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Simultaneous determination of urinary 1- and 2-naphthols, 3- and 9-phenanthrols, and 1-pyrenol in coke oven workers

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A method was developed for simultaneous quantification of urinary 1- and 2-naphthols, 3- and 9-phenanthrols and 1-pyrenol using gas chromatography with mass spectrometry (GC-MS). This method was applied to urine samples from coke oven workers ($n = 28$) and controls ($n = 22$) from Northern China. Geometric mean levels of urinary 1-naphthol (58.8 $\mu g\, l^{-1}$), 2-naphthol (34.1 $\mu g\, l^{-1}$), 3-phenanthrol (7.35 $\mu g\, l^{-1}$), 9-phenanthrol (1.28 $\mu g\, l^{-1}$) and 1-pyrenol (25.4 $\mu g\, l^{-1}$) were significantly higher among coke oven workers than controls. All the substances tested were highest among top-of-oven workers, who had 15-fold higher 1-naphthol, eight-fold higher 2-naphthol and 20-fold higher 1-pyrenol levels compared with controls. Using multiple linear regression models, 72.5% of the variation in 1- and 2-naphthol and 82.8% of the variation in 1-pyrenol were explained by the concentration of naphthalene or pyrene in the urine, the work category and the smoking intensity. Cigarette consumption significantly contributed to levels of urinary 1-pyrenol and naphthols, particularly 2-naphthol. A negative relationship between work category and the ratio of naphthols/1-pyrenol was observed among smokers. Our results suggest that urinary naphthols and phenanthrols reflect polycyclic aromatic hydrocarbon (PAH) exposure as well as the widely used 1-pyrenol, and that interactions between cigarette smoking and PAH exposure result in different patterns of metabolism for individual PAHs.

Keywords: naphthol, phenanthrol, pyrenol, naphthalene, pyrene, PAH, urine.

Introduction

Polycyclic aromatic hydrocarbons (PAHs) comprise a class of chemicals produced by incomplete combustion of organic materials. Occupational exposures to PAHs have been associated with cancers of the lung, skin and bladder (Lloyd 1971, Armstrong *et al*. 1994, Tremblay *et al*. 1995, Mastrangelo *et al*. 1996, Boffetta *et al*. 1997). Coke oven workers are exposed to particularly high levels of PAHs via both inhalation of fumes and absorption through intact skin (Grimmer *et al*. 1993, Pyy *et al*. 1997); these exposures are thought to be responsible for four- to 10-fold increases in the incidence of lung cancer in this population (Lloyd 1971, Boffetta *et al*. 1997). More modest PAH exposures are encountered in other

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industries (e.g. aluminium and iron production, use of coal tar and creosote) and in the ambient environment, where cigarette smoking and engine exhausts are the primary sources (Hoepfner *et al*. 1987, Armstrong *et al*. 1994, Tremblay *et al*. 1995).

Most PAHs are ultimately metabolized to phenols that can be excreted in urine, often following conjugation. Several phenolic urinary metabolites have been used as biomarkers of PAH exposure, notably naphthols, phenanthrols and 1-pyrenol (Jansen *et al*. 1995, Grimmer *et al*. 1997, Jongeneelen 1997). Of these, 1-pyrenol, a metabolic product of pyrene, has been most widely used for human studies (reviewed by Dor *et al*. 1999, Bouchard and Viau 1999). However, naphthols and phenanthrols are derived from vapour-phase PAHs (i.e. naphthalene and phenanthrene, respectively) that tend to be much more abundant than pyrene in a given environment (Bjørseth *et al*. 1978, 1981, Hansen *et al*. 1991, Buchet *et al*. 1992). Furthermore, while pyrene is thought to mimic the uptake of particle-bound PAHs through both the lungs and the skin (VanRooij *et al*. 1993, Strickland and Kang 1999), it has been suggested that naphthalene is a more specific marker for the inhalation of PAHs (Jansen *et al*. 1995, Kim *et al*. 2001). For these reasons, it is potentially useful to measure both 1-pyrenol *and* the naphthols or phenanthrols to gauge the magnitude and potential routes of PAH exposure.

The few studies reporting parallel measurements of urinary 1-pyrenol, the naphthols and/or the phenanthrols are summarized in Table 1. Grimmer *et al*. (1993, 1997) used gas chromatography with either flame ionization detection (GC-FID) or mass spectrometry (GC-MS) to measure phenanthrols and 1-pyrenol; however, their assay required a large urine volume (150–200 ml) and extensive clean-up prior to quantification. Most of the other methods employed high performance liquid chromatography (HPLC) with fluorescence detection, based largely on the assay for 1-pyrenol reported by Jongeneelen *et al*. (1987). However, this methodology cannot be easily adapted for simultaneous measurement of urinary naphthols and/or phenanthrols, due to limited sensitivity for naphthols (Hansen *et al*. 1994, 1995, Heikkilä *et al*. 1995, Hollender *et al*. 2000) and the poor resolution of some phenanthrols (Lintelmann *et al*. 1994, Angerer *et al*. 1997). Kim *et al*. (1999, 2001) overcame some of these problems by employing separate HPLC/fluorescence assays for 1-pyrenol and the naphthols. In a recent report, Bouchard *et al*. (2001) determined urinary naphthols and 1-pyrenol using GC-MS and reported similar detection limits to those of Kim *et al*. (2001), but the volume of urine used was not reported. The most sensitive method for simultaneous measurement of 1-pyrenol, naphthols and phenanthrols was reported by Gmeiner *et al*. (1998), who employed solid-phase microextraction (SPME) followed by *in situ* derivatization and GC-MS. However, the special apparatus used for this assay is not available to most laboratories.

