Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1062 (2005) 31–37
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Solid-phase extraction of esculetin from the ash bark of Chinese traditional medicine by using molecularly imprinted polymers

Shu-Guo Hu, Li Li, Xi-Wen He*

State Key Laboratory of Functional Polymer Materials for Adsorption and Separation, Department of Chemistry, College of Chemistry, Nankai University, 94 Weijin Road, Tianjin 300071, PR China

Received 26 June 2004; received in revised form 10 November 2004; accepted 12 November 2004

Abstract

A molecularly imprinted polymer solid-phase extraction method is used to extract esculetin from the ash bark of Chinese traditional medicine. Ratio of ethanol and water as washing solution were investigated. Data of accumulative adsorption on molecularly imprinted polymers from the continuous loading experiment suggests that there are two different kinds of recognition sites in molecularly imprinted polymers. By selecting the washing and eluting solution a scheme was designed to separate esculetin and its analogues including esculin, coumarin, 7-methoxylcoumarin and daphnetin. Finally, by applying the revised scheme esculetin was extracted from the ash bark of Chinese traditional medicine that was purchased from two big drugstores, respectively, with both molecularly imprinted polymers and non-molecularly imprinted polymers.

Keywords: Solid-phase extraction; Molecularly imprinted polymers; Esculetin; Esculin; Coumarin; 7-methoxylcoumarin; Daphnetin; Chinese traditional medicine

1. Introduction

Reactive oxygen species (ROS) are thought to be the causative agents of various diseases, including cardiovascular diseases, and even accelerating factors in the aging process [1,2]. If excess ROS are not eliminated by biological antioxidant defense systems, they cause oxidative damage to in vivo components such as lipids, proteins, and DNA. Coumarins comprise a group of phenolic compounds widely distributed in natural plants, and they have recently attracted much attention because of their broad pharmacological activities [3]. Among these are the arachidonic acid cascade [4]. ELT also inhibits the differentiation of human leukemia HL-60 cells [5], which has been used extensively to gain insight into the processes of myeloid cell differentiation and their control mechanisms. Furthermore, ELT shows scavenging activity against ROS such as superoxide radicals [6] and hydroxyl radicals [7], and inhibits lipid peroxidation in rat livers [8]. More recently, ELT has been reported to inhibit oxidative damage induced by *tert*-butyl hydroperoxide in rat liver [9]. ELT is also found to be an active ingredient in some Chinese traditional medicines used to fight cough, phlegm, asthma, and inflammation. Generally, ELT as well as many other derivatives of coumarin co-exist in the ash bark of CTM. It is difficult to extract and separate ELT from the ash bark using conventional method because of low content and the complex matrix in the ash bark.

Traditionally, separation of active component in CTM is tedious and inefficient due to poor affinity and selectivity of conventional materials (e.g., silica-gel, polyamide, ion-exchange types and reversed-phase column) [10]. Molecularly imprinted polymers (MIPs) is a new kind of sorbent with high selectivity in solid-phase extraction (SPE) [11–14] and has been applied in different fields [15–20]. In 1940,

* Corresponding author. Present address: College of Chemistry, Nankai University, Tianjin, China Tel.: +86 2223504461; fax: +86 2223502458.
E-mail address: xiwenhe@nankai.edu.cn (X.-W. He).

0021-9673/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2004.11.036

Pauling first proposed the hypothesis about MIPs. But the development of molecularly imprinting technology was slow up to 1970s after the originating work was accomplished by Vlatakis et al. [21] and Wulff [22]. The principle of MIPs is described as templates (imprinted molecules) are first allowed to form complex with functional monomers by non-covalent bonds or covalent bonds in a solvent or mixed solvents. Subsequently, functional monomers are cross-linked around templates. After polymerization, templates are removed from the polymers, and leaving the specific cavities in regard to the shape and functionality. These cavities can selectively rebind templates. Polymers keep specific cavities sterically as templates, so the selectivity of MIPs for rebinding templates is high. Chemically and mechanically stable MIPs can rebind the specific molecule from structural analogues with high selectivity comparable to that of antibodies, enzymes or other native biological structures. MIPs as the sorbent for SPE have shown much better specificity compared to the conventional sorbents.

In this work, ratio of water and ethanol in the washing solution and ratio of water, ethanol and DMF in the eluting solution on the separation and recovery of ELT were investigated. A final scheme was designed based on these studies to separate and recovery ELT from its analogues which coexist in the ash bark of CTM. In addition, the adsorption efficiency of ELT on MIPs and non-molecularly imprinted polymers (NMIPs) was compared.

