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Study on the molecularly imprinted polymers with methyl-testosterone as the template

Minli Yang, Wancheng Gu, Li Sun, Feng Zhang, Yun Ling, Xiaogang Chu*, Daning Wang

Institute of Food Safety, Chinese Academy of Inspection and Quarantine, No 3, Gaobeidian North Road, Chaoyang District, Beijing 100123, PR China

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ABSTRACT

Molecularly imprinted polymers (MIPs) using methyl-testosterone as the template, methacrylic acid (MAA) as the monomer and ethylene glycol dimethacrylate (EDMA) as the crosslinker were prepared by precipitation polymerization. The morphology of the obtained particles was characterized by scanning electron microscopy (SEM) and the pore size was measured by BET. Then, the specificity and selectivity of the MIPs were evaluated using the equilibrium rebinding experiments. Besides, the MIPs were also used as the stationary phase of HPLC column and the retention behaviour to the template and analogues was confirmed using HPLC–MS–MS. Finally, the real application of the methyl-testosterone imprinted polymers was evaluated using SPE procedure with the spiked tap water and lake water. The results indicated that the prepared methyl-testosterone imprinted polymer showed specific rebinding ability to its template and could retain the template strongly compared with other structural analogues. At the same time, the MIPs could be used as SPE column to enrich methyl-testosterone in the lake water and show broad prospects in real samples.

1. Introduction

Molecular imprinting technology (MIT) has been developed rapidly during the last decades [1–3]. Since the highly specific interaction between the template and the polymer is similar to that between antibody and antigen, hormone and receptor, enzyme and substrate, molecularly imprinted polymers (MIPs) can be used in many different fields, such as the stationary phase in HPLC [4–6] and SPE [7–9] for separation, recognition element in biosensors [10,11], catalysts and mimic antibodies [12,13].

Up to now, many different methods have been developed for the preparation of MIPs [14–17]. The widely used methods, bulk polymerization, the template, monomer, crosslinker and initiator are added to a glass tube and begin to polymerize initiated by thermal or photo (UV) radiation. After polymerization, the monolith obtained needs to be crushed, ground, then the particles with certain size distribution can be directly used as the stationary phase in HPLC and SPE. This process is easy to operate, but time consuming and labour intensive, typically less than 50% of the ground polymer is recovered as useable particles. The rebinding cavities could be destroyed during the crushing and grinding procedures and irregular particles generally give less efficient column packing for chromatography. Compared with bulk polymerization, precipitation polymerization is a more economical and labour-saving method often used for preparing MIPs with uniform shape and low-dispense phase distribution in good yield [14]. The particles obtained are sub-micron scale (0.3–10 μm) and monodispersed. The method is based on the precipitation of the polymeric chains out of the solvent in the form of particles as they grow more and more insoluble in an organic continuous medium [18]. The procedures are the same as those in bulk polymerization except in much more diluted porogen. The particles obtained can be used directly, the grinding and crushing procedures are not necessary. In this case, particles are prevented from coalescence by the rigidity obtained from the crosslinking of the polymer, so there is no need of any extra stabiliser.

Methyl-testosterone is a kind of synthesized metabolic steroid which has been widely used in stockbreeding and aquatic products as a growth promoting agent since last century. Methyl-testosterone will produce serious side effects such as liver poisoning, embryo poisoning and cancer due to its slow metabolism and strong accumulation in the body. Therefore, EU started to prohibit the use of steroidal hormone to feed the livestock since 1988. In China methyl-testosterone was also forbidden as the growth promoting agent since 1999. Rapid and accurate determination of the veterinary drugs residue in livestock products is a key step to prevent the situation that a few enterprises still use illegally to seek the interests thoroughly. Therefore, it is necessary to establish the simple, rapid, sensitive determination method with low cost. Until now, there are many reports on the detection technologies of methyl-testosterone including HPLC [19,20], GC–MS [21,22], LC–MS [23], ELISA and so on. Among these methods, the proce-

* Corresponding author. Tel.: +86 10 85778904; fax: +86 10 85791012.
E-mail addresses: xgchu@vip.sina.com, minli_yang@hotmail.com (X. Chu).

