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Preparation and application of solid-phase microextraction fiber based on molecularly imprinted polymer for determination of anabolic steroids in complicated samples

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ABSTRACT

A relatively selective, chemically and physically robust SPME fiber was developed in a simple way with testosterone-imprinted polymer, and then directly coupled with gas chromatography–mass spectrometry (GC–MS) for selective extraction and analysis of anabolic steroids. The factors influencing polymerization (i.e., cross-linker, polymerization solvent, polymerization time) were optimized in detail and the polymer was characterized by scanning electron microscope, infrared spectrometer and thermogravimetric analyzer. Furthermore, the extraction performance of the MIP-coated SPME fibers such as extraction ability and selectivity was evaluated. Moreover, the interaction mode between target analytes and fiber coating was deducted. Finally, the method for extraction and determination of androsterone, stanolone, androstenedione and methyltestosterone by the homemade MIP-coated SPME fibers with GC–MS was obtained. It was applied to the simultaneous analysis of four anabolic steroids in the spiked human urine with the satisfactory recoveries.

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1. Introduction

Molecular imprinting [1,2] is a technique for the preparation of synthetic polymer with predetermined selectivity for a desired template molecules, and the molecularly imprinted polymer (MIP) is highly stable polymer possessing recognition sites within the polymer matrix that are adapted to the three-dimensional shape and functionalities of an analyte of interest [3].

MIP possesses dazzling advantages over their biological counterparts and has drawn much attention in a lot of aspects of analytical chemistry [4,5], such as stationary phases for liquid chromatography [6,7], capillary electrochromatography [8], sensors [9–11], quartz crystal microbalance [12,13], solid-phase extraction [14–17], membrane separation [18], solid-phase microextraction [19] and so on.

Solid-phase microextraction (SPME), which was firstly introduced by Pawliszyn's group [20], is considered as one of the most promising pretreatment methods because it combines the extraction, concentration, and sample introduction in one simple step. It greatly reduces preparation time and increases sensitivity simultaneously over conventional extraction techniques [21–24]. SPME is based on the partitioning of target analytes between the sample and fiber coating, therefore, the fiber coating is the key factor of the SPME technique. In order to achieve higher applicability for various compounds, many materials have been utilized as the coating [25–27].

The required selectivity in SPME could be provided with utilizing MIP as the material of SPME fiber coating with its advantages of high selectivity, reusability, physical and chemical stability over a wide range of pH, solvents and temperature, and easy preparation characteristics. Thus, the field of utilizing MIP as the material of SPME fiber coating has drawn attention in the past decade and most reports are coupled directly to high performance liquid chromatography (HPLC) [28–33]. Actually, MIP as the material of SPME fiber coating and directly coupling to gas chromatography–mass spectrometry (GC–MS) could perfectly inosculate the sample preparation, separation, detection and structure interpretation. Djozan et al. [34–36] have investigated the MIP-coated SPME fibers, which were directly coupled to gas chromatography (GC) and GC–MS, and demonstrated the possibility of selective extraction of template molecules and analogous compounds. Zeng et al. [37] have developed a SPME fiber immobilizing on a stainless steel wire in a simple way and directly coupling to GC.

Most synthetic anabolic steroids are derived from testosterone and have very similar molecular structures [38], therefore, they are very suitable for the study of MIP. Furthermore, the use of

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steroids in formal games constitutes unfair competition and can be harmful to the physical and mental health of human beings under aberrant use. Hence, anabolic steroids have been included in the International Olympic Committee doping list due to their illegal use in some sports [39]. GC–MS has been used as an official method to detect the illegal anabolic steroids used in sports. However, GC–MS methods always require complicated and time-consuming sample preparations. Consequently, sensitive and selective pretreatment techniques are urgent required for the determination of trace anabolic steroids in urine or blood serum. As far as we known, there are no publications about utilizing testosterone-imprinted polymer as material of SPME fiber coating.

In this paper, we aimed to develop a kind of selective, exclusive, chemically and physically robust SPME fibers for anabolic steroids in a simple way and at low cost from testosterone-imprinted polymer. The fibers were subsequently used for extraction of anabolic steroids and then analyzed with GC–MS. With this goal in mind, functional monomer methacrylic acid (MAA), cross-linker trimethylol-propanetrimethacrylate (TRIM) and template molecules testosterone were used to prepare for the SPME fibers through a thermal radical copolymerization procedure. The fibers were monolith and flexible enough to be placed in a homemade syringe and inserted into GC and/or GC–MS injection port. Finally, a method for the determination of androsterone, stanolone, androstenedione and methyltestosterone by the MIP-coated SPME fibers coupling to GC–MS was developed. This analytical method possessed the obvious simplicity in comparison with the conventional extraction techniques, and was applied to the simultaneous analysis of four anabolic steroids in the spiked human urine.

2. Experimental

2.1. Chemicals

Androsterone, stanolone, androstenedione, azo(bis)-isobutyronitrile (AIBN) were purchased from Sigma (St. Louis, Missouri, USA). Methyltestosterone and tamoxifen were purchased from National Institute for the Control of Pharmaceuticals and Biological Products (Beijing, China). Testosterone was obtained from JiuBang Chemical Co. Ltd. (Shanghai, China) and lambda-cyhalothrin was kindly provided by Fujian Provincial Central inspection Institute (Fuzhou, China). MAA was purchased from Aldrich (Milwaukee, WI, USA). TRIM was obtained from TCI (Tokyo, Japan). Ethylene dimethacrylate (EDMA) was purchased from Acros (NJ, USA). 3-(Methacryloxy) propyltrimethoxysilane was obtained from Alfa Aesar (Ward hill, MA, USA). Methanol was HPLC grade and all other reagents used were analytical grade.

A stock standard mixture containing androsterone, stanolone, androstenedione and methyltestosterone (2.00 mg/mL, respectively) was prepared in methanol and stored at 4 °C. The working standard solutions of lower concentration were prepared by the serial dilution of the stock solutions and renewed every month. Milli-Q ultrapure water (Millipore, Bedford, USA) was used throughout the experiments.

2.2. GC–MS analysis

All the gas chromatographic experiments were carried out on an Agilent 6890N-5973i GC–MSD (Hewlett-Packard, Palo Alto, CA, USA) and a 30 m × 0.25 mm ID, 0.25 μm film thickness HP-5MS GC column (Agilent Scientific, USA) was employed. The injector temperature was maintained at 240 °C. Column flow was set at 1.0 mL/min using ultra-purified helium (>99.999%) as carrier gas. The oven temperature was set at 100 °C for 5 min, then at ramp rate of 25 °C/min increased to 250 °C, and held for 7 min, and then heated at 30 °C/min to 310 °C. The electron impact (EI) ion source, quadrupole mass analyzer, and the interface temperature were maintained at 230 °C, 150 °C and 300 °C, respectively. Splitless injection with a 12 min solvent delay was employed and the injection volume was 1.0 μL. Electron impact ionization (70 eV) was utilized. The mass spectra of target compounds were obtained and quantified in the selected ion monitoring (SIM) mode. Ions for monitoring androsterone, stanolone, androstenedione and methyltestosterone at m/z were the ion fraction groups of (290, 107 and 246), (231, 290 and 55), (286, 124 and 244) and (124, 43 and 302), respectively, on the basis of results obtained from an initial full scan mode with scan range from m/z 50 to 400. The quantitation was based on the Extracted Ion Chromatograms (EICs), and the corresponding quantitative ions for androsterone, stanolone, androstenedione and methyltestosterone were m/z 290, 231, 286 and 302, respectively.

2.3. Fiber preparation

The commercial silica fibers (Yongnian Optical Fiber Factory, Yongnian, Heibei Province, China) with diameter of 140 μm were cut to 20.0 cm in length and immersed in acetone for 5 min to remove the polyimide on their surfaces, then rinsed with water and dried. After that, the fibers were immersed in 1.00 mol/L sodium hydroxide solution for 60 min at room temperature, and rinsed with water. And then the fibers were immersed in 1.00 mol/L hydrochloric acid for 60 min at room temperature. Finally, the fibers were washed with water and dried in an oven at 150 °C for 1 h. Prior to the coating procedure, the silylation was achieved by immersing the above fibers into a 10% (v/v) 3-(methacryloxy) propyltrimethoxysilane solution in acetone at room temperature for 2 h. Then the fibers were washed with methanol and dried at room temperature.

2.4. Polymer preparation

The MIP was prepared through the thermal radical copolymerization of MAA and TRIM in the presence of testosterone as template molecules. To achieve this purpose, 0.12 mmol testosterone and 10 μL MAA were dissolved in 1.2 mL acetonitrile in a 1.5 mL glass tube to prepare for a testosterone pre-polymer solution. After 12 h [31,32], 150 μL TRIM and 1.536 mg AIBN were added and dissolved adequately. And then, the mixture was deoxygenized with a stream of nitrogen for 5 min. Subsequently, the silylated fibers were inserted in the tube and sealed immediately and placed in a DHG-9030A oven (Yiheng, Shanghai, China) to perform the polymerization at 60 °C for 8 h. The non-imprinted polymer (NIP)-coated SPME fibers were also prepared according to the above procedures except for the absence of testosterone during polymerization. Pulled out cautiously from the tubes, the fibers with MIP or NIP coating on the surface were obtained. Finally, the fibers were soaked in 0.5 mL of 10% (v/v) acetic acid solution in methanol for 30 min to remove the template molecules. This procedure was performed repetitiously until no response of testosterone when the fibers were coupled to GC–MS.

2.5. Characterization of the MIP-coated SPME fibers

The surface characteristic study of the MIP-coated SPME fiber coating was monitored by environmental scanning electron micrography with an XL-30 scanning electron microscope (Philips, Netherlands). The infrared absorption spectrum of coating between 400 and 4000 cm^{-1} was obtained in a 360 FTIR spectrometer (Nicolet, America). The thermogravimetric analysis was performed in a STA449C thermogravimetric analyzer (Netzsch, Germany) under inert atmosphere (Ar), over the temperature range of 50–400 °C (heating rate of 10 °C/min). The Brunauer–Emmett–Teller (BET)

surface areas were determined by nitrogen adsorption–desorption isotherm measurements at 77 K with an ASAP2020M apparatus (Micromeritics Instrument Corp., America).

2.6. SPME procedure

The MIP-coated SPME fiber was assembled in a homemade SPME device based on the normal GC microsyringe, and 20 mL spiked sample extract was placed into a 25 mL glass vial used as sample container. Prior to the sample extraction, the MIP- or NIP-coated SPME fiber should be conditioned in 10% (v/v) acetic acid solution in methanol for 5 min. Subsequently, the MIP- or NIP-coated SPME fiber was immersed into the spiked sample to extract analytes for 30 min (unless specially mentioned) at 40 °C with stirring speed 500 rpm, and then pulled out. Finally, the fiber was air-dried for 3 min, and thermally desorbed by directly inserting the fiber into the GC injector set at 240 °C for 1 min.

It should be noted that, variables that affect the extraction performance of the MIP-coated SPME fibers, nature of interactions between target analytes and fiber coating, extraction ability and selectivity of the MIP-coated SPME fibers, and factors of SPME method were investigated in spiked water samples.

2.7. Sample preparation

Spiked water samples were prepared everyday by adding appropriate volume of anabolic steroids standard solution into 20 mL water for SPME extraction.

The urine samples were used for method validation and application. They were donated by several volunteers and stored in the refrigerator at −20 °C. All urine samples were added NaCl to make the final concentration of NaCl to be 0.175 g/mL, then centrifuged at 4500 rpm for 15 min, and then filtered through a 0.22 μm membrane filter. Finally, 20 mL clear solution was directly transferred into a 25 mL serum bottle and spiked with different volumes of analytes mixed solution for SPME extraction. The blank urine samples for SPME extraction were prepared using identical procedures without the addition of the anabolic steroids mixed standard solution.

3. Results and discussion

3.1. Preparation of MIP-coated SPME fibers

The properties of the MIP and NIP depend on the composition of the polymer (i.e., types and concentrations of template molecules, functional monomer, cross-linker, initiator and polymerization solvent). In this part, the optimization results were determined by chromatographic peak area of four anabolic steroids and scanning electron micrographics (SEM) of the prepared MIP-coated SPME fibers.

The correct selection of template molecules and functional monomer is important in molecular imprinting. In this research, testosterone and MAA were used as the template molecules and functional monomer, respectively. The cross-linker provides imprinted polymer with high mechanical stability and chemical inertness. TRIM and EDMA, the most frequently used in the MIP preparation, were selected (Fig. 1). The results indicated that the coating prepared with TRIM had better morphological structure (Fig. 1d) and extraction ability. Therefore, TRIM was selected as the cross-linker in the following experiments.

Polymerization solvent, which influences preparation feasibility, polymer morphology and extraction reproducibility, is another important factor for effective molecular recognition. To optimize solvent, polymerization was carried out in the commonly used solvents, such as chloroform, acetonitrile and toluene (Fig. 2). It