2. Material and method

2.1. Chemicals

ELT was purchased from Fluka. Esculine (ECL), daphnetin (DNT), 7-methoxycoumarin (7-CMR) were purchased from National Institute for the Control of Pharmaceutical and Biological Products (NICPBP, Beijing, China). Coumarin (CMR) was purchased from GuangFu Research Institute of Refinement Chemical Engineering. Their chemical structures are shown in Fig. 1. Ethylene glycol dimethacrylate (EGDMA) was purchased from Shuzhou Anli Chemical & Engineering Co. Ltd. Acrylamide was purchased from Research Institute of Chemical Reagent in Tianjin. Azo-*N*,*N*,9-diisobutyronitrile (AIBN) was supplied by Special Chemical Reagent Factory of Nankai University. Sodium hydroxid from Tiantai Reagent Factory, Ethanol, acetic acid, methanol, and *N*,*N*-dimethylformamide (DMF) from Chemical Reagent Ltd. of Tianjin were analytical grade. Methanol, chloroform and ether from Guangfu Research Institute of Chemical Reagent were HPLC grade. Water is double de-ionized.

2.2. Preparation of MIPs and NMIPs

A 0.1781 g (1 mmol) ELT, 0.2843 g (4 mmol) acrylamide, 4 g (about 20 mmol) EGDMA and 15 mg AIBN were added to a 25-ml conical flask containing 14 ml ethanol. The solution was purged with nitrogen and sonicated for 5 min and then was transferred to an ampere tube. The ampere tube sealed under vacuum and placed in the water bath at 60 °C for 24 h. Polymers formed in the ampere tube were ground into fine particles using a mortar and pestle. Polymer particles of 40–65 μm were sieved, collected and washed with ethanol–acetic acid (8:2, v/v) and ethanol to remove the template (ELT). The same procedure was used to prepared NMIPs except that the template (ELT) was not used.

2.3. Analysis equipment

HPLC analysis was performed using an Agilent 1100 liquid chromatograph (USA) with a multiple wavelength detector. Separations were carried out on a 250 mm × 4.6 mm × 5 μm Kromasil (Chromatographic Science and Technology Company, Tianjin, China) C_{18} column. The mobile-phase was a mixture of methanol and water (55:45,v/v, pH 2.9, H_3PO_4; pH was adjusted by a pH meter) and the flow rate was maintained at 1 ml min^{-1}. A Finnigan-Advantage mass spectrometry (MS, USA) system with an ESI (electrospray ionization) interface was used for identifying analytes by offline mode. MS conditions were as follows: sheath gas flow rate 15 arb, aux/sweep bas flow rate 0 arb,

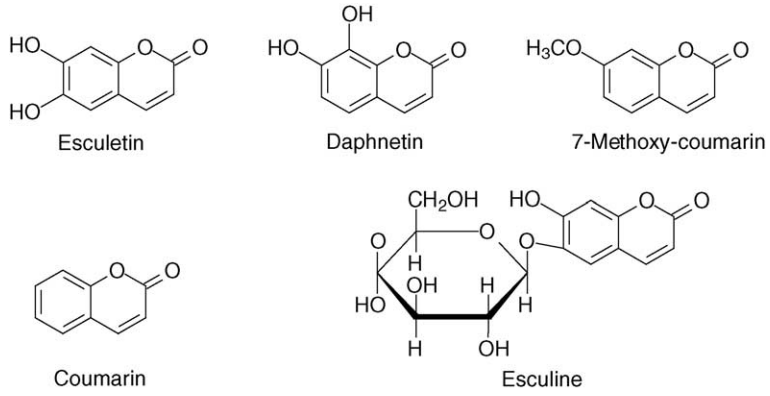

Fig. 1. Chemical structure of analogues.

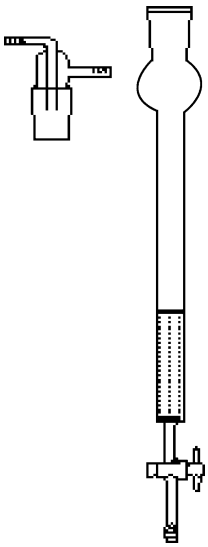

Fig. 2. Sketch map of home-made SPE column.

spray voltage 4.80 kV, capillary temperature 230.00 °C, capillary voltage −10.00 V, scan times 66,000 amu s^{-1} and tube lens offset 55.00 V. A shimadzu UV-240 detector (Japan) was used in accumulative adsorption experiment.

