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Selective solid-phase extraction of cholesterol using molecularly imprinted polymers and its application in different biological samples

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Abstract

Non-covalent molecularly imprinted polymers (MIPs) of cholesterol were prepared by UV initiated polymerization. A polymer that had the highest binding selectivity and capability was used as solid-phase extraction (SPE) sorbents for direct extraction of cholesterol from different biological samples (human serum, cow milk, yolk, shrimp, pork and beef). The extraction conditions of molecularly imprinted SPE (MISPE) were optimized and the optimum protocol was: conditioning MISPE cartridges with *n*-hexane, loading with *n*-hexane, washing with *n*-hexane and *n*-hexane:toluene = 9:1, respectively, then eluting with chloroform:ethanol:acetic acid = 3:1:1. Cholesterol MISPE selectively recognized, effectively trapped and pre-concentrated cholesterol over a concentration range of 10–80 μg/mL. Recoveries ranged from 80.6% to 92.7%, with R.S.D. lower than 9.8%. Under the optimal condition, MISPE recoveries of spiked human serum, yolk, cow milk, shrimp, pork and beef were 91.1%, 80.4%, 86.6%, 78.2%, 81.4% and 80.1%, respectively. Compared with C18 SPE, almost all of the matrix interferences were removed after MISPE, and better baselines and higher selectivity were achieved.

Keywords: Molecularly imprinted polymers; Solid-phase extraction; Cholesterol; Biological samples

1. Introduction

Cholesterol is a steroid that plays important roles in developing cardiovascular diseases. Correctly quantified cholesterol concentration in biological and food samples is in great need. Presently, chromatography (either gas chromatography or high pressure liquid chromatography) is standard cholesterol detection method [1]. But as complexity matrix, sample pretreatment for biological samples is necessary. Steroid analogs and cholesterol oxidation products are normal interferences in chromatography analysis. Solid-phase extraction (SPE) has become a routine sample preparation technique and is also used in cholesterol analysis [1,2]. SPE is high load, high recovery, enhanced reproducibility, automation capability, and wide spectrum of stationary phases available. But conventional SPE is low selectivity. As the typically used sorbents (alkylsilicas, styrene-divinylbenzene, graphitised carbon black, etc.) are not selective, a large amount of matrix interferences is extracted simultaneously with the target analyte. This decreases the SPE separation and enrichment efficiency [3,4].

High selective molecular recognition-based separation methods such as immunosorbents (IS) would be a good solution to this problem. IS immobilizes antibody of target analyte on an adequate solid support. By using the special molecular recognition between antibody and antigen, the extracts obtained from IS were almost completely free of co-extractives. IS have been successfully used to separate a great variety of analytes in different samples [5–7]. Unfortunately, antibody isolation is expensive, time-consuming, and easily denatured in the presence of organic solvents. These drawbacks limited the usage of IS on sample pretreatment.

Molecularly imprinted polymers (MIPs) are synthetic cross-linked polymers formed by the presence of a target molecule (template). The template is then removed by washing, which produce a cavity with molecular recognition sites that can bind selectivity to the original template [8–30]. MIPs are highly selective to capture the target analyte as the antibody. But as artificial polymers, MIPs are easy and rapid to prepare, very stable in

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harsh conditions (organic solvents, strong acids, etc.), and allow the usage of a great variety of binding/eluting conditions without the risk of losing binding activity. MIPs have been used in SPE that is known as molecularly imprinted solid-phase extractions (MISPE). MISPE has been successfully used in determination of many analytes in different biological samples such as beverages, animal feeds, serum, urine [11–23].

By far, binding characters of different cholesterol imprinted MIPs have been studied widely [24–29]. Selectivity of different cholesterol imprinted MIPs show that these MIPs can recognize and bind cholesterol more specifically than cholesterol oxidation products [24] and other steroid analogs [25–29]. MIPs are also used for removing of cholesterol from an intestinal mimicking medium [30]. These selective recognition and binding properties of cholesterol imprinted MIPs offer the possibility for their usage in sample pretreatment. In this report, cholesterol imprinted polymers were used in MISPE. Under the optimal conditioning, loading, washing and eluting protocols, MISPE was successfully applied to the extraction of cholesterol from different biological and food samples (human serum, cow milk, yolk, shrimp, pork and beef) for the first time.

2. Experiment

2.1. Chemicals and reagents

Cholesterol, methacrylic acid (MAA), and ethylene glycol dimethacrylate (EDMA), BSFTA (derivatization grade) were from Sigma (St. Louis, MO). Azobisisobutyronitrile (AIBN), HPLC grade organic solvents (chloroform, toluene, methanol, ethanol, acetic acid, *n*-hexane and acetonitrile) were from Tianjin chemical reagent company, China. MAA and EDGMA were purified prior to use via general distillation methods in vacuo under argon protection to remove the polymerization inhibitor. AIBN was recrystallised from methanol and then dried at room temperature in vacuum prior to use. Toluene and chloroform was distilled before use. The standard cholesterol serum was from total cholesterol kit (Zhongsheng Beikong Biotech Company, China) with cholesterol concentration of 4.99 mmol/L (1.93 mg/mL).

2.2. Apparatus and analytical conditions

Agilient model HP6890 gas chromatography with a flame ionization detector (FID) was used. The temperature of the injection port was 260 °C and that of the detector was 280 °C. Separation were carried out on a HP-5 column (30 cm × 0.32 mm × 0.25 μm). The separation condition was optimized with respecting to the column temperature. The final optimum column temperature was held at 240 °C for 10 min, raised at a rate of 5 °C/min to 280 °C and held for 10 min. N_2 was used as the carrier gas at an average linear velocity of 1.1 mL/min, the split ratio was 2:1.

All samples were evaporated to dryness at 45 °C under a stream of N_2. The residues were dissolved in pyridine, trimethylsilylated with BSTFA and analyzed by GC. Derivatization of estradiol and estriol was carried out with BSTFA at 80 °C for 1 h, while derivatization of cholesterol was carried out with BSTFA at 25 °C for 15 min. The linearity range of detection was 10–200 μmol, $R^2 = 0.9976$, limit of quantitation (LOQ) was 0.5 μmol, related standard deviation (R.S.D.) was 1.9% intra-day and 3.4% inter-day.

2.3. Preparation of MIPs

Table 1 showed the volume of porogen used for the preparation of MIPs and NIPs in this study. For a general polymerizing procedure, 1 mmol template cholesterol, monomer MAA (6 mmol), cross-linker EGDMA (35 mmol) and free-radical initiator AIBN (30 mg) were dissolved in different volume of porogen (chloroform:toluene = 1:7, v:v). The solution was degassed in an ultrasonic bath for 5 min then sparged with oxygen-free nitrogen for 10 min. Polymerization was occurred by keeping in 4 °C for 24 h under a UV lamp at 365 ± 5 nm. After polymerization, the polymers were ground. A 30–60 μm size fractions of the particles were collected. Removal of the imprinted cholesterol from the MIPs particles was accomplished through sonication in washing reagents (chloroform:acetic acid = 4:1, v:v) for 10 h while changing the washing reagents every 2 h, followed by a Soxhlet extraction with acetonitrile for 16 h. Non-imprinted polymers (NIPs) were synthesized and treated simultaneously under the same conditions without adding a template.

2.4. Binding capability of MIPs

A 2, 0–0.5 mmol/L cholesterol toluene solution was added to 20 mg MIPs. The samples were shaken at 25 °C for 24 h. Cholesterol concentration on the supernatant (free cholesterol) was analyzed by GC. The amount of cholesterol bound to the imprinted particles was calculated by subtracting the free

Table 1
Preparation conditions and binding characters of MIPs and NIPs

Polymer	Porogen (ml)	Cholesterol (mmol)	Equation	K_d ($\times 10^{-5}$ mol/L)	B_{max} ($\times 10^{-2}$ mmol/g)
MIP1	4	1	$B/F = -57.752 \times 10^5 B + 0.0223$	1.37	0.378
MIP2	6	1	$B/F = -45.399 \times 10^5 B + 0.017$	2.45	0.377
MIP3	8	1	$B/F = -46.674 \times 10^5 B + 0.0213$	1.51	0.446
MIP4	12	1	$B/F = -37.778 \times 10^5 B + 0.0116$	2.11	0.305
NIP1	4	0	$B/F = -62.104 \times 10^5 B + 0.0113$	1.88	0.186
NIP2	6	0	$B/F = -50.275 \times 10^5 B + 0.0091$	1.92	0.179
NIP3	8	0	$B/F = -56.477 \times 10^5 B + 0.0105$	1.86	0.19
NIP4	12	0	$B/F = -68.171 \times 10^5 B + 0.0117$	1.89	0.18

amount of cholesterol from its initially added amount of cholesterol. Scatchard plot was constructed by plotting the ratios of bound to free cholesterol concentration against the bound concentration. The dissociation constant and the maximum absorption capability were determined from the equation, $(B/[\text{free}]) = -(B/K_d) + (B_{max}/K_d)$, where K_d is the equilibrium dissociation constant, B the concentration of bound cholesterol, [free] the concentration of free cholesterol, and B_{max} is the maximum absorption capability [8]. The dissociation constant (K_d) was figured out from Scatchard plot.

2.5. Selectivity of MIPs

A range of structural analogues of cholesterol (2 mL cholesterol, estradiol and estriol solution, 50 μmol/L in toluene) were added to 20 mg MIPs separately [8]. The samples were shaken at 25 °C for 24 h. Concentration of cholesterol, estradiol and estriol in the supernatant was analyzed by GC.

IPB (imprinting-induced promotion of binding, $IPB = (C_{mip} - C_{nmip})/C_{nmip}$) was used to demonstrate the specificity of the cholesterol MIPs due to the molecular imprinted effect. C_{mip} was the amount of the analyte that was bound to MIP, and C_{nmip} was the corresponding value for the non-imprinted NIP [26]

2.6. MISPE

2.6.1. Saponification

Saponification was carried out to remove fat and extract non-saponifed chemicals (including cholesterol) from samples.

Human serum: human serum samples from anonymous patients were obtained from TONGJI Hospital, Wuhan, China. To 25 μL serum, 500 μL ethanol and 60 μL 8.9 mol/L potassium hydroxide solution were added. The mixture was vortex mixed and kept in 50 °C for 1 h. A 0.5 mL water and 1 mL *n*-hexane were added to the saponified supernatant. The mixture was vortex mixed, and 1 mL organic supernatant was used for MISPE.

Cow milk: To 10 mL milk, 10 mL 200 g/L potassium hydroxide ethanol solution was added. The mixture was vortex mixed and kept in 80 °C for 30 min. Then 5 mL water and 25 mL *n*-hexane were added to the saponified supernatant. The mixture was vortex mixed, and then 1 mL organic supernatant was applied to MISPE.

Yolk, shrimp, pork and beef: 10 mL water was added to 1.5 g yolk, 5 g shrimp, 10 g pork and 10 g beef to form the original samples. To 10 mL original samples, 30 mL 95% ethanol and 20 mL 50% potassium hydroxide solution were added. The mixture was vortex mixed and kept in 60 °C for 1 h. Six microlitres water and 10 mL *n*-hexane were added to 12 mL saponified supernatant. The mixture was vortex, and 1 mL organic supernatant was used for MISPE.

2.6.2. Optimization of MISPE with standard solutions

Empty SPE cartridges (3 mL) were packed with 100 mg MIPs or NIPs. Before each use, the sorbents were conditioned first. Extraction experiments consisted of loading the MISPE column with 1 mL 50 μmol/L cholesterol or 1 mL spiked (50 μg/mL) saponified solution of biological samples. The extraction protocols were optimized and the optimum condition was: conditioning MISPE cartridges with 5 mL *n*-hexane, loading with 1 mL *n*-hexane, washing the cartridges with 1 mL *n*-hexane and 1 mL *n*-hexane:toluene = 9:1, respectively, then eluting with 3 mL chloroform:ethanol:acetic acid = 3:1:1. All the applied fractions were collected and detected by GC. Each sample was assayed (MISPE and GC) three consecutive times.

Fifty micrograms per millilitre was chosen as spiked concentration because it was close to the cholesterol concentration in 1 mL original saponified samples (from 39.2 to 79.6 μg/mL).

2.6.3. SPE procedure on C18

One microlitre spiked saponified yolk samples were also applied to commercial C18 columns (Supelclean LC-18, Supelco). C18 SPE extraction conditions were optimized by modifying Johnson's protocol [2]. The optimum condition was: conditioning the column by 5 mL water and 5 mL methanol, respectively, loading with *n*-hexane, washing with 1 mL *n*-hexane, and eluting with 3 mL methnol:H_2O = 80:20 (v:v).

