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Esmaeel Soleimani*

Benzene, toluene, ethylbenzene, and xylene: Current analytical techniques and approaches for biological monitoring

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Abstract: Benzene, toluene, ethylbenzene, and xylene (BTEX) are a group of volatile organic compounds that human exposure to them may result in the development of some diseases, including cancer. Biological monitoring plays an important role in exposure assessment of workers occupationally exposed to chemicals. Several metabolites have been proposed for biological monitoring of individuals who are exposed to BTEX. There are a variety of extraction methods and analytical techniques for the determination of unmetabolized BTEX in exhaled air and their urinary metabolites. The present study aimed to summarize and review the toxicokinetics of BTEX and sample preparation and analytical methods for their measurement. Metabolites of BTEX are discussed to find out reliable ones for biological monitoring of workers exposed to these chemicals. In addition, analytical methods for unmetabolized BTEX in exhaled air and their metabolites were reviewed in order to obtain a comparison between them in term of selectivity, sensitivity, simplicity, time, environmental-friendly and cost. Given the recent trends in sample preparation, including miniaturization, automation, high-throughput performance, and on-line coupling with analytical instrument, it seems that microextraction techniques, especially microextraction by packed sorbents are the methods of choice for the determination of the BTEX metabolites.

Keywords: BTEX, toxicokinetics, exhaled breath, urine, biological monitoring, analytical methods

***Corresponding author: Esmaeel Soleimani,** Department of Occupational Health and Safety Engineering, School of Health, Shiraz University of Medical Sciences, Shiraz, Iran, email: esoleimani61@gmail.com

Abbreviations

ACGIH	American Conference of Governmental Industrial Hygienists
ATSDR	agency for toxic substance and disease registry
BEIs	biological exposure indices
BM	biological monitoring
BTEX	benzene, toluene, ethylbenzene, and xylene
CAR	carboxen
DI	direct immersion
EPA	Environmental Protection Agency
ECD	electron-capture detector
EME	electromembrane extraction
FID	flame ionization detector
GSH	glutathione
HA	hippuric acid
HF-LPME	hollow-fiber liquid-phase microextraction
HPLC	high-performance liquid chromatography
HS	head space
IARC	International Agency for Research on Cancer
IC	ion chromatography
LC-MS/MS	liquid chromatography–tandem mass spectrometry
LC-QqTOFMS	liquid chromatography/hybrid quadrupole time-of-flight mass spectrophotometry
LLE	liquid–liquid extraction
LLLME	single drop liquid–liquid-liquid microextraction
MHA	methylhippuric acids
MEPS	microextraction by packed sorbet
METs	microextraction techniques
MOF-MEPS	metal-organic frameworks (MOF) in MEPS

MIPs	molecularly imprinted polymers
MIMEPS	molecularly imprinted polymer in MEPS
o-cresol	ortho-cresol
OELs	occupational exposure limits
PA	polyacrylate
PAD	photodiode array detector
p-cresol:	para cresol
PDMS	polydimethelsiloxane
PDLLME	partitioned dispersive liquid–liquid microextraction
PGA	phenylglyoxylic acid
PMA	S-phenylmercapturic acid
RPM	round per minute
SPE	solid phase extraction
SLM	supported liquid membrane
SPME	solid phase microextraction
SAX	strong anion exchange
TLV	threshold limit value
TWA	time-weighted average
tt-MA	trans,trans-muconic acid
VOCs	volatile organic compounds

1 Introduction

BTEX are volatile organic compounds (VOCs), consisting benzene, toluene, ethylbenzene, and xylene. BTEX are present in the air of typical urban environments, emitted by everything from paint to vehicle exhaust and industrial emissions. Gas and petroleum refining industries and petrochemical plants are important workplaces where the exposure to benzene is apparent. Exposure of the general population occurs because of breathing air containing benzene. Thus, a subject may be exposed to benzene both occupationally and environmentally [1]. Benzene can evaporate into air very quickly and the main route of exposure to it is via inhalation, however, it can also enter the body through gastrointestinal tract and slightly across the skin [2,3]. Benzene is an established cause of adult leukemia and has been classified as a group I carcinogen (carcinogenic to humans) by International Agency for Research on Cancer (IARC) and US Environmental Protection Agency (EPA) [4,5]. The American Conference of Governmental Industrial Hygienists (ACGIH) proposed a threshold limit value–time-weighted average (TLV-TWA) of 0.5 ppm for benzene in occupationally exposed workers (Table 1).

Toluene is widely used in industrial processes such as the production of paints, paint thinners, adhesives, fingernail polishes, lacquers, and rubber [6]. Environmental exposure to toluene may occur from many sources such as drinking water, food, air, and consumer products [6]. Toluene has been classified as a group 3 carcinogen (not classifiable as to its carcinogenicity to humans) by the IARC [5]. The ACGIH proposed TLV-TWA of 20 ppm for toluene in occupationally exposed workers (Table 1).

Some people are exposed to ethylbenzene in their occupations, and many are exposed to this chemical every day through routine activities such as the use of automobiles, boats, aircraft, and gasoline-powered tools and equipment. Gas and oil workers, varnish workers, spray painters, and individuals involved in gluing operations may be exposed to ethylbenzene [7]. In addition to inhalation, exposure to ethylbenzene may occur through skin contact [8]. Ethylbenzene has been classified as group 2B carcinogens (possibly carcinogenic to humans) by IARC [5]. The ACGIH proposed TLV-TWA of 20 ppm for ethyl benzene in subjects with occupational exposure (Table 1).

The term xylene is used to describe the three isomers of xylene in which the methyl groups vary on the benzene ring, including meta-xylene, ortho-xylene, and para-xylene. Xylene is primarily used as a solvent in various industries such as printing, rubber, and leather industries. It is also used as a cleaning agent, a paint thinner, and in varnishes as well as in production of plastic bottles and polyester. The general population may be exposed to xylene through drinking water and/or eating xylene-contaminated food [9]. Similar to toluene, xylene has been classified as a group 3 carcinogen (not classifiable as to its carcinogenicity to humans) by the IARC [5]. The ACGIH proposed TLV-TWA of 100 ppm for xylene in subjects with occupational exposure (Table 1).

Human exposure to BTEX may result in the development of some diseases. For instance, high-level benzene exposure leads to aplastic anemia and leukemia in exposed workers [10,11]. Characterization of human exposure to these chemicals is a necessary step in the determination of health risk and its management, as well as, in environmental and occupational epidemiological studies. In the field of industrial hygiene, exposure assessment and risk assessment are inextricably mixed such that they cannot be reasonably separated. Considering that the health risk following exposure to a toxic chemical can be estimated by multiplying the exposure by the toxicity (health risk = exposure × toxicity), the evaluation of exposure is fully half the health risk assessment, in the world of industrial hygiene. Therefore, the assessment of individuals' exposure to chemicals is critically important for the management and prevention of adverse health effects [12,13].