2.4. SPE column

The home-made SPE column (150 mm × 10 mm) is shown in Fig. 2. SPE column was packed with 400 mg of MIPs (NMIPs), with a glass-wool frit on the top of polymers. In SPE operation, each volume of loading, washing and eluting was 5 ml. Solution passed through the column under the pressure of N_2, and eluents were analyzed by HPLC (Data in Fig. 3 were also acquired by HPLC.). When there was a switch during SPE operation from aqueous solution to organic solution, the column was blown with N_2 for 10 min prior to the switch.

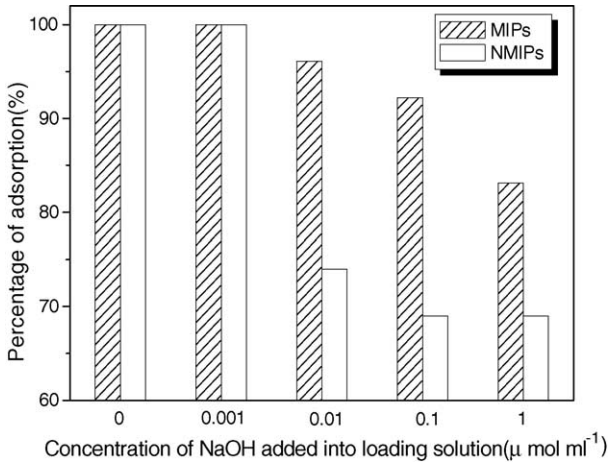

Fig. 3. Effect on percentages of adsorption on MIPs and NMIPs with the pH of loading solution increased. The concentration of 5 ml loading solution was 0.1 μmol ml^{-1}.

2.5. Accumulative adsorption experiment

Aqueous solution (115 ml) including 0.1 μmol ml^{-1} of ELT and 0.001 μmol ml^{-1} of NaOH was continually loaded into MIPs and NMIPs column, respectively. The average velocity of flow was 0.2–0.25 ml min^{-1}. Each 5 ml of effluent was detected by UV detector.

The linear range of ELT was 0.005–0.2 μmol ml^{-1} ($Y = -0.00418 + 11.38723X$, $R = 0.99998$).

2.6. Extraction of ash bark of CTM

The ash bark was purchased from two drugstores TongJunGe and Pujitang, respectively, in China. The ash bark was crushed into powder, 0.5 g of the ash bark and 50 ml of water were added into 100-ml round-bottom flask and the dispersion was boiled for 2 h using circulation reflux. The supernatant was filtrated with 0.45 μm film and 10 ml of filtrate was diluted to 100 ml with water. Diluted liquid (5 ml) extract was loaded into column, and then the column was washed with 2 × 5 ml water (pH 8) and 2 × 5 ml the mixture of ethanol and water (1:9, v/v); the column was blown for 10 min with N_2, then washed with 5 ml ether: chloroform (1:9, v/v); the column was blown for 2 min with N_2, then eluted with 2 × 5 ml the mixture of water, ethanol and DMF (5:4:1, v/v/v) and 10 ml ethanol. If the volume of residues lacked in 5 ml, the corresponding solution was added. The diluted liquid extract of ash bark from different drugstores was tested three times, respectively. In the quantitative aspect, the aqueous solutions of pure ELT of which the content was similar to the samples were used as the standard samples. The standard samples and samples were tested four times and two times, respectively, and their average values were used. The concentration of ELT detection limit was 178 ng ml^{-1}.

3. Results and discussion

3.1. Adsorption studies

In China generally people take the aqueous solution of CTM that is boiled for a while, so in this work the aqueous solution was selected as the extraction and loading solution. The effect of adsorption of ELT on MIPs and NMIPs was examined adding different concentration of NaOH to loading aqueous solution. In Fig. 3, the results show the percentages of adsorption of ELT decreased on MIPs and NMIPs column with the concentration of NaOH added into loading solution increased. When the concentration of NaOH was lower than 1×10^{-3}, ELT was adsorbed completely on MIPs and NMIPs; with the concentration of NaOH increased adsorption percentage decreased both on MIPs and NMIPs, but adsorption amount of ELT on NMIPs was lower than MIPs. It explains that with the concentration of NaOH increased, the hydroxyl groups on ELT change into the oxygenic negative ion and the H-bond interaction between polymers and ELT is

