and GC interface were 250 and 280°C and ionizing voltage was 70 eV. In the SIM mode, we used *m/z* 162, 133 for nicotine and *m/z* 87, 165, 136 for nicotine-d3 detection.

Calibration

Calibration standards (0–12.5 μg/mL, nine points) were prepared in a methanol and octanol mixture (3:1 v/v). The concentration of the internal standard spiked in calibration standard samples and hair sample extracts was 1 ng/μL for isotope dilution analysis. A calibration curve was determined from the nicotine amount spiked into the GC injector and the corresponding response factor (ratio of peak area of nicotine to the isotope). The relationship between the response factor and the known amount of nicotine was evaluated by simple linear regression.

Quality Control Assessment

For quality control, hair nicotine measurement bias and precision were determined. For this evaluation, two sets of five hair samples were spiked with 0.67 and 2.33 ng/mg. Hair samples were prepared by spiking 200 or 700 μL of 0.1 μg/mL nicotine solution onto 30 mg of hair. Bias was evaluated by calculating recovery (%), and precision was determined as the relative standard deviation (%) from the five replicate analyses of the two sets. In addition to spiked samples, fresh checking standards (the same as the calibration standard of 0.5 ng/μL, $n = 8$ per batch) were analyzed with each batch and reagent blank samples were also used to determine the LOD and blank-corrected hair nicotine concentration. The LOD was determined by multiplying the standard deviation of background nicotine concentrations in the blank samples ($n = 8$) and the Student's t-value appropriate for a 99% confidence level with $n - 1$ degrees of freedom. The t-value corresponds to a cut-off value of the upper 1% of the t distribution with specified degrees of freedom, which were seven in this current study (Miller and Miller 1988).

Hair Sample Analysis

To apply our optimized extraction parameters and analytical procedures, we analyzed nicotine concentration in hair samples collected from nonsmoking mothers and children in 31 countries (40 mothers and 40 children per country; Wipfli *et al*., 2008). A minimum of 30 mg of 3 cm long hair was cut near the hair root from the rear of each participant's scalp. Collected samples were stored in a clean envelope marked to show the cut end of the hair and transported to the laboratory. Samples were stored in a refrigerator until they were analyzed to avoid any contamination. A total of 10% of the hair samples from each batch underwent duplicate analyses. Using the participants' self-reported questionnaires, we identified the number of household smokers and how many cigarettes were smoked per day in each household.

Statistical Analysis

Statistical analyses were performed using SAS (version 9.1; SAS Institute Inc., Cary, NC, USA). We compared the distribution of extraction efficiencies at various time points using the Wilcoxon rank sum test. The differences among log-transformed hair nicotine concentrations by categories of the number of cigarette smoked per day were evaluated using the analysis of variance (ANOVA) with a multi-comparison test option of Bonferroni at the significance level of 0.05. We also performed a nonparametric test to compare median hair nicotine concentrations between the women and children.

Results and Discussion

Optimization of Analytical Method

Hair nicotine optimization studies were conducted using the GC/MS operating conditions described above. Nicotine, along with its corresponding internal standards, was detected at 4 min as a retention time. Example chromatograms are presented in Fig. 1.

Effect of extraction time. Figure 2 shows the extraction efficiency at various sample shaking times. The extraction efficiency increased dramatically from 10 to 30 min. Extraction efficiency was relatively constant at about 90% after 30 min of shaking time. The Wilcoxon rank sum test results indicated that the recovery of nicotine concentration at 30 min (87%) did not vary from 60 (91%), 90 (88%) or 120 (85%) minutes. However, we selected 60 min as the final extraction time because the highest recovery (91%) was obtained at that time point.

