

## Separation of Cholesterol from Other Steroids Using Molecularly Imprinted Polymer Prepared by Seeded Suspension Polymerization

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Micron-sized particles of cholesterol-imprinted polymers were synthesized by seeded suspension polymerization in a mixture of 2-propanol and water using polystyrene microbeads as the seeds. Methacrylic acid was employed as the functional monomer to form complexes with template (cholesterol), along with ethylene glycol dimethacrylate as the crosslinker. After removal of template molecules, the columns ( $H = 15$  cm,  $D_i = 0.46$  cm) packed with cholesterol-imprinted polymers were effective for the chromatographic separation of cholesterol from other steroids. When the sample of steroids was eluted isocratically at a flow-rate of  $Q = 0.5$  mL min<sup>-1</sup>, using a mixture of acetonitrile and water ( $\Psi = 95:5$ ) as the mobile phase, the retention times for estrone,  $\beta$ -estradiol and cholesterol were respectively  $\tau = 5.3$ , 12.3 and 17.2 min. The average retention times were  $\bar{\tau} = 5.3$ , 10.9 and 16.7 min respectively for estrone, progesterone and cholesterol in samples. The separation was based on the specific binding of cholesterol to recognition sites formed on the imprinted polymers. A separation factor of 1.6 for cholesterol and  $\beta$ -estradiol was obtained. The chromatographic efficiency was dependent on the mobile phase composition. Reducing the water content in the non-polar mobile phase to zero could significantly enhance the separation. Compared with particles from bulk polymerization, the column packed with cholesterol-imprinted particles from seeded suspension polymerization had a higher chromatographic efficiency and the advantage of micro-analysis.

### Key words:

Molecular imprinting, seeded suspension polymerization, cholesterol, molecularly imprinted polymer

## Introduction

Molecularly imprinted polymers (MIPs) have gained great interest in the past decade and their widespread applications have become important, as shown in recent reviews.<sup>1–4</sup> Both covalent and non-covalent methods have been used for preparing the MIPs. The covalent imprinting involves the use of covalently template-bound monomer, which becomes fixed in their spatial arrangement by co-polymerization with crosslinking monomer. In non-covalent imprinting, molecules of functional monomer assemble around the template by non-covalent interactions to form the recognition site after co-polymerization with crosslinker. Most of the MIPs are prepared by the bulk polymerization with either covalent or non-covalent method. In comparison with bulk polymerization for synthesizing porous molecularly imprinted polymer (MIP) that

needs to be ground before use, the use of a two-phase system like suspension polymerization can yield MIP particles in one step. This paper describes the synthesis of cholesterol-imprinted polymers by a seeded suspension polymerization in a mixture of 2-propanol and water using polystyrene particles as the seed particles.

Cholesterol was first printed in a bulk polymer by Whitcombe and co-workers employing the covalent imprinting method.<sup>5</sup> They synthesized 4-vinylphenyl carbonate ester as the covalently template-bound monomer, which is efficiently cleaved hydrolytically, with the loss of carbon dioxide, resulting in a recognition site that interacts with the print molecule through hydrogen bonding. Non-covalent imprinting of cholesterol was also promising in the polymer of acrylic acid, 2-hydroxyethylmethacrylate, or  $\beta$ -cyclodextrin, or even copolymers of these compounds.<sup>6–8</sup> Most of cholesterol-imprinted polymers prepared by the non-covalent method involved the polymerization initiated

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with gamma rays.<sup>9–11</sup> These cholesterol-imprinted polymers were conventional “bulk” polymers prepared in the presence of a porogenic solvent by either covalent or non-covalent method. Methods other than bulk polymerization have been used, e.g., preparing cholesterol-imprinted polymers by aqueous suspension polymerization was investigated by the preparation of ethylene glycol dimethacrylate and divinylbenzene-based beads imprinted using cholesteryl(4-vinyl)-phenyl carbonate as the template.<sup>12–13</sup> Submicron core-shell polymer particles, with molecularly imprinted shells, were prepared by a two-stage polymerization process.<sup>14–15</sup> Others used steroids as template to prepared MIPs.<sup>16–18</sup> In this study, molecularly imprinted polymer particles were prepared so that they could be ready for use as chromatographic support.

