



Original Article

Urinary *Trans*, *Trans*-Muconic Acid is Not a Reliable Biomarker for Low-level Environmental and Occupational Benzene Exposures

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ABSTRACT

Background: Benzene is a known occupational and environmental pollutant. Its urinary metabolite *trans*, *trans*-muconic acid (*tt*-MA) has been introduced by some environmental and occupational health regulatory associations as a biological index for the assessment of benzene exposure; however, recently, doubts have been raised about the specificity of *tt*-MA for low-level benzene exposures. In the present study, we investigated the association between urinary levels of *tt*-MA and inhalational exposure to benzene in different exposure groups.

Methods: Benzene exposure was assessed by personal air sampling. Collected benzene on charcoal tube was extracted by carbon disulfide and determined by a gas chromatograph (gas chromatography with a flame ionization detector). Urinary *tt*-MA was extracted by a strong anion-exchange column and determined with high-performance liquid chromatography–UV.

Results: Urinary levels of *tt*-MA in intensive benzene exposure groups (chemical workers and police officers) were significantly higher than other groups (urban and rural residents), but its levels in the last two groups with significant different exposure levels (mean = 0.081 ppm and 0.019 ppm, respectively) showed no significant difference (mean = 388 µg/g creatinine and 282 µg/g, respectively; $p < 0.05$). Before work shift, urine samples of workers and police officers showed a high amount of *tt*-MA and its levels in rural residents' samples were not zero.

Conclusion: Our results suggest that *tt*-MA may not be a reliable biomarker for monitoring low-level (below 0.5 ppm) benzene exposures.

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1. Introduction

Benzene is an important industrial chemical used worldwide for the production of other chemicals and solvents such as styrene, cumene, and cyclohexane. It is also used in the manufacture of some types of plastics, rubbers, lubricants, dyes, detergents, drugs, and pesticides.

Although the International Program on Chemical Safety in 1993 reported that about 60% of workers are exposed to benzene at levels lower than 0.1 ppm and only 1% of them are exposed to levels higher than 10 ppm [1], workers in different industries and workplaces are among the higher-risk groups exposed to benzene.

Benzene is emitted into the environment from industrial processes, gasoline, engine exhausts, and combustion of organic materials, causing significant exposure of the general population. It is

reported that about 1% of benzene is found in gasoline in most countries [2,3]. Many foodstuff and consumer products, along with tobacco smoke (about 87 µg/cigarette), contain considerable levels of benzene. The average background level of benzene intake for nonsmokers has been estimated to be about 0.5 mg/d [4]. Significant environmental distribution of benzene and its dangerous health consequences have raised deep concerns. It has been reported that benzene's concentrations in the environmental air range from 0.5 µg/m³ in uninhabited regions to 200–300 µg/m³ in urban environments of large cities and capitals [5,6].

Benzene can either be absorbed through the respiratory system or have considerable dermal absorption, due to its lipophilic structure. Therefore, the individual uptake of benzene among different people can be highly variable, depending on the rate of pulmonary ventilation and some other factors [7,8]. As a result,

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ambient air monitoring by itself could not reliably assess benzene exposure. Alternative ways for comprehensive exposure assessment are biological monitoring methods that determine exact entrance of pollutants into the human body. In the case of benzene, the question is whether or not some effective biological indicators exist and which one is suitable for biological monitoring of low benzene exposure.

In addition, measurement of the unmetabolized benzene excreted in urine (which represents about 1% of the absorbed quantity) has been proposed as a useful biomarker for benzene exposure [9], but a recent study has claimed that, at very low occupational or environmental exposure to benzene, urinary benzene is less valid than urinary metabolites of benzene, even if both are strongly influenced by smoking habit [10].

Traditionally, the most common method used for biological monitoring of benzene exposure is based on the measurement of different benzene metabolites in the blood and urine as well as adducts of reactive benzene metabolites with cellular component such as DNA [11,12].