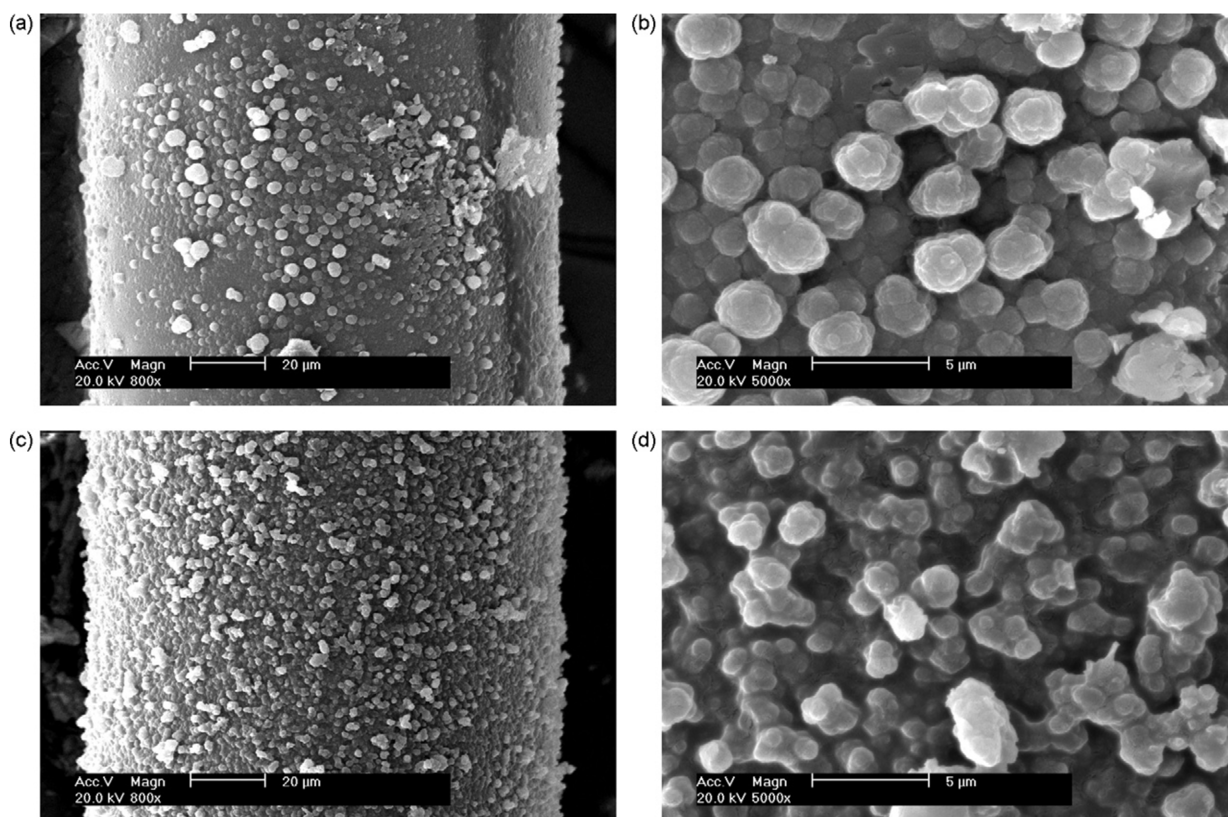

Fig. 1. SEM images of prepared MIP-coated SPME fiber, 40 μL MAA, 0.12 mmol testosterone, 1.536 mg AIBN and 150 μL (a and b) EDMA and (c and d) TRIM were dissolved in 1.2 mL toluene and performed the polymerization at 60 °C for 12 h.

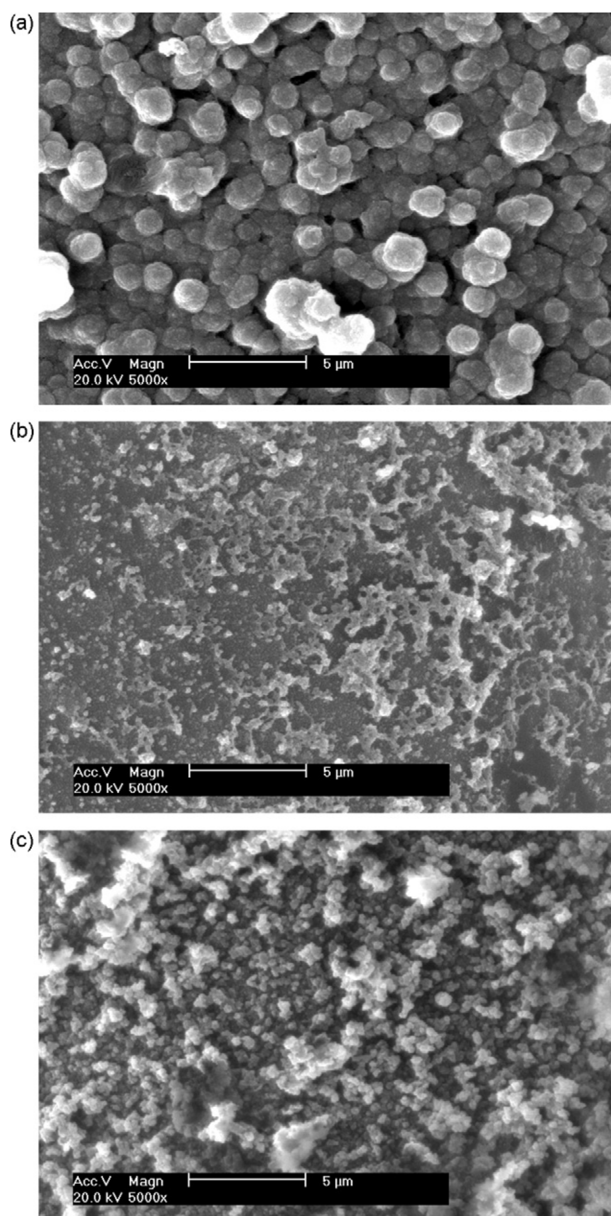

Fig. 2. SEM images of prepared MIP-coated SPME fiber, 40 μL MAA, 0.12 mmol testosterone, 1.536 mg AIBN and 150 μL TRIM were dissolved in 1.2 mL (a) toluene, (b) chloroform, (c) acetonitrile and performed the polymerization at 60 °C for 12 h.

was found that polymerization could not take place in chloroform, and when acetonitrile and toluene were used, there were homogeneous and dense coating on the fibers. Furthermore, MIP-coated SPME fibers prepared with acetonitrile had better extraction reproducibility and better morphological structure (Fig. 2c). Therefore, acetonitrile was selected as polymerization solvent.

Also, quantity of template molecules (testosterone) in pre-polymer solution was optimized. Table 1 demonstrated the variation of extraction efficiency of prepared fibers versus different quantity of template molecules in pre-polymer solution (because of the same responses of four anabolic steroids and the limited space of the manuscript, androsterone was selected as model in Table 1). From the results, fiber ability increased with amounts of template molecules and reached a maximum at 0.12 mmol.

