



## **A dispersive solid phase extraction of hippuric acid in urine by silica nanocomposite supported onto silica gel prior to HPLC**

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### **Abstract**

In this study, a novel adsorbent material based on silica nanocomposite supported silica gel was successfully developed for extraction of hippuric acid. This adsorbent material was prepared via sol-gel method using 3-(propylmethacrylate)trimethoxysilane/silica nanocomposite (SN) acetic acid as catalyst, hippuric acid (HA) as template, acetonitrile as solvent and supported by silica gel. The morphology of SN was observed using field emission scanning electron microscopy (FESEM) while texture properties by N<sub>2</sub> adsorption analysis. The silica nanocomposite was employed as a sorbent for extraction of hippuric acid. The effect of precursor amount, and template quantity on the sorption characteristic of SN was evaluated. Adsorption studies were conducted to identify the SN with the best sorption capacity for the extraction of hippuric acid. The adsorption carried out using 10 mL of sample or hippuric acid standard (200 mg/L), 0.2 g SN, 15 minutes stirring time and at pH 6. The HA

was then desorbed into 10 mL methanol: acetic acid (9:1) solution by ultrasonication for 10 minutes. The SN prepared using 800 µL of 3-(propylmethacrylate)trimethoxysilane), (0.30 mmol/L of hippuric acid), 700µL acetonitrile as solvent and 600 µL of acetic acid was found to exhibit better sorption for hippuric acid. The results show percentage recovery around 97.9-99.6% and relative standard deviation at 0.4-4.6%, respectively. The silica nanocomposite supported with silica gel performance validation were analysed by high performance liquid chromatography (HPLC) at 225 nm of UV detector.

**Keywords:** Silica nanocomposite, hippuric acid, sol-gel, High performance liquid chromatography.

## Introduction

Toluene has been the most widely used organic solvent in the industries in this decade<sup>(1,2)</sup>. It has been reported that exposure to high concentration 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<sup>(3-5)</sup>. Exposure to high concentration of toluene in the workplace is one of the main risks that often occur in industrial sector. Toluene metabolizes in the human body and excreted in urine as hippuric acid, benzyl mercapturic acid and sulphate glucuronic acid conjugates (cresol). One of the ways to detect worker's exposure to toluene are using hippuric acid, *o*-cresol and benzyl mercapturic acid as a biomarker by screening blood and urine. Inoue et al., (2007) defined the biomarker of toluene exposure excreted in urine are 82.3% hippuric acid, 0.06% *o*-cresol and 0.03% benzylmercapturic acid<sup>(6,7)</sup>. Therefore, studies were strictly concentrated on hippuric acid metabolite as this a higher metabolite excreted in urine sample.

For extraction of urine sample, liquid-liquid extraction (LLE) was used for sample preparation method. However, this method was complicated and involved a multiple steps, whereby the sample should be acidified and basified then extracted with ethyl acetate followed by centrifugation. Evaporation step would take part for sample pre-concentration step and organic layer was taken and injected into HPLC<sup>(8)</sup>. The main drawbacks of this method are time consuming, require large amount of organic solvent and low sensitivity.

Nowadays, solid phase extraction is commonly used as an extraction medium including

hippuric acid extraction. Therefore, solid phase extraction (SPE) is performed using disposable cartridges containing 500 mg of strong basic anion-exchange material (SAX), which is preconditioned with 3 mL methanol and 3 mL water. After applying 1-2 mL urine, the cartridge is washed with 3 mL 1% aqueous acetic acid. The hippuric acid is eluted with 3-4 mL of 10% aqueous acetic acid <sup>(9)</sup>. Nevertheless, the SPE sorbent is costly and limited shelf-life.

Thus, the drawbacks has led to the current quest by researchers to produce or to find new extraction techniques. A surface modification of a polysulfone membrane (MIP PSM) by molecularly imprinted sol-gel was developed by Moein *et al*; 2014 <sup>(10)</sup>. Later, this research was expanded using hollow fiber polysulfone membrane <sup>(11, 12)</sup> and utilized for online extraction of hippuric acid in biological matrices for detection of lung cancer. Even though, MIP PSM and MIP hollow fiber PSM are very good membrane extractor, PSM and hollow fiber PSM itself is very expensive. In addition, these membranes are not strong and tough because they can be broken by the glass surface on the membranes.

Nanocomposite silica has emerged as a popular alternative and it offers certain advantages. They can be synthesized by using facile methods; are inert and exhibit good stability. Their existence in the form of spheres, wires and sheets renders them suitable for varied purposes. Their porosity can be tuned to alter surface areas and they can be easily functionalized to display different chemical groups. Nanocomposite silica on the account of their sorbent properties, porous nature and increased surface area allow more effective extraction <sup>(13)</sup>.

In spite of emerging interest in nanocomposite silica, to the best of our knowledge, no surface modification using nanocomposite silica has yet been reported for the separation and determination of hippuric acid. The purpose of present work is to synthesize silica nanocomposite via sol-gel method and supported onto activated silica gel. This novel silica nanocomposite supported with silica gel would be a spectacular material to extract hippuric acid from urine sample.

In this study, hippuric acid was used as template, 3-(propylmethacrylate)trimethoxysilane as a precursor and acetic acid as catalyst. Under optimized conditions, nanocomposite silica was used as adsorbent for selective extraction of hippuric acid from urine sample. Moreover, the selectivity of prepared nanocomposite silica supported onto silica gel was evaluated by determination of hippuric acid in urine sample coupled with HPLC. The extraction performance of developed silica nanocomposite supported by silica gel was evaluated and its

physical properties were characterized by FESEM, N<sub>2</sub> adsorption, and FTIR. The developed nanocomposite silica supported by silica gel offered an alternative method of clean up and determination of hippuric acid, carrying the benefit of simplicity, low cost and sufficient sensitivity, without any large volumes of organic solvent and robust.

## **Material and methods**

### **Materials and chemicals**

Hippuric acid (HA) and 3-(propylmethacrylate)trimethoxysilane (3-PMTMOS), with purity 98% were purchased by Sigma Aldrich (United State of America) while acetonitrile (ACN), acetic acid, hydrochloric acid (HCL), silica gel and sodium hydroxide were supplied from Merck (Darmstadt, German).