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dure of sample pre-treatment is complex and time-consuming, the derivation step is necessary in some methods. Molecularly imprinted polymers (MIPs) can be used for the separation of structural analogues due to their high selectivity. He et al. [24] have prepared testosterone imprinted silica using an ionic liquid 1-butyl-3-methylimidazolium tetrafluoroborate ([Bmim]BF4) as a new type of solvent and the imprinted silica was characterized by FT-IR spectroscopy, N_2 gas adsorption–desorption isotherm and the high-resolution transmission electron microscopy (TEM). Whereas, there was no report on the preparation of MIPs with methyl-testosterone as the template and using HPLC–MS–MS as the characterization means. In this work, the MIPs with methyl-testosterone as the template were synthesized using precipitation polymerization. The morphology of the obtained particles was characterized by scanning electron microscopy (SEM) and the pore size was measured by BET. Then, the specificity and selectivity of the MIPs were evaluated using the equilibrium rebinding experiments. Besides, the MIPs were also used as the stationary phase of HPLC column and SPE cartridge for the enrichment of methyl-testosterone from real samples. In addition, the retention behaviour was confirmed using HPLC–MS–MS.

2. Experimental

2.1. Chemical and materials

Methyl-testosterone, testosterone propionate, 19-nortesterone, progesterone and medroxyprogesterone acetate were purchased from Acros. Methacrylic acid (MAA) was from Sigma–Aldrich (Taufkirchen, Germany). Ethylene glycol dimethacrylate (EDMA), 2,2′-azobis(2-isobutyronitrile) (AIBN) and acetonitrile (ACN) were from Merck (Darmstadt, Germany). MAA was distilled under vacuum to remove the stabilizers before use. All solutions were prepared using ultrapure water, obtained by reverse osmosis including UV treatment (Milli-RO 5 Plus, Milli-Q185 Plus, Millipore, Eschborn, Germany). The structure of the compounds related was shown in Fig. 1.

2.2. Preparation of MIPs

The template methyl-testosterone (1 mmol)) and the monomer (MAA, 4 mmol) were weighed and put into acetonitrile of different volume. After the mixture was shaken for 5 h in the thermostat oscillator, the crosslinker EDMA (20 mmol) and the initiator AIBN (100 mg) were added to the solution. Then purging with nitrogen for 10 min, the round bottomed flasks were placed in a thermostatted water bath (Yuyao, China) at 60 °C for 24 h. The resultant solution was dried using nitrogen and the particles were collected for further use. The blank polymers were synthesized in the absence of the template.

2.3. Characterization of the particles

The type of the scanning electron microscope (SEM) instrument is JSM-5800 (JEOL Co. Japan) with a detector system and software. Magnification time: 10,000.

The parameter used for the measurements:

voltage = 20 kV.
current = 200 pA.
distance = 25 mm.

The specific surface area of the particles was determined by nitrogen gas adsorption measurements using BET (ASAP2010, Micromeritics Co., USA).

2.4. Equilibrium adsorption experiments

The rebinding capability of MIPs was tested using equilibrium adsorption experiments. An amount of 20 mg of the imprinted polymer particles was weighed into 8 ml Teflon tube. Then 5.0 ml of methyl-testosterone solution in acetonitrile with known concentration (0.05–1.50 mmol/l) was mixed with the polymers. The mixture was incubated on a horizontal shaker (SA-31, Japan) for 12 h at room temperature, then centrifuged at 10,000 rpm for 20 min, the supernatant was collected and filtrated through 0.45 μm membrane. The concentration of methyl-testosterone in the filtrate was measured by Agilent 1100 HPLC system which consists of a G-1379A degasser, a G-1311A quatpump, a G-1313A ALS autosampler, a G1316A column temperature oven and a G-1315B DAD. The amount of methyl-testosterone bound to the polymers was calculated by subtracting the amount of free methyl-testosterone from the initial concentration. The maximum adsorption amount Q_{max} and equilibrium dissociation constant K_d were calculated according to the concentration change before and after the adsorption.