In addition, various volume ratios of functional monomer (MAA) with cross-linker (TRIM) were discussed (see Table 1). The results indicated that the extraction ability of the MIP-coated SPME fibers

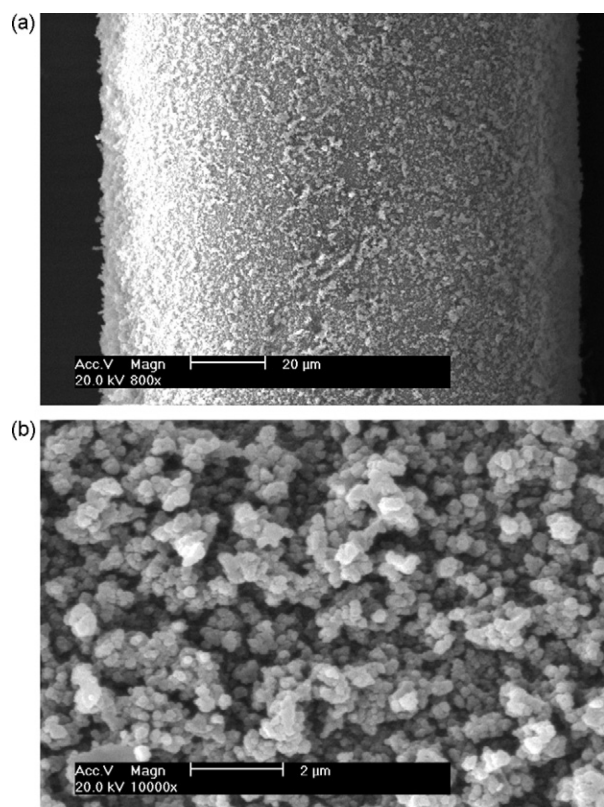

Fig. 3. SEM images of prepared MIP-coated SPME fiber. (a) and (b) show different magnifications (preparation conditions of MIP coating as following: monomer, 10 μL MAA; cross-linker, 150 μL TRIM; quantity of template molecules (testosterone), 0.12 mmol; solvent, 1.2 mL acetonitrile; initiator, 1.536 mg AIBN; polymerization time, 8 h; temperature, 60 °C).

prepared with the volume ratio of 1:15 got a maximum chromatographic peak area, while, higher volume ratio of MAA with TRIM showed poor extraction behavior. It was probably due to the enhancement in non-homogenous polymerization and decrease of molecular imprinting sites of fibers. Thus, the volume ratio of MAA with TRIM at 1:15 (i.e., molar ratio of 1:4) was selected owing to the best extraction capability as well as the best morphological structure.

The polymerization time could affect fiber porosity and fiber preparation reproducibility, since it controlled the degree of cross-linking of the polymeric network. Thus, new fibers were prepared with different polymerization time (see Table 1). The extraction ability of MIP-coated SPME fibers increased with polymerization time and reached a maximum at 8 h.

To sum up, the testosterone-imprinted polymer was synthesized by using 0.12 mmol of testosterone as template molecules, 10 μL MAA as functional monomer, 150 μL TRIM as the cross-linker, 1.2 mL acetonitrile as polymerization solvent, and AIBN as initiator. The MIP was prepared through the thermal radical copolymerization at 60 °C for 8 h.

3.2. Characterization and extraction performance of the MIP-coated SPME fibers

3.2.1. Morphological structure of MIP-coated SPME fibers

The morphological structure of the testosterone-imprinted polymer coating could be assessed from Fig. 3, which showed the SEM of the MIP-coated SPME fibers under the magnifications of 800 (a) and 10,000 (b). The figures showed that the coating possessed a highly cross-linked, porous and homogeneous structure on the surface of the MIP layer. These results revealed that the

Table 1
Optimization of quantity of template molecules, volume of MAA and polymerization time.[a].

Quantity of template molecules		Volume of MAA		Polymerization time	
Testosterone (mmol)	Peak area[a]	MAA (μL)[b]	Peak area[a]	Time (h)	Peak area[a]
0.06	483,308	5	8,104,583	3	7,072,874
0.08	951,889	**10**	**8,922,676**	6	7,777,562
0.10	1,596,544	15	7,270,012	7	9,759,945
0.12	**2,177,492**	20	5,668,669	**8**	**10,862,802**
0.14	1,269,212	25	4,790,805	9	10,408,804
–	–	30	3,484,144	10	8,267,889
–	–	35	2,532,386	11	7,610,576
–	–	40	2,143,057	12	7,865,758
–	–	50	1,542,158	15	6,138,715
–	–	–	–	18	6,224,688
–	–	–	–	21	5,739,650

[a]GC–MS conditions as in Section 2.2. SPME conditions: pH, 7.00; NaCl concentrations, 0 g/mL; other SPME conditions as in Section 2.6. Concentration of androsterone was 0.1 μg/mL.
[b]TRIM was 150 μL.
The bold values were maximum peak areas of androsterone.

MIP-coated SPME fibers were advantageous to enhance extraction performance.

Seven fibers were prepared simultaneously with the same polymerization solution, and the average thickness of them was 3.1 μm with relative standard deviation (RSD) of 8.7%.

3.2.2. Infrared spectra of MIP coating

The infrared (KBr pellet) spectra of MIP and NIP coatings were shown in Fig. 4. The main functional groups of the predicted MIP and NIP coatings could be found with corresponding infrared absorption peaks. A broad absorption band at 3410 cm^{-1} on the MIP coating corresponded to several overlapped peaks of infrared absorption such as the stretching vibration of O–H bonds of MAA molecules (functional monomer), the hydrogen bonding and electrostatic binding interactions between testosterone and MAA. However, for NIP, just a weaker absorption peak around 3420 cm^{-1} was discovered because of the lack of hydrogen bonding or electrostatic binding interaction between testosterone and MAA. The sharp and intensified peaks at 1570 and 1410 cm^{-1} were observed on both coatings, owing to the stretching vibration of residual vinylic C=C bonds and the in-plane bending vibration of C–H bonds on methyl groups, respectively. Other absorption peaks matched both MIP and NIP coating: 2960 or 2970 cm^{-1} (stretching vibration of C–H bonds on methyl groups); 1730 cm^{-1} (stretching vibration of C=O bonds on carbonyl groups); 1150 cm^{-1} (stretching vibration of C–O and C–C).

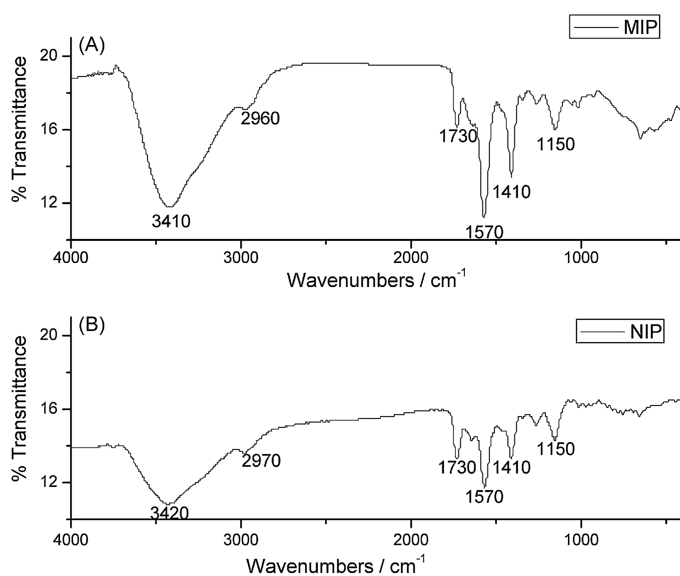

Fig. 4. Infrared (KBr pellet) spectra for MIP (top) and NIP (bottom) SPME coatings. Polymerization conditions as in Fig. 3.

