ELSEVIER

Journal of Chromatography B, 717 (1998) 179–199

JOURNAL OF CHROMATOGRAPHY B

Review

Analysis and evaluation of *trans*,*trans*-muconic acid as a biomarker for benzene exposure

Gerhard Scherer*, Thomas Renner, Michael Meger

Analytisch-biologisches Forschungslabor ABF, Goethestrasse 20, 80336 Munich, Germany

Abstract

Benzene is an important industrial chemical and, due to its occurrence in mineral oil and its formation in many combustion processes, a widespread environmental pollutant. Since benzene is hematoxic and has been classified as a human carcinogen, monitoring and control of benzene exposure is of importance. Although *trans*,*trans*-muconic acid (*tt*MA) was identified as a urinary metabolite of benzene at the beginning of this century, only recently has its application as a biomarker for occupational and environmental benzene exposure been investigated. The range of metabolic conversion of benzene to *tt*MA is about 2–25% and dependent on the benzene exposure level, simultaneous exposure to toluene, and probably also to genetic factors. For the quantitation of *tt*MA in urine, HPLC methods using UV and diode array detection as well as GC methods combined with MS or FID detection have been described. Sample pretreatment for both HPLC and GC analysis comprises centrifugation and enrichment by solid-phase extraction on anion-exchange sorbents. Described derivatization procedures prior to GC analysis include reaction with N,O-bis(trimethysilyl)acetamide, N,O-bis(trimethylsilyl)trifluoroacetamide, pentafluorobenzyl bromide and borontrifluoride–methanol. Reported limits of detection for HPLC methods range from 0.1 to 0.003 mg l^{-1}, whereas those reported for GC methods are 0.03–0.01 mg l^{-1}. Due to its higher specificity, GC methods appear to be more suitable for determination of low urinary *tt*MA levels caused by environmental exposure to benzene. In studies with occupational exposure to benzene (>0.1 ppm), good correlations between urinary *tt*MA excretion and benzene levels in breathing air are observed. From the reported regressions for these variables, mean excretion rates of *tt*MA of 1.9 mg g^{-1} creatinine or 2.5 mg l^{-1} at an exposure dose of 1 ppm over 8 h can be calculated. The smoking-related increase in urinary *tt*MA excretion reported in twelve studies ranged from 0.022 to 0.2 mg g^{-1} creatinine. Only a few studies have investigated the effect of exposure to environmental levels of benzene (<0.01 ppm) on urinary *tt*MA excretion. A trend for slightly increased *tt*MA levels in subjects living in areas with high automobile traffic density was observed, whereas exposure to environmental tobacco smoke did not significantly increase the urinary *tt*MA excretion. It is concluded that urinary *tt*MA is a suitable biomarker for benzene exposure at occupational levels as low as 0.1 ppm. Biomonitoring of exposure to environmental benzene levels (<0.01 ppm) using urinary *tt*MA appears to be possible only if the ingestion of dietary sorbic acid, another precursor to urinary *tt*MA, is taken into account.

Keywords: Reviews; Muconic acid; Benzene

Contents

*Corresponding author.

0378-4347/98/$19.00
PII: S0378-4347(98)00065-6

1. Introduction

Although *trans*,*trans*-muconic acid (*tt*MA, *trans*,*trans*-2,4-hexadienedioic acid, CAS 3588-17-8) has been reported as a urinary metabolite of benzene metabolism since the beginning of this century [1], its importance as a biological marker of occupational and environmental exposure to benzene has been recognized only in the last decade. The classification of benzene as a human carcinogen by the International Agency for Research on Cancer (IARC) [2] has resulted in the reduction of occupational exposure levels and, as a consequence, led to the need for improved biomonitoring techniques.

The occupational threshold limit value (TLV) of benzene (expressed as the 8-h time weighted average (TWA_8)) has been lowered to 0.5–1 ppm (1 ppm= 3.2 mg m^{-3}) in many industrialized countries in this decade. Recently, a TWA_8 of 0.3 ppm has been proposed by the American Conference of Governmental Industrial Hygienists (ACGIH) [3]. Today, the major exposure at the workplace occurs during production, distribution and handling of gasoline, in which short-time peak concentrations often exceed 1 ppm. Due to its high volatility, benzene is ubiquitously distributed in ambient air. The predominant sources are emissions from automobiles, the petrochemical industry, and pyrolysis of organic materials. Outdoor levels range from 0.5 to 6 $\mu g\, m^{-3}$ in rural regions [4] and are predicted to be <0.1 $\mu g\, m^{-3}$ in remote areas such as high mountains. In cities and industrial centers with high traffic density, benzene concentrations of 7.4–210.6 $\mu g\, m^{-3}$ are found [4]. Indoor levels of benzene are normally higher than those outside, and may range from 5.8 to 120 $\mu g\, m^{-3}$ [4]. Environmental tobacco smoke (ETS) may also contribute to indoor air benzene levels. The mainstream smoke of a cigarette contains up to 73 μg benzene [5]; therefore, smokers exhibit higher mean personal exposures to benzene than nonsmokers. Nutritional factors are estimated to contribute only about 1% to the total intake of benzene which almost exclusively originates from ambient air [6]. This estimate is in contrast to previous assumptions of the National Research Council (NRC) [7] and the IARC [2] who reported benzene levels ranging from 24 to 60 $\mu g\, m^{-3}$ in the exhalate of nonexposed individuals, suggesting that their uptake might be of dietary (or environmental) origin.

Apart from benzene, the only other known precursor of *tt*MA is *trans*,*trans*-2,4-hexadienoic acid (sorbic acid) [8,9], which is a widely used preservative in food products. Sorbic acid concentrations in

various foodstuffs range from 2 to more than 200 mg kg^{-1} [10]. The average daily dietary intake is estimated to be 29.4 mg sorbic acid [11]. However, the contribution of sorbic acid to the background levels of *tt*MA in urine is still unclear.

Benzene, at least at high exposure doses, constitutes a health risk. Although the toxicological relevance of low dose exposures to benzene is as yet unclear, biomonitoring of low level exposures to benzene by suitable biomarkers is of both occupational and environmental importance. In this review, we discuss the analytical methods available for determination of urinary *tt*MA. In addition, we present and evaluate data for urinary *tt*MA excretion after exposure to benzene from different sources. Special emphasis is given to the evaluation of *tt*MA as a biomarker for low, environmental exposure to benzene.

2. Metabolism, toxicology and potential biomarkers of benzene

2.1. Historical background

The historical background of the chemistry and biochemistry of muconic acid has recently been reviewed by Ducos and Gaudin [12]. Muconic acid as a metabolite of benzene was first described by Jaffé in 1909 [1], who orally administered benzene to rabbits and dogs and recovered about 0.3% of the dose in urine as muconic acid. Fuchs and Soos in 1916 [13] were the first to isolate muconic acid from human urine samples of leukemia patients who had been treated with a total dose of 72 g benzene (3–5 g/day). These authors found 0.11% of the applied dose as muconic acid in urine. Parke and Williams in 1953 [14] reported the first metabolic study on benzene after administering [^{14}C]-benzene to rabbits. The total recovery of benzene was 84–89%, with 32.6% of the dose recovered in urine as phenol, catechol, hydroquinone, 1,2,4-benzenetriol, *trans*, *trans*-muconic acid and L-phenylmercapturic acid, 44.5% of the dose recovered in expired air as benzene (43%) and carbon dioxide (1.5%) and 5–10% recovered in the feces and tissues. Parke and Williams [15] also showed that *tt*MA was not formed from phenol as a precursor and that *cis*,*cis*-muconic acid is not an intermediate in the metabolic pathway to *tt*MA. The latter observation confirmed a previous study by Drummond and Finar [16].

In 1985, Gad-El Karim et al. [17] described for the first time the use of ether extraction coupled with high-performance liquid chromatography (HPLC) for quantitation of urinary *tt*MA. They also reported that phenol, hydroquinone, and catechol were not precursors to *tt*MA. These findings led Gad-El Karim et al. to suggest that urinary *tt*MA is a specific biomarker for low levels of exposure to benzene.

2.2. Metabolic pathway to trans,trans-muconic acid

The main route for the uptake of benzene both at the workplace and from the environment is via inhalation. At some workplaces, transdermal absorption of benzene may also contribute significantly to the exposure dose. About 50 to >90% of inhaled benzene is absorbed [18,19]. Benzene is primarily metabolized in the liver to a series of ring-hydroxylated and conjugated metabolites as well as ring-opened products which are excreted in urine [20] (Fig. 1). About 12% of benzene uptake is exhaled unchanged and 0.1–0.2% appears unchanged in urine [18].

The first step in benzene metabolism is the formation of the reactive intermediate benzene epoxide which is supposed to be in equilibrium with the unstable oxepin. The benzene epoxide–oxepin system is probably formed enzymatically by cytochrome P450 2E1 via a reactive hydroxyl radical pathway [21]. The reactive intermediates of benzene (epoxide–oxepin) may be subject to at least four different metabolic pathways: (i) nonenzymatic rearrangement of benzene epoxide–oxepin to phenol which can be further hydroxylated to hydroquinone, catechol and 1,2,4-trihydroxybenzene. The hydroxylated metabolites are excreted into the urine unconjugated or as glucuronides or sulfates. Phenol is the main metabolite of benzene (13–50% of the total dose), whereas hydroquinone (5%), catechol (1.3–1.6%), and 1,2,4-trihydroxybenzene are minor metabolites [9,18,19]. Hydroquinone can be oxidized to *p*-benzoquinone, the precursor to 2,5-dihydroxy-phenylmercapturic acid. Alternatively, *p*-benzoquinone may covalently bind to the DNA bases guanine, adenine and cytidine

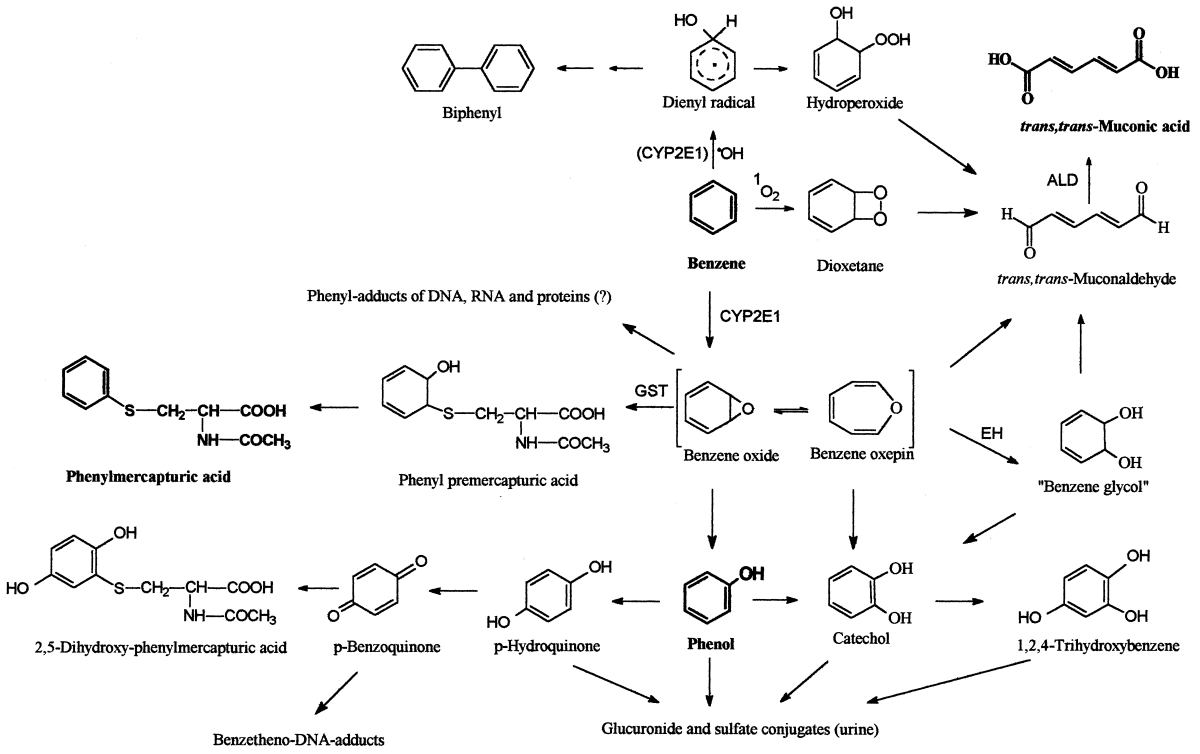

Fig. 1. Metabolic pathways of benzene (according to Sabourin et al. [64], Witz et al. [27], and Johansson et al. [21], modified). Compounds commonly used as biomarkers for benzene exposure are shown in bold characters. Abbreviations: CYP2E1, cytochrome P450 2E1; GST, glutathione-*S*-transferase; EH, epoxide hydrolase.