Urine metabolites that might serve as indices of occupational or environmental exposures to benzene include phenol, hydroquinone, *trans*, *trans*-Muconic acid (*tt*-MA), and *S*-phenylmercapturic acid [13]. Among them, urinary *tt*-MA is increasingly recognized as a reliable and relatively convenient biomarker [14–16]. The compound *tt*-MA is an open-ring (nonphenolic) metabolite of benzene [17]. About 2% of the total benzene uptake in humans is excreted via urine as *tt*-MA, and its excretion half-life is reported to be < 6 hours [18]. Despite the low metabolic conversion of benzene to *tt*-MA, this metabolite appears to be more suitable than phenolic metabolites, and the American Conference of Governmental Industrial Hygienists (ACGIH) has recommended it as an appropriate biomarker for the assessment of occupational exposure to benzene (above 0.25 ppm), with limit values (biological exposure indices) of 500 µg/g creatinine [19].

Several competing metabolic pathways have been proposed for benzene metabolism, and recent studies indicated that some of these pathways were saturated at specific exposure levels and some other activated in certain conditions [11]. By contrast, the specificity of *tt*-MA as a biomarker for low-level benzene exposure may be limited because it is also reported to be a metabolite of sorbic acid, a widely used food preservative [20,21]. Owing to these findings, recently some doubts have been raised about the specificity of *tt*-MA as a reliable biomarker for low-level benzene exposure [22]. Therefore, in the present study, urinary concentrations of *tt*-MA in different groups with various benzene exposure levels have been measured and compared, to investigate the reliability of this index for biological monitoring of low-level benzene exposures.

2. Materials and methods

2.1. Participants and study protocol

For comprehensive evaluation of selectivity of *tt*-MA as a biomarker of benzene exposure, four study groups were selected: (1) 61 workers of a chemical company (producer of linear alkyl benzene); (2) 52 police officers of a large city (Ahvaz, Iran); (3) 73 urban residents of a large city (Ahvaz, Iran); and (4) 75 rural residents. All participants were 22–45-year-old males. In the case of the chemical company, all the workers participated in the study, but for other study groups, the participants were selected randomly. For all participants, inhalational exposure to benzene and urinary levels of *tt*-MA (in morning and evening urine samples) were determined on the same day. Necessary information (demographic characteristics, smoking, medical and occupational history, special diet, home location, work shift, etc.) was collected

by a questionnaire before sampling, and reviewed by the investigator for identifying and avoiding confounding factors. Ahvaz University of Medical Sciences Ethics Review Committee, Ahvaz, Iran has approved the study proposal (ID: 2301), and all participants provided written informed consent before participation.

2.2. Benzene exposure monitoring

Inhalational benzene exposure for all study participants was monitored by active personal sampling. Benzene was collected by a sampling train consisting of a pump (SKC Inc., Eighty Four, PA., USA) and a charcoal tube [SKC number 226-01, 100 mg; SKC; National Institute of Occupational Safety and Health (NIOSH) approved]. Flow rates of the pump were calibrated prior to each run using a wet test meter. The charcoal tube was clipped on the collar and the pump was installed on the belt of all participants. At the end of the sampling time, the charcoal tubes were sealed and sent to the laboratory for analyses. The samples were kept at 4°C prior to desorption with carbon disulfide (benzene free) and analysis. The analysis method was according to the NIOSH method (method numbers: 1500 and 1501), which is based on gas chromatography with a flame ionization detector. To eliminate possible intensive dermal exposures of chemical company workers, it was necessary for them to use suitable personal protective equipment.

For urban residents, police officers, and rural residents (with regard to their low-level exposures), sampling was continued for 8 h/d (from 9 AM to 5 PM), but for chemical workers with higher benzene exposure levels, multiple sampling during work shift (30-minute–4-hour samples based on environmental benzene concentrations and job condition) were used, and by this means, time-weighted average of benzene exposure for all study groups was calculated.