3.2.3. Thermogravimetric analysis of MIP coating

In injection and desorption process of MIP for GC analysis, the thermal stability of SPME fiber coating is considerably important. Thermogravimetric analysis (TGA) of polymer revealed that the polymer was thermally stable up to 290 °C for both coatings. Since the injector temperature of GC or GC–MS was commonly controlled at 290 °C or below, therefore, both the MIP and NIP coatings had good thermal stability, and they were suitable for GC analysis.

3.2.4. Chemical stability of MIP-coated SPME fibers

The prepared MIP-coated SPME fibers were separately immersed in 1.00 mol/L of HCl, 1.00 mol/L of NaOH, hexane, toluene, chloroform, methanol, 10% acetic acid in methanol and distilled water to investigate the chemical stability. After keeping in those solutions for 2 h at room temperature, MIP coatings were found to remain good surface quality without desquamating, cracking and swelling. Then these fibers were used for extraction and desorption of four anabolic steroids in spiked water solutions, and no measurable degradation of extraction ability was observed. It could be deduced that the proposed MIP-coated SPME fibers were chemically stable in strong acid, base and organic solvents. The result further demonstrated the characteristics and superiority of MIP.

3.2.5. Solvent effects

According to digressive sequence of polarity index [40], solvents stretching across a range of polarities including hexane, toluene, dichloromethane, acetone, methanol, and water were employed to investigate the nature of interaction between target analytes and fiber coating [37]. The experiments were designed by preparing the anabolic steroids mixed standard solution in the above-mentioned solvents at the concentration of 0.1 μg/mL for SPME. For hexane and toluene, there was no detectable peak of analytes, while for dichloromethane, acetone and methanol, the polyimide was easily removed, and then obstructed the homemade SPME device. Therefore, the simplest solvent, water, was used for taking place of polar organic solvents and made the extraction from human urine feasible.

MIP-coated SPME fibers in spiked water maintained high extraction ability, which indicated that the hydrogen bonding interaction between analytes and polymer was weak. Therefore, it could be initially deduced that the interaction of anabolic steroids with the MAA groups was mainly based on ionic interaction via electrostatic forces.

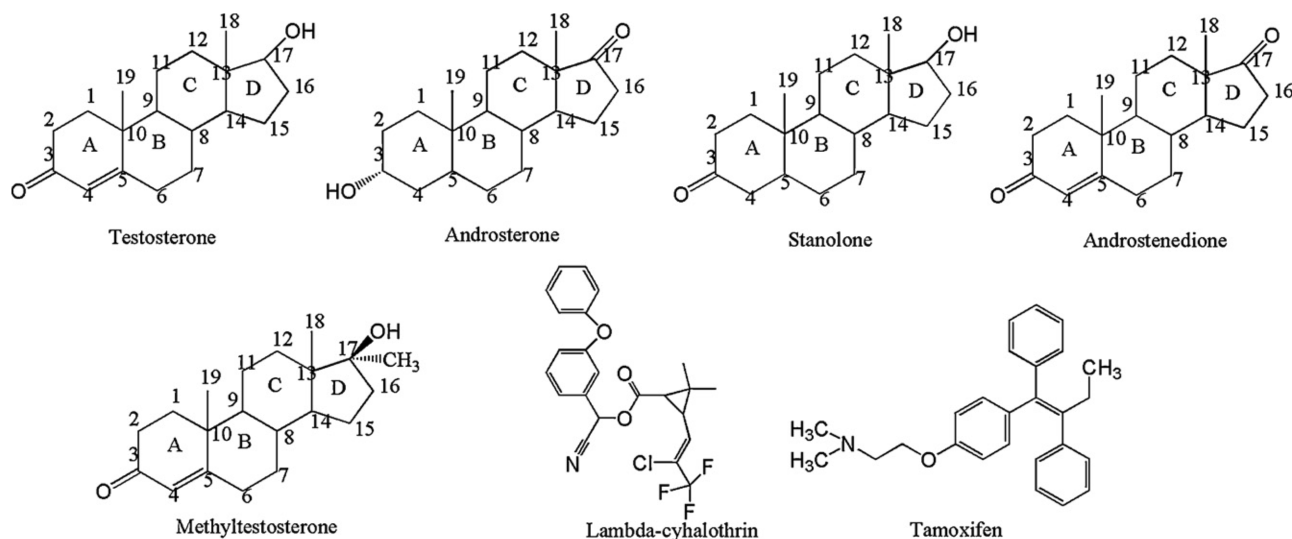

Fig. 5. Structures of the studied molecules.

3.2.6. Effect of pH

To further discuss the nature of interaction between target analytes and fiber coating, pH of aqueous solution were carefully adjusted in the range of 4.00–9.00 (adjusted them with 0.05 mol/L K_2HPO_4 and 0.05 mol/L NaH_2PO_4). The results showed extraction efficiency of MIP-coated SPME fibers increased with pH from 4.00 to 6.89, then decreased from 6.89 to 9.00. The results also indicated that molecularity of anabolic steroids were suitable for extraction. Therefore, electrostatic binding was expected to be formed between anabolic steroids and MAA groups as a key interaction for binding site construction. However, it should be kept in mind that, besides electrostatic binding, hydrogen bonding and hydrophobic interactions of analyte with MIP are also expected to occur [22].

3.2.7. Extraction ability

In this study, four anabolic steroids containing hydrophobic tetracyclic perhydro-1,2-cyclopentanophenanthrene ring in their molecular structures (the structures were shown in Fig. 5, the others were two reference compounds) were selected as model compounds to investigate the extraction performance of the novel MIP coating. The extraction ability of the MIP-coated SPME fibers was evaluated by comparing extraction of model compounds from 20 mL spiked water sample at the concentration of 5 μg/mL with direct injection of 1 μL model compounds at the same concentration. As shown in Fig. 6, obvious enhancement of the peak areas was found in the SPME chromatogram, which indicated obvious extraction efficiency of the MIP-coated SPME fibers for anabolic steroids. The enrichment factor was calculated as the mass of analyte extracted over that obtained by direct injection. The value was 41.3, 974.3, 19.0 and 20.9 for androsterone, stanolone, androstenedione and methyltestosterone, respectively.

Moreover, the enrichment factor of stanolone was much higher than those of androstenedione and methyltestosterone. The higher enrichment factor was attributed mainly to the strong interaction between fiber coating and analytes via electrostatic forces in a polar solvent. For stanolone, existence of hydroxyl group in its molecular structure could enhance electrostatic binding interaction with carboxy groups in the MIP-coated SPME fibers, and then increase extraction efficiency. However, for methyltestosterone, existence of methyl group near hydroxy group on C17 could hinder the electrostatic binding interaction. It could be also discovered that the extraction amounts of androsterone and stanolone were comparable for the MIP-coated SPME fibers. A possible explanation was that androsterone and stanolone had identical number of hydroxyl group, carbonyl group except the distribution substitute on ring A or ring D, then introduced similar interaction of electrostatic forces.

These results confirmed the considerable selectivity of the prepared MIP-coated SPME fibers. From these results, it could be concluded that the proposed fibers possessed good applicability to selectively extract the mentioned compounds from freshly prepared aqueous samples.

