Journal of Chromatography B, 877 (2009) 2945–2951

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Post-derivatization procedure for determination of hippuric acid after extraction by an automated micro solid phase extraction system and monitoring by gas chromatography

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ARTICLE INFO

Article history:
Received 5 May 2009
Accepted 24 June 2009
Available online 30 June 2009

Keywords:
Gas chromatography
Derivatization
Hippuric acid
Micro solid phase extraction
Urine sample

ABSTRACT

A rapid, simple and high sensitive method is described here for extraction of hippuric acid (HA) from human urine samples by using an automated micro solid phase extraction system (μ-SPE). However in order to increase sensitivity of gas chromatography with flame ionization detector, a post derivatization procedure was developed. In this work, a polypyrrole was synthesized by chemical oxidation of the pyrrole monomer in non-aqueous solution and applied as an excellent and efficient sorbent for μ-SPE. The calibration curves were linear in the range of 0.018–8.95 μg mL^{-1} for HA, in both water and urine samples with correlation coefficients 0.9973 and 0.9946, respectively; limits of detections were 12.1 ng mL^{-1} and 16.5 ng mL^{-1}, respectively. This method was successfully used to analyze trace amounts of HA in human urine samples without any interference from coexisting substances.

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1. Introduction

Trans-cinnamaldehyde is the main component of the essential oil in cinnamon bark and it is a popular food flavoring in Iran. Cinnamon bark is frequently used as a stomachic, an antipyretic and an antiallergic drug. It is well known that the trans-cinnamaldehyde has antifungal [1], antibacterial [2] and antitermitic [3] activities. Less is known about the human toxicity of trans-cinnamaldehyde and only limited to primary irritant, skin sensitization [4] and/or allergic reactions to perfume [5]. Trans-cinnamaldehyde is a reactive conjugated allyl aldehyde, and is rapidly oxidized to cinnamic acid when exposed to air. Also, cinnamyl anthranilate and 3,4,5-trimethoxy-cinnamaldehyde are two other related compounds to cinnamaldehyde that have been reported to induce tumors in experimental animals [6,7]. Mereto et al. demonstrated that the high doses of cinnamaldehyde in rats induced genetic alterations at the chromosomal level, and therefore, induced micronuclei in rodent liver [8]. However, due to structural similarity of cinnamaldehyde to cinnamic acid, cinnamyl anthranilate and 3,4,5-trimethoxy-cinnamaldehyde, recently the toxicity and carcinogenicity effects of cinnamaldehyde have been considered [9]. According to Teuchy et al., trans-cinnamaldehyde is oxidized to cinnamic acid and excreted in the urine as hippuric acid (HA) [10]. Peters and Caldwell in a study conducted on metabolism of trans-cinnamaldehyde in male and female Fischer 344 rats and CD1 mice found out that trans-cinnamaldehyde is excreted primarily as hippuric acid in the urine [11]. They suggested that HA is arising from the oxidation of cinnamaldehyde to cinnamic acid followed by β-oxidation of the side-chain to yield 3-hydroxy-3-phenyl propionic acid. Subsequent loss of a two-carbon fragment yields benzoic acid, which is further conjugated with glycine to yield HA [11]. A toxicokinetic study of cinnamaldehyde in male and female F344 rats given 50 mg kg^{-1}, 250 mg kg^{-1}, or 500 mg kg^{-1} by gavages demonstrated that excretion of HA was highly correlated (R = 0.999) with the dose of cinnamaldehyde [12]. In addition, Hoskins shows that the oral administration of cinnamic acid in humans also resulted in the excretion of HA in the urine [13]. To the best of our knowledge there is not any literature concerned with the analysis of cinnamaldehyde and/or cinnamic acid in human blood. Therefore, the best estimation for assessment of exposure to cinnamic acid and cinnamaldehyde is the quantitative determination of their metabolites, HA excreted in urine, which show a good correlation between the level of exposure and amount of metabolites excreted. There are several methods used for analyzing urinary HA, such as spectrophotometry [14], high-performance liquid chromatography (HPLC) with UV detector [15–19], liquid chromatography with tandem mass spectrometry [20], immunochromatography with ELISA [21], micellar electrokinetic capillary chromatography with UV detector [22], gas chromatography (GC) with mass spectrometer [23–26] and FID detectrors [27,28] and capillary zone electrophoresis with UV detector [29]. Because HA has a carboxylic group, the direct and efficient

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doi:10.1016/j.jchromb.2009.06.036