Table 1: Occupational exposure limits (OELs) and biological exposure indices (BEIs) for BTEX

Chemicals	TLV-TWA[a] (ppm)	PEL-TWA[b] (ppm)	REL-TWA[c] (ppm)	Metabolites	BEIs[d]	Sampling time
Benzene	0.5	1.0	0.1	tt-MA	500 µg/g creatinine	End of shift
				PMA	25 µg/g creatinine	End of shift
Toluene	20	200	100	Toluene in blood	0.02 mg/L	Prior to last shift of workweek
				Toluene in urine	0.03 mg/L	End of shift
				o-cresol	0.3 mg/g creatinine	End of shift
Ethylbenzene	20	100	100	MA + PGA	0.15 g/g creatinine	End of shift at end of workweek
Xylene	100	100	100	MHA	1.5 g/g creatinine	End of shift

[a]ACGIH – threshold limit value-time weighted average
[b]OSHA – permissible exposure limit-time weighted average
[c]NIOSH – recommended exposure limit-time weighted average
[d]BEIs – biological exposure indices
tt-MA – trans,trans-muconic acid; MA – mandelic acid; MHA-methylhippuric acid; PGA – phenylglyoxylic acid; o-cresol – ortho-cresol; PMA – S-phenylmercapturic acid;

Occupational exposure to chemicals can be assessed using air monitoring and/or biological monitoring (BM). In the former, air samples are collected in the breathing zones of the workers on a sampling medium in an active or passive way [14]. In active sampling, a pump is used to pull air through the sampling media that captures the contaminant of interest. On the other hand, in passive sampling, containers or badges which collect chemicals based on diffusion are used. Air monitoring makes it possible to determine the airborne levels of contaminants that a subject is exposed via inhalation [15-17]. A worker's TWA exposure to a chemical is calculated as follow:

$$TWA = \frac{\sum_{i=1}^{n} C_i \times T_i}{T} \quad (1)$$

where: *Ci* is the chemical concentration (ppm or mg/m^3) in each air sample, *Ti* is the time over which measurement took place (min), and *T* is duration of work shift (e.g., 480 min for a standard work shift). This calculated TWA is then compared with the corresponding ACGIH TLV-TWA for that chemical. TLV-TWA is defined as "a concentration for a conventional 8 h workday and a 40 h workweek, to which it is believed that nearly all workers may be repeatedly exposed, day after day, for a working lifetime without adverse effect".

However, air monitoring suffers from some limitations and it is not always a good predictor of actual worker's exposure to chemicals. For instance, some chemicals can be absorbed through the skin or gastrointestinal tract [2,18,19]. In addition, local exhaust ventilation and the use of personal protective equipment such as respirators affect the levels of inhalation exposure to chemicals [18,20]. Air monitoring techniques generally determine the external dose of a worker or a group of workers. The external dose reflects the level of a chemical that is actually found in air, water, soil or even food. However, to assess the health effects of a toxicant, the internal dose is of importance. For a given toxicant, external dose is not necessarily equivalent to the internal dose. The internal dose is a fraction of the external dose and represents the level of toxicant that passed through physical and physiological barriers in the body such as the skin or lung epithelia. Therefore, besides air monitoring, BM is recommended to be considered as complement to exposure assessment by air sampling to determine the relative contributions from all exposure routes when assessing the total subject's exposure [21]. The results of BM are compared to BEIs®. The occupational exposure limits (OELs) and BEIs® for BTEX are shown in Table 1.

Given the above, it is apparent that exposure assessment plays an important role in the management and prevention of health risks in individuals exposed to chemicals. To have a good exposure assessment, we need valid and reliable metabolites and suitable analytical methods for the determination of them in biological matrices. At first, a review is presented on the toxicokinetics of BETX. Then, several analytical methods for the determination of BTEX in exhaled air and their metabolites in urine are discussed. The primary focus is on the techniques reported in the last 15 years for BTEX metabolites.

2 Toxicokinetics of BTEX

Inhalation is the major route of exposure to BTEX. In this section, the absorption, distribution, biotransformation and excretion of BTEX following inhalation exposure are presented.

2.1 Absorption

While it can enter the body through gastrointestinal tract and slightly across the skin [2,3], the main route of exposure to benzene is inhalation. Benzene is rapidly absorbed from the respiratory tract. In the first few minutes of exposure, the highest absorption (70-80%) occurs, but it decreased rapidly with time [22]. Studies have shown that the respiratory uptake of benzene is about 47-52% depending on the level of exposure [23,24]. Toluene is rapidly absorbed following inhalation exposure as shown by the appearance of it in the blood within 10–15 min of exposure [25]. However, absorption via gastrointestinal tract and skin is much slower. The respiratory uptake of toluene is higher during exercise than at rest [26,27].

Similar to benzene and toluene, ethylbenzene is rapidly absorbed through the respiratory tract [28,29]. Tardif et al. reported a steady state blood:alveolar air concentration ratio of about 30 within 60 min of initiating exposure [29]. In a study of volunteers exposed to 23-85 ppm of ethylbenzene for 8 h, Bardodej and Bardodejova showed that they retain 64% of the inspired vapor and only trace amounts were detected in expired air at the end of the exposure period [30]. The main absorption of xylenes occurs following inhalation and oral exposures. About 60% of inhaled xylene is retained and about 90% of ingested xylene is absorbed. Dermal absorption occurs to a much lesser extent [9]. It seems that no difference exists between men and women in pulmonary retention of xylenes [31]. The retained isomers seem to have similar absorption (63.6% on average), regardless of exposure duration or dose [32]. Other studies reported that between 49.8% and 72.8% of inhaled xylene is retained [33-35].

2.2 Distribution

Once absorbed into the blood, benzene is distributed throughout the body. It is lipophilic and thus a high distribution to fatty tissue might be expected. In subjects exposed to benzene via inhalation, the chemical has been detected in blood, brain, liver, kidney, stomach, bile, abdominal fat, and urine [24,36]. In humans, benzene crosses the placenta and its level in the cord blood is equal to or greater than that in maternal blood [37].

Both human and experimental studies showed that a positive correlation exist between toluene concentrations in alveolar air and in blood [25,38]. *In vitro* and *in vivo* data indicated that toluene is distributed between the plasma and red blood cells (RBCs) at approximately 1:1 and 1:2 ratios, respectively [39]. Absorbed dose is distributed to lipid-rich and highly vascular tissues such as the brain, liver and lung [40,41].

The retention of ethylbenzene in adipose tissue is estimated to be 5% of the total uptake [42]. To the best of our knowledge, there is no study concerning the distribution of ethylbenzene in humans following exposure to this chemical alone. However, some studies suggest that the partitioning of ethylbenzene from air into adipose tissue in humans is similar to that observed in rats [43,44]. Once absorbed, xylene is rapidly distributed throughout the body through the systemic circulation. The xylene isomers have similar distributions in the body. In the blood, it is primarily bound to serum proteins and the main tissue for its accumulation is adipose tissue (range from 4% to 10% of the absorbed dose) [42,45,46]. Experimental studies show that m-or p-xylene and their metabolites are mainly distributed in lipid-rich tissues such as the brain and fat and well-perfused organs such as the liver and kidney [47,48]. p- and o-xylene readily cross the placenta and distributed in amniotic fluid and embryonic and fetal tissues [49,50].

2.3 Biotransformation

Following inhalation exposure, the major route for excretion of unmetabolized BTEX is via exhalation. Absorbed BTEX undergo a complex metabolism pathway, converting them to their certain metabolites.

2.3.1 Benzene

Benzene is mainly excreted as metabolites in urine and unmetabolized via the lungs. Only small amounts of the unchanged benzene are excreted in urine [51,52]. The metabolism pathway of benzene is concentration-dependent. At low concentrations, the metabolism is rapid and benzene is excreted predominantly as conjugated metabolites in urine. At higher concentrations, the metabolic pathways appear to become saturated and the majority of absorbed dose is excreted unmetabolized in exhaled air. The metabolic pathways for benzene can be found in the studies of Nebert et al. [53] and Ross [54]. Several pathways are involved in the metabolism of benzene. In the first step, benzene is oxidized through the cytochrome P-450 2E1 (CYP2E1) to form benzene oxide [55]. The major initial metabolite of benzene is phenol [28] which is oxidized to catechol or hydroquinone in the presence of CYP2E1. Then, catechol or hydroquinone is oxidized to 1,2- and 1,4-benzoquinone via myeloperoxidase, respectively [53]. It is also possible that catechol or hydroquinone convert

to 1,2,4-benzenetriol via CYP2E1 catalysis. Each of these phenolic metabolites (phenol, catechol, hydroquinone, and 1,2,4-benzenetriol) can undergo sulfonic or glucuronic conjugation [53,56]. Besides the above mentioned pathways, benzene oxide can undergo two other reactions. First, it can react with glutathione (GSH) to form S-phenylmercapturic acid (PMA) [57,58]. Second, in an iron-catalyzed ring-opening reaction, it converted to trans,trans-muconic acid (tt-MA) [53,54,59].