The selectivity of the MIPs was evaluated by comparing the equilibrium adsorption ability to the template with that to the structural analogues. The solution of testosterone propionate, 19-nortesterone, progesterone and medroxyprogesterone acetate with the concentration of 1.0 mmol/l was prepared. Then 5 ml solution mentioned above was mixed together with 20 mg polymer particles in Teflon tubes. The rebinding procedure was similar as the former description. The distribution coefficient was calculated according to the equation of $K = C_P/C_S$, where C_P is the substrate concentration adsorbed on the polymer, C_S is the substrate concentration remaining in the solution. Recognition factor was calculated according to the equation $\beta = K_{MIP}/K_{BP}$, where K_{MIP} is the distribution coefficient of the MIPs and K_{BP} is that of the blank polymer.

The same procedures were applied to the blank polymers in order to compare with the MIPs.

2.5. HPLC–MS–MS analysis

Besides the equilibrium adsorption experiments, the retention behaviour of MIPs was also evaluated using HPLC coupled with tandem mass spectrometry due to its high sensitivity instead of HPLC only. The instrument used is Agilent 1200 (USA) consisting of a G1378B degasser, a G1312B binary pump, a G1367D HiP-ALS SL autosampler, and a G1316B TCC SL column temperature oven, together with API 5000 mass spectrometry.

The collected polymers were packed into stainless steel column (100 mm × 4.6 mm i.d., Beijing, China). The column was washed thoroughly with methanol-acetic acid (9:1, v/v) to remove the template until the stable baseline was achieved. HPLC–MS–MS was carried out at room temperature at a flow rate of 0.15 ml/min and the mobile phase was pure acetonitrile (A) and 0.1% formic acid (B). The experimental parameters including composition of the mobile phase, declustering potential (DP), collision energy (CE) were optimized to achieve the highest intensity of the analytes. Then the mixture of methyl-testosterone, testosterone propionate, 19-nortesterone, progesterone (PS) and medroxyprogesterone acetate was injected, the concentration of each compound was 300 ng/ml and the injection volume was 2 μl. The column temperature was 30 °C.

The MS–MS parameters were as follows: Capillary voltage was 5.5 kV. Ion source temperature was 550 °C. The cone gas velocity was 100 psi. The desolvation gas velocity was 600 psi. The collision gas was N_2. The collision pressure was 2.5×10^{-5} Pa with ESI + MRM analyze mode. The exact parameters for each compound are listed in Table 1.

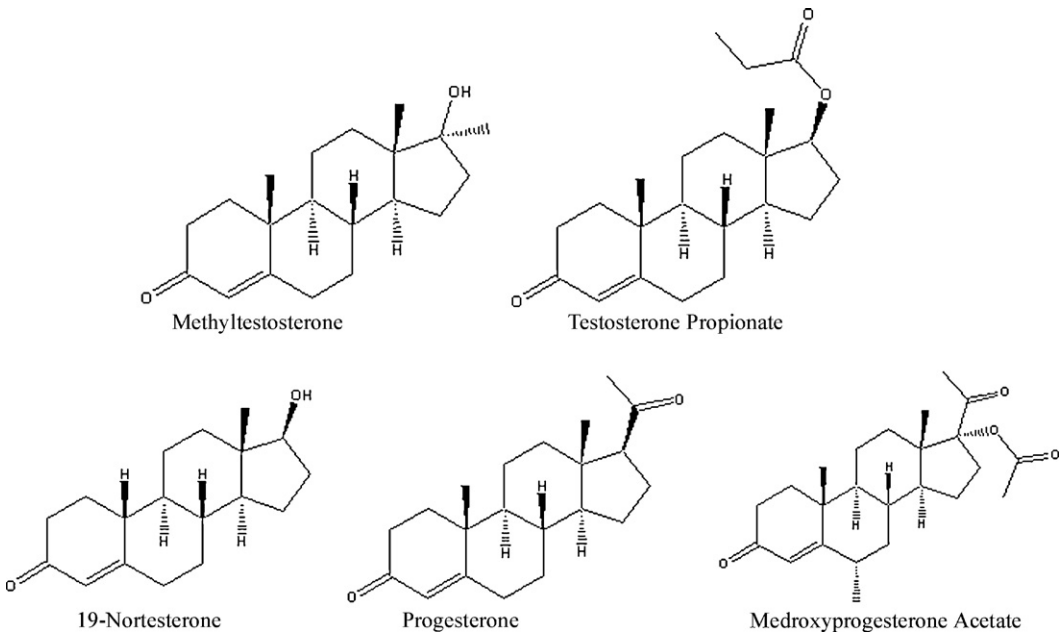

Fig. 1. Structure of the template and other analogues.

