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# A novel diagnostic *in situ* derivatization kit for the simultaneous determination of 14 biomarkers of exposure to benzene, toluene, ethyl benzene and xylenes in human urine by isotope dilution liquid chromatography tandem mass spectrometry and kit optimization using response surface methodology

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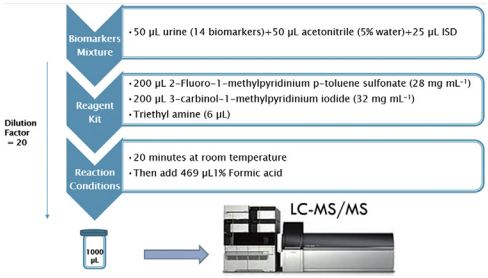
## HIGHLIGHTS

- In situ derivatization-based LC-MS/MS method for BTEX metabolites.
- Charge-reversal derivatization of phenols and carboxylic acids.
- Comprehensive urine BTEX metabolite panel for occupational exposure assessment.

## GRAPHICAL ABSTRACT

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## ABSTRACT

Metabolite profiling can be used as a diagnostic measure for both short and long term co-exposure by individuals to benzene, toluene, ethylbenzene and xylenes (BTEX). A novel one pot derivatization *in situ* kit (OPDISK) was developed and optimized using a multivariate approach based on central composite design. The OPDISK was designed to simultaneously derivatize, in a urine sample matrix, a series of fourteen carboxylic acid and phenol-bearing urinary metabolites of BTEX to enhance their chromatographic analysis and sensitivity for detection by liquid chromatography – electrospray ionization – tandem mass spectrometry (LC-ESI-MS/MS). Using the reagent kit, the less responsive functional units on the molecules were converted to permanently positively-charged functional units. The kit was composed of three components, 2-fluoro-1-methylpyridinium p-toluenesulfonate (FMP), 3-carbinol-1-methylpyridinium iodide (CMP) and triethylamine (TEA) as a basic catalyst and, only after diluting a urine sample 20 fold with acetonitrile, was applied under mild conditions of room temperature and short reaction time of 20 min. The derivatized biomarkers were then directly analyzed using isotope dilution LC-ESI-MS/MS. The method was sensitive (limit of detection on column ranged from 1.4 pg to 3.1 ng), accurate (mean accuracy from 85% to 114%), and precise (mean coefficient of variation from 1% to 14%). The method results indicated a good linearity ($R^2 \geq 0.990$) for all metabolites. ClinChek® urine control samples were used successfully to demonstrate the accuracy of the method.



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## 1. Introduction

Benzene, toluene, ethylbenzene, and xylenes (BTEX) are monoaromatic hydrocarbons, which are ubiquitous in the surrounding environment. They are extensively used as solvents, as raw materials for the synthesis of pharmaceuticals and many other organic compounds, and as vital components of many industrial products including gasoline, paints, pesticides, plastics, and glues. Humans can be easily exposed to BTEX compounds through inhalation, skin absorption, and/or ingestion of contaminated food and water [1]. Due to their high volatilities, BTEX vapors can easily contaminate air, water, and soil. BTEX exposure is not limited only to outdoor environments. Many studies have shown that indoor environments (e.g. furniture polishes, paintings, and household products) can provide high exposure levels [2,3].

According to the International agency for research on cancer (IARC), benzene is a group 1 carcinogen to humans [4], where increased incidence of leukemia has been reported. Ethylbenzene is indicated to be a possible human carcinogen [5]. Toluene and xylenes are not carcinogenic based on the current evidence, but they are known to have neurotoxic effects [6]. Because of toxic and carcinogenic effects of BTEX, the American Conference of Governmental Industrial Hygienists (ACGIH) has recommended that urinary metabolites be used for the biological monitoring of occupational human exposure to BTEX [7]. These urinary metabolites are useful biomarkers of internal dose for occupational and environmental exposure as they reflect absorption by all routes (inhalation, dermal, and ingestion) and they have the advantages of long half-life, high specificity, low volatility, and the non-invasive sampling of urine.

These metabolites were reported in literature due to their strong correlation with exposure levels across a wide range of BTEX concentrations and include: t,t-muconic acid (MU), S-phenylmercapturic acid (PMA), phenol, and catechol for benzene; hippuric acid (HA), S-benzylmercapturic acid (BMA), o-cresol, and p-cresol for toluene; mandelic acid (MA) and phenylglyoxylic acid (PGA) for ethylbenzene; and methylhippuric acids (MHA) for xylenes. Benzoic acid (BA) is not reported as a direct biomarker of exposure to BTEX but its intake as benzoate, which is a common food preservative, can affect urinary background levels of HA (glycine conjugate of benzoic acid) in the human population. Lord et al. summarized different interpretation scenarios for different BA/HA ratios, other than those caused by exposure to BTEX [8]. Hence, we added BA to our list of biomarkers to provide a method that can be adopted to obtain a complete diagnostic picture in toxicological studies. However, HA is not the only one of these metabolites which suffers from background levels in urine of non-exposed individuals. MU can be formed from dietary sorbic acid preservative [9]. BMA can be derived from benzyl alcohol which is present in many cosmetic products [10]. HA can be found in peaches, fruit juices, sodas, ketchup, and due to dietary intake of benzoate preservatives [11]. This necessitates simultaneous determination of multiple metabolites for each parent compound and not relying solely on a specific biomarker. Moreover, adopting a multiple biomarkers approach will help to achieve more accuracy and specificity for evidence-based decisions, to provide a pattern match for single or co-exposures, to show co-exposure interaction effects on delay, suppression, or enhancement of formation, and to capture the variability through the studied population.

Several analytical methods have been published for the determination of urinary metabolites of one or more of the BTEX compounds. This includes those based on paper and thin-layer chromatography [12,13], gas chromatography-flame ionization detection (GC-FID) [14,15], GC-MS [16], HPLC-UV [17–19], and HPLC-MS [20]. However, few studies reported the simultaneous determination of urinary metabolites derived from all BTEX compounds. Simultaneous determination of several metabolites would be particularly useful in cases of co-exposures. Approaches which have adopted simultaneous determination include capillary electrophoresis [21], HPLC-UV [22,23], HPLC-MS [24–26], GC-MS [27] and solid-phase microextraction followed by HPLC or GC-MS [28]. Despite being comprehensive in covering the determination of concurrent metabolites, these approaches have focused only on a representative metabolite or two for each of the BTEX compounds. Some of BTEX urinary metabolites were not incorporated in each of the reported simultaneous determination methods and most of the reported methods focused only on either the carboxylic or phenolic metabolites to target as biomarkers. Further, some of the aforementioned studies used some kind of biological sample pretreatment and clean-up steps such as liquid-liquid extraction, solid-phase extraction (SPE), and in case of GC, a derivatization procedure is usually used for these polar metabolites. These traditional sample preparation methods are usually labor-intensive and can lead to sample loss, increased total analysis time, low recovery, and exposure to contaminants. This is most likely due to the lack of adequate specificity and/or their presence at very small concentrations in urine, especially at low exposure levels to BTEX. Recent sample preparation methods, using restricted access media or online SPE columns for online trapping and pre-concentration, require additional hardware for automated column switching and expertise. Hence, a gap occurs in understanding the mechanism of toxicity and metabolite formation caused by single or co-exposure to BTEX compounds. Studies have shown that the relative proportions of urinary metabolites are affected by co-exposure, as well as by the concentration, rate, and route of exposure. For instance, co-exposure to toluene and xylenes has been reported to reduce PMA in urine [29].

The goal of this work was to develop and optimize a novel combination of a one-pot derivatization *in situ* [30] kit (OPDISK) that can be directly integrated with LC-ESI-MS/MS for the simultaneous quantitation of 14 phenolic and carboxylic biomarkers of BTEX in human urine. Our developed method accommodates small volumes of urine samples (50 μL) and can be fully automated and used as a single analytical platform to achieve the necessary short analysis time, high throughput, sensitivity, and specificity to qualify as a reliable broadly aimed tool for bio-monitoring in routine screening programs, large epidemiological, toxicological, and clinical studies, thus substituting a combination of analytical platforms for short and long term effects of single and multiple BTEX exposures. This will allow better understanding of the effects of exposure to BTEX compounds. Eventually, undertaking the necessary preventive measures and evidence based practices by public health practitioners and health authorities will minimize exposure and related harmful health effects.

## 2. Experimental

### 2.1. Reagents and chemicals

Triethylamine (TEA), phenol, 1,2-dihydroxybenzene, *o*-cresol, *p*-cresol, *trans*, *trans*-muconic acid (MU), hippuric acid (HA), 2-methylhippuric acid (2MHA), 3-methylhippuric acid (3MHA), 4-methylhippuric acid (4MHA), phenylglyoxylic acid (PGA), *DL*-mandelic acid (MA), iodomethane, 3-pyridinemethanol, and Surine™ negative urine control were purchased from Sigma–Aldrich (St. Louis, MO). *DL*-phenylmercaprturic acids (PMA), *DL*-benzylmercapturic acid (BMA), 2-fluoro-1-methylpyridinium *p*-toluenesulfonate (FMP) and LC-MS grade formic acid were purchased from TCI America (Tokyo, Japan). Phenylglyoxylic Acid-d5 was purchased from Toronto research chemicals. N-acetyl-S-benzyl-2,3,4,5,6-d5-

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