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SHORT COMMUNICATION

Solid Extraction of Caffeine and Theophylline from Green Tea by Molecular Imprinted Polymers

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Abstract–This paper involves a feasibility study on using molecular imprinted polymers as the sorbent materials in solid phase extraction for caffeine and theophylline from green tea. Two kinds of MIPs, with caffeine-theophylline mixture and pentoxifylline-theophylline mixture as the templates respectively, MAA as the monomer, EDMA as the crosslinker and AIBN as the initiator, were applied to this purpose. Mixture solution of caffeine and theophylline (1 μg/ml in acetonitrile) was applied to the solid extraction cartridges following a load, wash and elute procedure with acetonitrile, methanol, methanol-acetic acid (90/10, v/v) as the solvents, respectively. This solid phase extraction protocol was applied for extraction of caffeine and theophylline from green tea. Comparison between the results obtained with the MIPs cartridges and a traditional C_{18} reversed-phase cartridge was made. It showed that the MIP-based sorbent on the solid phase extraction was comparable with that of C_{18} material. HPLC analysis using a C_{18} column (5 μm, 250× 4.6 mm from Rstech corporation), methanol: water (60 : 40, v/v) as the mobile phase at a flow rate of 0.6 ml/min was applied for the quantitative determination.

Key words: Molecular Imprinted Polymers, Solid Phase Extraction, Caffeine, Theophylline

INTRODUCTION

Molecular imprinted polymers (MIPs) exhibiting high selectivity and affinity to the predetermined molecule (template) are now seeing a fast growing research. The special binding sites are formed by the self-assembly of the template with functional group and the monomer, followed by a crosslinked co-polymerization. After the polymerization, the template is removed from the polymer, leaving recognition sites that, in terms of size, shape and functionality, are complementary to the template. So, ideally, the resulting MIP can selectively re-bind the template in preference to other closely related structures [Nicholls et al., 1995; Zhou et al., 1999; Sajonz et al., 1998; Owens et al., 1999; Chen et al., 2001; Zheng et al., 2002]. Most MIPs are prepared by non-covalent imprinting and the common systems are based on commodity methacrylic monomers, such as methacrylic acid (MAA) because its carboxyl group is the most common hydrogen-bonding and acidic functional group in molecular imprinting, cross-linked with ethyleneglycol dimethacrylate (EDMA).

Solid phase extraction (SPE) can be used to isolate and pre-concentrate the analytes in complex samples. This technique is more rapid, simple, economical and environment-friendly than the traditional liquid-liquid extraction. The materials used in SPE are usually based on the non-specific binding of the targets, which often suffers some shortcomings, such as low specificity and selectivity [Baggiani et al., 2001]. In recent years, solid-phase extraction involving molecular imprinted polymers (MISPE) have been proved to be successful applications [Blomgre et al., 2002; Meng and Liu, 2001; Theodoridis and Manesiotis, 2002; Jodlbauer et al., 2002; Kugimiya and Takeuchi, 1999] for its features of high selectivity, ease of synthesis, low cost for preparation and workability under different conditions especially that of harsh pH and organic solvents.

Recently green tea as a healthy beverage has attracted more and more attention for its anticancer [Chi, 1997; Melissa et al., 2000] and antioxidation activities [Liebert et al., 1999]. The main components contained in green tea are polyphenols (named catechins) [Pelillo et al., 2002], flavonols [Wang and Helliwell, 2000], methylxanthines [Zuo et al., 2002], etc. Different methods for extracting the active components contained in green tea have been developed [Pan et al., 2003; Chiehming et al., 2000]. Yet new attempts with high purity and selectivity are still under progress. During the past few years, xanthines including theophylline and caffeine represent a group of templates of great interest in MIPs [Baggiani et al., 1997; Mullett and Lai, 1999; Kobayashi et al., 2001; Theodoridis and Manesiotis, 2002]. MIPs as a kind of selective sorbent material have many successful applications in solid phase extraction, but its application on extracting certain active components directly from natural plant is relatively few [Xie et al., 2001].