to yield benzetheno adducts with low binding indices [20]. (ii) Benzene epoxide may be hydrolysed by epoxide hydrolase generating benzene dihydrodiol (also termed 'benzene glycol') which can be enzymatically dehydrogenated to catechol or ring-opened to form *trans,trans*-muconaldehyde, the precursor to *tt*MA [22]. As a further pathway the epoxidation of benzene dihydrodiol to anti-dihydrodiol-epoxide has been postulated [23]. (iii) Benzene epoxide can be conjugated with glutathione ultimately yielding the urinary metabolite *S*-phenylmercapturic acid (PheMA). Only 0.1–0.5% of the total benzene dose is excreted as PheMA [18,24]. (iv) Finally, benzene epoxide can covalently bind to cellular macromolecules such as DNA, RNA and proteins to form phenyl-adducts. N^7-Phenylguanine has been determined in trace amounts in urine of workers exposed to benzene [25]. However, Edman degradation of haemoglobin from individuals exposed to benzene does not yield detectable amounts of phenylvaline [26].

The formation of *trans,trans*-muconaldehyde from benzene and its further metabolism has been investigated primarily by the group of Witz ([22], for review see [27]). Apart from the above mentioned pathway via benzene dihydrodiol, this group has postulated a number of further metabolic pathways to yield *trans,trans*-muconaldehyde (Fig. 2). The epoxidated oxepin may be rearranged to muconaldehyde. Furthermore, 1-hydroxy-2-hydroperoxy-3,5-cyclohexadiene may be formed from benzene by reaction with hydroxyl radicals and subsequent peroxidation prior to ring-opening to yield muconaldehyde. Finally, benzene dioxetane formed by reaction of benzene with singlet oxygen is also a potential precursor of muconaldehyde. Experimental evidence from studies with microsomes and perfused liver suggests that reactive oxygen species may play an important role

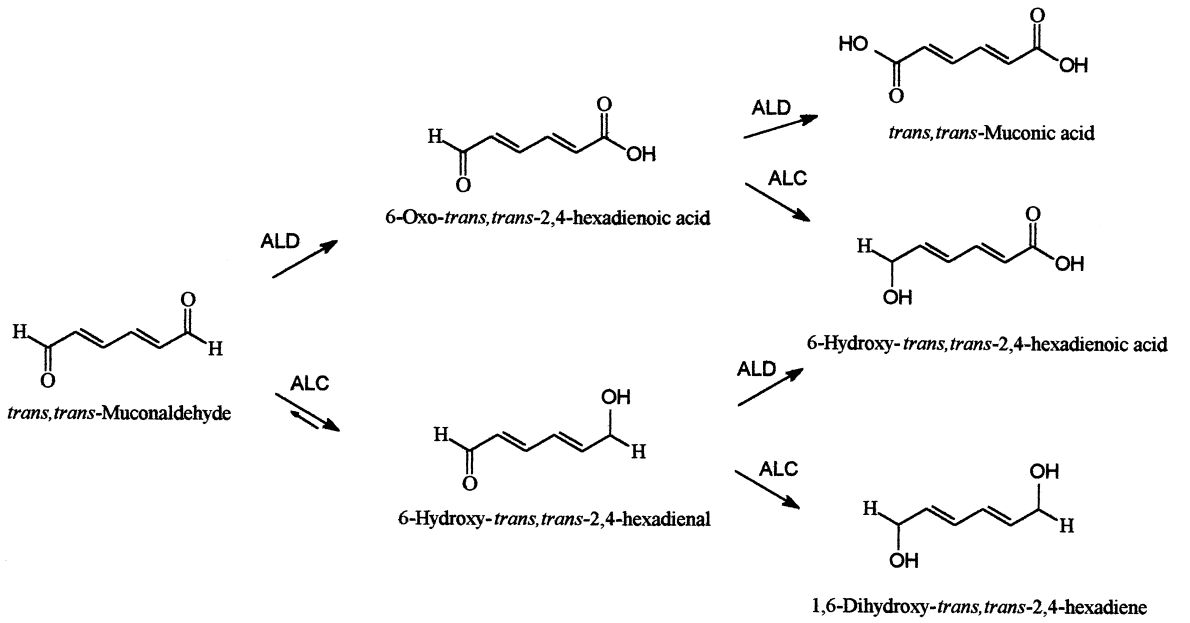

Fig. 2. Metabolic pathways of *trans,trans*-muconaldehyde (according to Witz et al. [27], modified). Abbreviations: ALD, aldehyde dehydrogenase; ALC, alcohol dehydrogenase.

in the ring-opening of benzene which most probably takes place in the liver [27].

trans,trans-Muconaldehyde is subjected to reduction by alcohol dehydrogenases to form alcohols, and oxidation by aldehyde dehydrogenases to yield carboxylic acids (Fig. 2) [27–29]. Among the products of muconaldehyde metabolism, only *tt*MA is a potential biomarker for benzene exposure. About 2–25% of the total benzene dose is excreted as *tt*MA in the urine [9,30,31]. The elimination halflife of *tt*MA in humans has been estimated to be 5 ± 2.3 h [24].

2.3. Factors modifying the urinary ttMA level

2.3.1. Level of benzene exposure

In 1953, Parke and Williams [14] observed that *tt*MA was inversely related to benzene exposure. Today, this effect is well established and applies to all species investigated, including man [32]. The conversion rate of benzene to urinary *tt*MA in man is found to be 1.9% at a TWA_8 exposure level of 10–100 ppm [9], 3.9 (1.9–7.3)% at a TWA_8 exposure level of $<$0.1–20 ppm [30] and 25 (7.2–58)% following 2 h exposure to 0.02–0.07 ppm benzene [31]. The mechanism by which the fraction of ring-breakage metabolites produced at high benzene doses is decreased remains unknown [32]. Exposure to benzene induces cytochrome P450 2E1, which is responsible for the first oxygenation step in benzene metabolism, but also for the generation of oxygen radicals [33]. Therefore, it is unlikely that the induction of cytochrome P450 2E1 is responsible for the observed nonlinear dose dependency of *tt*MA formation. This is in line with data obtained by Schrenk et al. [34], showing that pretreatment of rats with methylcholanthrene (which decreases hepatic CYP2E1) and with isopropanol (an inducer of CYP2E1) does not significantly change the amount of *tt*MA formed by in vitro incubation with isolated hepatocytes.

2.3.2. Coexposure to toluene

Coexposure to toluene is reported to inhibit *tt*MA formation from benzene in rats [35] and humans [9]. This effect has been observed at benzene and toluene concentrations in the ppm range. Since concomitant exposure to benzene and toluene occurs at many

workplaces, coexposure to toluene has to be taken into account when evaluating urinary *tt*MA excretion as a biomarker for occupational benzene exposure. Whether this effect is also of importance at the 100-fold lower levels of environmental benzene and toluene exposure remains to be determined.

2.3.3. Genetic susceptibility

Johnson and Lucier [36] speculated that genetic polymorphism in benzene metabolism might be responsible for the consistent observation that a small proportion of the general population have urinary *tt*MA levels compatible with those seen in persons occupationally exposed to >1 ppm benzene. In a recent study, Gobba et al. [37] found a bimodal distribution of urinary *tt*MA excretion in a group of 80 bus drivers. The mean ratio between *tt*MA and unchanged benzene in urine of the two subgroups amounted to 0.15 and 0.85. Based on this ratio, the authors classified their subjects as 'poor' and 'efficient' *tt*MA metabolizers, respectively. If confirmed, this finding has implications on the evaluation of urinary *tt*MA as an index for benzene exposure. Furthermore, given the hemotoxicity of muconaldehyde [27], the precusor of *tt*MA, a high ratio of *tt*MA:unchanged benzene in urine would indicate increased susceptibility to benzene toxicity [37]. The molecular basis for this polymorphism is as yet unknown.

2.3.4. Pregnancy

Melikian et al. [38] reported an interesting, but as yet unconfirmed effect of pregnancy on the urinary excretion of *tt*MA. The ratio between urinary *tt*MA and cotinine (a marker for tobacco smoke exposure) was higher for pregnant smokers (0.24) than in nonpregnant smokers (0.13). Assuming that smoking was the major source of benzene in these females, *tt*MA formation was 2-fold greater in pregnant than in nonpregnant women. The authors speculate that this could be due to an increase in metabolism of benzene to *tt*MA during pregnancy or, alternatively, that the percentage of benzene metabolized to *tt*MA may be greater at low than at high smoking-related benzene doses [38].

2.3.5. Sorbic acid

More than three decades ago, Westöö [8] observed that mice administered [1-^{14}C]-sorbic acid excreted 0.2–0.6% of the radioactivity as muconic acid. Since sorbic acid is a common food preservative [39] and a fungistatic agent used in pharmaceutical and cosmetic preparations, it is important to take into consideration the contribution of urinary *tt*MA originating from sorbic acid, particularly when evaluating environmental benzene exposure. The conversion rate of sorbic acid to *tt*MA in humans has been investigated by several working groups [40–42]. Ducos et al. [40] reported metabolic rates of 0.13 and 0.18% for two subjects. Ruppert et al. [41] found a mean conversion rate of 0.12 (0.08–0.19) % in eight nonsmokers. Pezzagno and Maestri [42] determined metabolic rates of 0.34% and 0.21% for two subjects. Whether sorbic acid represents a significant confounding factor for urinary *tt*MA excretion as a biomarker of low benzene exposure, depends primarily on the amount of sorbic acid ingested with the diet. Van Dokkum et al. [43] assumed that the average daily intake of sorbic acid ranges from 3 to 30 mg. Based on this assumption, dietary sorbic acid would account for 10–50% of *tt*MA background excretion in nonsmokers and for 5–25% in smokers [41]. In contrast to this, Pezzagno and Maestri [42] assumed that 0.3–0.5 g sorbic acid might be ingested daily, leading to an excretion of 1 mg *tt*MA, a quantity corresponding to a benzene exposure at 1 ppm over about 8 h. More information on the daily intake of sorbic acid is needed for a valid estimation of the contribution of sorbic acid to the urinary *tt*MA background level. In order to correct the amount of *tt*MA in urine from this confounding factor, measuring sorbic acid in urine may be a possibility since it is known that about 0.7% of sorbic acid administered to mice is excreted in urine [8].

2.4. Toxicological aspects

Benzene has been classified as a human carcinogen [44]. At high exposure levels (upper ppm range), benzene was found to have radiomimetic properties. Benzene in high doses may lead to progressive degeneration of the bone marrow and induce aplastic anaemia and leukaemia [33,45]. The hematotoxicity of benzene is believed to be mediated by benzene metabolites and possibly other intermediates including reactive oxygen species [27]. Several different mechanisms may be involved in the toxicity of benzene: covalent binding of *p*-benzo-

quinone to macromolecules (including DNA), cross-linking of proteins or DNA by *trans,trans*-muconaldehyde, depletion of cellular glutathione (an important antioxidant) by *p*-benzoquinone and *trans, trans*-muconaldehyde, oxygen radical production by redox cycling from *p*-benzoquinone, and benzene-induced expression of CYP P450 2E1 [27,33]. A synergistic effect in terms of bone marrow toxicity has been reported for *p*-benzoquinone and *trans, trans*-muconaldehyde [46]. *trans,trans*-Muconaldehyde is also cytotoxic and weakly mutagenic in bacterial and mammalian cells, whereas *tt*MA is >100 times less toxic than *trans,trans*-muconaldehyde and not mutagenic [47,48]. The latter finding is in contrast to a previous report claiming that *tt*MA is mutagenic in an *E. coli* assay [49].

