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Solid-Phase Extraction of Catechin Compounds From Green Tea by Catechin Molecular Imprinted Polymers

M. TIAN, H. ZHANG and K.H. ROW*

Department of Chemical Engineering, Inha University, 253 Yonghyun-Dong, Nam-Ku, Incheon 402-751, South Korea

*Corresponding author: Fax: +82 32 872 0959; Tel: +82 32 860 7470; E-mail: rowkho@inha.ac.kr

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Catechin compounds, (+) catechin (+C), epicatechin (EC), (-) epigallocatechin (EGC) and (-) epigallocatechin gallate (EGCG) were extracted from green tea by hot water and then separated using molecular imprinted polymer (MIP) sorbents in solid-phase extraction (SPE). The MIPs were synthesized using catechin templates, methacrylic acid (MAA) monomer, ethylene glycol dimethacrylate (EGDMA) cross-linker and α,α′-azobis(isobutyronitrile) (AIBN) initiator. Total catechin adsorption isotherms for catechin-MIPs and non-template imprinted polymer (NIP) were compared, allowing a suitable catechin-MIP (EGCG-MIP) sorbent to be selected for the separation of catechin compounds from green tea extract solution by an optimized SPE procedure and the recovery of (-) epigallocatechin, (+) catechin, epicatechin and (-) epigallocatechin gallate were 73.2, 91.7, 80.5 and 86.9 %, respectively.

Key Words: Solid phase extraction, Molecular imprinted polymers, Catechin compounds, Green tea.

INTRODUCTION

Green tea, a common beverage produced from dried fresh tea leaves, contains characteristic polyphenolic compounds: polysaccharides, flavonoids, vitamins B, C and E, R-amino butyric acid, catechins and fluoride. Tea polyphenols have beneficial effects such as sequestrating metal ions, scavenging reactive oxygen species,[1] reducing cholesterol, relieving hypertension and protecting against cardiovascular disease and cancers of such as the bladder, prostate, esophagus and stomach[2,3]. Catechins, such as epigallocatechin (EGC), catechin (C), epicatechin (EC) and epigallocatechin gallate (EGCG) are important polyphenolic components of green tea extract and been studied for their strong sulfate effects and anticancer functions[4].

Molecular imprinting has been applied to chiral separation[5,6], solid extraction,[7,8] bio-mimic sensor,[9,10] and membrane separation[11,12]. Molecular imprinted polymers (MIP) can be prepared by covalent and non-covalent methods; the latter has been widely used owing to its relative simplicity[13]. Solid-phase extraction involving molecular imprinted polymers (MISPE) has been successfully applied to biological and environmental samples[14-17]. They have demonstrated high selectivity, facile synthesis, low preparation cost and workability under a variety of conditions, *e.g.*, harsh pH and organic solvents. The use of MIPs in SPE is advantageous when selective extraction is required, as commonly used sorbents lack selectivity. MISPE can pre-concentrate the analyte and remove other compounds from the sample matrix[18].

Fig. 1 shows the chemical structure of catechins and schematic diagram of the synthesis of catechin-MIPs.

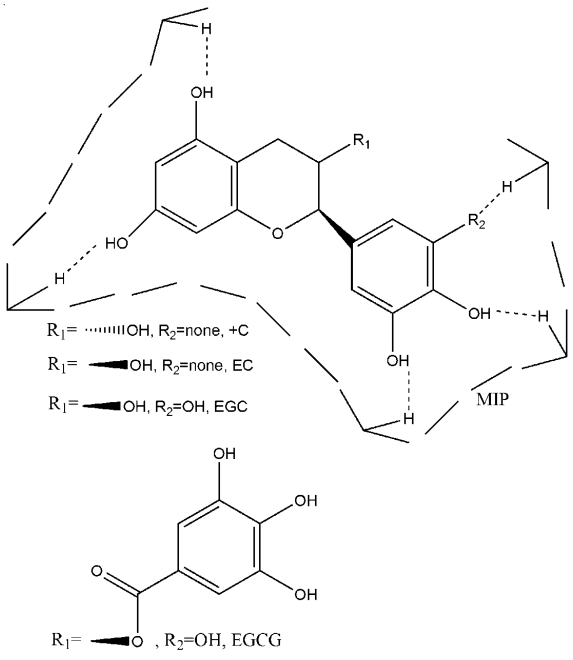

Fig. 1. Chemical structure of catechins and schematic diagram of the synthesis of catechin-MIPs

Xanthines such as caffeine have been researched as potential templates for MIP[19-22]. Molecular imprinted polymers have been used as selective sorbents in solid phase extraction, but work about the efficient and direct separation of active components by MIPs is not exhaustive and several potential adsorbents remain unstudied[23].

This work reports the use of NIP and certain MIPs (+C-MIP, EC-MIP and EGCG-MIP) in the solid-phase extraction and separation of catechins from green tea. Their catechin adsorption isotherms were also investigated. Optimizing the loading volume and the washing and elution solvents for SPE resulted in the successful separation and purified of the catechins by the catechin-MIPs. EGCG-MIP was the most efficient adsorbent of catechins from the green tea extract solution.

EXPERIMENTAL

Green tea was purchased from Enshi (Hubei, China). Theobromine, theophylline, caffeine, methacrylic acid (MAA), (+) catechin (+C), epicatechin (EC), (-) epigallocatechin (EGC) and (-) epigallocatechin gallate (EGCG) were purchased from Sigma (ST Louis, MO, USA). α,α′-Azobis(isobutyronitrile) (AIBN) was obtained from Junsei Chemical Co. Ltd. (Japan). Ethylene glycol dimethacrylate (EGDMA) was from Fluka (Buchs, Switzerland). All the above reagents were used without further treatment. Methanol and *n*-heptane were from Duksan Pure Chemical Co. Ltd. (Ansan, Korea). Distilled water was filtered using a vacuum pump (Division of Millipore, Waters, Milford, MA, USA) and a filter (HA-0.45, Division of Millipore, Bedford, MA, USA). All the solvents were of HPLC or analytical grade. All the samples were filtered (MFS-25, 0.2 μm TF, