Biomarkers, 2011; 16(4): 334–345
© 2011 Informa UK, Ltd.
ISSN 1354-750X print/ISSN 1366-5804 online
DOI: 10.3109/1354750X.2011.561499

informa
healthcare

RESEARCH ARTICLE

A quantitative approach to evaluate urinary benzene and S-phenylmercapturic acid as biomarkers of low benzene exposure

Silvia Fustinoni[1], Laura Campo[1], Rosa Mercadante[1], Dario Consonni[1], Danuta Mielzynska[2], and Pier Alberto Bertazzi[1]

[1]University of Milan, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Department of Occupational and Environmental Medicine, Milano, Italy and [2]Institute for Ecology of Industrial Area, Katowice, Poland

Abstract

Context: Benzene is a ubiquitous pollutant; smoking habit, genetic polymorphisms, and analytical difficulties impact the identification of the best biomarker.

Objective: To apply a systematic quantitative approach to evaluate urinary benzene (BEN-U) and S-phenylmercapturic acid (SPMA) as biomarkers of low benzene exposures.

Methods: Seventy-one blue collar refinery workers, 97 white collar refinery workers and 108 general population subjects were included. Intrinsic characteristics, sampling and analytical issues were compared.

Results: BEN-U and SPMA were detected in 99% and 78% of samples, which correlated with benzene exposure ($r = 0.456$ and $r = 0.636$, respectively) and with urinary cotinine ($r = 0.630$ and $r = 0.570$, respectively). Intrinsic characteristics were similar for the two biomarkers: specificity (0.64 and 0.69 for BEN-U and SPMA), sensitivity (0.74 and 0.83), as well as intra- and inter-individual variability (150% and >14 for both).

Conclusion: BEN-U and SPMA show similar intrinsic characteristics; analytical issues in detecting SPMA suggest that BEN-U is more convenient for investigating low exposure levels.

Keywords: Environmental pollution/Ecotoxicology, mass spectroscopy, chemical carcinogenesis

Introduction

Benzene, although banned from several industrial processes and common products, is still a critical molecule in the chemical industry. Benzene is obtained from oil and widely used as intermediate in the synthesis of several chemicals, including styrene. In addition, it is an impurity (<1% in the EU) in the aromatic hydrocarbon mixture added to gasoline as an antiknock agent. The presence of benzene in the general environment is due to incomplete combustion of organic matter, including gasoline, coal, woods and tobacco. For this reason, it is practically ubiquitous worldwide, and automotive exhaust fumes and smoking represent major sources of exposure for the general population.

Benzene is classified as a known human carcinogen by the International Agency for Research on Cancer (IARC, 1987) and is labeled H350 (may cause cancer to humans) according to European regulation No. 1272/2008 (European Commission, 2008). Due to its toxicity, airborne benzene exposure is regulated in many countries. For example, 1 ppm (3.2 mg/m^3) is the occupational limit in the EU (European Commission, 1997). A time-weighted average concentration of 0.5 ppm (1.6 mg/m^3) during an 8-h work shift is recommended as a threshold limit value by the American Conference of Governmental Industrial Hygienists (ACGIH, 2009). Besides occupational exposures, exposure in the general population is a public concern issue. This exposure is

Address for Correspondence: Dr. Silvia Fustinoni PhD, Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Department of Occupational and Environmental Medicine, Via S. Barnaba, 8, Milano, 20122 Italy. Tel: +39 0250320158. Fax: +39 0250320111. E-mail: silvia.fustinoni@unimi.it

(Received 23 July 2010; accepted 06 February 2011)

now regulated by guidelines for air quality; for example, 5 μg/m^3 benzene has been enforced as a mean calendar year limit in the EU from 2010 (European Commission, 2000), and biomonitoring in the general population has been recognized as an important public health issue (European Commission, 2004).

For biological monitoring of occupational benzene exposure, urinary *t,t*-muconic acid (MA) and S-phenylmercapturic acid (SPMA) are recommended as determinants in the biological exposure indices list of ACGIH, with 500 μg/g creatinine and 25 μg/g creatinine, respectively, in samples collected at the end of the shift (ACGIH, 2009). For both, the B notation warns that the marker is usually present in a significant amount in biological specimens collected from subjects who have not been exposed occupationally. Urinary MA (2 mg/L) and SPMA (0.045 mg/g creatinine), and benzene in the blood (5 μg/L), are listed as exposure equivalents for carcinogenic substances (EKA) by the Deutsche Forschungsgemeinschaft (DFG), for samples collected at the end of exposure or end of shift, corresponding to an airborne benzene level of 1 ppm (3.2 mg/m^3) (DFG, 2009). No differentiation between smokers and nonsmokers is reported in either list, although several studies have shown the influence of smoking on benzene biomarkers levels, which may be the result of testing at low air exposures, but not at exposures close to occupational exposure limits.

In the last several decades, several studies on biological monitoring of benzene exposure focused on non-traditional occupational settings, as in traffic policemen (Fustinoni et al., 1995; Crebelli et al., 2001; Fustinoni et al., 2005; Manini et al., 2008; Barbieri et al., 2008), gasoline station attendants (Inoue et al., 2001; Fustinoni et al., 2005; Lovreglio et al., 2010), public transportation drivers (Fustinoni et al., 2005; Manini et al, 2006), and other more traditional work settings such as petrochemical and refinery plants (Hoet et al., 2009; Carrieri et al., 2010) where improved working conditions have lowered occupational exposures significantly. These studies demonstrated the usefulness of SPMA and suggest that urinary benzene (BEN-U) is a new biomarker suitable to assess low exposures, i.e. exposures below occupational limit values (Fustinoni et al., 2005; Barbieri et al., 2008; Lovreglio et al., 2010; Fustinoni et al., 2010a).

Given the need to focus on exposure levels consistent with those found in current working and living environments, the present study aimed to evaluate SPMA and BEN-U as biomarkers of low exposures, using a systematic quantitative approach, that is actually lacking in the extensive literature on benzene biomonitoring. The study was performed on three groups of subjects with nominal exposure spanning over a wide range of concentrations. The comparison between SPMA and BEN-U was performed according to the criteria suggested by Dor and coworkers (Dor et al., 1999), so that both sampling conditions and analytical techniques, and the intrinsic characteristics of the biomarkers were considered. Among the intrinsic characteristics, we estimated background level, specificity, sensitivity, inter-individual variability, and correlation with air exposure; moreover, due to the availability of multiple determinations in individuals in the general population, it was possible to estimate intra-subject variability. Additionally, the influence of smoking status on biomarker levels was evaluated. As far as sampling conditions and analytical techniques, we summarized the information on sample collection, storage, and analysis, considering both the results obtained in the present work, and information from our previous experiences (Fustinoni et al., 1999; Fustinoni et al., 2000; Fustinoni et al., 2010b). To measure BEN-U and SPMA gas chromatography/mass spectrometry (GC/MS) and liquid chromatography/triple quadruple mass spectrometry (LC/MS/MS) assays were applied (Fustinoni et al., 1999; Fustinoni et al., 2010b).

Methods

Study population

The subjects were 65 male and 6 female workers employed as blue collar workers in the Polish refinery industry (B). A group of matched white collar workers (office workers from the same plant) was recruited as internal controls (W), for a total of 97 subjects. For subjects B, but not for subjects W, a single personal air sample was collected for benzene determination during a work shift; for both subjects B and W, a spot urine sample was collected at the end of the monitored work shift. An additional group of 108 subjects belonging to the general population working and living in the city of Milan, Italy, or in nearby areas, was recruited as a convenient external control (C); they were chosen because they are not exposed to benzene occupationally; for these subjects, personal exposure to benzene was monitored for a 5-h period of a working day, while subjects were performing their usual activities; three spot urine samples were also collected. A complete description of these subjects, together with their personal exposure to air benzene and excretion of BEN-U, as geometric mean value of multiple determinations, were reported previously (Fustinoni et al., 2010a). Information about work activities, demographic and lifestyle factors, and medical histories were obtained using a questionnaire administered by occupational physicians. The study was authorized by the appropriate ethics committee. All subjects were informed about the aims and the protocol of the study, and provided written consent to be included as human subjects.

