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Validation of an HPLC/MS/MS method with isotopic dilution for quantitative determination of *trans*,*trans*-muconic acid in urine samples of workers exposed to low benzene concentrations

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ABSTRACT

Urinary *trans*,*trans*-muconic acid (*t*,*t*-MA), a biomarker of benzene exposure, is usually determined by HPLC methods with detection by either UV or, more recently, electrospray tandem mass spectrometry. However, not all these methods have been fully validated for quantitative analysis. This paper presents an HPLC/MS/MS method for reliable quantitative determination of *t*,*t*-MA that uses a commercial deuterium-labeled isotope as internal standard; the matrix effect has been evaluated and LOD is 0.22 μg/L. We used this method to test 200 urine samples, 175 of them collected at end-of-shift from workers in an oil refinery.
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1. Introduction

Several analytical methods have been proposed for the determination of *trans*,*trans*-muconic acid (*t*,*t*-MA) in human urine. This metabolite of benzene was suggested by the American Conference of Governmental Industrial Hygienists as an indicator of occupational exposure, with a limit set at 500 μg/g of creatinine [1]. Almost all today's HPLC methods use strong anionic exchange cartridges for sample cleaning and reverse-phase column separation, with UV detection at λ 259 nm [2–6]. New methods have been proposed, that still use SPE for urine clean-up and HPLC separation, but instead of UV they employ electrospray tandem mass spectrometry (MS) detection, a technique widely applied for the determination of biomarkers on account of its higher sensitivity and specificity [7–11].

Two main problems are encountered in the HPLC/MS/MS determination of *t*,*t* MA in human urine: (i) the urine contains several other organic acids [12,13] that are co-extracted in the SPE sample purification phase and can generate ions isobaric with the analyte [14], and (ii) the matrix effect [15]. The matrix effect changes the instrumental response of the analyte when it is dissolved in urine compared to pure solvent, lowering the accuracy in quantitative analysis; this can be overcome by using an isotopic labeled internal standard. Some methods have been validated for quantitative analysis with isotopic dilution using as internal standard a $^{13}C_6$ labeled *t*,*t*-MA, biosynthetically prepared by injecting $^{13}C_6$ benzene into rats [7,8] or synthetized starting from $^{13}C_6$ labeled adipic acid [9]. These solutions, however, are not applicable in all laboratories that do routine biological monitoring of workers.

In Ref. [10] quantitation is done by the external standard method, but the matrix effect is still there and in addition "a very high peak due to an unidentified compound coelutes with *t*,*t*-MA within a narrow retention time range and provides a signal in the same MRM channel (m/z 141 → 97)". Another report gives validation data for quantitative analysis using an internal standard directly added to the urine samples without any clean-up procedure [11].

Some authors propose simultaneous detection for *t*,*t*-MA and *S*-phenylmercapturic acid (SPMA) [10,19], the alternative biomarker proposed by the ACGIH for benzene exposure. However, SPMA determination is not reliable unless strong acid hydrolysis of the urine samples is done before analysis [20,21], but *t*,*t*-MA is not stable in these conditions (see Section 3.6).

The aim of the present study was to modify existing analytical methods for *t*,*t*-MA in order to obtain a well-separated, narrow chromatographic peak for the analyte, to minimize all matrix interferences on MS/MS detection, and to achieve complete validation using a commercially available deuterium-labeled isotope as internal standard (*t*,*t*-MA-d_4), in order to have a tool for routine quantitation of *t*,*t*-MA in the urine of workers occupationally exposed to airborne benzene levels below the TLV-TWA of 0.5 ppm [1].

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2. Experimental

2.1. Chemicals and supplies

The analytical reference standard of *t*,*t*-MA was purchased from Sigma–Aldrich (Milan, Italy). The deuterated internal standard *t*,*t*-MA-d4 was obtained from CDN Isotopes Inc. (Pointe-Claire, Quebec, Canada). Glacial acetic acid (100%; Merck, Darmstadt, Germany) was used for preparing the mobile phase and for the SPE, with purified water from a Milli-Q Plus system (Millipore, Milford, MA, USA). Methanol for LC/MS and for SPE was supplied by J.Y. Baker (Deventer, Holland). Sodium phosphate dibasic and potassium phosphate monobasic for preparation of the buffer for SPE were purchased from Sigma–Aldrich (Milan, Italy). Discovery DSC-SAX tubes (6 mL, 500 mg) for SPE were supplied by SUPELCO (Bellafonte, PA, USA), and the SPE vacuum manifold by Waters (Milford, MA, USA). Anotop 10 LC syringe filter devices (0.2 μm pore size, 10 mm diameter) were purchased from Whatman Inc. (Maidstone, UK). A Phenomenex Sinergy 4U Fusion RP C-18 column (150 × 4.6, 80A) was supplied by Chemtek Analytica s.r.l. (Bologna, Italy), and used throughout the study. Control human urine samples for standard calibration curves and quality control samples were obtained from healthy, non-smoking volunteers.