### **Silica gel Activation**

The silica gel surfaces were activated by combination of 10 g of the silica gel with 100 mL of 6 mol/L hydrochloric acid. The resultant mixture was refluxed under stirring for 10 hours. The solid product was filtered and washed repeatedly with double deionized water to neutrality and dried at 110 °C for 10 hours<sup>(14, 15)</sup>.

### **Synthesis of silica nanocomposite supported by silica gel**

The sol-gel nanocomposite solution, which was used for modification of the silica gel was optimized carefully by adjusting the amount of the precursor and template amount as listed in Table 1. A mixture of 800 μL of 3-PMTMOS as precursor and 700 μL hippuric acid (HA) (0.3mmolL<sup>-1</sup>) as template molecule was sonicated for 30 minutes to prepare the silica nanocomposite (SN) sol-gel solution with the maximum binding probability. Then, (600μL) acetic acid was slowly added stepwise (200μL each step) and sonicated for another 10 minutes. This was followed by dropwise addition of 200μL distilled water to start hydrolysis process and the solution was kept in this state for 30 minutes. Finally, to form silica nanocomposite sol-gel layer supported onto silica gel, it was immersed in activated silica gel and leave it the solution two days to create the maximum binding between silica nanocomposite and silica gel. For polycondensation process, the silica nanocomposite supported by silica gel was subjected to a temperature at 105°C for 8 hours. After completing these steps, this silica nanocomposite supported by silica gel was washed with a mixture of methanol: acetic acid (9:1) by soxhleet extraction for 24 hours to remove the trapped and

create a porous selective surface <sup>(10–12, 16)</sup>.

### **Physical characterization of silica nanocomposite**

Physical characterization of the silica nanocomposite were performed using field emission scanning electron microscopy (FESEM), Nitrogen Adsorption and Fourier Transform Infrared (FTIR). The surface morphology and elemental composition of the sample was analyzed using FESEM 6701 F microscope with energy of 15.0 V couple with EDX analyzer. The grinded sample was sputtered aluminum stub that covered with carbon cement tape and coated with platinum. The stub morphology scanning was obtained under different magnification to obtain clear surface images of the samples. Nitrogen adsorption porosimetry measurements were performed on an ASAP 2010 accelerated surface area and porosimetry analyser (Micromeritics 3Flex Version 3.01 Instrument Corporation, Norcross, GA). Prior to measurements, 300–400 mg portions of sample were degassed overnight at 100°C under high vacuum. The specific surface areas (S) were calculated using standard BET method and specific pore volumes ( $V_p$ ) and average diameters ( $d_p$ ) using BJG theory. Fourier transform infrared spectra of activated silica, silica nanocomposite unleached and silica nanocomposite leached were done using potassium bromide (KBr) pellet technique and recorded in the range of 400–4000  $\text{cm}^{-1}$  by Perkin Elmer spectrometer (model 707, Germany).

### **Adsorption and Desorption Study**

A stock solution of hippuric acid at a concentration of 200 mg/L was prepared by acetonitrile-distilled water (10:90 v/v) and stored at 4°C in the refrigerator. 0.2 g of silica nanocomposite (SN) was weighed and used as an extraction medium. Prior conducting adsorption study, the SN was immersed with a mixture of methanol/acetic acid (8:2) for 1 hours to remove the hippuric acid template and activate the surface. The SN was placed in the stock solution (200 mg/L) and stirred for 15 minutes. The concentration of the hippuric acid after adsorption by the SN was determined by measuring the supernatant absorbance at 225 nm using high performance liquid chromatography (HPLC). The amount of hippuric acid bound to the SN was calculated by subtracting the amount of free hippuric acid from the initial amount added in the mixture <sup>(17)</sup>. For the desorption procedure, SN was sonicated with several solvents on ultrasonic water bath for 10 minutes.

The chromatographic system was equipped with a 150 mm x 2.0 mm i.d. reversed phase C18 column (Thermo Scientific, USA) using HPLC-UV Waters. Separation was carried out in isocratic mode using a mobile phase of distilled water, acetonitrile and acetic acid

(84:16:0.025). The mobile phase flow rate was fixed at 1.0 mL/min. UV detection of analyte was at 225 nm<sup>(18)</sup>.

### **Silica nanocomposite performance Validation**

The linearity and determination of the limit of detection (LOD) and limit of quantification (LOQ) of SN leached and SN without template were performed by preparing a standard stock solution of hippuric acid at a concentration of 1000 mg/L in acetonitrile-distilled water. Standard solution was prepared at concentration 10 mg/L, 50 mg/L, 100 mg/L, 150 mg/L and 200 mg/L. Blank urine sample were spiked with 5 mg/L, 10 mg/L, 50 mg/L and 100 mg/L hippuric acid standard, then it was extracted by SN procedure and analyzed by HPLC-UV at 225 nm. Calibration curves with linear regressions were prepared by plotting the peak area value of standards against spiked analyte concentrations.