Thus, it appears that *trans,trans*-muconaldehyde is probably a major toxic metabolite of benzene while *tt*MA is a detoxification product of benzene metabolism.

3. Analytical methods

trans,trans-Muconic acid (molecular mass 142.11) crystallizes from water as colourless prisms [melting point 301°C (decomposition), boiling point 320°C]. It is barely soluble in cold water (0.2 g l^{-1} at 15°C), but freely soluble in hot alcohol and glacial acetic acid. The UV-absorption maxima are at 251, 259, and 264 nm (0.1 *M* NaOH) [50]. As a dicarbonic acid it should show two pK_a values at 3–4 and 5–6. However, no pK_a values for *tt*MA could be found in the literature.

3.1. Sample collection and storage

For biomonitoring purposes, postworkshift (or postexposure) spot urine samples or 24-h samples are suitable. In general, samples are stored without further preservation, although, in some cases 1% glacial acid [51] or 1% of 6 *M* hydrochloric acid [52] are added. Samples are normally analyzed within 48 h, or stored frozen at −20 to −25°C. Urinary *tt*MA is reported to be stable for at least 2 weeks at 20°C without preservation [40,53], or 1 month when stored at −20°C with acid preservation [52]. Melikian et al. [38] reported no significant change in the levels of *tt*MA in urine after storage in the dark at −20°C over a period of 9 months. In order to remove any particulate matter, urine samples can be centrifuged for 10 min at 1200 *g* prior to further clean-up steps [54].

3.2. Sample pretreatment

Either liquid–liquid extraction (LLE) or solid-phase extraction (SPE) are employed for sample pretreatment. For LLE, urine samples are centrifuged and acidified by addition of ascorbic acid [55], formic acid [56] or hydrochloric acid [31]. The acidified urine (1–2 ml) is extracted 2–3 times with 3–20 ml of diethyl ether. The diethyl ether layers are combined and evaporated to dryness at ambient temperature under reduced pressure [57] or under a stream of nitrogen [31,56]. The residue is redissolved in a defined volume of the mobile phase for HPLC separation or in a solvent containing the derivatizing reagent for GC analysis. Although LLE of *tt*MA from urine is not very time-consuming and does not require more organic solvent than SPE, the latter is used more frequently, due to its easier handling combined with high reproducibility and recovery for the enrichment of *tt*MA as well as the robustness of the technique [38,40,51].

SPE is performed using disposable cartridges containing 500 mg of strong basic anion-exchange material (SAX), which is preconditioned with 3 ml methanol and 3 ml water. After applying 1–2 ml urine, the cartridge is washed with 3 ml 1% aqueous acetic acid. The *tt*MA is eluted with 3–4 ml of 10% aqueous acetic acid. For HPLC application, the eluate is adjusted to a defined volume before injection. If GC analysis is performed, the eluate is evaporated to dryness, redissolved, and the residue derivatized.

More reproducible recovery rates were found when urine samples were adjusted to pH 7–10 [24,30], 4.9–5.1 [58], or 4.5–5.7 [59] prior to application on SAX columns.

3.3. Analysis by high-performance liquid chromatography

HPLC is the most commonly used technique for the determination of urinary *tt*MA. This is mainly due to its good separation performance with polar, nonvolatile compounds, without the necessity of

derivatization, and the reasonable selective detection with single wavelength UV absorbance or diode array. The pretreated sample (5–100 μl) is injected onto a reversed-phase (C_{18}) HPLC column. The analytes are eluted isocratically with 1% aqueous acetic acid–methanol (90:10, v/v) and detected at a wavelength (246–271 nm) close to the UV absorption maximum of *tt*MA [9,40,51,55,58,60]. Under these conditions, a slight sample enrichment is achieved at the head of the column because the elution strength of the mobile phase is higher than that of the solvent of the injected sample. *tt*MA is eluted within 5–10 column volumes, but the time for the total run normally has to be extended in order to remove late eluting components of the matrix. This can be overcome by the application of gradient elution with mixtures of 1% acetic acid and methanol [29]. Vanillic acid is used as an internal standard in order to control recovery during sample preparation [52,61], but the present method is sufficiently reliable to allow external standardization [40]. In addition, hippuric acid, which is normally present in urine extracts, can be used as retention time marker for the identification of *tt*MA when present at low levels [53]. Table 1 summarizes the experimental conditions for sample pretreatment and *tt*MA analysis by HPLC.

Further improvements of the HPLC separation of *tt*MA can be achieved by using a gradient elution [62], acidified sodium acetate as a mobile phase buffer [59] and diode array detection (DAD) [59]. To improve the LOD (0.01–3 mg l^{-1}), a column switching technique can be used which enables the injection of a 200-μl SPE extract onto a reversed-phase precolumn. After washing the precolumn and column switching to the analytical HPLC column, the LOD can be decreased to 0.003 mg l^{-1} [42,54,63].

A reversed-phase ion-pair separation (RP-IP–HPLC) method using tetrabutylammonium hydrogen sulfate (TBAS) as an ion-pair reagent a gradient of water, methanol and tetrahydrofuran as the mobile phase has been reported [64]; however, it is not suitable for routine application. Ion chromatography, although suitable for the separation of *tt*MA [65], has not yet been applied for the trace analysis of *tt*MA in urine. Since both hydrophobic reversed-phase and ion-exchange interactions influence the chromatographic properties of *tt*MA [66], various mobile phase parameters including pH, ionic strength, type of buffer, amount and type of organic modifier are important in determining analyte mobility. By optimizing these parameters, *tt*MA can be almost completely separated from matrix components by isocratic elution, but further identification by DAD or LC–MS appears to be essential.

3.4. Analysis by gas chromatography

Several methods have been described to determine *tt*MA by gas chromatography. GC has the advantage of a higher resolution capacity than HPLC, and matrix interferences can easily be eliminated. Additionally, the application of mass detection ensures the identification of the analytes and leads to more reliable results. However, derivatization is necessary for GC separation of *tt*MA requiring an extra sample preparation step which is an additional source for diminished recovery rates and increased methodological variation.

Application of SPE with anion-exchange materials prior to analysis by GC requires complete evaporation of the aqueous eluate. For this purpose, the use of a centrifuging evaporator became common [59,68,69].

Volatile derivatives of *tt*MA for GC analysis can be formed by reaction with N,O-bis-(trimethylsilyl)acetamide (BSA) in dimethylformamide [56,70] or N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) [58] yielding trimethylsilyl esters, with pentafluorobenzyl bromide in the presence of triethylamine [38,68,71] yielding pentafluorobenzyl esters and with borontrifluoride–methanol [69] or diazomethane [31,58] yielding dimethylesters.

For control of recovery during complete sample preparation [d_4]-*tt*MA [56,70], 2-bromohexanoic acid [68,69] or *trans*,*trans*-2,5-dimethyl-2,4-hexadienoic acid (DMHA) [58] have been used as internal standards.

Various detection methods have been used including flame ionization detection (FID) [31,58], mass spectrometric (MS) detection [56,68,69] or mass detection with negative ion chemical ionization (NICI-MS) [38]. Details of GC methods reported in the literature are summarized in Table 2.

Table 1
Conditions for sample pretreatment and HPLC separation of *trans,trans*-muconic acid in urine

Author, year	Sample pretreatment[a]	HPLC column	Flow-rate (ml min^{-1})	Mobile phase[b]	Column temperature[c] (°C)	Detection wavelength (nm)	Injection volume[c] (μl)
Gad-El Karim et al., 1985 [55]	LLE: 1 ml urine (pH 3) 2×20 ml diethyl ether	Spherisorb ODS (250×5 mm, 5 μm)	0.4	1% HAc–MeOH (90:10, v/v)	N.R.	254	N.R.
Sabourin et al.,1988 [64]	No sample pretreatment	Alltech C_{18} (259×4.6 mm, 10 μm)	1.0	Gradient of aqueous TBAS, MeOH and THF	N.R.	265	100
Inoue et al., 1989 [9]	No sample pretreatment	Spherisorb ODS (250×4 mm, 5 μm)	1.0	1% HAc–MeOH (90:10, v/v)	N.R.	265	5–10
Schad et al., 1990 [57]	LLE: 1 ml urine (+4 ml H_2O)3×5 ml diethyl ether	Nucleosil 100 $5C_{18}$ (250+4×4 mm, 5 μm)	1.0	10 m*M* H_3PO_4–MeOH (70:30, v/v)	30	271	100
Ducos et al.,1990 [40]	SPE: 1 ml urine 500 mg Bond Elute SAX-SPE	LiChrosorb C_{18} (250×4.6 mm,5 μm)	1.0	1% HAc–MeOH (90:10, v/v)	N.R.	259	10
Schad et al., 1992 [67]	SPE: 4 ml urine 500 mg Bond Elute SAX-SPE	Nucleosil ODS (250×4.6 mm, 5 μm)	1.0	5 m*M* H_3PO_4–MeOH (70:30, v/v)	N.R.	270	20
Goon et al., 1992 [29]	No sample pretreatment	Reversed-phase column	1.0	Gradient of 1% HAc and MeOH	N.R.	246	N.R.
Lee et al., 1993 [52]	SPE: 1 ml urine (+1 ml 0.5 *M* Tris[d]) 300 mg Dowex 1 anion-exchanger	Partisphere $5C_{18}$ (110×4.7 mm, 5 μm)	1–1.5	HAc–MeOH–5 m*M* NaAc (10:100:890, v/v)	N.R	265	5
Melikian et al., 1993 [60]	SPE: 1 ml urine 500 mg Fisher Scientific PrepSep SAX	LiChrosorb C_{18} (250×4.6 mm, 10 μm)	1.0	1% HAc–MeOH (90:10, v/v); gradient	N.R.	264	20
Bartczak et al., 1994 [58]	SPE: 1 ml urine (pH 4.9–5.1) 500 mg Bond Elute SAX-SPE	LiChrosorb RP_{18} (250×4.6 mm, 5 μm)	1.0	1% HAc–MeOH (90:10, v/v)	N.R.	255	30
Rauscher et al., 1994 [51]	SPE: 2 ml urine (acidified) 500 mg SAX SPE-cartridge	Hypersil ODS (100+20×2.1 mm, 5 μm)	0.2	1% HAc–MeOH (90:10, v/v)	N.R.	259	10
Maestri et al., 1995 [54]	SPE: 1 ml urine 500 mg Isolute SAX precolumn[e]: Resolve C_{18} (5×4.6 mm)	Hypersil ODS (250×4.6 mm, 3 μm)	0.7	1% HAc–MeOH (94.5:5.5, v/v)	25	259	200[e]
Boogaard et al., 1995 [24]	SPE: 1 ml urine (pH: 7–10) SAX	Spherisorb 5 ODS-2 (100×2.1 mm, 5 μm)	1.0	1% HAc–MeOH (80–20, v/v)	20	259	20
Boogaard et al., 1996 [30]	SPE: 1 ml urine (pH: 7–10) SAX	Spherisorb 5 ODS-2 (100×2.1 mm, 5 μm)	1.0	1% HAc–MeOH (80:20, v/v)	20	259	20
Buratti et al., 1996 [62]	SPE: 2 ml urine 500 mg Supelco SAX-NR_4^+	Supelcosil C_{18} (50+20×4.6 mm, 3 μm)	Gradient	HCOOH–THF–H_2O (14:17:969, v/v)	N.R.	263	40
Ghittori et al., 1996 [63]	SPE: 1 ml urine 500 mg Bond Elute SAX-SPE precolumn[e]: C_{18} (5 mm)	Hypersil ODS (250×4.6 mm, 3 μm)	0.7	1% HAc–MeOH (94.5:5.5, v/v)	25	259	200[e]
Weaver et al., 1996 [59]	SPE: 1 ml urine (pH 4.5–5.7) 500 mg Fisher Scientific PrepSep SAX	Altima C_{18} (250×4.6 mm, 5 μm)	1.0	1.8 m*M* NaAc (pH 3, HAc) +10% MeOH	40	DAD[f]	20

[a] LLE, liquid–liquid extraction; SPE, solid-phase extraction.
[b] HAc: acetic acid; MeOH: methanol; TBAS: tetrabutylammonium hydrogen sulfate; THF: tetrahydrofuran; NaAc: sodium acetate.
[c] N.R.: not reported.
[d] Tris: tris-(hydroxymethyl)-aminomethane.
[e] Column switching technique was used.
[f] DAD: diode array detection.