In the current study we used a simple GC-MS assay to measure urinary naphthols, phenanthrols and 1-pyrenol in urine samples from a sample of coke oven workers and controls. We examined the correlation of the levels of these urinary metabolites as well as the effects of the PAH exposure category and smoking intensity on metabolite production.

Table 1. Methods used for the analysis of naphthols and/or phenanthrols as well as 1-pyrenol in urine.

		Naphthols			Phenanthrols			1-Pyrenol		
Reference	Method	Volume (ml)	LOD ($\mu g\ l^{-1}$)	CV (%)	Volume (ml)	LOD ($\mu g\ l^{-1}$)	CV (%)	Volume (ml)	LOD ($\mu g\ l^{-1}$)	CV (%)
Grimmer *et al*. (1993)	GC/FID	NM	NM	NM	200	NR	5.0–34.2	200	NR	4.8
Lintelmann *et al*. (1994)	HPLC/fluorescence	NM	NM	NM	20–25	< 0.0002	5.5–7.6	20–25	< 0.0002	7.6
Grimmer *et al*. (1997)	GC-MS	NM	NM	NM	150	NR	6.06–7.67	150	NR	4.99
Angerer and Schaller (1999)	HPLC/fluorescence	NM	NM	NM	5	0.30–0.50	5.10–12.3	5	0.10	7.60–9.20
Hansen *et al*. (1993, 1994, 1995)	HPLC/fluorescence[a]	10	5.76	NR	NM	NM	NM	15	0.30	NR
Kim *et al*. (1999), Kim *et al.* (2001)	HPLC/fluorescence[a]	1–3	0.13	1.44–6.30	NM	NM	NM	10–25[b]	0.11[b]	12.6[b]
Gmeiner *et al*. (1998)	SPME/GC-MS[a]	5	0.03	NR	5	0.05–0.11	NR	5	0.05	NR
Hollender *et al*. (2000)	HPLC/fluorescence	20	12.4	NR	20	0.01	NR	20	0.004	NR
Bouchard *et al*. (2001)	GC-MS[a]	NR	0.10	3.63–4.16	NM	NM	NM	NR	0.10	8.20
Present study	GC-MS	2	0.28–0.33	5–10	2	0.45–2.64	16.5–27	2	1.31	17

LOD, limit of detection; CV, coefficient of variation; GC-FID, gas chromatography with flame ionization detection; HPLC, high performance liquid chromatography; GC-MS, gas chromatography with mass spectrometry; SPME, solid-phase-microextraction; NM, analyte not measured; NR, not reported.

[a] Separate assays/injections were applied for each group of metabolites.

[b] Based on Jongeneelen *et al*. (1987).

Materials and methods

Chemicals and supplies

1-Naphthol (99+%), 2-naphthol (99+%), [$^2H_7$]1-naphthol (97atom%D), 9-phenanthrol (tech.) and 1-pyrenol (98%) were obtained from Aldrich Chemical Co. (Milwaukeee, Wisconsin, USA). [$^2H_9$]1-Pyrenol (D, 98%) was obtained from Cambridge Isotope Laboratories, Inc. (Andover, Massachusetts, USA). 3-Phenanthrol (99+%) was obtained from Promochem GmbH (Wesel, Germany). β-Glucuronidase/sulphatase (type H-2 from *Helix pomatia*; β-glucuronidase activity 105 000 units ml^{-1} and sulphatase activity 4300 units ml^{-1}) was obtained from Sigma Chemical Co. (St Louis, Missouri, USA). Tri-Sil TBT was obtained from Pierce Chemical (Rockford, Illinois, USA). Ethyl acetate (analytical reagent) and hexane (nanograde) were obtained from Mallinckrodt Baker, Inc. (Paris, Kentucky, USA).

Subjects and urine sampling

Urine from unexposed laboratory volunteers was used for calibration purposes as needed. Spot urine samples were obtained at the end of a workweek (4 days of 8 h/day) from 28 coke oven workers (two women and 26 men, 17 of whom were smokers) and from 22 clerical workers (three women and 19 men, three of whom were smokers) in the iron and steel industry of northern China. All urine samples were maintained at −80°C prior to analysis. Of the coke oven workers, 15 were top-of-oven workers and 13 were side or bottom workers. Data from environmental measurements of benzene-soluble matter (BSM) were available from historical records of the same coke ovens during the period from 1986 to 1990. Operating conditions and work practices during this period were similar to those at the time of urine sampling (June 2000). Based on these measurements, bottom and side workers had lower exposures (0.18 and 0.27 mg BSM m^{-3}, respectively) than top-of-oven workers (1.37 mg BSM m^{-3}). Data on cigarette smoking were obtained by questionnaire.