## Materials and methods

### Materials

All chemicals were of analytical reagent grade. Methacrylic acid (99 %) and ethylene glycol dimethacrylate (EGDMA, 98 %) were obtained from Merck (Darmstadt, Germany). Styrene (99 %), sodium dodecyl sulfate (SDS), and NaNO<sub>2</sub> were purchased from Riedel-de Haen (Germany). Polyvinylpyrrolidone (PVP, K-30), 2,2'-Azobisisobutyronitrile (AIBN) and 2-propanol were from TCI (Tokyo, Japan). Methanol, alcohol, acetone and acetonitrile were of HPLC grade and obtained from TEDIA (Fairfield, OH, USA). Cholesterol (95 %), progesterone (98 %), estrone (99 %) and  $\beta$ -estradiol (98 %) were obtained from Sigma (St. Louis, MO, USA). Water was double de-ionized throughout the Milli-Q system (Millipore, Bedford, MA, USA).

### Preparation of MIP particles

Polystyrene seeds were prepared by the method of dispersion polymerization in ethanol with 2,2'-azobisisobutyronitrile (AIBN) and polyvinylpyrrolidone (PVP) as the initiator and stabilizer, respectively. The monomer solution was prepared by mixing 10 mL of styrene and  $m = 1.3$  g PVP with 80 mL of ethanol in a flask. AIBN ( $m = 0.091$  g) was dissolved in 80 mL of ethanol and mixed with the monomer solution. After the nitrogen purge, free radical polymerization was carried out under stirring (130 rpm) at  $\theta = 60$  °C for  $t = 24$  h. The resultant PS beads were washed with methanol and distilled water, and then dried in a vacuum oven at room temperature.

The cholesterol-imprinted polymer was prepared by the method of seeded suspension polymerization. Into 100 mL of 2-propanol and H<sub>2</sub>O ( $\Psi =$

3:1) mixture, 1g polystyrene seeds,  $m = 0.5$  g PVP, 0.25 g SDS, and 0.01 g NaNO<sub>2</sub>, were dispersed using ultrasonication. This mixture was shaken (140 rpm) for 24 h at room temperature and then mixed with a solution containing  $m = 0.387$  g cholesterol,  $V = 0.682$  mL methacrylic acid, 4.72 mL ethylene glycol dimethacrylate and 50 mg AIBN that was prepared in a 50 mL flask under ultrasonication and purged with nitrogen. The resultant two-phase suspension mixture was allowed polymerization under stirring (130 rpm) at  $\theta = 4$  °C coupling with UV-radiation for  $t = 24$  h. The UV lamp had a power of 100 W and a wavelength of  $\lambda = 365$  nm. The UV-initiated polymerization at low temperature could be replaced by a thermal-initiated method, i.e., by just elevating the temperature to  $\theta = 60$  °C for polymerization. The particle diameter and size distribution were determined by scanning electron microscopy (SEM) using a scanning electron microscope (Hitachi S-4800).

For the sake of comparison, cholesterol-imprinted polymer particles were also prepared in a single step by the method of bulk polymerization at low temperature. Briefly, cholesterol ( $m = 0.387$  g), methacrylic acid ( $V = 0.682$  mL), EGDMA ( $V = 4.72$  mL), and AIBN ( $m = 0.05$  g) were dissolved in  $V = 7.5$  mL of chloroform in a conical Erlenmeyer flask. After degassing and nitrogen purging, the flask was sealed and allowed to polymerize at  $\theta = 4$  °C for  $t = 6$  h under UV ( $\lambda = 365$  nm, 100 W lamp) irradiation. The resultant MIP polymer was ground and sieved. Only the fraction of powders having a particle diameter ranging from  $d_p = 25$  to 44  $\mu$ m was collected for packing a chromatographic column. Referenced non-imprinted polymers were prepared with the same procedures for seeded suspension polymerization and bulk polymerization methods except no print molecule was present. Those non-imprinted polymers were also packed into columns for the blank tests.