3. Results and discussion

3.1. Preparation and evaluation of NIPs and MIPs

Binding characters of different NIPs and MIPs (Table 1, Fig. 2) showed that MIP3 had the highest binding capability. It was clear that 8 mL porogen (0.6 times of total reaction volume) seemed to be the best porogen volume. Less or more porogen would get lower binding capability. These may because that polymer synthesized in small porogen volume would have less special cavities, denser polymer structure and higher rigidity, which would decrease binding capability. On the other sides, polymers synthesized in larger porogen volume would have larger cavities but less rigidity. After the template was removed, more substantial number of the cavities might shrink, which decreased polymer's binding capability [7]. At the same time, similar binding characters were observed among NIPs that prepared using different porogen volume.

High ligand selectivity and affinity are characteristics of MIPs. The IBP of estradiol, estriol and cholesterol (Fig. 1(a)) to MIP1, MIP2, MIP3, MIP4 showed that the MIPs had medium cross-reactivity with estradiol (from 29.4% to 41.7%) and estriol (from 26.9% to 47.6%), but the highest binding selectivity was toward cholesterol (Table 2). Cholesterol imprinted MIPs bond cholesterol more selectively than steroid analogs. These results were similar with previously published reports [26–27].

Table 2
IPB (%) of various compounds on MIPs and NIPs

Solution	MIP1	MIP2	MIP3	MIP4
Cholesterol	178	162.5	204.8	117.2
Estradiol	33.7	41.7	29.4	33.5
Estriol	46.8	47.6	33.9	26.9