2.2. Preparation of urine samples

Urine samples were collected in sterile polypropylene containers and stored at −20 °C until analysis. A 3-mL portion of each sample was treated with 1 mL of phosphate buffer (1.179 g of KH_2PO_4 and 4.303 g of Na_2HPO_4 in 1 L of water, pH 7.4), and 60 μL of deuterated internal standard solution in methanol (*t*,*t*-MA-d4, 10 mg/L); SPE cartridges were previously conditioned with 3 mL of methanol and 3 mL of 0.1% (v/v) acetic acid in water; and after loading the samples, the cartridges were washed with 3 mL of 0.1% (v/v) acetic acid in water then 3 mL of methanol, and finally eluted with 3 mL of 10% (v/v) acetic acid. The eluate was filtered on a 0.2 μm syringe filter, and 20 μL injected into the HPLC–MS/MS system. Each sample was tested in duplicate.

2.3. Validation of the analytical method

Five independent sets of calibration curves, in methanol and in urine, were analyzed on five different days, three of them not consecutive. Each pair of urine and matrix calibration curves was prepared from a different donor. Samples were analyzed in duplicate and the average was used. The results were used to establish the performance of the method.

2.4. Preparation of standard solutions in methanol

Five mg of *t*,*t*-MA were weighed and dissolved in 250 mL of methanol to obtain a standard solution of 20 mg/L (A). One further dilution was prepared at the concentration of 10 mg/L (B). A stock standard solution containing 100 mg/L of internal standard was obtained by weighing 10 mg of *t*,*t*-MA-d_4 and dissolving it in 100 mL methanol (solution C). By mixing suitable amounts of B and C, five independent calibration curves in methanol were prepared, each consisting of five calibration standards in the range 20–1000 μg/L of *t*,*t*-MA and containing 200 μg/L of internal standard, which were analyzed on five different days. Solutions A, B and C were stored in the dark at 4 °C for subsequent use.

2.5. Preparation of urine standards and quality control samples

Five different calibration curves, each using the urine from a different, healthy, non-smoking donor, were prepared and analyzed on separate days. Each calibration curve consisted of one "blank" urine sample, five calibration standards with additions of 20, 100, 250, 500, and 1000 μg/L of *t*,*t*-MA, and two independent replicates of quality control samples with 50, 200 and 800 μg/L added, each sample containing 200 μg/L of deuterated internal standard. Since *t*,*t*-MA is endogenous in urine, blank urine samples do not exist and therefore we call the samples from non-smoking, non-occupationally exposed subjects, without added *t*,*t*-MA, our "blanks". Five independent replicates of the quality control samples were also prepared and tested the same day in order to assess intra-day variability. Samples were prepared by spiking 3 mL of urine with suitable amounts of solutions A or B and with 60 μL of C. All samples were purified with SPE, as described for unknown urine samples (see Section 2.2).

2.6. Preparation of matrix standards

In order to determine the matrix effect of different urines on the instrumental response, five blank urine samples (3 mL) were submitted to the SPE procedure and the eluates spiked with suitable amounts of solutions A, B and C in order to reach the same final concentrations of the five urine calibration standard above described. This was repeated for the five urines (from different donors).

2.7. HPLC–MS/MS conditions

The urine samples and calibration and matrix standards were analyzed on a Series 200 LC quaternary pump (PerkinElmer, Norwalk, CT, USA) using a 150 mm × 4.6 mm, 80A Phenomenex Sinergy 4U Fusion RP C-18 analytical column.