Both tt-MA and PMA are valid and sensitive biomarkers. However, in low concentrations of airborne benzene (8 h TWA exposure of less than 0.3 ppm), PMA is a reliable indicator because of its superior specificity. In addition, since PMA has a longer elimination half-life, it is a more reliable biomarker than tt-MA for unusual work schedules (e.g. 12 h work shifts). However, at higher concentrations (8 h TWA exposure of higher than 1 ppm), tt-MA is reliable and may even be preferred because its determination is easier than PMA [60]. The ACGIH suggested both tt-MA and PMA as indicators of benzene exposure and proposed BEIs of 500 μg/g creatinine and 25 μg/g creatinine, respectively, for them in workers occupationally exposed to benzene [21] (Table 1).

2.3.2 Toluene

CYP-catalyzed methyl and ring hydroxylations are the initial steps in the metabolism of toluene. Formation of benzyl alcohol via methyl hydroxylation is the prominent first step in human liver microsomes [61,62]. On the other hand, the formation of ortho- or para-cresols (o-cresol and p-cresol) as a result of ring hydroxylation usually represents less than 5 percent of total metabolite formation. The CYP2E1 and CYP1A2 are the most active CYP isozymes in the formation of benzyl alcohol or o- and p-cresols, respectively. However, this depends on the level of toluene exposure [61]. At low levels, CYP2E1 contributes to benzyl alcohol and p-cresol formation and CYP1A1/2 contributes to the formation of o- and p-cresol. On the other hand, at higher levels of toluene, CYP2B1 and CYP2C11/6 contributes to the formation of benzyl alcohol and o- and p-cresol. During two steps of alcohol dehydrogenase and aldehyde dehydrogenase, benzyl alcohol is converted to benzoic acid which can be conjugated with glycine to form hippuric acid (HA), catalyzed by acyl-CoA synthetase and acyl-CoA: amino acid N-acyltransferase [63]. HA represents 83-94% of urinary metabolites of toluene in rats. Benzoic acid can also conjugate with glucuronic acid and forms benzoyl glucuronide which can account for 3-9% of urinary metabolites of toluene [63].

As mentioned above, HA is the major urinary metabolite of toluene. However, because of non-occupational sources of HA, including sodium benzoate (a food preservative) and fruits, it cannot be considered as a reliable indicator if 50 ppm airborne concentration of toluene is not present [64]. This concentration is 2.5 times higher than the TLV-TWA for toluene (20 ppm) [21]. Ducos et al. showed that although o-cresol showed a good correlation with atmospheric levels of toluene, corrected o-cresol has a greater variability than urinary toluene and is more sensitive to smoking habits, as well. They concluded that urinary toluene is very interesting surrogate to o-cresol and could be considered as a biomarker of choice for toluene exposure [65]. The ACGIH proposed toluene in blood (0.02 mg/L) and urine (0.03 mg/L), and o-cresol (0.3 mg/g creatinine) in urine of workers occupationally exposed to toluene as indicators of exposure to this chemical [21] (Table 1).

2.3.3 Ethylbenzene

Liver microsomal enzymes participate in ethylbenzene hydroxylation. The initial step, catalyzed by CYP isoforms CYP2E1 and CYP2B6, is hydroxylation of the side chain of ethylbenzene to form 1-phenylethanol which is directly excreted to urine (mainly glucuronides) or further oxidized to form acetophenone. Both 1-phenylethanol and acetophenone can be excreted in the urine as minor metabolites or converted to subsequent metabolites. A further oxidation process results in the sequential formation of 2-hydroxyacetophenone, 1-phenyl-1,2-ethanediol, mandelic acid (MA), and phenylglyoxylic acid (PGA). In individuals with inhalational exposure to ethylbenzene, MA (about 70%) and PGA (about 25%) are the major metabolites [28,29,66]. In contrast, only 4.6% of the absorbed dose of ethylbenzene is excreted as MA following skin exposure [67]. ACGIH proposed the sum of MA and PGA in urine (0.15 g/g creatinine) as indicator of occupational exposure to ethylbenzene [21] (Table 1).

2.3.4 Xylene

Liver microsomal enzymes (mixed function oxidases) participate in xylene hydroxylation. The oxidation of a side-chain methyl group of xylene to form methylbenzoic acids and then conjugating them with glycine to yield methylhippuric acids (MHA) is predominant, accounting approximately all of the absorbed dose of xylene [68-70]. In another metabolic pathway that accounts for <10% of

the absorbed dose, xylene eliminates unchanged in the exhaled breath and in the urine and further metabolized to form methylbenzyl alcohols, o-toluic acid glucuronide, xylene mercapturic acid and dimethylphenols [71]. The ACGIH proposed MHA in the end of shift urine samples (1.5 g/g creatinine) as an indicator of occupational exposure to this chemical [21] (Table 1).

2.4 Excretion

Exhalation is the major route for excretion of unmetabolized BTEX. Excretion of benzene via the lungs is reported to be 16.4-41.6% with no sex-related differences [22,23]. The highest excretion rate is observed during the first hour of the exposure [22]. Absorbed benzene is also excreted in humans via biotransformation to phenol and tt-MA followed by urinary excretion of sulfates and glucuronides derivatives [53,54].

In acute inhalation exposure, absorbed toluene is excreted predominately in the urine as metabolites and, to a lesser extent (7-20% of absorbed toluene), as unmetabolized form in exhaled air and urine [72,73]. The majority of absorbed toluene is rapidly eliminated from the body [74,75] and a smaller portion (that which accumulated in adipose tissues) is slowly eliminated [73,74,76]. Excretion of unchanged toluene in urine is a minor elimination route based on mass balance. However, elimination kinetics data are consistent with the proposed use of unchanged toluene for BM of workers occupationally exposed to this solvent [21,65]. Pierce et al. in the analysis of toluene elimination via exhaled air (as unmetabolized toluene) and in urine (as metabolites) in male subjects exposed to 50 ppm $^{2}H_{8}$-toluene for 2 h showed the distribution of the total dose as follows: 13% ^{2}H8-toluene in exhaled air, and 75% $^{2}H_{5}$-hippuric acid, 0.31% $^{2}H_{7}$-o-cresol, 0.53% $^{2}H_{7}$-m-cresol, and 11% $^{2}H_{7}$-p-cresol in urine [76].

Ethylbenzene is rapidly biotransformed and eliminated from the body as urinary metabolites. Elimination of ethylbenzene via exhaled air showed multi-phasic kinetics with an early-phase half time of less than 1 h [29]. This rate of elimination is significantly faster than the elimination rate of the urinary metabolites. For instance, MA and PGA are excreted in the urine with a half-life of about 3-5 h and 10-12 h, respectively [28,29]. Elimination of MA showed biphasic kinetics, a relatively rapid phase (half-life of 3.1 h) and a slower one (half-life of 25 h) [77]. In ethylbenzene-exposed human subjects, the highest excretion rate of the urinary metabolites observed 6-10 h after the exposure [77].