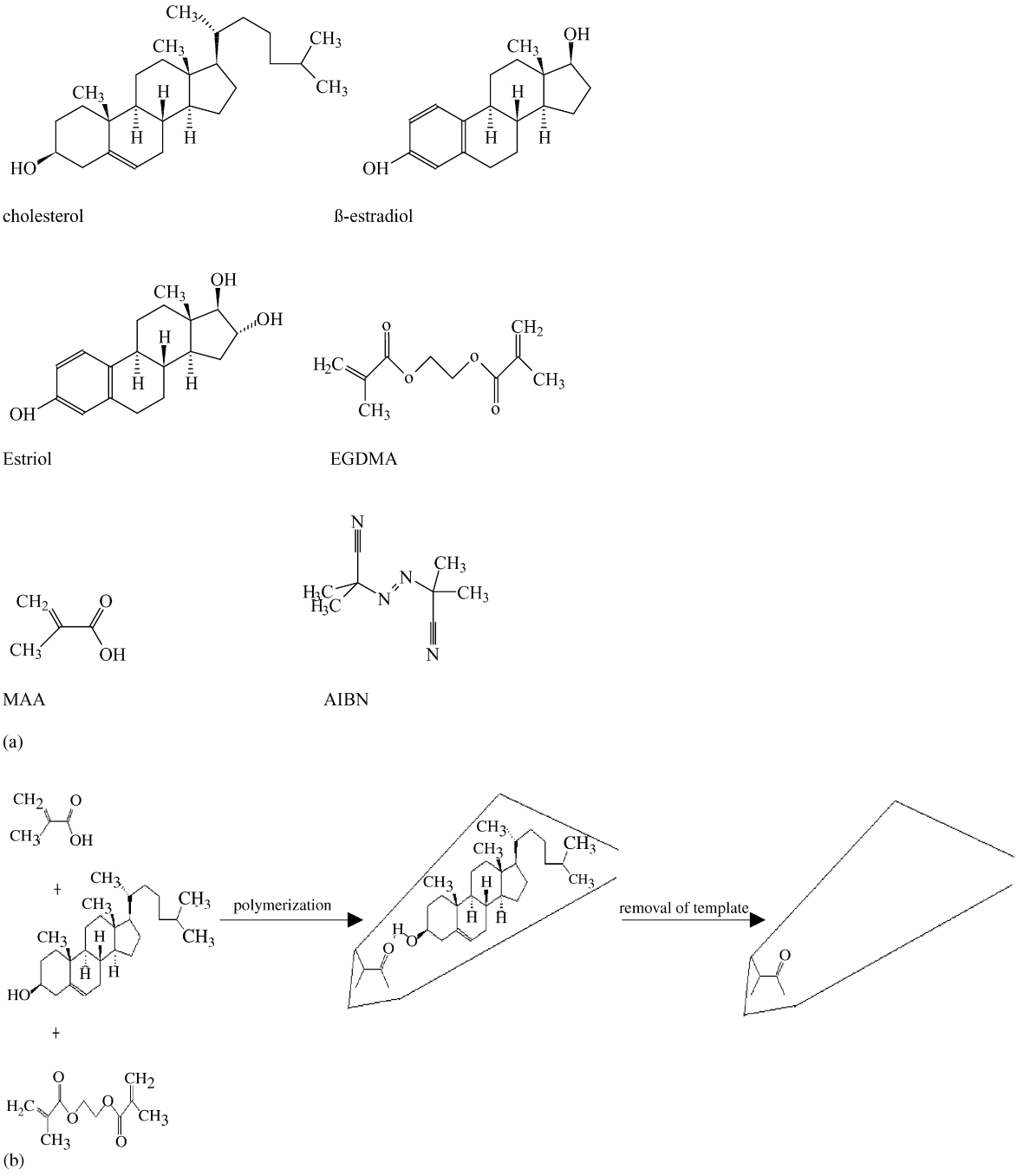

Fig. 1. (a) The structure of chemicals used in this research. (b) Scheme of non-covalent cholesterol polymerization using MAA as monomer.

3.2. Optimization of MISPE protocols

Fifty micromoles per litre cholesterol standard solutions were applied to MIPs and NIPs in order to find the optimized MISPE protocol. As the binding characters of all NIPs were similar (Table 1, Fig. 2), and 8 mL porogen was the best porogen volume. Only NIP3 was used. Three different polarity solvents (toluene, *n*-hexane and ethanol) were utilized in the loading step in order to find the best loading solvent. All loaded cholesterol was retained when using *n*-hexane loading. A large amount of cholesterol was not retained by either the MIPs (45.6% for MIP1, 53.9% for MIP2, 64.4% for MIP3, 49.2% for MIP4) or the NIP (41.8% for NIP3) when using toluene loading. Less than 10% loaded cholesterol retained in all polymers when using ethanol loading. Even the loaded cholesterol concentration was reduced to 26 μmol/L, the recoveries of loading step did not lower when toluene or ethanol was used as loading solvent. Therefore, *n*-hexane was selected as loading solvent for further investigations.

Optimization of the washing procedure is critical in MISPE. The selectivity of MISPE is generally obtained by the introduction of a selective washing procedure in order to

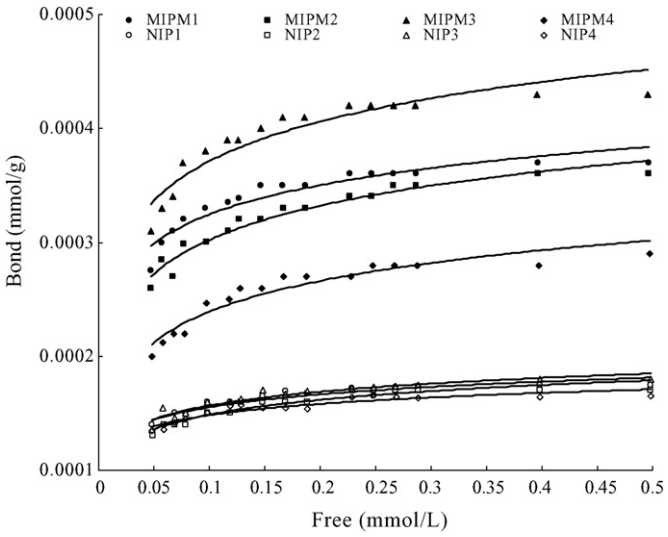

Fig. 2. Binding isotherm of MIPs and NIPs.