Personal benzene exposure

For subjects B, air benzene was sampled by an active sampler (Gillian Pump), connected to a cartridge containing Chromosorb 106, operating at 100 mL/min flow rate (NIOSH, 2003). For subjects C, air benzene was

sampled using the passive sampler Radiello equipped with a 35–50 mesh charcoal cartridge (Sigma-Aldrich, 2010). In both cases, the sampling device was worn by the subjects, positioning the cartridge near the breathing zone. For subjects B, sampling was performed during the work shift (typically from 8:00 a.m. to 16:30 p.m.). For subjects C, sampling was performed during the morning (typically from 8:00 a.m. to 13:00 p.m.). Benzene in samplers was determined by CS_2 desorption followed by gas chromatography/flame ionization detector (GC/FID) analysis for subjects B (NIOSH, 2003), or by GC/MS for subjects C, according to the cartridge manufacturer's procedure (Sigma-Aldrich, 2010). The limit of quantification (LOQ) was 0.02 mg/m^3 (0.006 ppm) for analysis performed by GC/FID and 0.001 mg/m^3 (0.0003 ppm) for analysis performed by GC/MS.

Biological monitoring

Specimen collection

For refinery workers (B and W), a urine spot sample was collected at the end of the work shift. For control subjects (C), spot urine samples were collected three times during the same week: before the beginning of air sampling, within 10 min of the end of air sampling and on Monday morning. Specimens were divided in aliquots for the determination of urinary biomarkers; for cotinine and SPMA, aliquots were poured into two plastic tubes for separate handling and conservation; for BEN-U, a 7-mL aliquot was poured into a pre-cleaned 8-mL glass vial, closed promptly with a rubber lid with a PTFE lining, and crimped with an aluminum seal. All samples were coded, frozen at –20°C, and delivered to the laboratory, where analyses were performed without knowledge of their origin.

SPMA

The determination of urinary SPMA was based on solid-phase extraction (SPE) followed by LC/MS/MS analysis in the presence of *DL*-S-phenylmercapturic-3,3-d_2 acid as the internal standard (CDN Isotopes Inc., Pointe-Claire, Quebec, Canada) following a previously published procedure (Fustinoni et al., 2010b). The LOQ was 0.1 μg/L. Each sample was run in duplicate, and the mean value was used for statistics. The between-run precision, assessed using duplicate of unknown samples (n = 168) (Rappaport, 1991), was 22.9%. The throughput was about 30 samples each day. A subsample of 145 urine samples from B and W subjects was analyzed by another laboratory, and the results were consistent, as previously reported (Fustinoni et al., 2010b).

BEN-U

BEN-U was detected by headspace solid-phase microextraction (SPME) followed by GC/MS analysis as described previously (Fustinoni et al., 2010b). The LOQ was 15 ng/L. The between-run precision, assessed using 46 duplicates of unknown samples (Rappaport, 1991), was 13.4%. The throughput was approximately 50 samples each day.

Urinary cotinine and creatinine

Urinary cotinine (COT-U), used to assess smoking habit, was determined by a LC/MS/MS, in the presence of (±)-cotinine-d_3 (N-methyl-d_3) as an internal standard (Meger et al., 2002). The LOQ was 25 μg/L. Within- and between-run precision, as the variation coefficient, were both <10%, and the accuracy was 86–101% of the theoretical value. Subjects with COT-U less than 100 μg/L were classified as nonsmokers, while subjects with COT-U equal to or greater than 100 μg/L were classified as smokers (Haufroid and Lison, 1998). Urinary creatinine was determined using Jaffe's colorimetric method (Kroll et al., 1986).

Statistical analyses

Statistical analyses were performed using SPSS version 15.0 (SPSS Inc. Chicago, IL). The relationship between benzene exposure and urinary biomarkers was assessed using correlation/regression techniques. A value corresponding to half the LOQ was assigned to measurements below the LOQ (Baccarelli et al., 2005). In C subjects, for which three urine samples were collected, statistical analyses were performed using BEN-U and SPMA levels determined in samples collected at the end of air monitoring; however, for the calculation of intra-individual variability, values of all three measurements were used. Moreover, to estimate inter-individual variability and background levels, the geometric mean value of three determinations was used. Since variables were positively skewed, we conducted analyses with parametric methods on log-transformed variables (ANOVA applying Bonferroni's correction, Pearson's correlation coefficient, multiple linear regression analyses). Least-squares linear regression analysis was used to estimate slope and intercepts of the relationship between variables. A multiple linear regression analysis was performed with the different urinary biomarkers as the dependent variable, and, as predictors, the exposure variables (air benzene and current exposure to cigarette smoke, measured as cotinine) and the concentration of urinary creatinine, which were associated with the biomarkers in some of the univariate analyses. The use of creatinine as independent variable allows the urinary analyte concentration to be adjusted appropriately for urinary creatinine and the statistical significance of other variables in the model to be independent of effects of creatinine concentration (Barr et al., 2005).

The final model had the following form:

$$\log(\text{Biomarker-U}) = \text{constant} + \log(\text{Benzene in air})*\beta_1 + \log(\text{cotinine})*\beta_2 + \log(\text{creatinine})*\beta_3$$

Background values of biomarkers were evaluated as the 95th percentile of the distribution of C. Using these background values, specificity, defined as the capability of the biomarker to classify correctly as "not exposed" (Correctly Classified Controls) the subjects belonging to the general population (True Controls), and sensitivity, the capability

to classify correctly as "exposed" (Correctly Classified Exposed) the refinery blue collars (True Exposed) were calculated with the following expressions:

$$\text{Specificity} = \frac{\text{Correctly Classified Controls}}{\text{True Controls}} \quad (1)$$

$$\text{Sensitivity} = \frac{\text{Correctly Classified Exposed}}{\text{True Exposed}} \quad (2)$$

Inter-individual variability of a biomarker was evaluated as the ratio between the 95th and 5th percentiles of the distribution in the C subjects. Intra-individual variability of a biomarker was evaluated using three determinations for each C subject, according to the equation:

$$\%CV = \sqrt{(e^{S^2_w} - 1)} \cdot 100$$

where S^2_w represents the within-subject variance component estimated as the error term in the two-way ANOVA (Rappaport, 1991). A two-tailed P value of 0.05 was considered significant.

Results

Urinary biomarkers

The characteristics of the study subjects are summarized in Table 1. C subjects were older than B and W subjects, while gender, and smoking habit, as determined by comparing smoker frequency and COT-U (median levels 1172, 1072 and 1374 μg/L in C, W and B, respectively), were similar in the three groups. The personal benzene exposure and urinary biomarker results, in subjects divided by exposure group and smoking habit, are reported in Table 2. Median benzene in air was 0.0040 mg/m^3 for B subjects, and 0.19 mg/m^3 in C subjects, a significantly higher value ($P < 0.001$). Considering single exposure group and dividing subjects according to smoking status, smokers had higher BEN-U than nonsmokers among B subjects ($P = 0.028$), and marginally higher among C subjects ($P = 0.058$).

BEN-U was above the LOQ in 99% of samples, with median levels of 550, 320, and 155 ng/L in B, W, and C subjects, respectively. According to Bonferroni's post hoc test, BEN-U was higher in B subjects than in W subjects ($P < 0.001$) and C subjects ($P = 0.010$), and higher in W subjects than in C subjects ($P = 0.005$). When only nonsmokers were considered, the difference between the groups was still significant: B or W subjects vs. C subjects ($P < 0.001$ for both comparisons), but not when comparing B and W subjects ($P = 0.184$). In smokers, B subjects had higher levels than C subjects ($P = 0.028$), but were not significantly different from W subjects ($P = 0.192$). In addition, among smokers, W and C subjects were similar ($P = 1.000$).