In humans, the majority (about 95%) of absorbed xylene is metabolized and excreted in the form of urinary metabolites (almost as MHA). The remaining 5% is eliminated unchanged in the exhaled air [68,74,78]. The excretion of MHA is fast and the major part of it is detected in the urine within 2 h of exposure. The excretion increases with time. Of total absorbed dose of xylene isomers, about 0.005% is excreted unchanged in the urine, and about 2% as xylenols [32]. The elimination of MHA is biphasic, with half-lives of 1 h for the rapid one and 20 h for the slower one [69,70]. The absorption of xylene increases with exercise and thus the urinary elimination of m-MHA and 2,4-xylenol increases [79].

3 Biological monitoring

BM, environmental monitoring, and health surveillance are important tools in the prevention of diseases caused by toxic agents in the general or occupational environment. BM is a tool to assess the levels of exposure to chemicals and corresponding health risks to workers. In BM, the concentration of a chemical determinant (the chemical itself, its metabolite(s), or a characteristic, reversible biochemical change induced by the chemical) is measured in the specimen (urine, blood, exhaled air) obtained from individuals who are exposed to chemicals [21,80]. BM is a measure of the overall uptake of a chemical by an individual. The ACGIH established reference guidelines of known biomarkers through the TLV® and BEI® [21]. According to the ACGIH, "BEI® represents the levels of determinants that are most likely to be observed in specimens collected from healthy workers who have been exposed to chemicals to the same extent as workers with inhalation exposure at the TLV-TWA [21]. BEI® generally indicates a concentration below which nearly all workers should not experience adverse health effects" [21]. The proposed BEIs by the ACGIH for BTEX are indicated in Table 1.

4 Analytical methods for biomonitoring of BTEX exposure

4.1 Methods for BTEX metabolites in urine

Table 2 presents several analytical methods that have been developed to analyze BTEX metabolites in urine. The most common analytical techniques are liquid chromatography with ultraviolet detection (HPLC-UV) [14,60,81-96], liquid chromatography–tandem mass spectrometry (LC-MS/MS)

Table 2: Comparison of analytical methods and sample preparation techniques for determination of the BTEX metabolites

Extraction technique	Analytical method	BTEX metabolites	Range (µg/mL)	LOD (µg/mL)	Precision (%RSD)	Recovery (%)	Extraction time (min)	Solvent consumption (mL)	Sample volume (mL)	Sorbent amount (mg)	Reference
LLE	GC-FID	O-cresol	0.0-2.3	0.04	1.9-2.2	ND	30	5.2	1.0	NA	[29]
LLE[a]	GC-MS	tt-MA, PMA, catechol, phenol, HQ	0.0-100	2-10	7.75-31.9	NR	120	3.2	0.5	NA	[109]
LLE[a]	GC-MS	HA, MHA, MA, PGA	0.05-25	8-20	3.8-9.9	92-104	50	6.5	0.2	NA	[111]
LLE[a]	HPLC-UV	MA,PGA	5-125	1.0	3.2-6.8	86.2-96.8	90	1.0	0.1	NA	[87]
LLE	HPLC-UV	MA	0.5-250	0.07	0.8-2.9	101	10	1.0	1.0	NA	[88]
LLE	HPLC-UV	HA, MHA	10-1000	4-6	5.3-6.1	ND	38	4.0	1.0	NA	[14]
Static headspace	GC-FID	Toluene	0.01-0.1	0.0005	ND	ND	NA	NA	5.0	NA	[65]
SPE	HPLC-UV	tt-MA, PMA	0.64 and 5.51	0.001	3.2 and 0.4	>95	8.0	8.0	1.0	500	[60]
SPE	HPLC-MS/MS	tt-MA	0.02-1	0.0005	1.7-8.8	>86	20	16	3.0	500	[101]
SPE[a]	GC-MS	HA, MHA, MA, tt-MA	0.003-20	0.003-0.01	3.2-24.8	>82.4	25	11	0.5	60	[89]
SPE[a]	GC-MS	tt-MA	0.01-1.0	0.01	7.4	93.3-106.3	60	15	2.0	500	[107]
MISPE[a]	GC-MS	tt-MA	0.125-2.0	0.037	3.6	83.4-94.8	18	13	1.0	150	[106]
MISPE	HPLC-UV	tt-MA	0.3-10	0.1	3.3-9.6	87-112	8	8.0	2.0	100	[86]
HF-LPME	HPLC-UV	tt-MA	0.05 to1.2	0.001	3.4-8.1	83-92	120	ND	ND	NA	[93]
IP-HF-LPME	HPLC-UV	tt-MA, HA, MA	0.001-20	0.0001-0.007	4.3-11.2	87-95	60	ND	ND	NA	[91]
LLLME	HPLC-UV	HA	0.001-0.4	0.0003	1.1-3.1	91.4-99.3	60	0.3	ND	NA	[81]
EME	LC–MS/MS	PMA, HA, MHA, PGA	0.002-10.0	0.0003-0.005	1.28-15.8	52.8-79	15	ND	0.8	NA	[118]
DI-SPME[a]	GC-MS	tt-MA,HA,MA, MHA,PGA	0.08-360	0.009-0.63	7.0-15	ND	40	NA	2.0	30µm PDMS and 85µm PA fibers	[111]

Table 2: (Continued)

Extraction technique	Analytical method	BTEX metabolites	Range (µg/mL)	LOD (µg/mL)	Precision (%RSD)	Recovery (%)	Extraction time (min)	Solvent consumption (mL)	Sample volume (mL)	Sorbent amount (mg)	Reference
HS-SPME[a]	GC-MS	tt-MA,HA,MA, MHA,PGA	0.08-360	0.004-026 MA,HA,PGA were not determined	7.0-15	ND	60	NA	2.0	30µm PDMS and 85µm PA fibers	[111]
SAX-MEPS	HPLC-UV	tt-MA	0.1-2.0	0.03	3.0-5.1	93.3-99.4	4	0.4	0.5	4.0	[82]
MIMEPS	HPLC-UV	tt-MA	0.015-2.0	0.015	3.5-6.6	89.8-91.6	4	1.0	0.5	4.0	[83]
MIMEPS	HPLC-UV	MA	0.2-20	0.2	3.8-5.1	88.9-91.6	5	1.7	1.0	4.0	[84]
MIMEPS	LC-MS/MS	HA	0.00018-0.2	0.0005	1.1-7.1	91-96	10	0.8	1.5	2.0	[99]
MOF-MEPS	HPLC-UV	tt-MA	0.01-50	0.001	2.1-6.2	89.9-96.8	5	2.0	ND	3.0	[95]
Dilute-inject	LC-MS/MS	tt-MA	0.2-0.5	0.07	3.5-6.6	ND	NA	NA	0.03	NA	[97]
Dilute-inject	LC-MS/MS	MA, PGA	20-2000	0.015, 0.02	<11	>82	NA	NA	10	NA	[100]
Dilute-inject	HPLC-UV	HA, MHA, MA, PGA,	30-5000	1.25-22	2.35-7.32	96.2-104.1	NA	8.0	1.0	NA	[90]

[a]Methods that require derivatization before the chromatographic analysis.