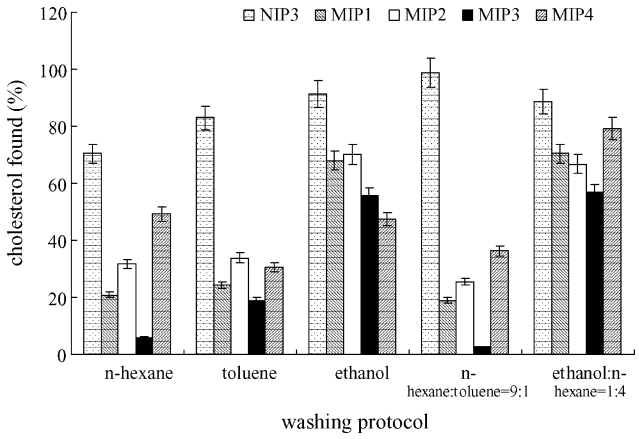

Fig. 3. Recoveries of cholesterol on MIPs cartridges using different washing procedure.

remove compounds retained only by non-specific interactions (Fig. 1(b)). Normally speaking, MIPs exhibit better molecular recognition in solvents used as porogen in the polymerization process [14–24]. Therefore, mixed solutions of different ratio of *n*-hexane, toluene and ethanol were performed in order to find the most appropriate washing ratio (Fig. 3). It was clear that both *n*-hexane and toluene:*n*-hexane = 1:9 can effectively disrupt the non-specific binding. Therefore, 1 mL *n*-hexane followed by 1 mL toluene:*n*-hexane = 1:9 were used as the washing solvents.

Finally, chloroform:ethanol:acetic acid = 3:1:1, methanol: acetic acid = 7:1, H_2O:acetonitrile = 1:19 and *n*-hexane: ethanol = 4:1 were applied as the eluting solvents (Fig. 4). Chloroform:ethanol:acetic acid = 3:1:1 got the highest eluting effect. Therefore, chloroform:ethanol:acetic acid = 3:1:1 was used as eluting solvent.

3.3. Specificity of MISPE

Fifty micromoles per litre cholesterol and 50 μmol/L estradiol *n*-hexane solution were extracted on MIPs and NIPs using the optimal MISPE protocol (Table 3). The recoveries of cholesterol on MIPs cartridges (from 71.2% to 101%) were much higher than that of estradiol (from 34.2% to 58.9%) and NIP3 (from 0.95% to 14.5%). All these indicated that cholesterol imprinted polymers can selectivity separate and enrich cholesterol from its analogues in MISPE.

As MIP3 exhibited the highest binding capability (Table 1) and binding selectivity (Table 2), the highest recovery of cholesterol and a medium binding to analogue estradiol (Table 3), it was chosen as MISPE sorbent for biological samples.

Under the optimal MISPE conditions, different concentrations of cholesterol standard solutions were loaded to MIP3 column. Table 4 showed that recoveries ranged from 80.6% to 92.7% for 10–80 μmol/L concentrations of cholesterol, with

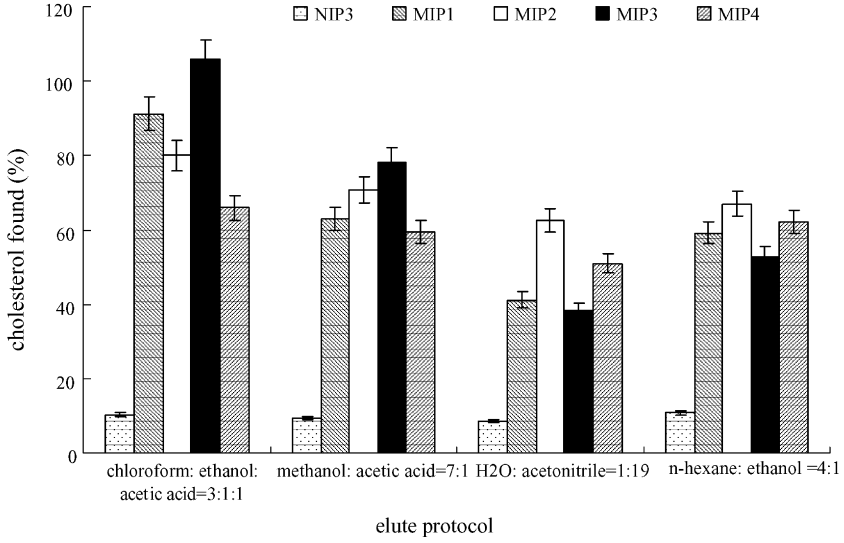

Fig. 4. Recoveries of cholesterol on MIPs cartridges using different eluting procedure.

