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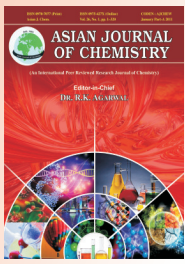

Vortex-Assisted Surfactant-Enhanced Emulsification Microextraction Combined with High Performance Liquid Chromatography-Fluorescence Detector for Determination of Nitrite in Urine

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A vortex-assisted surfactant-enhanced emulsification microextraction (VASEME) combined with high performance liquid chromatography-fluorescence detector (HPLC-FLD) procedure was developed for the determination of trace nitrite in human urine. The method is based on the selective reaction of nitrite with *o*-phenylenediamine in acid media to form benzotriazole, which exhibited strong fluorescence at 568 nm with excitation at 420 nm in alkaline medium. The fluorescence of system was enhanced by hydroxypropyl-β-cyclodextrin (HP-β-CD) through complexation. The nonionic surfactant Triton X-114 was adopted as emulsifier and *n*-octanol as the extraction solvent and vortex-mix was applied to assist emulsification in vortex-assisted surfactant-enhanced emulsification microextraction. Under the optimal conditions: the linearity was observed in the range of 4 to 80 ng/mL and the correlation coefficient (r) was 0.996. The relative standard deviations were below 3.7 % (n = 5). The limit of detection (LOD) based on a signal-to-noise ratio (S/N) of 3 was 0.08 ng/mL and a signal-to-noise ratio (S/N) of 10 the quantification limits (LOQ) was 0.314 ng/mL. Good recoveries (≥ 85 %) were obtained when the proposed method was applied to determine nitrite in human urine samples.

Keywords: Surfactant, Vortex, Microextraction, High performance liquid chromatography, Fluorescence, Nitrite, Urine.

INTRODUCTION

Nitric oxide (NO), a signaling molecule, plays an important role in regulating vascular tone, neurotransmission, host immunity, nutrient metabolism and whole body homeostasis[1]. Nitric oxide formation is increased in bladder inflammatory conditions[2,3], but it is difficult to determine NO because of its low content in picomolar to nanomolar ranges and a very short half-life[4]. Urinary nitrite is the major end product of local NO formation in the urinary tract since there are no known mechanisms for nitrite conversion into nitrate in the urinary tract[5]. Infected urine may contain nitrite as a result of bacterial nitrate reductase activity and detection of nitrite in urine is routinely used in the diagnosis of bacterial cystitis[6]. Sensitive and simple methods are required in order to measure low levels of nitrite in urine following formation of small amounts of NO.

Different analytical methodologies have been developed for the analysis of nitrite in various matrices, including spectrophotometry[7,8], spectrofluorimetry[9-11], high performance liquid chromatography[12-15], ion-chromatography[16-18], gas chromatography[19,20] and capillary electrophoresis[21]. Among these methods, spectrophotometry and chromatography are widely used because the instrumentations are common available and analytical procedures are simple enough.

Sample preparation plays an important role in the nitrite ion analysis in complex matrices (*e.g.* urine). Ionic liquid-dispersive liquid-liquid microextraction (ILDLLME)[22], solid phase extraction (SPE)[23,24], cloud point extraction (CPE)[25,26] were reported for isolating and enrichment of nitrite ion from some water, fruit and biological samples.

In this work, vortex-assisted surfactant-enhanced emulsification microextraction (VASEME) was used as a sample preparation method on the determination of trace nitrite in urine samples, which could reduce interference from the matrix. Compared with the repored studies[12,14,17,20,27], VASEME-HPLC-FLD can obtain higher sensitivity and simple sample treatment. Several factors affecting extraction were tested, such as kind and volume of extraction solvent and emulsifier, pH of extraction system and vortex time.

EXPERIMENTAL

The analyte was performed using Agilent 1100 HPLC equipped with a vacuum degasser, an auto sampler, a quaternary pump and a fluorescence detector (Agilent, USA). A reversed-phase Agilent TC-C18 column (5 μm, 150 mm × 4.6 mm) was used for separation. A vortex oscillator (Shanghai, China) was used to assist the microextraction procedure. A centrifuge (Shanghai, China) was used for the phase separation process.

Reagents and solution: All reagents were at least of analytical grade unless otherwise noted. Stock standard solution of nitrite (200 μg mL^{-1}) was prepared by dissolving 15 mg of dried sodium nitrite (105 °C for 1 h, Tianjin, China) in 50 mL of water and kept under 4 °C. Working solution was prepared by diluting the stock solution. *o*-Phenylenediamine (OPD) solution (0.025 %, w/v) was prepared by dissolving 25 mg of *o*-phenylenediamine (Shanghai, China) in 100 mL of ethanol. 0.15 M of hydroxypropyl-β-cyclodextrin (HP-β-CD) (Shanghai, China) was prepared by dissolving appropriate quantity of HP-β-CD in water. Hydrochloric acid (1 M) and sodium hydroxide (1.09 M) were used to adjust the pH of the system. N-butanol, isobutanol, *n*-octanol and isooctanol were purchased from Aladdin (Shanghai, China). Triton X-114, Triton X-100 and TMN-6 were purchased from Sigma (St. Louis, Mo., USA). Methanol (HPLC grade) was obtained from Merck KGaA (Darmstadt, Germany). Purified water was obtained using an Aike water purification system (Chengdu, China) and used as experimental water throughout the research.

Sample preparation: Fresh matutinal human urine samples were provided by healthy volunteer. Before analysis, the sample was kept at 4 °C. A 10 mL urine sample spiked with the target analyte was centrifuged at 3500 rpm for 10 min to remove any insoluble material. Then 3 mL clear supernatant was diluted to 5 mL (for decreasing matrix effects), which was prepared for the next experiment.

Vortex-assisted surfactant-enhanced emulsification microextraction (VASEME) procedure: To a 10 mL centrifuge tube, a certain amount of aqueous sample solution spiked with the target analytes, 160 μL of 1 M HCl and 200 μL of 0.025 % *o*-phenylenediamine were added, respectively. The mixture was allowed to stand for 10 min at room temperature. Then, 164 μL of 1.09 M NaOH was added to make the system alkaline and 200 μL of 0.15 M HP-β-CD was added. The appropriate amount of *n*-octanol and TritonX-114 were added. The mixture was vortex-mixed for 1 min and the solution became cloudy. The phase separation was finished by centrifugation at 3500 rpm for 5 min and two clear phases formed. Target analyte was enriched into the upper phase with a small volume and 10 μL of enriched phase was directly injected into the HPLC system for analysis.

Chromatographic conditions: For the analysis of nitrite, a reversed-phase Agilent TC-C18 column (5 μm, 150 mm × 4.6 mm) was used for separation and the fluorescence intensity was measured at 568 nm with excitation wavelength at 420 nm. The mobile phase was methanol-water(40:60, v/v) and the flow rate was maintained at 1 mL/min. The column temperature was 30 °C.

RESULTS AND DISCUSSION

Selection of extraction solvent: Organic solvents are selected on the basis of extraction capability of interested compounds and good chromatography behavior. The selection of appropriate microextraction solvent plays an important role to get a high sensitivity in VASEME. Thus, *n*-butanol, isobutanol, *n*-octanol, isooctanol were investigated as extraction solvent under the same experimental conditions. *n*-butanol, isobutanol could not form two phases after centrifugation. *n*-Octanol, isooctanol could separate into two phases after centrifugation, but *n*-octanol was the better enrichment factor and extraction efficiency (Fig. 1).

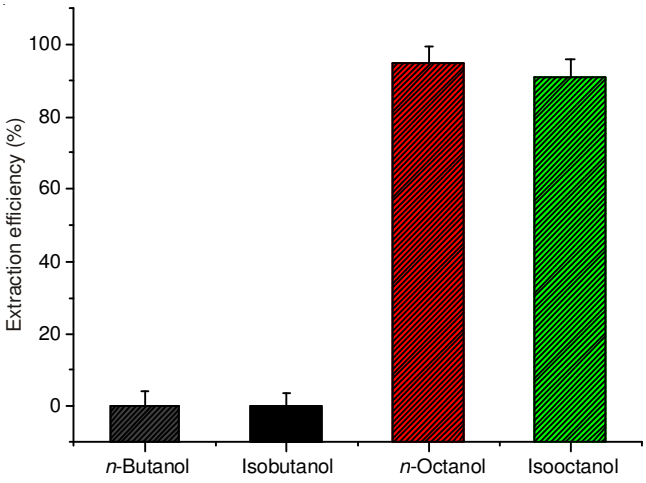

Fig. 1. Kind of extraction solvent

Volume of extraction solvent: To examine the effect of extraction solvent volume, 5 mL aqueous solution spiked with the target analyte by same VASEME procedures was studied by using different volumes of extraction solvent. Fig. 2 shows the curve of extraction efficiency *versus* volume of extraction solvent. 500 μL *n*-octanol was chosen in the extraction.