[97-102], HPLC with photodiode array detector (HPLC-PAD) [103,104], gas chromatography (GC) with mass spectrometry (GC-MS) [105-111], GC with flame ionization detector (GC-FID) [112,113] or electron-capture detector (GC-ECD) [113], liquid chromatography/hybrid quadrupole time-of-flight mass spectrometry (LC-QqTOFMS) [114], and ion chromatography (IC) [115]. Some of these methods are develop specifically to measure only one of the BTEX metabolites [14,60,81-84,86,88,94-99,106,107], whereas others were developed to simultaneous determination of two or more metabolites of BTEX [85,89,90,105,108-111,114]. Most of these analytical methods use liquid–liquid extraction (LLE) [14,87,88,109,110,113] or conventional solid phase extraction (SPE) [86,92,103,105,106,112] for sample preparation. However, microextraction techniques (METs) such as hollow-fiber liquid-phase microextraction (LPME) [91,93], single drop liquid–liquid-liquid microextraction (LLLME) [81], partitioned dispersive liquid–liquid microextraction (PDLLME) [96], solid phase microextraction (SPME) [111] and microextraction by packed sorbet (MEPS) [82-84,99] are also used in recent years. In addition, the "dilute-and-shoot" approach without need for a sample preparation step is used [97,98,100,102]. Recently, a method has been developed to direct extraction of HA and MHA from untreated urine samples using a probe impregnated with molecularly imprinted polymers (MIPs) and subsequent analysis by micellar electrokinetic chromatography [116].

4.1.1 LLE and conventional SPE methods

In LLE, an extraction solvent (phase B) is used to extract the analytes of interest in the matrix (phase A). Phase B is usually an organic solvent while phase A is aqueous. An extraction can be performed if the analyte has favorable solubility in the extracting solvent. In recent decays, LLE has been used in several studies for the determination of BTEX metabolites [14,87,88,109,110,113]. Most of this studies provides reliable methods for the determination of tt-MA [109], MA [87,88,110,113], PGA [87,110], PMA [109], HA [14,110], and MHA [110] in urine samples, even in the cases of low concentrations of BTEX in air.

Conventional SPE methods have also been developed for determination of BTEX metabolites [60,86,92,101,103,105-107]. The quaternary ammonium ion exchange resin (SAX-SPE) is the most frequently used procedure for the extraction of tt-MA from urine [60,92]. Briefly, SPE sorbent is conditioned with 3 mL of methanol and 3 mL of 0.1% (v/v) aqueous acetic acid. After sample loading, the column is washed with 3 mL of 1% (v/v) aqueous acetic acid. Finally, tt-MA is eluted with 3 to 4 mL of 10% (v/v) aqueous acetic acid [60,92,101]. MAX anion exchange column for the simultaneous extraction of urinary tt-MA, MA, and HA [105] and amine anion exchange column for tt-MA [107] are another SPE methods for BTEX metabolites.

Because of the low selectivity of conventional SPE sorbents (C2, C8, C18, SAX, MAX, etc.), more selective sorbents such as MIPs in SPE columns (MISPE) have been used in recent years, for the determination of urinary metabolites [86,106]. In MISPE, the extraction procedure is similar to the conventional SPE columns, as described already above. However, MISPE is more selective and environmental friendly than the conventional SPE.

4.1.2 METs

In recent years, a number of METs have been developed for the determination of BTEX metabolites, including LLLME [81], HF-LPME [91,93], SPME [111], PDLLME [96] and MEPS [82-84,95,99].

4.1.2.1 LLLME

LLLME technique involves a series of two reversible extractions. In three phases LLLME, analytes are first extracted from an aqueous donor phase into a thin layer organic membrane phase (in their neutral form) and then back-extracted into an aqueous acceptor phase [81,117]. Toulabi et al. have developed a single drop LLLME method for the determination of HA in human urine and serum samples [81]. Briefly, 5 mL of aqueous sample solution (adjusted to pH 3) containing HA is transferred to a 5-mL volumetric flask. Then, 300 μL of butyl acetate:ethyl acetate (1:2, v/v) is added to the top of the aqueous phase. A 10 μL syringe is used for suspending the microdrop (7 μL, adjusted pH to 11) to the organic phase during extraction and also for injection into the HPLC injection valve after extraction. To provide sufficient stirring extraction, a magnetic stirring bar (8.5 mm × 3.0 mm) is placed into the solution (700 rpm). After 45 min, the microdrop is retracted back into the syringe and immediately transferred to the injection valve of HPLC-UV for analysis.

4.1.2.2 HF-LPME

Recently, a method based on HF-LPME has been developed by Bahrami et al. for the determination of tt-MA, HA, and MA in urine samples [91]. This technique is performed in the following steps: 1) the HF membranes are manually cut

to provide 8.8 cm equal pieces with an internal volume of 24 µL; 2) to remove any contamination, the pieces are washed with acetone for 5 min in an ultrasonic bath and then dried in air; 3) the receiver phase loads into a 25 µL Hamilton syringe and then the syringe's needle is inserted into the lumen of the HF; 4) to fill the pores, the HF is immersed in 10% (w/v) of Aliquat-336 solution as an ion-pair reagent for 15 s and then rinsed with distilled water for 10 s to remove excessive organic solvent; 5) while one end of the HF is closed using an aluminum foil, the acceptor phase is injected into the other end of the HF and then it bent to an U-shape configuration; 6) to extract the analytes, the U-shape HF is immersed in a 12 mL sample vial containing 10 mL of the aqueous sample. The extraction efficiency increased with increasing the stirring up to 800 rpm; 7) after 60 min, the end of the HF is cut and the acceptor phase which now contains the analytes is withdrawn into the syringe and injected into HPLC for analysis [91].

4.1.2.3 Electromembrane extraction (EME)

The EME is a MET based on HF-LPME for the extraction of charged compounds. In this sample preparation technique, analytes extracted using a few amounts (microliters) of an organic solvent. As compared with the conventional HF-LPME, the EME technique has some advantageous, including higher extraction efficiency, effective sample clean-up, analyte enrichment, good selectivity, and shorter extraction time. Suh et al. have developed an EME technique for the simultaneous determination of BTEX metabolites including PMA, MA, MHA, and PGA [118]. For this, about 0.8 mL of donor solution was transferred into a vial. A 3.2 cm piece of HF is used to prepare supported liquid membrane (SLM). To create the SLM, one end of the HF is closed by mechanical pressure and then dipped in 1-octanol for one min. The other end of the HF is connected to a pipette tip that is inserted through the vial's rubber cap. By using a 100 µL micro-syringe, 40 µL of the acceptor solution (5 mM ammonium acetate, pH 10.2) are introduced into the lumen of the fiber. Then, a cathode is placed into the sample and an anode is introduced into the fiber's lumen. After connecting the electrodes to a power supply, the vial is placed on a stirrer at a speed of 1500 rpm. The extraction was continued for 15 min at 300 V voltage and then the acceptor solution was transferred for LC–MS/MS analysis [118].

4.1.2.4 SPME

Pacenti et al. have developed a method for the determination of HA, MHA, MA, tt-MA and PGA directly in the urine employing derivatization with trimethyloxonium tetrafluoroborate and sequential extraction by head space (HS) and direct immersion (DI) SPME [111]. In this method, 2 mL of urine are transferred into a 10 mL vial and after derivatization of the acids, the urine samples undergo SPME. For the extraction procedure, the syringe injector of the SPME unit equipped with 30 µm polydimethelsiloxane (PDMS) and 85 µm polyacrylate (PA) fibers is used. For HS-SPME, the vials are placed at 50°C in a temperature-controlled agitator. Before automatically introduce of the fiber into the vial for 20 min in static conditions, a pulsed agitation is carried out for 30 min incubation time. For DI-SPME the fiber is directly immersed in a 10 mL vial for 20 min at 60°C, under continuous agitation at 500 rpm. For desorbing the analytes, the fiber is introduced into the GC injection port and maintained at 300°C for 4 min [111].