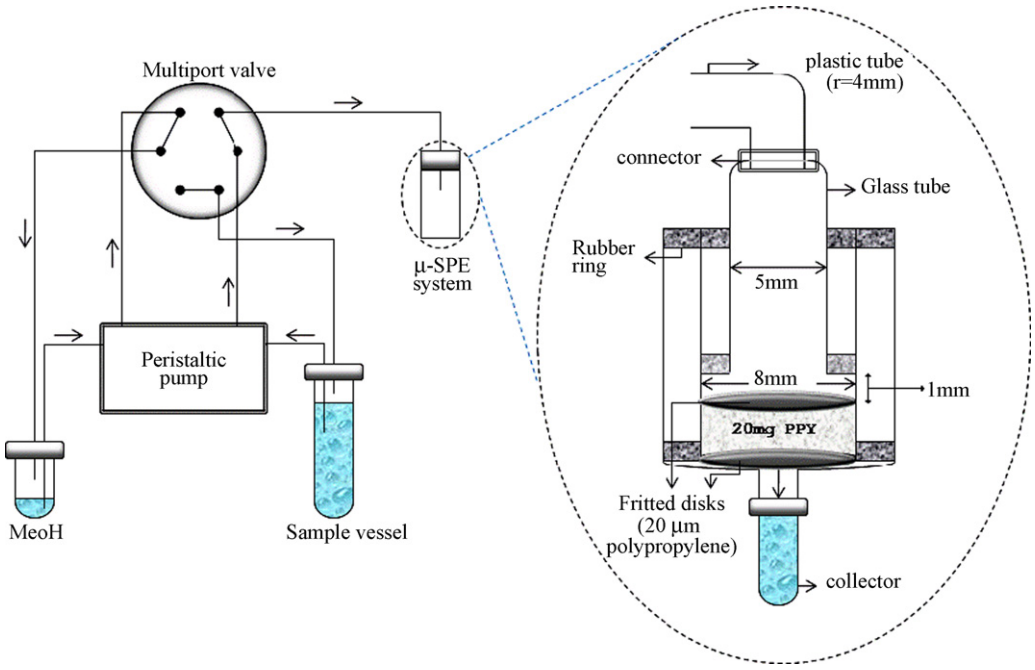

Fig. 1. Scheme of micro solid phase system.

analysis of HA is not suitable by GC and needs one-step derivatization. Methylation [27,28,30] and extractive isopropylation [27] have been used for the derivatization and GC separation of HA and its derivatives. Methylation with diazomethane requires highly toxic reagent and the silyl derivatives are moisture sensitive. Methylation with hydrogen chloride in methanol (HCl–MeOH), a low-cost and less toxic reagent, gives good peak shape and complete separation of HA and its derivatives on the GC [30]. In order to increase the sensitivity of procedure for determining the low concentration of HA, we used solid phase extraction method. Recently in our laboratory we developed a new automated micro solid phase extraction based on polypyrrole as a suitable sorbent for extraction of pesticides [31]. Due to multifunctional properties of polypyrrole, it has attracted a great interest in the development of chemical stationary phase for the separation and extraction of non-polar, polar and ionic compounds [32,33]. The polypyrrole extracted the analytes via the π–π, acid–base and hydrogen bonding interactions. In spite of wide application of SPE with different type of sorbents, it is established that the SPE is time-consuming (wetting, washing, conditioning, loading, cleaning and elution [34]), and requires high purity of organic solvents [35] that are potentially toxic and expensive. Recently, the micro solid phase extraction (μ-SPE) was developed for extracting per-*O*-methylated monosaccharides and disaccharides [36], persistent organic pollutants in tissue samples [37], acidic drugs in wastewaters [38], etc. The automated μ-SPE devices have some advantages over traditional SPE [31,39–41]. The purpose of the present study was to develop an on-line automatic method for extraction of HA from urine samples using a μ-SPE with polypyrrole system, post derivatization of hippuric acid by conversion to its methyl ester form with methanol in hydrochloric acid and monitoring by GC.

2. Experimental

2.1. Chemical reagents

All chemicals used were from commercial sources. Methanol (MeOH), ethanol (EtOH), acetonitrile (ACN), ethyl acetate (EA), hydrochloride acid (HCl), ortho-phosphoric acid (H_3PO_4), boric acid (HBO_2), sodium hydroxide, hippuric acid (HA), and $FeCl_3{\cdot}9H_2O$ were purchased from Merck (Darmstadt, Germany). Stock solution of 0.1 mol L^{-1} of HA was prepared in absolute methanol. Pyrrole was obtained from Fluka (Buchs Switzerland) and was used as received without any distillation.

2.2. Apparatus

For optimization of analytical conditions a Varian CP-3800 (Varian, Walnut Creek, CA, USA) gas chromatograph equipped with an automatic split-splitless injector and a FID system was used. A fused silica capillary column (CP-Sil 8 CB) with 30 m × 0.25 mm i.d. and 0.25 μm film thickness, supplied by Varian (Chrompack capillary column No.CP8752) was used with helium as carrier gas at a constant pressure of 10 psi. According to the instrument manual of Varian CP-3800 GC at this pressure the column efficiency reaches its maximum level. Other GC conditions employed for determination of HA were: injector temperature 280 °C, column temperature program 80 °C (hold for 1 min), 20 °C min^{-1} to 140 °C (hold for 1 min), 20 °C min^{-1} to 280 °C and stay at 280 °C for 10 min. FID Temperature was 280 °C. pH measurements were performed with a model 780 Metrohm (Switzerland) pH meter using a combined glass electrode. A Windaus two-channel peristaltic pump model D-38678 was used for simultaneous pumping of sample and eluent. A four-way injection valve (Supelco Rheodyne Model 5020) was used as a multiport valve.

2.3. Polypyrrole preparation

The polypyrrole was prepared according to literature [31] as follows: 0.67 g of pyrrole was dissolved in 10 mL ACN and was added slowly to 20 mL ACN containing 6.48 g (0.02 mol) $FeCl_3{\cdot}9H_2O$ by slow stirring (150 rpm at room temperature). A black precipitate with small and homogenized particle size was formed and after half an hour stirring of reaction mixture, the solid product was filtered and well washed with water, ACN and MeOH several times. The sorbent was placed in a beaker containing double distilled water for 24 h, then filtered, dried and stored in a glass dark vessel.

2.4. μ-SPE system

The μ-SPE system used to extract HA is prepared as follows: A fritted disk of polypropylene was placed in one side end of a cartridge (5 mm diameter) containing a lure tip. The cartridge was filled with 20 mg of synthesized polypyrrole particles, packed and fixed by another fritted disk (5 mm diameter). A glass tube (3 mm diameter) was used to introduce the sample and eluent to the cartridge. All sections of this system were fixed by using rubber rings (Fig. 1).

2.5. Urine sample preparation and extraction of HA

The urine samples were collected from healthy volunteers just drinking the cinnamon-tea after 5 h in the morning. Any precipitated material in exactly 10 mL of the samples was removed using centrifugation at 4500 rpm for 10 min, and then the supernatant of the urine (0.1 mL) was considered for analysis with the proposed procedure as follows: firstly the μ-SPE system was preconditioned with 3.0 mL MeOH and 3.0 mL of Britton–Robinson buffer (3.0×10^{-3} mol L^{-1}, pH 2); then an aliquot of the sample (0.1 mL) was pipetted into a 10 mL volumetric flask and diluted with Britton–Robinson buffer (3.0×10^{-3} mol L^{-1}, pH 2) up to the mark line. The samples were subsequently passed automatically through the preconditioned cartridge containing 20 mg of polypyrrole with a flow-rate of 3.0 mL min^{-1}. Then the cartridge was washed with 1.0 mL of Britton–Robinson buffer (3.0×10^{-3} mol L^{-1}, pH 2), 0.5 mL of double distilled water, and dried by passing of air. Finally, the HA was automatically stripped with 1.0 mL of MeOH at flow-rate of 1.0 mL min^{-1} by changing the valve of μ-SPE system.

2.6. Derivatization procedure

After μ-SPE of HA, the extract was collected in a 1.5 mL of micro centrifuge tube, and the organic phase was evaporated to dryness under a compressed N_2 flow at 70 °C temperature. The residue was redissolved in 150 μL of MeOH containing 0.33 M HCl; the cap of micro centrifuge tube was sealed and placed in a water bath at 70 °C for 20 min. Then the mixture was dried by N_2 at 70 °C and the residue was dissolved in 50 μL of MeOH. Finally 1.0 μL was injected to the GC systems with adopted split ratio of 80.

