Exposure to BTEX and Ethers in Petrol Station Attendants and Proposal of Biological Exposure Equivalents for Urinary Benzene and MTBE

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ABSTRACT

Objective: To assess exposure to benzene (BEN) and other aromatic compounds (toluene, ethylbenzene, *m*+*p*-xylene, *o*-xylene) (BTEX), methyl *tert*-butyl ether (MTBE), and ethyl *tert*-butyl ether (ETBE) in petrol station workers using air sampling and biological monitoring and to propose biological equivalents to occupational limit values.

Methods: Eighty-nine petrol station workers and 90 control subjects were investigated. Personal exposure to airborne BTEX and ethers was assessed during a mid-week shift; urine samples were collected at the beginning of the work week, prior to and at the end of air sampling.

Results: Petrol station workers had median airborne exposures to benzene and MTBE of 59 and 408 $\mu\text{g m}^{-3}$, respectively, with urinary benzene (BEN-U) and MTBE (MTBE-U) of 339 and 780 ng l⁻¹, respectively. Concentrations in petrol station workers were higher than in control subjects. There were significant positive correlations between airborne exposure and the corresponding biological marker, with Pearson's correlation coefficient (*r*) values of 0.437 and 0.865 for benzene and MTBE, respectively. There was also a strong correlation between airborne benzene and urinary MTBE (*r* = 0.835). Multiple linear regression analysis showed that the urinary levels of benzene were influenced by personal airborne exposure, urinary creatinine, and tobacco smoking [determination coefficient (*R*²) 0.572], while MTBE-U was influenced only by personal exposure (*R*² = 0.741).

Conclusions: BEN-U and MTBE-U are sensitive and specific biomarkers of low occupational exposures. We propose using BEN-U as biomarker of exposure to benzene in nonsmokers and suggest 1457 ng l⁻¹ in end shift urine samples as biological exposure equivalent to the EU occupational limit value of 1 p.p.m.; for both smokers and nonsmokers, MTBE-U may be proposed as a surrogate biomarker of benzene exposure, with a biological exposure equivalent of 22 $\mu\text{g l}^{-1}$ in end shift samples. For MTBE exposure, we suggest the use of MTBE-U with a biological exposure equivalent of 22 $\mu\text{g l}^{-1}$ corresponding to the occupational limit value of 50 p.p.m.

KEYWORDS: biological exposure equivalent; biological monitoring; biomarkers; exposure assessment; petrol station workers; urinary benzene; urinary MTBE

INTRODUCTION

Petrol (gasoline) is a complex mixture of hundreds of volatile hydrocarbons, predominantly alkanes, cycloalkanes, and alkenes, produced from the refining of oil. Additives are also added to improve the quality of the petrol and to ensure that specifications are met (EFOA, 2005). European legislation regulates automotive emissions and fuel composition to meet air quality targets: the fuel quality directive 98/70/EC was the first to require all EU countries to use lead free petrol (EC, 1998). A more recent directive, 2009/30/EC, introduced a mechanism to monitor and reduce greenhouse gas emissions and also amended Directive 98/70/EC regarding specifications for petrol, diesel, and gas-oil allowing ethers with five or more carbon atoms per molecule, such as methyl *tert*-butyl ether (MTBE), ethyl *tert*-butyl ether (ETBE), and *tert*-amyl methyl ether (TAME), to be blended up to a maximum of 22% by volume (EC, 2009). In addition, this directive allows fuel to contain aromatic hydrocarbons up to a maximum of 35% (by volume) of toluene, ethylbenzene, and xylene, although benzene must be less than 1% v/v.

As reported in the latest published EU Fuel Quality Monitoring Report, more than 15 million tons of automotive fuels are dispensed in Italy every year, all with a Research Octane Number (RON) of grade 95. Taking into account seasonal variation, the average composition of typical petrol dispensed in Italy is 30.7% aromatics, 5% ethers, and 0.8% benzene (Hill *et al.*, 2006).

The International Agency for Research on Cancer (IARC) has classified both petrol and petrol engine exhaust as possibly carcinogenic to humans (group 2B) (IARC, 2013). Among the components of petrol, benzene is a known carcinogen to humans (group 1) (IARC, 1987) and is a category 1A (H350) carcinogen according to the European Commission (EU) (EC, 2008). Ethylbenzene is classified in group 2B (IARC, 2000), while toluene, xylenes, and MTBE are in group 3 (not classifiable as to their carcinogenicity to humans) (IARC, 1999a,b). Ethylbenzene, toluene, xylenes, and MTBE are not classified as carcinogens according to the European Commission (EC, 2008).

Workers in many occupations may be exposed to unburned petrol vapors and engine exhaust, among them (petrol) filling station attendants, traffic policemen, car park attendants, professional drivers, street

vendors, and car mechanics. Filling station attendants, in particular, are subject to exposure to the volatile organic compounds from petrol vapors as well as exhaust emissions from customer's vehicles and passing traffic (IARC, 2013).

Because of the ubiquity and particular toxicity of benzene, occupational exposure to this compound is regulated in many countries. The EU occupational limit value for benzene is $3200 \mu\text{g m}^{-3}$ (1 p.p.m.) (EC, 2004). The American Conference of Governmental Industrial Hygienists (ACGIH) recommends a threshold limit value (TLV) as the time-weighted average (TWA), during an 8 h work shift, of $1600 \mu\text{g m}^{-3}$ (0.5 p.p.m.) (ACGIH, 2012). In Germany, the Committee for Hazardous Substances (AGS Committee) has proposed a tolerable risk of 4:1000 and an acceptable risk of 4:10 000 (changing to 4:100 000 no later than 2018), applicable over a working lifetime of 40 years with continuous exposure every working day (BauA, 2008). For benzene, the tolerable risk corresponds to a concentration of $1900 \mu\text{g m}^{-3}$, and the acceptable risk to a concentration of $200 \mu\text{g m}^{-3}$ ($20 \mu\text{g m}^{-3}$ by 2018) (BauA, 2012). In the Netherlands, a health-based occupational exposure limit has been recently recommended, corresponding to an airborne concentration of $700 \mu\text{g m}^{-3}$ (Health Council of the Netherlands, 2014). Indicative occupational limit values for an 8 h exposure have been established in the EU for xylenes and toluene (221 and 192 mg m^{-3} , respectively) (EC, 2000, 2006). Limit values as the TLV-TWA are recommended by ACGIH for toluene, ethylbenzene and xylenes (74.5 , 87 and 434 mg m^{-3} , respectively), as well as for MTBE, ETBE, and TAME (180 , 21 , and 83 mg m^{-3} , respectively) (ACGIH, 2012).