Table 3
Recovery of cholesterol and estrodiol using optical MISPE protocol on different MIPs column ($n = 3$)

Fraction	Recovery (%)									
	NIP3		MIP1		MIP2		MIP3		MIP4	
	Cholesterol	Estradiol	Cholesterol	Estradiol	Cholesterol	Estradiol	Cholesterol	Estradiol	Cholesterol	Estradiol
Load, 1 mL, hexane	n.d.[a]	0.4 ± 0.7	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]	1.9 ± 1.2
Washing 1, 1 mL, hexane	19.8 ± 2.4	28.25 ± 5.94	n.d.[a]	12.2 ± 7.1	n.d.[a]	14.8 ± 3.5	n.d.[a]	16.7 ± 6.8	12.8 ± 4.2	19.6 ± 2.9
Washing 2, 1 mL, hexane:toluene = 9:1	67.9 ± 6.3	68.5 ± 4.5	12.6 ± 4.1	27.4 ± 5.2	15.7 ± 5.4	31.3 ± 5.7	8.7 ± 1.9	29.6 ± 4.7	21.6 ± 4.9	39.4 ± 3.4
Elute, 3 mL, methanol	16.5 ± 2.2	0.95 ± 1.3	87.1 ± 6.4	57.6 ± 3.8	78.4 ± 7.2	58.9 ± 4.9	101 ± 7.6	46.4 ± 5.8	71.2 ± 7.1	34.2 ± 6.2
Total	104.2 ± 3.9	98.1 ± 3.2	99.7 ± 4.5	97.2 ± 6.2	94.1 ± 5.8	105 ± 5.0	109.7 ± 4.9	92.6 ± 5.4	105.6 ± 5.2	95.1 ± 4.4

[a] Not detected triplicate experiments were performed for each polymer.

Table 4
Repeatability and recovery of MISPE on cholesterol using MIP3 column

Background concentration (μg/mL)	Spiked concentration (μg/mL)	Repeatability (R.S.D.%, $n = 5$)		Recovery[a] (%, $n = 5$)
		Intra-day	Inter-day	
0	10	4.3	9.8	80.6
	20	5.4	7.9	86.8
	40	4.7	7.3	90.2
	80	5.2	6.9	92.7

[a] Recovery = (measured spiked sample concentration − measured blank sample concentration)/initial spiked sample concentration × 100%.

R.S.D. lower than 9.8%. MISPE assay was quite reproducible and accurate.

3.4. Selective extraction of cholesterol from complex matrices

In order to investigate the potential of MIPs for the selective entrapment of target analyte from complex matrices, spiked (50 μg/mL) human serum, cow milk, yolk, shrimp, pork, beef samples were applied to MIP3 using the optimum MISPE protocol. Satisfactory sample clean-up was achieved by the MISPE extraction (Table 5). The average recoveries were reproducible and in agreement with the recoveries of standard solutions. These showed the high affinity and high binding capability of MIP3 for cholesterol purification and enrichment, and high agreement and repeatable of MISPE on tested biological samples.

In order to evaluate the enriching capability of MISPE, 10 mL diluted spiked (5 μg/mL) serum samples were applied to MISPE using the optimum protocol. Cholesterol recoveries were a little bit difference in two concentration serum samples (91.1% for undiluted and 88.9% for diluted samples). This suggested that acceptable cholesterol recoveries could be maintained despite the usage of a 10 times diluted sample extraction. This feature may have important implication for trace analytes in biological and environmental samples which normally require the processing of large sample volumes.

Chromatogram of yolk sample after saponification (Fig. 5(a)), saponification followed by C18 SPE (Fig. 5(b)) and saponification followed by MISPE (MIP3) (Fig. 5(c)) showed that almost all the entire matrix interferences were removed after MISPE, and the yolk extracts following MISPE had better baselines, better recovery and higher selectivity than that obtained after C18 SPE. It confirmed that satisfactory sample clean-up was achieved by the MISPE.