3. Result and discussion

Polypyrrole is one of the most multifunctional materials that are widely used in various scientific fields [42]. The preparation, characterization and properties of polypyrrole depend on its preparation conditions [43]. Polypyrrole has high extraction efficiency toward polar and aromatic compounds. High efficiency can also be achieved for anionic compounds due to the ion exchange property of polypyrrole. For example in a study by Wu et al. organoarsenic compounds were extracted from aqueous samples using in-tube solid phase microextraction coated with polypyrrole. In this study, it was shown that the PPY coated capillary, exhibited better extraction efficiency for most of the compounds studied, especially for the anionic species due to the inherent multifunctionality of pyrrole polymer [44]. However these multifunction interactive properties of polypyrrole such as non-polar interactions, acid–base interactions, dipole–dipole interactions, ion exchange properties and H-bonding make it a unique polymer when used as sorbent in solid phase and micro solid phase extraction systems. Bagheri and Mohammadi [35] applied polypyrrole as a sorbent for SPE of environmental pollutants from water. Wu et al. [45] applied polypyrrole for in-tube solid phase microextraction in order to determine the amphetamine, methamphetamine and their methylenedioxy derivatives in urine and hair. Yu and Lai [46] used polypyrrole modified stainless steel

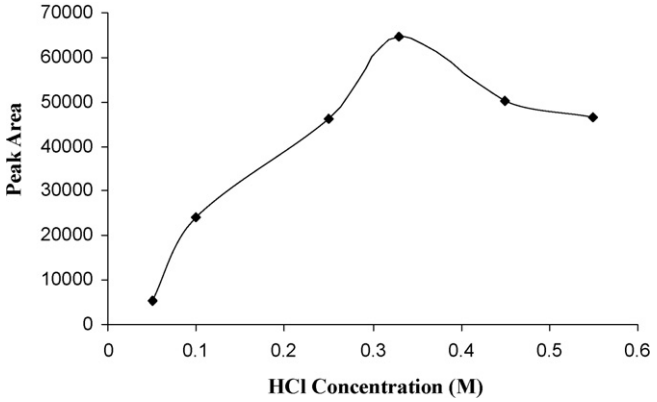

Fig. 2. The effect of HCl concentration on the derivatization of HA (the final derivatized residue was dissolved in 100 μL of MeOH).

frits for on-line micro solid phase extraction of Ochratoxin A. To study metabolites in urine, the sample preparation is a crucial task since metabolites often appear at very low concentrations present in complex matrices containing potential interfering such as proteins, salts and other small organic molecules of less interest. The challenge of the analytical chemist is therefore to extract the highest possible amount of analytes, preferably with a rapid, simple and automated method. HA is an acidic molecule, therefore, for μ-SPE of HA, several experimental parameters were optimized to achieve the highest efficiency determination and extraction. These parameters include derivatization conditions, pH of sample, type of striping solvent, flow-rate of sampling and breakthrough volume. The rate of elution was constant (1.0 mL min^{-1}) throughout the experiment; therefore it will not be discussed in detail.

3.1. Derivatization of HA

In all pervious studies when HA was analyzed by gas chromatography, difficulties were encountered in sensitivity and reproducibility, because of high boiling point, adsorption and interaction with the column caused poor peak signals [23–28]. Thus, one-step chemical derivatization reaction was carried out as an essential step for analyzing HA by GC. However, some of derivatizing reagents that have already been used are carcinogen or moisture sensitive; therefore, in this study, in order to define a significant peak of HA by GC, the carboxyl group of HA was methylated by MeOH–HCl solution. For optimization of derivative conditions, the reaction was carried out with different concentrations of HCl and incubation for 20 min at 70 °C. As it is observed from Fig. 2, the methylation and sensitivity of HA was completed at 0.33 M of HCl–MeOH within 20 min. At higher concentrations of 0.33 mol L^{-1} of HCl the peak area of HA decreased. This is due to the cleavage of amide bond and decomposition of HA at high concentration of HCl. This derivatization is stable and completely reproducible under 0.33 mol L^{-1} HCl–MeOH at 70 °C. Therefore the HCl concentration of 0.33 mol L^{-1} in MeOH was chosen for derivatization of HA at 70 °C. Recently Kataoka et al. used the same procedure for derivatization of HA and its derivatives [25].