2.3. Measurement of urinary levels of *tt*-MA

Each participant provided two (morning and evening) 5 mL urine samples. The first sampling was conducted in the early morning (the 1st urination of day), and the second samples were collected at the end of the 8-hour work shift (for police officers and chemical workers) or at the end of 8-hour inhalation exposure sampling (for urban and rural residents). These samples were collected in polypropylene tubes, preserved with 100 µL of 6 mol/L hydrochloric acid, and were immediately transported to the laboratory to be stored at –20°C until the day of analysis. The levels of *tt*-MA were measured by high-performance liquid chromatography according to the method described by Lee et al [23] with some modifications. In this method, vanilic acid was used as the internal standard. Samples from all study groups were analyzed in the same series, without the knowledge of exposure status. Urinary concentrations of *tt*-MA were corrected for creatinine content. The concentration of *tt*-MA in the samples was determined using a calibration curve, which was prepared by spiking of different concentrations of *tt*-MA (10,100,1000 and 10,000 µg/L) in a blank urine sample.

Analysis was carried out by high-performance liquid chromatography (JASCO Corporation, Tokyo, Japan) with a reversed-phase C18 column [250 × 5 mm² (i.d.) with 5-µm particle size; Merck, Darmstadt, Germany]. The flow rate was equal to 1.2 mL/min. The mobile phase consisted of (per liter) 10 mL of acetic acid, 100 mL of methanol, and 890 mL of 5 mmol/L sodium acetate (pH, 3.5). The detector (UV/visible) was set at a wavelength of 259 nm.

Creatinine levels of the urine samples were determined by Golestan Hospital Medical Diagnostic Laboratory (Ahvaz, Iran) by its commercial kit (Pars Azmon, Tehran, Iran) and spectrophotometer at a wavelength of 500 nm according to the manufacturer's instruction.

Table 1
Characteristics of participants and study groups

Study group	Participants	Final number of samples*	Smoker	Consumption of preservative-containing food [†]
Chemical company workers	61	57	31	8
Police officers	52	51	12	5
Urban residents	73	69	22	12
Rural residents	75	71	21	1

* Both morning and evening samples were provided correctly.

[†] Eating a meal containing sorbic acid-preserved foods within 24 hours prior to sampling.

2.4. Statistical analysis

The relationship between benzene exposure and urinary levels of *tt*-MA was assessed by making comparisons among the different study groups and subgroups, using correlation/regression techniques. The *t* test and analysis of variance with a confidence level of ≤ 0.05 were applied to determine whether there was a significant difference between one or more confounding factors and different variables. The criterion for significance was set at $p < 0.05$. All statistical analyses were carried out by the SPSS software (version 18.0 for Windows; SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Participants and study groups

As mentioned above, during the study some samples were lost. Final numbers of samples, along with other characteristics of the participants and study groups, are presented in Table 1.

3.2. Inhalational benzene exposure

Method limits of detection were 0.01 ppm. The within-day coefficient of variation was 4–9% and between-day coefficient of variation 8–13%. Time-weighted average levels of benzene exposure [mean \pm standard deviation (SD)] for different study groups are presented in Fig. 1. Exposure levels for study groups were quite different. While benzene exposure among chemical workers rises to 3.5 ppm (mean \pm SD = 1.56 \pm 0.34 ppm), its mean \pm SD level for rural residents was 0.019 \pm 0.016 ppm.

3.3. Urinary levels of *tt*-MA

The *tt*-MA and internal standard were detected at 5.2 minutes and 10.2 minutes, respectively. Linearity between 10 μ g/L and 10,000 μ g/L was very good ($r^2 = 0.982$); limits of detection and quantification were 0.53 μ g/L and 2.11 μ g/L, respectively. The mean coefficients of variation for replicate analysis with spiked samples were 7.4% (repeatability) and 11.2% (between-day precision); the mean recovery of *tt*-MA in this method was 86.1%.

Evening urine samples of chemical company workers showed the highest concentration of *tt*-MA, which rose to 4,500 μ g/g creatinine (for some workers who were exposed to benzene while washing tanks). In both morning and evening samples, the mean urinary levels of *tt*-MA in chemical company workers' urine samples were significantly higher than those of the other study groups. However, these levels in other study groups showed no significant differences. The mean urinary concentrations of *tt*-MA for smokers