2.6. SPE procedure for the determination of methyl-testosterone in real samples

To evaluate the analytical applicability of the prepared MIPs, the SPE procedure for the determination of methyl-testosterone in tap water and lake water was carried out. First, a PTFE frit (porosity 10 μm, Merck) was placed on the bottom of the empty polypropylene SPE cartridge (6 ml, Dikma, USA). Then a slurry of 500 mg of MIPs in 5.0 ml of methanol was filled into the cartridge and another frit was placed on the top of the cartridge to cover the MIPs. Before the water samples were processed, the cartridge was preconditioned with 5.0 ml of methanol. Meanwhile, a C18 SPE column was loaded at the same condition for comparison.

Tap water was collected from the tap of the laboratory without pretreatment. Lake water samples were collected in amber glass bottles from rivers in Beijing and filtered through 0.2 μm nylon membrane which was 47 mm in diameter (Pall, New York, USA). The samples were stored in the dark at 4 °C and then analyzed within 48 h. Since the low solubility of methyl-testosterone in pure water, the spiked sample was prepared using the mixture of acetonitrile and water (1:9, v/v) with the concentration of 200 μg/L methyl-testosterone. After loading 50 ml spiked samples, the loaded columns were washed with 5 ml of methanol–water (1:1, v/v), then the analyte was eluted with 10 ml methanol-acetic acid (7:3, v/v) for three times. The obtained extract was evaporated to dryness with rotary evaporator, reconstituted in acetonitrile to a final volume of 1.0 ml. The analytes in the washing solution and the eluate were analyzed using normal HPLC method with C18 column and DAD detector.

Table 1
Parent/daughter ions used in MRM.

Compound	Parent ion (*m*/*z*)	Daughter ion (*m*/*z*)	Collision energy (eV)	Declustering potential (V)
Methyl-testosterone	303.3	96.9	40	130
Testosterone propionate	345.2	109.0	40	130
19-Nortesterone	275.2	109.1	52	130
Progesterone	315.1	97.0	52	130
Medroxyprogesterone	345.1	123.1	40	130

3. Results and discussions

3.1. Characterization of the MIPs

The effect of the solvent volume, initiator amount on the size of the particles was investigated. The same components including 1 mmol methyl-testosterone, 4 mmol MAA and 20 mmol EGDMA were added to acetonitrile of different volume (20, 100, 200, 350, 500 and 600 ml). All the particles obtained were characterized using SEM and the results indicated that the diameter of the particles decreased with the increasing porogen volume which was in accordance with the results described by Tong [25–28]. Such phenomenon was resulted from the fact that under dilute conditions, less oligomers and nuclei were formed during the polymerization and the flow ability of the polymer increased. Therefore, less radical monomer and cross-linkers diffused to the surface of nuclei and grew up into the particles with smaller diameter. The imprinted monolith was obtained in 20 ml acetonitrile, when the solvent volume increased to 200 ml, the particle diameter was about 1.5–1.7 μm, and when the solvent volume increased to 500 ml, the diameter decreased to 400–500 nm. The morphology of the MIPs prepared in 200 ml (MIP1) and 500 ml acetonitrile (MIP2) was shown in Fig. 2. The morphology of the corresponding blank polymers was similar as the MIPs (figures not shown). The values of surface area, pore size and volume were listed in Table 2. The data indicated that MIPs prepared in 500 ml acetonitrile possessed the largest surface area due to its smaller particle diameter, and all the values of the MIPs were much higher than those of the blank polymers. For the polymers prepared in 200 ml acetonitrile, the same trend was observed although the difference between the MIPs and the blank polymers was not so obvious. Besides the influence of

Table 2
Results of BET characterization.