### Chromatography of cholesterol and other steroids

The resultant MIP particles from seeded suspension polymerization were washed with methanol and distilled water, and then dried in a vacuum oven at room temperature. The MIP particles were then directly packed into  $H = 15$  cm,  $D_i = 0.46$  cm columns. While, the MIP particles prepared from bulk polymerization were packed into  $H = 25$  cm,  $D_i = 0.46$  cm columns.

Samples were injected into the column and eluted isocratically at a flow rate of  $Q = 0.5$  mL min<sup>-1</sup>. If not specified otherwise, the mobile phase was a mixture of acetonitrile and water ( $\Psi = 95:5$ ). The temperature was kept at  $\theta = 25$  °C. Measuring

the absorbance at  $\lambda = 210$  nm constantly monitored the effluent solution. Toluene was used as the non-retained component for the determination of the void fraction for each column. Capacity factor ( $k'$ ) was calculated according to the equation as  $k' = (\tau_R - \tau_0)/\tau_0$ , where  $\tau_R$  is the retention time of the analyte, and  $\tau_0$  is the retention time of the non-retained component (toluene). The plate number was calculated based on the formula:  $N = 5.54 (\tau_R/b_{0.5h})^2$ , where  $b_{0.5h}$  is the peak width at half-height. The selectivity factor ( $\alpha$ ) was defined as the ratio of capacity factor of cholesterol to that of  $\beta$ -estradiol.

## Results and discussion

### Chromatographic separation of cholesterol

The seeded suspension polymerization was found to be promising for the preparation of cholesterol-imprinted polymers. Although the polystyrene seeds were spherical with an average diameter of  $\bar{d} = 1.5$   $\mu\text{m}$ , the resultant molecularly imprinted polymer particles were relatively larger and irregular in shape. Before polymerization, polystyrene beads were enlarged by adsorbing cholesterol, methacrylic acid, and EGDMA to form the oil phase suspended in aqueous medium. Free radical polymerization of functional monomer and cross-linker surrounding the template could be either UV-initiated at low temperature or thermally initiated at high temperature. It is believed the new polymerization occurred mainly on the surface of the seeds, although a small portion of seeds could form aggregates. However, the resultant imprinted polymer particles were still smaller. Observations on scanning electron microscope revealed that particles possess almost a diameter less than  $\bar{d}_p = 5$   $\mu\text{m}$ . Prepared particles were all passed through the sieve for retaining particles larger than 25  $\mu\text{m}$  and ready for direct packing into a column.

The column packed with cholesterol-imprinted polymers prepared by seeded suspension polymerization at either low or high temperature was effective for the separation of cholesterol from other steroids. Fig. 1 shows the chromatograms using the column packed with MIP prepared at  $\theta = 4$   $^\circ\text{C}$  under the initiation of UV irradiation. The average retention times for estrone, progesterone and cholesterol in the samples with a total concentration of  $\gamma = 10$   $\text{mg L}^{-1}$  were  $\bar{\tau} = 5.3$ , 10.9 and 16.7 min, respectively. When the samples containing estrone,  $\beta$ -estradiol and cholesterol with a total concentration of  $\gamma = 10$   $\text{mg L}^{-1}$  were applied to the column, the average retention times were  $\bar{\tau} = 5.3$ , 12.3 and 17.2 min. The separation behaviors of these steroids were very similar on the column packed with MIPs

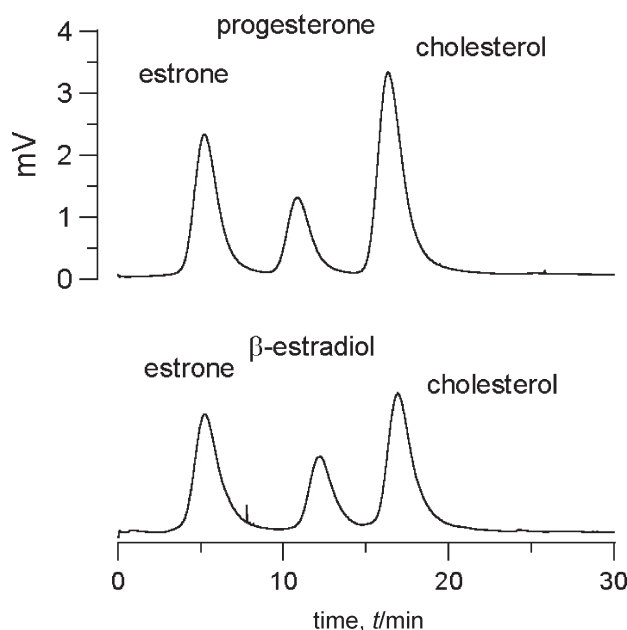


Fig. 1 – Separation of cholesterol from other steroids using cholesterol-imprinted polymer prepared by seeded suspension polymerization using UV-initiation at low temperature. Upper: three-component mixture of estrone, progesterone and cholesterol ( $\gamma = 3$ , 2, and 5  $\text{g L}^{-1}$ ). Lower: mixture of estrone,  $\beta$ -estradiol and cholesterol ( $\gamma = 3$ , 2, and 5  $\text{g L}^{-1}$ )

that were prepared by seeded suspension at  $\theta = 60$   $^\circ\text{C}$  under thermal initiation.

During chromatography, the cholesterol, the print molecule, in the sample competed with others for the binding sites on the particles, mainly the recognition sites left by the print molecules. As expected, cholesterol was strongly retained by the MIP due to the shape complementary, and came out last from the column. This specific adsorption was enforced by weak interactions mainly hydrogen bonding between hydroxyl group in cholesterol and functional group of MMA in polymer. However, the hydrophobic conditions of the mobile phase ( $\varphi = 95$  % acetonitrile) would break down the hydrogen bonding, and cholesterol would be finally eluted out from the column.

Since the mobile phase consisted of a water-soluble solvent (acetonitrile) and small amount ( $\varphi = 5$  %) of water, the non-polar solvent like toluene would be eluted out from the column with the mobile phase. However, the cholesterol-like steroids having either a hydroxyl (estrone and  $\beta$ -estradiol) or C=O group (progesterone) in the C3 position could interact to the immobilized acid groups introduced by MAA. Therefore, these steroids could stay in the MIPs column for longer times, in comparison with toluene that was regarded as a non-retained compound.

The column with MIP particles from low-temperature and high-temperature seeded suspension

polymerization had the same separation factors for resolution of  $\beta$ -estradiol and cholesterol (1.6) and a similar chromatographic efficiency. However, chromatography on MIP particles from low-temperature was more reproducible. In the preparation of MIPs, complexes of template, functional monomer (methacrylic acid) and crosslinker (ethylene glycol dimethacrylate) were self-assembled on seed particles during the polymerization process. A lower temperature would favor the stabilization of these complexes, which would result in correct recognition sites after polymerization and removal of template.

### Comparison with MIP prepared by bulk polymerization

As shown in Fig. 1,  $\beta$ -estradiol had the closest retention time with cholesterol, because its structure is much similar to that of cholesterol. Samples containing different combinations of  $\beta$ -estradiol and cholesterol concentrations were then applied to the columns for the examination of chromatographic efficiency using prepared MIPs. The results are summarized in Table 1.  $\beta$ -Estradiol has also a hydroxyl group at C3 position but differs from cholesterol by substituting the branched aliphatic group at C17 with a hydroxyl group (17- $\beta$ -hydroxyl) and lacking methyl group at C19 position. Because of these differences,  $\beta$ -estradiol could be well separated from cholesterol in every sample applied to column packed with MIPs, prepared by either UV- or thermal-initiated method. The separation was obviously based on the selective adsorption of print molecules to recognition sites formed on the imprinted polymers. That is, the selectivity in the chromatography was mainly based on the affinity difference between solutes due to their shape complementary to the cavity created by template printing.