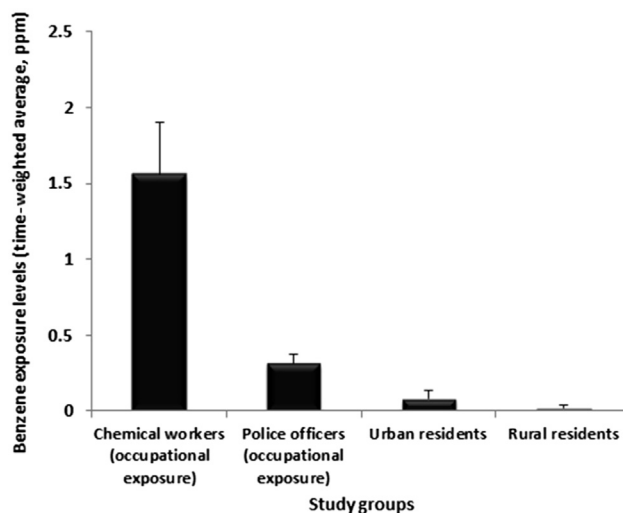


Fig. 1. Comparing the time-weighted average of benzene exposure levels (ppm) among four different study groups. Data are presented as mean \pm SD.

were significantly higher than those for nonsmokers in all study groups (Fig. 2 and Table 2).

Urinary levels of *tt*-MA in morning and evening samples showed a significant difference only for chemical company workers and police officers, and evening levels were significantly higher than morning levels ($p < 0.05$; Fig. 3). Urinary levels of *tt*-MA in rural resident urine samples were not zero (even for nonsmokers). The urinary *tt*-MA levels of participants consuming preservative-containing foods (such as sausage and canned foods) were separately analyzed. Except the chemical company workers, the mean urinary levels of *tt*-MA for preservative-containing food-consuming participants in all study groups were significantly higher than those for other participants in their respective study groups ($p < 0.05$; Fig. 4).

4. Discussion and conclusion

Benzene, as one of the most widely used industrial solvents and a ubiquitous environmental pollutant, has very serious effects on human health. Some of its destructive effects such as chromosomal damage, depression of the immune system [24–26], progressive degeneration of the bone marrow, and induction of aplastic anemia and leukemia [16] resulted in its classification as a human carcinogen by the International Agency for Research on Cancer [27] and, consequently, in a reduction of its acceptable occupational exposure levels. Its occupational threshold limit value has been lowered to 0.5–1 ppm by many occupational health organizations such as NIOSH and Occupational Safety and Health Administration.

Hence, monitoring of its exposure in various populations seems to be of utmost importance. Although it is more than two decades that ACGIH and some other occupational and environmental organizations introduced *tt*-MA as a selective and sensitive biomarker for monitoring of benzene exposure, recently some evidence has been presented about the insufficiency of this index for monitoring low-level benzene exposures. For example, a study by Weaver and colleagues [21] showed that *tt*-MA is a specific biomarker for benzene only for exposure levels above 0.5 ppm. However, a study on major industries with benzene exposure revealed that 60% of workers currently have exposure levels below 0.1 ppm [28].

The results of this study showed that the time-weighted average of benzene exposure among chemical company workers is about three times higher than the levels recommended by ACGIH and also