One of the major advantages of MIPs is their high chemical robustness, providing the opportunity to reactivate under relatively harsh conditions for multiple usages. The recoveries of MISPE diminished if three consecutive extractions were per-

Table 5
Recovery of cholesterol in different biological matrix after extracted with MIP3 ($n = 5$)

Matrix	Cholesterol concentration (μg/mL saponified sample)	Added (μg)	Recovery[a] (%)	R.S.D. (%)
Cholesterol standard serum	48.3	50	92.7	6.9
Human serum	52.4	50	91.1	7.6
Yolk	79.6	50	80.4	9.4
Milk	39.2	50	86.6	6.1
Shrimp	56.4	50	78.2	5.1
Pork	55	50	81.4	4.7
Beef	45.9	50	80.1	4.2

[a] Recovery = (measured spiked sample concentration − measured blank sample concentration)/initial spiked sample concentration × 100%.

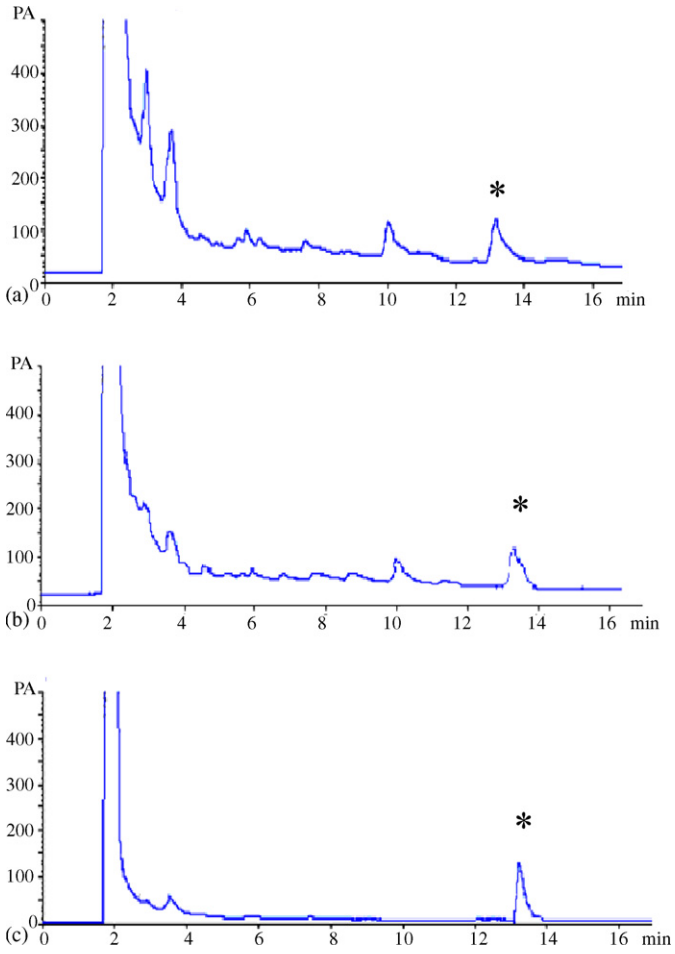

Fig. 5. (a) gas chromatography of yolk sample after saponification; (b) yolk samples after saponification followed by C18 SPE; (c) yolk samples after saponification followed by after MISPE. Spiked concentration: 50 μg/mL.

formed in the same cartridge. In such a case, washing the MIPs cartridge with an excess 5 mL eluting solvent followed by 20 mL conditioning solvent before another loading sufficed for MIPs regeneration. The affinity binding capability regained without largely affected.

The ability of MIP3 to discriminate between the cholesterol and high amounts of interference in complex biological samples confirmed the suitability of MIP3 for a wide range of applications in the biological pretreatment. It is important to stress that all of the samples in this work were analyzed using the same cartridge in 3 weeks and no losses were detected.

4. Conclusion

In this study, a UV initiated imprinted polymer (MIP3) using cholesterol as template was used as sorbents for MISPE on several biological samples. The results indicated that the MIPs exhibited high binding capability and selectivity, and higher recoveries in MISPE when complex biological saponified samples applied directly. Optimum loading, washing and eluting protocols were critical for the best MISPE procedure. With an optimized protocol, a high selectivity can be obtained from all the tested biological samples, and better recoveries than C18 SPE can be presented. MISPE had good precision and accuracy. The high extraction efficiency of MISPE from different complex matrices suggested that it was a practicable solution for sample preparation in routine analysis of cholesterol in biological samples.

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