broken gradually. In other words the interaction of ELT and polymers were broken gradually with the pH of solution increased. On the other hand, we found that the adsorption percentage of ELT on NMIPs decreased more quickly than MIPs. It shows NMIPs adsorb ELT by H-bond, but MIPs are likely to recognize ELT by not only H-bond but also the suitable three-dimensional cavities. Considering adsorption amount and solubility of ELT (ELT is easy to dissolve in alkaline solution.), the aqueous solution including 0.001 μmol ml^{-1} of NaOH was selected as final loading solution. In order to investigate adsorption amount of ELT on MIPs and NMIPs column, 115 ml aqueous solution including 0.1 μmol ml^{-1} of ELT was continually loaded into MIPs and NMIPs column respectively. Results are shown in Fig. 4. For NMIPs when the accumulative volume of loading solution reached 50 ml, the adsorption percentage of ELT in the final 5 ml of loading solution (45–50 ml) is 17.9%. When the accumulative volume of loading solution was 90 ml, the adsorption percentage of ELT in the final 5 ml of loading solution (85–90 ml) was about 10.8% and did not change much with more loading of ELT solution. It shows that the adsorption nearly came up to saturation. For MIPs when the accumulative volume of loading solution reached 50 ml, the adsorption percentage of ELT in the final 5 ml of loading solution (45–50 ml) is about 69.3%; with the volume of loading increased, the detected concentration increased, when the accumulative volume of loading solution reached 115 ml, the detected adsorption percentage of ELT in the final 5 ml of loading solution (110–115 ml) is about 33.8%. We found for MIPs when the accumulative volume of loading solution increased from 90 to 95 ml the adsorption percentage of ELT in the corresponding 5 ml loading solutions decreased sharply (more than 10%). This result suggests that recognition sites and the sizes of cavities on MIPs are inhomogeneous. Matsui et al. [23] etc. mentioned that cavities on MIPs are classified into three kinds—macropore, mesopore (>20 Å) and micropore (<20 Å), and the mass transfer rate in macropore and mesopore is faster than in micropore. From Fig. 4, we presume that before 90 ml the adsorption of

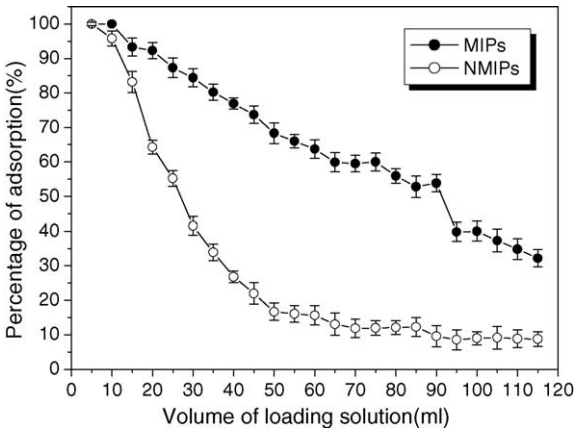

Fig. 4. Adsorption of ELT on MIPs and NMIPs column in continuous loading experiments. The detailed procedure was in the part of experiment. Data are from average value of two same experiments.

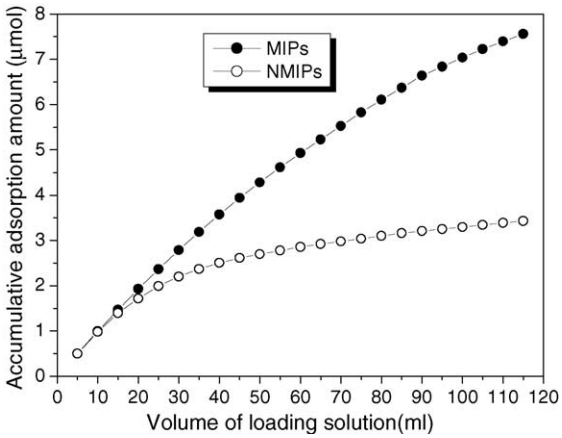

Fig. 5. Accumulative adsorption curves of ELT on MIPs and NMIPs corresponding to Fig. 4.

ELT occurs on recognition sites with high affinity in macropores and mesopores in MIPs; after 90 ml the adsorption of ELT occurs on recognition sites with low affinity in micropores. Fig. 5 shows the accumulative adsorption of ELT on MIPs and NMIPs. When the volume of loading solution was 115 ml the accumulative adsorption amount of ELT on MIPs is about twice than on NMIPs. And for NMIPs the adsorption was saturated nearly; for MIPs the adsorption amount would increase continually with the loading solution added. It explains that adsorption amount of ELT on MIPs is much more than on NMIPs. In this work, aqueous solution was selected as the loading solution, which resulted that non-selective adsorption on MIPs was serious because of hydrophobic action of ELT on polymers (The molecular structure of ELT has hydrophobic parent cycle of coumarin in Fig. 1.). So, in order to reduce non-selectivity adsorption MIPs and NMIPs column were washed by ratio of ethanol and water. The results are shown in Fig. 6. On MIPs column when the percentage of ethanol in the mixture was not beyond 10%, only about 1% of ELT was eluted from column; on NMIPs column with the percentage of ethanol increased, the recoveries of ELT