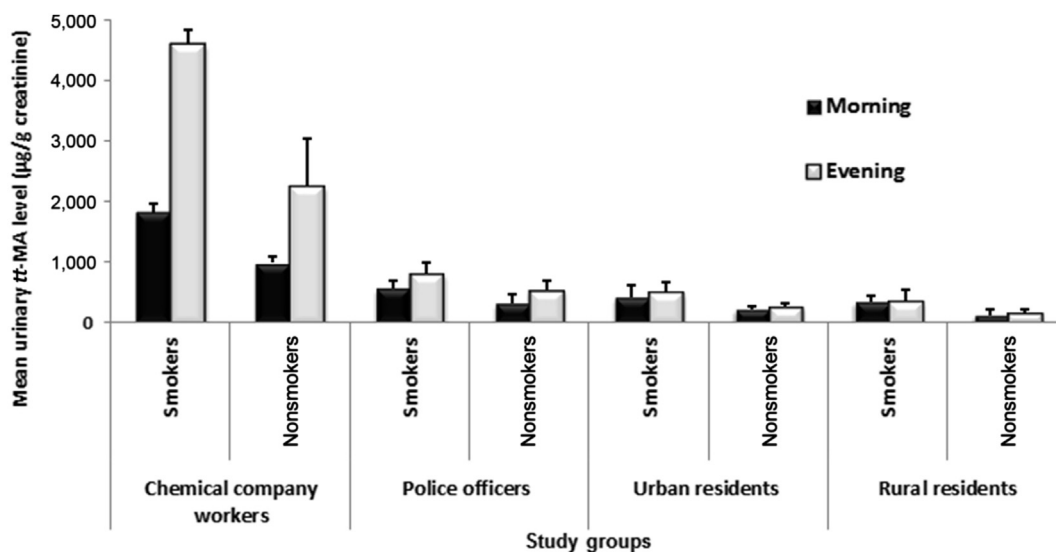


Fig. 2. Comparing mean urinary levels of *tt*-MA (µg/g creatinine) in different study groups based on their groups and smoking status. Data are presented as mean ± SD. *tt*-MA *trans*, *trans*-muconic acid.

Table 2

Levels of *tt*-MA in analyzed urine samples

Study group	Smoking status	Urine sample	<i>tt</i> -MA (µg/g creatinine)
Chemical company workers	Smokers	Morning	1,824 ± 147
		Evening	4,616 ± 225
	Nonsmokers	Morning	987 ± 111
		Evening	2,271 ± 780
Police officers	Smokers	Morning	571 ± 120
		Evening	820 ± 187
	Nonsmokers	Morning	321 ± 145
		Evening	544 ± 160
Urban residents	Smokers	Morning	425 ± 198
		Evening	501 ± 161
	Nonsmokers	Morning	224 ± 55
		Evening	275 ± 57
Rural residents	Smokers	Morning	352 ± 102
		Evening	351 ± 201
	Nonsmokers	Morning	125 ± 87
		Evening	160 ± 52

Data are presented as mean ± SD from three independent experiments. *tt*-MA *trans*, *trans*-muconic acid.

the standard of Iran (0.5 ppm). Despite the fact that environmental exposure levels of other study groups (police officers, and urban and rural residents) were not very high (below 0.5 ppm and for some cases close to zero), *tt*-MA was found in all urine samples, confirming that benzene can be a ubiquitous environmental pollutant and there should be other sources for *tt*-MA in the urine. As seen in Fig. 2, the SD of benzene exposure levels in all study groups, especially for chemical company workers, is relatively high, which might be a consequence of the differences in the nature of the tasks, locations, and sampling conditions.

Following monitoring of benzene exposure in a study, it was estimated that an 8-hour exposure to 1 ppm benzene would result in *tt*-MA levels ranging from 500 µg/g creatinine to 1,500 µg/g creatinine, while another study evaluated these values to be about 1,900 µg/g creatinine [16]. In the present study, the mean urinary levels of *tt*-MA measured in the evening samples of chemical workers (3,443 µg/g creatinine) was statistically different from those of other three study groups (police officers, and urban and rural residents), but the mean *tt*-MA levels of these three groups showed no significant between-group differences ($p < 0.05$).

Urinary *tt*-MA in chemical company workers with exposure levels above 1 ppm showed a good linear regression with exposure levels ($r^2 = 0.904$, data not shown), but in the other study groups (with exposure levels below 0.5 ppm) this correlation was not seen. Considering the results of exposure levels and urinary *tt*-MA concentration in the four study groups, it can be concluded that for exposure levels below 0.5 ppm, inhalational exposure levels and urinary *tt*-MA concentration do not have a linear correlation, and *tt*-MA cannot be used as a sensitive and reliable biomarker for low-level benzene exposure.

No significant difference in the urinary *tt*-MA level of morning and evening samples of urban and rural residents ($p < 0.05$) and the presence of a considerable amount of *tt*-MA in the morning samples of workers and police officers (2,352 µg/g creatinine and 419 µg/g creatinine, respectively) indicated that, following exposure to benzene and its distribution in the body, a large amount of benzene may accumulate in some body organs and tissues (such as fat tissues), and over time, it may redistribute, metabolize, and excrete from the body in the form of *tt*-MA. Dermal absorption (particularly for chemical workers) can be another important source of delayed appearance of *tt*-MA in urine.