As shown in Table 1, the interaction of  $\beta$ -estradiol to carboxylic groups of immobilized methacrylic acid of the imprinted polymer contrib-

uted a capacity factor of  $k' = 1.6$  or  $3.1$ , depending on the preparation polymerization method. Capacity factors for cholesterol in the columns with MIPs prepared by seeded suspension and bulk polymerizations were  $k' = 4.6$  and  $3.1$ , respectively. However, the binding of cholesterol and  $\beta$ -estradiol to non-imprinted polymers, prepared by seeded suspension or bulk polymerization, was very difficult under the elution conditions. On the column packed with non-imprinted polymers from seeded suspension, capacity factors for cholesterol and  $\beta$ -estradiol were  $k' = 0.05$  and  $0.04$ , respectively. Capacity factors for these two compounds on the non-imprinted polymers by bulk polymerization were also much smaller (all less than  $k' = 0.05$ ), suggesting that the interaction of hydroxyl groups on cholesterol and  $\beta$ -estradiol to carboxylic groups of the non-imprinted polymer was very weak under the elution conditions (95–100 % acetonitrile). The shape complementary to the cavity created by template printing likely dominated the binding of cholesterol and  $\beta$ -estradiol onto the imprinted polymer. These results revealed that the molecularly imprinted polymers exhibiting high affinity and selectivity to the template molecule were naturally affined separation matrices.

The seeded suspension polymerization method employed in this work yielded MIP particles in one step. In contrast, the bulk polymerization could end up with the production of porous MIPs that need to be ground before use. The column packed with ground MIPs particles ( $d_p = 25\text{--}44\ \mu\text{m}$ ) from bulk polymerization was also found to be effective for the separation of cholesterol and  $\beta$ -estradiol. As shown in Table 1, this column resulted in different retentions and selective factor. Cholesterol and  $\beta$ -estradiol were separated very well ( $\alpha = 3.1$ ) in this column, but were accompanied with a peak broadening (small  $N$ ). Compared with MIPs prepared by bulk polymerization, imprinted polymer particles prepared from seeded suspension polymerization had a much lower capacity for cholesterol

Table 1 – Chromatographic separation of cholesterol and  $\beta$ -estradiol using MIPs prepared with different polymerization methods. The mobile phase was a mixture of acetonitrile and water ( $\Psi = 95:5$ ,  $\varphi/\%$ ).

Preparation method	Retention time, $\tau$ / capacity factor, $k'$		Plate number $N$	$\alpha$ ( $k'_C / k'_{\beta E}$ )
	$\beta$ -estradiol $k'_{\beta E}$	cholesterol $k'_C$		
seeded suspension <sup>a</sup>	11.3 (2.9)	16.3 (4.6)	619	1.6
bulk polymerization <sup>b</sup>	5.1 (1.0)	10.2 (3.1)	224	3.1

<sup>a</sup> The length of columns was  $l = 15\ \text{cm}$ ; capacity factors were calculated using the retention times for the non-retained compound (toluene) which was determined to be  $\tau = 2.9\ \text{min}$  using MIPs prepared at either low or high temperature. Concentrations of cholesterol and  $\beta$ -estradiol were totally  $\gamma = 1\ \text{g L}^{-1}$  in each sample.

<sup>b</sup> Column length was  $l = 25\ \text{cm}$ ; the retention time for toluene was  $\tau = 2.5\ \text{min}$ . Concentrations of cholesterol and  $\beta$ -estradiol were totally  $\gamma = 1\ \text{g L}^{-1}$  in each sample.



adsorption due to a much smaller number of recognition sites generated on the particles. Therefore, the columns packed with MIPs from seeded suspension polymerization were useful for micro-preparative analysis. Fig. 2 shows the peak area and peak height as functions of cholesterol concentration in a 5  $\mu\text{L}$ -sample. Both the peak area and peak height were found to be proportional to the sample load up to ca.  $\gamma = 9 \text{ mg L}^{-1}$  (corresponding to 45 ng) as shown in Fig. 2. This linearity suggests that the column is potential for quantitative microanalysis.

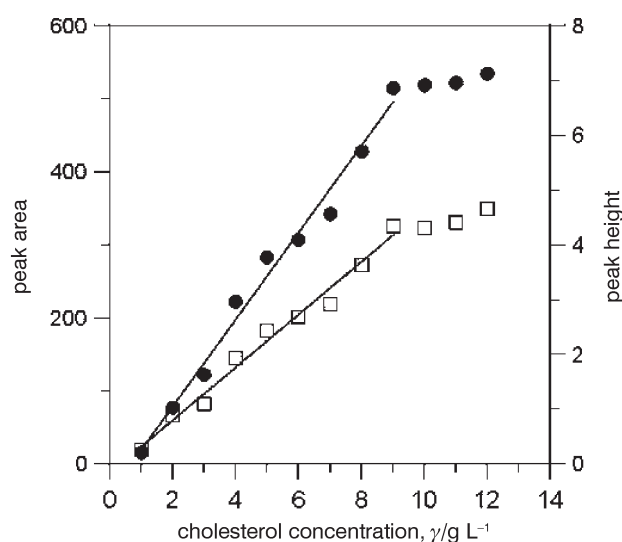


Fig. 2 – Effect of sample concentration on the peak area (●) and peak height (□) of the eluted peak for print molecule (cholesterol). The mobile phase was a mixture of acetonitrile and water ( $\Psi = 95:5$ , %). The column was packed with MIPs prepared by seeded suspension polymerization using UV-initiation at low temperature.

### Influence of mobile phase composition

Although the use of acetonitrile and water mixture in the volume ratio of  $\Psi = 95:5$  was found successful for the separation of cholesterol from other steroids, this mobile phase composition was subject to be optimal. The mobile phase composition could be changed to yield higher chromatographic efficiencies. The separation could be enhanced, for example, by reducing the water content to minimal. Fig. 3 shows the separation of cholesterol and  $\beta$ -estradiol using pure acetonitrile or chloroform as the mobile phase for elution. For the non-printed compound  $\beta$ -estradiol, behaving like in a reversed phase mode, it would come out from the column sooner if the mobile phase became more non-polar. Therefore, the retention time for  $\beta$ -estradiol was shorter when chloroform was used instead of acetonitrile, because the former is more non-polar.

Fig. 4 shows the effects of the mobile phase composition on the chromatographic separation of

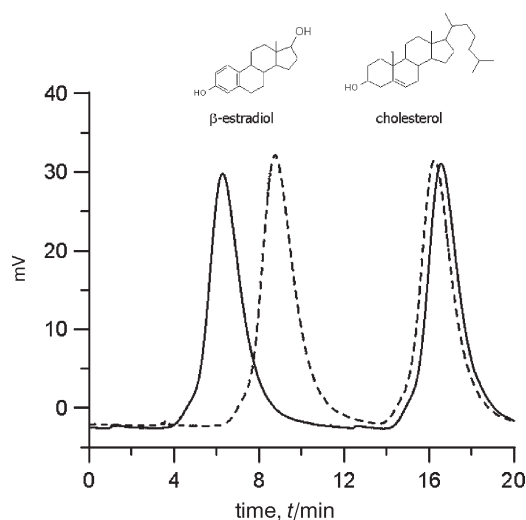


Fig. 3 – Influence of mobile phase on the chromatographic separation of cholesterol and  $\beta$ -estradiol on MIP particles made by seeded suspension polymerization using UV-initiation at low temperature. The mobile phase was 100 % acetonitrile (dashed line) and 100 % chloroform (solid line); the sample ( $V = 5 \mu\text{L}$ ) contained  $\gamma = 5 \text{ g L}^{-1}$  of  $\beta$ -estradiol and  $\gamma = 5 \text{ g L}^{-1}$  of cholesterol.