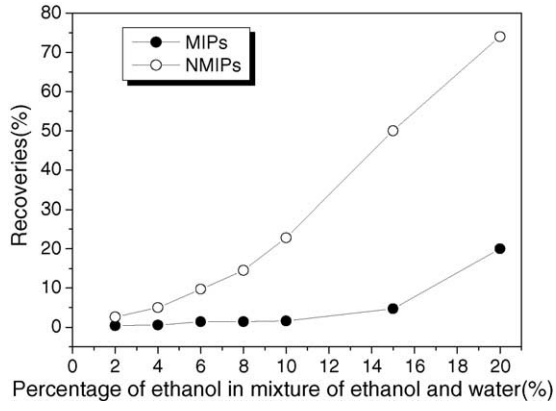

Fig. 6. Recoveries of ELT on MIPs and NMIPs by using different percentages of water and ethanol as washing solution. The concentration of each loading solution was 0.1 μmol ml^{-1}. The volume of loading and washing is 5 ml, respectively.

Table 1
The scheme of separating ELT and four analogues

Loading	5 ml mixture solution including ELT, ECL, DNT, 7-CMR, CMR and 0.001 μmol ml^{-1} of NaOH (the concentration of each component is 0.1 μmol ml^{-1})
Washing 1	5 ml aqueous solution including 0.001 μmol ml^{-1} of NaOH
Washing 2	5 ml aqueous solution including 10% ethanol
Washing 3	5 ml chloroform solution including 10% ether
Eluting 1	5 ml mixture solution including water, ethanol and DMF (4:5:1, v/v/v)
Eluting 2	5 ml mixture solution including water, ethanol and DMF (4:5:1, v/v/v)
Eluting 3	10 ml ethanol

eluted increased quickly. When the percentage of ethanol was 10%, the recovery of ELT was 22.8%; when the percentage of ethanol was 20%, the recovery was 74.0% on NMIPs (The recovery was 20.0% on MIPs.). It explains that MIPs show higher affinity for ELT than NMIPs. In order to keep high recovery of ELT, the aqueous solution including 10% ethanol was selected as washing solution in next separation and extraction experiment.

3.2. Method development of the SPE procedure

In the ash bark, there are many different and complex components, such as ECL, CMR, 7-CMR, DNT and etc. Furthermore, majority of compounds in the ash bark are derivatives of CMR. It is a key for extraction of ELT from the ash bark to separate ELT from derivatives of CMR. So, four derivatives of CMR were selected to test the selectivity of MIPs and NMIPs. The designed scheme including washing and eluting condition and results are shown in Table 1 and Fig. 7. ECL is characteristic of a kind of esters with hydrophilicity and large molecular volume; DNT is characteristic of a kind of position

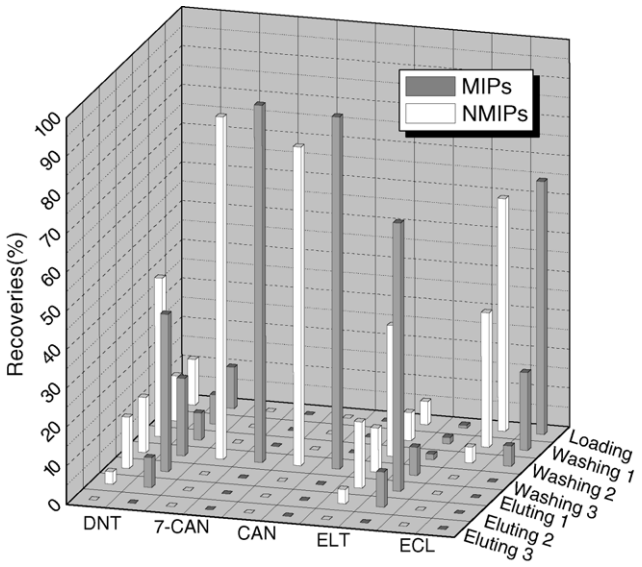

Fig. 7. ELT and four analogues were separated on MIPs and NMIPs column according to the scheme in Table 1.