To further investigate the extraction ability of the MIP-coated fiber for the anabolic steroids selected, the commercial PDMS fiber with coating thickness of 100 and 30 μm were selected as the representatives of traditional fibers based on "like dissolves like" principle and used for comparison. The results (Fig. 7) illustrate that the extraction efficiency for selected anabolic steroids by the MIP-coated fiber was much higher than that of the PDMS fiber. The higher extraction efficiency was attributed mainly to the interaction between fiber coating and analytes. Finally, nitrogen sorption

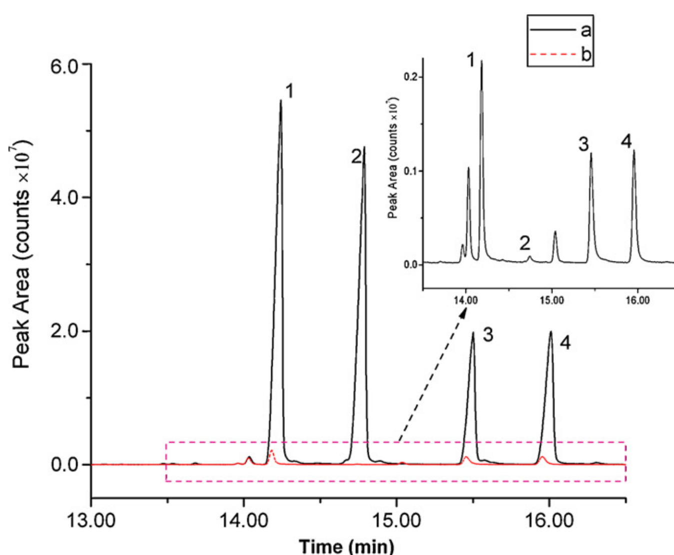

Fig. 6. Comparison of chromatograms between (a) the direct injection of 1.0 μL of each of four anabolic steroids at 5 μg/mL and (b) the MIP-coated SPME fiber of the same concentration (peaks: (1) androsterone; (2) stanolone; (3) androstenedione; (4) methyltestosterone).

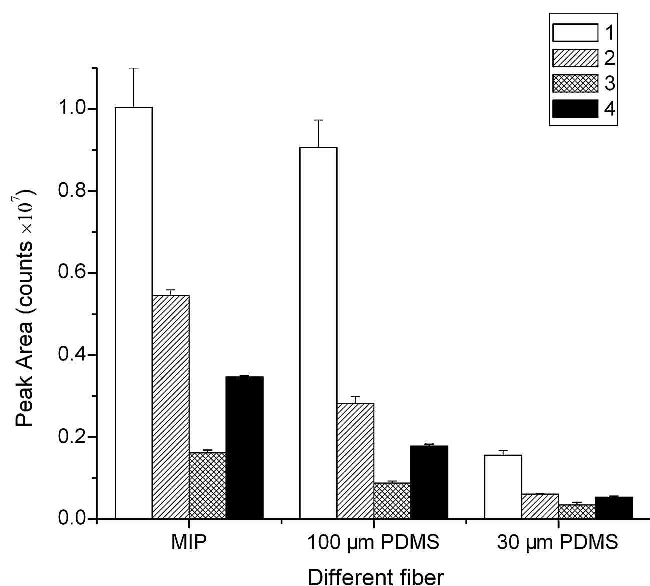

Fig. 7. Comparison of extraction amounts using a MIP-coated fiber, a commercial 30 μm PDMS fiber and a commercial 100 μm PDMS fiber.
The concentration of (1) androsterone, (2) stanolone, (3) androstenedione and (4) methyltestosterone was 0.1 μg/mL, respectively. GC–MS and SPME conditions as in Table 1.

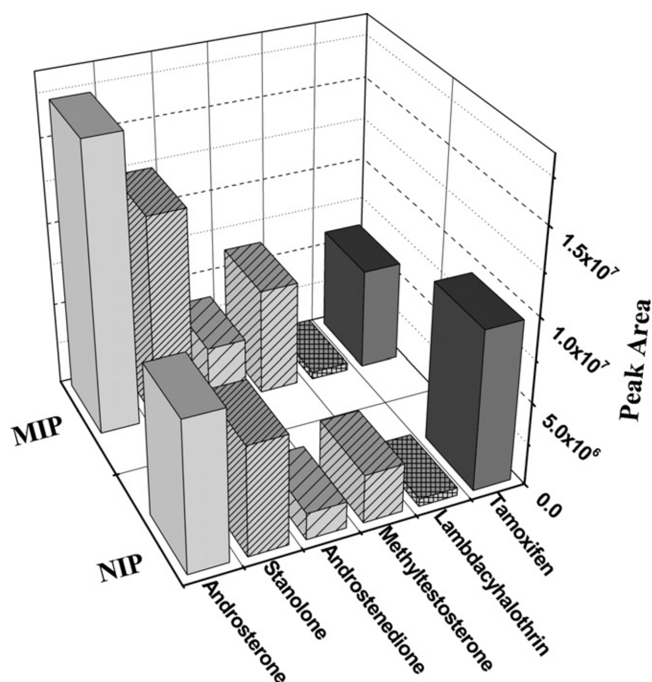

Fig. 8. Extraction amounts of four anabolic steroids, tamoxifen and lambda-cyhalothrin with MIP- and NIP-coated SPME fibers at 0.1 μg/mL level. GC–MS conditions as in Table 1. SPME conditions: extraction time, 20 min; other SPME conditions as in Table 1.

measurement on the MIP was performed and the BET surface area of MIP was found to be 248.3 m^2/g.

3.2.8. Extraction selectivity of MIP-coated SPME fibers

Theoretical prediction of selectivity of MIP for different compounds is quite complex due to their differences in polarity, hydrophobicity, dipole, ionization state and shape or conformational effects [35]. Therefore, it's indispensable to carry out experimental investigation of selectivity of MIP-coated SPME fibers. For this purpose, the MIP- and NIP-coated SPME fibers were separately used for extraction of 0.1 μg/mL of four anabolic steroids and two reference compounds, tamoxifen and lambda-cyhalothrin (with structures as shown in Fig. 5) from aqueous solutions. The results were showed in Fig. 8 by means of chromatographic peak area.

Obviously, the chromatographic peak areas of four anabolic steroids with the MIP-coated SPME fibers were much higher than those of the NIP-coated SPME fibers. The results indicated that the fibers revealed higher selectivity to the structural analogues of testosterone. It was mainly attributed to the recognition of MIP on molecular size compared with template molecules, electrostatic forces between carboxylic groups in the MIP coating and hydroxyl groups, carbonyl groups in four anabolic steroids at similar positions.

For two reference compounds, tamoxifen contained tertiary amine group, ether linkage, and lambda-cyhalothrin contained carbonyl group, ether linkage, therefore, they were reactive to form electrostatic forces with the MIP coating to some extent, and hence tamoxifen and lambda-cyhalothrin could be extracted with the MIP- or NIP-coated SPME fibers. However, the chromatographic peak areas of tamoxifen and lambda-cyhalothrin extracted by MIP-coated SPME fibers were lower than those of NIP-coated SPME fibers because of the differences of structures between two reference compounds and template molecules.