Polymer	Specific surface area (m^2/g)	Pore volume (cm^3/g)	Pore diameter (nm)
MIP1	17.0526	0.059291	13.2244
BP1	13.8753	0.045095	12.3256
MIP2	37.1001	0.188106	19.2230
BP2	25.6273	0.086520	12.8255

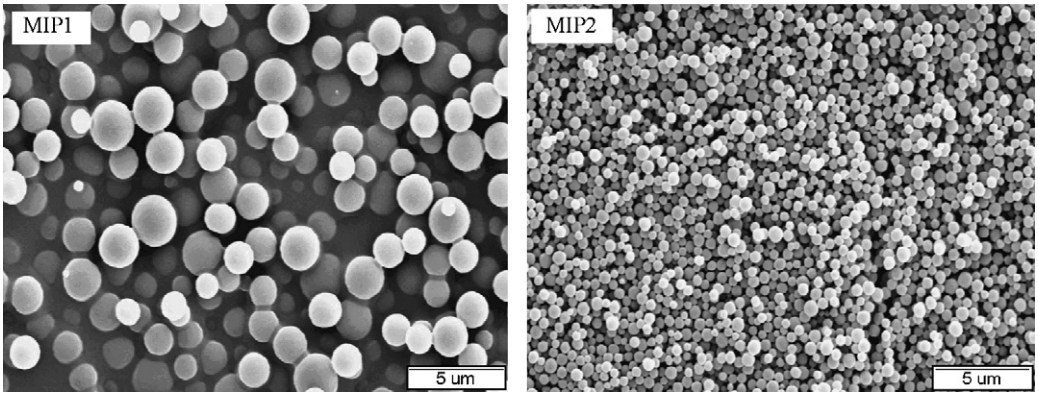

Fig. 2. SEM images of the methyl-testosterone MIP prepared in 200 ml (MIP1) and 500 ml (MIP2) acetonitrile.

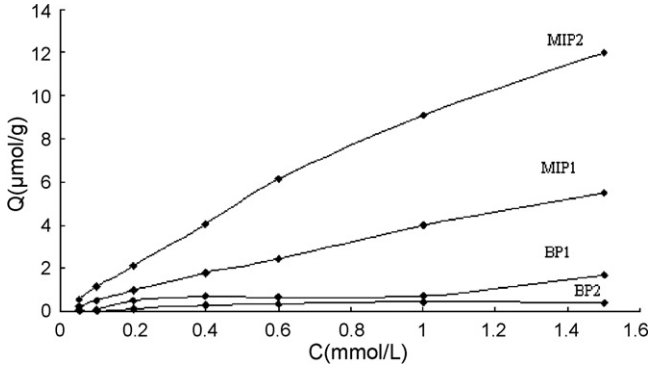

Fig. 3. Adsorption isotherms of the MIP and BP at 35 °C.

the porogen volume on the morphology has been embodied in the table, the difference between the MIPs and the blank polymers was also shown. All the values of specific surface area, pore size and volume of the MIPs were higher than those of the corresponding blank polymers, which resulted in the higher rebinding capacity of the MIPs than that of the blank polymers.

3.2. Rebinding capacity and selectivity of MIPs

The equilibrium adsorption experiments were carried out to evaluate the rebinding ability of the prepared MIPs. The rebinding properties of MIP1, MIP2 and the corresponding blank polymers BP1, BP2 were determined by measuring the methyl-testosterone uptake over a range of concentrations from 0.05 to 1.50 mmol/l. Scatchard plots were constructed for a constant mass of polymer and the maximum rebinding capacity and the dissociation constants of the polymers were calculated by Scatchard equation: $Q/C = (Q_{max} - Q)/K_d$.

The plot of Q vs. C was shown in Fig. 3. The results of the prepared polymers were listed in Table 3.

The results demonstrated (Fig. 2 and Table 3) that both the Q_{max} and K_d for the imprinted polymers were higher than those for the blank polymers, which was consistent with the BET results. For the MIP2, higher rebinding capacity was observed compared with MIP1 due to its higher specific surface area. The plots of Q–C were almost linear and might be composed of one straight line, thus indicating that the recognition sites in these imprinted polymers are uniform in nature.

