Determination of urinary trans,trans-muconic acid reference values in the general Tunisian population

Abstract. We wish to determine the urinary trans,trans-muconic acid reference values in the Tunisian general population, and evaluate the impact of several factors (age, gender, tobacco...) on these reference values. Urine samples were collected from 182 healthy Tunisian subjects who had not been occupationally exposed to benzene. This determination was performed by solid phase extraction sampling technique together with high performance liquid chromatography-photodiode array detector. Trans,trans-muconic values ranged from 0.003 to 0.618 mg/g creatinine, the 95% reference interval was: 0.004-0.36, and the 90% confidence interval of the upper reference limit was: 0.24-0.62 mg/g creatinine. Urinary trans,trans-muconic levels were significantly higher among smokers. Significant differences were also observed for the < 20 and the ≥ 40 age groups. As a result urinary trans,trans-muconic background levels allow the biomonitoring of workers occupationally exposed to benzene at levels as low as 0.5 ppm. Age and tobacco, but not gender may affect the trans,trans-muconic reference values.

Key words: benzene, biomonitoring, general population, reference values, trans,trans-muconic acid

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Aromatic hydrocarbons such as benzene, toluene and xylenes are ubiquitous environmental pollutants. Although benzene is a well known and established human carcinogen [1], it's still used in chemical manufacturing industries. Moreover, benzene is naturally present in the crude petroleum and often blended into motor fuels to enhance octane ratings, generating subsequent emissions from vehicle exhausts. Furthermore, benzene can also be generated from incomplete combustion of natural materials (forest fire, volcanoes...) and from tobacco smoke, a major source of indoor exposure to benzene [2]. The general population is therefore exposed to this highly toxic pollutant.

Recently, the time waited average threshold limit values of benzene and several other solvents have been lowered. The levels of biomarkers became closer to the background values in the general population. Since the biological monitoring of exposure is based on the comparison of the monitoring data with reference values (RVs) determined in population that is not occupationally exposed to the pollutant, the knowledge of RVs and factors which influence them is more and more required for an accurate interpretation of the performed biological measures. Numerous studies including general population have been conducted. However, for the most part of these studies, the objective was to study the increase of the biomarker concentration in the exposed workers, rather than the determination of the RV itself.

The aim of the present study was the determination of RVs of trans,trans-muconic acid (t,t-MA) in occupationally unexposed reference Tunisian subjects and the evaluation of the impact of several factors (age, gender, tobacco...) on these levels.

**Materials and methods**

**Subjects and exclusion criteria**

A total of 230 individuals non-occupationally exposed to solvents were recruited with informed consent. For all subjects, information about work conditions, district of residence, smoking habits, alcohol consumption, sport practice, odd jobs, personal medical antecedents and medicine intake had been collected by questionnaire. Subjects suffering from renal or hepatic illness, diabetes, arterial hypertension, asthma; and those taking any medicine were excluded. After urinary creatinine determination and, biochemical and hematological analysis, individuals having urinary creatinine values outside the range 0.5-2.5 g/L, and those having a simultaneous occurrence of two abnormal biochemical or hematological values were excluded. Data from 182 selected reference subjects were evaluated (120 males and 62 females), aged 6-56 years (mean ± SD = 28.9 ± 12.5).

**Biological samples**

Blood samples were drawn using a vacuum collection system in two 5 mL tubes with EDTA for hematological analysis and without anticoagulant for the determination of transaminases (AST and ALT), urea, creatinine, alkaline phosphatase, creatinine kinase and gamma-glutamyltransferase.

Spot urine samples were collected in 160 mL flasks and stored at –20°C if not immediately analyzed.

**Measurement of urinary t,t-MA**

Measurement of t,t-MA was performed according to the method of Ducos et al. [3], slightly modified: 1 mL of urine sample was submitted to a clean-up procedure with a Bond-Elut solid phase extraction column filled with 500 mg Sax previously conditioned by 3 mL methanol and 3 mL distilled water. The column was later washed with 3 mL acetic acid solution (1%). Finally, t,t-MA was eluted with 3 mL acetic acid solution (10%). The extract was adjusted to 5 mL with distilled water. 20 μL of this solution was injected into the HPLC system, which consisted of a system controller (Waters 600), a gradient HPLC pump (Waters 600), a column oven (Waters 600) and a photodiode array detector (PDA) (Waters 996). Chromatographic separation was performed on a 250 cm x 4.6 mm x 5 μm column (Waters Spherisorb ODS2), with a 4.6 mm x 10 mm x 5 μm guard column (Waters Spherisorb ODS2). The columns were heated at 35°C. The mobile phase was a 1% acetic acid/methanol solution (90:10 V/V) with a flow rate of 1 mL/min. The use of diode array detector and a created spectra database allowed peak purity checking and spectrum peak identification. The analytical single derived channel wavelength for peak detection was set at 264 nm. The detection limit of the method derived from a minimum signal to noise ratio of 3 was 0.02 mg/L.