Table 2
Conditions for sample pretreatment and GC analysis of *trans,trans*-muconic acid in urine

Author, year	Sample pretreatment	I.S.[a]	Derivatization reagent (solvent)[b]	GC column	GC temperature program	Carrier gas[c]	Injection[c]	Detected ion[d] (*m*/z)	Injection volume[c] (μl)
Bechtold et al., 1991 [56]	LLE: 2 ml urine (+100 μl 88% HCOOH) 2×3 ml diethyl ether	$[d_4]$-*tt*MA	BSA (DMF)	HP Ultra-1 (25 m, 0.25 mm, 0.5 μm)	Initial 80°C, 1 min; 12°C min^{-1}: 265°C	N.R.	Splitless, split on: 0.5 min	271 (*tt*MA) 275 (I.S.)	N.R.
Bartczak et al., 1994 [58]	LLE: 5 ml urine (+1 ml conc. HCl) 2×5 ml diethyl ether	DMHA	Diazomethane (diethyl ether)	DB5 (30 m, 0.25 mm, 0.25 μm)	Initial 50°C; 15°C min^{-1}: 280°C	N_2	N.R.	FID	1
	LLE: urine (+ HCOOH) diethyl ether	$[^{13}C_6]$-*tt*MA	BSTFA	HP Ultra-1 (15 m, 0.2 mm)	Initial 80°C, 2 min; 12°C min^{-1}: 170°C, 1 min; 230°C, 1 min	N.R.	N.R.	271 (*tt*MA) 277 (I.S.)	N.R.
Rauscher et al., 1994 [68]	SPE: 2 ml urine (acidified) 500 mg SAX Speed Vac	2-Bromo-hexanoic acid	$C_6F_5CH_2Br$ (heptane, triethylamine)	HP Ultra-2 (50 m, 0.22 mm, 0.25 μm)	Initial 100°C, 1 min, 10°C min^{-1}: 280°C, 20 min	He	N.R.	502, 321 (*tt*MA)	N.R.
Ruppert et al., 1995 [69]	SPE: 2 ml urine 500 mg Baker bond NR_4^+ Speed Vac	2-Bromo-hexanoic acid	BF_3–methanol (methanol)	HP Ultra-5 (30 m, 0.25 mm, 0.25 μm)	Initial 80°C, 1 min; 20°C min^{-1}: 280°C, 5 min	He	Splitless, split on: 0.5 min	139, 170,(*tt*MA), 154 (I.S.)	1
Weaver et al., 1996 [59]	SPE: 1 ml urine (pH 4.5–5.7) 500 mg FS[e] PrepSep SAX, Speed Vac	None	BSA (DMF)	HP-1 (25 m, 0.2 mm, 0.11 μm)	Initial 100°C, 1 min; 12°C min^{-1}: 265°C	N.R.	N.R.	271 (*tt*MA)	1
Yu et al., 1996 [31]	LLE: 5 ml urine (+1 ml conc. HCl) 2×5 ml diethyl ether	None	Diazomethane (diethyl ether)	DB5(30 m, 0.25 mm, 0.25 μm)	Initial 80°C, 2 min; 12°C min^{-1}: 170°C, 1 min; 250°C, 1 min	N_2; H_2–air[f] (FID)	N.R.	FID	1

[a] DMHA: *tt*-2,5-dimethyl-2,4-hexadienoic acid
[b] BSA: N,O-bis(trimethylsilyl)acetamide; DMF: dimethylformamide; BSTFA: N,O-bis(trimethylsily)trifluoroacetamide; $C_6F_5CH_2Br$: pentafluorobenzyl bromide; BF_3: borontrifluoride.
[c] N.R.: not reported.
[d] Monitored ion of mass detection (*m*/z), if not otherwise stated.
[e] FS: Fisher Scientific.
[f] Burning gas for the flame ionisation detector.

3.5. Comparison and evaluation of the analytical methods

Analysis of urinary *tt*MA for biomonitoring purposes requires analytical procedures which reliably determine this single analyte present in a complex matrix. When monitoring low occupational or environmental exposures to benzene, urinary *tt*MA concentrations are often close to the LOD. Table 3 shows the performance of the reported methods.

Unfortunately, the LOD and recovery rates were not determined in an identical way in all studies and in some reports methods for determining the LOD were not described. Due to poor separation of *tt*MA from matrix interference in some HPLC methods, several attempts have been made to reconfirm the results by GC, reanalysis by HPLC under improved chromatographic conditions (e.g. gradient elution), and application of a more specific detector (e.g. DAD). Analytical quality assurance, even in small sample series, appears to be necessary. The application of GC–MS for the determination of *tt*MA at low levels (<0.1 mg l^{-1}) is advisable. Combining a column switching technique with HPLC (LC-HPLC) results in a sensitivity similar to GC methods [54]. Since Melikian et al. [60] observed a decreasing recovery with increased *tt*MA concentrations (0.1 mg l^{-1}: 98%; 0.2 mg l^{-1}: 93%; 0.4 mg l^{-1}: 90%; 1.0

Table 3
Methodological characteristics and some statistical data of methods reported for the determination of *trans,trans*-muconic acid in urine

Author, year	Sample preparation	Analysis method[a]	Recovery[b] (%)	LOD[b] (mg l^{-1})	Linearity[b] (mg l^{-1})	Precision[b,c]
Gad-El Karim et al., 1985 [55]	LLE	HPLC–UV	37	N.R.	N.R.	N.R.
Sabourin et al., 1988 [64]	–	HPLC–UV	N.R.	N.R.	N.R.	N.R.
Inoue et al., 1989 [9]	–	HPLC–UV	N.R.	0.1	N.R.	5.0% (1.3 mg l^{-1}, $n=10$)
Schad et al., 1990 [57]	LLE	HPLC–UV	90	N.R.	N.R.	N.R.
Ducos et al., 1990 [40]	SPE	HPLC–UV	80	0.05–0.1	N.R.	5.4% (1.0 mg l^{-1}, $n=5$)
Schad et al., 1992 [67]	SPE	HPLC–UV	N.R.	3	N.R.	N.R.
Goon et al., 1992 [29]	–	HPLC–UV	N.R.	N.R.	N.R.	N.R.
Lee et al., 1993 [52]	SPE	HPLC–UV	90	N.R.	0.025–5	7%
Melikian et al., 1993 [60]	SPE	HPLC–UV	98	0.02	N.R.	N.R.
Bartczak et al., 1994 [58]	SPE	HPLC–UV	92	0.02	0.05–5	7% (0.09 mg l^{-1}, $n=6$)
Rauscher et al., 1994 [51]	SPE	HPLC–UV	N.R.	N.R.	0.05–20	N.R.
Popp et al., 1994 [72]	SPE	HPLC–UV	N.R.	N.R.	0.05–10	2.9% (4.5 mg l^{-1})
Maestri et al., 1995 [54]	SPE	LC-HPLC–UV	90	0.003	N.R.	4%
Boogaard et al., 1995 [24]	SPE	HPLC–UV	95	0.01	N.R.	3.2% (0.64 mg l^{-1}, $n=10$)
Boogaard et al., 1996 [30]	SPE	HPLC–UV	95	0.01	N.R.	11% (1.1 mg l^{-1}, $n=60$)
Buratti et al., 1996 [62]	SPE	HPLC–UV	95	0.002	0.006–2	N.R.
Ghittori et al., 1996 [63]	SPE	LC-HPLC–UV	91	0.003	N.R.	3.8% inter-assay
Weaver et al., 1996 [59]	SPE	HPLC–DAD	95	0.02	0.078–5	14% (0.04 mg l^{-1}, $n=3$)
Ong et al., 1996 [73]	SPE	HPLC–UV	90	0.025	N.R.	<10%
Gobba et al., 1997 [37]	SPE	HPLC–UV	97	0.01	N.R.	4.2% inter-assay
Bechtold et al., 1991 [56]	LLE	GC–MS	105	0.01	0.1–25	N.R.
Bartczak et al., 1994 [58]	LLE	GC–FID	92	0.02	0.03–1.2	9.7% (0.04 mg l^{-1}, $n=5$)
Rauscher et al., 1994 [68]	SPE	GC–MS	N.R.	0.01	0.05–10	6% (2.5 mg l^{-1}, $n=6$)
Ruppert et al., 1995 [69]	SPE	GC–MS	97	0.01	0.01–1	7.4% (0.06 mg l^{-1}, $n=17$)
Yu et al., 1996 [31]	LLE	GC–FID	N.R.	0.03	N.R.	N.R.

[a] LC-HPLC–UV: HPLC with column switching technique.
[b] N.R.: not reported.
[c] Percentages refer to intra-assay precision, if not stated otherwise; the concentration in parentheses indicates the level at which precision was determined.

mg l^{-1}: 80%), the addition of an internal standard with similar chemical and physical properties (e.g. adipinic acid, 2-bromohexanoic acid) before sample pretreatment is essential.

4. Application of *tt*MA as a biomarker for benzene exposure

4.1. Occupational exposure

The threshold limit value (TLV) of benzene at the workplace has been lowered in most industrialized countries since it has become clear that benzene is hematotoxic and a human carcinogen. The EC benzene directive [74] sets the TWA_8 at 5 ppm (16 mg m^{-3}) and provides an action level of 1 ppm (3.2 mg m^{-3}). The German technical exposure limit (TRK) for benzene was lowered in 1993 from 5 ppm to 1 ppm for workplaces in general, and to 2.5 ppm for some special occupations [75]. The American Conference of Government Industrial Hygienists (ACGIH) proposed in 1990 to lower the TLV for benzene from 1 to 0.1 ppm [76]. This TLV has not been adopted and the ACGIH revised its proposal to 0.3 ppm in 1994 [3]. The reduction of the TLV of benzene required the replacement of the commonly used phenol in urine as a biomarker for benzene exposure by more specific markers [77,78]. Inoue et al. in 1989 [9] were the first to apply *tt*MA in urine as a biomarker for occupational benzene exposure. Since then, a number of studies using *tt*MA for biomonitoring of benzene exposure at different workplaces have been published (Table 4). With one exception [56], HPLC methods were used for the determination of *tt*MA in urine of exposed workers.