cholesterol and  $\beta$ -estradiol. To elute the cholesterol and steroids from the column, hydrophobic but water-soluble solvent like acetonitrile or chloroform was used as main component of the mobile phase. As the water content in the mobile phase increased, the retention of cholesterol did not change much, but the retention for  $\beta$ -estradiol increased quite significantly. This resulted in a decrease in the selectivity with water content in the mobile phase containing either acetonitrile or chloroform, as shown in Fig. 4. The influence of water content was very

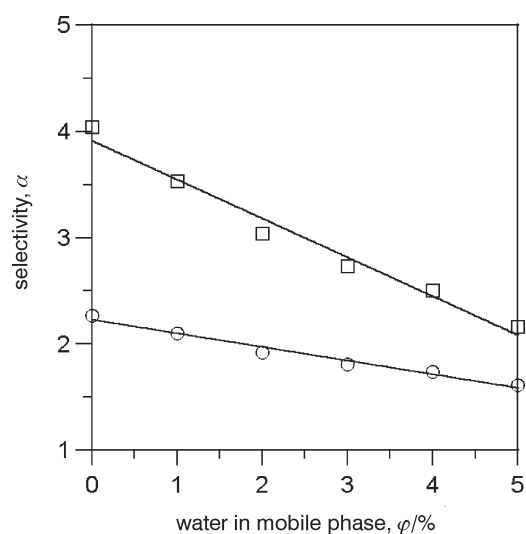


Fig. 4 – Influence of water concentration in the mobile phase consisting of chloroform (□) or acetonitrile (○) on the selectivity factor between cholesterol and  $\beta$ -estradiol. The column was packed with MIPs prepared by seeded suspension polymerization using UV-initiation at low temperature.

significant in the chloroform system. The more water in the mobile phase, the less the hydrophobic of the eluent. The hydrophobic steroid  $\beta$ -estradiol thus came out from the column later when the mobile phase was less hydrophobic. For the non-polar toluene having no affinity to the MIP and change, the acetonitrile to chloroform as the mobile phase did not change its retention.

## Conclusion

In this paper, imprinted polymers were synthesized by suspension polymerization using cholesterol as template and their molecular recognition properties were studied. The column packed with cholesterol-imprinted polymers prepared by seeded suspension polymerization at either low or high temperature was effective for the separation of cholesterol from other steroids. MIPs were applied as stationary phase of liquid chromatography, which proves that recognition ability can be ascribed to the imprinting process. The influence of recognition conditions on the retentivity and selectivity of the MIP demonstrated that hydrogen bonding between substrates and the binding sites played an important role. Our experience shows that this method could be used directly for the determination of cholesterol in foods residue with low cost and high speed.

## ACKNOWLEDGEMENT

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## List of symbols

$b$	– peak width, mm
$d$	– diameter, $\mu\text{m}$
$d_p$	– particle diameter, $\mu\text{m}$
$\bar{d}$	– average diameter, $\mu\text{m}$
$D_i$	– inner diameter of column, cm
$H$	– height of column, cm
$k'$	– capacity factor

$l$	– length of column, cm
$m$	– mass, g
$N$	– plate number
$Q$	– volume flow rate, $\text{mL min}^{-1}$
$t$	– time
$V$	– volume, $\mu\text{L}$
$\alpha$	– selectivity
$\gamma$	– mass concentration, $\text{mg L}^{-1}$
$\theta$	– temperature, $^{\circ}\text{C}$
$\lambda$	– wavelength, nm
$\tau$	– retention time, min
$\bar{\tau}$	– average retention time, min
$\varphi$	– volume fraction, %
$\Psi$	– volume ratio

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