Analysis of urinary naphthols, phenanthrols and 1-pyrenol

Urine samples were brought to room temperature before analysis, and 50 μl of a solution containing 2 μg l^{-1} of [$^2H_7$]1-naphthol and [$^2H_9$]1-pyrenol in hexane were added to 2 ml of urine in 8 ml glass vials. After adjusting the pH to 5.0 with 50 μl of 2 N sodium acetate, urine was incubated with 10 μl of β-glucuronidase/sulphatase at 37°C for 17 h. The sample was extracted twice using 4 ml ethyl acetate, which was then dried with anhydrous sodium sulphate. Following evaporation under nitrogen, the residue was reconstituted in 190 μl of hexane in a 2 ml auto-injector vial containing a 200 μl insert. Then 10 μl of a silylation cocktail (Tri-Sil TBT) was added, and the vial was capped immediately and incubated at 70°C for 30 min (based on Seidel *et al*. 1993). The trimethylsilyl ethers of the analytes were analysed by GC-MS in electron ionization mode. A HP 5980 II series gas chromatograph coupled to a HP 5971-A mass selective detector was used with a DB-1 fused silica capillary column (60 m, 0.245 mm internal diameter, 0.25 μm film thickness) and helium carrier gas at a flow rate of 1 ml min^{-1}. The injector and MS transfer-line temperatures were 250°C and 280°C, respectively. The ion source temperature was between 168°C and 174°C. The GC oven was held at 75°C for 1 min and was then increased at 8°C min^{-1} to 200°C where it was held for 8 min and then increased at 7°C min^{-1} to 230°C where it was held for 14 min, and finally increased at 8°C min^{-1} to 240°C where it was held for 30 min. Molecular ions at mass to charge ratios (m/z) of 216 (1- and 2-naphthol), 223 ([$^2H_7$]1-naphthol), 266 (3- and 9-phenanthrol), 290 (1-pyrenol) and 299 ([$^2H_9$]1-pyrenol) were monitored. Retention times were as follows: 1-naphthol, 23.25 min; 2-naphthol, 23.85 min; [$^2H_7$]1-naphthol, 23.18 min; 9-phenanthrol, 41.47 min; 3-phenanthrol, 42.78 min; 1-pyrenol, 62.63 min; [$^2H_9$]1-pyrenol, 62.29 min. Quantification was based on peak area ratios of the analytes to the internal standards. [$^2H_7$]1-Naphthol was used as the internal standard for 1- and 2-naphthol and 3- and 9-phenanthrol, and [$^2H_9$]1-pyrenol was used as the internal standard for 1-pyrenol. A chromatogram obtained in selected ion monitoring mode from an exposed subject (top-of-oven worker) is presented in Figure 1.

For quality control purposes, pooled control urine from laboratory volunteers was spiked with known concentrations of standards, divided into 4 ml aliquots and stored at −80°C. These samples were analysed together with each batch of samples to serve as positive controls. Calibration curves were obtained with standard solutions prepared with urine from a control subject at concentrations of 0.5, 1, 2, 5, 10, and 20 μg l^{-1} and analysed in the same manner as the samples. A linear relationship was observed between the abundances of the trimethylsilyl ethers and the concentrations of analytes over this range.

Determination of recoveries, precision, storage stability and limits of detection

To estimate the recoveries of PAH metabolites from urine, duplicate samples of naphthols, phenanthrols and 1-pyrenol were prepared at concentrations of 2, 5, 10 and 20 μg l^{-1} in control urine

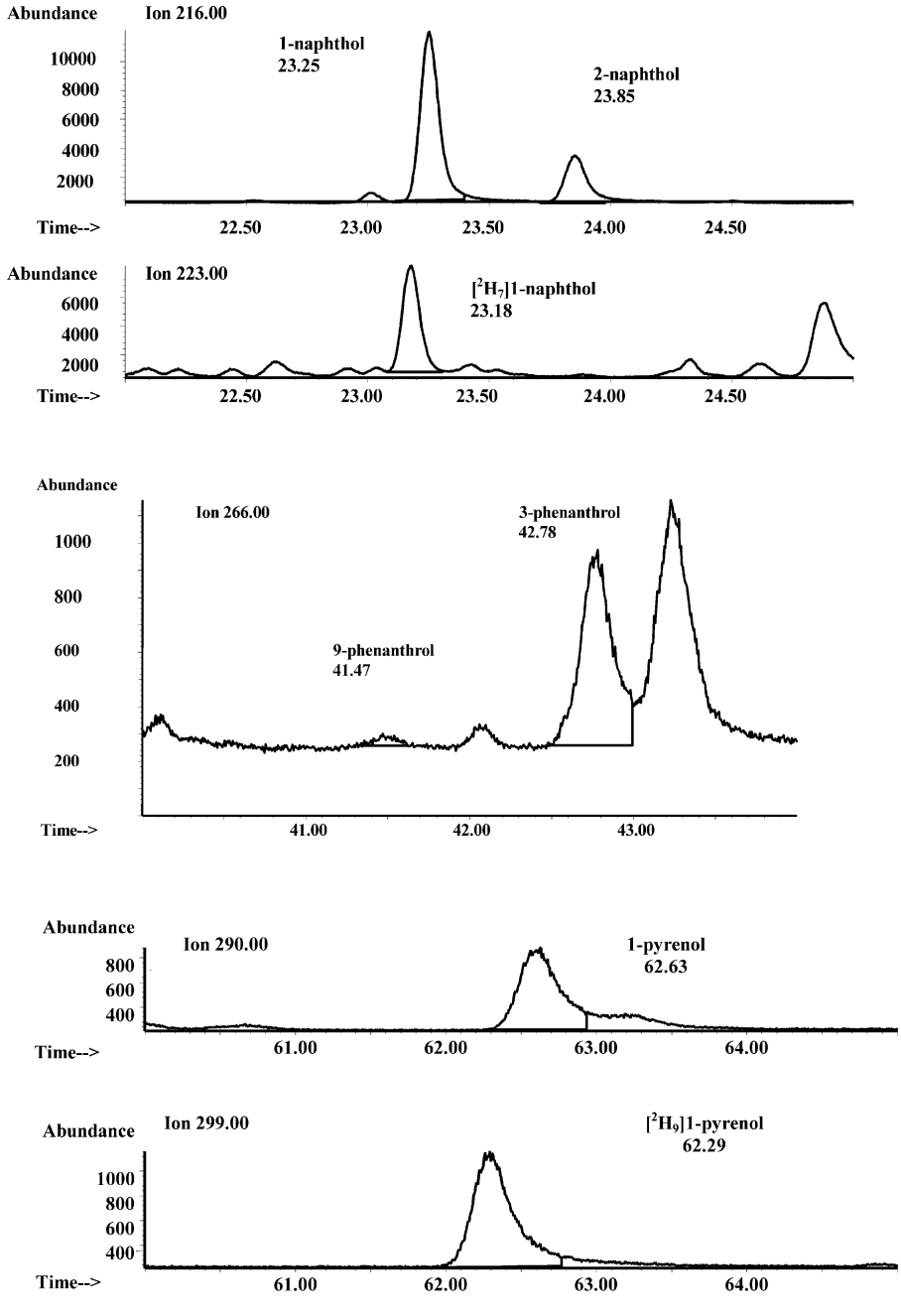

Figure 1. Chromatograms of urinary naphthols, phenanthrols and 1-pyrenol obtained from an exposed subject (top-of-oven worker).

and then put through the assay. Since no effect of concentration was discerned by one-way analysis of variance (ANOVA), recoveries were estimated after pooling data over all the concentrations.