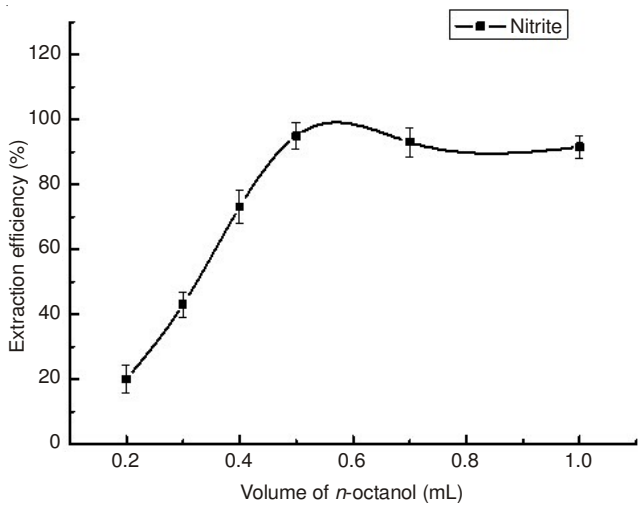

Fig. 2. Volume of extraction solvent

Selection of emulsifier: In VASEME, selecting an appropriate emulsifier is important, since emulsifier should be miscible with both microextraction solvent and aqueous sample. Surfactants contain both hydrophobic groups and hydrophilic groups and the insoluble hydrophobic group may extend into the organic phase, while the water soluble head group remains in the water phase. Surfactant is often used as an effective and economic emulsifier, phase dispersant and solubilizer. For the sake of acquiring the most suitable emulsifier, nonionic surfactant including Triton X-114, Triton X-100 and TMN-6 were tested. Triton X-114 was chosen as emulsifier with the better extraction efficiency (Fig. 3).

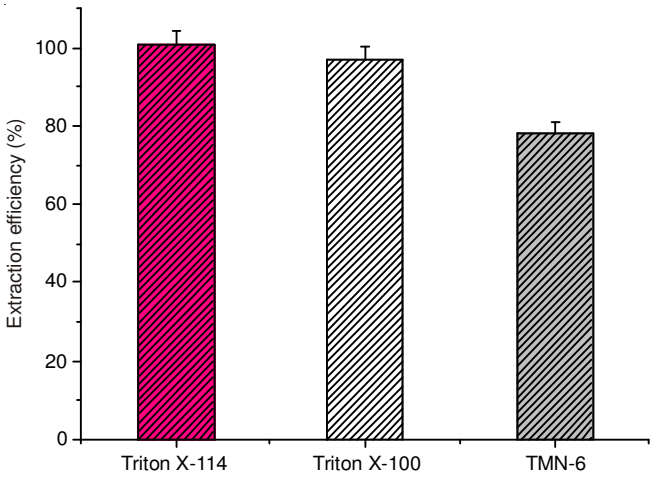

Fig. 3. Kind of emulsifier

Volume of emulsifier: Influence of variation of the volume of Triton X-114 on extraction efficiency was tested (Fig. 4). According to the curve, at first the extraction efficiency increases and then decreases by increasing the volume of Triton X-114. It seems, at a low volume of Triton X-114, cloudy state is not formed well, thereby, the extraction efficiency decreases. At the high volume of Triton X-114, phase separation did not quite finish, therefore, the extraction efficiency decreases. In this study, 20 µL Triton X-114 was chosen to obtain good extraction efficiency.

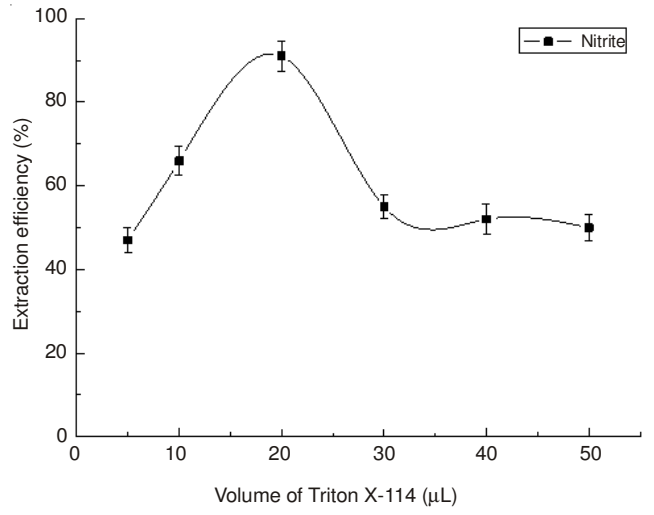

Fig. 4. Volume of emulsifier

Effect of pH: The reaction product benzotriazole exhibited very weak fluorescence in acidic solution. When the medium was made alkaline with NaOH solution, the fluorescence intensity was enhanced. In this study, pH between 7 and 14 were studied on the extraction efficiency of VASEME (Fig. 5) and pH 11-12 was selected as suitable pH value for extraction.

