MICRO-SOLID PHASE EXTRACTION METHODS BASED ON MOLECULARLY IMPRINTED POLYMER FOR DETERMINATION OF TOLUENE METABOLITES IN URINE SAMPLES

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DEDICATION

Dedicated goes to

My mother and father, **Madianah Binti Abu and Zinalibdin Bin Abdul Rahim**, whose sacrifice, prayer;
My **Brothers and sisters**, whose support and encouragement;
And
My wife, **Tity Ernie Suryati Binti Ahmad**, My Children, **Nur Insyirah Binti Mohamad Raizul, Muhammad Fahimy Bin Mohamad Raizul, Nur Sumayyah Binti Mohamad Raizu**l whose patience;
Lead me to achieve my doctoral degree.
Not forgotten My late son, **Muhammad Raqimy Bin Mohamad Raizul,** your memory strengthens my soul

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ABSTRACT

Toluene is one of the most widely used solvents in various industries. Workers exposure to toluene can be monitored using hippuric acid and *o*-cresol as urine biomarkers for biological exposure index. However, extraction of biological fluids often results in sample emulsification in liquid-liquid extraction (LLE) while also reducing the efficiency of sorbent materials in solid phase extraction (SPE). Therefore, it is imperative to solve the emulsification problem and enhance the reusability and selectivity of SPE sorbent. In this study, a new SPE method was developed using molecularly imprinted polymers (MIPs) based on hippuric acid (MISPE-HA) and *o*-cresol (MISPE-Cre). The MIPs were prepared by sol-gel polymerization of hippuric acid (0.3 mmol) or *o*-cresol (0.5 mmol), 3-propyltrimethoxymethacrylate (800 µL), ethanol (12 mL), tetraethoxysilane (TEOS) (12 mL) and catalyst (400 µL of hydrochloric acid). BET analysis for MISPE-HA and MISPE-Cre demonstrated surface areas of 388.64 m^2/g and 574.90 m^2/g, with pore volumes of 0.4638 cm^3/g and 0.5314 cm^3/g, respectively. Furthermore, both MISPE samples displayed pore diameters of 4.70 nm and 2.20 nm, respectively, which proved the presence of mesoporosity in their structures. MISPE-HA showed a higher adsorption capacity (9.67 mg/g) compared to MISPE-Cre (0.99 mg/g). The results indicated that the kinetics of adsorption obeyed well the pseudo-second order model and Scatchard plot for adsorption isotherm. In order to improve analyte mobility and reusability, new dispersive solid phase extraction (DISPE) methods were developed based on surface imprinted hippuric acid grafted onto silica gel (MIP@SiO_2-HA) and surface imprinted *o*-cresol grafted onto silica gel (MIP@SiO_2-Cre). The MIP@SiO_2 materials were prepared by hippuric acid (0.3 mmol) or *o*-cresol (0.5 mmol), 3-propyltrimethoxymethacrylate (800 µL), tetraethoxysilane (10 mL), activated silica gel (0.1 g) and acetic acid (0.01 mol/L). Nitrogen adsorption showed that both MIP@SiO_2-HA and MIP@SiO_2-Cre were mesoporous materials with pore diameters of 4.70 nm and 4.76 nm, respectively. BET study showed that, MIP@SiO_2-HA had a surface area of 122.10 m^2/g and pore volume of 0.4638 cm^3/g, while MIP@SiO_2-Cre exhibited surface area of 106.90 m^2/g and pore volume of 0.1991 cm^3/g. MIP@SiO_2-HA indicated a good adsorption capacity (9.68 mg/g) compared to that of MIP@SiO_2-Cre, which had a low adsorption capacity of 0.99 mg/g. MIP@SiO_2 was also found to be fitted well with pseudo-second order model for kinetic adsorption and Scatchard plot for adsorption isotherm. The MIP@SiO_2 and MISPE method validation indicated good limit of detection (LOD) and good limit of quantification (LOQ) with relative standard deviation (RSD) less than 5% and high extraction recovery more than 90%, which complied with ISO 17025:2005. The results demonstrated that these adsorbents have potential applications in the determination of toluene metabolites in urine samples due to the improved selectivity, better elution ability for target molecule and high reusability.

ABSTRAK

Toluena adalah salah satu pelarut yang digunakan secara meluas dalam pelbagai industri. Pendedahan pekerja terhadap toluena boleh dipantau menggunakan asid hipurik dan *o*-kresol sebagai biopenanda urin bagi indeks pendedahan biologi. Walau bagaimanapun, pengekstrakan cecair biologi sering mengakibatkan pengemulsian sampel dalam pengekstrakan cecair-cecair, di samping itu juga mengurangkan kecekapan bahan penjerap dalam pengekstrakan fasa pepejal (SPE). Oleh itu, adalah amat penting bagi mengatasi masalah pengemulsian dan meningkatkan kebolehgunaan dan kepilihan penjerap SPE. Dalam kajian ini, kaedah SPE baharu telah dibangunkan menggunakan polimer cetakan molekul (MIP) berasaskan asid hipurik (MISPE-HA) dan *o*-kresol (MISPE-Cre). Bahan MIPs telah disediakan secara pempolimeran sol-gel asid hipurik (0.3 mmol) atau *o*-kresol (0.5 mmol), 3-propiltrimetoksimetakrilat (800 µL), etanol (12 mL), tetraetoksisilana (TEOS) (12 mL) dan mangkin (400 µL asid hidroklorik). Analisis BET bagi MISPE-HA dan MISPE-Cre menunjukkan luas permukaan 388.64 m^2/g dan 574.90 m^2/g, dengan isipadu liang masing-masing sebanyak 0.4638 cm^3/g dan 0.5314 cm^3/g. Tambahan lagi, kedua-dua sampel MISPE memaparkan diameter liang masing-masing 4.70 nm dan 2.20 nm, yang membuktikan kehadiran mesoliang di dalam strukturnya. MISPE-HA menunjukkan kapasiti penjerapan lebih tinggi (9.67 mg/g) berbanding dengan MISPE-Cre (0.99 mg/g). Hasil kajian menunjukkan kinetik penjerapan mematuhi dengan baik model tertib pseudo-kedua dan taburan Scatchard bagi isoterma penjerapan. Bagi memperbaiki pergerakan analit dan kebolehgunaan semula, kaedah baharu pengekstrakan sebaran fasa pepejal (DISPE) telah dibangunkan berdasarkan cetakan asid hipurik tercantum di permukaan gel silika (MIP@SiO_2-HA) dan cetakan *o*-kresol tercantum di permukaan gel silika (MIP@SiO_2-Cre). MIP@SiO_2 disediakan menggunakan asid hipurik (0.3 mmol) atau *o*-kresol (0.5 mmol), 3- propiltrimetoksimetakrilat (800 µL), tetraetoksisilana (10 mL), gel silika teraktif (0.1 g) dan asid asetik (0.01 mol /L). Penjerapan nitrogen menunjukkan bahawa kedua-dua MIP@SiO_2-HA dan MIP@SiO_2-Cre adalah bahan mesoliang dengan diameter liang masing-masing adalah 4.70 nm dan 4.76 nm. Kajian BET menunjukkan MIP@SiO_2-HA mempunyai luas permukaan 122.10 m^2/g dan isipadu liang 0.4638 cm^3/g manakala MIP@SiO_2-Cre mempamerkan luas permukaan 106.90 m^2/g dan isipadu liang 0.1991 cm^3/g. MIP@SiO_2-HA menunjukkan kapasiti penjerapan yang baik (9.68 mg/g) berbanding dengan MIP@SiO_2-Cre yang mempunyai kapasiti penjerapan yang rendah iaitu 0.99 mg/g. MIP@SiO_2 juga didapati sesuai dipadankan dengan tertib pseudo-kedua bagi penjerapan kinetik dan taburan Scatchard untuk isoterma penjerapan. Validasi kaedah bagi MIP@SiO_2 dan MISPE menunjukkan had pengesanan (LOD) dan had kuantifikasi (LOQ) yang baik dengan sisihan piawai relatif (RSD) kurang daripada 5% dan perolehan semula lebih daripada 90%, yang mematuhi standard ISO17025:2005. Keputusan menunjukkan bahan penjerap ini berpotensi diaplikasikan bagi penentuan metabolit toluena dalam sampel urin kerana kepilihan yang bertambah baik, keupayaan elusi molekul sasaran yang lebih baik dan kebolehgunaan semula yang tinggi.

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LIST OF ABBREVATION

USECHH	-	Use and Standards of Exposure of Chemicals Hazardous to Health
OSHA	-	Occupational Safety and Health Act
OEL	-	Occupational exposure limits
NIOSH	-	National Institute Occupational Safety and Health
BEI	-	Biological exposure index
PEL	-	Permissible exposure limit
ACGIH	-	American Conference of Governmental Industrial Hygienists
DOSH	-	Department of Occupational Safety and Health
SAX	-	Strong basic anion exchange
SPE	-	Solid Phase Extraction
MIP	-	Molecularly imprinted polymer
SPME	-	Solid Phase Microextraction
LLE	-	Liquid-liquid Extraction
MISPE	-	Molecularly imprinted solid phase extraction
HPLC-UV	-	High performance liquid chromatography-ultra violet
GC-FID	-	Gas chromatography- flame ionization detector
MIP@SiO_2	-	Molecularly imprinted surface grafted silica gel
3-PMTMOS	-	3-(propylmethacrylate)trimethoxysilane
TEOS	-	Tetraethyl orthosilicate

FTIR - Fourier Transform Infra-red
FESEM-EDX - Field Emission Scanning Electron Microscopy-Energy dispersive X-ray
XRD - X-ray Diffraction
TGA-DTA - Thermogravimetry Analysis – Differential Thermal Analysis
BET - Brunauer–Emmett–Teller
NGO - Non-governmental association
MEK - Methyl ethyl ketone
AIHA - American Industrial Hygiene Association
TLV - Threshold Limit Value
SBMA - S-benzyl mercapturic acid
TWA - Time Weighted Average
WHO - World Health Organization
UV-Vis - Ultra Violet - Visible
Pyr - Pyridine
N.R - Not reported
HCl - Hydrochloric acid
Na_2SO_4 - Sodium sulphate
HA - Hippuric acid
MeOH - Methanol
ACT - Acetone
ACN - Acetonitrile
HOAC - Acetic acid
NaCl - Sodium chloride
LLLME - Liquid-liquid-liquid Microextraction
CW/DVB - Carbowax-divinylbenzene

DI-SPME - Direct immersion – Solid phase microextraction
CLRP - Controlled/living free radical polymerization
SiO_2 - Silica oxide
APTES - 3-aminopropyl triethoxysilane
PTMOS - phenyltriethoxy silane
NSAID - non-steroidal anti-inflammatory drug
TMSPMA - 3-(trimethoxysilyl)propylmethacrylate
NIX - none imprinted xerogel
MIX - Molecularly imprinted xerogel
PSM - Polysulfone membrane
MHSM - Molecularly imprinted sol gel hollow fibre membrane
RSD - Relative standard deviation
MAA - Methacrylic acid
EGDMA - Ethylene glycol dimethacrylic
NGDA - Nordihydroguaiaretic acid
SMIP - Surface molecularly imprinted polymers
MISPE-HA - Molecularly imprinted polymer- hippuric acid
NISPE-HA - Non-imprinted polymer -hippuric acid
MISPE-Cre - Molecularly imprinted polymer- o-cresol
NISPE-Cre - Non-imprinted polymer – o-cresol
LOD - Limit of detection
LOQ - Limit of quantification
IPA - Isopropanol
MeOH - Methanol
EtOH - Ethanol
N_2 - Nitrogen

He	-	Helium
Na_2SO_4	-	Sodium sulphate
JCPDS	-	Joint Committee on Powder Diffraction Standard

LIST OF SYMBOLS

ppm - part per million
mg/L - milligram per liter
g/g - gram per gram
µg/L - microgram per liter
nm - nanometer
mg/mL - milligram per milliliter
mL - milliliter
µL - microliter
mg - milligram
mL/min - milliliter per minute
M - molar
pmol/mL - picomol per milliliter
cm - centimeter
mm - millimeter
°C - degree Celsius
Θ - theta
α - alpha
β - beta
Å - angstrom
π - pi
nmol/L - nanomole per liter
ng/mL - nanogram per milliliter
γ - gamma

mg/g	-	milligram per gram
µg/mg	-	microgram per milligram
µm	-	micrometer
mol/L	-	mole per liter
mmol	-	millimole
m^2/g	-	meter square per gram
cm^3/g	-	centimeter square per gram

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CHAPTER 1

INTRODUCTION

1.1 Background of Research

Workplace chemical exposure has been a huge problem for most industries handling chemicals hazardous to health. Numerous chemicals has been widely used as solvent in industries electric and electronic industry, printing factory, shoe factory, chemical industry and paint industry. Among the solvents, xylene (Chen *et al.*, 1994; Ohashi *et al.*, 2006), toluene (Kawai *et al.*, 1996; Caldwell *et al.*, 2000; Cok *et al.*, 2003) and benzene (Rauscher *et al.*, 1994; Ghittori *et al.*, 1995; Inoue *et al.*, 2000) are the common solvents used. These chemicals are listed as hazardous and carcinogens under Use and Standards of Exposure of Chemicals Hazardous to Health Regulation 2000 which is enforced by Department of Occupational Safety and Health (DOSH) Malaysia (OSHA Act 514, 1994).

Occupational exposure limits (OEL) are important features for control of substances hazardous to health. Personal air sampling is routinely conducted on workers to determine the level of exposures.

These samples are collected and analyzed using validated sampling and analytical methods (NIOSH Manual of Analystical Methods, 2017). Besides that, biological exposure monitoring to chemicals in the workplace is an important component of exposure assessment. As biological monitoring is generally described as the collection of specimens of tissue or body fluid, for estimating the chemical composition of the body's internal environment, it should be employed in conjunction with ambient air monitoring. Judgments on the acceptable level of a chemical or its metabolite in biological samples are based on reference value of biological exposure index (BEI).

Toluene has been one of the most widely used organic solvent in the industries since this 21st century (Moon *et al.*, 2001; Inoue *et al.*, 2004). It has been reported that exposure to high concentrations of toluene will lead to a series of diseases such as acute and chronic respiratory effects, functional alterations of the central nervous system, mucous and dermal irritations and chromosome aberrations (Ogata *et al.*, 1969; King *et al.*, 1981; Chen *et al.*, 1994). The exposure to high concentrations of toluene has been reported by many researchers (Ogata and Taguchi, 1986, 1987; Inoue *et al.*, 1991; Ohashi *et al.*, 2006; Ukai *et al.*, 2007) by detecting toluene exposure through screening of worker's blood and urine.

Occupational Safety and Health Act (OSHA) and National Institute Occupational Safety and Health (NIOSH) of America have declared the permissible exposure limit (PEL) for toluene in general industry as 200 mg/L and 100 mg/L, respectively (National Institute

Occupational, Safety and Health, 1977). However, the American Conference of Governmental Industrial Hygienists (ACGIH) strictly aligned the OEL on toluene as 20 mg/L (American Conference of Govermental Industrial Hygienists, 1995). In Malaysia, the OEL for toluene is 50 mg/L under USECHH 2000. The current American Conference of Governmental Industrial Hygienists Biological Exposure Index (ACGIH BEI) values for toluene are 0.5 mg/L *o*-cresol and 1.6 g/g creatinine hippuric acid in post-shift urine sample. Nevertheless, BEI for toluene is not established in the Guidelines on Medical Surveillance in Malaysia but indirect measurement of creatinine value is used (Guidelines on Medical Surveillance, 2009).

It has been noted by many researchers that the main biomarkers for toluene exposure are hippuric acid and *o*-cresol. Ukai *et al.*, 2007 studied on hippuric acid and *o*-cresol as biomarker of choice for long term occupational toluene exposure. However, for a short term exposure, the better indicator in urine are unmetabolized toluene and benzylmercapturic acid. They analyzed five urine workers who were occupationally exposed to 50 mg/L toluene per hour and the results were as follows: hippuric acid 30 mg/L, *o*-cresol 15.4 µg/L, benzylmercapturic acid 1.51 µg/L, unmetabolized toluene 1.47µg/L and benzyl alcohol 0.15 µg/L. The hippuric acid and *o*-cresol are in compliance with BEI.

Blood, plasma and urine are much more complex than many other samples due to the presence of proteins, salts and various organic compounds with similar chemistry to the analytes of interest. Thus, the extraction methods for biological samples have been difficult and complicated. In addition, the extraction of biological fluids has always been challenging, frequently produced the emulsification of the solvents and destruction of sorbent packs. Liquid-liquid extraction is a common method via a NIOSH test method (NIOSH Manual of Analytical Method 5th Edition, 2017) for toluene metabolites extraction using ethyl acetate or diethyl ether as solvent for extraction of hippuric and cresol isomer. However, this method is tedious and multiple step and emulsification occurred when the biological sample matrix is dirty. Another extraction method was established via solid phase extraction (SPE) using strong basic anion exchange (SAX) and polymerically bonded octadecyl (C18) as sorbent pack for extraction of hippuric acid and cresol isomer (Rauscher *et al.*, 1994; Bieniek, 1996). Biological fluids containing thousands of chemical and dirty sample will destroy the SPE sorbent pack. Besides that, SAX cartridges is very expensive and increase the cost of extraction. Thus, a new extraction technique should be developed using lower volume of sample and solvent complying to the green chemistry extraction.

One of the alternative ways to extract and pre-concentrate the toluene metabolites is using molecularly imprinted polymer (MIP). In addition, MIP offer higher selectivity for the analysis of complex samples. MIP is a new generic technology for the introduction of recognition properties into synthetic polymers. Nowadays, this technique has attracted attention of scientists engaged in the separation and extraction of biological fluids. This is due to the many advantages in comparison to SPE, solid phase microextraction

(SPME) and liquid-liquid extraction (LLE) which include superior stability, low cost, and ease of preparation (Moein *et al.*, 2014b).

The need of this study is to develop simple, fast and green extraction methods for determination of toluene metabolites. MIP was synthesized via sol-gel polymerization and used for a packed sorbent for molecularly imprinted solid phase extraction (MISPE). Another extraction technique was developed using synthesized molecularly surface imprinting via sol-gel polymerization grafted into silica gel (MIP@SiO_2). This technique improved the recognition sensitivity, higher binding capacity, faster mass transfer and binding kinetic, and the elution ability for target molecule. Among support particles, activated silica gel shows promising characteristics due to the high stability, chemical inertness and non- swelling properties. These materials were applied to the extraction of toluene metabolites from urine sample prior to high performance liquid chromatography-ultra violet (HPLC-UV) and gas chromatography- flame ionization detector (GC-FID). The reproducibility, extraction performance, sensitivity and selectivity were investigated.

1.2 Problem Statements

Rapid industrial development in Malaysia contributes to the national economic growth and prosperity. However, this development also contributes to environmental problems either in workplaces or environments. In reality, production processes in factories nowadays are still lacking in occupational, safety and health in their working environments. Thus, the workers are easily exposed to chemical hazards, especially solvent used in the production line, due to the lack of enforcement to protect the workers in the workplace or due to the ignorance of the employer.

In Malaysia, DOSH was established to enforce the OSHA 1994 for the protection of workers in the workplace. One of the subsection of this Act describes the responsibility of employers to formulate and implement the safe system of work. Monitoring BEI via biological sample such as blood and urine is one of the mechanisms to ensure the health of workers handling chemical hazardous to health.

For monitoring BEI, urine is the simplest sample to collect from workers. However, urine itself contains a large number of chemicals and emulsification upon addition of solvent is one of the challenges for extraction of the targeted analyte in urine sample (El-Beqqali and Abdel-Rehim, 2016). Thus, a selective sorbent is required to extract analyte from urine samples prior to analysis. SPE is the most useful sample preparation method via a simple extraction step and without emulsification of sample, but it is relatively expensive, limited life-time, requires large amounts of organic solvent and carry over can be a problem(Moein *et al.*, 2014b). These problems led to the current quest by researchers for new extraction techniques.

In recent years, MIPs in extraction technique have attained considerable attention because of its ability to increase the selectivity of the sample preparation process. MIP prepared via sol-gel polymerization have significant potential whereby this technique exhibited a lower total uptake with significantly lower non-specific binding. Besides, sol-gels do not exhibit the same degree of swelling as other MIPs prepared by other methods (Farrington and Regan, 2009). Most MIP bulk polymerization result in low binding capacity, poor sites accessibility to target molecule and irregular shape. Surface imprinting technology was developed to avoid the common limitation of bulk polymerization (Liao *et al.*, 2016). The fundamental strategy of surface imprinting was to locate the imprinting sites on the surface of materials, which improved the recognition sensitivity and the elution ability for target molecules, and the prepared MIPs produced high stability, chemical inertness and non-swelling properties.

This research is targeted to prepare the MISPE and MIP@SiO_2 for extraction of toluene metabolites from urine samples. The use of these materials in developing techniques for extraction, detection and degradation of pesticides has been reported (Giraldo *et al.*, 2007; Taira *et al.*, 2012; Yang *et al.*, 2013). However, to date, there has been no report on the use of these materials for extraction of toluene metabolites. Thus, this research takes an initiative to synthesis these materials for extraction of toluene metabolites.

1.3 Objectives of Research

The aim of this research is to prepare materials for the extraction of selected toluene metabolites, namely hippuric acid and *o*-cresol from urine samples by using MISPE and MIP@SiO_2. The specific objectives of the research are the following:

1. To prepare and characterize molecularly imprinted polymer based on hippuric acid for solid phase extraction (MISPE-HA) and surface molecularly imprinted polymer based on hippuric acid grafted on silica gel (MIP@SiO_2-HA) via sol-gel polymerization.

2. To develop extraction methods based on MISPE-HA and MIP@SiO_2-HA combined with HPLC-UV for the determination of hippuric acid in urine samples.