For occupational exposure to benzene, urinary trans,trans-muconic acid (tt-MA), and S-phenylmercapturic acid (SPMA), measured in end-of-shift samples, are recommended by ACGIH, with $500 \mu\text{g g}^{-1}$ creatinine and $25 \mu\text{g g}^{-1}$ creatinine, respectively as biological exposure indices (BEI) equivalent to the TLV-TWA. For both, a B notation warns that the marker is usually present in a significant amount in biological specimens collected from subjects without occupational exposure. For toluene exposure, urinary *o*-cresol and toluene are indicated with BEI of 0.3 mg g^{-1} creatinine (B notation) and $30 \mu\text{g l}^{-1}$, respectively. For ethylbenzene and xylene exposure, mandelic acid

plus phenylglyossilic acid (0.7 g g^{-1} creatinine, with Ns and Sq notations, indicating 'nonspecific' and 'semi-quantitative', respectively) and methylippuric acid are listed (1.5 g g^{-1} creatinine), respectively (ACGIH, 2012). No such indices have been proposed for ethers. Our previous studies, conducted on petrol station attendants, traffic policeman, and office workers, have demonstrated that urinary benzene (BEN-U) and MTBE (MTBE-U) are specific and sensitive biomarkers of exposure (Fustinoni *et al.*, 2005; Campo *et al.*, 2011) even if biological limit values have not been established yet.

In the present study, the exposure to benzene and other aromatic compounds and ethers (and MTBE in particular) was investigated in filling station attendants using personal air sampling and biological monitoring. The goal of this investigation was to identify suitable biomarkers of benzene and petrol vapor exposure. Differently from our previous studies, a multiple sample collection design was used to investigate the biomarker excretion profiles. The biological exposure equivalents for MTBE-U and BEN-U were proposed for the first time by using the relationship between airborne chemicals, BEN-U, and MTBE-U.

MATERIALS AND METHODS

Study population and sampling

The study population consisted of 179 healthy male adults (≥ 18 years old). Of these, 89 were petrol station workers (workers) and 90 were selected from the general population (control subjects), all working and living in the northwest area of the Milan province, within a 25 km distance from Milan city center. Control subjects, partly described in previous studies (Fustinoni *et al.*, 2010a, 2011), were matched with workers with regard to age and smoking habit. Data regarding personal characteristics, health status, active and environmental tobacco smoke, use of products containing solvents such as varnish and glue in the last 24 h, means of transport, and time to get to the job place were collected through a questionnaire administered by trained interviewers. Written informed consent was collected from each participant in the study. Health data were collected and processed according to the eHealth Task Force Report ('Ilves report on e-health') (EC, 2012). Personal data were treated according to the Directive 95/46/EC of the European

Parliament and of the Council of 24 October 1995 (EC, 1995). Samples were coded and handled without knowledge of their origin.

Individual personal exposure to airborne benzene, toluene, ethylbenzene, and xylenes (BTEX) and ethers was monitored for about 5 h during the working day (beginning 5:30–9:30 and ending 11:00–14:30), in the second part of the work week. Air was sampled using a passive sampler, Radiello, worn by subjects in the respiratory zone and equipped with a 35–50 mesh charcoal cartridge (Supelco, Sigma-Aldrich, Milano, Italy). At the end of the sampling period, the cartridge was sealed in the appropriate glass tube, and stored in a clean box at room temperature until analysis. Cartridges were analyzed within 30 days of collection, according to the manufacturer's instruction.

Urine spot samples were collected three times during the same week: (i) on Monday morning, before work began (between 5:30 and 9:30) that is after 2 days of absence from work (baseline, BL); (ii) on the day of air sampling, before initiation of the shift, and at the same time of the beginning of the air sampling (before sampling, BS, i.e. on Thursday between 5:30 and 9:30); and (iii) within 10 min of the end of the air sampling (end of sampling, ES, i.e. on Thursday between 11:00 and 14:30). Urine was collected in disposable polyurethane bottles. A disposable syringe was used to immediately place two 7-ml aliquots in two pre-sealed storage vials (Fustinoni *et al.*, 2009), one for the determination of urinary BTEX, the other for the determination of urinary ethers. Specimens were then refrigerated at 4°C and delivered to the laboratory within 12 h. Once in the laboratory, urine samples were divided into several aliquots for the analysis of the other biomarkers. All aliquots were kept at -20°C and analyzed, according to biomarkers' stability, within 60 days.

Personal air sampling

Determination of airborne BTEX, MTBE, and ETBE
Airborne benzene (BEN-A), toluene (TOL-A), ethylbenzene (EtBen-A), *m+p*-xylene (*m+p*-XYL-A), *o*-xylene (*o*-XYL-A), (all together BTEX-A), MTBE (MTBE-A), and ETBE (ETBE-A) were measured using a GC-MS method (Fustinoni *et al.*, 2010a). The limit of quantification (LOQ) was $8 \mu\text{g l}^{-1}$ for all analytes. Considering the average sampling time and the uptake rates of each analyte, this concentration was

estimated as corresponding to airborne levels of $0.8 \mu\text{g m}^{-3}$ for MTBE-A and ETBE-A, and $1.0 \mu\text{g m}^{-3}$ for the remaining target chemicals.

Biological monitoring

Determination of urinary BTEX, MTBE, and ETBE
BEN-U, toluene (TOL-U), ethylbenzene (EtBen-U), *m*+*p*-xylene (*m*+*p*-XYL-U), and *o*-xylene (*o*-XYL-U) were determined by headspace solid-phase microextraction (HS-SPME) followed by GC-MS analysis according to published methods (Fustinoni *et al.*, 1999; Fustinoni *et al.*, 2010b) with some modifications (Fustinoni *et al.*, 2010a). The LOQ was 15 ng l^{-1} for all analytes.