In this work, by using a mixture template protocol, we developed new MIPs showing higher selectivity for both caffeine and theophylline. These MIPs were used as SPE sorbents for extraction of caffeine and theophylline from green tea.

EXPERIMENTAL

1. Chemicals

Caffeine, theophylline, and methacrylic acid (MAA) were purchased from Sigma (ST Louis, MO, USA). α,α'-Azobis (isobutyronitrile) (AIBN) was the product of Junsei Chemical Co., Ltd. (Japan). Ethylene glycol dimethacrylate (EDMA) was from Fluka (Buchs, Switzerland). All the above reagents were used directly without fur-

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ther treatment. Acetonitrile, methanol and chloroform were all of HPLC grade and from Duksan Pure Chemical Co., LTD (Ansan, Korea). Acetic acid (analytical grade) was from Oriental Chemical Industries (Incheon, Korea). Green tea was purchased in a domestic market of Seoul, Korea. Doubly distilled water was filtered by decompressing pump (Division of Millipore, Waters) and filter (FH-0.5 μm).

2. Polymer Preparations

In a 250 ml two-neck glass flask, monomer (MAA), crosslinker (EGDMA), initiator (AIBN), porogen and different templates, 1 mmol pentoxifylline for P1, 1 mmol caffeine for P2, 0.5 mmol theophylline for P3, 0.5 mmol pentoxifylline+0.25 mmol theophylline for PT and 0.5 mmol caffeine+0.25 mmol theophylline for CT, respectively, were added. The reaction mixture was put in supersonication for 10 min, sparged with helium for 10 min to remove oxygen, then vacuumed for 10 min and sealed under vacuum. Polymerization was performed in a water bath at the temperature that was maintained at 60 °C for 24 h. After the polymerization, the bulk polymer was taken out from the reaction flask and put into an oven for drying. The dried polymer was grounded into particles and passed through a 35 μm sieve; small particles were removed by repeated sedimentations with water. By these procedures, particles of 25 μm-35 μm were collected. For chromatographic evaluation of the MIPs, the dried particles were packed into a 3.9×150 mm Waters stainless steel column. Methanol-acetic acid (90 : 10, v/v) was first used as the mobile phase at a flow rate of 0.3 ml/min for 4 hours to remove the template, then only acetonitrile was used as the mobile phase. UV wavelength was set at 270 nm. The retention factor (k') was calculated as $(t-t_0)/t_0$, where t is the retention time of the compound, and t_0 the dead time of the column and determined by acetone as the marker. Blank polymer was prepared following the same procedure just in the absence of template.

3. HPLC Analysis

HPLC was used for quantitative analysis of the SPE results. The liquid chromatography system contains a Waters 600 s Multisolvent Delivery System and a Waters 616 pump (Waters, Milford, MA, USA), a detector of Waters 2487 Dual Absorbance (Waters, Milford, MA, USA) and Rheodyne injection valve (20 μl sample loop). Millennium 3.2 (Waters, Milford, MA, USA) was used as data acquisition system. Quantitative determination was based on a C_{18} column (5 μm, 250×4.6 mm from Rstech corporation), methanol : water (60 : 40, v/v) as the mobile phase at a flow rate of 0.6 ml/min, injection volume 5 μl, UV wavelength was set at 270 nm.