The elution followed the scheme reported in Table 1, using methanol (phase A) and acetic acid 0.1% (v/v) in water (phase B), flow rate 0.8 mL/min. Total run time was 15 min. In these conditions, the retention time of *t*,*t*-MA is around 10.2 min and that of the internal standard 10.1 min (Fig. 1). The total run time was 15 min.

The ion source of the AB/MDS Sciex API 4000 triple quadrupole mass spectrometer can accept a mobile phase flow up to 1 mL/min and therefore the HPLC eluate was fed entirely into the turbo ion spray (TIS) probe. The source temperature was set at 500 °C. The instrument was calibrated using polypropylene glycol and the resolution was adjusted to a peak width (FWHM) of 0.7 Th over the range of *m*/*z* 100–1000. Detection was done in the negative ion, multiple reaction monitoring (MRM) mode, and parameters were optimized for the analytes by the automated "Infusion Quantitative Optimization" procedure and subsequently refined by flow injection analysis (FIA) using the pure standards. The following *m*/*z* ion combinations (precursor → product) were monitored and the single reaction monitoring (SRM) transitions (negative mode) were as follows: *m*/*z* 141 → *m*/*z* 97 for *t*,*t*-MA and *m*/*z* 145 → *m*/*z* 100 for the deuterated internal standard. All values are summarized in Fig. 2. Version 1.4 of the Analyst® software was employed for instrument control and data acquisition.

Table 1
HPLC elution scheme

Step	Time (min)	% A phase	% B phase
Equilibration	3	10	90
Injection	0		
Isocratic	2	10	90
Linear gradient	13	90	10
Total run time	15		

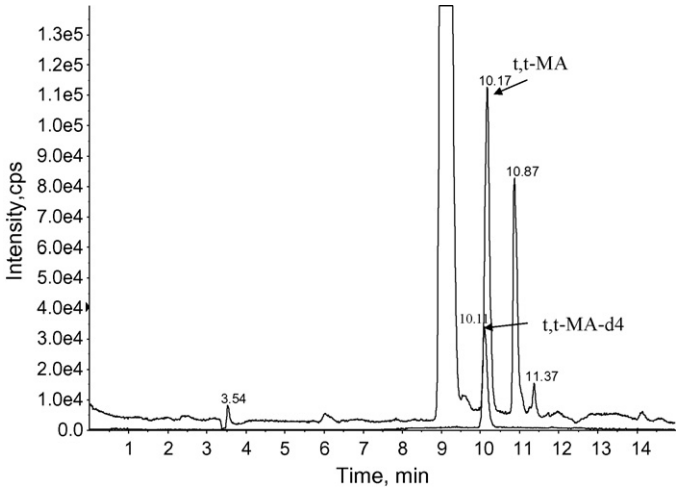
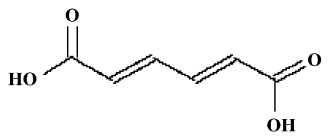


Fig. 1. Typical multiple reaction monitoring (MRM) chromatogram of a urine calibration standard containing 50 μg/L of *trans*,*trans*-muconic acid and 200 μg/L internal standard (*t*,*t*-MA-d4). cps: counts per second.



trans, trans - Muconic acid		
Precursor ion	**Q1 Mass (m/z)**	**- 141.00**
Product ion	**Q3 Mass (m/z)**	**- 97.00**
HOOCCD=CDCD=CDCOOH		
trans, trans - Muconic - d_4 acid		
Precursor ion	**Q1 Mass (m/z)**	**- 145.00**
Product ion	**Q3 Mass (m/z)**	**- 100.00**
Common Parameters		
Dwell time	***msec***	***200***
CUR (curtain gas)	***(arbitrary unit)***	***20.00***
GS1 (gas1)	***arbitrary unit)***	***40***
GS2 (gas2)	***arbitrary unit)***	***50***
CAD (collisionally activated dissociation)	***(arbitrary unit)***	***5.00***
EP (collision cell entrance potential)	***(ΔV)***	***-10.00***
CXP (collision cell exit potential)	***(ΔV)***	***-7.00***
CE (collision energy value)	***(ΔV)***	***-12***
DP (cluster-breaking orifice voltage)	***(ΔV)***	***- 35***

Fig. 2. Mass spectrometry API 4000 experimental conditions.

2.8. Data processing

The peak areas generated by the samples were integrated by the 1.4 Analyst® software.