Table 4
Urinary *tt*MA levels after occupational exposure (postshift) to benzene and in nonexposed controls

Author, year	Method (LOD)[a]	Occupation	Exposure level (ppm benzene)[b]	*N*	*tt*MA in urine of exposed workers[b,c]	Correlation[d] and excretion at 1 ppm[e]	Controls	*N*	*tt*MA in urine of nonexposed[b,c]
Inoue et al., 1989 [9]	HPLC (0.1 mg l^{-1})	Shoemakers and painters	Men: 11.1 32.6 60.2 Women: 18.1 42.4 76.4	24 20 15 19 37 29	11.37 mg g^{-1} 47.50 mg g^{-1} 90.10 mg g^{-1} 26.85 mg g^{-1} 45.78 mg g^{-1} 94.94 mg g^{-1}	r=0.860 3.34 mg g^{-1} r=0.858 1.88 mg g^{-1}	Nonexposed workers (some were exposed to up to 7 ppm benzene)	213	<LOD in 137 of 213 subjects
Ducos et al., 1990 [40]	HPLC (0.05–0.1 mg l^{-1})	Workers in a perfume factory	(13–198)	3 (each for 5 d)	10–95 mg g^{-1}	–	Administrative staff in the factory	5	0.16 mg l^{-1}
Bechtold et al., 1991 [56]	GC–MS (0.01 mg l^{-1})	Workers from a rubber factory and a Chinese glue factory	4.4	14	6.2±3.1 mg g^{-1}	r=0.9	Nonexposed workers from alternate locations	8	0.27±0.21 mg g^{-1}
Ducos et al., 1992 [53]	HPLC (0.04 mg l^{-1})	Workers in the perfume industry	9.0 (0.1–75)	23	8.9 (0.1–47.9) mg g^{-1}	r=0.905 1.17 mg l^{-1}	Administrative staff from 3 factories	79	0.13 (<0.04–0.66) mg l^{-1}
Rauscher et al., 1993 [79,83]	HPLC (0.05 mg l^{-1})	Workers				r=0.765 4.06 mg l^{-1}	Nonexposed workers from two plants (<0.08 ppm benzene)	105	a: 0.58±0.80 mg l^{-1} b: 0.59±1.17 mg l^{-1}
		A: oil refinery	0.10±0.16	23	2.79±4.56 mg l^{-1}				
		B: styrene production	0.24±0.24	25	1.19±0.98 mg l^{-1}				
		C: oil refinery	0.50±0.55	32	1.78±2.55 mg l^{-1}				
		D: car mechanics	0.81±0.96	26	1.64±1.32 mg l^{-1}				
Lauwerys et al., 1994 [81]	HPLC (0.05–0.1 mg l^{-1})	Employees working in garages and at coke ovens	Up to 2 ppm	38	at 0.5 ppm: 0.8 mg g^{-1} at 1 ppm: 1.4 mg g^{-1}	r=0.81 1.41 mg g^{-1}	Subjects exposed to <0.01 ppm benzene	35	G.M.:0.130 mg g^{-1} (smokers) 0.06 mg g^{-1} (nonsmokers)
Popp et al., 1994 [72]	HPLC (0.1 mg l^{-1})	Car mechanics	0.8 (max: 3.9)	20	1.28±1.14 mg g^{-1}	r=0.54	Nonexposed car mechanics	6	0.69±0.39 mg g^{-1}
Ong et al., 1995 [19]	HPLC (0.025 mg l^{-1})	Car mechanics, petrol pump attendants workers in a shoe manufacturing plant			G.M. (G.S.D.)	r^2=0.80 1.58 mg g^{-1}	Nonexposed hospital staff or graduate students (all nonsmokers)	40	G.M. (G.S.D.): 0.11 (0.07)
			<1	26	0.36 (0.22) mg g^{-1}				
			1–5	27	4.59 (5.9) mg g^{-1}				
			>5	11	20.89 (11.3)				

Table 4. Continued

Author, year	Method (LOD)[a]	Occupation	Exposure level (ppm benzene)[b]	*N*	*tt*MA in urine of exposed workers[b,c]	Correlation[d] and excretion at 1 ppm[e]	Controls	*N*	*tt*MA in urine of nonexposed[b,c]
Boogaard and Sittert, 1995 [24]	HPLC (0.01 mg l^{-1})	Workers in the petrochemical industry	Median: <0.1–17	188	1 ppm:1.74 mg g^{-1} (calculated)	r=0.959 1.74 mg g^{-1}	Nonexposed workers at the technical and medial departments	55	0.058 (S.E.: 0.013) mg g^{-1} for 14 smokers, <LOD for most nonsmokers
Ghittori et al., 1995 [80]	HPLC (0.05–0.1 mg l^{-1})	Workers in a chemical plant	G.M. (G.S.D.):0.1	145	1 ppm:2.01 mg g^{-1} (calculated)	r=0.58 2.01 mg g^{-1}	Nonexposed workers	40	0.228 mg g^{-1} (smokers) 0.062 mg g^{-1} (nonsmokers)
Ghittori et al., 1996 [63]	HPLC (0.003 mg l^{-1})	Workers in a chemical plant	G.M. (G.S.D.):0.078	171	1 ppm:0.71 mg g^{-1} (calculated)	r=0.614 0.71 mg g^{-1}	Administrative staff from another plant	100	0.225 mg g^{-1} (smokers) 0.114 mg g^{-1} (nonsmokers)
Ong et al., 1996 [73]	HPLC (0.025 mg l^{-1})	Workers from five large petroleum refineries in Singapore	0.12 0.46	103 28	0.10±0.03 mg g^{-1} 0.63±0.04 mg g^{-1}	r=0.55 0.145 mg g^{-1}	Nonexposed, nonsmoking workers (0.014 ppm benzene)	40	0.14±0.07 mg g^{-1}
Kivistö et al., 1997 [82]	HPLC (0.04 mg l^{-1})	Workers in a benzene processing plant and a cokery	1.3 (0.09–11.7) 1.6 (0.06–14.7)	18 20	1.56±1.42 mg l^{-1} 5.40±8.81 mg l^{-1}	r=0.87 3.33 mg l^{-1}	Referents from rural villages (exposed to 0.009 ppm benzene)	29	0.128±0.242 mg l^{-1}
Gobba et al., 1997 [37]	HPLC (0.01 mg l^{-1})	Bus drivers	10–1000 μg m^{-3} (estimated)	77	0.297±0.370 mg g^{-1} (bimodal distribution)		Administrative staff (nonsmokers)	35	0.162 (0.010–0.637) mg g^{-1}

[a] LOD: Limit of detection.

[b] Values are arithmetic means±standard deviation, if not otherwise stated; S.E.: Standard error of the mean; G.M. (G.S.D.): Geometric mean (geometric standard deviation).

[c] mg g^{-1}: milligrams *tt*MA per gram creatinine.

[d] Coefficient of correlation between urinary *tt*MA and benzene exposure level.

[e] Excretion of *tt*MA was calculated using the reported equations for the (log-)linear regression between *tt*MA excretion and benzene exposure.

The LODs of the methods applied were in the range of 0.1–0.01 mg l^{-1}. Studies reporting the lowest occupational exposure to benzene (<1 ppm) [63,72,73,79,80] also showed the weakest correlations ($r<0.8$) between the benzene exposure level and urinary *tt*MA excretion, indicating that some analytical difficulties and/or confounding factors at low urinary *tt*MA levels were present. A similar conclusion must be drawn from the *tt*MA concentrations observed in urine of nonexposed subjects. The reported mean levels for *tt*MA in nonexposed control subjects show approximately a 10-fold variation which can hardly be explained by the influence of smoking alone (Table 4).

Most of the studies on occupational benzene exposure supplied equations for a linear or log-linear regression between urinary *tt*MA excretion and the TWA of the benzene exposure over a working day. We used these equations to calculate the *tt*MA excretion at an exposure level of 1 ppm (Table 4). Based on data of five studies [9,19,24,63,80], exposure to 1 ppm benzene over one working shift leads to creatinine-standardized *tt*MA excretion rates of 0.71–3.34 mg g^{-1} (mean: 1.88 mg g^{-1}). From the log-linear regression of one study [73] an excretion of 0.145 mg g^{-1} is derived. However, we suppose that the reported equation is erroneous. According to equations reported in four studies [53,79,81,82], the calculated postshift *tt*MA concentrations range from 1.17 to 4.06 (mean: 2.49) mg l^{-1}. The German exposure equivalent value (EKA) for postshift urinary *tt*MA is 1.6, 2, 3, 5 and 7 mg l^{-1} for benzene exposure levels of 0.6, 1, 2, 4 and 6 ppm [75,78]. The exposure equivalent for 1 ppm benzene (2 mg l^{-1}) is close to the average calculated from the four studies (2.49 mg l^{-1}). However, there is a rather high variation in the reported data which could have several reasons: linear regressions do not take into account the nonlinearity of the relationship between the benzene exposure dose and the fraction converted to *tt*MA [9,32]. From the studies summarized in Table 4, no clear trend for higher urinary *tt*MA levels at the 1 ppm benzene exposure can be realized for those studies with low occupational exposure to benzene [63,73,79–81] compared to those with high occupational exposure to benzene [9,24,53,82]. Finally, coexposure to toluene which has been shown to inhibit *tt*MA formation from benzene [9,35] has only rarely been taken into account, although it has to be assumed that benzene and toluene are both present at many workplaces.

4.2. Smoking

Benzene concentrations in mainstream smoke (the smoke inhaled by the smoker) range from 6–8 μg/cigarette for ultra and low yield cigarettes [84,85] and 36–68 μg/cigarette for medium to high yield filter and nonfilter cigarettes [84]. An average smoker (20 cigarettes/day) may inhale 0.72–1.36 mg benzene per day due to smoking. This corresponds to an 8 h exposure to 0.03–0.05 ppm benzene assuming a breathing rate of 1 m^3 h^{-1} and a complete absorption of benzene. With somewhat different assumptions, Hoffmann et al. [86] estimated that the smoking-related benzene exposure comes close to the proposed TWA_8 of 0.1 ppm benzene at workplaces, resulting in an amount of 1.54 mg benzene inhaled per day (smoking 20 cigarettes/day: 0.6–1.46 mg/day). The amount of *tt*MA in mainstream smoke is negligible (<0.01 μg/cigarette) [60]. With a conversion rate of benzene to urinary *tt*MA of 2–25% [9,30,31], smoking should, on average, increase the daily *tt*MA excretion by 0.026–0.62 mg (considering the molar mass ratio of *tt*MA:benzene of 142:78). The lower limit of this estimate is at or below the LOD of currently available analytical methods for urinary *tt*MA. In all but two [30,79] of fourteen studies on smoking and *tt*MA excretion, significantly elevated *tt*MA levels were found in smokers (Table 5). The ratio of the urinary *tt*MA level between smokers and nonsmokers ranged from 1.4 to 4.8. The additional amount of *tt*MA excreted by smokers varied from 0.022 to 0.20 (mean: 0.1) mg g^{-1} creatinine. Assuming that the creatinine excretion, on average, is 1.5 g/day [87], this corresponds to a smoking related daily *tt*MA excretion of 0.03–0.30 (mean: 0.15) mg, which is well within the estimated range of 0.026–0.62. One study in Table 5 was omitted from this estimation because rather high urinary *tt*MA levels of 0.96 and 0.44 mg l^{-1} for smokers and nonsmokers, respectively, were reported [68]. The authors stated that they found an unspecific response causing higher apparent *tt*MA levels in single cases when using the HPLC method.