Based on within-subject variance components of the log-transformed measurements of urinary naphthols, phenanthrols and 1-pyrenol following one-way ANOVA of measurements from duplicate

aliquots of urine from 11 coke oven workers, coefficients of variations (CVs) were estimated as follows:

$$\text{estimated CV} = \sqrt{e^{s_W^2} - 1}$$

where s_W^2 represents the estimated within-subject variance component (Rappaport 1991).

Storage stability was assessed using two 100 ml batches of pooled control urine that had been spiked with 1-and 2-naphthol, 3- and 9-phenanthrol and 1-pyrenol at a final concentration of 10 $\mu g\,l^{-1}$. First, 2 ml aliquots of these pooled samples were stored in plastic vials at -80°C. Duplicate samples were put through the assay immediately after preparation and after 1 week, 1 month, 2 months and 6 months of storage. Multiple comparisons were performed to investigate the differences in mean analyte concentrations measured at different time points.

Limits of detection (LODs) for the naphthols and 1-pyrenol were calculated based on a signal-to-noise ratio of three. We were not able to determine LODs for the phenanthrols because of interfering peaks originating from the reagents. Thus, limits of quantification (LOQs) were estimated for each phenanthrol (in blank water samples put through the assay) as the estimated mean peak area of the interfering peak plus two SDs.

Statistical analyses

All statistical analyses were performed using SAS system software (V. 8.1, SAS Institute, Cary, North Carolina, USA) at a significance level (two-tailed) of 0.05. Due to high variability and skewness of urinary analyte levels, all tests were performed after (natural) logarithmic transformation and data were summarized as geometric means and geometric standard deviations. Measurements below the LOD were replaced with $\text{LOD}/\sqrt{2}$ prior to statistical analysis (Hornung and Reed 1990). Multiple comparisons were applied to investigate the effect of storage time on concentrations of urinary metabolites and to test the differences in logged mean levels of urinary metabolites categorized by work category and cigarette smoking status (one-and two-way ANOVA using Proc GLM of SAS with Bonferroni correction). Median levels of urinary metabolites were also compared by the Wilcoxon rank-sum test (using NPAR1WAY procedure of SAS) with respect to work category and cigarette smoking status. Very consistent results were obtained with the Wilcoxon rank sum test and the two-way ANOVA. Results of the two-way ANOVA procedure, which simultaneously tests for both main effects (work category and smoking) and their interaction, are reported. Pearson correlation coefficients for pairs of (logged) urinary metabolites were estimated in exposed and control subjects separately.

Levels of urinary naphthalene and pyrene were available (Waidyanatha *et al*., in press). Since unmetabolized urinary PAHs partition from blood into urine and are not subject to the vagaries associated with metabolism, we assumed that they reflect external exposures during periods just prior to urine collection. Multiple linear regression (using Proc Reg of SAS) was used to investigate the contributions of (logged) naphthalene or pyrene concentration in urine (as measures of external exposure), work category (using dummy variables), packs of cigarettes/day, sex and age on (logged) levels of 1- and 2-naphthol and 1-pyrenol. Urinary phenanthrols were excluded from multivariate analyses because levels in control subjects were below the LOQs. Backward elimination was used, with retention of variables at a p value < 0.1. Possible outliers were investigated via studentized residuals and influence, as indicated by Cook's distance (COOKD); no observations were excluded as outliers based on these criteria. Similar models were used to investigate the effect of work category and smoking intensity on the ratios of 1- and 2-naphthol to 1-pyrenol.

Adjustment for urinary creatinine

Urinary creatinine was determined colorimetrically using a commercial kit based on Jaffe's reaction (The Creatinine Companion, Exocell Inc, Pennsylvania, USA). Mean levels of urinary creatinine for the exposed and control subjects were 12.7 and 12.1 $\text{mmol}\,l^{-1}$, respectively. Previous reports suggest that correction for urinary creatinine can be useful for substances that are eliminated primarily by glomerular filtration and are not reabsorbed by the tubules; however, such corrections may be inappropriate in situations involving concentration-dependent passive elimination or reabsorption (Boeniger *et al*. 1993, Waidyanatha *et al*. 2001). Thus, unmetabolized naphthalene and pyrene were not adjusted for urinary creatinine levels. Creatinine-adjusted levels of urinary metabolites were highly correlated with unadjusted levels (Pearson coefficients between 0.82 and 0.97). Statistical analyses were performed before and after the adjustment for urinary creatinine and the results were compared.

Results

Recovery, precision, stability and LODs

Recoveries of spiked urine samples (data pooled for determinations at 2, 5, 10 and 20 $\mu g\ l^{-1}$) were as follows ($\pm$SE): 1-naphthol, 97.0 $\pm$ 2.12%; 2-naphthol, 99.8 $\pm$ 1.94%; 9-phenanthrol, 93.1 $\pm$ 2.81%; 3-phenanthrol, 103 $\pm$ 2.30%; 1-pyrenol, 99.4 $\pm$ 2.69%. The CVs for duplicate samples ($n = 22$ pairs) were: 1-naphthol, 4.69%; 2-naphthol, 9.56%; 3-phenanthrol, 16.5%; 9-phenanthrol, 27.0%; 1-pyrenol, 17.0%.