4. Solid Phase Extraction

Commercial SPE cartridges were emptied from their packing materials. Next the cartridge tube and frits were thoroughly cleaned and dried. 0.1862 g of the corresponding polymers was packed dry in the cartridges and the upper first were placed on top. The used C_{18} SPE cartridge (with 200 mg packing material) was a product from Altech (Deerfield, U.S.A). Before the extraction, the MIP cartridges were treated with methanol/acetic acid (90 : 10, v/v), 3×3 ml, followed by methanol 3×3 ml. C_{18} and blank polymer was treated only by methanol 3×3 ml. Extraction experiments consisted of loading the SPE cartridges with a mixture of caffeine and theophylline (concentrations are 1 μg/ml, respectively) or a solvent extraction sample of green tea, the loading volumes were 1 ml, respectively; washing with 1 ml methanol, eluting with 1ml mixture of methanol and acetic acid (90 : 10, v/v). All the applied fractions were collected and evaporated to dryness. The residues were reconstituted to solution with 100 μl methanol and analyzed on HPLC. Quantitative determinations were based on the constructed calibration curves: $y=7.86\times10^3+1.76\times10^7x$, r=0.998, for caffeine and $y=6.43\times10^3+2.04\times10^7x$, r=0.998, for theophylline, respectively.

5. Solvent Extraction of Green Tea

Solvent extraction from green tea was prepared as reported before [Kang et al., 2000]. 5 g of green tea was extracted by 150 ml doubly distilled water with continuously stirring under 50 °C for 4 h. The extraction was filtered by 0.2 μm, 25 mm syringe filter from Altech (Deerield, U.S.A.). Then the extraction was diluted with water (1 ml green tea extraction+9 ml distilled water) when it was applied for loading on the SPE cartridges.

RESULTS AND DISCUSSIONS

1. Selectivity of the MIPs

Different MIPs were prepared in this work. First, single template, i.e., pentoxifylline, caffeine or theophylline, was used. Then, two kinds of mixtures, pentoxifylline-theophylline, and caffeine-theophylline, were used as the templates, respectively. The selectivity of the MIPs and also that of a blank are listed in Table 1. Though very small difference existed in the molecule structures of the three applied templates (Fig. 1), different selectivity of the single template MIPs (P1, P2 and P3) can be noticed. Among them, theophylline

Table 1. Selectivity of the studied compounds, pentoxifylline, caffeine, theophylline and theobromine on imprinted and blank polymers

Polymer	Template	Compound	Retention factor (k)
P1	Pentoxifylline	Pentoxifylline	0.405
		Caffeine	0.580
		Theophylline	1.47
		Theobromine	1.83
P2	Caffeine	Pentoxifylline	0.329
		Caffeine	0.672
		Theophylline	1.59
		Theobromine	1.87
P3	Theophylline	Pentoxifylline	0.483
		Caffeine	0.736
		Theophylline	3.79
		Theobromine	2.40
PT	Pentoxifylline	Pentoxifylline	0.707
	+theophylline	Caffeine	1.05
		Theophylline	6.74
		Theobromine	3.89
CT	Caffeine	Pentoxifylline	0.508
	+theophylline	Caffeine	0.874
		Theophylline	3.89
		Theobromine	2.46
Blank	-	Pentoxifylline	0.243
		Caffeine	0.402
		Theophylline	1.22
		Theobromine	1.52

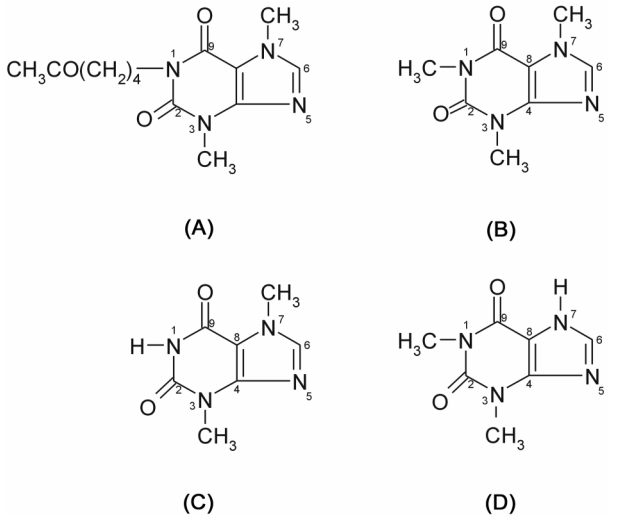

Fig. 1. Molecular structure of pentoxifylline (A), caffeine (B), theophylline (C) and theobromine (D).