Table 5
Urinary *tt*MA levels in smokers and nonsmokers

Author, year	Method (LOD)[a]	Smokers	*N*	*tt*MA in urine of smokers[b,c]	Correlation[d]	Nonsmokers	*N*	*tt*MA in urine of nonsmokers[b,c]
Melikian et al., 1993 [60]	HPLC (0.02 mg l^{-1})	Males, mean urinary cotinine: 1.6±0.30 mg g^{-1}	42	0.29 mg g^{-1}***	Cotinine (urine): r=0.55	Males, mean urinary cotinine: 0.006±0.003 mg g^{-1}	42	0.09 mg g^{-1} (< LOD in 12 subjects)
Lee et al., 1993 [52]	HPLC (0.025 mg l^{-1})	Hospital staff, no benzene exposure	35	0.19 (0.06–0.43)mg g^{-1}***	No correlation to number of cigarettes	Hospital staff, no benzene exposure	23	0.14 (0.01–0.029) mg g^{-1}
Melikian et al., 1994 [38]	HPLC (0.02 mg l^{-1})	Male smokers	42	0.22±0.03 mg g^{-1}***	–	Male nonsmokers	42	0.06±0.05 mg g^{-1}
		Female smokers	53	0.24±0.02 mg g^{-1}***		Female nonsmokers	37	0.05±0.007 mg g^{-1}
		Pregnant smokers	63	0.22±0.03 mg g^{-1}**		Pregnant nonsmokers	34	0.06±0.02 mg g^{-1}
Rauscher et al., 1994 [68]	HPLC (0.05 mg l^{-1})	Subjects from the general population	22	0.96±1.84 mg l^{-1}	–	Subjects from the general population	58	0.44±0.73 mg l^{-1}
Lauwerys et al., 1994 [81]	HPLC (0.05–0.1 mg l^{-1})	Males	14	G.M.:0.130 mg g^{-1}*	–	Males	21	G.M.:0.06 mg g^{-1}
Ong et al., 1994 [61]	HPLC (0.025 mg l^{-1})	Subjects with no known benzene exposure	46	0.19±0.09 mg g^{-1}*	Cotinine (urine): r=0.47	Subjects with no known benzene exposure	40	0.14±0.07 mg g^{-1}
Ruppert et al., 1995 [69]	GC–MS (0.01 mg l^{-1})	Males, age: 22–42 years	10	0.090±0.035 mg g^{-1}*	–	Males, age: 22–42 years	10	0.054±0.017 mg g^{-1}
Ghittori et al., 1995 [80]	HPLC (0.05–0.01 mg l^{-1})	Workers, mean age: 40 years	20	0.228±0.139 mg g^{-1}*	–	Workers, mean age 40 years	20	0.062±0.043 mg g^{-1}
Ghittori et al., 1996 [63]	HPLC (0.003 mg l^{-1})	Staff exposed to <10 ppb benzene	50	0.255±0.149 mg g^{-1}*	Number of cigarettes: r=0.738	Staff exposed to <10 ppb benzene	50	0.114±0.063 mg g^{-1}
Boogaard and van Sittert, 1996 [30]	HPLC (70 nM ≈0.01 mg l^{-1})	Employees without potential benzene exposure, moderate smokers	14	0.058 (S.E.: 0.013) mg g^{-1}	–	Employees without potential benzene exposure	38	0.036 (S.E.: 0.016 mg g^{-1}
Buratti et al., 1996 [62]	HPLC (LOD: 1.9 μg l^{-1} LOQ: 6.2 μg l^{-1})	Healthy male subjects, working in an urban environment	30	0.100±0.045 μg g^{-1}***	Association to number of cigarettes/day	Healthy male subjects, working in an urban environment	81	0.050±0.036 μg g^{-1}
Ruppert et al., 1997 [41]	GC–MS (0.01 mg l^{-1})	Smokers from a household study	32	Median (range): 0.13 (0.06–0.39)* mg g^{-1}	–	Nonsmokers from a household study	82	Median (range): 0.065 (0.02–0.59) mg g^{-1}
Kivistö et al., 1997 [82]	HPLC (0.04 mg l^{-1})	Smoking referents from a rural village	19	0.199±0.313 mg l^{-1}*	–	Nonsmoking referents from a rural village	20	<0.043±0.213 mg l^{-1}
Renner et al., (in preparation)	GC–MS (0.01 mg l^{-1})	Smokers from the general population	24	0.063±0.046 mg g^{-1}*	Cotinine (urine): r=0.61	Nonsmokers from the general population	43	0.044±0.036 mg g^{-1}

[a] LOD: Limit of detection; LOQ: Limit of quantitation.
[b] Values are arithmetic means±standard deviation, if not otherwise stated; S.E.: standard error of the mean; G.M.: Geometric mean.
[c] mg g^{-1}: milligrams *tt*MA per gram creatinine; statistical significance: *: P<0.05, **: P<0.01, ***: P<0.001 (for differences between smokers and nonsmokers).
[d] Coefficient of correlation between urinary *tt*MA and indicator of smoking intensity.

4.3. Environmental exposure

Small amounts of benzene occur ubiquitously in the environment. Human daily intake of benzene from food and water has been estimated to be 0.02 and 0.015 μg/day, respectively, which would be negligible compared to the benzene exposure originating from ambient air [6]. However, a dietary intake of benzene as high as 250 μg/day has also been estimated [7]. Emissions from traffic exhausts have been identified as a main source for environmental benzene exposure. In Germany, mean benzene concentrations of 1–10 and 10–20 $\mu g\,m^{-3}$ in ambient air of rural and urban areas, respectively, have been reported [88,89]. In the US, the total exposure assessment methodology (TEAM) study revealed an average benzene level in ambient air of 15 $\mu g\,m^{-3}$ [90]. In areas with dense traffic ambient and at gasoline stations, benzene concentrations can increase well above 100 $\mu g\,m^{-3}$ and 10 000 $\mu g\,m^{-3}$, respectively [91]. ETS is considered to be an additional source for benzene exposure in the general population [92]. In 200 nonsmoking homes in the US, a mean benzene concentration of 7 $\mu g\,m^{-3}$ compared to 10.5 $\mu g\,m^{-3}$ in 300 homes with at least one smoker was reported [93]. Median

Table 6
Urinary *tt*MA levels in subjects exposed to environmental levels of benzene

Author, year	Method (LOD)[a]	Subjects	Source of exposure	Variables for exposure	*N*	*tt*MA in urine[b,c]
Scherer et al., 1995 [95]	GC–MS ($0.01\ mg\,l^{-1}$)	Nonsmokers from 30 households age: 4–80 years	ETS	Living in nonsmoking homes	39	92 (27–328) $\mu g\,g^{-1}$
				Living in smoking homes	43	126 (21–594) $\mu g\,g^{-1}$
			Automobile traffic	Suburban, nonsmoking home	24	73 (27–266) $\mu g\,g^{-1}$ A
				City, nonsmoking home	15	124 (31–328) $\mu g\,g^{-1}$ A
				Suburban, smoking home	23	116 (21–402) $\mu g\,g^{-1}$
				City, smoking home	20	138 (31–594) $\mu g\,g^{-1}$
Weaver et al., 1996 [59]	HPLC ($0.016\ mg\,l^{-1}$)	Children (4.3±1.6 years) participating in a lead poisoning study	ETS	Urinary cotinine ≤44 ng/ml	39	64±3 $\mu g\,g^{-1}$ d
				Urinary cotinine >44 ng/ml	39	91±3 $\mu g\,g^{-1}$ d
			Automobile traffic	Time spent near the street <60 min	33	50±3 $\mu g\,g^{-1}$ A
				Time spent near the street ≥60 min	35	102±4 $\mu g\,g^{-1}$ A
Yu et al., 1996 [31]	GC–FID ($0.029\ mg\,l^{-1}$)	Female volunteers participating in an experimental exposure study	ETS	Nonexposure days	5	34–74 μg (total amount excreted)
				Exposure days (2 h in an ETS filled room, benzene level: 60–224 $\mu g\,m^{-3}$)	5	42–95 μg (total amount excreted)
Buratti et al., 1996 [62]	HPLC (LOD: $1.9\ \mu g\,l^{-1}$ LOQ: $6.2\ \mu g\,l^{-1}$)	Healthy male subjects working in an urban environment	ETS	Subjects without ETS exposure	60	76±49 μg^{-1}
				Subjects with ETS exposure	22	77±60 μg^{-1}
Renner et al., (in preparation)	GC–MS ($0.01\ mg\,l^{-1}$)	Nonsmokers from the general population	ETS	Subjects reporting no or only minor exposure to ETS	21	32±22 $\mu g\,g^{-1}$
				Subjects reporting exposure to ETS	22	54±40 $\mu g\,g^{-1}$

[a] LOD: Limit of detection; LOQ: Limit of quantitation.

[b] Values are arithmetic means±standard deviation, if not otherwise stated.

[c] $mg\,g^{-1}$: milligrams *tt*MA per gram creatinine; Values within one study marked with the same capital letter are significantly different, $P<0.05$.

[d] Calculated from natural-log-transformed values presented as mean±S.D. in the original paper [59].

benzene levels in 230 nonsmoking and smoking homes in Germany were 6.9 and 9.3 $\mu g\,m^{-3}$, respectively [94].

Assuming a breathing rate of 20 m^3/day [6], complete absorption of benzene, and a 25% conversion rate of benzene to urinary *tt*MA [31], exposure to an average concentration of 20 $\mu g\,m^{-3}$ benzene in ambient air (corresponding to an area with rather dense automotive traffic) would result in a (maximum) urinary *tt*MA excretion of 180 μg/day. At a background exposure of 5 $\mu g\,m^{-3}$, the *tt*MA excretion would be about 45 μg/day. With an ETS-related increase in indoor benzene concentration of 5 $\mu g\,m^{-3}$ and a daily exposure duration of 8 h, an upper limit for benzene uptake of 40 μg/day can be calculated (a breathing rate of 1 $m^3\,h^{-1}$ is assumed) resulting in an urinary *tt*MA excretion of 18 μg/day. It is clear from this rough estimate that real-life ETS exposure is unlikely to lead to a measurable increase in urinary *tt*MA excretion. This is in agreement with four field studies ([59,62,95], Renner et al., in preparation] which found no significant increase in *tt*MA excretion in ETS exposed nonsmokers (Table 6) compared to nonsmokers not exposed to ETS. In a study with high experimental ETS exposure (benzene level in indoor air: 60–224 $\mu g\,m^{-3}$), the total amount of *tt*MA excreted increased, on average, only by about 15 μg after exposure to ETS compared to the pre-exposure background excretion [31].

The influence of automobile traffic density on the *tt*MA level was investigated in two studies [59,95] (Table 6). Small but significant increases were observed for nonsmokers living or staying in areas with higher traffic density. However, the investigations are too small and the effects reported inconsistent so that no firm conclusions may be drawn.

The background excretion level for *tt*MA in nonsmokers, neither exposed to traffic exhaust nor to ETS, ranges from 32–92 $\mu g\,g^{-1}$ (Table 6) (corresponding to a *tt*MA excretion of 48–138 μg/day, when a daily creatinine excretion of 1.5 g is assumed). This is higher than would have been estimated as a background excretion for *tt*MA (45 μg/day). In our view, two major sources may contribute to the level and variation of the *tt*MA background in urine and thus limit the suitability of *tt*MA as a biomarker for environmental benzene exposure: the ingestion of unknown amounts of sorbic acid in the diet (see Section 2.3.5) and the potential contamination of the food with benzene [6,7]. In addition, the as yet unclear genetic polymorphism in benzene metabolism [37] may also increase the variability in urinary *tt*MA levels.

4.4. Comparison with other biomarkers for benzene exposure

The biological monitoring of occupational and environmental exposure to benzene has been discussed in detail in three recent reviews [18,78,96]. Potential biomarkers for benzene exposure can be divided into three groups: (i) nonmetabolized benzene in exhaled air, blood and urine, (ii) benzene metabolites in urine such as ring-hydroxylated compounds (phenol), ring-opened compounds (*tt*MA) and glutathione adducts (*S*-phenylmercapturic acid), (iii) adducts with DNA (N^7-phenylguanine), haemoglobin or albumin (N-phenylvaline, *S*-phenylcysteine). For human biomonitoring purposes, only markers of the first two groups have been applied. In Table 7, coefficients of correlation between various biomarkers for benzene and *tt*MA as well as between these biomarkers and the benzene exposure levels are listed. Correlations between all biomarkers (except for phenol due to its low specificity at exposure levels below 5 ppm) and the benzene levels in air are usually strong ($r>0.5$) at occupational exposure levels (>0.1 ppm). In only a few studies [73,79,80] are correlations reported for low benzene exposure approaching environmental levels.

Urinary *tt*MA has been regarded as a suitable biomarker for exposure to benzene down to levels of 1 ppm [96] or even lower (0.5 ppm [78], 0.3 ppm [73]). In the range of environmental benzene exposure (<0.01 ppm), other biomarkers such as unmetabolized benzene in exhalate, blood or urine as well as *S*-phenylmercapturic acid in urine appear to be more specific [18,78,96]. However, these markers have other disadvantages: the very short initial half-life of about 1 h for benzene in exhalate and blood requires timely sampling; the potential contamination with benzene when measuring the parent compound in body fluids or exhalate, particularly at low levels, requires special precautions during sampling and analysis; the analytical method for *S*-phenylmercapturic acid in urine is highly sophisticated [78].