Spiked urine samples (10 $\mu g\ l^{-1}$) that were analysed over a period of 6 months indicated no differences in mean levels of urinary metabolites at different time points ($p > 0.05$). Following 6 months of storage, urinary metabolites were recovered at the following percentages of their initial measurements: 1-naphthol, 88.7%; 2-naphthol, 107%; 9-phenanthrol, 82.9%; 3-phenanthrol, 112%; 1-pyrenol, 120%.

The estimated LODs were as follows: 1-naphthol, 0.330 $\mu g\ l^{-1}$, 2-naphthol, 0.280 $\mu g\ l^{-1}$, 1-pyrenol, 1.31 $\mu g\ l^{-1}$. LOQs for 3-phenanthrol and 9-phenanthrol were 2.64 $\mu g\ l^{-1}$ and 0.453 $\mu g\ l^{-1}$, respectively. As shown in Table 2, the naphthols were detected in all exposed and control subjects. 9-Phenanthrol was below the LOQ in all the controls and five exposed subjects, 3-phenanthrol was below the LOQ in 20 controls and six exposed subjects, and 1-pyrenol was below the LOD in 13 controls and one exposed subject. The LOD of 1.31 $\mu g\ l^{-1}$ for 1-pyrenol corresponds approximately to 0.5 $\mu mol\ mol^{-1}$ creatinine, which corresponds to the upper 95th percentile of control measurements in many countries outside China (Levin 1995). Therefore, it is not clear whether this method can be generally used in control subjects without modification (e.g. increased urine volume).

Effects of exposure category and smoking

Summary statistics of all analyses are shown in Table 2 (data not adjusted for urinary creatinine) for subjects categorized as clerical workers (controls), bottom and side workers, and top-of-oven workers. Geometric mean levels of all metabolites were higher among exposed subjects (in both exposure categories) compared with controls ($p < 0.05$). Levels of all analytes were higher in top-of-oven workers than in side and bottom workers; however, the differences were not statistically significant ($p > 0.05$). Similar results were observed when measurements were adjusted for urinary creatinine, except that the geometric mean of urinary 1-pyrenol was significantly higher for top-of-oven workers than for side and bottom workers ($p < 0.05$).

Urinary analytes in each exposure category were examined with respect to smoking status. As shown in Figure 2, a marked effect of exposure and a lesser effect of cigarette smoking were observed. Among controls, urinary 1- and 2-naphthol levels were five- and six-fold higher, respectively, in smokers than in non-smokers, and the difference was significant for 2-naphthol ($p < 0.05$). Levels of urinary 1- and 2-naphthol were also higher in smokers than non-smokers for side and bottom workers and top-of-oven workers; however, the differences were not

Table 2. Levels of urinary metabolites among coke oven workers and controls: geometric mean ± geometric SD of analyte levels and number of samples below LOD (or LOQ) by work category.

	Clerical workers ($n = 22$)		Bottom and side workers ($n = 13$)		Top-of-oven workers ($n = 15$)	
Urinary metabolite	Level ($\mu g\ l^{-1}$)	Number < LOD	Level ($\mu g\ l^{-1}$)	Number < LOD	Level ($\mu g\ l^{-1}$)	Number < LOD
1-Naphthol	3.96 ± 2.51	0	30.0 ± 2.63⋆	0	58.5 ± 2.98⋆	0
2-Naphthol	4.18 ± 2.46	0	26.9 ± 2.07⋆	0	34.1 ± 3.05⋆	0
9-Phenanthrol	—	22	1.04 ± 2.47⋆	2	1.28 ± 2.93⋆	3
3-Phenanthrol	—	20	4.55 ± 2.05⋆	4	7.35 ± 3.02⋆	2
1-Pyrenol	1.28 ± 1.72	13	12.0 ± 2.72⋆	1	25.4 ± 3.13⋆	0

⋆ p value < 0.05 for test of equal geometric means against controls (multiple comparisons of logged data with Bonferroni's correction).