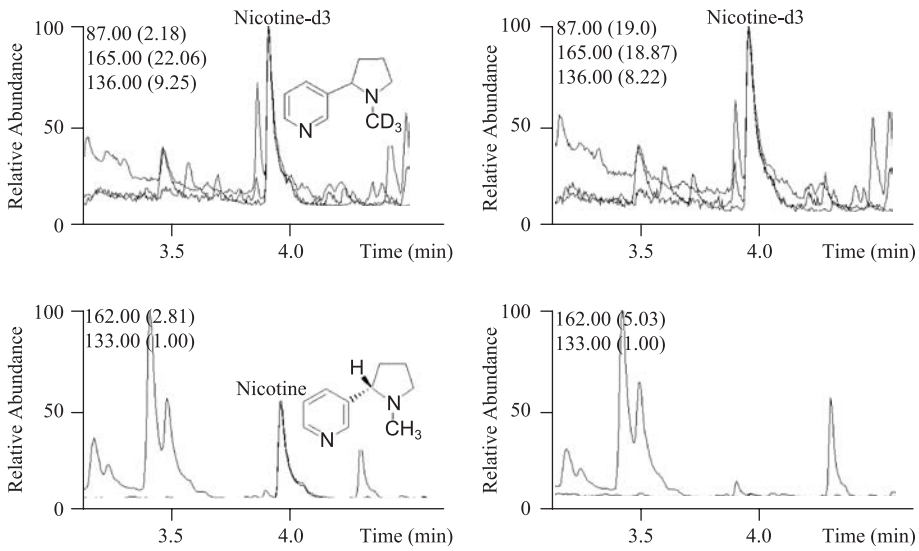

Figure 1. Chromatograms of nicotine (m/z 162, 133) extracted from smoker's (left) and nonsmoker's (right) hair samples and spiked nicotine-d3 (m/z 87, 165, 136).

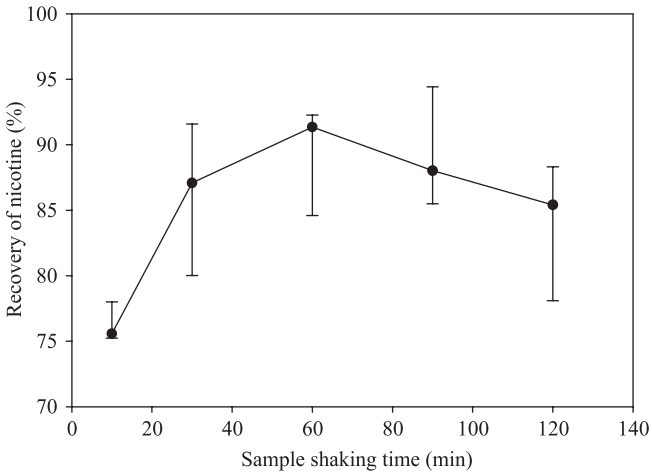

Figure 2. Recovery of nicotine (median ± interquartile range, %) by hair sample extraction shaking time.

Effect of extraction solvent. We compared extraction efficiency using two solvents, dichloromethane and diethyl ether. No significant difference in extraction efficiency for nicotine was evident between the two solvents (data not shown). We chose to use diethyl ether as our extraction solvent due to its higher rate of volatilization.

Repeated extraction. From our extract efficiency tests with three time consecutive extraction trials, we determined that 60–78 and 84–91% of nicotine could be extracted by single and double extraction, respectively, for both smokers' hair or nonsmokers' hair samples with nicotine spiked (Fig. 3). Therefore, we applied the double extraction on our field hair samples collected from 31 countries.

The calibration curves showed a strong relationship between instrument responses and known amounts of standard samples spiked into the instrument. The coefficients of determination (r^2) were 0.99 or higher in the applicable calibration ranges. The LOD estimated from the blank samples was 0.02 ng/mg for a 30 mg hair sample. The limit of detection was comparable to that (0.05 ng/mg) reported by Mahoney and Al-Delaimy (2001).

Theoretically, an isotope is just different in mass compared with the original but it has almost exactly the same physicochemical properties as the original. Therefore, our analytical method using a nicotine isotope during the entire procedures of hair digestion, nicotine extraction and instrument analysis should reduce quantification errors resulting from choosing a nonisotope as an internal standard or missing an internal standard during hair digestion or extraction steps.

Analysis of Qualitative Control Samples

Bias and precision of measurements were evaluated using both checking standards (equal to the calibration standard of 0.5 ng/mL, $n = 8$, within batch, and n = 144, between batch) and hair samples spiked with two different concentrations (i.e. 0.67 and 3.33 ng/mg, $n = 5$, within batch, $n = 30$ between batches). The recoveries from the checking standards were 97.1 and 107.9% for within and between batches with 8% RSD, respectively (Table 1). The median recoveries from the nicotine spiked hair samples within batches were 84.8 and 88.1% for the two concentration levels, respectively; recoveries from between batches were 73 and 83%. Precisions ranged from 6.3% for 3.3 ng/mg nicotine spiked hair samples with-batch to 21% for 0.67 ng/mg between-batches. Our results were comparable to those reported by Mahoney and Al-Delaimy (2001). Their reported mean recoveries were 95.3% for 4.1 ng and 93.1% for 32.4 ng of nicotine standard spiked. Their estimates of precision ranged from 4.7% for 25.7 ng/mg to 10.2% for 0.25 ng/mg hair nicotine samples within-batch, and 8.7% for 22.5 ng/mg to 19.8 % for 0.32 ng/mg between-batches.