Analytical features: In order to investigate the applicability of the proposed method in urine samples, several factors

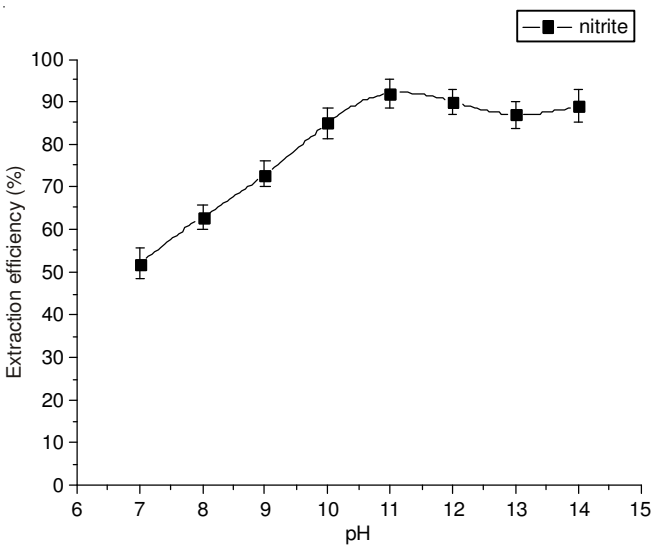

Fig. 5. Effect of pH

including linear range, regression equation, correlation coefficient and detection limit were evaluated under the optimum conditions. Good linear relationship was observed in the concentration range from 4 to 80 ng/mL and the correlation coefficient (r) was 0.996. The limit of detection (LOD) based on a signal-to-noise ratio (S/N) of 3 was 0.08 ng/mL and a signal-to-noise ratio (S/N) of 10 the quantification limits (LOQ) was 0.314 ng/mL. The relative standard deviations (RSDs) were below 3.7 % (n = 5) (Table-1).

Analysis of real samples: Real urine samples were analyzed to assess their content in nitrite. Fig. 6 showed the obtained chromatograms of the spiked urine sample. After VASEME, the trace nitrite was enriched into a small volume phase, which can remove the interference from the matrix and higher sensitivity was obtained (Fig. 6 (B)). In order to validate the recovery, precision and stability of the proposed method, the urine samples were spiked with the analyte at different concentration levels analyzed using the same VASEME-HPLC-

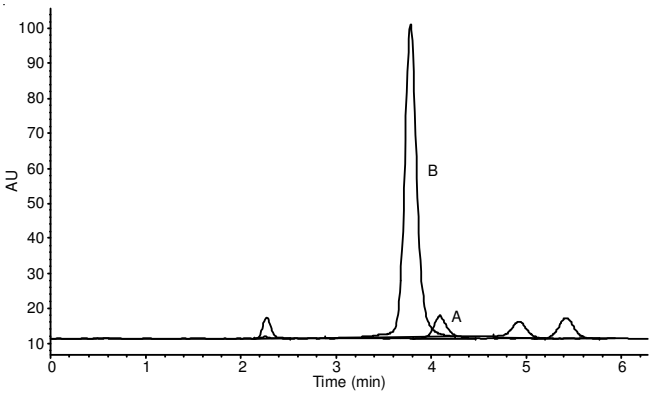

Fig. 6. Typical chromatogram of urine: (A) urine spiked with nitrite (40 ng/mL) without VASEME; (B) urine spiked with nitrite (40 ng/mL) after VASEME

TABLE-1
QUANTITATIVE RESULTS OF THE PROPOSED VASEME-HPLC-FLD METHOD

Analyte	Linear equation	Linearity (ng/mL)	r	RSD % (n = 5)	LOD (ng/mL)	LOQ (ng/mL)
Nitrite	y = 224.88x-7.8376	4-80	0.996	3.7	0.08	0.314

FLD procedure. The intra-day precision was analyzed by performing the determinations five times in the same day. The inter-day precision was analyzed by repeating the determinations five times on three different days. Recoveries and intra- and inter-day precision were calculated and listed in Table-2. The spiked recoveries were between 85-92.6 % with the RSD of 4.3-5.5 %.

TABLE-2
INTRA-AND INTER-DAY PRECISION AND RECOVERY FOR QUANTITATION OF NITRITE

	Added (ng/mL)	Recovery (%) (RSD %)	Intra-day precision (n = 5) RSD %	Inter-day precision (n = 3) RSD %
Nitrite	–	–	–	–
	10	85.0 (4.9)	3.7	6.1
	30	87.7 (4.3)	4.1	5.7
	60	92.6 (5.5)	4.3	6.3

Conclusion

This paper presents an VASEME-HPLC-FLD method for determining trace nitrite in urine sample with high sensitivity. Compared with several other methods, VASEME-HPLC-FLD could obtain lower limits of detection (Table-3). The method is easily available equipments, easy sample preparation and better performance. The good recoveries achieved in the real sample studies revealed the promising practical utility.

TABLE-3
COMPARED LODS OF DIFFERENT METHODS FOR DETERMINATION OF NITRITE

Method	LOD(ng/mL)	References
HPLC-FLD	0.5	12
HPLC-UV/visible	3.69×10^5	14
S.D	13.6	17
IC	30	20
GC-MS	0.001	27
VASEME	0.08	This work

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