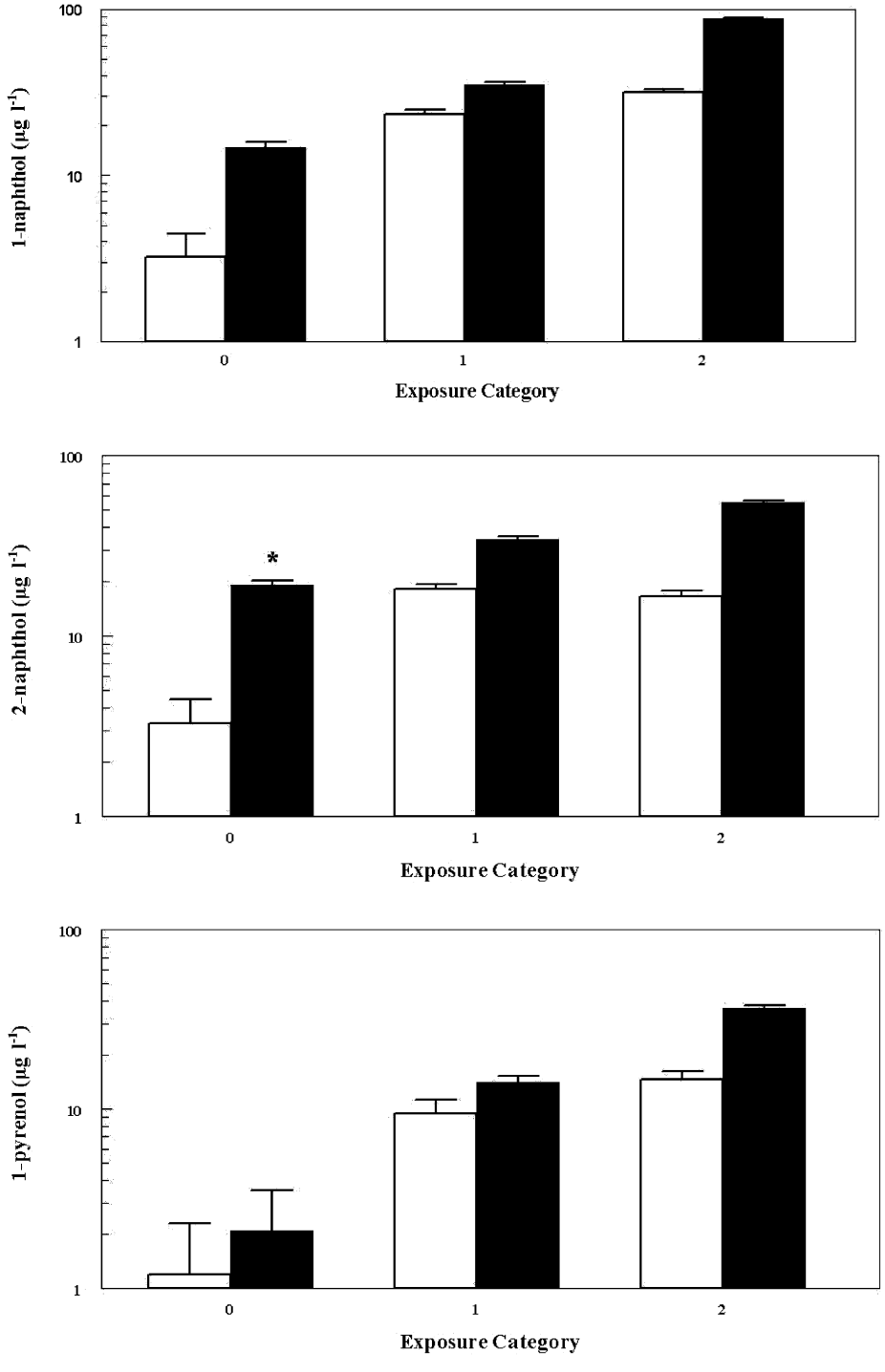

Figure 2. Levels of urinary metabolites aggregated by exposure category (0, clerical workers; 1, side and bottom workers; 2, top-of-oven workers) and smoking status (open bars, non-smokers; filled bars, smokers). Geometric means and SEs are shown. * p value < 0.05 for test of equal means against non-smokers within same exposure category (multiple comparisons of logged data with two-way ANOVA and Bonferroni correction).

Table 3. Coefficients and R^2 values of final regression models of urinary metabolites among all subjects ($n = 50$).

Response variable	Overall R^2	p value	Predictor variable	Coefficient	p value	ΔR^2
1-Naphthol, ln(μg l^{-1})	0.725	< 0.0001	Intercept	1.37	< 0.0001	
			Top-of-oven worker	1.96	< 0.0001	0.333
			Side or bottom worker	1.47	< 0.0001	0.282
			Smoking intensity (packs/day)	0.676	0.009	0.057
			Urinary naphthalene, ln(μg l^{-1})	0.299	0.005	0.053
2-Naphthol, ln(μg l^{-1})	0.725	< 0.0001	Intercept	1.40	< 0.0001	
			Top-of-oven worker	1.30	< 0.0001	0.231
			Side or bottom worker	1.25	< 0.0001	0.315
			Smoking intensity (packs/day)	0.808	0.0005	0.104
			Urinary naphthalene, ln(μg l^{-1})	0.308	0.001	0.075
1-Pyrenol, ln(μg l^{-1})	0.828	< 0.0001	Intercept	4.80	< 0.0001	
			Top-of-oven worker	1.93	< 0.001	0.376
			Side or bottom worker	1.32	< 0.0001	0.321
			Smoking intensity (packs/day)	0.600	0.005	0.035
			Urinary pyrene, ln(μg l^{-1})	0.671	< 0.0001	0.096

significant ($p > 0.05$). In each exposure category, urinary 1-pyrenol was approximately two-fold higher among smokers, but the differences were not significant ($p > 0.05$). Measurements of urinary phenanthrols were not different between smokers and non-smokers in coke oven workers. Levels of urinary metabolites were compared with respect to work category after adjusting for cigarette smoking status (two-way ANOVA). In general, levels of urinary metabolites were higher among coke oven workers than controls for smokers and non-smokers, but the differences were not all significant. Among smokers, geometric mean levels of urinary 1-pyrenol and 3- and 9-phenanthrol were significantly higher ($p < 0.05$) among top-of-oven workers when compared with controls, and 1-pyrenol was significantly higher among side and bottom workers when compared with controls ($p < 0.05$). Among non-smokers, geometric mean levels of urinary 1- and 2-naphthol and 1-pyrenol were significantly higher among top-of-oven workers when compared with side or bottom workers and controls ($p < 0.05$). For all comparisons, consistent results were obtained when metabolites were adjusted for urinary creatinine.

Correlation between urinary analytes

The linear correlations between the various PAH metabolites were investigated separately for exposed workers and controls. All of the metabolites showed strong correlations among exposed workers (Pearson $r = 0.74$–0.87, $p < 0.0001$). Among the control subjects, urinary 1- and 2-naphthol were strongly correlated with each other ($r = 0.82$, $p < 0.0001$) and moderately correlated with 1-pyrenol ($r = 0.55$, $p = 0.008$ for 1-naphthol, and $r = 0.48$, $p = 0.025$ for 2-naphthol).

Table 4. Coefficients and R^2 values of regression models of logged ratios of naphthols/1-pyrenol.