4.1.2.5 PDLLME

In this method, polar analytes are extracted into polar dispersive solvents and subsequently to organic extractant droplets according to their partition coefficients. Recently, a PDLLME method has been developed for the determination of tt-MA in urine samples [96]. Briefly, to a 10 mL screw cap conical test tube containing 5 mL of urine sample, 200 µL of extraction solvent and 2000 µL of disperser solvent are added. Then, the mixture is gently shaken until a cloudy solution formed in the test tube. In the next step, the mixture is centrifuged at 5000 rpm for 10 min. About 100 µL of the sediment phase is transferred to a new tube and dried under a gentle stream of nitrogen. The residue reconstituted in 100 µL of methanol and 20 µL is injected to HPLC [96].

4.1.2.6 MEPS

MEPS is a high throughput, simple and straightforward sample preparation technique developed in 2004 by AbdelRehim et al. [119,120]. MEPS overcomes the inherent drawbacks of conventional SPE columns [121]. In recent years, MEPS has been emerged as a powerful tool in bioanalysis with an increasing range of applications [121-124]. To the best of our knowledge, there are five methods based on MEPS for determination of BTEX metabolites [82-84,95,99]. Soleimani et al. have developed extraction methods using SAX or MIP in MEPS (SAX-MEPS or MIMEPS) for the determination of tt-MA [82,83] and MA [84] in urine samples. Moein et al. have developed a MIMEPS method for the determination of HA in plasma and urine samples [99]. More recently, Rahimpoor et al. have developed zirconium-based metal–organic frameworks (MOF) in MEPS (MOF- MEPS) for the extraction of urinary tt-MA [95].

The MEPS protocols are easy and straightforward (Figure 1). For determination of tt-MA or MA using MIMEPS, approximately 4 mg of the MIPs are packed between two polyethylene frits inside a 250 µL Hamilton syringe. Before using for the first time, the MIPs are conditioned with 3×100 µL of ethanol (for tt-MA) or methanol (for MA), followed by 3×100 µL of water (for both tt-MA and MA). The sample is percolated through the MIP sorbent five times (5×100 µL) for tt-MA and eight times (5×100 µL) for MA at the speed of 10µL/s (extract-discard mode). Then, the MIPs are washed with 1×100 µL of water (for both tt-MA and MA). In the next step, the analytes are eluted by 2×100 µL of ethanol-acetic acid (80:20, v/v) for tt-MA and 2×100 µL of methanol-acetic acid (80:20, v/v) for MA. An aliquot of 10 µL is injected into the HPLC system. To avoid carry-over, the MIPs were cleaned after each extraction using 4×150 µL of the elution solution, followed by 4×150 µL of the washing solution [83,84]. The SAX-MEPS procedure for the determination of tt-MA [82] is similar to that of MIMEPS.

For the determination of HA [99], approximately 2 mg of MIP membrane are packed between two frits inside a Hamilton 250-µL syringe. The MIP membrane is first conditioned with 100 µL of distillated water. Then, the sample is loaded by taking ten replicate 100-µL aliquots of the diluted plasma sample (10×100 µL) through the MIP membrane. Then, the MIP membrane sorbent is washed with 1×100 µL of ultra-pure water/formic acid (0.1%) (90:10). The analyte is eluted with 100 µL of acetonitrile/methanol (50:50) and 30 µL of the solution are injected into a LC-MS/MS system. After each extraction, the MIP membrane sorbent is washed using 3×100 µL of the elution solution followed by 3×100 µL of the washing solution to avoid carryover [99] (Figure 1).

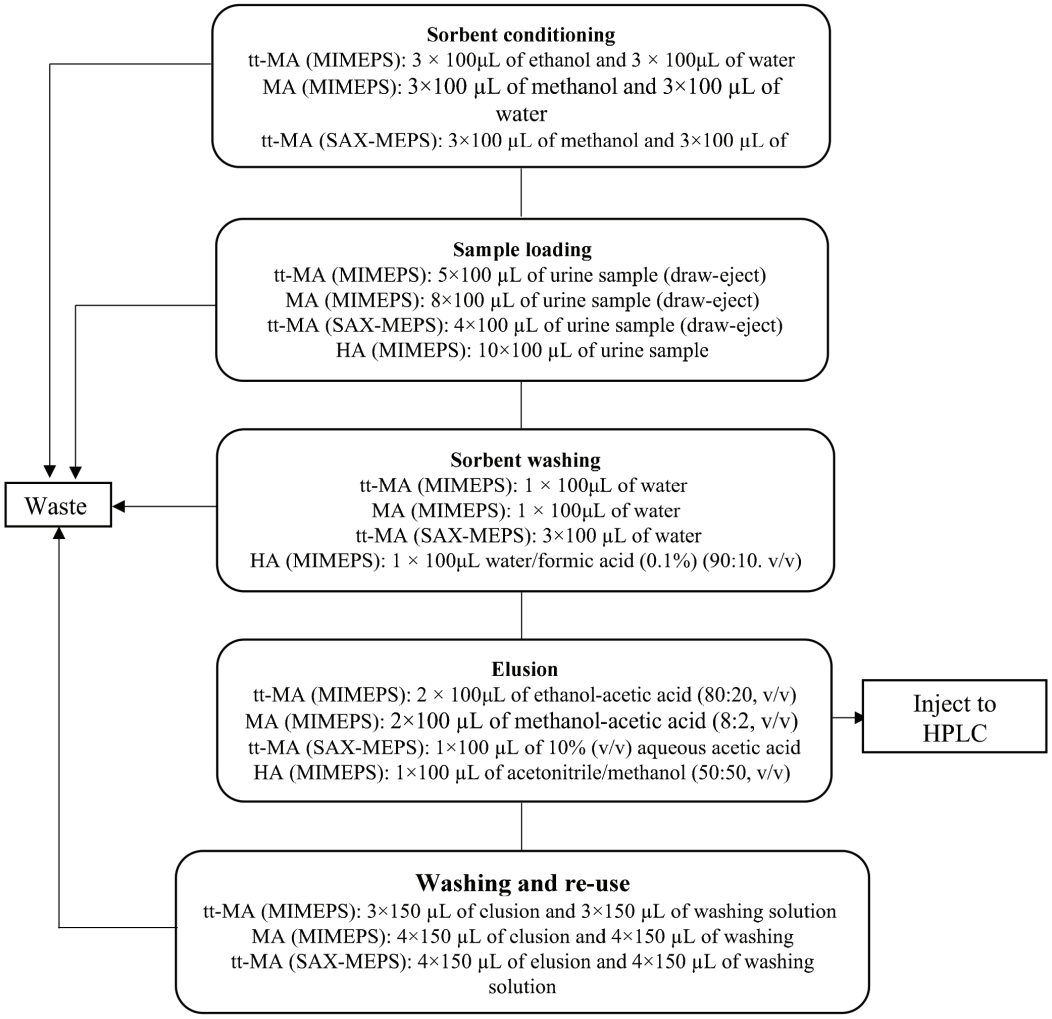

Figure 1: SAX-MEPS and MIMEPS protocols for the extraction of tt-MA [82,83], MIMEPS protocol for MA [84], and MIMEPS protocol for HA [99] in urine.