**Statistical analysis**

Reference intervals were established by the calculation of the interval covering 95% of the distribution of reference values and 90% confidence intervals around the limits as suggested by the International Federation of Clinical Chemistry [4]. The normality of the results were tested by using the Kolmogorov-Smirnov (KS) statistics; and between subgroups comparison was performed by using the Mann-Whitney’s U-test and by student’s t test for the log-transformed data.
Results and discussion

Study population

The Tunisian age distribution assessed by the National Institute of Statistics in 2004 [5] is similar to that of the world general population as defined by the World Health Organization [6] (figure 1). More than 60% of both populations are aged from 15 to 59 years. People who belong to this age interval group are considered as “active”.

Since RVs are determined for comparison with t,t-MA concentrations measured in occupationally exposed subjects, the major part of the studied sample (89%) was aged within the 15-59 interval. Nevertheless, for a better study of possible age influence on urinary excretion of t,t-MA, the studied group was divided into 3 subgroups: less than 20 years old (< 20), from 20 to 39 (20-39) and 40 or older (≥ 40).

Females represent 49.9% of the Tunisian population. However, they don’t exceed 24.2% of the total “fully active” population [5]. In the study group, sex ratio was 1.94, with 34.1% females.

With regard to living area, 78.8% of the selected reference subjects lived in the capital Tunis and in the second largest Tunisian city of Sfax, whereas 21.2% lived in small towns and rural areas.

Age, sex and main other characteristics of the studied population are depicted in figure 2.

Assessment of reference ranges

t,t-MA concentrations ranged from < 0.02 to 0.93 mg/L. These concentrations are higher than the values reported by Ducos et al. [7]. Concentrations measured in 145 subjects non-occupationally exposed to benzene never exceeded 0.7 mg/L. In contrast, Inoue et al. [8] found a largest value as high as 2.0 mg/L.

Since fluid intake can highly influence levels of biomarkers in urine [9], it’s recommended to correct values for creatinine [10]. Creatinine adjustment significantly reduces intra-individual variation [11]. The world health organization [12] recommends using only urine samples with a creatinine concentration of 0.3-3 g/L, for the biological monitoring of exposure at the work place. Rosenberg et al. stated that metabolites measures in urine samples with creatinine concentration out of the range 0.5-3 g/L were unreliable [9]. In the current study, t,t-MA values were measured only in urine samples with creatinine concentration between 0.5 and 2.5 g/L, as recommended by the German Federal Environment Agency [10]. However, creatinine adjustment may introduce other variation due to body surface area, gender, age, and diet [13]. Most of these criteria will be discussed later in this paper.

The distribution of the corrected t,t-MA concentrations, the mean and median values are reported in table 1. In case the t,t-MA value was below the detection limit, the result was replaced by a value of half the detection limit (60 of the 182 analyzed urine samples).

According to KS test, data distribution was significantly non-normal (p < 0.001), with a right-tail skew (figure 3). The non-parametric method was therefore used to analyze the results.

Table 1. Reference values and intervals of urinary t,t-MA (mg/g creatinine).

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Sample</th>
<th>Tunisian</th>
<th>World</th>
</tr>
</thead>
<tbody>
<tr>
<td>≥ 60</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15 to 59</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 to 14</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 to 4</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 1. Distribution of ages in the studied sample with regard to the general Tunisian and world populations.

Figure 2. Characteristics of the studied population. M: males; F: females; BMI: body mass index; C: city; T: small town or countryside; R: residential; I: industrial.

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<table>
<thead>
<tr>
<th>Age</th>
<th>n = 182</th>
<th>Range</th>
<th>95% RI</th>
<th>Mean [SD]</th>
<th>Median [CI]</th>
</tr>
</thead>
<tbody>
<tr>
<td>t,t-MA</td>
<td>0.003-0.618</td>
<td>0.004-0.36</td>
<td>0.06[0.07]</td>
<td>0.03 [0.24-0.62]</td>
<td></td>
</tr>
</tbody>
</table>
The observed values ranged from 0.003 to 0.618 mg/g creatinine. The upper reference limit (URL), in a 90% confidence interval was 0.62 mg/g creatinine. These values are in complete concordance with the Italian RV interval (i.e. 0.01-0.605 mg/g creatinine) [14]. Several other studies related in the literature reported highest values very consistent with our results (i.e. 0.604 and 0.625 mg/g creatinine) [15, 16]. Also, Ruppert et al. [17] reported t,t-MA concentrations ranging from 0.02 to 0.59 mg/g creatinine, in 114 subjects non-occupationally exposed to benzene.