Response variable	Overall R^2	p value	Predictor variable	Coefficient	p value	ΔR^2
ln(2-naphthol/1-pyrenol) in all subjects ($n = 50$)	0.253	0.0035	Intercept	1.13	< 0.0001	
			Top-of-oven worker	-1.04	0.0003	0.175
			Side or bottom worker	-0.52	0.063	0.034
			Smoking intensity (packs/day)	0.353	0.11	0.044
ln(combined naphthols/1-pyrenol) in smokers ($n = 20$)	0.546	0.001	Intercept	2.80	< 0.0001	
			Top-of-oven worker	-1.36	0.0003	0.533
			Side or bottom worker	-1.17	0.002	0.013
ln(1-naphthol/1-pyrenol) in smokers ($n = 20$)	0.337	0.031	Intercept	1.95	< 0.0001	
			Top-of-oven worker	-1.07	0.01	0.307
			Side or bottom worker	-1.03	0.02	0.030
ln(2-naphthol/1-pyrenol) in smokers ($n = 20$)	0.659	0.0001	Intercept	2.22	< 0.0001	
			Top-of-oven worker	-1.81	< 0.0001	0.658
			Side or bottom worker	-1.32	0.0007	0.001

Multiple linear regression models

The contributions of different covariates on levels of urinary naphthols and 1-pyrenol were investigated. Sex and age were not significant predictors of these urinary metabolites and were dropped from all models. Final models included urinary levels of the parent PAH (naphthalene or pyrene), work category and packs of cigarettes/day. As shown in Table 3, these variables explained about 73% of the variation for urinary 1- and 2-naphthols and 83% for 1-pyrenol.

The (logged) ratios of urinary naphthols to urinary 1-pyrenol were also investigated with respect to exposure category and smoking intensity. Among all subjects, these variables did not explain significant amounts of the variability of ln(1-naphthol/1-pyrenol) or ln[(1-naphthol+2-naphthol)/1-pyrenol] ($p > 0.05$; data not shown), but explained 25.3% of the variation of ln(2-naphthol/1-pyrenol) (Table 4). However, after stratification by smoking status, a strong negative relationship was observed between work category and ln[(1-naphthol+2-naphthol)/1-pyrenol] among smokers ($R^2 = 0.546$, $p = 0.001$; Table 4) but not among non-smokers ($R^2 = 0.06$, $p > 0.05$; data not shown). A negative relationship was also observed between work category and ln(1-naphthol/1-pyrenol) among smokers ($R^2 = 0.34$, $p = 0.03$; Table 4) but not among non-smokers ($R^2 = 0.01$, $p = 0.86$; data not shown). For the (logged) ratio of 2-naphthol/1-pyrenol, work category explained 65.9% of the variability among smokers ($p = 0.0001$; Table 4), but only 16.7% of the variability among non-smokers ($p = 0.084$; data not shown).

Models of urinary metabolites were also investigated after the adjustment for urinary creatinine (parent PAHs were not adjusted). In these models, urinary naphthalene levels were not significantly associated with urinary 1- and 2-naphthol ($p > 0.05$). Final models, including work category and packs of cigarettes/day as predictors, explained 66% and 70% of the variations in creatinine-adjusted measurements of urinary 1- and 2-naphthol, respectively. The final model of creatinine-adjusted urinary 1-pyrenol (urinary pyrene, work category, packs of cigarettes/day) explained 75% of the variation. We observed higher estimated values of the error variance for linear models of 1-naphthol and 1-pyrenol after adjustment for urinary creatinine (mean squared errors [MSEs] for unadjusted versus adjusted measurements were: 1-naphthol, 0.72 versus 0.86; 2-naphthol, 0.55 versus 0.52; 1-pyrenol, 0.48 versus 0.67.

Discussion

We report here a simple GC-MS assay to simultaneously measure naphthols, phenanthrols and 1-pyrenol in 2 ml of urine. The method is functionally similar to that of Yang *et al*. (1999), who employed GC-MS, enzymatic hydrolysis and derivatization with pentafluorobenzylbromide to measure urinary 1- and 2-naphthol in 1 ml of urine. By adding internal standards and using a simple derivatization technique, we were able to streamline the method while also extending it to the phenanthrols and 1-pyrenol. The assay was sufficiently sensitive to detect naphthols in all of the control subjects and 1-pyrenol in approximately 41% (nine out of 22) of the controls. Due to unresolved impurities in the reagents, the method had insufficient sensitivity to quantify 3- and 9-phenanthrol in control

subjects; however, these phenanthrols were measured in most of the coke oven workers. The estimated CVs for urinary metabolites in the present study tended to be higher than those reported previously in much larger volumes of urine, particularly for 1-pyrenol (Table 1). Although the sensitivity and precision of the current assay were sufficient for our needs and for studies of the naphthols more generally, future applications involving 1-pyrenol or the phenanthrols could be well served by using 5–10 ml of urine, rather than 2 ml as used in the current assay.

Previous reports have indicated that 1-pyrenol is stable in urine at -20°C for more than 6 months (Jongeneelen *et al*. 1987, Bouchard and Viau 1999). In our study, mean levels of urinary 1- and 2-naphthol, 3- and 9-phenanthrol and 1-pyrenol did not change significantly during 6 months of storage at -80°C. However, these experiments were conducted with urine samples that had been spiked with unconjugated forms of the metabolites. Since most of these metabolites are excreted *in vivo* as sulphate/glucuronide conjugates, stabilities and recoveries in actual samples may have higher variation due to the hydrolysis step.