Urinary MTBE (MTBE-U) and ETBE (ETBE-U) were determined by HS-SPME-GC-MS (Scibetta *et al.*, 2007). The LOQs were 10 ng l^{-1} for MTBE-U and 15 ng l^{-1} for ETBE-U.

Determination of urinary tt-MA and SPMA

Determination of urinary tt-MA and SPMA concentrations were based on solid-phase extraction followed by liquid chromatography-tandem mass spectrometry (LC-MS-MS) analysis (Fustinoni *et al.*, 2011). The LOQs were 20 and $0.1 \mu\text{g l}^{-1}$ for tt-MA and SPMA, respectively.

Urinary cotinine and creatinine

Urinary cotinine (COT-U), a biomarker of tobacco smoking, was measured in BL samples by LC-MS-MS. The LOQ was $10 \mu\text{g l}^{-1}$ (Fustinoni *et al.*, 2013). Subjects with COT-U below $100 \mu\text{g l}^{-1}$ were classified as 'nonsmokers', while subjects with COT-U equal to or above $100 \mu\text{g l}^{-1}$ were classified as 'smokers' (Haufrond and Lison, 1988).

Urinary creatinine (crt) was determined using Jaffe's colorimetric method (Kroll *et al.*, 1986). The creatinine value was used to assure sample validity, excluding samples with excessive physiologic dilution or concentration according to the $0.3 \text{ g l}^{-1} \leq \text{creatinine} \leq 3.0 \text{ g l}^{-1}$ range (WHO, 1996).

Statistical analysis

Statistical analysis was performed using the SPSS 20.0 package for Windows (SPSS Statistics, IBM Italia). A value corresponding to one-half of the quantification limit was assigned to measurements below analytical quantification.

Data on airborne chemicals and urinary biomarkers were decimal log-transformed to ensure normal distribution. Student's *t*-test or analysis of variance were applied to compare two or more groups of independent samples, Pearson's correlations were used to test the associations between variables, and the chi-square test was used to compare the percentage distribution among groups. For analytes with less than 50% of the data above the LOQ, the chi-square test was used.

Data were included in simple and multiple linear regression models (only for biomarkers with more than 50% of samples above the LOQ). The multiple regression model evaluated the effect of airborne exposure ($\mu\text{g m}^{-3}$), urinary creatinine (g l^{-1}), smoking habit (COT-U, $\mu\text{g l}^{-1}$), age (years), and body mass index (BMI) (kg/m^2) on the ES urinary level of each biomarker (ng l^{-1}), assumed to be a dependent variable. An interaction term between the airborne exposure and the smoking status was added to each model. The final model was:

$\text{Log Urinary biomarker} = \text{constant} + \beta_1 (\text{log personal exposure}) + \beta_2 (\text{log creatinine}) + \beta_3 (\text{log cotinine}) + \beta_4 \text{age} + \beta_5 \text{BMI} + \beta_6 (\text{log personal exposure} \times \text{smoking status})$.

To calculate the biological exposure equivalents corresponding to the existing occupational limit values for ES BEN-U (only for nonsmokers), or ES MTBE-U (all subjects), simple regression models with BEN-A or MTBE-A as independent variables and ES BEN-U or ES MTBE-U as dependent variables were used.

A *P* value ≤ 0.05 was considered significant.

RESULTS

Study population

Table 1 reports personal and job characteristics of the study subjects. Results for tobacco smoking are reported based on questionnaire answers and on measurement of COT-U. When the COT-U cutoff ($\geq 100 \mu\text{g l}^{-1}$) was applied, 21 subjects (12%) were reclassified (12 petrol station workers and 9 controls), among which 18 were reclassified as smokers and 3 as nonsmokers. A discrepancy between the declared and the actual smoking habits has been repeatedly reported, especially for active smokers who may underestimate how often they smoke (Connor Gorber, 2009). For these reasons, COT-U was used as the classification parameter as recommended by the

Table 1. Summary of selected characteristics of the study subjects

Characteristics	Petrol station workers	Control subjects	P*
Number of subjects, <i>N</i>	89	90	
Age, mean (min – max) year	44 (19–71)	44 (18–72)	0.967
Body mass index, mean (min – max) kg m ⁻²	25.9 (19.2–36.5)	26.2 (17.7–36.5)	0.638
Smokers ^a , <i>N</i> (%)	30 (33.7%)	31 (34.4%)	0.917 ^b
Smokers (COT-U > 100 µg l ⁻¹), <i>N</i> (%)	40 (55.0%)	36 (40.0%)	0.503 ^b
Cigarette/day (smokers), mean (min – max) <i>N</i>	16 (3–30)	17 (3–31)	0.763
Cigarettes smoked before the shift, mean (min – max) <i>N</i>	1 (0–3)	1 (0–5)	0.090
Cigarettes smoked during the shift, mean (min – max) <i>N</i>	5 (0–10)	5 (0–10)	0.952
COT-U (smokers), mean (min – max) µg l ⁻¹	1515 (113–4439)	1417 (103–3405)	0.444
Job environment, <i>N</i>			
Only indoor	12	11	<0.01 ^b
Only outdoor	52	75	
Indoor and outdoor	25	4	
Job location, <i>N</i>			
Urban	34	0	<0.01 ^b
Suburban	28	90	
Extra urban	27	0	
Time to get to the job place, mean (min – max) minutes	11 (1–45)	15 (2–80)	0.008
Means of transport to get to the job place, <i>N</i>	Car/motorbike/ bike/on foot/bus	76/8/5/0/1	0.032
Dispensed petrol, mean (min – max) l	1261 (113–6000)	na	
Number of petrol stations, <i>N</i>	46	na	
Active petrol pumps for station, mean (min – max) <i>N</i>	4 (1–14)	na	

na, not applicable;

^aFrom questionnaire.^bchi-square test.**P* values represent significance of t-test or chi-square test for comparison between study groups.

Subcommittee on Biochemical Verification ([SRNT Subcommittee on Biochemical Verification, 2002](#)).