Finally, in order to discuss differences of extraction ability among MIP-coated SPME fibers, five fibers were used for extracting 0.1 μg/mL anabolic steroids mixed standard solution and the RSDs were lower than 11.2% for all four anabolic steroids.

3.3. Optimization of the SPME method

The effect of solution temperature on the extraction efficiency was discussed from 30 to 70 °C. Extraction efficiency steadily increased in the range of 30–40 °C, and then decreased in the range from 40–70 °C. The results were shown in Fig. 9a. To achieve higher sensitivity, an extraction temperature of 40 °C was selected for subsequent work.

Stirring was employed to accelerate the mass transfer, increase extraction efficiency and reduce extraction time. From Fig. 9b, there was an increase in extraction efficiency with stirring rate increasing from 100 to 500 rpm. At higher rate, there was no positive effect on the extraction efficiency. The results indicated that the diffusion rate of analytes reached the maximum at 500 rpm and the extraction equilibrium was established. On the basis of these observations, a stirring rate of 500 rpm was selected.

As mentioned in Section 3.2.6, high responses from pH 4.00 to 6.89 can be achieved for all four anabolic steroids, whereas responses decreased when the pH of sample solution increased from 6.89 to 9.00. And finally pH 6.89 was selected in the subsequent experiment considering the analytical responses of all the anabolic steroids.

The effect of salt addition was investigated by adding NaCl into the sample solution in a series of concentration range from 0 to 0.25 g/mL (see Fig. 9c) and a final concentration of 0.175 g/mL was selected for further work. The effect of extraction time was studied in the time range of 10–60 min, which was shown in Fig. 9d. It could be seen from the figure that the responses of all target analytes increased gradually with increasing of extraction time from 10 to 40 min and remained no obvious increase for an additional extraction time. For the sake of time-saving, an extraction time period of 40 min was selected for further work.

The desorption temperature and desorption time in the hot GC injector were studied over the range 220–250 °C and 20–80 s, respectively. It was observed that desorption efficiency was increased with the temperature and reached a maximum at 240 °C.

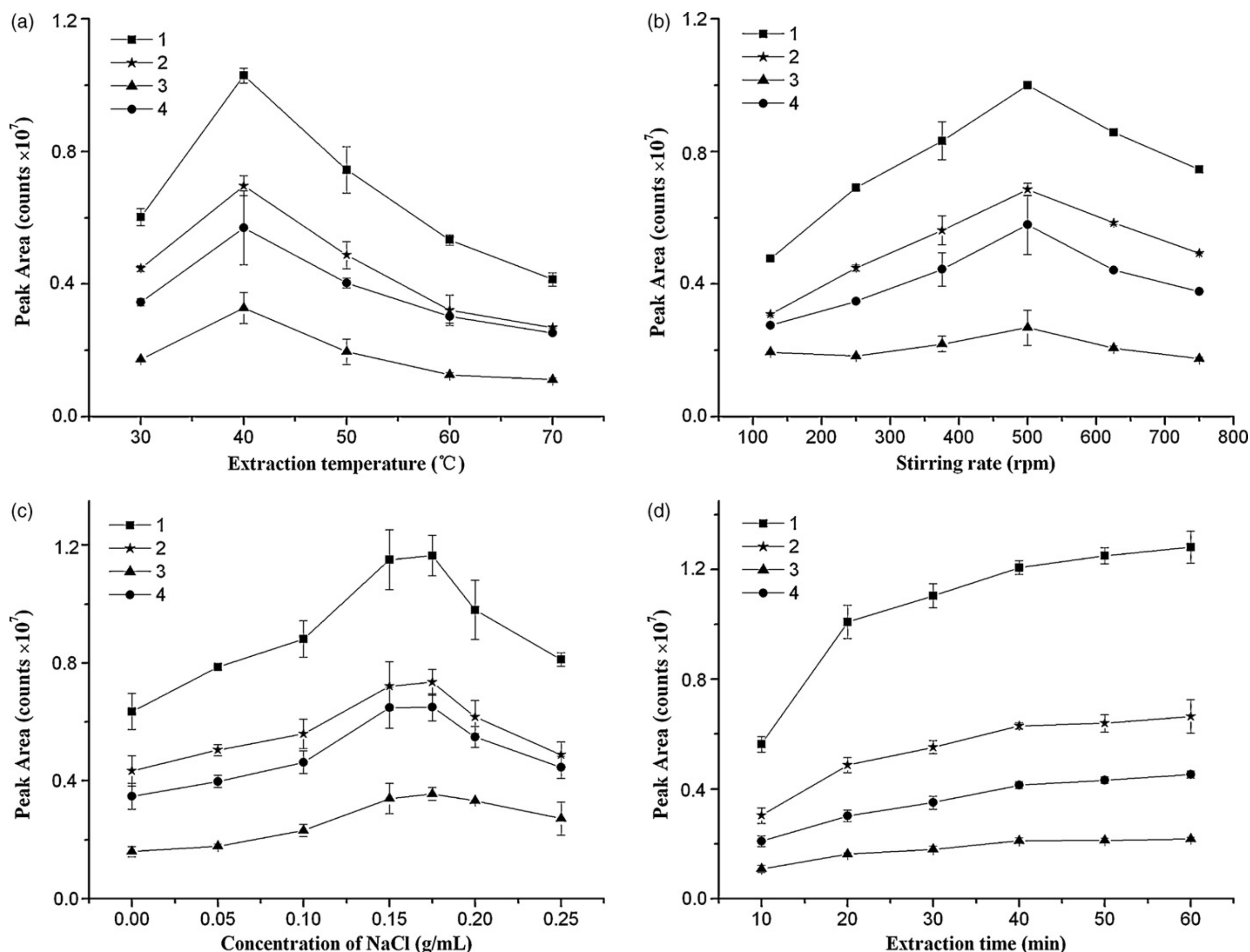

Fig. 9. Optimization of the extraction conditions: (a) extraction temperature, (b) stirring rate, (c) concentration of NaCl and (d) extraction time. The concentration of (1) androsterone; (2) stanolone; (3) androstenedione and (4) methyltestosterone was 0.1 μg/mL, respectively. Other SPME and GC–MS conditions as in Table 1.

Comparing the responses of target analytes when desorption time ranged from 20 to 60 s, the desorption amounts increased with the increasing desorption time, and then remained unaffected for longer time. Based on these results, 240 °C and 60 s were chosen as the optimum desorption temperature and time, respectively.

To sum up, the optimum conditions of extraction and desorption were as follows: in 20 mL spiked water solution including 0.175 g/mL NaCl, the MIP-coated SPME fiber extracted analytes for 40 min with 500 rpm stirring rate at 40 °C, and desorption was performed at 240 °C for 60 s.