3.2. Effect of pH

The magnitude of the distribution constant of analyte between sample solution and sorbent can be influenced by the sample pH [43,47,48]. Therefore, in this study, the effect of pH on the extraction of HA was evaluated using a series of 3.0×10^{-3} mol L^{-1} of Briton–Robinson buffer solution with pH 2.0–11.0. As shown in Fig. 3, the extraction efficiency of polypyrrole to the HA decreased

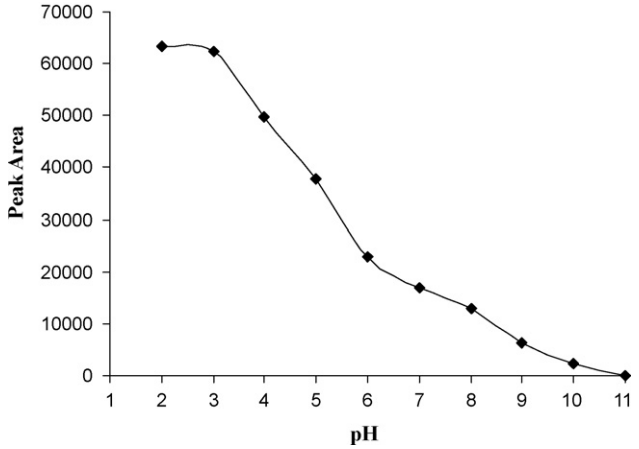

Fig. 3. The effect of sample solution pH on the extraction of HA from sample (the final derivatized residue was dissolved in 100 μL of MeOH).

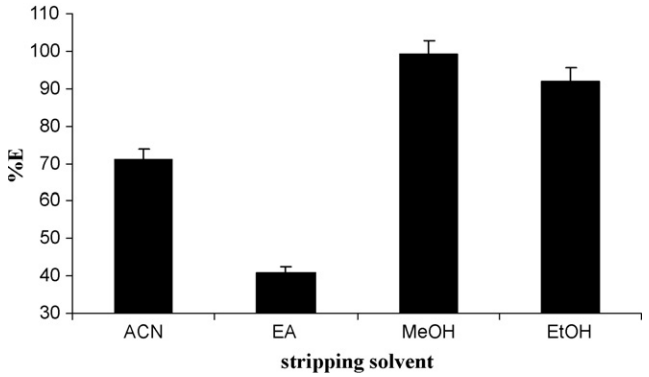

Fig. 4. The effect of stripping solvent on the elution of HA from μ-SPE (the final derivatized residue was dissolved in 100 μL of MeOH).

significantly when the sample pH increased from 3.0 to 11.0. This may be explained by the fact that HA is a weak acid and by adjusting the pH to the acidic level; it can be maintained in a neutral form for better extraction by the polypyrrole on μ-SPE. However, the HA was expected to be more efficiently extracted in its anionic form than the neutral form, based on the anion versus cation exchange properties of polypyrrole [47], but a higher recovery was found at low pH (2.0). This result suggested that the extraction of HA by the polypyrrole might not be controlled by the ion exchange properties of polypyrrole, but it is controlled by hydrophobic interactions and H-bonding. Therefore, a 3.0×10^{-3} mol L^{-1} of Briton–Robinson buffer with pH 2.0 was selected in this work for all samples.

3.3. Effect of salting out

The salting out effect has been commonly used in solid phase, liquid–liquid extraction methods. Generally, salt addition can decrease the solubility of analytes in the aqueous phase (and can also reduce the solubility of organic solvents in water) while enhancing their partitioning into the organic phase. To improve the extraction of HA, we tried salting out of sample with sodium chloride. The efficiency of extraction of HA from aqueous solution containing different amounts of sodium chloride (0–10%, w/v) was investigated. However, salt addition had an adverse effect on the overall efficiency of the method and was therefore abandoned. This may be due to the reduction of active site of sorbent. Djozan et al. [49] reported the same salting out behavior for the extraction of methamphetamine form aqueous solution at neutral pH.

3.4. Effect of stripping solvent

The HA is relatively hydrophobic and its solubility in organic solvents is better than water [28], therefore, it may not be easily desorbed. Thus, a suitable organic solvent was needed for this process. The HA could be desorbed from the sorbent with organic stripping phase by simply switching the six-port valve to the inject position. Therefore, various organic solvents such as EA, ACN, EtOH and MeOH were tested (Fig. 4).

As it is observed from Fig. 4 MeOH was able to completely desorb HA from the sorbent, therefore, MeOH was selected as stripping solvent.

3.5. Breakthrough volume

An important parameter to control in SPE is the breakthrough volume, which is the maximum sample volume that should be percolated through a given mass of sorbent after which analytes start to elute from the sorbent resulting in non-quantitative recoveries. The breakthrough volume is strongly correlated to the chromatographic retention of the analyte on the same sorbent and depends on the nature of both the sorbent and the trace analyte, as well as on the mass of sorbent considered and the analyte concentration in the sample. This volume may be determined experimentally or estimated using several methods [50]. In order to determine the volume of the sample that can be concentrated with acceptable recovery for HA, the breakthrough volumes were measured. Therefore, different volumes (20 mL, 50 mL, 75 mL and 100 mL) of sample with pH 2.0 (3.0×10^{-3} mol L^{-1} of Briton–Robinson buffer) were spiked with 1.0 mL solution containing 1.5 μg mL^{-1} of HA, and passed throughout the cartridge with a velocity of 3.0 mL min^{-1}, then the trapped HA was stripped with 1.0 mL of MeOH and follow the proposed procedure was followed. The results revealed that the acceptable recoveries were obtained for HA by using 75 mL of sample.

3.6. Sample flow-rate

In on-line μ-SPE, the sorption of HA into the stationary phase is a dynamic process and flow-rate of sample is an important factor in SPE efficiency. Therefore the sample flow-rate should be optimized to ensure quantitative retention along with minimization of the time required for sample processing. This parameter may have a direct effect on the breakthrough volume, and elevated flow-rates may reduce the breakthrough volume [50,51]

In this work, the flow-rate of stripping solvent (MeOH) was selected 1.0 mL min^{-1} throughout the study. The flow-rate used to introduce the sample was stepwise increased between 0.4 mL min^{-1} and 10 mL min^{-1} until the efficiency extraction of μ-SPE system was reduced (Fig. 5). As it can be observed from Fig. 5 the loading flow-rate of 3.0 mL min^{-1} was selected for the next steps.

3.7. Evaluation of extraction efficiency of μ-SPE

Urine usually contains a lot of organic and inorganic contaminates which may affect the results of clinical urinary analysis. Therefore the efficiency of μ-SPE system for extraction of analyte from urine and water sample was studied. According to the experimental process from both water and urine samples, the extracted HA should be absorbed and desorbed with μ-SPE system by switching the six-port valve. The entire μ-SPE extraction, washing and stripping processes were accomplished automatically within 7.0 min. As in solid phase micro extraction (SPME), the amount of analyte extracted and the extraction efficiency of μ-SPE depend on the interactions between the HA and polypyrrole, which include π–π, electrostatic and hydrophobic interactions [52]. The

Table 1
Extraction efficiency of μ-SPE of HA from buffered water and urine samples.

Sample type	Added (μg mL^{-1})[a]	Amount of analyte extracted	Extraction efficiency(%)[b]
Water	0.1	0.99	99.0
	0.2	2.0	100.0
	0.3	3.01	100.3
Urine	0.1	1.00	100.0
	0.2	1.98	99.0
	0.3	2.99	99.6

[a] 10 mL of sample (pH 2.0; 0.003 mol L^{-1} Britton–Robinson buffer) containing different amount of HA (μg mL^{-1}), amount of HA extracted (n_A) as in Eq. (1).
[b] The percentages of extracted amount of analyte by μ-SPE procedure were measured by Eq. (2).

Table 2
Characteristics parameters of calibration and analytical features of determination of HA, using the proposed procedure.

Sample	L.R.[a]	L.E.	R^2	RSD% (n = 4)	RR% (n = 4)	LOD	LOQ
Water	0.018–8.95	Y = 13792X + 3213.6	0.9973	1.2	100.5	12.1	40.3
Urine	0.018–8.95	Y = 13932X + 3393.7	0.9946	1.56	99.8	16.5	55

[a] 10 mL of sample (pH 2.0; 0.003 M Britton–Robinson buffer) containing different amount of HA (μg mL^{-1}), after extraction and derivatization the final volume was 50 μL and 1.0 μL was injected to the GC. L.R. (μg mL^{-1}): Linear Range; L.E. (μg mL^{-1}): Linear Equation; RR%: Relative Recovery; LOD (ng mL^{-1}): Limit of Detection; LOQ (ng mL^{-1}): Limit of Quantification.