No differences were found between the two groups for age, BMI, and smoking habit.

Among petrol station workers, 12 subjects worked exclusively indoor as cashiers, 25 exclusively outdoor as pump attendants and 52 had mixed indoor/outdoor tasks. Subjects working outdoor were involved both in filling up the customer's car tanks with petrol and in the supervision and support of customers filling up their cars. Most of controls worked outdoor and all of them worked in suburban locations. Control subjects needed more time to get to the job place. Most of the study subjects used cars to reach the job place. No use of products containing solvents such as varnish and glue was reported by study subjects.

Personal exposure to BTEX and ethers

Personal exposure to airborne BTEX and ethers was much higher in petrol station workers than in controls (Table 2). In petrol station workers, the most abundant compound was TOL-A (median 236, maximum 4136 $\mu\text{g m}^{-3}$), followed by *m+p*-XYL-A (73, 92 650 $\mu\text{g m}^{-3}$), BEN-A (59, 3246 $\mu\text{g m}^{-3}$), *o*-XYL-A (26, 92 650 $\mu\text{g m}^{-3}$), and EtBen-A (23, 34 820 $\mu\text{g m}^{-3}$); as regards ethers, MTBE-A (408, 57 616 $\mu\text{g m}^{-3}$) was the most abundant analyte, followed by ETBE-A (3.1, 835 $\mu\text{g m}^{-3}$).

Tobacco smoking increased personal exposure to BEN-A in controls (5 versus 4 $\mu\text{g m}^{-3}$, $P = 0.032$); and marginally increased exposure to BEN-A and ETBE-A in workers (71 versus 47 $\mu\text{g m}^{-3}$ $P = 0.082$; and 4.0 versus 2.3 $\mu\text{g m}^{-3}$ $P = 0.070$, respectively).

Comparing the different job tasks, petrol pump attendants, working both in highway and in urban road stations, were exposed to the highest BEN-A and MTBE-A levels (BEN-A 6 $\mu\text{g m}^{-3}$, 47 $\mu\text{g m}^{-3}$ and 71 $\mu\text{g m}^{-3}$ and MTBE-A 32.2 $\mu\text{g m}^{-3}$, 298.6 $\mu\text{g m}^{-3}$ and 619.1 $\mu\text{g m}^{-3}$ in cashiers, subjects with mixed tasks and petrol pump attendants, respectively, $P < 0.001$) (see Supplementary Fig. S1). The highest BEN-A exposure (3246 $\mu\text{g m}^{-3}$) was observed for a petrol pump attendant involved in a fuel loading operation.

Biological monitoring

Concentrations of BEN-U were significantly higher in workers than in controls (Table 2), in all sampling periods. The other aromatic compounds were generally higher in BL and ES samples from workers. MTBE-U was higher in BS and ES samples from workers, with

levels that were 5.8- and 9.8-fold higher, respectively, than in controls. ETBE was detectable in a higher percentage of BS and ES samples from workers than from control subjects.

Generally, higher levels of urinary analytes were found in petrol pump attendants than in cashiers or those workers with mixed jobs. However, these differences were significant only for some analytes or at specific sampling time of the week (for example, ES MTBE-U 426 ng l^{-1} , 636 ng l^{-1} and 1308 ng l^{-1} in cashiers, subjects with mixed tasks, and petrol pump attendants, respectively, $P < 0.001$). In particular, BEN-U was not significantly different among the three groups (Supplementary Fig. S1). BEN-U in pump attendants and in workers with mixed jobs was higher than in controls, while no difference was found between cashiers and controls.

BEN-U was always higher in smokers, regardless of whether they were petrol station workers or control subjects. Concentrations of other aromatic compounds were also higher in petrol station workers who smoked, compared to nonsmokers (but only in BL and ES samples, with an approximate 2-fold increase). In controls, however, only TOL-U (BL and BS samples) was found to be higher in smokers. No differences in MTBE-U or ETBE-U concentrations were found (Supplementary Table S1).

BEN-U was always higher in petrol station workers than controls, with the notable exception of ES samples for smokers, where no difference was found (Supplementary Table S1). Considering the job task, BEN-U in nonsmoking subjects was not different between cashiers and controls, while it was higher in mixed jobs and in pump attendants than in controls. Again, no difference was found between cashiers and controls, regardless the smoking habit.

In nonsmoking petrol station workers, BEN-U was higher in ES samples than in BS or BL samples, while in smoking workers ES BEN-U was lower than BS BEN-U. In nonsmoking control subjects, both BL and ES BEN-U were higher than BS BEN-U, while in smoking controls only ES BEN-U was higher than BS BEN-U (Supplementary Fig. S2A). Similar trends were observed for the other urinary aromatic analytes. MTBE-U in workers increased during the work week ($P < 0.001$), in control subjects ES and BL sample levels were higher than BS levels ($P < 0.05$) (Supplementary Fig. S2B).

Table 2. Personal exposure to airborne pollutants and concentrations of urinary biomarkers in the two study groups.