## **Results and Discussion**

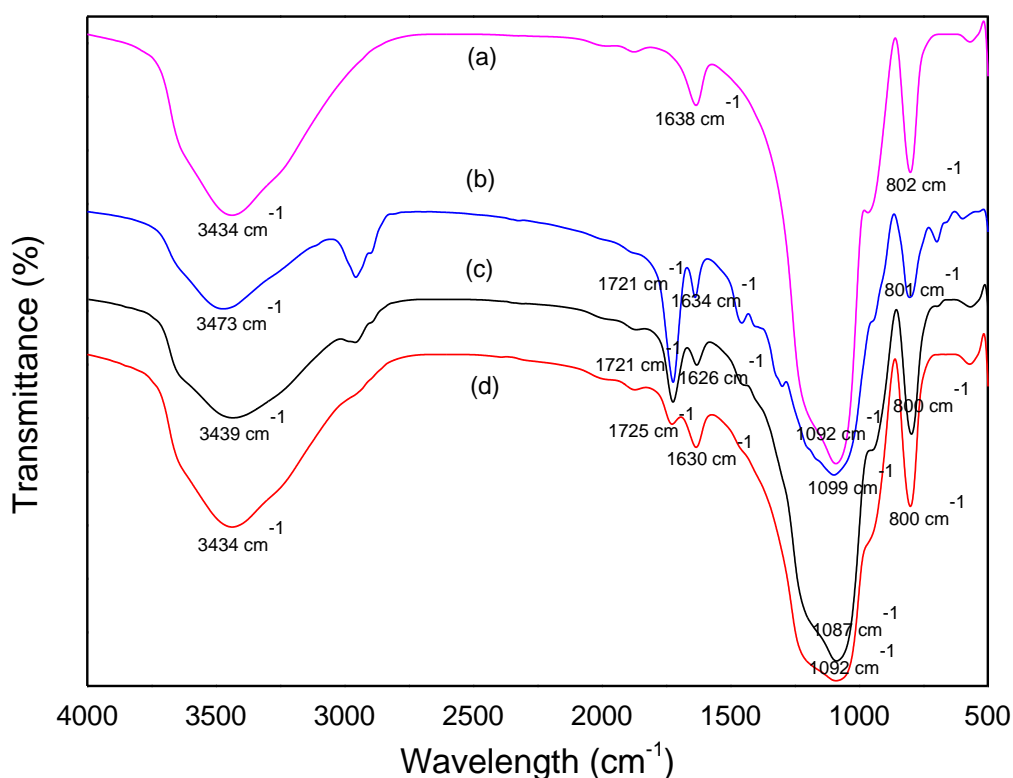
### **Characterization of Silica nanocomposite**

#### **Fourier Transform Infrared**

The infrared spectra of activated silica, silica nanocomposite without template, silica nanocomposite unleached and silica nanocomposite leached was shown in Figure 1. The spectrum was displayed almost a similar characteristic peaks, indicating similarity in the backbone structured of silica gel, however the intensity of absorbance was slightly different between the silica nanocomposite and activated silica. All silica nanocomposite were illustrated the parallel peaks around 1626-1634 cm<sup>-1</sup> for O-H vibration, C=O stretching at around 1721-1729 cm<sup>-1</sup> and the bands around 800 cm<sup>-1</sup> resulted the Si-O vibrations. For, Si-O-Si stretching of activated silica, SN leached and SN without template showed similar peaks at around 1087-1092 cm<sup>-1</sup>. In addition the similar O-H stretching peaks for activated silica, SN leached ad SN without template were found at 3433-3438 cm<sup>-1</sup> (16, 19).

The interesting of absorption bands for activated silica was found on O-H vibration at 1638 cm<sup>-1</sup> because of activated silica gel not coated with any silane groups and no bonding was found on the spectrum. SN unleached displayed the interesting characteristics for Si-O-Si stretching vibration at 1099 cm<sup>-1</sup>, absorption bands at 2962 cm<sup>-1</sup> represented the stretching vibration of CH<sub>3</sub> and absorption band at 3473 cm<sup>-1</sup> are assigned to the O-H vibration. This clearly prove that the template binding existed because of the shifting of absorption band for

Si-O-Si<sup>(16, 20)</sup>. However, for the O-H stretching shifting are very significant compared other spectrums. In addition, the CH<sub>3</sub> stretching absorption bands is very dominant. It can be conclude that the specific binding between template and propyl methacrylate group from precursor is exist



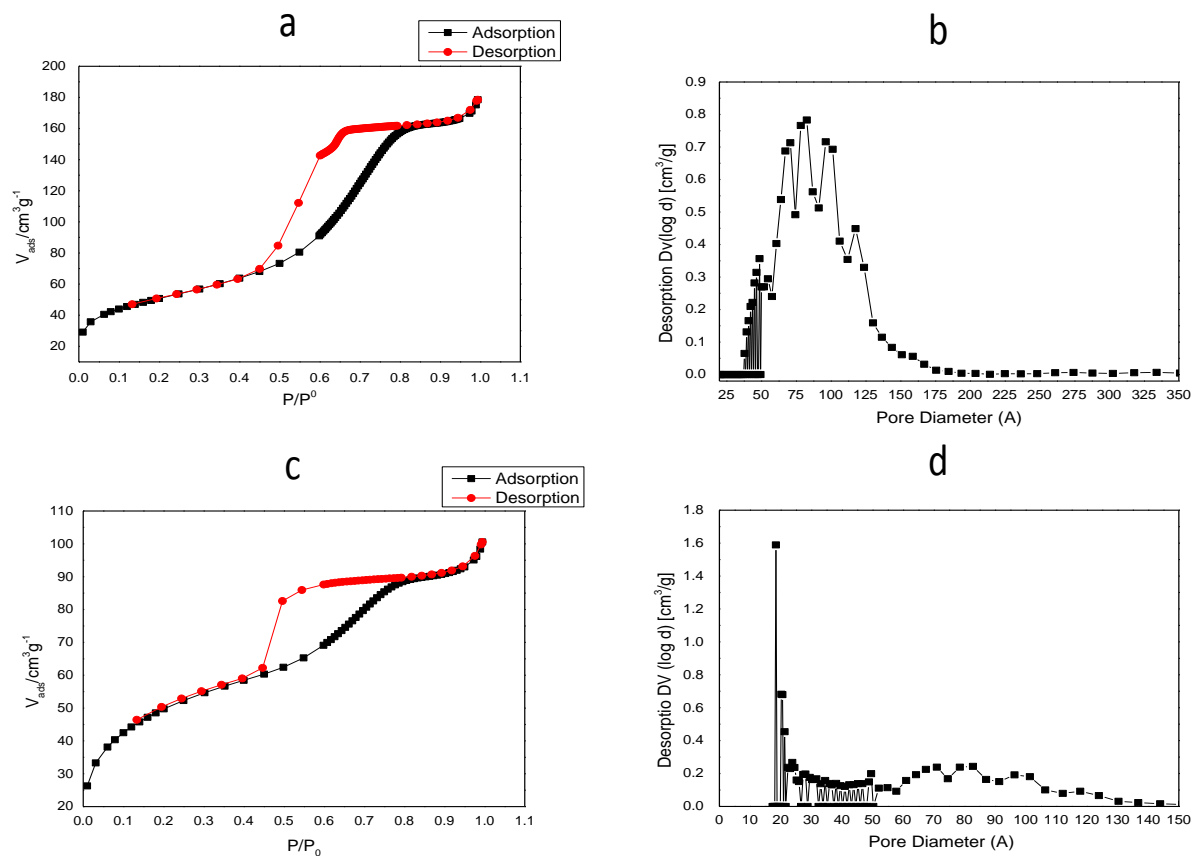
**Figure 1. Fourier Transform Infrared (FTIR) spectrums of (a) activated silica, (b) SN unleached, (c) SN without template and (d) SN Leached.**