Duplicate analysis was performed on 10% of hair samples from each batch. The correlation coefficient between the primary and the duplicate analyses was 0.97 ($n = 172$; Fig. 4) and the median

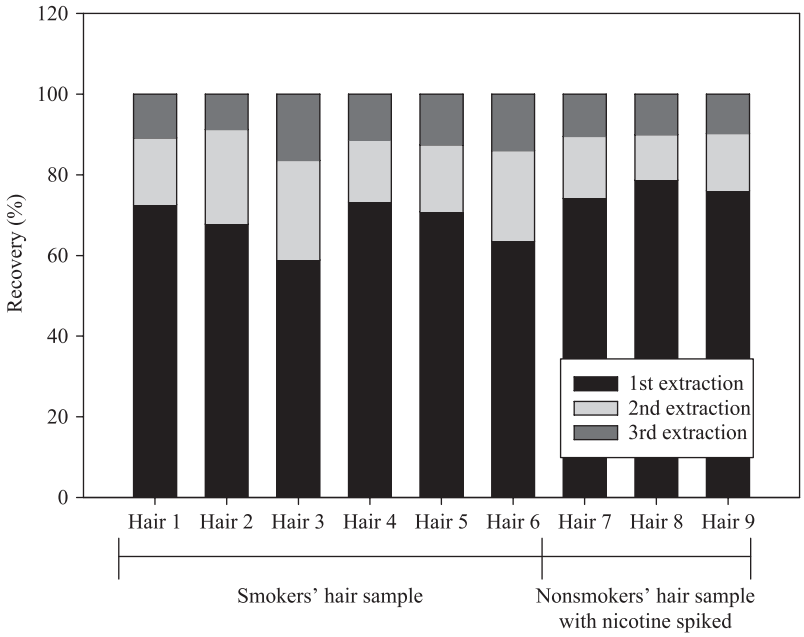

Figure 3. Plot of nicotine extraction efficiency for repeated extractions.

Table 1. Summary of precisions and recoveries obtained with an isotope dilution method of GC/MS

			Within-batch				Between-batch		
	Spiked concentration	*n*	Observed concentration (mean ± SD)	Precision (%)	Recovery (%)	*n*	Observed concentration (mean ± SD)	Precision (%)	Recovery (%)
Checking standard (ng/mL)	0.50	8	0.49 ± 0.04	8.5	97.1	18	0.51 ± 0.04	8.01	107.9
Hair nicotine (ng/mg)	0.67	5	0.57 ± 0.05	8.5	84.8	6	0.49 ± 0.10	20.9	73.5
	3.33	5	2.93 ± 0.18	6.3	88.1	6	2.78 ± 0.33	11.9	83.3

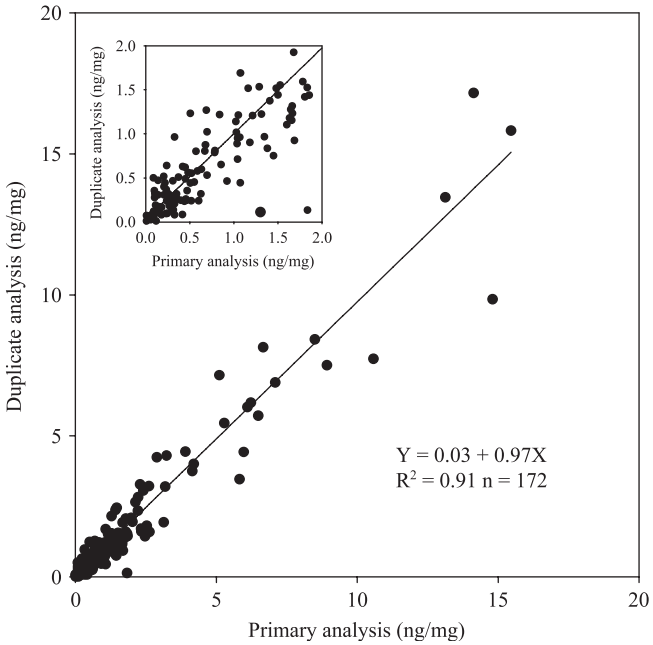

Figure 4. Scatter plot comparing the concentration of hair nicotine in duplicate experiments using samples from 31 countries with the GC/MS isotope dilution method.