Table 7
Comparison between *tt*MA and other biomarkers for exposure to benzene

Author, year	Range of benzene exposure (ppm)	Variables correlated[a]	Coefficient of correlation[b]
Ducos et al., 1992 [53]	0.1–75	*tt*MA in urine vs. benzene in air	$r=0.905^{***}$ ($N=105$)
		Phenol in urine vs. benzene in air	$r=0.828^{***}$ ($N=105$)
		*tt*MA in urine vs. phenol in urine	$r=0.857^{***}$ ($N=105$)
Rauscher et al., 1993 [79]	0.10–0.81 (means)	*tt*MA in urine vs. benzene in blood	$r=0.416^{*}$ ($N=106$)
Popp et al., 1994 [72]	Max.: 3.9	*tt*MA in urine vs. benzene in air	$r=0.54^{*}$ ($N=20$)
	Mean: 0.8	PheMA in urine vs. benzene in air	$r=0.81^{***}$ ($N=20$)
		Benzene in blood vs. benzene in air	$r=0.44$ ($N=20$)
Boogaard and van Sittert, 1995 [24]	0.01–200	*tt*MA in urine vs. benzene in air	$r=0.959^{***}$ ($N=58$)
		PheMA in urine vs. benzene in air	$r=0.968^{***}$ ($N=58$)
		*tt*MA in urine vs. PheMA in urine	$r=0.795^{***}$ ($N=188$)
Ghittori et al., 1995 [80]	0.01–30	*tt*MA in urine vs. benzene in air	$r=0.58^{***}$ ($N=145$)
		PheMA in urine vs. benzene in air	$r=0.74^{***}$ ($N=145$)
		Benzene in urine vs. benzene in air	$r=0.66^{***}$ ($N=145$)
		*tt*MA in urine vs. PheMA in urine	$r=0.57^{*}$? ($N=145$)
		*tt*MA in urine vs. benzene in urine	$r=0.57^{*}$? ($N=145$)
	0.01–0.5	*tt*MA in urine vs. benzene in air	$r=0.56^{***}$ ($N=122$)
		PheMA in urine vs. benzene in air	$r=0.63^{***}$ ($N=123$)
		Benzene in urine vs. benzene in air	$r=0.54^{***}$ ($N=124$)
Ong et al., 1995 [19]	>5	*tt*MA in urine vs. benzene in air	$r=0.76^{***}$ ($N=11$)
		Benzene in urine vs. benzene in air	$r=0.22$ ($N=11$)
		Phenol in urine vs. benzene in air	$r=0.70^{*}$ ($N=11$)
	1–5	*tt*MA in urine vs. benzene in air	$r=0.75^{***}$ ($N=27$)
		Benzene in urine vs. benzene in air	$r=0.70^{**}$ ($N=27$)
		Phenol in urine vs. benzene in air	$r=0.50$ ($N=27$)
	<1	*tt*MA in urine vs. benzene in air	$r=0.82^{***}$ ($N=26$)
		Phenol in urine vs. benzene in air	$r=0.14$ ($N=26$)
Ong et al., 1996 [73]	>0.25	*tt*MA in urine vs. benzene in air	$r=0.55^{*}$ ($N=28$)
		Benzene in blood vs. benzene in air	$r=0.44$ ($N=12$)
		Benzene in urine vs. benzene in air	$r=0.71^{**}$ ($N=19$)
		Phenol in urine vs. benzene in air	$r=0.32$ ($N=28$)
	>0.25	*tt*MA in urine vs. benzene in air	$r=0.14$ ($N=103$)
		Benzene in blood vs. benzene in air	$r=0.12$ ($N=49$)
		Benzene in urine vs. benzene in air	$r=0.35^{*}$ ($N=100$)
		Phenol in urine vs. benzene in air	$r=0.18$ ($N=103$)
Kivistö et al., 1997 [82]	0.03–14.7	*tt*MA in urine vs. benzene in air	$r=0.87$ *? ($N=39$)
		Benzene in blood vs. benzene in air	$r=0.96$ *? ($N=46$)
		Benzene in urine vs. benzene in air	$r=0.97$ *? ($N=13$)
		Benzene in exhalate vs. benzene in air	$r=0.95$ *? ($N=20$)
		PheMA in urine vs. benzene in air	$r=0.97$ *? ($N=41$)
		*tt*MA in urine vs. benzene in blood	$r=0.86$ *? ($N=43$)
		*tt*MA in urine vs. PheMA in urine	$r=0.84$ *? ($N=43$)
		*tt*MA in urine vs. benzene in urine	$r=0.96$ *? ($N=14$)
		*tt*MA in urine vs. benzene in exhalate	$r=0.84$ *? ($N=18$)
	<1	*tt*MA in urine vs. benzene in air	$r=0.80$ *? ($N=29$)
		Benzene in blood vs. benzene in air	$r=0.79$ *? ($N=36$)
		PheMA in urine vs. benzene in air	$r=0.39$ *? ($N=31$)
Renner et al. (in preparation)	<0.01 (estimated)	*tt*MA in urine vs. PheMA in urine	$r=0.38^{**}$ ($N=67$)

[a] In some studies the variables were log-transformed before correlation; PheMA: *S*-phenylmercapturic acid.
[b] Statistical significance: *: $P<0.05$, **: $P<0.01$, ***: $P<0.001$; *?: level of significance not indicated in the article.

We have recently determined *tt*MA and *S*-phenylmercapturic acid in urine of environmentally exposed subjects from the general population and found a weak but significant association between both biomarkers ($r=0.38$) [Renner et al., in preparation]. However, we excluded two nonexposed nonsmokers who exhibited unusually high *tt*MA (>1000 μg/day) but low *S*-phenylmercapturic acid excretions. We believe that intake of high amounts of dietary sorbic acid probably caused the elevated urinary *tt*MA excretion in the nonexposed subjects. Therefore, in our view *tt*MA in urine is also a suitable biomarker for environmental benzene exposure, as long as the intake of sorbic acid is adequately taken into account.

5. Conclusions

Due to its carcinogenic and hematotoxic properties together with its widespread occurrence, benzene is an important occupational and environmental contaminant. Dependent on the benzene exposure level, metabolic conversion and excretion as *tt*MA in urine amounts to 2–25% of the benzene dose. Other factors such as simultaneous exposure to toluene, metabolic polymorphisms, and sorbic acid used as a food preservative may influence the background level of urinary *tt*MA excretion. Although HPLC methods are sensitive enough to allow determination of urinary *tt*MA in concentrations as low as 0.05–0.1 $mg\,l^{-1}$, a requirement for determination of occupational exposure to benzene, GC–MS with LODs of 0.01 $mg\,l^{-1}$ is preferable for the biomonitoring of subjects exposed to environmental levels of benzene. The use of an internal standard in order to control the recovery of *tt*MA during sample preparation is recommended for both HPLC and GC analysis. The use of a reference compound, suitable as a retention time marker in HPLC is also advisable.

Urinary *tt*MA has been widely used for occupational biomonitoring of benzene exposure at the workplace. Strong correlations are usually observed between *tt*MA excretion in urine and occupational benzene levels above 0.1 ppm. A TWA_8 exposure to 1 ppm benzene results, on average, in the excretion of 1.9 mg *tt*MA g^{-1} creatinine or 2.5 $mg\,l^{-1}$ urine. In almost all studies investigating the relationship between urinary *tt*MA and smoking, a significant increase in urinary *tt*MA is observed in smokers compared to nonsmoksers, ranging from 0.022 to 0.2 $mg\,g^{-1}$ creatinine.

Environmental exposure to ambient benzene slightly increased *tt*MA levels in urine of subjects living in areas with a high traffic density while exposure to benzene in ETS appears to be too small to be measurable by urinary *tt*MA excretion.

In comparison to other biomarkers for benzene exposure, urinary *tt*MA has advantages in terms of specificity, sensitivity and simplicity of analysis at occupational exposure levels above 0.1 ppm benzene. However, for biomonitoring benzene exposure in the environmental range (<0.01 ppm), several potential confounding factors including dietary sorbic acid intake and metabolic polymorphism in benzene conversion to *tt*MA need to be taken into consideration.

References

[1] M. Jaffé, Z. Physiol. Chem. 62 (1909) 58.
[2] International Agency for Research on Cancer, IARC Monographs on the evaluation of the carcinogenic risk of chemicals to humans. Some industrial chemicals and dyestuffs. Benzene, Vol. 29, IARC, Lyon, 1982.
[3] American Conference of Governmental Industrial Hygienists (ACGIH). Threshold limit values and biological exposure indices for 1994–1995; Cincinnati, OH.
[4] S. Fuselli, in: M. Imbriani, S. Ghittori, G. Pezzagno, E. Capodaglio (Editors), Advances in Occupational Medicine and Rehabilitation. Update on Benzene, Fondazione Salvatore Maugeri Edizioni, Pavia, 1995, p. 9.
[5] K.D. Brunnemann, M.R. Kagan, J.E. Cox, D. Hoffmann, Exp. Pathol. 37 (1989) 108.
[6] H.A. Hattemer-Frey, C.C. Travis, M.L. Land, Environ. Res. 53 (1990) 221.
[7] National Research Council. Drinking water and health; Washington DC, National Academy of Sciences Press, Vol. 3, pp. 80–86, 261–262.
[8] G. Westöö, Acta Chem. Scand. 18 (1964) 1373.
[9] O. Inoue, K. Seiji, H. Nakatsuka, T. Watanabe, S.-N. Yin, G.-L. Li, S.-X. Cai, C. Jin, M. Ikeda, Br. J. Ind. Med. 46 (1989) 122.
[10] D. Kaniansky, M. Masár, J. Madajová, J. Chromatogr. A 677 (1994) 179.
[11] Ministry of Agriculture, Fisheries and Food, Dietary intake of food additives in the UK, HMSO, London, 1993.
[12] P. Ducos, R. Gaudin, in: M. Imbriani, S. Ghittori, G. Pezzagno, E. Capodaglio (Editors), Advances in Occupational Medicine and Rehabilitation, Fondazione Salvatore Maugeri Edizioni, Pavia, 1995, p. 189.