SPMA was above LOQ in all B subjects, in 93% of W subjects, and only in 50% of C subjects, with median levels of <0.10, 0.69, and 1.02 μg/L SPMA and <0.10, 0.40, and 0.65 μg/g creatinine in B, W, and C subjects, respectively. SPMA (μg/L) was higher in B or W subjects compared to C subjects ($P < 0.001$ for both comparisons), but did not differ significantly between B and W subjects ($P = 0.100$). SPMA adjusted for creatinine was higher in B subjects than in W subjects ($P < 0.001$) and in C subjects ($P = 0.023$), and was higher in W subjects than in C subjects ($P < 0.001$). In nonsmokers, SPMA was higher in B or W subjects than in C subjects ($P < 0.001$ for both comparisons), while the difference between B and W subjects was not significant ($P = 0.184$). In smokers, levels in B or W subjects were higher than in C subjects ($P < 0.001$ for both comparisons), while levels in W and C subjects were similar ($P = 1.000$). Similar results were obtained when comparing SPMA levels expressed as μg/g creatinine. In each exposure group, biomarkers were always higher in smokers than in nonsmokers ($P < 0.001$). Box plots of personal exposure to benzene, BEN-U and SPMA in subjects divided according to exposure group and smoking habit are reported in Figure 1.

Pearson's correlations and simple linear regressions

Pearson's correlations between the investigated parameters (log-transformed) are reported in Table 3. The correlations with benzene in the air (B and C subjects) were significant for both BEN-U ($r = 0.456$) and SPMA ($r = 0.636$). The correlations were higher in nonsmokers ($r = 0.627$ and 0.759, respectively) and lower in smokers

Table 1. Summary of selected characteristics of the study subjects.

	General population subjects (C)	Refinery white collar workers (W)	Refinery blue collar workers (B)
N. of subjects	108	97	71
Age† (years)	44.8 ± 11.2	38.4 ± 9.5	38.2 ± 11
Gender			
N. Female (%)	7 (6.5%)	11 (11.3%)	6 (8.5%)
N. Male (%)	101 (93.5%)	86 (88.7%)	65 (91.5%)
Cigarette smoking			
N. Smokers (%)	43 (39.8%)	42 (43.3%)	38 (53.5%)
N. cigarettes/d†	12.5 ± 8.7	18.6 ± 6.3	20.1 ± 8.1
Urinary cotinine in smokers* (μg/L)	1172 (119–2936)	1072 (223–2345)	1374 (172–3021)

†Mean ± SD.
*Median (5th–95th percentile).

Table 2. Personal exposure to airborne benzene, and levels of exposure biomarkers in the study subjects, divided according to job title and smoking habit.

		General population subjects (C)			Refinery white collar workers (W)			Refinery blue collar workers (B)		
		Median (5th–95th percentile)	*N*	*N* > LOQ	Median (5th–95th percentile)	*N*	*N* > LOQ	Median (5th–95th percentile)	*N*	*N* > LOQ
Benzene air (mg/m^3)	All subjects	0.0040 (0.0015–0.0161)	108	106	—	97	—	0.19[a] (0.06–2.31)	71	70
	Nonsmokers	0.0037 (0.0014–0.0160)	65	63	—	55	—	0.14[a] (0.06–2.20)	33	33
	Smokers	0.0052[c] (0.0025–0.0161)	43	43	—	42	—	0.25[a] (0.06–2.67)	38	37
BEN-U (ng/L)	All subjects	155 (54–2554)	108	108	320[a] (83–2316)	97	96	550[a,b] (117–7487)	71	70
	Nonsmokers	90 (51–373)	65	65	218[a] (78–704)	55	54	308[a] (110–1471)	33	32
	Smokers	603[c] (73–4961)	43	43	550[c] (250–2761)	42	42	879[a,c] (175–18218)	38	38
SPMA (μg/L)	All subjects	<0.10 (<0.10–2.17)	108	54	0.69[a] (<0.10–3.18)	97	90	1.02[a] (0.14–6.98)	71	71
	Nonsmokers	<0.10 (<0.10–0.25)	65	19	0.40[a] (<0.10–1.24)	55	48	0.44[a] (0.11–3.89)	33	33
	Smokers	0.33[c] (<0.10–3.22)	43	35	1.50[a,c] (0.47–4.21)	42	42	1.39[a,c] (0.31–10.12)	38	38
SPMA (μg/g creatinine)	All subjects	<0.10 (<0.10–1.79)	108	54	0.40[a] (<0.10–2.29)	97	90	0.65[a,b] (0.12–5.30)	71	71
	Nonsmokers	<0.10 (<0.10–0.18)	65	19	0.25[a] (<0.10–0.89)	55	48	0.31[a] (<0.10–1.64)	33	33
	Smokers	0.24[c] (<0.10–1.89)	43	35	0.97[a,c] (0.28–2.59)	42	42	1.02[a,c] (0.43–8.31)	38	38

[a]Significantly higher (P <0.05) than subjects from the general population.
[b]Significantly higher (P <0.05) than refinery white collar workers.
[c]Significantly higher (P <0.05) than nonsmokers.

(r = 0.266 and 0.519, respectively). For urinary biomarkers, significant correlations were found between SPMA and BEN-U (r = 0.735 for all subjects). Benzene biomarkers correlated with cotinine, a measure of smoking habit, when all subjects were considered together and in smokers only, but not in nonsmokers. Also, benzene levels measured in air samples correlated with cotinine in all subjects, suggesting that smoking impacts air exposure, in addition to the internal dose of benzene. Interestingly, both SPMA and BEN-U levels correlated to some extent with urinary creatinine, a marker of kidney excretion activity and urine dilution. Scatter plots and linear regressions between log-transformed BEN-U or SPMA and benzene air, and between BEN-U and SPMA are reported in Figures 2A, B, and C.

Multiple linear regression analysis

The effect of personal benzene exposure, smoking habit, as determined by COT-U, and creatinine on urinary biomarkers was investigated by multiple linear regression analysis (Table 4). This analysis was performed on C and B subjects (178 subjects totally) for which data on benzene air levels were available. The models, described in the Statistical Analyses subsection of the Methods section, were statistically significant ($P < 0.001$ for both biomarkers), with determination coefficients (R^2) of 0.549 for BEN-U and 0.621 for SPMA. All predictors were significant for both biomarkers ($P \leq 0.009$). When comparing the different contributions of independent variables, for BEN-U, the most relevant factor was cotinine ($r_p = 0.646$), followed by benzene air levels ($r_p = 0.437$) and creatinine ($r_p = 0.272$). For SPMA, the most relevant factor was log benzene air levels ($r_p = 0.646$), followed by cotinine ($r_p = 0.597$) and creatinine ($r_p = 0.195$).

Summary of characteristics of BEN-U and SPMA

The sampling conditions, analytical techniques and intrinsic characteristics of BEN-U and SPMA are summarized in Table 4. The section on sampling conditions and analytical techniques reports data obtained in the present work (precision, sample throughput, and percentage of quantifiable samples), but also related to previous studies (Fustinoni et al., 1999; Fustinoni et al., 2000; Fustinoni et al., 2010b). The section on intrinsic characteristics summarizes the outcome of the study; they were obtained from original data, and from data previously acquired (for BEN-U levels in subjects C, see also Fustinoni et al., 2010a). On the basis of Equations 1 and 2, specificity (Sp) and sensitivity (Se) values were estimated. When only nonsmokers were considered, Sp and Se were 0.86 and 0.88 for BEN-U and 0.91 and 0.90 for SPMA (see Table 5).