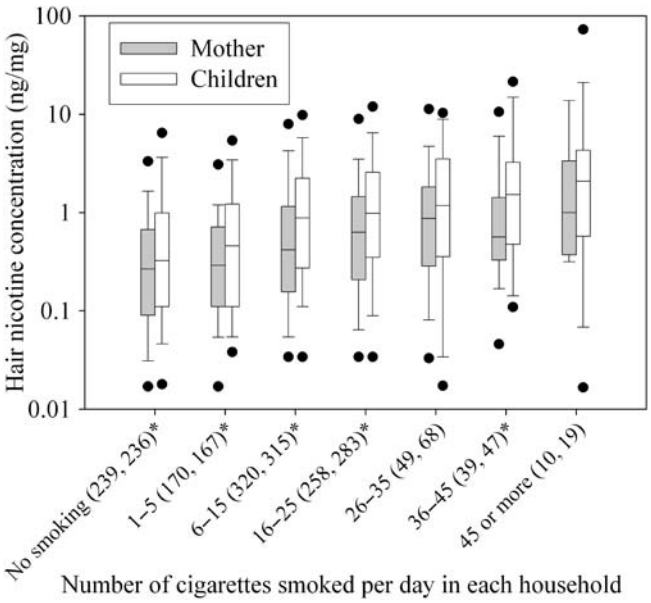

Figure 5. Hair nicotine concentration distribution by range of the number of cigarettes smoked per day in each household among SHS-exposed study participants from 31 countries. The top and bottom of the box indicate the inter quartile range (from 25th to 75th percentile) and the vertical line indicates the median. Whiskers indicate the 10th and 90th percentiles. Numbers in the parentheses represent the number of hair samples from women and children per each category. Asterisk indicates that children's median hair nicotine concentration was statistically significantly higher than the median concentrations of the women's in each category.

relative difference between the two values over the average of the two values was 24%, indicating a high level of precision within each sample using our optimized method and analytical protocol.

Analysis of Field Hair Samples

In a study in 31 countries, hair nicotine concentrations from women and children exposed to SHS varied in relation to the reported number of cigarettes smoked per day in the household (Fig. 5). The median concentrations by category for women and children who were exposed to SHS in the household were 0.42 and 0.88 ng/mg for six to 15 cigarettes; 0.87 and 1.18 ng/mg for 26 to 35 cigarettes; and 1.00 and 2.08 ng/mg for 46 or more cigarettes, respectively. The median hair nicotine concentration from households with little ('0 to 5' cigarettes) or no SHS exposure were significantly lower ($p < 0.05$) than median values of hair nicotine for mothers and children with greater reported SHS exposure.

The median values of children's hair nicotine concentrations were consistently higher than those of the mothers' hair nicotine concentrations across all categories of the number of cigarettes smoked. Except for the categories 'No smoking', '26–35' and '46 or more cigarettes smoked', the differences were statistically significant ($p < 0.05$). Differing behavior patterns of children and adults could result in increased opportunity for children to inhale SHS (Weaver *et al*., 1998; Willers *et al*., 1995). Physiological differences between children and adults could result in greater body burdens for children from similar SHS exposure (Weaver *et al*., 1998; Willers *et al*., 1995). Willers *et al*. (1995) measured urinary cotinine as a biomarker of SHS in 14 children (age 4–11 years) and in seven adults who were exposed to SHS at an air nicotine level of 110 mg/m^3 for 2 h in a bus. In this study, the estimated mean nicotine dose in children (2.3 μg/kg body weight) was 35% larger than that in adults (1.7 μg/kg body weight), at the same level of SHS exposure.

Figure 5 suggests a positive association between hair nicotine concentrations and the number of cigarettes smoked in a household. Other studies have reported a similar concentration range

and exposure–response relationships of hair nicotine concentration of children with the number of cigarette smoked per day by other household members (Al-Delaimy *et al*., 2000; Sorensen *et al*., 2007). Al-Delaimy *et al*. (2000) reported that median hair nicotine concentrations from children who were exposed to SHS from 0 ($n = 24$), 1–9 ($n = 23$), 10–19 ($n = 31$) and 20 or more ($n = 34$) cigarettes smoked per day by household adults were 0.001, 0.1, 0.22 and 2.4 ng/mg, respectively (estimated from Fig. 1 of their paper). Sorensen *et al*. (2007) reported that mean hair nicotine concentration for infants not exposed to SHS from household smoking was 1.2 ± 2.4 ng/mg ($n = 301$), while the value for infants ($n = 56$) who were exposed to SHS from 1 to 9 cigarettes smoked in the household was 5.6 ± 6.0 ng/mg. More results of data analysis from our study are described in a separate paper (Wipfl *et al*., 2008).

Our study demonstrates the utility of an optimized GC/MS isotope dilution method for measuring a wide range of hair nicotine concentrations (0.01–100 ng/mg) in a large number of non-smokers ($n = 2480$) who are potentially exposed to SHS. With this method, we can analyze more than 40 hair samples per day, documenting its suitability for routine analysis of large batches. By analyzing 3 cm of hair, representing about 3 months of hair growth, we were able to capture longer-term exposure and distinguish exposure status among women and children with varying long-term exposures. Additional investigations are needed to evaluate the effects of hair treatments on hair nicotine levels and inter-individual variation of hair nicotine concentrations over various intervals of time with a controlled SHS exposure source strength.

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