isomers of ELT; CMR and 7-CMR are characteristic of a kind of analogues with hydrophobicity and small molecular volume, which is the reason for selecting these four compounds. In Fig. 7 because of large molecular volume and good water-solubility, ECL was retained weakly in the loading step and eluted completely in the washing 2 step. CMR and 7-CMR were retained strongly due to their hydrophobicity, so low polarity solvent such as ether and chloroform were selected in washing 3 step. Ether, ratio of ethanol and chloroform were tried to elute CMR and 7-CMR in our experiment. We found that ether was effective on eluting CMR and 7-CMR, but about half part of ELT was also eluted with 5 ml ether. And CMR and 7-CMR were not eluted completely using 5 ml of chloroform solution including low percentage of ethanol. But if the percentage of ethanol in the mixture of ethanol and chloroform was increased, ELT was eluted too. Finally, chloroform solution including 10% ether was selected as the washing 3 solution. Under this condition CMR and 7-CMR were eluted completely and ELT was only eluted about 7% on MIPs (33.8% on NMIPs). Molecular structures of DNT and ELT are very similar which resulted DNT was adsorbed more strongly than other derivatives of CMR on MIPs. But from Fig. 7, it clearly shows that MIPs retained ELT more strongly than DNT in loading and washing steps. In loading step, about 10% of DNT was found on MIPs. In eluting step, recovery of DNT was lower than ELT too on MIPs. We also found that adsorption activities of ECL, CMR and 7-CMR were similar on MIPs and NMIPs. And MIPs show higher affinity for DNT and ELT than NMIPs. Especially, it is obvious that 58.4% of ELT was eluted on NMIPs and 11% on MIPs respectively in loading and washing steps. The final recovery of ELT on MIPs and NMIPs was 78.4 and 20.8%, respectively, in the eluting 1 and 2 steps. The recovery of DNT on MIPs and NMIPs was 48.3 and 16.4%, respectively. We are not afraid that DNT will be co-extracted with ELT, because in the ash bark content of DNT is much lower than ELT, DNT will be eluted completely using this design scheme in next experiment. On the other hand, we realized that ELT was found in loading and washing 1 step in the procedure of testing mixed sample and not found in the procedure of testing single sample. At first, we presumed that adsorption amount of ELT on MIPs column reduced because of loading mixed sample. But we also found amount of ELT increased and amount of ECL decreased in loading and washing 1 step solution with the lapse of time. It explains that ECL hydrolyzed to ELT gradually. So, we think that a few amount of ELT found in loading and washing 1 step was from the hydrolysate of ECL and not from mixed sample. By testing, we are sure that ECL will not hydrolyze in an hour. So, it will not disturb determination of ELT if samples are tested in an hour. In washing 3 step, we found that amount of CMR and 7-CMR would decrease if washing 3 solution was dissolved renewedly with ethanol after dried by air. It shows CMR and 7-CMR will sublime with solvent together if corresponding washing solution is dried by air. So, washing solution including CMR and 7-CMR was tested directly by HPLC.

In eluting step, DMF with strong polarity was selected as the additive in ethanol. Ratio of DMF and ethanol were tried, but mixture of DMF and ethanol was not effective to elute ELT. Eluting MIPs column with 5 ml of ethanol solution including 20% DMF, the recovery of ELT was about 70%. When the percentage of DMF was increased to 50% the recovery did not increase accordingly. It is interesting that when the low percentage of water existed in the mixture of ethanol and DMF as eluting solution, the recovery increased. But if eluting solution included no ethanol the recovery was still low. So, ratio of DMF, ethanol and water was tried, and when ratio of DMF, ethanol and water was 1:5:4 (v/v/v), the recovery was about 85 and 94% on MIPs and NMIPs, respectively.

We think that the probable mechanism of eluting ELT from polymers is describe as: in comparison with water the polarity of ethanol is weaker so ethanol does not afford good conditions for DMF before adding water; the solubility of ELT is poor in water so even if ratio of water is increased, the recovery will not be enhanced; in addition ethanol may increase the solubility of ELT and reduce the hydrophobic of ELT on polymers. We also tried to elute ELT with ethanol including 5% trifuoroacetic acid (TFA) or triethylamine (TEA), but the peak of sample in HPLC was interfered by TFA and TEA (Eluting solution could not be dried by air pump because ELT will sublime with the solvent.). On the other hand because the acidity of TFA is too strong which resulted that it was troublesome to regenerate MIPs column (The functional monomer is acrylamide in this work.).