The selectivity of the prepared MIPs was also evaluated by comparing their rebinding capacity to the template and to the structural analogues. Testosterone propionate, 19-nortesterone, progesterone, medroxyprogesterone were chosen and the concentration of each was 1.0 mmol/l. The values of K and β were listed in Table 4. As the data listed in Table 4 shown, the highest K_{MIP} value and the relatively low K_{BP} value for the template was observed in MIP2, resulting in the highest β value and indicating the strongest specific interaction between the template and MIP2.

3.3. Retention behaviour of MIPs using HPLC–MS–MS

Besides carrying out the equilibrium adsorption experiments, the retention behaviour of the MIPs was also confirmed by HPLC. Since the DAD was not so sensitive and the analogues cannot be distinguished by HPLC, HPLC–MS–MS was used to characterize the retention behaviour of the MIPs for its high sensitivity. The diameter of MIP2 was so small and would result in the high pressure, therefore, only the prepared MIP1 and the corresponding blank polymers were packed into the HPLC columns and evaluated using HPLC–MS–MS. In the analysis, the analytes with relatively low concentration were injected in order that the specific interaction of the MIPs could be shown obviously. The mode of Multiple Reaction Monitoring (MRM) was applied for its high sensitivity. The parent/daughter ion pair for each analyte was shown in Table 1 and the retention behaviours of the MIPs for each structural analogue were shown in Fig. 4. The values of capacity factors (k') and imprinting factor (IF) were listed in Table 5.

As Fig. 4 and Table 5 indicate, on the MIP1 packed column, the capacity factor k' of the template on the MIPs was 0.37 and the imprinting factor IF value was up to 5.76, much higher than the values for other four analogues. The strongest retention for the template was observed and almost the similar retention time was shown for the other analogues. But the quite different phenomenon was demonstrated on the column packed with the blank polymers

Table 3
The Q_{max} and K_d values of the polymers.

Polymer	Q_{max} (μmol g^{-1})	K_d (μmol mL^{-1})	Equation
MIP1	19.4105	4.2808	$Q/C = 4.5343 - 0.2336Q$
BP1	3.3566	1.9350	$Q/C = 1.7347 - 0.5168Q$
MIP2	44.1643	4.0866	$Q/C = 10.807 - 0.2447Q$
BP2	0.7482	0.8925	$Q/C = 0.8384 - 1.1205Q$

Table 4
Selective rebinding of different substrates on MIP and BP.

Substrate	K-MIP1	K-BP1	β	K-MIP2	K-BP2	β
Methyl-testosterone	3.70	0.66	5.61	8.69	0.39	22.28
Testosterone propionate	1.60	0.62	2.58	3.25	0.72	4.51
Medroxyprogesterone	1.46	0.98	1.49	1.53	0.42	3.64
19-Nortesterone	2.32	1.24	1.87	2.06	0.45	4.58
Progesterone	2.20	0.51	4.31	1.97	0.24	8.21

Table 5
k' and IF values of the analogues on HPLC columns.

	Methyl-testosterone	Testosterone propionate	19-Nortesterone	Progesterone	Medroxyprogesterone
k'_{MIP}	0.373	0.016	0.1	0.058	0.013
k'_{BP}	0.064	0.007	0.048	0.016	0.005
IF[a]	5.76	2.18	2.10	3.58	2.54

[a] IF value was calculated as the ratio of k'_{MIP} to k'_{BP}.