Using data from C subjects (three determinations available for each subject), we estimated background values and intra- and inter-individual variability. Background was calculated by considering the distribution of the geometric mean values of three determinations; it was greatly impacted by smoking habit. For BEN-U, the background was 180 and 2700 ng/L in nonsmokers and smokers, respectively, and for SPMA, the background was 0.21 and 1.83 μg/L in nonsmokers and smokers, respectively. Inter-individual variability, estimated as the ratio of the 95th to the 5th percentile, was 3-fold in nonsmokers and 32-fold in smokers for BEN-U, and greater than 2-fold in nonsmokers and greater than 18-fold in smokers for SPMA. Intra-individual variability was comparable in smokers and nonsmokers (150% for BEN-U and greater than 158% for SPMA). Estimates for SPMA suffer from a lack of precision due to a large percentage of data below the LOQ. The correlations of the biomarkers with air

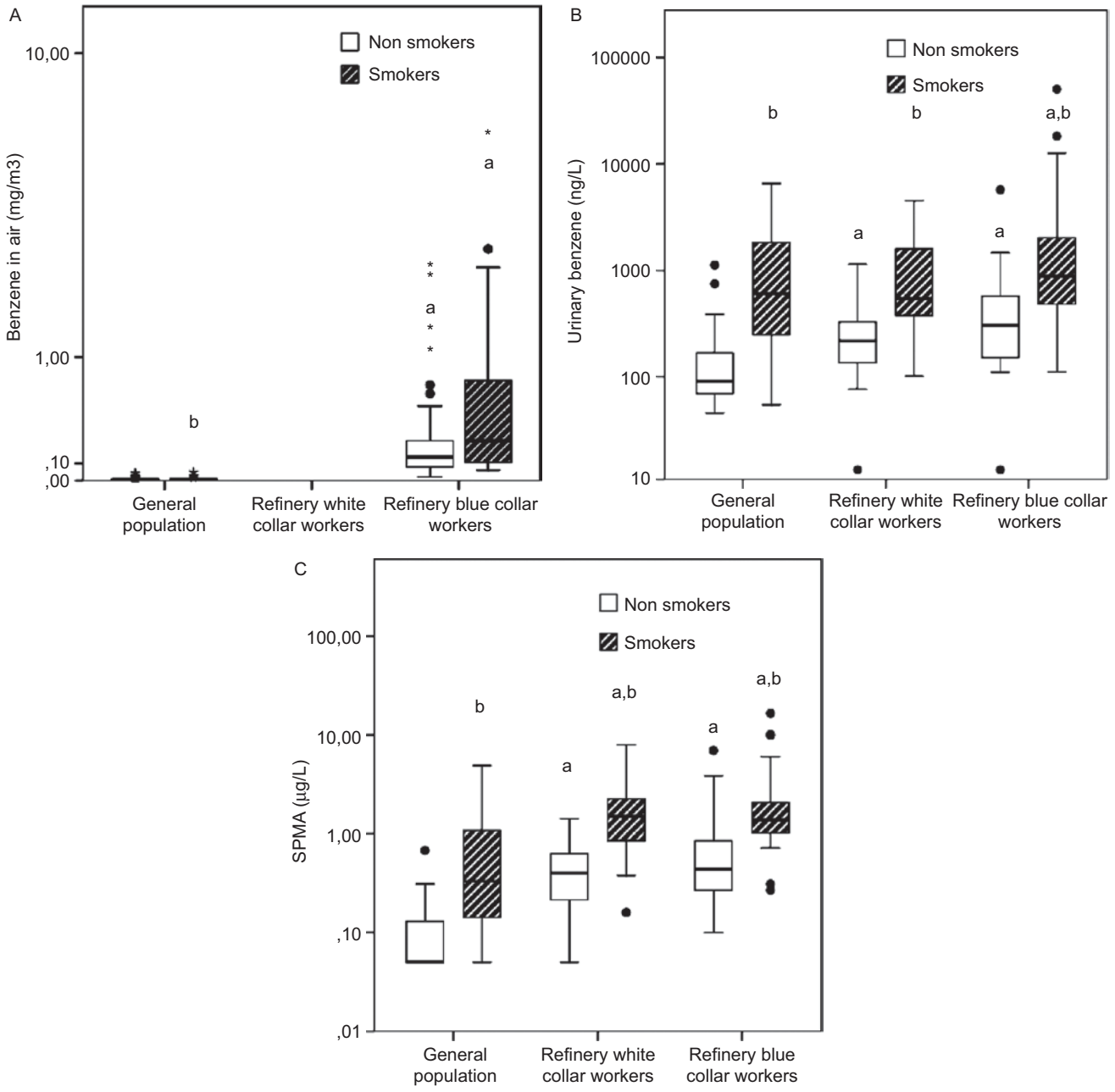

Figure 1. Box plot of benzene air levels (1A), BEN-U (1B) and SPMA (1C) in subjects divided according to exposure group and smoking habit. [a]significantly higher ($P < 0.05$) than subjects from the general population; [b]significantly higher ($P < 0.05$) than nonsmokers.

benzene (see above paragraph *Pearson's correlations and simple linear regressions*) were significant for both BEN-U ($r = 0.456$) and SPMA ($r = 0.636$), and higher in nonsmokers ($r = 0.627$ and 0.759, respectively) than in smokers ($r = 0.266$ and 0.519, respectively).

Discussion

In the present investigation, BEN-U and SPMA were evaluated as biomarkers of exposure to low levels of benzene. In refinery workers, the median personal benzene exposure (Table 2) was well below the occupational limit value of 1 ppm (or 3.2 mg/m^3) set by the EU, and 0.5 ppm (or 1.6 mg/m^3) suggested by the ACGIH; however, 1% and 10% of subjects exceeded the 1 ppm and 0.5 ppm limit values, respectively. This exposure was 3- to 10-fold higher than that observed in gasoline station attendants, traffic policemen, and bus drivers (Fustinoni et al., 2005; Barbieri et al., 2008; Lovreglio et al., 2010), and 20- to 100-fold higher than that of the general population (Fustinoni et al., 2005 and 2010a; Lovreglio et al., 2010; Lin et al., 2008). BEN-U and SPMA were higher in refinery workers than in subjects from the general population. Considering all subjects, B subjects had higher BEN-U and SPMA (expressed as µg/g creatinine) than W subjects (see Table 2). Such differences were not observed when subjects were divided according to smoking status; while for smokers this is probably due to the confounding effect of

Table 3. Pearson's correlations between airborne benzene and exposure biomarkers in all subjects or in subjects divided according to smoking habits.

		log BEN-U (ng/L)	log SPMA (μg/L)	log SPMA (μg/g creatinine)	log cotinine (μg/L)	log creatinine (g/L)
Log benzene air (mg/m^3)	All subjects (178)	0.456**	0.636**	0.628**	0.229**	−0.071
	Nonsmokers (98)	0.627**	0.759**	0.717**	−0.144	0.030
	Smokers (80)	0.266*	0.519**	0.551**	0.212	−0.112
Log BEN-U (ng/L)	All subjects (276)		0.735**	0.667**	0.630**	0.118*
	Nonsmokers (153)		0.575**	0.456**	−0.010	0.295**
	Smokers (123)		0.661**	0.562**	0.439**	0.161
Log SPMA (μg/L)	All subjects (276)			0.933**	0.570**	0.101
	Nonsmokers (153)			0.916**	−0.127	0.221**
	Smokers (123)			0.894**	0.389**	0.154
Log SPMA (μg/g creatinine)	All subjects (276)				0.584**	−0.264**
	Nonsmokers (153)				−0.144	−0.191*
	Smokers (123)				0.372**	−0.305**
Log cotinine (μg/L)	All subjects (276)					−0.088
	Nonsmokers (153)					−0.082
	Smokers (123)					0.020

*Correlation is significant at the 0.05 level (2-tailed), **correlation is significant at the 0.01 level (2-tailed).

smoking, for nonsmokers the lack of significance in spite of the differences observed between groups (median BEN-U 308, 218, and 90 ng/L in B, W, and C; SPMA 0.31, 0.25, <0.10 μg/g creatinine) could be attributable to the small sample size. The effect of smoking status revealed a significant increase of both BEN-U and SPMA in smokers compared to nonsmokers, in agreement with previous observations. Particularly in B subjects, the increase was roughly 2.8-fold for BEN-U, and 3.1-fold for SPMA. The increase in biomarker levels attributable to occupational exposure was 3.5-fold for BEN-U and more than 6.5-fold for SPMA. The comparable effect on the levels of benzene biomarkers exerted by these sources proves that, even at air exposures over occupational limit values, smoking represents a major source of benzene absorption, and suggests that the differentiation between smokers and nonsmokers should be considered in setting the occupational limit values for biological monitoring.