3.3. Extraction of ELT from ash bark

It is known that the components of CTM are complex and amount of different constituents are uncertain because of different producing area and depositing time. To enhance adsorption amount and solve problem of hydrolysis of ECL, extraction liquid from the ash bark was loaded directly ($1\,\mu mol\,ml^{-1}$ of ELT without adding NaOH was tried to load on MIPs, no ELT was found in the effluent.). Considering non-selectivity adsorption increased by loading samples directly, volumes of washing 1 and washing 2 solution were increased one times and other steps were same as the scheme of Table 1. The results shows that amount of ELT found in the ash bark of CTM is 2.16 ± 0.13 (from the big store Tongjunge) and 1.38 ± 0.12 (from the big store Pujitang) $\mu mol\,g^{-1}$, respectively. It is obvious the amount of ELT from drugstore Tongjunge is more than from drugstore Pujitang. We estimate that it relates to producing area and depositing time. Because the content of ELT in ash bark can be affected by habitat climate; in addition ECL in the ash bark tardily hydrolyzes to ELT with the lapse of depositing time that may enhance amount of ELT too. Fig. 8(2) shows the chromatogram of enriching and separating ELT using MIPs and NMIPs, respectively. From Fig. 7, we know that changes of the volumes of washing 1 and 2 will affect the recovery of ELT heavily on NMIPs and hardly on MIPs, which is verified in Fig. 8(2). In Fig. 8(2), the recovery of ELT was very low on NMIPs and about 10% of the recovery of ELT on MIPs. Fig. 8(1), shows the chromatogram of the liquid extract without extraction. It shows that components in liquid extract were very complex. Moreover comparing Fig. 8(1) with Fig. 8(2A) the content of interferents was much higher than ELT. But after MIPs column was washed with a serial of suitable solution, interferents were removed mostly and the only main peak of ELT was found in final eluting solution in Fig. 8(2A) that could be verified by the mass chromatogram in Fig. 9. It explains that the scheme designed was effective to enrich and separate ELT from the ash bark of TCM.

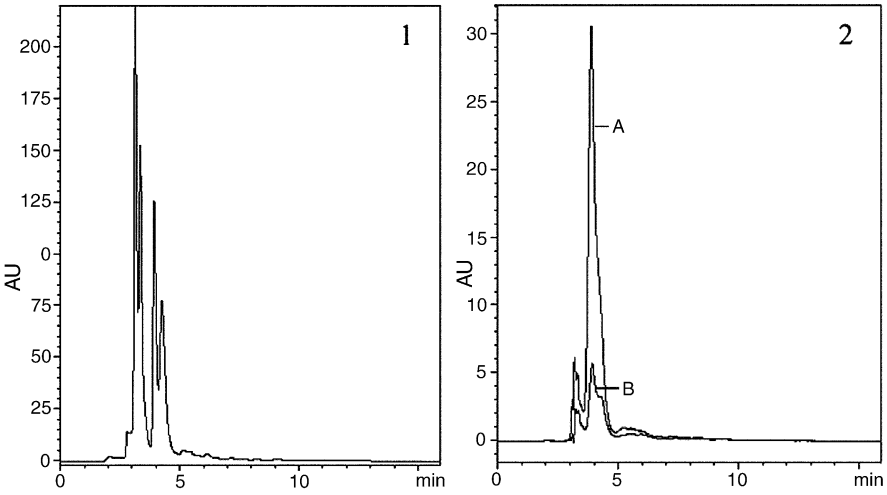

Fig. 8. (1) The chromatogram of liquid extract of the ash bark without extraction at 350 nm. (2) A and B were the chromatograms of enriching and separating ELT from liquid extract of the ash bark using MIPs and NMIPs SPE column, respectively. The detailed process was in the part of the experiment.

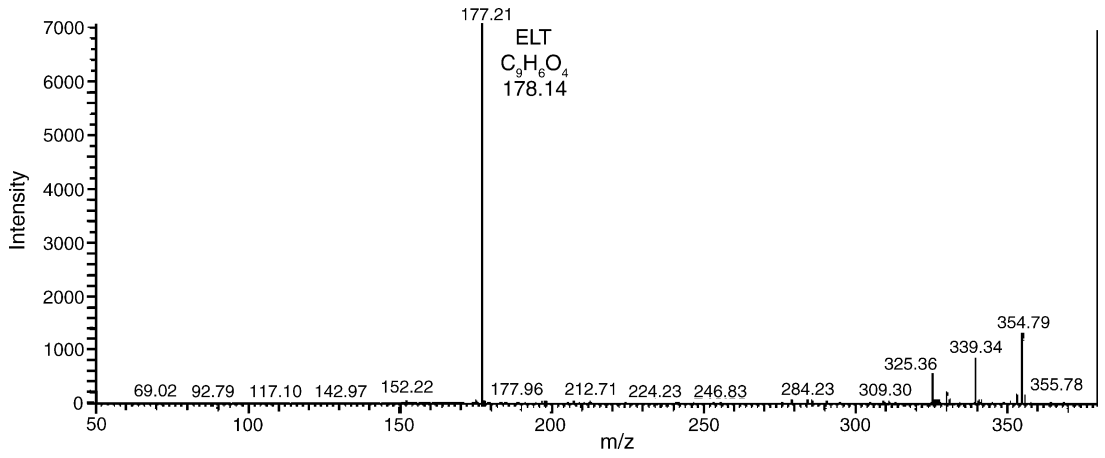

Fig. 9. The mass spectrum corresponding to (2A). The MS condition was shown in the part of experiment.