It has been known that *tt*-MA is also a metabolite of sorbic acid. Weaver and colleagues [21] reported that sorbic acid-preserved foods have the potential to cause substantial interference with *tt*-MA as a biomarker for both occupational and environmental benzene exposure. The average peak of urinary *tt*-MA levels after ingestion of some sorbic acid-preserved foods (such as Hostess cupcakes and Sunny Delight) was 452 µg/g creatinine and 675.3 µg/g creatinine, respectively, which are greater than the predicted values for 0.1–0.5 ppm benzene exposure and in some cases even above the predicted values for 1 ppm benzene exposure [29]. In our study, according to the information extracted from questioners, some participants consume sorbic acid-preserved foods (such as canned foods and sausage). As mentioned above, the results of *tt*-MA measurements in urine samples of these participants were analyzed separately. The results showed that except the chemical company workers, the mean levels of urinary *tt*-MA in all groups were about two-fold higher than mean levels of their respective groups. Based on the results of previous studies, increased urinary *tt*-MA levels in these participants were predictable, but what we should take into

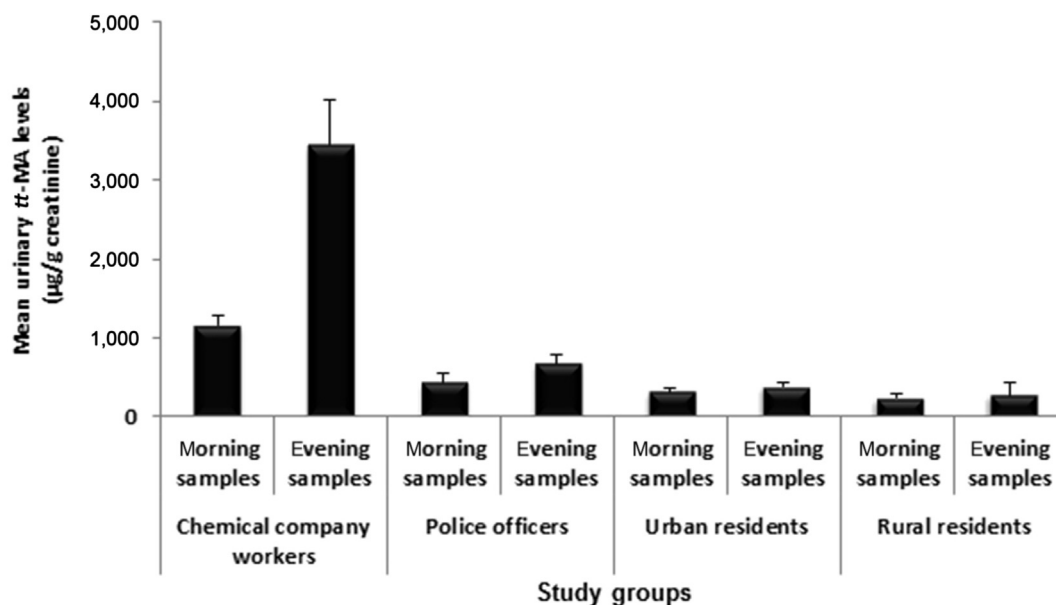


Fig. 3. Comparing mean urinary levels of *tt*-MA ($\mu\text{g/g}$ creatinine) in morning and evening samples of all study groups. Data are presented as mean \pm SD. *tt*-MA *trans*, *trans*-muconic acid.