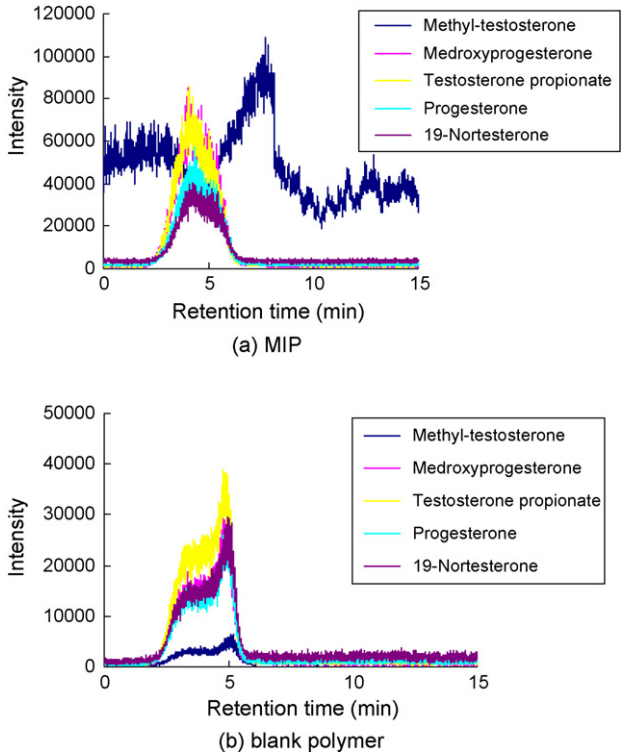

Fig. 4. XIC chromatograms of the compounds on MIPs (a) and blank polymers (b).

that no retention behaviour was shown for each analyte including the template and the analogues. By comparing the different retention behaviour on the two columns, the conclusion that there was the specific interaction between the template and the MIPs can be drawn.

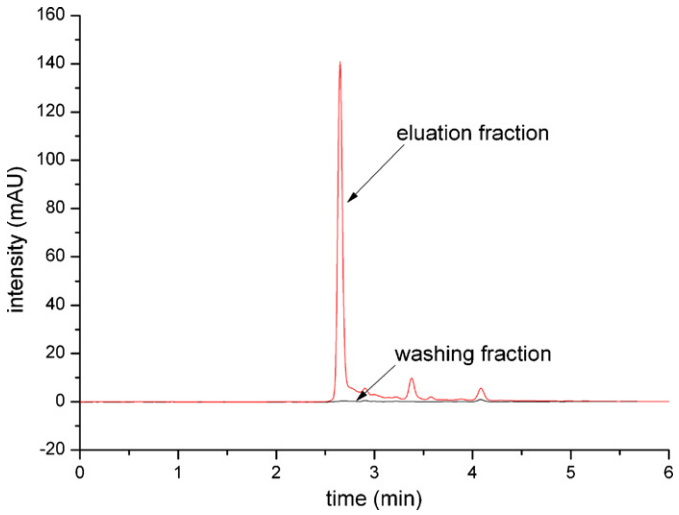

Fig. 5. Comparison of UV spectra for the washing fraction and elution fraction from the MIPs SPE cartridge (volume and loading concentration: 50 ml × 200 μg/l in ACN–water (1:9, v/v)).

3.4. SPE application of methyl-testosterone imprinted polymers in real samples

The aim of this study was to test the synthesized polymers with SPE experiments using the real samples. In the present work, MIP2 packed column and a C18 SPE column were employed for preliminary extraction of the analyte from tap water and lake water samples, which were spiked with methyl-testosterone at the concentration of 200 μg/l. Taking into consideration the low solubility of methyl-testosterone in water and easy adsorption on the wall of container, all the water samples were spiked with 10% (v/v) ACN. After the loaded columns were washed with 5 ml of methanol–water (1:1, v/v), most of the water-soluble interfering substances could be removed, methyl-testosterone was not detectable in the washing solutions of all the columns when perco-