4. Conclusion

MIPs as the sorbent for SPE have been applied widely in these years. But the report that MIPs are applied to extract the active component in the CTM directly is few [10,24]. In this work, using MIPs-SPE column, we separate ELT from four kinds of representative compounds that may consist in the ash bark of CTM successfully by selecting washing and eluting solution. And possible recognition mechanism of MIPs is discussed using the data of accumulative adsorption. Finally, ELT is extracted from the ash bark of CTM that was purchased from two different drugstores successfully. Comparing with NMIPs, MIPs show higher selectivity to ELT. Thus, SPE using MIPs as the sorbent is a good method to separate ELT from the complex CTM directly. It may be a new and good way to enrich and separate the active component from the complex biosample in the future.

Acknowledgements

The project is supported by the National Nature Science Foundation of China (nos. 20375017 and 20175009), the Doctoral Foundation of Education Ministry of China (no. 20020055002) and the Natural Science Foundation of Tianjin (no. 033603511).

References

[1] A.A. Horton, S. Fairhurst, Crit. Rev. Toxicol. 18 (1987) 27.
[2] H. Esterbauer, K.H. Cheeseman, Chem. Phys. Lipids 45 (1987) 103.
[3] T. Kaneko, N. Baba, M. Matsuo, Chem. Biol. Interact. 142 (2003) 239.
[4] T. Neichi, Y. Koshihara, S. Murota, Biochim. Biophys. Acta 753 (1983) 130.
[5] S. Kawaii, Y. Tomono, E. Katase, K. Ogawa, M. Yano, Anticancer Res. 20 (2000) 2505.
[6] W.S. Chang, C.C. Lin, S.C. Chuang, H.C. Chiang, Am. J. Chin. Med. 24 (1996) 11.
[7] K. Hiramoto, N. Ojima, K. Sako, K. Kikugawa, Biol. Pharm. Bull. 19 (1996) 558.
[8] S.M. Aragón, J.M. Benedí, A.M. Villar, Gerontology 44 (1998) 21.
[9] W.L. Lin, C.J. Wang, Y.Y. Tsai, C.L. Liu, J.M. Hwang, T.H. Tseng, Arch. Toxicol. 74 (2000) 467.
[10] L.L. Zhu, X.J. Xu, J. Chromatogr. A 991 (2003) 151.
[11] I. Ferreer, D. Barceló, Trends Anal. Chem. 18 (1999) 180.
[12] L.I. Andersson, J. Chromatrogr. B 739 (2000) 163.
[13] F. Lanza, B. Sellergren, Chromatographia 53 (2001) 599.
[14] L. Andersson, Bioseparation 10 (2002) 353.
[15] F. Chapuis, V. Pichon, F. Lanza, S. Sellergren, M.-C. Hennion, J. Chromatogr. A 999 (2003) 23.
[16] K. Möller, C. Crescenzi, U. Nilsson, Anal. Bioanal. Chem. 378 (2004) 197.
[17] S.G. Hu, S.W. Wang, X.W. He, Analyst 128 (2003) 1485.
[18] P.C.L. Edward, Y.F. Sherry, Microchem. J. 75 (2003) 159.
[19] A. Molmelh, R. Weiss, B. Mizaikoff, J. Agric. Food Chem. 50 (2002) 1804.
[20] M. Zhang, J. Xie, Q. Zhou, G. Chen, Z. Liu, J. Chromatrogr. A 984 (2003) 173.
[21] G. Vlatakis, L.I. Andersson, R. Muller, Nature (Lond.) 361 (1993) 645.
[22] G. Wulff, Anew. Chem. Int. Ed. Engl. 34 (1995) 1812.
[23] J. Matsui, F. Kuniyuki, T. Takeuchi, Anal. Chem. 72 (2000) 1810.
[24] J.P. Lai, X.W. He, Y. Jiang, F. Chen, Anal. Bioanal. Chem 375 (2003) 264.