### Nitrogen Adsorption Analysis

Figure 2 (a) and (c) shows the nitrogen adsorption and desorption isotherms of silica nanocomposite of SN leached and SN without template. The physisorption isotherms found to be Type IV, which indicates the existence of mesoporosity with high energy of adsorption cavities in silica nanocomposite (SN) leached and SN without template, respectively. Characteristics of Type IV indicates the presence of its hysteresis loop which is appeared related to the capillary condensation occurred in the mesopore structures. SN leached and SN without template tend to exhibit the hysteresis loop of a type H2. Type H2 indicates its pores have a narrow neck with wide bodies, generally known as ‘ink-bottle shapes’ pores<sup>(21, 22)</sup>.

The pore size distribution curves from desorption of pore diameters and apertures of SN

without template (b) and SN leached (d) (illustrated in Figure 2). The condensation and evaporation processes occurred at relative pressures related by the Kelvin equation to the diameters of pores and openings, correspondingly. During the adsorption process, the gas condensates inside the pores at particular pressure values depending on the cavity diameters but, during the desorption process, evaporation starts from the meniscus at pore's entrance when the relative pressure corresponds to the aperture size. The cavities created in SN without template was showed maxima homogenous pore openings in a range of pore diameters (78.50-82.79 Å) or (7.85-8.27 nm); (0.7663 – 0.7829 cm<sup>3</sup>/g). However, for SN leached was illustrated well-defined maxima homogenous pore openings in a range of pore diameters (18.44-20.23 Å) or (1.84- 2.02 nm); (0.6816 – 1.5887 cm<sup>3</sup>/g). Figures 2(c) and 2(d) was classified as in the mesopores region (2nm<pore diameter<50nm). The higher pore diameter of SN leached were displayed the higher imprinting effect of templates <sup>(23)</sup>.



**Figure 2. Nitrogen adsorption isotherms of silica nanocomposite without template (a) and silica nanocomposite leached (b) and pore size distribution curves were plotted from BJH desorption for silica nanocomposite (c) and silica nanocomposite leached.**



According to the fundamental of physisorption, it is practical to categorize pores based on their sizes. Therefore, Table 1 describes that SN without template and SN leached was categorized as mesopores (2-50 nm). Surface area, pore volume and pore diameter of SN without template were showed higher value than SN leached. Table 1 below was tabulated the BET surface area, external surface area, pore volume and pore diameter for SN without template and SN leached.

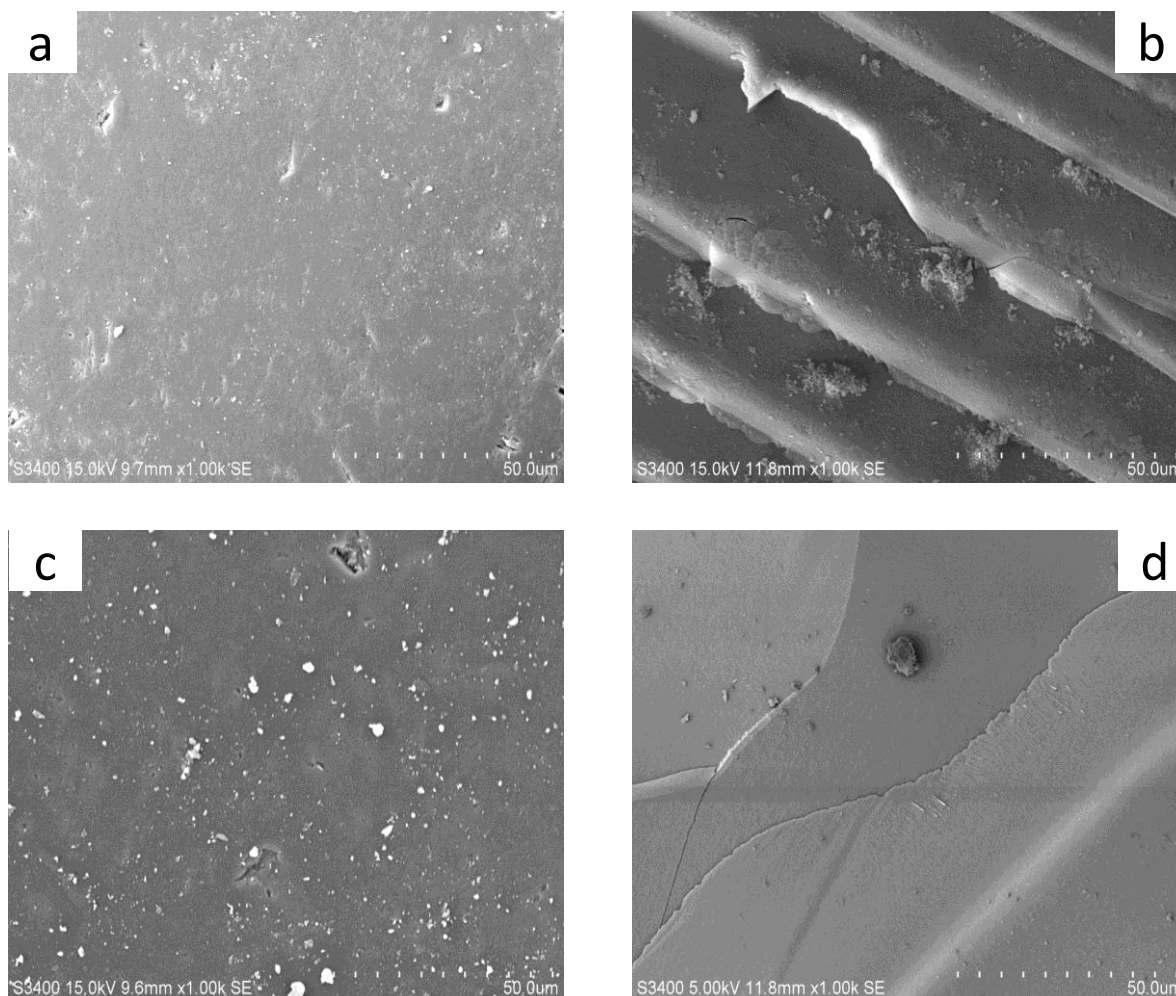
**Table 1. Surface area and pore analysis of SN without template and SN leached by nitrogen adsorption.**