[13] D. Fuchs, A. Soòs, Z. Physiol. Chem. 98 (1916) 11.
[14] D.V. Parke, R.T. Williams, Biochem. J. 54 (1953) 231.
[15] D.V. Parke, R.T. Williams, Biochem. J. 51 (1951) 339.
[16] J.C. Drummond, I.L. Finar, Biochem. J. 32 (1938) 79.
[17] M.M. Gad-El-Karim, V.M. Sadagopa Ramanujam, M.S. Legator, Xenobiotica 15 (1985) 211.
[18] C.-N. Ong, B.-L. Lee, J. Chromatogr. B 660 (1994) 1.
[19] C.N. Ong, P.W. Kok, B.L. Lee, C.Y. Shi, H.Y. Ong, K.S. Chia, C.S. Lee, X.W. Luo, Occup. Environ. Med. 52 (1995) 528.
[20] R. Snyder, C.C. Hedli, Environ. Health Perspect. 104 (1996) 1165.
[21] I. Johansson, M. Ingelman-Sundberg, J. Biol. Chem. 258 (1983) 7311.
[22] L. Latriano, B.D. Goldstein, G. Witz, Proc. Natl. Acad. Sci. USA 83 (1986) 8356.
[23] H. Glatt, R. Padykula, G.A. Berchtold, G. Ludewig, K.-L. Platt, J. Klein, F. Oesch, Environ. Health Perspect. 82 (1989) 81.
[24] P.J. Boogaard, N.J. Van Sittert, Occup. Environ. Med. 52 (1995) 611.
[25] K. Norpoth, W. Stücker, E. Krewet, G. Müller, Int. Arch. Occup. Environ. Health 60 (1988) 163.
[26] M. Bader, G. Lehnert, J. Angerer, Int. Arch. Occup. Environ. Health 65 (1994) 411.
[27] G. Witz, Z. Zhang, B.D. Goldstein, Environ. Health Perspect. 104 (1996) 1195.
[28] T.A. Kirley, B.D. Goldstein, W.M. Maniara, G. Witz, Toxicol. Appl. Pharmacol. 100 (1989) 360.
[29] D. Goon, X. Cheng, J.A. Ruth, D.R. Petersen, D. Ross, Toxicol. Appl. Pharmacol. 114 (1992) 147.
[30] P.J. Boogaard, N.J. Van Sittert, Environ. Health Perspect. 104 (1996) 1151.
[31] R. Yu, C.P. Weisel, J. Toxicol. Environ. Health 48 (1996) 453.
[32] R.F. Henderson, Environ. Health Perspect. 104 (1996) 1173.
[33] A. Yardley-Jones, D. Anderson, D.V. Parke, Br. J. Ind. Med. 48 (1991) 437.
[34] D. Schrenk, A. Orzechowski, L.R. Schwarz, R. Snyder, B. Burchell, M. Ingelman-Sundberg, K.W. Bock, Environ. Health Perspect. 104 (1996) 1183.
[35] M.T. Brondeau, P. Ducos, R. Gaudin, G. Morel, P. Bonnet, J. de Ceaurriz, Toxicol. Lett. 61 (1992) 311.
[36] E.S. Johnson, G. Lucier, Am. J. Ind. Med. 21 (1992) 749.
[37] F. Gobba, S. Rovesti, P. Borella, R. Vivoli, E. Caselgrandi, G. Vivoli, Sci. Total Environ. 199 (1997) 41.
[38] A.A. Melikian, A.K. Prahalad, R.H. Secker-Walker, Cancer Epidemiol. Biomarkers Prev. 3 (1994) 239.
[39] E. Lück, Food Add. Contam. 7 (1990) 711.
[40] P. Ducos, R. Gaudin, A. Robert, J.M. Francin, C. Maire, Int. Arch. Occup. Environ. Health 62 (1990) 529.
[41] T. Ruppert, G. Scherer, A.R. Tricker, F. Adlkofer, Int. Arch. Occup. Environ. Health 69 (1997) 247.
[42] G. Pezzagno, L. Maestri, Indoor Built Environ. 6 (1997) 12.
[43] W. van Dokkum, R.H. de Vos, F.A. Cloughley, K.F.A.M. Hulshof, F. Dukel, J.A. Wijsman, Br. J. Nutr. 48 (1982) 223.
[44] International Agency for Research on Cancer, IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Overall Evaluations of Carcinogenicity: An updating of IARC Monographs, IARC, Lyon, 1987, p. 120.
[45] G.F. Kalf, R. Snyder, in: M. Imbriani, S. Ghittori, G. Pezzagno, E. Capodaglio (Editors), Advances in Occupational Medicine and Rehabilitation, Fondazione Salvatore Maugeri Edizioni, Pavia, 1995, p. 39.
[46] R. Snyder, E. Dimitruadis, R. Guy, P. Hu, K. Cooper, H. Bauer, G. Witz, B.D. Goldstein, Environ. Health Perspect. 82 (1989) 31.
[47] H. Glatt, G. Witz, Mutagenesis 5 (1990) 263.
[48] G. Witz, S.C. Gad, R.R. Tice, Y. Oshiro, C.E. Piper, B.D. Goldstein, Mutat. Res. 240 (1990) 295.
[49] T.G. Rossman, C.B. Klein, C.A. Snyder, Environ. Health Perspect. 81 (1989) 77.
[50] M. Windholz, in: M. Windholz, S. Budavari, R.F. Blumetti, E.S. Otterbein (Editors), The Merck Index, 10th edition, Merck, Rayway, NJ, 1983, p. 902.
[51] D. Rauscher, G. Lehnert, J. Angerer, Clin. Chem. 40 (1994) 1468.
[52] B.-L. Lee, A.-L. New, P.-W. Kok, H.-Y. Ong, C.-Y. Shi, C.-N. Ong, Clin. Chem. 39 (1993) 1788.
[53] P. Ducos, R. Gaudin, J. Bel, C. Maire, J.M. Francin, A. Robert, P. Wild, Int. Arch. Occup. Environ. Health 64 (1992) 309.
[54] L. Maestri, S. Ghittori, M.L. Fiorentino, M. Imbriani, Med. Lav. 86 (1995) 40.
[55] M.M. Gad-El Karim, V.M.S. Ramanujam, M.S. Legator, Xenobiotica 15 (1985) 211.
[56] W.E. Bechtold, G. Lucier, L.S. Birnbaum, S.N. Yin, G.L. Li, R.F. Henderson, Am. Ind. Hyg. Assoc. J. 52 (1991) 473.
[57] H. Schad, F. Schäfer, L. Weber, in: F. Schuckmann, S. Schopper-Jochum (Editors), Verhandlungen der Deutschen Gesellschaft für Arbeitsmedizin e.V, Gentner Verlag, Stuttgart, 1990, p. 263.
[58] A. Bartczak, S.A. Kline, R. Yu, C.P. Weisel, B.D. Goldstein, G. Witz, W.E. Bechtold, J. Toxicol. Environ. Health 42 (1994) 245.
[59] V.M. Weaver, C.T. Davoli, P.J. Heller, A. Fitzwilliam, H.L. Peters, J. Sunyer, S.E. Murphy, G.W. Goldstein, J.D. Groopman, Environ. Health Perspect. 104 (1996) 318.
[60] A.A. Melikian, A.K. Prahalad, D. Hoffmann, Cancer Epidemiol. Biomarkers Prev. 2 (1993) 47.
[61] C.N. Ong, B.L. Lee, C.Y. Shi, H.Y. Ong, H.P. Lee, Int. J. Cancer 59 (1994) 177.
[62] M. Buratti, S. Fustinoni, A. Colombi, J. Chromatogr. A 677 (1996) 257.
[63] S. Ghittori, L. Maestri, L. Rolandi, L. Lodola, M.L. Fiorention, M. Imbriani, Appl. Occup. Environ. Hyg. 11 (1996) 187.
[64] P.J. Sabourin, W.E. Bechtold, R.F. Henderson, Anal. Biochem. 170 (1988) 316.
[65] W. Elbert, J. Hahn, M. Lerch, Int. J. Environ. Anal. Chem. 35 (1989) 149.
[66] J.J. Johnston, W.M. Draper, R.D. Stephens, J. Chromatogr. Sci. 29 (1991) 511.

[67] H. Schad, F. Schäfer, L. Weber, H.J. Seidel, J. Chromatogr. 593 (1992) 147.
[68] D. Rauscher, M. Bader, J. Angerer. The *t,t*-muconic acid excretion of the general population caused by environmental benzene exposures; Poster presented at the 1st International Congress on Environmental Medicine, February 23–26, Duisburg, 1994.
[69] T. Ruppert, G. Scherer, A.R. Tricker, D. Rauscher, F. Adlkofer, J. Chromatogr. B 666 (1995) 71.
[70] W.E. Bechtold, R.F. Henderson, J. Toxicol. Environ. Health 40 (1993) 377.
[71] J. Begerow, J. Angerer, Fresenius J. Anal. Chem. 366 (1990) 42.
[72] W. Popp, D. Rauscher, G. Müller, J. Angerer, K. Norpoth, Int. Arch. Occup. Environ. Health 66 (1994) 1.
[73] C.N. Ong, P.W. Kok, H.Y. Ong, C.Y. Shi, B.L. Lee, W.H. Phoon, K.T. Tan, Occup. Environ. Med. 53 (1996) 328.
[74] Environmental Health Executive. Occupational exposure limits; London: HMSO (EH40/94).
[75] Deutsche Forschungsgemeinschaft (DFG), Maximale Arbeitsplatzkonzentrationen und Biologische Arbeitsstofftoleranzwerte 1994, VCH Verlagsgesellschaft mbH, Weinheim, 1994.
[76] American Conference of Governmental Industrial Hygienists, Applied Occupational and Environmental Hygiene, 5 (1990) 453.
[77] D. Rauscher, K.H. Schaller, J. Angerer, in: R. Kessler (Editor), Arbeitsmedizinische und umweltmedizinische Aspekte zu Altlasten. - Bewertung und Bewältigung, Gentner Verlag, Stuttgart, 1994, p. 377.
[78] K.H. Schaller, in: M. Imbriani, S. Ghittori, G. Pezzagno, E. Capodaglio (Editors), Advances in Occupational Medicine and Rehabilitation, Fondazione Salvatore Maugeri Edizioni, Pavia, 1995, p. 135.
[79] D. Rauscher, G. Scherer, K.H. Schaller, J. Angerer. *t,t*-muconic acid as a biomarker for an occupationally and environmentally caused benzene exposure; Poster presented at The 24th International Congress on Occupational Health, September 9–October 1, Nice, 1993.
[80] S. Ghittori, L. Maestri, M.L. Fiorentino, M. Imbriani, Int. Arch. Occup. Environ. Health 67 (1995) 195.
[81] R.R. Lauwerys, J.-P. Buchet, F. Andrien, Am. J. Ind. Med. 25 (1994) 297.
[82] H. Kivistö, K. Pekari, K. Peltonen, J. Svinhufvud, T. Veidebaum, M. Sorsa, A. Aitio, Sci. Total Environ. 199 (1997) 49.
[83] D. Rauscher, G. Lehnert, J. Angerer. Biomonitoring of occupational and environmental benzene exposures by measuring the *trans*, *trans*-muconic acid excretion in urine; Poster presented at The First Arnold O. Beckman IFCC European Conference on Environmental Toxicology; Biomarkers of Chemical Exposure, June 16–18, Munich, 1993.
[84] K.D. Brunnemann, M.R. Kagan, J.E. Cox, D. Hoffmann, Carcinogenesis 11 (1990) 1863.
[85] G.D. Byrd, K.W. Fowler, R.D. Hicks, M.E. Lovette, M.F. Borgerding, J. Chromatogr. 503 (1990) 359.
[86] D. Hoffmann, A.A. Melikian, E.L. Wynder, Prev. Med. 25 (1996) 14.
[87] M.F. Boeniger, L.K. Lowry, J. Rosenberg, Am. Ind. Hyg. Assoc. J. 54 (1993) 615.
[88] J. Müller, Staub-Reinhalt. Luft 51 (1991) 147.
[89] H.W. Chriske, A. Brockhaus, U. Ewers, Arbeitsmed. Sozialmed. Präventivmed. 26 (1991) 483.
[90] T.D. Hartwell, E.D. Pellizzari, R.L. Perritt, R.W. Whitmore, H.S. Zelon, L. Wallace, Atm. Environ. 21 (1987) 2413.
[91] F. Adlkofer, G. Scherer, C. Conze, J. Angerer, G. Lehnert, J. Cancer Res. Clin. Oncol. 116 (1990) 591.
[92] L.A. Wallace, E.D. Pellizari, T.D. Martwell, R. Perritt, R. Ziegenfus, Arch. Environ. Health 42 (1987) 272.
[93] L.A. Wallace, Environ. Health Perspect. 82 (1989) 165.
[94] C. Krause, W. Mailahn, R. Nagel, C. Schulz, B. Seifert, D. Ullrich, in: B. Seifert, H. Esdorn, M. Fischer, H. Rüden, J. Wegner (Editors), Indoor Air '87. Proceedings of the 4th International Conference on Indoor Air Quality and Climate. Volatile Organic Compounds, Combustion Gases, Particles and Fibres, Microbiological Agents, Institute for Water, Soil and Air Hygiene, Berlin, 1987, p. 102.
[95] G. Scherer, T. Ruppert, H. Daube, I. Kossien, K. Riedel, A.R. Tricker, F. Adlkofer, Environ. Int. 21 (1995) 779.
[96] A.M. Medeiros, M.G. Bird, G. Witz, J. Toxicol. Environ. Health 51 (1997) 519.