3. To prepare and characterize molecularly imprinted polymer based on *o*-cresol for solid phase extraction (MISPE-Cre) and surface molecularly imprinted polymer based on *o*-cresol grafted on silica gel (MIP@SiO_2-Cre) via sol-gel polymerization.

4. To develop extraction methods based on MISPE-Cre and MIP@SiO_2-Cre combined with GC-FID for the determination of *o*-cresol in urine samples.

1.4 Scopes of the Research

This work studies on the development of dispersive solid phase extraction (DSPE) utilizing new micro-solid phase sorbents, namely MISPE and MIP@SiO_2. The performances of the developed methods in the determination of toluene metabolites in urine samples were examined.

The first part of this research embarked on synthesis of MIP for sorbent pack of SPE via sol-gel polymerization using template molecule hippuric acid and *o*-cresol. In the second part of this research, molecularly imprinted polymer surface grafted on silica gel was prepared using sol-gel polymerization of hippuric acid and o-cresol for extraction of toluene metabolites via DSPE. Preparation of MISPE and MIP@SiO_2 materials were investigated.

In this research, all sorbents types were synthesized by sol-gel method. This method were chosen as it provides one step reaction process at room temperature which can generate material ready for fast shaping. In this study, 3-(propylmethacrylate)trimethoxysilane (3-PMTMOS) was used as the precursor and the results were compared to those of previously investigated precursors such as tetraethoxy silane (TEOS) and 3-(triethoxysilyl)-propylamine (Moein *et al.*, 2015b). The efficiency of adsorption for both materials were examined and calculated by the extraction of hippuric acid and *o*-cresol.

The physical properties of the materials were characterized using Fourier Transform Infra-red (FTIR), Field Emission Scanning Electrone Microscopy - Energy Dispersive X-ray analysis (FESEM-EDX) , X-ray Diffraction (XRD), Thermogravimetry Analysis-Differential Thermal analysis (TGA-DTA), Nitrogen Adsorption and Brunauer–Emmett–Teller (BET) surface area analysis.

Several important extraction parameters such as extraction time, solid to liquid ratio, pH, solvent, selectivity and repeatability were optimized. Binding kinetic, adsorption isotherm and adsorption mechanism were studied and explored to ensure the adsorption type and capacity of the materials. Moreover, the performance of the developed method were evaluated, validated and applied to the determination of these toluene metabolites in urine samples.

1.5 Significance of the Research

NIOSH Manual of Analytical Methods 8301 used LLE as the sample preparation method for the determination of hippuric acid, *o*-cresol and methyl hippuric acid in urine. However, this sample preparation method involved multiple steps and suffers from some drawbacks which are tedious, time consuming and poor detection limit. The results showed that *p*-cresol and *o*-cresol isomers eluted together by this method (Matsui *et al.*, 1978). Other extraction techniques used disposable SPE catridges containing strong basic anion-exchange material (SAX), which was preconditioned with methanol and water. Lower urine sample volume was introduced and eluted with 10% aqueous acetic acid (Rauscher *et al.*, 1994).

The urinary cresol is present in urine sample usually as sulfate or glucuronic acid conjugates. Hydrolysis and neutralization procedures are needed for changing this sulfate or glucuronic acid conjugates to cresol isomer. Acid hydrolysis was the first step encountered to produce unconjugated *o*-cresol and extracted with methylene chloride, concentrated by evaporation and the sample was analysed by gas chromatograpy-flame ionization detector (Truchon *et al.*, 1996). However for SPE, acid hydrolysis is still required in this step followed by adjusting the mixture to a basic condition with sodium hydroxide. A SPE catridge was conditioned by methanol and distilled water and eluted with acetonitrile-methanol (l:1 v/v) (Bieniek, 1996).

For SPE method, the packed sorbent used for hippuric acid (SAX) and *o*-cresol (C18) are relatively expensive. Biological sample can effect the sorbent physical properties and the sorbent deteriorated after handling a few samples. It was reported this will reduce the efficiency of packed sorbent. (Moein *et al.*, 2014a). Besides that, SPE has other disadvantages such as limited life-time, high detection limit, incomplete removal of interferences and sample carry (Toulabi *et al.*, 2010).

Ukai *et al.,* 2007 reported that the urinary toluene metabolites of hippuric acid 30 mg/L, *o*-cresol 15.4 µg/L, benzylmercapturic acid 1.51 µg/L, unmetabolized toluene 1.47µg/L and benzyl alcohol 0.15

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