Silica nanocomposite	BET surface area, $S_{\text{BET}}$ ( $\text{m}^2/\text{g}$ ) <sup>a</sup>	External Surface area, $S_t$ ( $\text{m}^2/\text{g}$ ) <sup>b</sup>	Pore volume ( $\text{cm}^3/\text{g}$ ) <sup>c</sup>	Pore Diameter ( $\text{\AA}$ ) <sup>d</sup>
SN leached	179.2	163.6	0.1284	39.76
SN without template	181.3	151.1	0.2843	49.04

<sup>a</sup>Surface area from multipoint BET value on a eleven-point linear plot, <sup>b</sup>External surface area obtained from  $t$ -plot, <sup>c</sup>BJH method cumulative desorption pore volume, <sup>d</sup>BJH method desorption pore diameter

### **Field Emission Scanning Electron Microscopy (FESEM)**

The field emission scanning electron microscopy (FESEM) was performed to study the characterization of surface morphology of SN without template and SN leached particles and cross section of their supported silica gel. Figure 3 (a) of surface FESEM images displayed by SN without template shown a smooth, slippy surface and cross section displayed in multi-layer. It is due to the no interaction between template and silica gel on surface imprinting. However, figure 3 (b) illustrating a rough, scraggy surface and single layer was found on cross section of SN with template. This condition indicates the template and the precursor has developed a bond and clocked on the layers. Hence, only a single layer was found at this cross section. Besides that, the rough and scraggy surface indicates by the cladding and filling actions of the crosslinking polymer layers which was formed in the process of HA surface imprinting.



**Figure 4. FESEM micrographs of (a) SN without template surface, (b) cross section of SN without template, (c) SN leached surface and (d) cross section of SN leached.**

### **Nanocomposite silica supported onto silica gel using sol gel method**

In the preparation process, some important parameters were carefully considered. Optimization of the proper ratio between template and precursor to create maximum binding network and better selectivity for silica nanocomposite is a critical issue. The results of this investigation showed that a HA 0.3 mmol/L :800  $\mu$ L precursor (SN C2 in Table 2) is the optimize ratio with the highest recovery and selectivity compared to other SN. The ratio of activated silica, SN C1, SN C2 unleached and SN C2 leached are select for further characterization due to the highest recovery.

**Table 2. Recovery result for preparation of silica nanocomposite supported onto silica gel with different amount of template and precursor.**

Silica nanocomposite	Template (mmol)	Solvent ACN ( $\mu\text{L}$ )	Precursor ( $\mu\text{L}$ )	Water ( $\mu\text{L}$ )	Acetic acid ( $\mu\text{L}$ )	Recovery (%)
SN A1	-	700	1000	200	600	28
SN A2	0.3	700	1000	200	600	92
SN B1	-	700	900	200	600	25
SN B2	0.3	700	900	200	600	95
SN C1	-	700	800	200	600	20
SN C2	0.3	700	800	200	600	98
SN D1	-	700	700	200	600	19
SN D2	0.3	700	700	200	600	88
SN E1	-	700	600	200	600	18
SN E2	0.3	700	600	200	600	84

Based on the tabulated table 2 all the silica nanocomposite has been successfully synthesized by sol gel polymerization. The preparation procedure via sol-gel method occurred in simple step which is hydrolysis and condensation. However, some of silica nanocomposite was not form fully gel because of the amount of precursor is not equivalent with amount of template. Because of this reason, the colloidal suspension is not occurred while in the process of synthesize this nanocomposite silica<sup>(24, 25)</sup>.

Nanocomposite silica using sol-gel method was successfully coated onto silica gel. This material should be immersing deeply to capture all the silica gel was fully coated onto nanocomposite silica sol-gel solution. The poly condensation process should executed with higher temperature to deliver sol-gel solution became solidify in the silica gel. Thus, the higher temperature will evaporate the water and acetic acid. Meanwhile, the bonding between 3-PMTMOS, hippuric acid and silica gel will be occur<sup>(10, 12)</sup>.

### **Optimization of Adsorption study**

To evaluate the ability of the silica nanocomposite supported by silica gel for extraction of hippuric acid. Basically, the first step extraction process is trying to adsorption/desorption the silica nanocomposite with the hippuric acid standard and established the optimization of different parameters. In addition, the effect of different parameter on extraction efficiency,

including solvent solution, stirring time, effect pH and solvent volume were investigated and optimized <sup>(26)</sup>.

### **Effect of pH**

To comprehensively evaluate the performance of silica nanocomposite, consideration was made in this research only pH for the first started. The pKa of the hippuric acid is reported as 3.62 <sup>(27)</sup>. This analyte is deprotonated in neutral and alkaline medium <sup>(28)</sup>. Hence, the pH of the sample solution was expected to be the significant component determining the adsorption performance. The pH values were investigated in the acidic range of 3.0 to 6.0 by adjusting the sample solution with 0.1 M HCl solution. On the other hand, the basic range was investigated in 7.0 to 11.0 by adjusting the sample solution with 0.1 M NaOH solution.

Also that, as depicted in Figure 5 (a) significant improvements in adsorption recovery was noted when the sample pH was increased from 5.0 to 6.0. The low recoveries observed at pH higher than 7.0 might probably due to the instability of the supported silica gel that affects the silica nanocomposite performance. A further increase of the pH to 8.0, 10 and 11 resulted in a gradual reduction of recovery as analyte started to be deprotonated. Figure 5 (a) illustrated the effect of pH for extraction condition.