The area of the *t*,*t*-MA peak of each blank urine sample was subtracted from the areas of the corresponding urine calibration standards and quality controls. The calibration curves were generated using linear regression analysis according to the equation $y = ax + b$, where y is the ratio between the area of *t*,*t*-MA calibration standards (after subtraction of the blank) to that of the internal standard, a is the slope of the regression line, x is the concentration of the analyte, and b is the intercept. The concentrations of the analyte in the unknown and quality control samples were calculated from the regression equation of the calibration curve and expressed as μg/L of urine.

2.9. Determination of the LOD and LLOQ

The limits of detection (LOD) and the lower limit of quantification (LLOQ) were defined as respectively three and ten times the standard deviation of the LC/MS/MS peak areas detected at the retention times of the analyte of interest in several blank urine samples (noise). As "blank" urine samples for *t*,*t*-MA are not available, noise standard deviation was calculated by the 1.4® Analyst in the immediate vicinity of the analyte peak. The corresponding concentrations were calculated from the ratio to the internal standard area on the calibration curve.

2.10. Biological monitoring of workers

We collected 280 end-shift urine samples from workers in an oil refinery and 53 spot samples from non-smoking external volunteers (group 1). Workers were divided into four groups: non-smokers not occupationally exposed to benzene (group 2), non-smokers occupationally exposed to benzene (group 3), smokers not occupationally exposed to benzene (group 4), smokers occupationally exposed to benzene (group 5). "Occupationally exposed" workers are subject to compulsory medical surveillance once a year, and airborne benzene levels are below the threshold of 0.5 ppm. Non-occupationally exposed workers are employed at the same site, but their tasks do not require medical surveillance. External volunteers were non-smokers living and working in a different place.

3. Results and discussion

3.1. HPLC–MS/MS analysis

The slight difference in retention time between *t*,*t*-MA and *t*,*t*-MA-d_4 (Fig. 1) is due to the deuterium isotope effect that causes partial resolution of the analyte from its internal standard [17]. The chromatogram of urine samples generated by the SRM transition m/z 141 → m/z 97 confirms the presence of a very high peak described by other groups at a retention time immediately before the *t*,*t*-MA peak [10], but in our case the chromatographic separation from *t*,*t*-MA was complete (Fig. 3).

The m/z 145 → m/z 101 transition suggested in Ref. [11] for *t*,*t*-MA-d_4, monitored in the same samples, produced very high background noise and a "dirty" peak at the retention time of the pure internal standard (Fig. 4). Therefore we selected a different transition (m/z 145 → m/z 100), which was the second most intense in the product ion mass spectrum of ion m/z 145 of *t*,*t*-MA-d4, and whose single ion monitoring (SIM) chromatogram in urine generates a single, clean peak (Fig. 5).

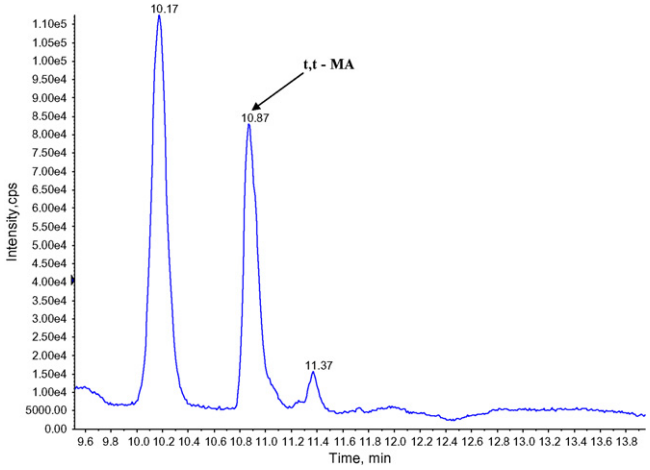


Fig. 3. SRM chromatogram for the transition −141 → −97 of a urine containing 50 μg/L *t*,*t*-MA.