imprinted MIP shows the highest selectivity to the template and the analogues. Caffeine imprinted MIP shows moderate selectivity, and that of pentoxifylline imprinted MIP is the lowest. This difference can be discussed in terms of the different basic characteristics of the template. It is known that when N-heterocycles were used as templates, it was thought that they could combine with the carboxylic acid (MAA) with the ring nitrogen through H-bond. The strength of the monomer-template interaction is therefore expected to be strongly influenced by the basicity of the template; the more basic template will interact more strongly with MAA producing a large population of sites and result in stronger selectivity for the template [Dauwe and Sellergen, 1996; Pap et al., 2002]. Among the three templates, the basicity of theophylline is strongest (pK_b 8.8), caffeine the second (pK_b 10.4), pentoxifylline is expected to be the weakest in basicity from the molecular structure. Because of its weak basicity, pentoxifylline can only form weak complex with MAA through H-bond. Hence only a small amount of selective sites were produced and most of the surface of the polymer remained nonselective. The results of caffeine and theophylline imprinted polymers are reasonable according to the above discussions. In case of mixture template imprinted polymers (PT, CT), one can see that the selectivity was higher than that of single template MIPs. This means a cooperative or sum effect was produced. From the molecular structure, theophylline shows the highest rigidity and pentoxifylline the lowest. As in known, a template with a high rigidity tends to fasten to the binding site, whereas the binding site formed by a template of lower rigidity shows higher flexibility. So in our work when the higher-rigidity theophylline and lower-rigidity pentoxifylline were mixed together as the template, a balance between the rigidity and flexibility of the binding sites was formed inside the polymer. This resulted in a cooperation effect and also higher accessibility to the binding sites, hence an increase in the affinity. Hence we choose PT and CT for the future SPE experiments. Furthermore, blank and commercial C_{18} were also applied for comparison purposes.

2. SPE Extraction of Caffeine and Theophylline

As a general rule, MIPs exhibit better molecular recognition in the solvent used as porogen during polymerization. It is rationalized that selective binding of the template to the MIP is enhanced in conditions similar to those occurring during the molecular self-assembly in the polymerization mixture. As acetonitrile is one of the porogen used during the preparation of MIPs, we chose acetonitrile as the loading solvent. Methanol and mixture of methanol/acetic acid (90 : 10, v/v) were used as the washing and eluting solvents, respectively. From Table 1, one can note that higher affinity and recovery can be obtained on the CT and PT cartridge than the blank. This is due to the specific binding of the MIPs to the template and the analogue. When comparing the results of MIPs with that of C_{18}, it can be seen that C_{18} has lower recovery to the target molecules. Methanol or acetonitrile containing a small percentage of acid, such as trifluoroacetic acid (TFA) [Martin et al., 1997; Olsen et al., 1999] and acetic acid [Rashik et al., 1997; Baggiani et al., 1999] have been used as eluting solvents to recover the template from the MIP-SPE cartridge. It is believed that the high polar solvent can interfere the specific binding, i.e., hydrogen bonding or electrostatic interaction between the template and the polymer. Otherwise the hydrophobic or reverse-phase interaction can be enhanced. This may explain the low recovery on the C_{18} SPE column in this work. Another example can be seen that a drastic recovery of caffeine on CT cartridge was obtained. This may be the result of the leakage of the template from the polymer. Since MIPs are made with large quantities of template, a small number of imprint molecules may remain in the resulting polymer, though after carefully extraction, they may leak later during SPE, thus interfering with trace analysis [Andersson et al., 1997; Rashik et al., 1997]. The other template theophylline did not show leakage from the recovery result. One can also see from Table 2 that when caffeine was substituted with pentoxifylline (PT), the recovery data of caffeine was reasonable. This is consistent with some authors' work, i.e., solving the leakage problem by using a structural analogue to the analyte of interest as the template for preparing the polymer, thus taking advantage of the crossreactivity of the MIPs [Andersson et al., 1997; Matsui et al., 2000]. In this case of PT MIP, pentoxifylline (template) can be

Table 2. Solid phase extraction of caffeine and theophylline with different sorbents

	CT[a]		PT[b]		C18		Blank	
	Caffeine	Theophylline	Caffeine	Theophylline	Caffeine	Theophylline	Caffeine	Theophylline
Loading (μg)	0.25	0.14	0.07	0.20	n.d[c]	n.d	n.d	0.24
Washing (μg)	0.85	0.72	0.86	0.72	0.40	0.44	0.81	0.61
Elution (μg)	1.25	0.28	0.16	0.11	0.26	0.25	n.d	n.d
Recovery (%)	235	114	109	103	66	69	81	85

[a]Caffeine-theophylline mips; [b]Pentoxifylline-theophylline mips; [c]Not detected.

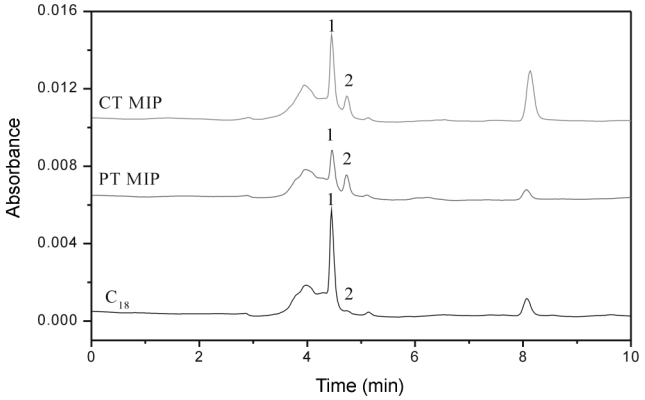

Fig. 2. Chromatograms of extraction results of green tea: collections after eluting the cartridge with methanol-acetic acid (90 : 10, v/v). HPLC analysis: C_{18} column (5 μm, 250×4.6 mm from Rstech corporation), methanol : water (60 : 40, v/v) as the mobile phase at a flow rate of 0.6 ml/min, injection volume: 5 μl. Peaks: 1=caffeine; 2=theophylline.

separated from caffeine in the chromatographic analysis (the retention time, $t_{caffeine}$=4.35 and $t_{pentoxifylline}$=4.92, respectively), so the existence of bleeding could not affect the analysis result.

3. SPE Extraction of Green Tea

The SPE extraction of green tea followed the same load-wash-elute procedure as did in the SPE extraction of caffeine and theophylline. But aqueous sample of green tea was used in the loading step. Fig. 2 illustrates the extraction results of CT MIP, PT MIP, and C_{18}. It is known the selective binding sites are mainly formed by hydrogen bonding or electrostatic force during the self-assembly between the template and monomer, while the other part of the MIP surface remains non-selective, namely hydrophobic. In this case, when aqueous sample was applied during the loading step, as water is a high polar solvent and shows strong hydrogen bonding ability, the selective binding of the MIPs was suppressed and the MIPs behaved as reversed-phase materials. So we can see similar extraction results between the applied SPE materials. On the other hand, one can conclude that the MIPs SPE materials used in this work are comparable with the conventional C_{18}.

CONCLUSION

In this work, different molecular imprinted polymers were prepared. Among them, the mixture template MIPs, caffeine-theophylline MIP (CT) and pentoxifylline-theophylline MIP (PT), showed higher selectivity to the target molecules. These mixture template MIPs were used for solid phase extraction of caffeine and theophylline and also sample of green tea. It was observed that higher affinity and recovery of caffeine and theophylline can be obtained on the CT and PT cartridge than the blank. When applied to the solid phase extraction of green tea, the MIPs materials behaved as reversed-phase, and the extraction results are comparable with the conventional C_{18}.

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