Urinary measurements of the metabolites of naphthalene, phenanthrene and pyrene were significantly higher among coke oven workers than unexposed controls in Northern China. The highest measurements were observed in urine samples from top-of-oven workers, where the levels of urinary 1- and 2-naphthol and 1-pyrenol were approximately 15-, 8- and 20-fold higher than control values, respectively. In the exposed group we observed strong linear correlations between urinary naphthols and phenanthrols with 1-pyrenol ($r = 0.74$–0.87, $p < 0.0001$), the widely accepted biomarker of exposure to PAHs. Weaker but still significant correlations were also observed between the naphthols and 1-pyrenol ($r = 0.48$–0.55, $p < 0.025$) among control subjects (phenanthrols could not be detected among controls due to the presence of interfering contaminants from the reagents). Thus, we conclude that metabolites of all these PAHs were related to a common source of exposure among coke oven workers, as were naphthols and 1-pyrenol among controls.

We wished to determine whether urinary naphthols and 1-pyrenol reflected urinary levels of the unmetabolized precursors (naphthalene and pyrene), as well as work category and smoking intensity. Using multiple linear regression models we found that these variables explained 73% of the variation in urinary naphthols and 83% of that for 1-pyrenol. The amounts of variation explained by predictor variables decreased in the order top-of-oven workers ($\Delta R^2 = 23$–38%), side and bottom workers ($\Delta R^2 = 28$–32%), cigarettes/day ($\Delta R^2 = 4$–10%), and unmetabolized naphthalene or pyrene ($\Delta R^2 = 5$–10%). We suspect that work category and the unmetabolized PAHs (which were assumed to reflect actual exposure levels) captured different predictive elements of the exposure milieu. That is, work category probably reflects job-specific factors such as temperature, route of exposure (air versus skin) and exercise level (Droz *et al*. 1991), while the unmetabolized PAHs reflect subject-specific variation in external exposure within a particular job (Kromhout *et al*. 1993).

The ranks of regression coefficients for work category were in the order top-of-oven workers > side and bottom workers > controls, for both urinary naphthols and 1-pyrenol. This is consistent with historical records of higher levels of BSM among

top-of-oven workers compared with side and bottom workers for the same coke ovens. The effect of work category was less obvious for 2-naphthol, where the coefficient for top-of-oven workers (1.30) was only slightly greater than that for side and bottom workers (1.25). This might reflect the greater effect of cigarette smoking, since the coefficient for smoking was higher for 2-naphthol (0.808) than for 1-naphthol (0.657) (see below). Our results confirm previous reports indicating that smoking contributed to urinary naphthols, particularly 2-naphthol, in unexposed subjects (Hoepfner *et al*. 1987, Buchet *et al*. 1992, Kim *et al*. 1999, Yang *et al*. 1999).

When urinary naphthols were adjusted for creatinine, urinary naphthalene was not a significant predictor of naphthol levels because the estimated error variance (MSE) was substantially higher than that for unadjusted measurements. These results indicate that adjustment for urinary creatinine can decrease the precision of assays, due to random errors associated with the measurement of creatinine (Boeniger *et al*. 1993), and suggest that such adjustments should not automatically be performed.

To determine whether the naphthols and 1-pyrenol were equally affected by predictor variables, we regressed the logged ratio of 1- and 2-naphthol and their sum on exposure category and smoking intensity (Table 4). When all subjects were considered together, work category and smoking intensity were significantly associated with ln(2-naphthol/1-pyrenol) but not with the logged ratios of either 1-naphthol/1-pyrenol or combined naphthols/1-pyrenol (data not shown). Among smokers, however, negative relationships were observed between work category and the logged ratios of combined naphthols/1-pyrenol, 1-naphthol/1-pyrenol and 2-naphthol/1-pyrenol, suggesting a decrease in these ratios as occupational exposure to PAHs increased. We investigated the logged ratio of urinary naphthalene/pyrene with respect to work category in a multiple linear regression model. No significant association was found between work category and the (logged) ratio of urinary naphthalene/pyrene ($R^2 = 0.02$, $p > 0.05$; data not shown), suggesting that the ratio of exposures to naphthalene and pyrene were not different in the two categories of coke oven workers. Although cigarette smoke tends to contain higher levels of naphthalene and phenanthrene than of pyrene (Witschi *et al*. 1997, Rustemeier *et al.* 2002), previous studies point to a decrease in urinary phenanthrols/1-pyrenol as the intensity of cigarette smoking increases (Jacob *et al*. 1999, Heudorf and Angerer 2001), a result similar to that seen for the naphthols in our study. A greater-than-additive effect of PAH exposure and smoking on urinary 1-pyrenol has been reported among coke oven workers, suggesting possible induction of pyrene metabolism by cigarette smoke (Jongeneelen *et al*. 1990, VanRooij *et al*. 1993). Additionally, the conversion of arene epoxides to dihydrodiols, a more significant step in the metabolism of naphthalene and phenanthrene than that of pyrene, was more efficient among persons with high PAH exposures (Jacob *et al*. 1999). Thus, the induction of alternative pathways by exposures to PAHs (increased formation of naphthalene dihydrodiols rather than naphthols) may have altered the ratio of urinary naphthols/1-pyrenol. In summary, our finding that the ratio of naphthols/1-pyrenol decreased among smokers but not among non-smokers supports the notion that there is an interaction between cigarette smoking and PAH exposure that

differentially affects metabolism of naphthalene and/or pyrene. However, we temper this speculation with knowledge that the smokers in our sample were unevenly distributed among the coke oven workers (17 smokers out of 28 [61%]) and controls (three smokers out of 22 [14%]).

Previous studies have reported that the skin is the main route of pyrene exposure among coke oven workers, with top-of-oven workers having the highest dermal exposures (VanRooij *et al*. 1993, Kuljukka *et al*. 1997). Following an intervention study, reduction in dermal exposures resulted in a significant decrease in urinary 1-pyrenol only among top-of-oven workers (Cho *et al*. 2000). Because air and dermal measurements were not available in the present study, we were unable to investigate the relative contributions of air and dermal contact on PAH metabolites.

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