Slightly lower RVs were observed in a recent study. 116 Brazilian reference subjects had t,t-MA values ranging from <0.03 to 0.55 mg/g creatinine [18]. However, less consistent greatest values were also reported by Melikian et al. [19] and Lee et al. [20]. The highest t,t-MA concentrations did not exceed 0.48 and 0.43 mg/g creatinine respectively. Furthermore, Waidyanatha et al. [21] observed a greatest value as low as 0.338 mg/g creatinine. Nevertheless, the number of subjects investigated in these studies was relatively low (i.e. 19-44).

Conversely, Boogaard et al. [22] reported a maximum t,t-MA background value as high as 0.71 mg/g creatinine. The variability of t,t-MA background level is probably due to genetic polymorphisms [23, 24] and dietary intake, particularly food preservative sorbic acid ingestion [25, 26]. In any case, in the biological monitoring of exposure to occupational environmental pollutants, measured values are often compared to the upper limit in the general population, especially for low exposure levels. The URL found in the present study is lower than the expected concentration of urinary t,t-MA in subjects exposed to an eight hours time weighted average (TWA) of 0.5 ppm benzene. Actually, in a previous study on exposure of tanker fillers and filling station attendants to benzene [27], the t,t-MA concentration calculated from the regression equation of the relation between benzene in breath zone air and post shift urinary t,t-MA levels was 0.82 and 1.57 mg/g creatinine, respectively for TWAs of 0.5 and 1 ppm benzene. These results were in good agreement with Lauwerys et al. [28] findings (0.8 and 1.4 mg/g creatinine). The American Conference of Governmental Industrial Hygienists [29] set the biological exposure index (BEI) for t,t-MA at 0.5 mg/g creatinine. This value seems relatively low with regard to background levels, especially among smokers. Actually, it has been established that tobacco smoke influences urinary t,t-MA levels. The smoking-related increase in t,t-MA excretion reported in twelve studies ranged from 0.022 to 0.2 mg/g creatinine [30].

In the present work, since the number of smokers (n = 50) is less than 119, non-parametric 90% confidence interval around the 95% reference interval limits could not be calculated [4]. Parametric calculations of reference values were therefore performed after logarithmic transformation of the data (table 2). Higher t,t-MA values were found in smokers with regard to non-smokers. Despite the lower number of samples from smokers, higher number of values outside the inter-quartile ranges were observed among this subgroup (figure 4), confirming the tendency towards increasing urinary t,t-MA concentrations in smokers.

<table>
<thead>
<tr>
<th>n</th>
<th>Range</th>
<th>95% RI</th>
<th>Mean</th>
<th>Median</th>
<th>URL 90% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smokers</td>
<td>50</td>
<td>[0.003-0.396]</td>
<td>[0.003-0.360]</td>
<td>0.03</td>
<td>[0.22-0.50]</td>
</tr>
<tr>
<td>Non-smokers</td>
<td>132</td>
<td>[0.003-0.399]</td>
<td>[0.003-0.362]</td>
<td>0.03</td>
<td>[0.22-0.50]</td>
</tr>
<tr>
<td>Females</td>
<td>62</td>
<td>[0.003-0.392]</td>
<td>[0.003-0.364]</td>
<td>0.03</td>
<td>[0.22-0.50]</td>
</tr>
<tr>
<td>Males</td>
<td>120</td>
<td>[0.003-0.392]</td>
<td>[0.003-0.364]</td>
<td>0.02</td>
<td>[0.22-0.50]</td>
</tr>
</tbody>
</table>

**Table 2. Reference values of urinary t,t-MA (mg/g creatinine) by tobacco smoking and gender.**

**Figure 3. Distribution of urinary t,t-MA (mg/g creatinine) reference values.**

**Figure 4.** Distribution of urinary t,t-MA (mg/g creatinine) for smokers and non-smokers.

Conversely, Boogaard et al. [22] reported a maximum t,t-MA background value as high as 0.71 mg/g creatinine. The variability of t,t-MA background level is probably due to genetic polymorphisms [23, 24] and dietary intake, particularly food preservative sorbic acid ingestion [25, 26].