### **Stirring time**

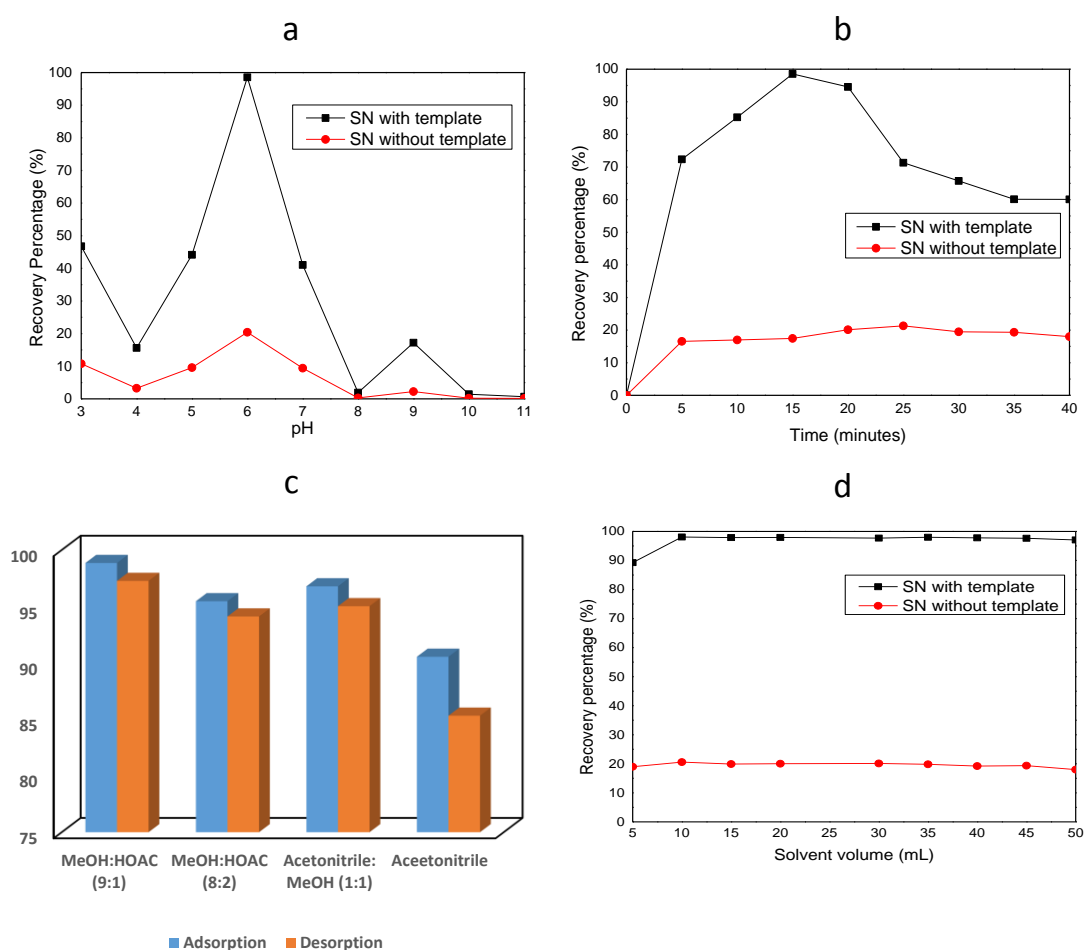
According to the results, stirring time the solution are seen to be important factors in the nanocomposite silica performance of hippuric acid extraction. The stirring time can be facilitating diffusion and mass transfer of the analyte from donor to acceptor phase. To this aim, stirring time in the range of 5-40 minutes was studied. Figure 5 (b) depicted that 15 minutes found to be the best stirring time for maximum extraction efficiency.

### **Solvent solution**

Organic solvents including methanol: acetic acid (9:1), methanol: acetic acid (8:2), acetonitrile: methanol (5:5) and acetonitrile were studied. The results show that the SN with template extraction amount of hippuric acid with methanol: acetic acid (9:1) was higher than those with other solvents. Probably, in low polarity solvents such as methanol: acetic acid, hydrogen- bond interactions of interferences are decreased. Moreover, better selectivity for the extraction of hippuric acid with SN with template was observed in methanol: acetic acid (9:1) at Figure 5 (c). Thus, methanol: acetic acid (9:1) was selected as the extraction solvent for further experiments.

## Effect of solvent volume

The effect of solvent volume on recovery of extraction hippuric acid by SN with template and SN without template was considered. For this aim, different ranges of solvent volume (5-45 mL) and the solvent used is methanol and acetic acid and the ratio (9:1). The results showed in Figure 5 (d) the extraction efficiency was at the maximum level for hippuric acid at volume of solvent 10 mL and increasing the solvent volume demonstrated the staged graph. Methanol and acetic acid have a strong hydrogen-bond interaction between the analyte because of that it can be extracted out from silica nanoparticle. However, increasingly the solvent volume did when the optimum condition not effect too much for recovery.



**Figure 5.(a) effect of pH for extraction condition (b) effect of stirring time for extraction condition (c) effect of different solvents (d) effect of solvent volume for extraction condition.**

### Enrichment factor

In order to evaluate the extraction efficiency of silica nanocomposite and obtain the optimized extraction conditions, enrichment factor (EF), extraction recovery (ER) and imprinting factor (IF) were studied and calculated in a manner similar to previous work by this researchers<sup>(10-12)</sup>.

**Table 3. The selectivity of SN with template for extraction of hippuric acid compared to SN without template (volume and concentration of compound were 10 mL, 100 mg/L, five determinations).**

Analyte	Enrichment factor (EF)		Imprinting factor (IF) SN with template/ SN without template
	SN with template	SN without template	
Hippuric Acid	0.98	0.19	5.2

In order to consider EF of SN with template and SN without template for extraction of hippuric acid were studied using the same process and the results are shown in Table 3. According to the results, SN with template showed good selectivity in the extraction of hippuric acid (IF was obtained at 5.2).