amount of analyte extracted and the extraction efficiency of SPME may be evaluated by the following equation [53]:

$$n_A = FA = \left(\frac{m}{A_d}\right) A \quad (1)$$

The amount of analyte extracted n_A can easily be obtained from experimental measurements with the above expression. n_A is the amount (mass) of analyte extracted by SPME, F is the detector response factor, which can be calculated by comparing the amount of analyte (m) injected to the area counts (A_d) obtained by liquid injection, A is the response obtained by SPME. Since, in μ-SPE, HA was desorbed with MeOH as an organic solvent, the volume of desorption solvent V_d and volume of solvent injected (Vi) into the GC were used to calculate the response factor F. The percentage extraction efficiency (E) was calculated as follows [31]:

$$\%E = \frac{n_A}{C_i} \quad (2)$$

where C_i is the initial concentration (n_A and C_i have the same dimensions). The buffered (Britton–Robinson, 3.0×10^{-3} mol L^{-1}, pH 2) samples of water and urine were spiked with three different amounts of the HA and were analyzed by the proposed method (Table 1). The results revealed that the recoveries of the spiked HA from urine samples are very closed to those obtained from water samples.

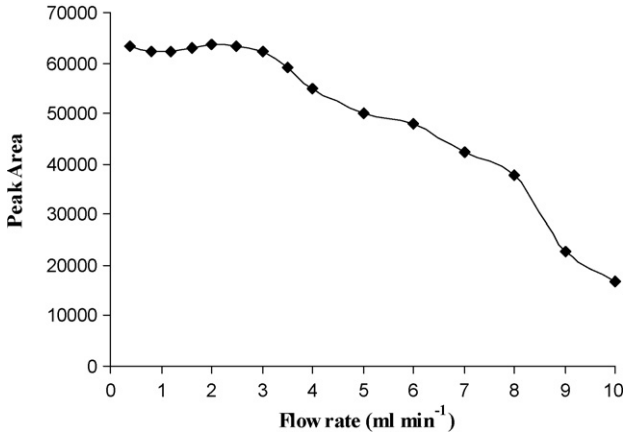

Fig. 5. The effect of flow-rate of sample on extraction efficiency of μ-SPE (the final derivatized residue was dissolved in 100 μL of MeOH).

3.8. Validation method

The GC-FID method for the determination of HA was validated by determining its performance characteristics regarding linearity, repeatability, selectivity and precision. To test the FID response linearity, a series of standard solutions of HA in the concentration range 0.0018–8.95 μg mL^{-1} was analyzed from water and urine solutions (at least seven samples covering the whole range were used). The relationship between peak area and concentration was linear for HA, as shown by the fact that the regression coefficients were higher than 0.99 in both water and urine samples. The regression equations and other characteristic parameters such as LOD, LOQ are shown in Table 2. The relative standard deviation (RSD) within a day, tested using four identical solutions of the HA at 2 μg mL^{-1} concentration level were less than 1.2% for water and 1.56% for urine solutions.

The analytical sensitivity was assessed by determination of the limit of detection (LOD) and the limit of quantification (LOQ). As defined by European Pharmacopoeia [54], the LOQ and LOD are the concentrations at which the signal to noise ratio is 3 or 10, respectively, and the results were given in Table 2. To determine the specificity of the method, three blank human urine samples were analyzed by proposed procedure. The chromatograms (not shown) obtained from the analysis of blank human urine samples did not show any peaks close to or at the retention time of the HA [55]. The μ-SPE method gave a concentration factor of 200 and is very higher than those reported previously (Table 3). The relative recovery was obtained from urine and water samples according to the literature [56] as follows: the blank urine and water samples were spiked at three concentration levels, analyzed according to the procedure outlined above, and their concentrations determined. For each concentration level, four replicate samples were used. Relative

Table 3
Comparison of the linear range and detection limits of pervious works with proposed work.

Analyte	Linear range (μg mL^{-1})	LOD (ng mL^{-1})	Ref.
HA	4.47–35.8	9.0×10^2	[15]
HA	8.95–179	21.5×10^2	[22]
HA	1.0–100	50.0×10^2	[23]
HA	0.05–25	1.7	[27]
HA	5.0–70	25.0×10^2	[28]
HA	62.5–2000	125.0×10^{21}	[55]
HA	0.018–8.95	16.5	This work