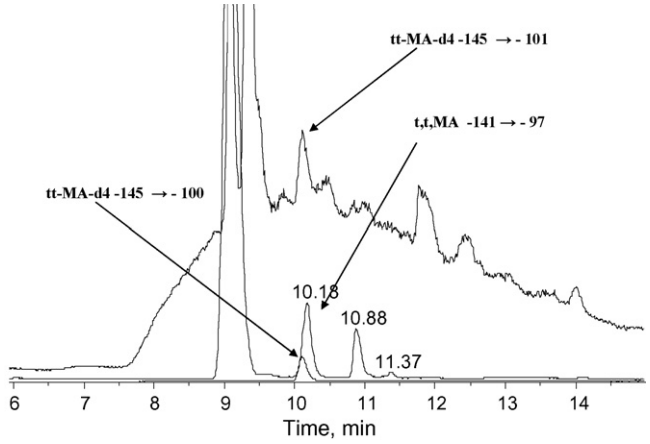


Fig. 4. Complete multiple reaction monitoring (MRM) chromatogram of a urine calibration standard containing 50 μg/L of *trans*,*trans*-muconic acid and 200 μg/L internal standard (*t*,*t*-MA-d4) with the three transitions: −141 → −97 for *t*,*t*-MA, −145 → −100 and −145 → −101 for the internal standard. cps: counts per second.

3.2. Calibration curves

Five calibration curves in urine and in methanol were analyzed. A linear regression analysis in the range 20–1000 μg/L of added *t*,*t*-MA and an ANOVA test yielded coefficients of determination R^2 always greater then 0.993. The goodness of the linear regression fit was tested by an *F* test always obtaining $p < 0.001$.

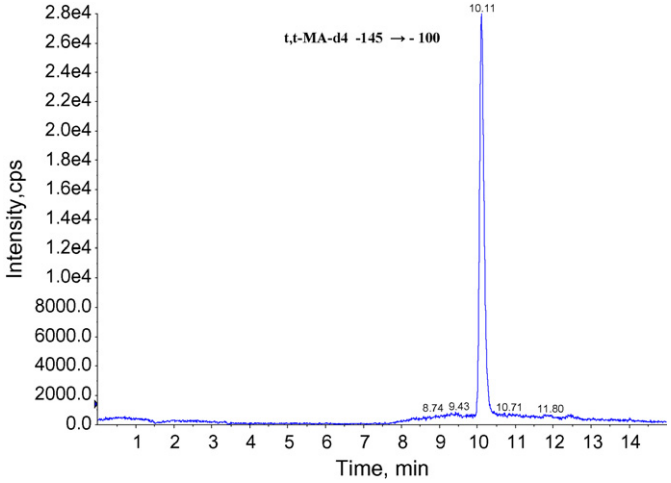


Fig. 5. SIM chromatogram for the transition −145 → −100 of a urine sample containing 200 μg/L of internal standard (*t*,*t*-MA-d4).

3.3. Limits of detection and quantitation

The LOD and LLOQ were respectively 0.5 and 1.5 μg/L. The chromatogram of a urine sample containing 1.8 μg/L of *t*,*t*-MA is reported in Fig. 6.

3.4. Matrix effect and SPE recovery

The matrix effect was evaluated for *t*,*t*-MA and for the deuterated internal standard following the procedure suggested by Matuszewski et al. [16]: the relative analyte response is expressed as the ratio (expressed as a percentage) between the *t*,*t*-MA peak areas of the matrix standards and those of the same concentration of standards dissolved in methanol, and we will call it "matrix effect recovery"; in these samples *t*,*t*-MA was not subjected to SPE and values were between 60 and 100%, confirming that the MS/MS response varies significantly between urine donors. SPE recovery was calculated by comparing the peak areas produced by analysis of the urine calibration standards (spiked before SPE) to those of the matrix standards (prepared with the same urine but spiked after SPE) and was more than 86% over five independent experiments. We defined "total recovery" the product of matrix effect recovery and SPE recovery, which equals the ratio between the peak areas of