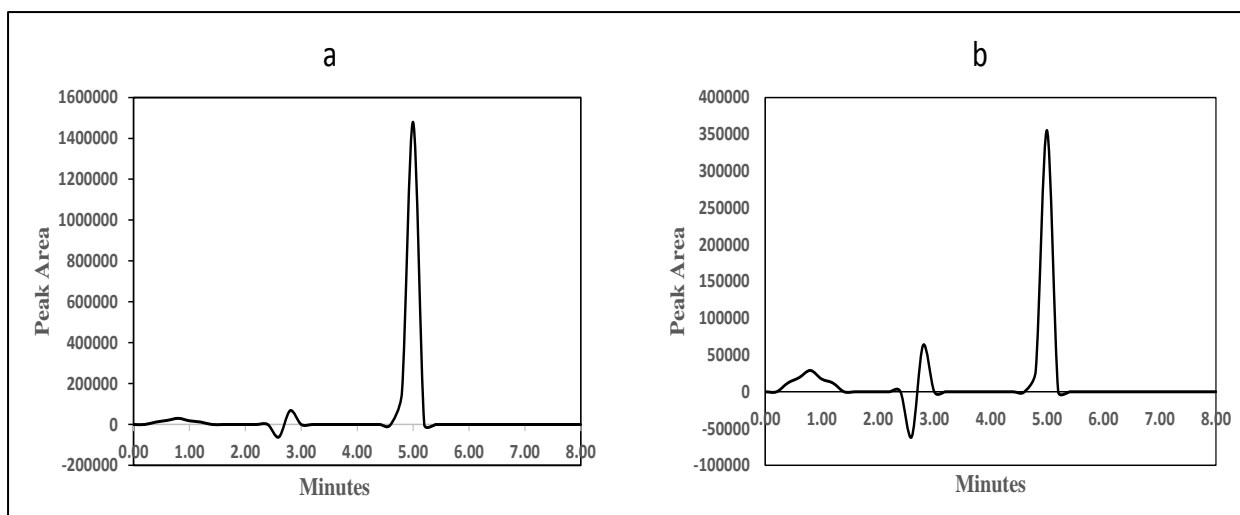
### Silica nanocomposite performance validation

Limit of detection (LOD), limit of quantification (LOQ) and relative standard deviation (RSD) were summarized in Table 4. The correlation coefficients  $r$  of the calibration curve method was  $\geq 0.9997$  for hippuric acid. The regression analyses between peak areas of hippuric acid compound versus concentration show satisfactory linearity in the range of 10 mg/L to 200 mg/L. The proposed silica nanocomposite provides a satisfactory and good LOD, LOQ and RSD for the detection of hippuric acid using standard calibration. The hippuric acid was extracted by SN using C18 HPLC column and detection at a wavelength of 225 nm by UV detector (Figure 6).

**Table 4. The LOD, LOQ and RSD values of hippuric acid using silica nanocomposite (SN)**

Analyte	LOD <sup>a</sup> (mg/L)	LOQ <sup>b</sup> (mg/L)	RSD <sup>c</sup> (%)	$r$
Hippuric acid	0.02	0.06	0.4-4.6	0.9997

<sup>a</sup>Limit of detection, <sup>b</sup>Limit of quantation and <sup>c</sup>Relative standard Deviation

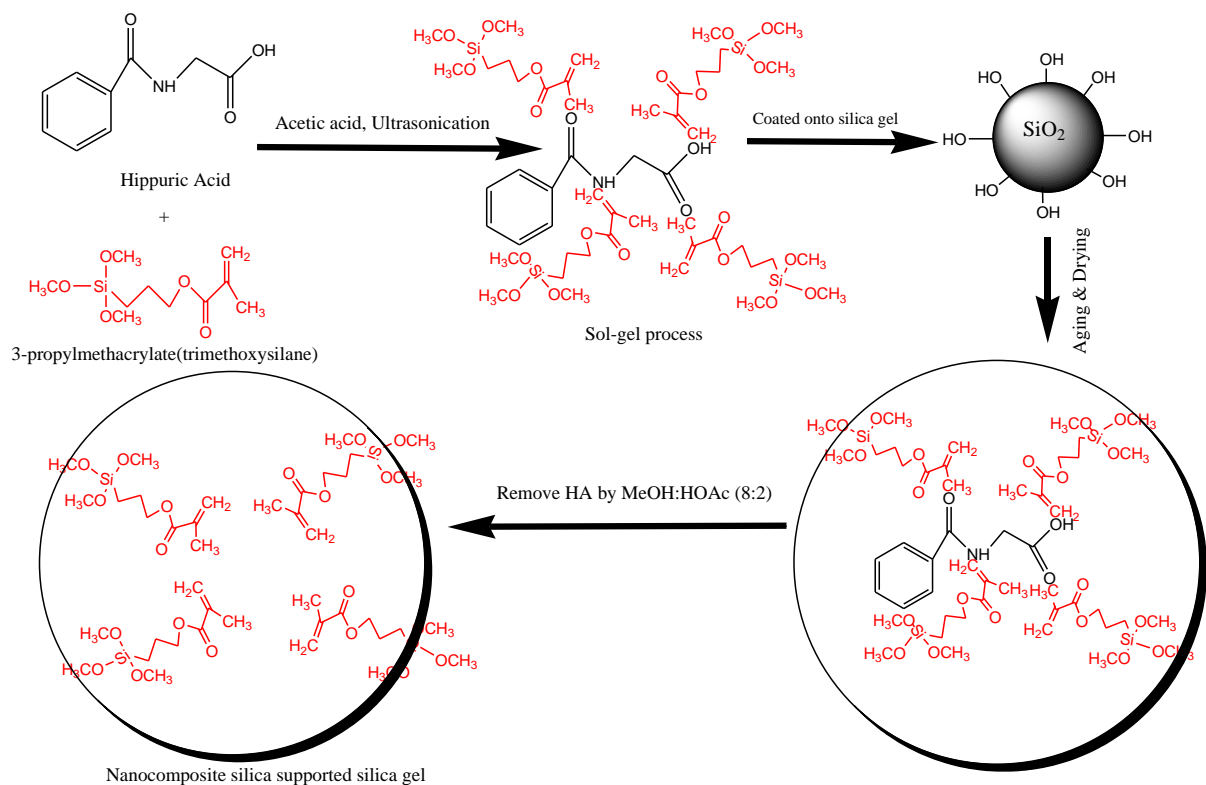


**Figure 6. HPLC condition: isocratic, UV detector at 225 nm, mobile phase acetonitrile: distilled water: Acetic Acid (16:84:0.025); 1.0 mL/min flow rate. HPLC separation of silica nanocomposite supported by silica gel (a) extraction with 200 mg/L of hippuric acid standard (b) extraction of spiked with 100 mg/L hippuric acid in urine sample.**