4.1.2.7 Dilute and shoot approach

Using MS/MS analysis, the sample is diluted and directly injected (dilute-and-shoot approach) to the chromatographic system without the need for sample preparation. There are some analytical methods using MS/MS analysis for the determination of BTEX metabolites [90,97,98]. For instance, Gagné has developed an UPLC-MS/MS analysis for determining urinary tt-MA. Briefly, 30 mL of urine is transferred to 1.5 mL Eppendorf tubes and then 970 mL of water and 0.1% formic acid (containing 2840 µg/L of d4-t,t-MA as internal standard) are added. The sample is vortexed and centrifuged at 14,000 rpm for 10 min. Then, 500 mL of the supernatant are transferred to the LC vial and analyzed by UPLC-MS/MS [97]. The multiple reaction monitoring (MRM) transition and conditions used for t,t-MA and the internal standard are listed in Table 3.

4.1.2.8 LC-QqTOFMS analysis

LC-QqTOFMS analysis has been developed for the simultaneous determination of tt-MA, HA, MHA, and PGA in urine samples [114]. This technique provides a sensitive and highly selective quantitative assessments of the analytes. The availability of full-scan mass spectra throughout each LC/TOF chromatogram and the accurate mass measurements provide qualitative information suitable to identify analytes present in the samples. Experimental conditions for the LC-QqTOFMS determination of the analytes is shown in Table 4.

4.2 Methods for unmetabolized BTEX in exhaled air

Determination of unmetabolized VOCs in exhaled air has attracted the attention of researchers in the fields of public health and medicine [125-139]. Table 5 shows analytical methods used for the determination of unmetabolized BTEX in exhaled air.

Table 3: The multiple reaction monitoring transition and conditions for tt-MA and internal standard [97]

Chemical	Cone (V)	Collision energy (V)	MRM transition (m/z)
t,t-MA Quantification MRM	22	10	141-97
t,t-MA Confirmation MRM	22	10	141-53
d4-t,t-MA (ISTD)	24	10	145-100

Table 4: Experimental conditions for the determination of selected analytes [114]

Analyte	Collision cell pulsing parameter		m/z precursor	DP	CE (V)
	IRD (µs)	IRW (µs)			
PGA	41.4	11.2	149.0	30	19
tt-MA	41.3	12.5	141.0	50	15
HA	39.9	11.2	178.0	30	18
MHA	45.2	13.9	192.0	26	15

IRD – ion release delay; IRW – ion release width; DP – declustering potential

In these techniques, GC-FID [131,132,134,137] or GC-MS [129,133,135,136,138-140] have been used. Different media are used for collection/adsorption of exhaled air, including solid sorbents [132,133,135,136,140], Haldane-Priestly tubes [141], SPME fibers [138,139,142], Tedlar bags [128,129,131,135,142], or canisters [143,144]. In some studies, a breath sampling tube and direct analysis has been developed [137]. Sample preparation methods include solvent extraction [145], thermal desorption/cryofocussing step prior to chromatographic analysis [35,136], headspace analysis [135] and SPME [138,139,142]. In recent years, SPME has been emerged as the most common method for the preparation of exhaled air samples.

4.2.1 SPME

During the last two decades, SPME has been used for the determination of BTEX in exhaled-air samples [138,139,142]. Some authors used this technique specifically to measure only one of BTEX [138,139], whereas simultaneous determination of two or more of them has also been reported [142]. In these studies, exhaled air samples are collected on Tedlar bags and then the SPME is used for the adsorption of the analytes. For instance, in a more recent study, Koureas et al. used SPME technique for the determination of 19 VOCs, including benzene, toluene and ethyl benzene in exhaled air samples [142]. Briefly, exhaled air samples are collected in Tedlar® bags and then an SPME manual holder with a 75 µm Carboxen-polydimethylsiloxane (CAR/PDMS)-coated fused silica fiber is used for adsorption of the analytes. The adsorption is performed for 25 min at room temperature followed by the desorption process for 5 min at 270°C. A same fiber is used for approximately 100 injections [142]. The characteristics of the SPME procedures in different studies are shown in Table 6.

Table 5: Analytical methods and sample preparation techniques for determination of the BTEX in exhaled air samples

Extraction technique	Analytical method	Analyte	Extraction time (min)	Range	LOD	Recovery (%)	Precision (%RSD)	Reference
Collection in bags, thermal desorption with cold trap	GC-MS	Benzene	ND	1.5-250 ng/L	1.5 ng/L of exhaled air	ND	<1.8	[129]
Collection in vials containing an adsorbing cartridge	GC-MS	Toluene	~ 5.0	0.05-50 µg/L	0.05 µg/L of exhaled air	91.4-98.1	3.8-9.95	[133]
Collection in bags, adsorption on silica gel, desorption to head space vial	GC-MS	Benzene	ND	0.2-29 ppb/5-L sample	0.1 ppb (5-L sample)	ND	1.0-3.0	[135]
SPME	GC-MS	Benzene	3.5	6.0-53.0 ppb	2.4 ppb	ND	3.2-11.0	[138]
SPME	GC-MS	Benzene	15.0	3.22-187.0 µg/m3	0.95 µg/m3	ND	2.9-7.4	[139]
Collection by spirometer onto charcoal tube, microwave desorption	GC-MS	Benzene, Toluene, EB, xylene	ND	2.5-100 ng/ tube	0.2 -3.0 ng/ tube	ND	16.9-25.5	[140]
Collection in a modified Haldane-Priestly tube, thermal desorption	GC-MS	Benzene, toluene, xylene	17.0	ND	1 nmol/L	ND	5.0-15.0	[141]
Collection in bags, SPME	GC-MS	Benzene, toluene, EB	30	0.024-46.4, 0.28-44.8, 0.29-45.8 ng/L air	0.11-0.66 ng/L	85.1-95.2	0.16, 0.28, 0.53 ng/l	[142]
Collection in canister, transfer to Tenax tube, thermal desorption	GC-FID	Benzene, toluene, xylene	NA	1-50 pmol	0.29-0.4 pmol	ND	4.5-9.0	[132]
Collection by spirometer into bags, adsorption on charcoal cloth, solvent extraction	GC-FID	Xylene	~ 30.0	0.21-4.16 µg/ mL	0.4 µg/l (5-L sample)	> 80	5.3	[145]

EB – ethylbenzene; NA – not applicable; ND – not determined

Table 6: The characteristics of the SPME procedure for determination of BTEX in exhaled air.

Fiber	Length of fiber (cm)	Extraction time (min)	Desorption time (min)	Adsorption temperature (°C)	Desorption temperature (°C)	Fiber use (Injections)	Reference
DVB/CAR/PDMS	2	0.5	3	ND	250	ND	[138]
DVB/CAR/PDMS	ND	0.5	3	ND	250	ND	[139]
CAR/PDMS	ND	25	5	RT	270	100	[142]

DVB/CAR/PDMS – divinylbenzene/carboxen/polydimethylsiloxane; ND – not determined; RT – room temperature

5 Discussion

Exhaled air and urine can be used as reliable and easy-to-collect media for BM of workers exposed to BETX. To date, many analytical methods have been developed for determination of unmetabolized BTEX or their urinary metabolites. Each method has its own limitations and advantages. Generally, the bottleneck of these analytical methods is sample preparation step. Several steps are involved in the development of an analytical method. The typical steps include sampling, sample preparation, and analysis. There might be several processes within the sample preparation itself, such as preconcentration and clean-up. Sample preparation is one of the most time-consuming and laborious steps in analytical techniques. The analytical performance is greatly affected by these steps in term of reliability, accuracy, time, and cost of analysis. In many analytical techniques, sampling and sample preparation accounts for most of the analysis time. On the other hand, today, there are more sophisticated analytical instruments and detection methodologies that have made extensive progress in analytical performance and lowered detection limits. Thus, sample preparation is the main bottleneck of reference techniques which use fast and high throughput sample analysis and detection procedures [123,124]. Therefore, the selection of a method for sample preparation should be made according to the following features: miniaturization, automation, minimum amount of solvent consumption and chemical waste, the highest recovery of the analyte, efficient removal of interfering compounds, online coupling with analytical instruments, easy-to-use, rapid, inexpensive, and environmental-friendly [121,122,146,147].