Pearson's correlation analysis (Table 3) between biomarkers and airborne benzene showed significant correlations. Such correlations improved when only nonsmokers were considered; this is in accordance with previous observations (Fustinoni et al., 2005 and 2010a; Lovreglio et al., 2010), and is explained by the fact that tobacco smoking represents an extra source of benzene intake, not properly captured by air measurements. BEN-U and SPMA correlated well with one another ($r = 0.735$), which indicates that they arise from the same exposure sources and similarly reflect internal benzene dose. In all subjects, and in smokers only, both BEN-U and SPMA correlated with COT-U, further supporting the effect of smoking. Also, urinary creatinine was positively associated with both BEN-U and SPMA to some extent, but, while creatinine adjustment is commonly used for SPMA (ACGIH, 2009; DFG, 2009), this is not the case for BEN-U, for which passive diffusion has been postulated for its renal elimination (Imbriani et al., 1986). In light of our result, this mechanism of excretion should be reconsidered.

Multiple regression analysis (Table 4) confirms the role of air exposure, smoking habit and urinary creatinine in determining the levels of BEN-U and SPMA, as reported above. The explained variability is relatively substantial, and higher for SPMA than for BEN-U (R^2 of 0.621 vs. 0.541). Other uncontrolled sources of variability include the variability associated with sampling and analytical assays, the daily variation in exposure levels, exposure to other solvents (affecting benzene absorption and/or metabolism), skin exposure, physiological characteristics of subjects, genetic polymorphism of metabolic enzymes, workload, and use of personal protective equipment. Concerning genetic polymorphism, previous studies showed that GSTM1, T1 and A1 have a significant role in inter-individual variability observed in SPMA excretion (Sørensen et al., 2004; Qu et al., 2005; Kim et al., 2007; Manini et al, 2010). BEN-U is affected by a CYP2E1 genetic polymorphism, even though its contribution to the variability was estimated to be only about 2% (Fustinoni et al., 2005).

When biomarkers properties are considered (Table 5), we recognize that sampling conditions can have a profound effect on the level of biomarkers. While no particular precaution is needed for urine collection for SPMA determination, BEN-U requires more precautions, as benzene will equilibrate between the urine and the air above the sample, which either decreases the analyte in the sample or enriches it, depending on the benzene concentration above the sample. The transfer of urine from the collection vessel into a sealed vapor-tight tube (to avoid the urine/air benzene exchange) should be performed as soon as possible and, in any case, within 15 min of micturition. Although data regarding the loss of benzene from the collection vessel are not available, a study on toluene showed that the loss was limited to

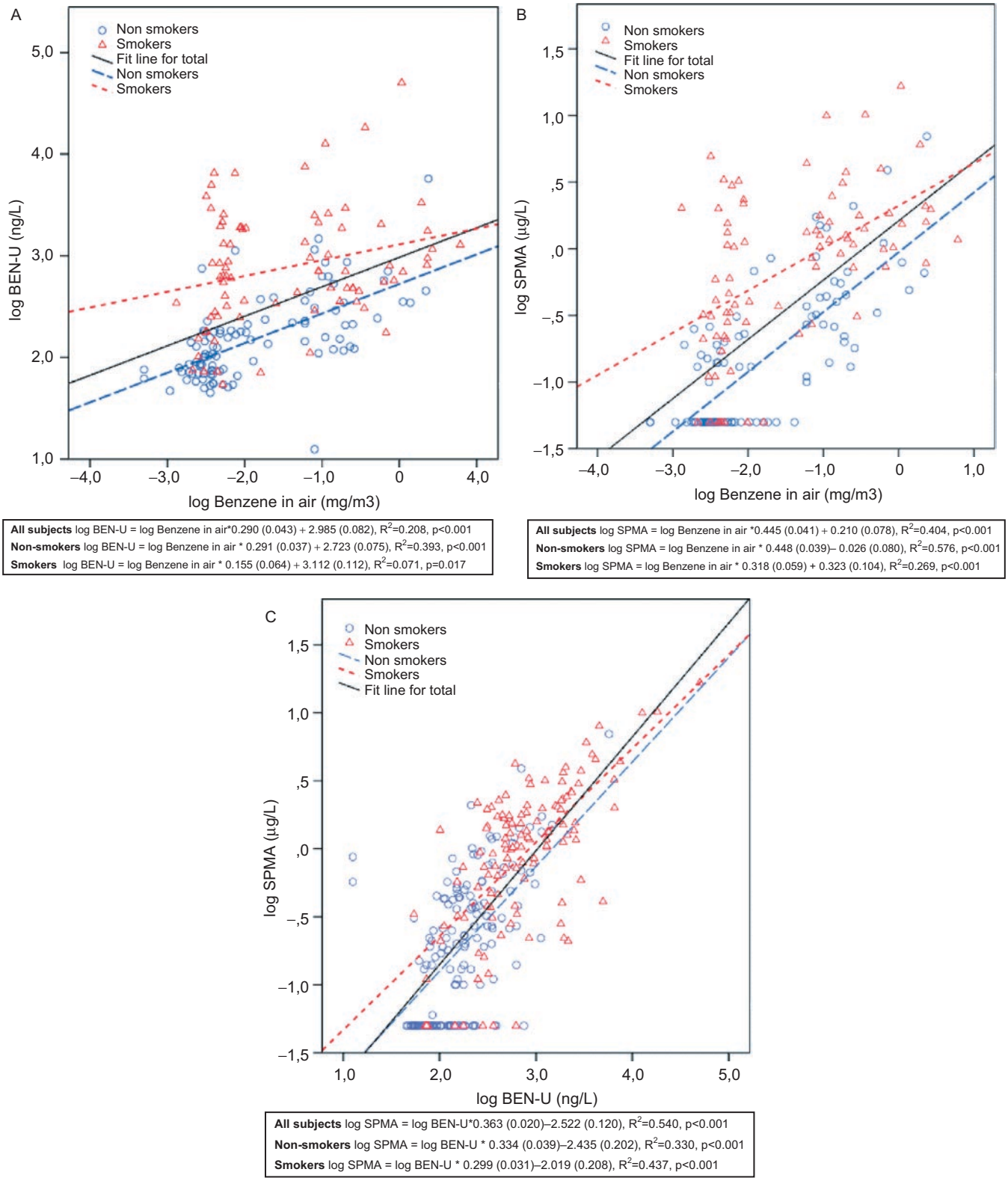

Figure 2. Scatter plot and linear regression lines comparing BEN-U or SPMA with benzene levels in the air (2A and 2B), and between BEN-U and SPMA (2C) in all subjects (solid line), in nonsmokers (circles, dashed line) and in smokers (triangles, dotted line).

less than 5% of its initial value when the transfer was performed within 20 min of micturition (Fustinoni et al., 2000). While BEN-U levels were consistent with reported values in similar environmental studies, SPMA levels were much lower than previously published values (Fustinoni et al., 2005), and in line with levels recently obtained by other authors using LC/MS/MS assays (Li et al., 2006; Schettgen et al., 2008). In general, a poor congruence among different studies reporting SPMA is observed (Waidyanatha et al., 2004; Fustinoni et al., 2005;

Table 4. Evaluation of airborne benzene exposure, smoking habit and creatinine concentration on the level of urinary biomarkers following multiple regression analysis: log (biomarker) = constant + log (benzene air) × β_1 + log cotinine × β_2 + log creatinine × β_3.