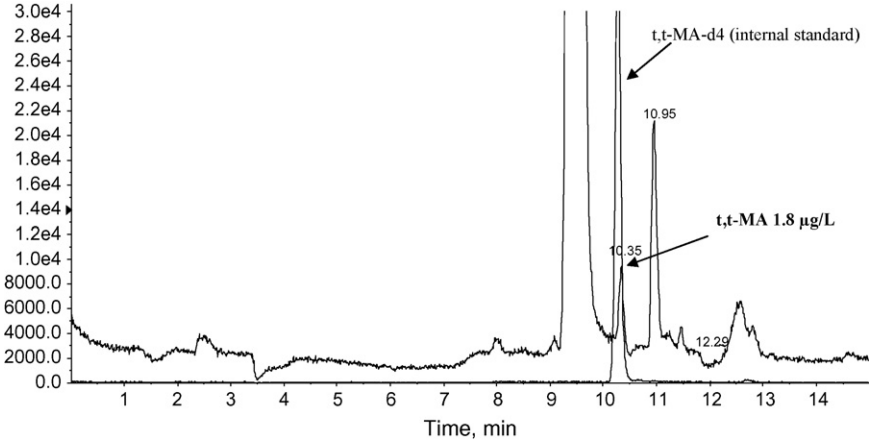


Fig. 6. Chromatogram of a urine sample containing 1.8 μg/L of *trans*,*trans*-muconic acid.

Table 2
Recovery due to solid-phase extraction (SPE) and matrix effect for *t,t*-MA and internal standard

Day/urine donor	Creatinine (g/L)	Baseline *t,t*-MA (μg/L)	SPE recovery (%)		Matrix effect recovery (%)		Total recovery (%)	
			t,t-MA	*t,t*-MA-d_4	*t,t*-MA	*t,t*-MA-d_4	*t,t*-MA	*t,t*-MA-d_4
1	0.7	40.4	108.8	94.9	68.1	76.2	74.1	72.3
2	0.3	5.0	88.2	87.2	100.9	100.2	89.0	87.3
3	1.0	54.2	102.6	103.2	60.9	69.2	62.5	71.4
4	2.7	42.1	105.0	109.0	60.0	58.0	63.0	63.2
5	0.5	35.5	86.0	84.0	78.0	76.0	67.1	63.8
Mean	1.1	35.4	98.1	95.7	72.5	75.9	72.2	73.5
Standard deviation	–	–	10.3	10.5	19.3	15.4	12.4	10.0
CV (%)	–	–	10.5	11.0	26.6	20.3	17.2	13.6

Table 3
Inter- and intra-assay accuracy and precision on urine quality controls

t,t-MA theoretical concentration	50 μg/L		200 μg/L		800 μg/L	
	Found concentration	Accuracy (%)	Found concentration	Accuracy (%)	Found concentration	Accuracy (%)
Mean of five replicates (different days)	49.9	99.9	206.9	103.5	776.4	97.1
Standard deviation (STD)	5.7	11.5	18.3	9.2	31.2	3.9
Interday % CV (STD/mean)	11.5	11.5	8.9	8.9	4.0	4.0
Mean of five replicates (same day)	49.8	99.7	176.5	88.3	745.0	93.1
Standard deviation (STD)	4.4	8.8	13.8	6.9	12.5	1.6
Intraday % CV (STD/mean)	8.8	8.8	7.8	7.8	1.7	1.7

the urine standards and those of the same concentrations of standards dissolved in methanol. Table 2 reports the complete results (the values are the average of five results for each urine, and the last row shows the means of the five experiments).

The best method of compensating for the matrix effect is the internal standard method for quantitative analysis. The best possible internal standard is a stable isotope-labeled compound that mimics the analyte's behavior better than any other analog molecule, and will therefore be subject to a very similar matrix effect. The matrix effect, if not compensated for, can affect the accuracy and precision of the method.

3.5. Accuracy and precision

The inter-day accuracy and precision were determined from the analysis on five independent QC samples at low, medium and high concentrations (50, 200 and 800 μg/L of added *t,t*-MA) tested over the 5 days of the validation study. The accuracy was determined by comparing the means of the concentrations found in the quality control samples with the theoretical values and presented as percentages, and ranged from 97.1 to 103.5%. Precision is expressed as the relative standard deviation (RSD) of the values found over the mean for each concentration (% CV).

The intra-day accuracy and precision were calculated by testing five independent replicates (five separate SPE columns) of low, medium and high-concentration QC samples (15 samples) on the same day. The accuracy, determined by comparing the means of the concentrations found in the quality control samples with the theoretical values, ranged from 88.3 to 99.7%. Results are summarized in Table 3.

3.6. Stability of t,t-MA

The stability of *t,t*-MA was verified frozen at −20 °C in sterile polypropylene containers by analyzing aliquots of the same sample from the collection day up to 6 months.

To investigate the feasibility of simultaneous detection of *t,t*-MA and SPMA [21], a 3-mL portion of each urine sample was treated with 1 mL of 9 M H_2SO_4 for 10 min, then NaOH 50% in water was added to reach a pH around 7. Unfortunately, this analysis showed that *t,t*-MA is totally degraded in these conditions.