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kers compared with nonsmokers. However, the difference between the values observed in the two subgroups was not significant, in line with Boogaard et al. [31] findings. Moreover, within the smokers subgroup, no correlation between the number of cigarettes smoked per day and levels of urinary t,t-MA excretion was observed. Nevertheless, when a student’s paired sample t-test was performed on log-transformed data, mean t,t-MA value in smokers was significantly higher than that in nonsmokers (p = 0.02).

Several studies reported poor but significant difference between values in smokers versus non-smokers [17, 18, 32]. Instead, other studies reported highly significant difference [19, 33, 34]. Furthermore, Taniguchi et al. [35] found significantly higher t,t-MA level in the 20 cigarettes/day or more smoking group than in the 1-19 cigarettes/day group (p < 0.001). The authors reported also, significantly higher t,t-MA level among subjects exposed to tobacco smoke (PS+) with regard to the unexposed group (PS-). However, the difference between values in the 1-19 cigarettes/day and the PS+ groups was not significant. These findings may explain our results, since 76% of our smokers group consumed less than 20 cigarettes/day. Unfortunately, information about eventual exposure to tobacco smoke (ETS) was not collected. Knowledge of ETS level could help for better interpretation of the data, although in most of the related studies, non-significant results were reported [17, 34, 36].

No correlation was found between gender and urinary t,t-MA concentrations. However, values were higher in males (table 2). This is most probably due to smoking habit. Actually, 94% of the studied females were nonsmokers, versus 62% in the males group. When gender influence is studied in the non-smoking subgroups of both sexes, concentrations ranges were found to be nearly the same (table 2) and, no statistically significant difference was found between the two subgroups, neither by Mann Whitney’s test, nor by the student’s t-test performed on the log-transformed data.

To our knowledge, a limited number of studies concerned the effect of gender on urinary t,t-MA excretion. In line with our results, Paula et al. [18] didn’t detect a significant effect of gender upon t,t-MA levels. On the other hand, Cocco et al. [32] reported significantly higher t,t-MA geometric mean among women (p < 0.05). Likewise, Kim et al. [37], using a complex model for studying relationships between levels of benzene metabolites with benzene exposure, noted that t,t-MA levels were 24% higher in females and decreased of 1.9% per year of life.

In the present study, a significant difference (p = 0.049) was found, only between concentrations in the < 20 and ≥ 40 years age subgroups (table 3). t,t-MA lower level in older subjects was probably due to the decline of the excretion of xenobiotics and their metabolites with aging, attributed to the hepatic blood flow reduction (0.5-1.5% per year from 25 years) and glomerular filtration rate diminution (1 mL/min per year from 40 years) [38]. Paula et al. [18], reported a significant difference between t,t-MA values in the age groups of 18-25 and > 36 years (p = 0.016). However, the authors reported a higher mean value in the adults group, although the highest registered value was observed in the 18-25 years group.

No statistical difference between t,t-MA values were observed in all the subgroups, neither by body mass index (BMI), nor by residence area (P > 0.05). Concentrations in subjects living in the largest Tunisian cities of Tunis and Sfax were not significantly different from values observed in small towns and countryside residents. Moreover, there was no correlation between t,t-MA values and the district of residence (industrial versus residential). In line with our results, Cocco et al. [32] didn’t find any association of urban traffic and residence near fuel station with elevated t,t-MA values. Instead, Ruppert et al. [17] reported significantly higher concentrations among non-smokers living in the city, with regard to non-smokers living in the suburbs (p < 0.05). Moreover, Staessen et
al. [39] reported higher t,t-MA concentrations in Belgian adolescents living in a polluted suburb with respect to the homologous reference population living in rural area (p = 0.02). Also, Amodio-Cocchieri et al. [40] found t,t-MA levels to be increased with proximity of the home to traffic. Other studies reported only slightly increased t,t-MA levels in subjects living in areas with high automobile traffic density, although environmental benzene concentrations were twice higher than that measured in rural area [36, 41].

### Conclusion

The mean reference value in the general Tunisian population is relatively low. However, the upper reference limit in 90% confidence interval was as high as 0.62 mg/g creatinine in smokers. This value is higher than the 0.5 mg/g creatinine BEI set by the ACGIH, but still allows the biological monitoring of occupational exposure to benzene at levels as low as 0.5 ppm. In non-smokers the URL didn’t exceed 0.4 mg/g creatinine. t,t-MA excretion levels were not influenced by gender, BMI or place of residence. A tendency towards t,t-MA increased levels were observed in the < 20 years group with regard to older subjects.

t,t-MA is an interesting and useful biomarker of benzene exposure down to 0.5 ppm. At lower exposure levels, the biomonitoring results have to be considered on a group basis. In any case, the smoking status of the monitored subjects should be known for a better interpretation of the results.

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### References