	Log BEN-U (ng/L)					Log SPMA (μg/L)				
	β	SE	t	P	r_p	β	SE	t	P	r_p
Constant	1.876	0.115	16.299	<0.001	—	−0.765	0.116	−6.591	<0.001	—
Log benzene air (mg/m^3)	0.214	0.033	6.417	<0.001	0.437	0.375	0.034	11.165	<0.001	0.646
Log cotinine (μg/L)	0.421	0.038	11.156	<0.001	0.646	0.374	0.038	9.828	<0.001	0.597
Log creatinine (g/L)	0.497	0.133	3.731	<0.001	0.272	0.353	0.134	2.627	0.009	0.195
Whole model R^2	0.549					0.621				
Whole model R^2_{adj}	0.541					0.614				
Whole model P	<0.001					<0.001				

Paci et al., 2007; Manini et al., 2008; Barbieri et al., 2008; Schettgen et al., 2008; Lovreglio et al., 2010; Carrieri et al., 2010). This may be due to analytical and/or storage and handling conditions.

BEN-U was assessed by headspace SPME followed by GC/MS in the presence of commercially available $^2H_6$-benzene as an internal standard; this assay is quite sensitive, specific, precise and accurate (Fustinoni et al., 1999; Fustinoni et al., 2010a). In particular, the LOQ is low enough to detect BEN-U in all subjects from the general population, and the assay has a precision of 13.4% and a throughput of about 50 samples each day (Table 5). A review of the literature showed that headspace GC/MS has been the technique of choice for the determination of BEN-U since the pioneering work of Ghittori and coworkers (Ghittori et al., 1993; Kok and Ong, 1994; Andreoli et al., 1999; Perbellini et al., 2002; Prado et al., 2004). The application of these assays yielded BEN-U levels comparable and coherent among different studies (Lagorio et al., 1998; Inoue et al., 2001; Waidyanatha et al., 2001; Kim et al., 2006; Manini et al., 2008; Barbieri et al., 2008; Hoet et al., 2009, and studies on the general population reviewed in Fustinoni et al., 2010a).

SPMA was assessed by SPE followed by LC/MS/MS in the presence of commercially available *DL*-S-phenylmercapturic-3,3-d_2 acid as the internal standard; this assay has a LOQ of 0.1 μg/L, which allowed us to quantify only about 50% of samples from the general population, and only 18% from nonsmokers (Table 5). Sample throughput and precision were 30 and 22.9 samples each day, lower than those found for BEN-U. Reviewing the literature for SPMA, we found that the assay applied for the determination of this marker has been changing for years. The first procedure applied GC/MS (detection limit ≥1 μg/L) to urine samples from occupationally exposed subjects (Stommel et al., 1989; Boogaard and van Sittert, 1995). An improved and simplified procedure based on LC/MS/MS after SPE (detection limit 0.4 μg/L; Melikian et al., 1999) was successively proposed. A chemiluminescent enzyme-linked immunosorbent assay (Aston et al., 2002), developed specifically to investigate exposure in occupational settings with air benzene close to limit values, was recently commercialized. Recently, other LC/MS/MS assays with an online sample pre-purification were developed (Lin et al., 2004; Li et al., 2006; Schettgen et al., 2008); they reduce sample handling and increase sensitivity (detection limit ≤0.1 μg/L), and were applied to assess SPMA in subjects belonging to the general population. In these studies, a percentage of undetected samples was reported (Li et al., 2006; Schettgen et al., 2008), showing that, especially in nonsmokers, SPMA is often below 0.1 μg/L. In summary, only methods using LC/MS/MS seem suitable for assessing SPMA concentrations in low occupational and/or environmental exposure scenarios. The instrumentation, besides being expensive, needs skilled technicians, and careful maintenance for optimal performance.

A further issue in using SPMA is the presence of both SPMA and pre-SPMA (Sabourin et al., 1988; Inoue et al., 2000) in urine. Recent works showed that urine treatment with strong or weak acid during sample storage, preparation, and/or analysis can affect the concentration of SPMA in samples, due to partial or total transformation of pre-SPMA into SPMA. The inter-individual variability in the excretion of the two forms, and the best condition to convert pre-SPMA into SPMA are currently under investigation (Tranfo et al., 2010; Sterz et al., 2010).

Considering intrinsic characteristics of biomarkers, higher specificity and sensitivity were associated with SPMA, although these parameters appear to be fairly consistent for BEN-U. It must be emphasized that specificity and sensitivity are closely related to the difference in exposure between the so-called "exposed" and "not exposed" subjects, and therefore they should not be considered constants, but rather are expected to change in different situations. Also, comparing the correlation between the biomarkers and personal air exposure, we found a higher correlation coefficient for SPMA than for BEN-U, even if the differences are small. Similar behavior between the two markers was associated with intra- and inter-individual variability and confounding effect of smoking status (Table 5).

Conclusions

In this work, BEN-U and SPMA were evaluated using a systematic quantitative approach. Comparing SPMA and BEN-U for sampling conditions and analytical techniques, we conclude that, although BEN-U presents critical sampling conditions, the analytical requirements for the determination of SPMA, and the continuing research for the identification of the best assay, make this marker

Table 5. Comparison between BEN-U (ng/L) and SPMA (μg/L) based on intrinsic characteristics of the biomarkers and sampling conditions and analytical techniques.

Biomarkers properties		BEN-U (ng/L)	SPMA (μg/L)
Sampling conditions and analytical techniques[a]	Nature of sample/invasivity	Urine/no	Urine/no
	Sample volume	1–2 mL	1–2 mL
	Sampling time	Within 20 min after the end of the work shift	At the end of the work shift
	Collection-storage containers	Gas tight vial	Normal tube
	Storage	Stable at least for 2 months at −20°C	Stable
	Contamination/loss of analytes during handling	Precaution	No problem
	Method	Simple and easy to replicate	Methods still in progress. Need for expensive equipment
	Instrument	GC/MS	LC/MS/MS
	Specificity of the analytical procedure	Very high	Very high
	Limit of quantification limit (LOQ)	15 ng/L, 100% samples above the LOQ in subjects from the general population	0.1 μg/L, 50% of samples above LOQ in subjects from the general population
	Precision	13.4%	22.9%
	Speed of the assay	About 50 samples/day	About 30 samples/day
Intrinsic characteristics	Significance	Exposure	Exposure
	Translation	Recent	Recent
	Background in the general population (95th percentile of the control distribution)[b]	All 1600 ng/L Nonsmokers 180 ng/L Smokers 2700 ng/L	All 1.41 μg/L Nonsmokers 0.21 μg/L Smokers 1.83 μg/L
	Specificity (capacity to correctly classify subjects without occupational exposure to benzene as "controls")	All 0.64 Nonsmokers 0.86 Smokers 0.56	All 0.69 Nonsmokers 0.91 Smokers 0.61
	Sensitivity (capacity to correctly classify subjects belonging to the occupational exposed group as "exposed")	All 0.74 Nonsmokers 0.88 Smokers 0.75	All 0.83 Nonsmokers 0.90 Smokers 0.80
	Correlation with air exposure[c]	All Pearson's $r = 0.456$ Nonsmokers Pearson's $r = 0.627$ Smokers Pearson's $r = 0.266$	All Pearson's $r = 0.636$ Nonsmokers Pearson's $r = 0.759$ Smokers Pearson's $r = 0.519$
	Intra-individual variability as CV% (three measurements for each subject belonging to the general population)[b]	All 150% Nonsmokers 151% Smokers 130%	All >158% Nonsmokers >143% Smokers >183%
	Inter-individual variability as ratio between 95th and 5th percentile of the distribution of the general population[b]	All 27 Nonsmokers 3 Smokers 32	All >14 Nonsmokers >2 Smokers >18
	Relevant confounding factors	Cigarette smoking	Cigarette smoking

[a]The results of this section are referred also to Fustinoni et al., 1999, Fustinoni et al., 2000, Fustinoni et al., 2010b.
[b]Parameters estimated using three measurements for each subject (C) (general population).
[c]Correlations obtained with data from subjects C (general population) and B (blue collar workers).

less suitable for the investigation of exposure in the general environment. On the other hand, the intrinsic characteristics, although more favorable for SPMA, are fairly good for both markers, and show that they can be both adopted to assess low occupational exposure to benzene.