### Proposed mechanism

Hippuric acid was selected as a template molecule and 3-(propylmethacrylate) trimethoxysilane (3-PMTMOS) as a precursor. In this case, propylmethacrylate moiety from precursor was responsible for Van Der Waals, dipole-dipole and hydrogen bond interaction with the template (17). This moiety plays a key role in creation of selective sites while trimethoxysilane groups in 3-PMTMOS acted as a cross-linkers between the template and the propylmethacrylate moiety <sup>(29)</sup>.

However, for silica gel and trimethoxysilane groups the propose binding on the Si-O-Si from both side. So, the trimethoxysilane was attacked the hydroxyl group from silica gel to form Si-O-Si and the result FTIR spectrum show that the hydroxyl group was decreased and Si-O-Si peak was found strong peak <sup>(14)</sup>. The propose mechanism of nanocomposite silica supported onto silica gel was illustrated in Figure 7.



**Figure 7. Propose mechanism for Nanocomposite silica supported onto silica gel.**

### Application for blank urine sample

The proposed method was applied to determine the concentration of hippuric acid spiked in urine samples and the obtained results were summarized in table 5. The tabulated results point out that 0.5 mg/L hippuric acid found in blank urine sample. It is consider normal hippuric acid containing in human body <sup>(30)</sup> and the obtained results for spiked urine samples are in good agreement with the respected values, showing satisfactory recovery of more than 98%.

**Table 5. Analytical results of hippuric acid in blank urine samples.**

Analyte	Spiked (mg/L)	Urine sample	
		Found mg/L	Recovery
Hippuric Acid	0	0.5	
	5	5.48	99.6
	10	10.37	98.7
	50	49.71	98.4
	100	98.40	97.9



## Method comparison

Several analytical methods for the determination of hippuric acid using GC-FID<sup>(31, 32)</sup>, HPLC<sup>(33)</sup>, GC-MS<sup>(34)</sup> and LC-MSMS<sup>(10, 11)</sup>. Nevertheless, problem do occurred on sample preparation using GC analysis whereby derivatization step should be included. In analytical work, time, labor consuming and the need for fast and simple sample preparation was required. In this study, silica nanocomposite supported onto silica gel provided a simple microextraction, selective and robust extractor medium. The proposed method, in some cases, appears to be superior to the other reported techniques for selective determination of hippuric acid including using LC-MSMS. Table 6 illustrates the linear range, limit of detection, recovery and relative standard deviation for latest researches.

**Table 6. Determination of hippuric acid using different methods.**

Method <sup>a</sup>	Extraction method <sup>b</sup>	Linear range	LOD (ng/mL)	Recovery (%)	RSD (%)	References
GC	LLE	5.0 -70 µg/mL	250	93.4	4.3	(31)
GC-MS	LLE	0.05-25µg/mL	65	92.7	3.6	(34)
HPLC	Centrifugation	2.0-32µg/mL	80	>92	0.36-5.7	(33)
LC-MSMS	MISM-MEPS	1-2000nmol/L	0.5	91-96	1.1-7.1	(10)
LC-MSMS	MSHM-LPME	1-2000nmol/L	0.5	89-93	1.2-4.5	(11)
HPLC	SN-silica gel-SPE	5- 200 µg/mL	20	>98	0.4-4.6	Present work

<sup>a</sup>GC, Gas Chromatography; GC-MS, Gas Chromatography-Mass Spectrometry; HPLC, High Performance Liquid Chromatography; LC-MS-MS, Liquid Chromatography-Mass Spectrometry-Mass Spectrometry.

<sup>b</sup>LLE, Liquid liquid extraction; MISM-MEPS, Molecularly imprinted polymer polysulfone membrane-microextraction packed sorbent; MSHM-LPME, Molecularly imprinted sol-gel hollow fiber membrane-liquid phase microextraction; Silica nanocomposite supported onto silica gel- solid phase extraction.

## Conclusion

In this work, a novel 3-(propylmethacrylate)trimethoxysilane/silica nanocomposite (SN) supported by silica gel was develop via sol-gel method. Physcial and chemical characterization analysis by using FTIR, FESEM and Nitrogen adsorption had provided

evidence of the chemical interaction, surface morphology and cavities of silica nanocomposite material, which are the key to fundamental imprinting studies.

This nanocomposite silica supported onto silica gel was applied for the selective extraction of hippuric acid. This materials used in liquid-phase microextraction technique for extraction of target analyte. The results of this work were studied by HPLC and some effective parameters in the extraction process such as organic extraction solvent, stirring time, pH and imprinted factor were investigated and optimized. The results showed the excellent accuracy, precision and sensitivity for this method.

For the future works, this nanocomposite silica would be apply for the extraction of hippuric acid in urine sample, whereby the real sample is collected from the medical surveillance sample.

### **Conflict of Interest**

They are no conflict interests were involved in this research.

### **Source of Funding**

Our gratitude goes to the Department of Chemistry Malaysia and the Public Relation Department of Malaysia for studentship and financial support to Mohamad Raizul Zinalibdin. Our institution, Universiti Teknologi Malaysia (UTM) is thankfully acknowledged for providing facilities in this study laboratory works. For my proofreader in this research, Miss Bashirah Fadzli and Madam Faridah Mohd Marsin your work is not forgotten.

### **Ethical Clearance**

Blank urine samples were taken by Department Chemistry of Malaysia, Johor Branch. Real urine samples will be taken by collaboration with Department of Occupational Safety & Health (DOSH)

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