Table 2 shows the comparison of the sample preparation techniques for the urinary BTEX metabolites. These methods have their own limitations and advantages over each other. The LLE methods suffer from some limitations, the most important of which are: (a) the need for solvents with high polarity that often yield emulsions, (b) incomplete phase separations, (c) less-than-quantitative recoveries, (d) use of expensive breakable glassware, (e) large volumes of organic solvents and the resulting extracts, usually in the range of milliliter (mL), (f) low selectivity and sensitivity, and (g) they are bench scale, complex, manual and time consuming. In addition, it is not easy to automate the LLE methods [122,123].

The disadvantages of LLE methods led researchers to resort to SPE technique because of its efficiency and selectivity. Conventional SPE method consists of four typical steps, including conditioning, sample loading, washing, and elusion. The lack of selectivity is the main drawback of conventional SPE sorbents (C2, C8, C18, SAX, MAX, etc.). Therefore, in recent years, the use of MIP in SPE columns (MISPE) have been used to overcome this limitation [148]. The advantage of the MISPE over conventional SPE is that it is more rapid, sensitive and selective. In addition, since the interferents are efficiently removed, the chromatographic run time is shorter and thus the analytical frequency is higher in comparison with conventional SPE [86,106]. SPE provides both higher recovery and good chromatography than LLE. It is obvious that SPE resolves many of the limitations of the LLE methods. However, SPE has its own limitations, especially when it compares with METs [82-84,94,96,121]. The amount of sorbent used is relatively high resulting in an extensive optimization of the typical steps in SPE, including conditioning, sample loading, washing and elusion. Therefore, in addition to a relatively high consumption of organic solvents and longer extraction time, there is not a convergence between the solvent volumes used to sample preparation and the sample volume needed for analysis by chromatographic techniques that require microliters (μL) of samples. Furthermore, the conventional SPE columns are single use and thus the total analysis cost is higher than methods that are reusable and can be used several times, such as MEPS [121-123].

Because of the inherent limitations of LLE and conventional SPE columns, and to provide maximum recovery of analytes, efficient removal of interferents, minimum or no consumption of solvents, automation, miniaturization, online coupling with analytical instruments, low-cost operation, and ease of use, METs

such as LPME [91,93], LLLME [81], DLLME [106], PDLLME [96], SPME [111], and MEPS [82-84,95,99] have been developed for determination of BTEX metabolites.

The LLLME and LPME methods are equilibrium extraction procedures and thus highest recovery can be achieved by the acceptor phase after equilibrium is obtained [81,91,93]. Similarly, in SPME, analytes partition between a fiber coating and the matrix until an equilibrium is achieved [111]. In LLLME, LPME, and SPME, the equilibrium constant and the equilibration time are influenced by pH, temperature, salt concentration and stirring [81,91,93,149,150]. As can be seen from Table 2, in addition to relatively low recoveries, the extraction times in LLLME [81], HF-LPME [93], IP-HF-LPME [91], PDLLME [96], HS-SPME [111], and DI-SPME [111] are about 60, 120, 60, ~30, 40, and 60 min, respectively. This is particularly important in the analysis of the high number of urine samples. SPME suffers from some limitations, including low recovery, different fiber's quality and length from batch to batch, sensitivity of the fiber to the nature of the matrix, and the inability of the fiber to withstand a complete run (analysis of standards, blanks, quality control samples, and real samples) [119]. In addition, in comparison with LLE and SPE methods, SPME has shown higher deviations [151].

A look at Table 2 shows that MEPS have some advantageous over other extraction techniques for the BTEX metabolites. In contrast to conventional SPE columns, in MEPS, the solid phase is inserted directly into the syringe, not into a separate column. Therefore, a separate robot is not further required to apply the sample into the sorbent bed. The amounts of sorbent in MEPS (2 to 4 mg) is about 30 to 225 times less than those used in SPE columns (60 to 500 mg). This small amount of the solid phase significantly reduces the volume of organic solvents and extraction time making MEPS as a rapid (about 6 to 30 times faster) and environmental friendly (about 10 to 30 times lower consumption of organic solvents) method for sample preparation. In addition, unlike single-use SPE columns, a sorbent bed can be used about 80 times [82-84,121]. The most important benefit of MEPS over other METs (e.g. LPME, LLLME, PDLLME, and SPME) is that it is more rapid (12 to 24 times faster) and robust. In addition, MEPS can be used on-line with the analytical instrument without any modification of the instrument [121,122].

Overall, there are several analytical methods based on LLE, SPE, and METs for the determination of the BTEX metabolites. Each of these techniques can be used for biomonitoring of individuals who are exposed to BTEX. However, given the recent trends in sample preparation, including miniaturization, automation, high-throughput performance, and on-line coupling with analytical instrument, it seems that MEPS can be a method of choice for the determination of the BTEX metabolites. MEPS is rapid, selective, sensitive, fast, non-expensive, and both user- and environmental friendly. MEPS combines the three steps of sample processing, extraction and injection fully automated as an on-line extraction device to GC or LC. In recent years, given the advantages of MEPS and selectivity and low cost of MIPs, the combination of them (MIMEPS) has been considered as the best technique in term of simplicity, selectivity, sensitivity, and environmentally friendly.

In addition to using sample preparation techniques for the extraction of the BTEX metabolites, dilute-and-shoot approach is also available. In this way, no sample preparation is required and the urine samples diluted and then injected directly to the chromatographic system [90,97,98]. The elimination of the sample preparation step and the improvement in the chromatographic runs provide a cheaper and faster analysis of urinary metabolites [97,98].

The presence of BTEX in exhaled air is considered as a selective and sensitive biomarker for evaluating recent exposure. Exhaled air offers some advantages over urine. It is easily accepted by the workers and the air sampling step is simple compared to urine. However, since it is not a common practice in BM, there is a lack of data on which to base the exhaled air analysis. In spite of the relatively high number of published papers on the toxicokinetics of BTEX in both humans and animals (see section of toxicokinetics of BTEX), little information exists on the relationship between the concentration of unmetabolized BTEX in the exhaled air and the levels of exposure. Therefore, in situations where there are high variations in the levels of airborne BTEX (e.g., occupational settings), the analysis of exhaled air for BM of workers is questionable. The relationship between the levels of BTEX in inhaled air and their urinary metabolites is well-known. The urinary metabolites of BTEX including tt-MA, PMA, o-cresol, MA, PGA and MHA are proposed by the ACGIH for BM of exposed workers. Several robust and high throughput METs and highly sensitive analytical techniques are existing for the determination of urinary BTEX metabolites. On the other hand, few studies have been published regarding sample preparation and analysis of unmetabolized BTEX in exhaled air. Unlike the urinary metabolites, data on the relationship between exposure to airborne BTEX and their levels in exhaled air is scare. The majority of analytical techniques for the determination of BTEX in exhaled air has been developed in the last three or more decades [35,129,135-137,141,143,144]. In recent years, few studies developing analytical techniques for exhaled air have been published [131,132,142]. Further studies are required to

establish such a relationship. The ACGIH did not offer the measurement of unmetabolized BTEX in the exhaled air for BM of exposed workers [21]. In addition, since relatively low levels of inhaled BTEX are excreted unmetabolized in the exhaled air, highly sensitive analytical techniques are required to determine these chemicals in exhaled breath.

Conflicts of interest: Authors declare no conflicts of interest.

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