Acknowledgments

We are indebted to the subjects who volunteered for the study.

Declaration of interest

The authors thank AISPO for financial support.

References

American Conference of Governmental Industrial Hygienists (ACGIH). (2009). TLVs and BEIs based on the documentation of the threshold limit values for chemical substances and physical agents and biological indices. Cincinnati, USA: ACGIH.

Andreoli R, Manini P, Bergamaschi E, Brustolin A, Mutti A. (1999). Solid-phase microextraction and gas chromatography-mass

spectrometry for determination of monoaromatic hydrocarbons in blood and urine: application to people exposed to air pollutants. *Chromatographia* 20:167–72.

Aston JP, Ball RL, Pople JE, Jones K, Cocker J. (2002). Development and validation of a competitive immunoassay for urinary S-phenylmercapturic acid and its application in benzene biological monitoring. *Biomarkers* 7:103–112.

Baccarelli A, Pfeiffer R, Consonni D, Pesatori AC, Bonzini M, Patterson DG Jr, Bertazzi PA, Landi MT. (2005). Handling of dioxin measurement data in the presence of non-detectable values: overview of available methods and their application in the Seveso chloracne study *Chemosphere* 60:898–906

Barbieri A, Violante FS, Sabatini L, Graziosi F, Mattioli S. (2008). Urinary biomarkers and low-level environmental benzene concentration: assessing occupational and general exposure. *Chemosphere* 74:64–69.

Barr DB, Wilder LC, Caudill SP, Gonzalez AJ, Needham LL, Pirkle JL. (2005). Urinary creatinine concentrations in the U.S. population: implications for urinary biologic monitoring measurements. *Environ Health Perspect* 113:192–200.

Boogaard PJ, van Sittert NJ. (1995). Biological monitoring of exposure to benzene: a comparison between S-phenylmercapturic acid, trans-trans-muconic acid, and phenol. *Occup Environ Health* 52:611–20.

Carrieri M, Tranfo G, Pigini D, Paci E, Salamon F, Scapellato ML, Fracasso ME, Manno M, Bartolucci GB. (2010). Correlation between environmental and biological monitoring of exposure to benzene in petrochemical industry operators. *Toxicol Lett* 192:17–21.

Crebelli R, Tomei F, Zijno A, Ghittori S, Imbriani M, Gamberale D, Martini A, Carere A. (2001). Exposure to benzene in urban workers: environmental and biological monitoring of traffic police in Rome. *Occup Environ Med* 58:165–171.

Deutsch Forschungsgemeinschaft (DFG). (2009). List of MAK and BAT values 2009. Report No 40, Weinheim: DFG, WILEY-VCH.

Dor F, Dab W, Empereur-Bissonnet P, Zmirou D. (1999). Validity of biomarkers in environmental health studies: the case of PAHs and benzene. *Crit Rev Toxicol* 29:129–168.

European Commission. (2008). Regulation (EC) No 1272/2008 of the European Parliament and the Council of 16 December 2008 on classification, labelling and packaging of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC, and amending Regulation (EC) No 1907/2006. Brussels, Belgium. Available online at: http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2008:353:0001:1355:EN:PDF

European Commission. (2004). Communication from the Commission to the Council, the European Parliament, the European Economic and Social Committee. "The European Environmental & Health and Action Plan 2004–2010" SEC (2004) 729. Brussels, Belgium.

European Commission. (2000). Directive 2000/69/EC (2000) of the European Parliament and of the Council of 16 November relating to limit values for benzene and carbon monoxide in ambient air. Brussels, Belgium.

European Commission. (1997). Council Directive 97/42/EC of 27 June 1997 amending for the first time Directive 90/394/EEC on protecting workers from risks related to exposure to carcinogens at work [Sixth Individual Directive according to Art. 16(1) of Directive 89/391/EEC]. Official Journal of the European Communities No. L179, 08.07.1997.

Fustinoni S, Buratti M, Giampiccolo R, Brambilla G, Foà V, Colombi A. (2000). Comparison between blood and urinary toluene as biomarkers of exposure to toluene. *Int Arch Occup Environ Health* 73:389–396.

Fustinoni S, Buratti M, Giampiccolo R, Colombi A. (1995). Biological and environmental monitoring of exposure to airborne benzene and other aromatic hydrocarbons in Milan traffic wardens. *Toxicol Lett* 77:387–392.

Fustinoni S, Rossella F, Campo L, Mercadante R, Bertazzi PA. (2010a). Urinary BTEX, MTBE and naphthalene as biomarkers to gain environmental exposure profiles of the general population. *Sci Total Environ* 408:2840–2849.

Fustinoni S, Campo L, Mercadante R, Manini P. (2010b). Methodological issues in biological monitoring of low exposures: the case of urinary benzene and S-phenylmercapturc acid. *J Chromatogr B* 878:2534–2540.

Fustinoni S, Consonni D, Campo L, Buratti M, Colombi A, Pesatori AC, Bonzini M, Bertazzi PA, Foà V, Garte S, Farmer PB, Levy LS, Pala M, Valerio F, Fontana V, Desideri A, Merlo DF. (2005). Monitoring low benzene exposure: comparative evaluation of urinary biomarkers, influence of cigarette smoking, and genetic polymorphisms. *Cancer Epidemiol Biomarkers Prev* 14:2237–2244.

Fustinoni S, Giampiccolo R, Pulvirenti S, Buratti M, Colombi A. (1999). Headspace solid-phase microextraction for the determination of benzene, toluene, ethylbenzene and xylenes in urine. *J Chromatogr B Biomed Sci Appl* 723:105–115.

Ghittori S, Fiorentino ML, Maestri L, Cordioli G, Imbriani M. (1993). Urinary excretion of unmetabolized benzene as an indicator of benzene exposure. *J Toxicol Environ Health* 38:233–243.

Haufroid V, Lison D. (1998). Urinary cotinine as a tobacco-smoke exposure index: a minireview. *Int Arch Occup Environ Health* 71:162–168.

Hoet P, De Smedt E, Ferrari M, Imbriani M, Maestri L, Negri S, De Wilde P, Lison D, Haufroid V. (2009). Evaluation of urinary biomarkers of exposure to benzene: correlation with blood benzene and influence of confounding factors. *Int Arch Occup Environ Health* 82:985–995.

IARC (International Agency for Research on Cancer). (1987). Benzene, In: *IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans, Suppl. 7*. Lyon, France: IARC.

Imbriani M, Ghittori S, Pezzagno G, Capodaglio E. (1986). Toluene and styrene in urine as biological exposure indices. *Appl Ind Hyg* 1:172–176.

Inoue O, Kanno E, Kakizaki M, Watanabe T, Higashikawa K, Ikeda M. (2000). Urinary phenylmercapturic acid as a marker of occupational exposure to benzene. *Ind Health* 38:195–204.

Inoue O, Kanno E, Yusa T, Kakizaki M, Watanabe T, Higashikawa K, Ikeda M. (2001). A simple HPLC method to determine urinary phenylmercapturic acid and its application to gasoline station attendants to biomonitor occupational exposure to benzene at less than 1 ppm. *Biomarkers* 6:190–203.