3.4. Validation

Under the optimum conditions, calibration curves were obtained by the MIP-coated SPME fiber for the four anabolic steroids in spiked water solutions and spiked human urine solutions, respectively. In spiked water solutions, calibration standards were prepared by concentration of 0.02, 0.05, 0.08, 0.10, 0.20, 0.50, 0.80, and 1.0 ng/mL. And in spiked human urine solutions, calibration standards were prepared by concentration of 0.08, 0.10, 0.20, 0.50, 0.80 ng/mL. Some analytical results, such as linear ranges, regression equations, detection limits of four anabolic steroids in spiked water and spiked human urine solutions, were listed in Table 2. The linearity was good for all analytes in the whole range of tested concentrations, as proved by the correlation coefficients (R^2) which were greater than 0.9910 for all curves. The detection limits for four anabolic steroids in spiked water solutions and spiked human urine solutions were in the range of 0.008–0.010 and 0.008–0.020 ng/mL, respectively. The intra-day precision of this SPME-GC–MS method was assessed with five spiked water solutions (0.50 ng/mL) and five spiked human urine solutions (0.50 ng/mL) on the same day, respectively. And the RSDs for four anabolic steroids varied from 8.3 to 13.6% and 9.4 to 14.9%, correspondingly. The results obtained confirmed the SPME method based on MIP-coated SPME fibers could be directly applied to real sample analysis.

3.5. Application

In order to validate the feasibility of the proposed method, extraction recoveries were assessed by spiking human urine solutions with trace mixed standards solutions (0.10, 0.50 and 0.80 ng/mL, respectively) and calculating with the regression equation of analytes in water matrix. The typical chromatograms of the blank and the spiked human urine samples by SPME were shown in Fig. 10. It could be seen from Fig. 10 that no significant compounds in the mixture of body fluids interfered with the determination of the four anabolic steroids. Also, the responses of all target ana-

Table 2
The regression equations, linearity, correlation coefficients and LOQs of the method.[a]

Analytes	Matrix	Regression equation[b] $y = ax + b$	Linearity (ng/mL)	Correlation coefficient (R^2)	LOQs (ng/mL)
Androsterone	Water	$y = 79861x + 4111.3$	0.02–1.0	0.9912	0.02
	Human urine	$y = 327619x - 29095$	0.08–0.80	0.9941	0.08
Stanolone	Water	$y = 59474x + 1750.6$	0.02–1.0	0.9955	0.02
	Human urine	$y = 257321x - 22022$	0.08–0.80	0.9938	0.08
Androstenedione	Water	$y = 60604x - 359.51$	0.02–0.80	0.9945	0.02
	Human urine	$y = 65493x - 4509.9$	0.08–0.80	0.9982	0.08
Methyltestosterone	Water	$y = 20234x + 635.76$	0.05–1.0	0.9980	0.05
	Human urine	$y = 59589x - 7083.9$	0.1–0.80	0.9910	0.10

[a] GC–MS conditions as in Section 2.2. SPME conditions: pH, 6.89; NaCl concentrations, 0.175 g/mL; extraction time, 40 min; other SPME conditions as in Section 2.6.
[b] The parameters, x and y, refer to the concentration of the target analytes (ng/mL) and the corresponding peak area.

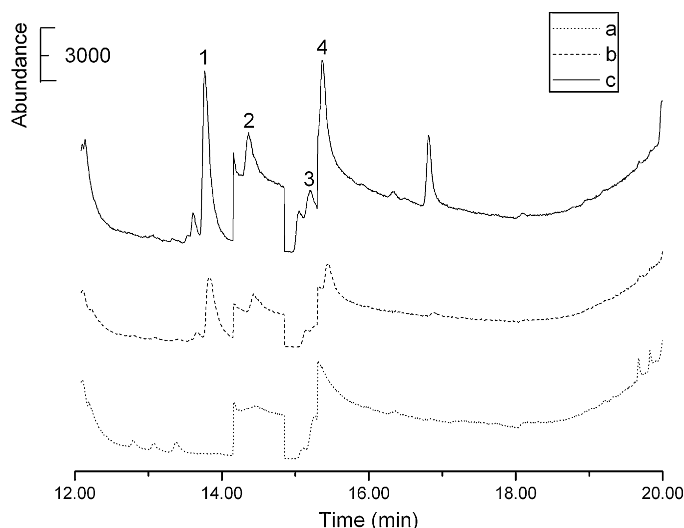

Fig. 10. SPME-GC–MS analyses of blank and spiked human urine with the 0.80 ng/mL of four anabolic steroids in SIM mode, and the test were performed under the optimum conditions of extractions. (a) Blank human urine extracted with MIP-coated SPME fiber, (b) spiked human urine extracted with commercial PDMS fiber (c) spiked human urine extracted with MIP-coated SPME fiber (peaks: (1) androsterone; (2) stanolone; (3) androstenedione; (4) methyltestosterone).

lytes extracted with MIP-coated SPME fiber were higher than those extracted with commercial PDMS fiber with coating thickness of 100 μm. The results indicated that the MIP-coated SPME fiber had better selectivity than PDMS.

According to the relationship between peak area and concentrations of analytes, recoveries and RSDs were calculated. The average recoveries of the anabolic steroids ranged from 80.1% to 108.4% with the RSDs ranging from 4.2% to 14.7%. These results completely satisfied the requirement of trace analysis of anabolic steroids (see Table 3). All the analytical results indicated that the simple SPME method proposed was very suitable for the simultaneous and rapid determination of trace androsterone, stanolone, androstenedione, and methyltestosterone in biological matrices and water solution samples without derivatization.

Table 3
Recoveries of spiked urine samples by SPME ($n = 3$).[a]

Analytes	Recoveries (% RSD, $n = 3$)		
	0.10 ng/mL	0.50 ng/mL	0.80 ng/mL
Androsterone	100.4 (14.3)	95.8 (4.2)	97.8 (8.9)
Stanolone	94.4 (13.8)	80.1 (11.4)	91.2 (7.3)
Androstenedione	108.4 (13.7)	88.1 (10.4)	87.9 (9.5)
Methyltestosterone	100.2 (14.7)	81.2 (10.2)	92.7 (10.3)

[a] GC–MS conditions as in Table 1.

4. Conclusion

In this paper, a novel testosterone MIP-coated SPME fiber was prepared. It was homogeneous, porous, selective, chemically and physically stable and could be directly inserted to GC–MS injection port. The average thickness of the MIP coatings was 3.1 μm with good fiber-to-fiber reproducibility. The proposed fiber exhibited high extraction efficiency towards anabolic steroids via electrostatic forces in polar solvents. Therefore, the sensitivity of anabolic steroids determination could be enhanced obviously by the MIP-coated SPME-GC–MS method. The detection limits of 0.008–0.010 ng/mL in spiked water solutions and 0.008–0.020 ng/mL in spiked human urine solutions were less than the maximum allowed limits of the WADA for anabolic steroids. In addition, the MIP-coated SPME-GC–MS method offered acceptable precision in the intra-day experiments and extraction recoveries of the anabolic steroids in spiked human urine solutions. In a word, the prepared MIP-coated SPME fiber was very suitable for coupling with GC–MS for simultaneous and rapid determination of trace analytes in biological matrices with negligible impurities interference.

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