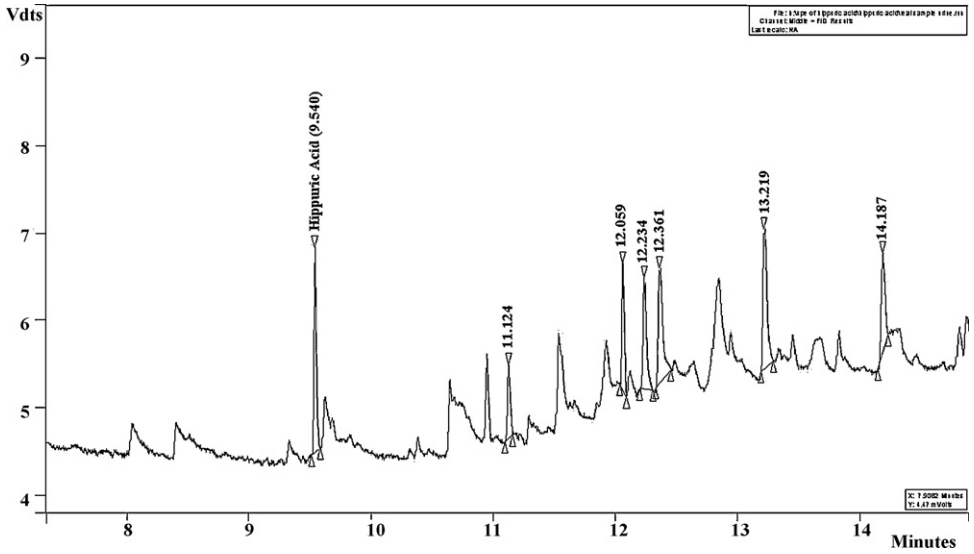

Fig. 6. A typical chromatogram of HA extracted from urine by proposed procedure at optimum conditions.

Table 4
Concentration of HA in three human urine samples (0.1 mL of urine were diluted to 10 mL with buffer (pH 2.0; 0.003 mol L^{-1} Britton–Robinson buffer).

Volunteers No.	Added ($\mu g\,mL^{-1}$)	Found ($\mu g\,mL^{-1}$)
1	0.0	15.6
	5.0	21.4
	10.0	25.5
2	0.0	18.2
	5.0	23.5
	10.0	28.1
3	0.0	12.5
	5.0	17.5
	10.0	22.1

recovery (sometimes called accuracy) was determined as the slope of a linear regression analysis of a plot of measured concentration versus spiked concentration. A 100% relative recovery is indicated by a slope of 1.00 (Table 4).

3.9. Analytical application

Hippuric acid was detected in the urine of three healthy volunteers. A typical chromatogram of urine analysis is shown as an example (Fig. 6). The quantitative results were placed in Table 4. From the data given in Table 4 the HA have a range of 12.5–18.2 $\mu g\,mL^{-1}$ at urine samples. In addition the human urine samples were spiked with appropriate volumes of HA solution to determine that the proposed procedure has no significant influence on the recovery of the HA from real samples and recovery is quantitative. In earlier studies, the average value which was obtained for HA was 0.222 mg mL^{-1} [57], 0.344 mg mL^{-1} [58], and 0.34 mg mL^{-1} [59]. This discrepancy between our quantitatively low results of HA in the present work and pervious studies [57–59] is due to the fact that the concentration of trans-cinnamaldehyde in cinnamon-tea is low (see Section 2.5).

4. Conclusion

In this work a polypyrrole was synthesized by chemical oxidation of the pyrrole monomer in ACN solution containing $FeCl_3$ and applied as an excellent and efficient sorbent for μ-SPE. According to Section 2.4, exactly 20 mg of synthesized polypyrrole particles was packed and fixed in a μ-SPE (Fig. 1). After treatment of urine samples, the pH of samples were adjusted by Britton–Robinson buffer (3.0×10^{-3} mol L^{-1}) at 2.0 and passed through the cartridge by flow-rate of 3.0 ml min^{-1}. After stripping of HA from sorbent by 1.0 mL of MeOH, the organic phase was evaporated to dryness under a compressed N_2 flow at 70 °C temperature. The residue was redissolved in 150 μL of MeOH containing 0.33 M HCl and placed in a water bath at 70 °C for 20 min. Then, the mixture was dried by N_2 at 70 °C and the residue was dissolved in 50 μL of MeOH. Finally 1.0 μL was injected to the GC systems with adopted split ratio of 80.

The results demonstrate that the μ-SPE analysis of HA from urine samples is an excellent method for estimation of exposure to cinnamic acid and cinnamaldehyde. In addition, this system offers a practical potential for extraction and determination of HA, having special advantages of high sensitivity, low detection limit, simplicity, and speed that have not been present together in the previously reported systems (Table 3). The derivatization procedure described here is even more sensitive than HPLC and even other GC–MS methods based on the silylation [24].

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