Airborne pollutants	Petrol station workers		Control subjects		Comparison between groups*		
	Median (5° – 95°); %>LOQ		Median (5° – 95°); %>LOQ		$P_{BL \text{ versus } BL}$	$P_{BS \text{ versus } BS}$	$P_{ES \text{ versus } ES}$
BEN-A $\mu\text{g m}^{-3}$	59 (5–284); 100		4 (1–18); 100			$P < 0.001$	
TOL-A $\mu\text{g m}^{-3}$	236 (24–1763); 100		26 (8–249); 100			$P < 0.001$	
EtBen-A $\mu\text{g m}^{-3}$	23 (4–176); 100		4 (2–23); 100			$P < 0.001$	
<i>m</i> + <i>p</i> -XYL-A $\mu\text{g m}^{-3}$	73 (11–608); 100		9 (4–78); 100			$P < 0.001$	
<i>o</i> -XYL-A $\mu\text{g m}^{-3}$	26 (4–235); 100		4 (2–29); 100			$P < 0.001$	
MTBE-A $\mu\text{g m}^{-3}$	408 (12–3088); 100		3.5 (<0.8–60); 96			$P < 0.001$	
ETBE-A $\mu\text{g m}^{-3}$	3.1 (<0.8–31.8); 80		<0.8 (<0.8–1); 7			$P < 0.001$	
Urinary biomarkers							
BEN-U ng l^{-1}	283 (63–4412); 100	337 (77–3891); 100	339 (90–2749); 100	146 (43–2232); 100	86 (50–1253) ^b ; 100	157 (54–2554) ^b ; 100	<0.001
SPMA $\mu\text{g l}^{-1}$	0.18 (<0.1–1.28); 71	0.23 (<0.1–1.90); 70	0.19 (<0.1–1.28); 67	0.11 (<0.1–1.00); 53	<0.10 (<0.1–1.16); 46	<0.1 (<0.1–0.99); 41	0.001 ^c
tt-MA $\mu\text{g l}^{-1}$	53 (<20–353); 80	67 (<20–422); 82	127 (27–522) ^{ab} ; 97	39 (<20–274); 70	72 (41–315) ^a ; 86	117 (<20–509) ^{ab} ; 94	0.394
TOL-U ng l^{-1}	480 (181–743); 100	439 (229–650); 100	597 (486–807) ^{ab} ; 100	251 (172–738); 100	461 (323–581) ^b ; 100	618 (500–890) ^{ab} ; 100	0.082
EtBen-U ng l^{-1}	77 (36–218); 100	112 (45–190) ^a ; 100	80 (27–169) ^b ; 100	72 (33–165); 100	90 (45–181) ^a ; 100	68 (37–155) ^b ; 100	0.559
<i>m</i> + <i>p</i> -XYL-U ng l^{-1}	213 (90–444); 100	196 (72–277) ^a ; 100	133 (83–242) ^{ab} ; 100	110 (63–237); 100	175 (111–246) ^a ; 100	118 (73–216) ^b ; 100	0.010
<i>o</i> -XYL-U ng l^{-1}	83 (32–162); 100	61 (24–115) ^a ; 100	49 (30–83) ^{ab} ; 100	37 (20–82); 100	60 (36–92) ^a ; 100	41 (25–77) ^b ; 100	0.004

Table 2. Continued

Airborne pollutants	Petrol station workers		Control subjects		Comparison between groups*				
	Median (5° – 95°); %>LOQ		Median (5° – 95°); %>LOQ						
MTBE-U ng l ⁻¹	81 (<10–365); 92	246 (25–1048) ^a ; 98	780 (124–5191) ^{ab} ; 99	70 (<10–219); 90	42 (<10–136) ^b ; 76	79 (<10–321) ^b ; 94	0.235	<0.001	<0.001
ETBE-U ng l ⁻¹	<15 (<15–25); 21	<15 (<15–42) ^b ; 48	<15 (<15–56) ^b ; 49	<15 (<15–30); 24	<15 (<15–44); 28	<15 (<15–54); 32	0.243 ^c	0.008 ^c	0.019 ^c

*P*_H = *H*-test; *P*_{ES} = *F*-test or chi-square test for the comparison between petrol station workers and controls in baseline, before-sampling and end-sampling samples, respectively.

$P_{\text{BL versus BL}} / P_{\text{ES versus ES}} = t\text{-test or chi-square test for the comparison between petrol station workers and controls in baseline, before-sampling and end-sampling samples, respectively.}$

^aDifferent than BL sample ($P < 0.05$).

^bDifferent than BS sample ($P < 0.05$).

^cChi-square test.

*Significance of t -test for comparison between study groups.

Considering benzene metabolites, for SPMA a higher number of samples above the LOQ was observed in workers than in controls (Table 2), with the exception of samples from smokers (Supplementary Table S1).

In workers and in control subjects tt-MA increased during the sampling week (Supplementary Fig. S2C), with ES tt-MA always higher than BL and BS tt-MA, while BL and BS tt-MA were different only in non-smokers (Supplementary Table S1). No significant trend was observed for SPMA (Supplementary Fig. S2D).

Higher levels of tt-MA and SPMA were generally found in petrol pump attendants than in station cashiers or those with mixed jobs, but the differences were not significant.

Pearson's correlations

Significant correlations were found among variables describing personal exposure (results not shown). BTEX-A were correlated each other ($0.808 \leq r \leq 0.989$, $P < 0.001$). Airborne ethers were correlated with BTEX-A, with r values ranging from 0.687 (ETBE-A versus TOL-A) to 0.962 (MTBE-A versus BEN-A). In workers, no significant correlation was found between personal exposure and job variables such as the amount of dispensed petrol on the day of sampling, or the number of active fuel pumps at the stations.

Significant correlations were also found among the different urinary biomarkers (results not shown), with better associations among analytes in samples collected at the same time interval during the week, with r values ranging from 0.147 (BS ETBE-U versus BS BEN-U) to 0.827 (ES SPMA versus ES BEN-U) ($P < 0.05$).

Airborne analytes were generally correlated with urinary analytes (Supplementary Table S2). BEN-A was correlated with all benzene biomarkers, with r values of 0.194, 0.376 and 0.437 for ES tt-MA, SPMA, and BEN-U, respectively. BEN-A was highly correlated with MTBE-U ($r = 0.630$ and 0.835 , in BS and ES samples, respectively). Positive correlations were found among all ES urinary analytes, except MTBE-U and *o*-XYL-U, and cotinine ($0.204 \leq r \leq 0.694$). In workers, no significant correlations were found between urinary analytes and job variables, even when examining only the petrol pump attendants.

Generally, correlations were weaker if analysis focused only on smokers or only on nonsmokers. The relationship between BEN-A and benzene biomarkers was the most affected by the smoking factor as r values were much higher when only nonsmokers were considered (Supplementary Table S2).

Multiple linear regression analysis

All multiple linear regression models were significant with R^2 as high as 0.741 (Table 3). For ES BEN-U, the model explained up to 57% of the variability and smoking was the most relevant predictive factor, with standardized beta (β_{std}) of 0.537 which is nearly twice that of the personal airborne exposure. For ES TOL-U, ES EtBEN-U and ES XYL-U the models explained much less of the variability with the highest amount being 16%. For benzene metabolites, the model explained 32 and 60% of the variability for ES tt-MA and ES SPMA, respectively; smoking was the most relevant predictive factor, with β_{std} values of 0.214 and 0.518, respectively, which are about 2-fold higher than that of personal airborne exposure. A significant negative interaction was found between BEN-A and the smoking status for both ES BEN-U and SPMA, showing that the relationship between BEN-A and these biomarkers is different in smokers and nonsmokers: in particular, the slope of the linear regression in smokers is much less steeper than in nonsmokers in the investigated exposure range (Fig. 1).

For urinary ethers, the model explained 74% for ES MTBE-U, with personal airborne exposure as the only relevant predictive factor (β_{std} 0.870). The multiple linear regression analysis predicting ES MTBE-U from BEN-A is also shown: the model explained about 70% of the variability with BEN-A as the only significant predictive factor (β_{std} 0.836).

Biological exposure equivalents

Figure 1 shows the regression lines and the correlation coefficients for BEN-A and ES BEN-U in subjects divided according to the smoking status (Fig. 1a) and for BEN-A and ES MTBE-U or MTBE-A and ES MTBE-U in all subjects (Fig. 1b,c, respectively).

Table 4 shows the tentative biological exposure equivalents for ES BEN-U in nonsmokers corresponding to a selection of occupational limit values. Levels

of 1116 and 1457 ng l⁻¹ were obtained corresponding to ACGIH TLV-TWA and EU occupational exposure limit, respectively. In smokers, the biological exposure equivalent was not calculated due to the lack of a significant correlation between ES BEN-U and BEN-A (Fig. 1a).

Moreover, given the robust linear relationship between ES MTBE-U and BEN-A (Fig. 1b) irrespective of the smoking status, biological exposure equivalents for ES MTBE-U, as surrogate biomarker of benzene exposure, were calculated with levels of about 12 and 22 µg l⁻¹ corresponding to ACGIH and EU benzene limit values, respectively.

Additionally, a biological exposure equivalent of 22 µg l⁻¹ for ES MTBE-U in correspondence of the MTBE-A TLV-TWA of 50 p.p.m. was calculated (Fig. 1c).

DISCUSSION

In this study, BTEX and ethers exposure has been investigated in petrol station attendants to evaluate urinary biomarkers and suggest biological exposure equivalents.

In workers, a personal median exposure to BEN-A of 59 µg m⁻³ was estimated. This value is far below the European occupational limit value of 3200 µg m⁻³. However, for one individual who was involved in a fuel loading operation on the day that sampling occurred, a BEN-A exposure exceeding the benzene limit value was measured (3246 µg m⁻³). When comparing BEN-A levels to the Dutch health-based occupational exposure limit and the German acceptable values, two subjects exceeded 700 µg m⁻³, nine subjects were exposed to levels >200 µg m⁻³, and the majority of subjects (75%) were exposed to levels >20 µg m⁻³, with those working as pump attendants exposed to the highest levels. These results show that occupational exposure to levels of benzene higher than existing occupational exposure limits may occur, notwithstanding preventive actions undertaken to limit the exposure such as the lowering of the maximum permissible percentage of benzene in gasoline and the aspiration system applied to the nozzle of the gasoline pump for vapor recovery.

BEN-A levels were similar to those measured in petrol station attendants from Milan in the year 2000 (Fustinoni *et al.*, 2005), but were more than twice than those reported in other Italian cities in the same year (Ghittori *et al.*, 2005; Carrieri *et al.*, 2006; Lovreglio

Table 3. Results of multiple linear regression analysis, with urinary ES biomarkers as the dependant variables. Results are expressed as adjusted determination coefficient (R^2 adj) and significance; and for each predictive variable as β , standardized β , partial correlation coefficient, and significance

Analyte	Total model R ² adj	$\beta \pm SE (\beta_{stand}), r_{part}$				
		Personal airborne exposure ($\mu\text{g m}^{-3}$)	Creatinine (g l^{-1})	Cotinine ($\mu\text{g l}^{-1}$)	Interaction	Constant
ES BEN-U	0.572***	0.344 $\pm$ 0.048*** (0.451); 0.437	0.532 $\pm$ 0.119*** (0.227); 0.192	0.537 $\pm$ 0.055*** (0.832); 0.627	-0.221 $\pm$ 0.065** (-0.333); 0.529	0.767 $\pm$ 0.265**
ES SPMA	0.605***	0.248 $\pm$ 0.044*** (0.347); 0.376	0.514 $\pm$ 0.107*** (0.234); 0.184	0.518 $\pm$ 0.050*** (0.856); 0.689	-0.163 $\pm$ 0.059** (-0.262); 0.566	-2.431 $\pm$ 0.239***
ES tt-MA	0.315***	0.084 $\pm$ 0.050* (0.134); 0.194	0.894 $\pm$ 0.124*** (0.464); 0.439	0.214 $\pm$ 0.058*** (0.403); 0.329	-0.040 $\pm$ 0.068,ns (-0.073); 0.288	1.237 $\pm$ 0.275***
ES TOL-U	0.164***	0.037 $\pm$ 0.009*** (0.334); 0.328	0.065 $\pm$ 0.024** (0.193); 0.189	0.032 $\pm$ 0.014** (0.340); 0.244	-0.010 $\pm$ 0.011, ns (-0.142); 0.263	2.630 $\pm$ 0.055***
ES EtBEN-U	0.098***	0.032 $\pm$ 0.034, ns (0.090); 0.133	0.281 $\pm$ 0.071*** (0.294); 0.293	0.044 $\pm$ 0.03, ns (0.168); 0.159	-0.003 $\pm$ 0.044, ns (-0.010); 0.172	1.781 $\pm$ 0.157***
ES m+p-XYL-U	0.107***	0.032 $\pm$ 0.019, ns (0.147); 0.106	0.029 $\pm$ 0.046, ns (0.046); 0.025	0.059 $\pm$ 0.023*** (0.341); 0.319	-0.004 $\pm$ 0.024, ns (-0.023); 0.295	1.804 $\pm$ 0.105***
ES o-XYL-U	0.033*	0.036 $\pm$ 0.026, ns (0.140); 0.210	0.003 $\pm$ 0.057, ns (0.004); 0.007	0.005 $\pm$ 0.025, ns (0.026); 0.123	0.031 $\pm$ 0.033, ns (0.132); 0.210	1.490 $\pm$ 0.126***
ES MTBE-U ^a	0.741**	0.533 $\pm$ 0.031*** (0.870); 0.865	0.057 $\pm$ 0.122, ns (0.018); 0.061	0.006 $\pm$ 0.051, ns (0.006); 0.085	-0.010 $\pm$ 0.042, ns (-0.017); 0.455	1.709 $\pm$ 0.268***
ES MTBE-U ^b	0.693***	0.842 $\pm$ 0.054*** (0.836); 0.835	0.008 $\pm$ 0.133, ns (0.003); 0.061	-0.068 $\pm$ 0.062, ns (-0.080); 0.085	0.015 $\pm$ 0.073, ns (0.018); 0.394	1.851 $\pm$ 0.296***

ns = not significant.

^aES MTBE-U predicted by MTBE-A.

^bES MTBE-U predicted by BEN-A.

*0.05 < P $\leq$ 0.1, **0.001 < P $\leq$ 0.05, *** P $\leq$ 0.001.

Figure 1 Scatter plots and regression lines according to the simple linear regression models: (a) ES BEN-U versus BEN-A (subjects stratified by the smoking status); (b) ES MTBE-U versus BEN-A (all subjects); (c) MTBE-U versus MTBE-A (all subjects).

et al., 2010; De Palma *et al.*, 2012). Higher values are likely because of the higher volume of fuel dispensed in the area of Milan (>850 000 tons of petrol in 2007), relative to the smaller Italian cities investigated (<200 000 tons in 2007) (Ministero Sviluppo Economico, 2007). Relatively few published studies report exposure to MTBE by filling station workers (Jo and Oh, 2001; Ghittori *et al.*, 2005). Most MTBE-exposure studies focus on truck drivers, pump maintenance workers, and workers involved in fuel loading (Saarinen *et al.*, 1998; Vainiotalo *et al.*, 2006); exposure to ETBE has seldom been investigated (Eitaki *et al.*, 2011). The MTBE-A levels measured in our study were half or one-third lower than those measured in the above cited occupational settings, but an order of magnitude higher than those reported in a previous Italian study, which is probably related to higher amounts of dispensed fuel (Ghittori *et al.*, 2005).

Achieving successful biological monitoring can be problematic due to the lack of validated biomarkers and/or biological limit values. Among the several analytes investigated, comparison to existing BEI is possible only for TOL-U, SPMA and tt-MA, whose median levels in ES samples from workers were ~50-fold, 100-fold, and 5-fold lower than the respective ACGIH BEI (ACGIH, 2012).

The influence of tobacco smoking was observed for all biomarkers, with the exclusion of U-MTBE, as previously reported (Fustinoni *et al.*, 2010a; Campo *et al.*, 2011), and of ETBE-U. In particular, smokers had BEN-U values that were 6.4- and 3.4-fold that of control and worker nonsmokers, respectively. Among the different benzene biomarkers, BEN-U was 2- to 3-fold higher, and SPMA was more frequently detected, in nonsmoking workers than in controls. These data show that the smoking contribution is actually higher than, or at least comparable to, occupational exposure to petrol in determining the internal dose of benzene in petrol station workers who smoke.

The effect of occupational exposure, combined with the smoking habit, personal characteristics, and different metabolism kinetics, leads to peculiar excretion patterns for the different analytes (Table 2, Supplementary Table S1, and Fig. S2). In nonsmoking workers, for example, ES BEN-U (Supplementary Fig. S2A) was higher than BL or BS BEN-U, which mirrored occupational exposure to BEN-A. However, the same trend was not true in

Table 4. Calculated biological exposure equivalents for ES BEN-U and ES MTBE-U corresponding to existing occupational limit values

Limit value	Airborne benzene	Biological exposure equivalents	
		ES BEN-U (ng l ⁻¹) (nonsmokers)	ES MTBE-U (µg l ⁻¹) (all subjects)
German Acceptable risk (by 2018)	20 µg m ⁻³	206	0.3
German Acceptable risk	200 µg m ⁻³	501	2
Dutch occupational exposure limit	700 µg m ⁻³	812	6
ACGIH TLV-TWA	1600 µg m ⁻³ (0.5 p.p.m.)	1116	12
German tolerable risk	1900 µg m ⁻³	1192	14
EU occupational exposure limit	3200 µg m ⁻³ (1 p.p.m.)	1457	22
Airborne MTBE		—	ES MTBE-U (µg l ⁻¹)
ACGIH TLV-TWA	180 mg m ⁻³ (50 p.p.m.)	—	22

smoking workers, for which ES BEN-U was lower than BS BEN-U; in contrast, ES BEN-U in control subjects was always higher than BS BEN-U, both in smokers and nonsmokers, probably mirroring environmental exposure occurring during the monitored period. A similar result was found in our previous study (Fustinoni *et al.*, 2005). The hypothesis that this behavior may be due to the different number of cigarettes smoked during the study period was not supported by the data collected by the questionnaires (Table 1). Another explanation may be linked to the nocturnal slowdown of the biochemical activity that leads to an increase of blood benzene mobilized from storage tissues that is reflected in early morning urine samples.

As regards tt-MA and SPMA, only an increase along the work week was observed for tt-MA (Supplementary Table S1, Figs S2C, and S2D). Among the biomarkers of the other aromatic hydrocarbons, no clear increase following occupational exposure and/or over the working week was observed, indicating these compounds were poor markers for assessing exposure to petrol vapors. Only for TOL-U an increase during the shift was observed (Supplementary Table S1). An investigation using this panel of aromatic compounds in petrol station workers has been reported in only one previous study, where similar conclusions were reached (De Palma *et al.*, 2012).

MTBE-U excretion reflected occupational exposure in workers, with a clear increase during both the daily work shift and over the work week, independent of the smoking factor (Supplementary Fig. S2B). The MTBE-U concentration rise from BL to BS samples is indicative of MTBE accumulation and suggests relatively slow excretion kinetics as the compound is mobilized from the adipose tissue. Indeed a volume distribution of about 300 l and an oil:blood partition coefficient of 14 have been estimated in healthy volunteers exposed to 5–75 MTBE p.p.m. in an exposure chamber (Nihlén *et al.*, 1998; Vainiotalo *et al.*, 2007). Assuming that the measured BS and ES concentrations are indicative of an average work shift, it can be concluded that the BS level (246 ng l⁻¹) accounts for about 30% of the previous ES level (780 ng l⁻¹). The significant correlation ($r = 0.668$, $P < 0.001$) found between BS MTBE-U and MTBE-A suggests a causal relationship between residual urinary MTBE and airborne exposure. These results agree with previously reported observations on gasoline pump maintenance workers and road-tanker drivers where the concentrations of the prior to the next shift samples were 4 to 32% of end-of-shift concentrations (Saarinen, 2002; Vainiotalo *et al.*, 2006). Considering (i) a median MTBE-A exposure of 408 µg m⁻³, (ii) 0.6 m³ h⁻¹ ventilation rate for a 5 h shift, (iii) 40% respiratory uptake (Nihlén *et al.*, 1998), and (iv) 0.3 l of urine excretion

in 5 h, we estimated that 0.05% of the inhaled dose was excreted as MTBE-U in the ES samples. Although this is an approximate calculation based on a single measurement in ES urine, it aligns with reported data showing 0.1–0.2% of MTBE in urinary cumulative excretion following experimental exposure of volunteers (Nihlén *et al.*, 1998; Vainiotalo *et al.*, 2006).

BEN-U levels reported here corroborate our previous study on petrol station attendants in the city of Milan (Fustinoni *et al.*, 2005) although we measured much lower median SPMA levels in the current investigation than in the previous study (0.19 versus 5.8 $\mu\text{g l}^{-1}$). The difference is due to the use, in the present study, of a specific and sensitive method based on LC-MS-MS, while the previous study utilized an immunoassay kit. Other studies provide supporting evidence for this conclusion (Lovreglio *et al.* 2010; De Palma *et al.*, 2012), and also support the need for ongoing development of new methods for this analysis (Fustinoni *et al.*, 2010b, 2011).

The MTBE-U levels were similar to those reported in the few studies focusing on the same occupational scenario (Ghittori *et al.*, 2005; De Palma *et al.*, 2012) and much lower than those reported in subjects employed in situations with higher exposure such as fuel loading (Saarinen *et al.*, 1998; Vainiotalo *et al.*, 2006). To the best of our knowledge, this is the first report on biological monitoring of occupational exposure to ETBE. We found urinary levels of ETBE to be quite low with 61% of samples below the LOQ. These data suggest that ETBE is not commonly blended into Italian petrol. Nevertheless ETBE-U was more frequently detected in workers than in controls, which suggests at least minimal occupational exposure.

Pearson's correlation and the multiple linear regression (Supplementary Table S2 and Table 3) showed that all urinary biomarkers were significantly correlated to the respective airborne pollutant, with the best correlations found for BEN-U and MTBE-U. Nevertheless, the variability explained for ES BEN-U and ES SPMA was 57 and 60%, respectively, and it was as low as 32% for ES-tt-MA. This indicates that some determinants relevant to explain the biomarker variability were not identified. Among them, a role may be played by genetic polymorphism of metabolite enzymes (especially for SPMA), diet (especially for tt-MA) (Ruppert *et al.*, 1997; Carbonari *et al.*, 2014), and dermal exposure.

Given the good correlations observed and the wide range of personal exposure levels (for BEN-A up to the highest occupational limit value) we derived tentative biological limit equivalents for the biomonitoring of occupational exposure to benzene and MTBE (Table 4). For benzene this is particularly relevant given the recent proposals for occupational limit values lower than 1 p.p.m. adopted in EU (BauA, 2012; Health Council of the Netherlands, 2014). This urges the adoption of sensitive and specific biomarkers such as BEN-U that is the best candidate among those available, and the identification of biological limit values for the interpretation of biomonitoring results in occupational hygiene. Given the relevant contribution of tobacco smoking in determining the body burden of benzene in smokers, at occupational exposures below 1 p.p.m., our proposal differentiates smokers and nonsmokers. In particular, for nonsmokers, a biological exposure equivalent of 1457 ng l^{-1} in urine samples collected at the end of the shift corresponding to the EU occupational limit value of 1 p.p.m. is proposed. For smokers, ES BEN-U is not a reliable biomarker of BEN-A exposure below 1 p.p.m. and so we propose the use of MTBE-U as a surrogate biomarker of benzene exposure in petrol station attendants to tackle the issue of tobacco smoking, as no influence of such habit on MTBE-U has been evidenced. In this case, a value of 22 $\mu\text{g l}^{-1}$ in urine samples collected at the end of the shift in the second part of the work week corresponding to an exposure to benzene of 1 p.p.m. is suggested. However it should be taken in account that, while the benzene content in petrol is rather constant, the content of MTBE is variable depending on the season, the country, and the blending mixture (Hill *et al.*, 2006). Finally, the excellent correlation between MTBE-U and MTBE-A allows us to propose the use of MTBE-U as biomarker of exposure to MTBE and to identify a biological exposure equivalent of 22 $\mu\text{g l}^{-1}$ in urine samples collected at the end of the shift in the second part of the work week corresponding to an exposure to MTBE of 50 p.p.m. (ACGIH TLV-TWA).

In conclusion, among the multiple biomarkers of occupational exposure to benzene and petrol vapors, BEN-U was found to be superior to tt-MA and SPMA for the better relationship with personal exposure and at differentiating exposed from unexposed subject. Our data show that MTBE-U is a promising biomarker of MTBE exposure and in general of the

studied components of petrol vapors. With the purpose to apply BEN-U and MTBE-U as biomarkers, biological exposure equivalents have been calculated for the existing occupational limit values.

SUPPLEMENTARY DATA

Supplementary data can be found at <http://annhyg.oxfordjournals.org/>.

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