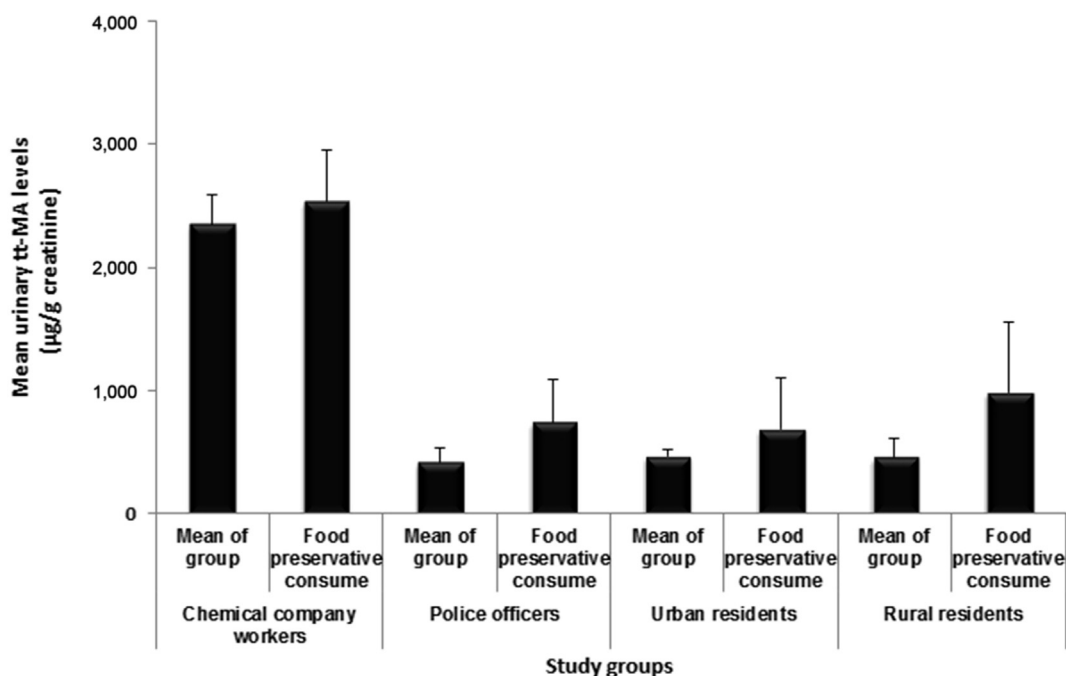


Fig. 4. Comparing mean urinary levels of *tt*-MA ($\mu\text{g/g}$ creatinine) in different study groups, based on preservative-containing food-consuming status. Data are presented as mean \pm SD. *tt*-MA *trans*, *trans*-muconic acid.

consideration is that nowadays preservative-containing foods are an integral component of most community's nutrition and diet, and will have a significant effect on urinary *tt*-MA; hence, *tt*-MA cannot be a selective and sensitive biomarker for benzene exposure. The other important point is that, in chemical workers' groups, the mean urinary *tt*-MA levels of preservative-containing food-consuming participants showed no significant difference with those of non-preservative-containing food-consuming individuals, which might be due to saturation of metabolic pathways.

Benzene is a component of environmental tobacco smoke, and the results of a study by Melikian and colleagues [30] showed that the levels of urinary *tt*-MA range from 20 $\mu\text{g/g}$ creatinine to 1,300 $\mu\text{g/g}$ creatinine, resulting in a mean of 290 ± 40 $\mu\text{g/g}$ creatinine in smokers, and corresponding values in nonsmokers ranged from "not detectable" to 520 $\mu\text{g/g}$ creatinine with an average of 90 ± 20 $\mu\text{g/g}$ creatinine (the levels of *tt*-MA in smokers were about 3 times higher than those in nonsmokers). The results of the present study are also in agreement with these findings. Levels of urinary *tt*-MA for smokers in all study groups except the chemical

company workers were about two times higher than those of nonsmokers. Based on information extracted from questionnaires, urinary levels of *tt*-MA showed a weak linear relation with the number of cigarettes smoked (data not shown). As usual, urinary levels of *tt*-MA in smokers and nonsmokers showed no significant difference due to high levels of occupational exposure to benzene ($p < 0.05$). Despite the introduction of *tt*-MA as a selective biomarker for occupational and environmental benzene exposure by ACGIH, NIOSH, and some other regulatory organizations, several factors such as exposure levels, smoking status, and diet may influence its urinary concentration significantly, in particular for low-level exposure (≤ 0.5 ppm), and if ACGIH would decide to reduce benzene's threshold limit value (threshold limit value–time-weighted average) to 0.3 ppm [31], *tt*-MA cannot be used as a reliable biomarker for biological monitoring of benzene any longer.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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