3.7. Biological monitoring of workers

As benzene is an ubiquitous environmental pollutant, *t,t*-MA was found in the urine of all subjects. The concentrations of *t,t*-MA divided by the creatinine concentration are reported in Table 4. Means and medians are reported, with the number of subjects in the group, standard deviations, minimum and maximum values and 5th and 95th percentiles of the distribution. The statistical dis-

Table 4
Values of *t,t*-MA measured in the end-shift urine samples of workers and controls

	Non-smoking controls	Non-smoking non-benzene exposed workers	Non-smoking benzene exposed workers	Smoking non-benzene exposed workers	Smoking benzene exposed workers
t,t-MA μg/g creatinine					
Group no.	1	2	3	4	5
N	53	70	70	70	70
Mean	46.9	52.0	67.3	147.4	145.8
Median	40.3	38.3	46.9	117.1	103.6
Standard deviation	23.7	64.4	55.9	138.3	131.0
Min	11.0	3.6	14.8	21.5	17.5
Max	107.8	475.7	322.3	927.9	664.9
5th percentile	18.0	12.3	17.2	39.9	23.1
95th percentile	107.8	134.3	157.7	329.6	432.7

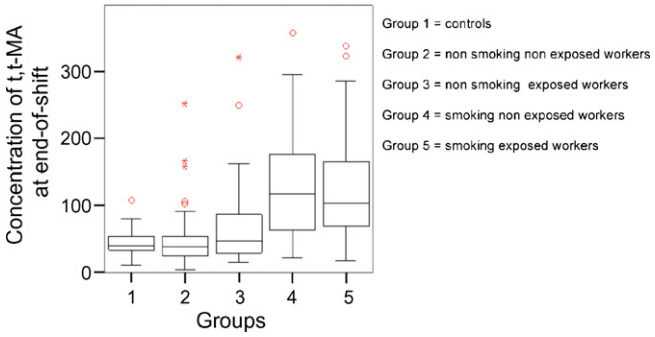


Fig. 7. Box-and-whisker plot of the distribution of the urine concentration (μg/L) of *t*,*t*-MA at end-of-shift in the five groups.

tribution of the end-shift *t*,*t*-MA results on workers and controls is described by the box-and-whisker plot in Fig. 7.

The gap between the means and medians is due to the higher results than can be produced by real higher benzene exposure (occupational or from cigarette smoking) or, in a few cases, by the interference of sorbic acid; in fact *t*,*t*-MA seems to be a metabolite for this substance too, a common food preservative found in the urine within 2 h of a meal [17,18].

The values for group 2 (non-smokers not occupationally exposed) did not differ (*T* test, $p = 0.29$) from those of external controls (group 1), indicating that the benzene exposure risk of the workers in this plant had been correctly assessed. The maximum is lower than the BEI of 500 μg/g of creatinine for non-smokers (groups 2 and 3), indicating a good level of risk containment, while on a group basis it is possible to discriminate between occupationally exposed and non-exposed subjects (*T* test, $p = 0.07$). Smoking is confirmed as an important source of benzene exposure and a significant confounding factor for the assessment of occupational exposure by biological monitoring, as there is not a statistically significant difference between groups 4 and 5.

4. Conclusions

The availability of new and powerful tools like MS/MS detection in analytical chemistry can lead to underestimates of the importance of sample purification and HPLC separation. When the aim is quantitation of an analyte dissolved in a complex matrix, bypassing these steps can lead to unreliable results.

The analytical method presented is an effective tool for the quantitative determination of *t*,*t*-MA in human urine, and is validated in terms of specificity, precision, accuracy, and limits of detection and quantitation. Possible sources of error such as matrix effect and interferences were taken into due consideration. We applied the isotopic dilution method, which is strongly recommended for quantitative HPLC–MS/MS determination, using a commercially available deuterium-labeled isotope of the analyte, which renders the method suitable for routine biological monitoring.

Analysis of biological samples from benzene-exposed workers showed that using *t*,*t*-MA as a biomarker of exposure, also thanks to the accuracy and specificity of this analytical method, one can distinguish groups with different exposure conditions: environmental, occupational, due to cigarette smoking, or their various combinations.

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