Kim S, Lan Q, Waidyanatha S, Chanock S, Johnson BA, Vermeulen R, Smith MT, Zhang L, Li G, Shen M, Yin S, Rothman N, Rappaport SM. (2007). Genetic polymorphisms and benzene metabolism in humans exposed to a wide range of air concentrations. *Pharmacogenet Genomics* 17:789–801.

Kim S, Vermeulen R, Waidyanatha S, Johnson BA, Lan Q, Rothman N, Smith MT, Zhang L, Li G, Shen M, Yin S, Rappaport SM. (2006). Using urinary biomarkers to elucidate dose-related patterns of human benzene metabolism. *Carcinogenesis* 27:772–781.

Kok PW, Ong CN. (1994). Blood and urinary benzene determined by headspace gas chromatography with photoionization detection: application in biological monitoring of low-level nonoccupational exposure. *Int Arch Occup Environ Health* 66:195–201.

Kroll MH, Chesler R, Hagengruber C, Blank DW, Kestner J, Rawe M. (1986). Automated determination of urinary creatinine without sample dilution: theory and practice. *Clin Chem* 32:446–452.

Lagorio S, Crebelli R, Ricciarello R, Conti L, Iavarone I, Zona A, Ghittori S, Carere A. (1998). Methodological issues in biomonitoring of low level exposure to benzene. *Occup Med (Lond)* 48:497–504.

Li Y, Li AC, Shi H, Junga H, Jiang X, Naidong W, Lauterbach JH. (2006). Determination of S-phenylmercapturic acid in human urine using an automated sample extraction and fast liquid chromatography-tandem mass spectrometric method. *Biomed Chromatogr* 20:597–604.

Lin LC, Shih JF, Shih TS, Li YL, Liao PC. (2004). An electrospray ionization tandem mass spectrometry based system with an online dual-loop cleanup device fro simultaneous quantitation of urinary benzene exposure biomarkers trans,trans-muconic acid and S-phenylmercapturic acid. *Rapid Comm Mass Spectrometry* 18:2743–2752.

Lin YS, Egeghy PP, Rappaport SM. (2008). Relationships between levels of volatile organic compounds in air and blood from the general population. *J Expo Sci Environ Epidemiol* 18:421–429.

Lovreglio P, Barbieri A, Carrieri M, Sabatini L, Fracasso ME, Doria D, Drago I, Basso A, D'Errico MN, Bartolucci GB, Violante FS, Soleo L. (2010). Validity of new biomarkers of internal dose for use in the biological monitoring of occupational and environmental exposure to low concentrations of benzene and toluene. *Int Arch Occup Environ Health* 83:341–356.

Meger M, Meger-Kossien I, Schuler-Metz A, Janket D, Scherer G. (2002). Simultaneous determination of nicotine and eight nicotine metabolites in urine of smokers using liquid chromatography-tandem mass spectrometry. *J Chromatogr b Analyt Technol Biomed Life Sci* 778:251–261.

Manini P, De Palma G, Andreoli R, Mozzoni P, Poli D, Goldoni M, Petyx M, Apostoli P, Mutti A. (2010). Occupational exposure to low levels of benzene: Biomarkers of exposure and nucleic acid oxidation and their modulation by polymorphic xenobiotic metabolizing enzymes. *Toxicol Lett* 193:229–235.

Manini P, De Palma G, Andreoli R, Poli D, Mozzoni P, Folesani G, Mutti A, Apostoli P. (2006). Environmental and biological monitoring of benzene exposure in a cohort of Italian taxi drivers. *Toxicol Lett* 167:142–151.

Manini P, De Palma G, Andreoli R, Poli D, Petyx M, Corradi M, Mutti A, Apostoli P. (2008). Biological monitoring of low benzene exposure in Italian traffic policemen. *Toxicol Lett* 181:25–30.

Melikian AA, O'Connor R, Prahalad AK, Hu P, Li H, Kagan M, Thompson S. (1999). Determination of the urinary benzene metabolites S-phenylmercapturic acid and trans,trans-muconic acid by liquid chromatography-tandem mass spectrometry. *Carcinogenesis* 20:719–726.

NIOSH (National Institute Occupational Safety and Health). (2003). Hydrocarbons, Aromatic. Method 1501. Available online at: http://www.cdc.gov/niosh/docs/2003-154/pdfs/1501.pdf. (Last accessed 31-12-2010).

Paci E, Pigini D, Cialdella AM, Faranda P, Tranfo G. (2007). Determination of free and total S-phenylmercapturic acid by HPLC/MS/MS in the biological monitoring of benzene exposure. *Biomarkers* 12:111–122.

Perbellini L, Pasini F, Romani S, Princivalle A, Brugnone F. (2002). Analysis of benzene, toluene, ethylbenzene and m-xylene in biological samples from the general population. *J Chromatogr B Analyt Technol Biomed Life Sci* 778:199–210.

Prado C, Garrido J, Periago JF. (2004). Urinary benzene determination by SPME/GC-MS. A study of variables by fractional factorial design and response surface methodology. *J Chromatogr B Analyt Technol Biomed Life Sci* 804:255–261.

Qu Q, Shore R, Li G, Su L, Jin X, Melikian AA, Roy N, Chen LC, Wirgin I, Cohen B, Yin S, Li Y, Mu R. (2005). Biomarkers of benzene; urinary metabolites in relation to individual genotype and personal exposure. *Chemico-Biological Inter* 153–154:85–95.

Rappaport SM. (1991). Assessment of long-term exposures to toxic substances in air. *Ann Occup Hyg* 35:61–121.

Sabourin PJ, Bechtold WE, Henderson RF. (1988). A high pressure liquid chromatographic method for the separation and quantitation of water-soluble radiolabeled benzene metabolites. *Anal Biochem* 170:316–327.

Schettgen T, Musiol A, Alt A, Kraus T. (2008). Fast determination of urinary S-phenylmercapturic acid (S-PMA) and S-benzylmercapturic acid (S-BMA) by column-switching liquid chromatography-tandem mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci* 863:283–292.

Sigma-Aldrich. (2010). Application Note Radiello, available online at: http://www.sigmaaldrich.com/etc/medialib/docs/Supelco/Application_Notes/radiello_d1_d6.Par.0001.File.tmp/radiello_d1_d6.pdf. (Last accessed 31-12-2010).

Sørensen M, Poole J, Autrup H, Muzyka V, Jensen A, Loft S, Knudsen LE. (2004). Benzene exposure assessed by metabolite excretion in Estonian oil shale mineworkers: influence of glutathione s-transferase polymorphisms. *Cancer Epidemiol Biomarkers Prev* 13:1729–1735.

Sterz K, Köhler D, Schettgen T, Scherer G. (2010). Enrichment and properties of urinary per-S-phenylmercapturic acid (pre-SPMA). *J Chromatogr B* (in press).

Stommel P, Müller G, Stücker W, Verkoyen C, Schöbel S, Norpoth K. (1989). Determination of S-phenylmercapturic acid in the urine–an improvement in the biological monitoring of benzene exposure. *Carcinogenesis* 10:279–282.

Tranfo G, Bartolucci GB, Pigini D, Paci E, Scapellato ML, Doria D, Manno M, Carrieri M. (2010). Comparison of hydrolysis and HPLC/MS/MS procedure with ELISA assay for the determination of S-phenylmercapturic acid as a biomarker of benzene exposure in human urine. *J Chromatogr B Analyt Technol Biomed Life Sci* 878:2529–2533.

Waidyanatha S, Rothman N, Fustinoni S, Smith MT, Hayes RB, Bechtold W, Dosemeci M, Guilan L, Yin S, Rappaport SM. (2001). Urinary benzene as a biomarker of exposure among occupationally exposed and unexposed subjects. *Carcinogenesis* 22:279–286.

Waidyanatha S, Rothman N, Li G, Smith MT, Yin S, Rappaport SM. (2004). Rapid determination of six urinary benzene metabolites in occupationally exposed and unexposed subjects. *Anal Biochem* 327:184–199.