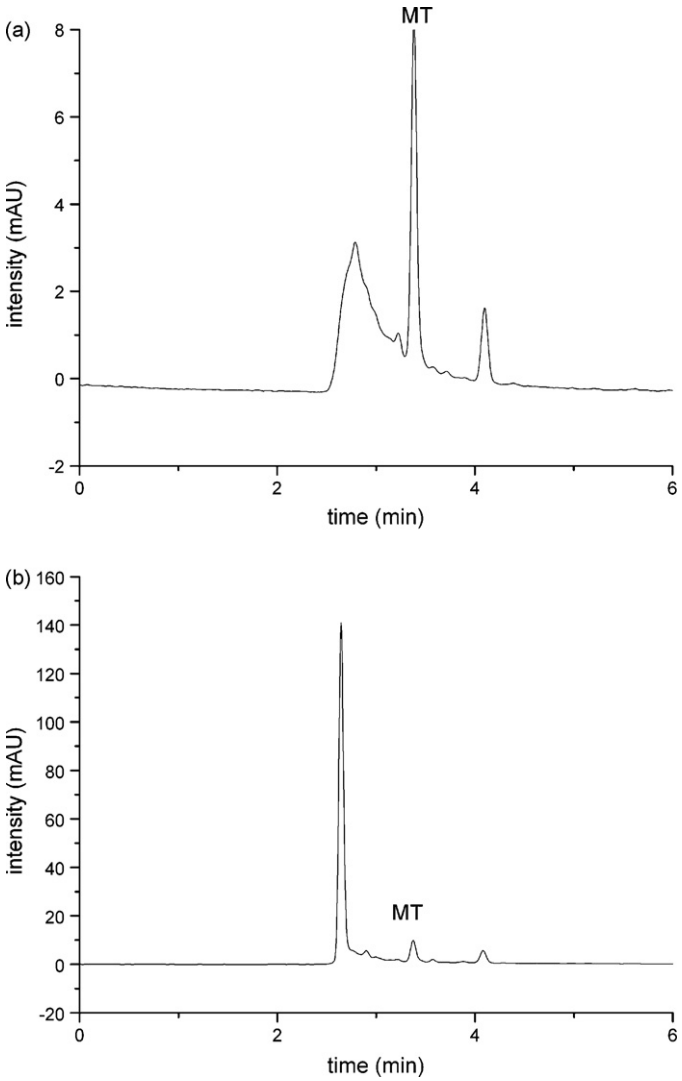

Fig. 6. Comparison of chromatograms of eluates obtained with C18 column (a) and MIPs column (b).

lating 50 ml of spiked sample. Then the columns were eluted with 10 ml methanol-acetic acid (7:3, v/v) for three times. The obtained extract was evaporated to dryness with rotary evaporator, reconstituted in acetonitrile to a final volume of 1.0 ml. The analytes in the washing solution and the eluate were analyzed using normal HPLC method with C18 column and DAD detector. The typical UV spectra of the washing fraction and elution fraction from MIPs SPE cartridge after loading 50 ml spiked lake water sample were shown in Fig. 5. It could be seen that no methyl-testosterone could be detected in the washing fraction, meanwhile the UV absorbance in the eluate was accordingly increased.

Fig. 6 shows the chromatograms of eluates obtained with MIPs column and C18 column, respectively. From Fig. 6, quite different phenomena were observed when using the different SPE columns. More water-soluble compounds (the first peak) were nonspecifically enriched on the MIPs, which should have been caused by the higher polarity of MAA polymer compared to C18 column. However, the recovery on the MIPs column was evidently higher ($84.7 \pm 4.8\%$, $n = 3$) than that on the C18 column ($67.8 \pm 5.6\%$, $n = 3$) for the lake water. On the other hand, the results showed that on the MIPs packed column, the recovery of tap water was $96.5 \pm 3.2\%$ ($n = 3$) while it was some lower for lake water ($84.7 \pm 6.6\%$, $n = 3$). Probably, the loss of analyte was ascribed to the relatively complex matrix of the lake water and its adsorption onto the colloidal fraction. MISPE was much easier to perform compared with the traditional extraction procedure and showed the satisfactory recoveries. In addition, the MIP reusability without any deterioration in performance was demonstrated for at least five repeated cycles.

4. Conclusions

Molecularly imprinted polymers (MIPs) using methyl-testosterone as the template, methacrylic acid (MAA) as the monomer and ethylene glycol dimethacrylate (EDMA) as the crosslinker were prepared by precipitation polymerization. The results obtained using scanning electron microscopy (SEM) and BET indicated that more porogen favored the formation of the particles with smaller pore diameter and larger specific surface area, meanwhile, these parameters of the methyl-testosterone imprinted polymers were larger than the corresponding blank polymers under the same condition. The morphological difference between the MIPs and the BP resulted in the different behavior in the equilibrium adsorption experiments. The subsequent HPLC–MS–MS results indicated that methyl-testosterone imprinted polymer can rebind the template selectively compared with other structural analogues. Besides, the prepared MIPs could be successfully applied to the enrichment of methyl-testosterone in the real samples.

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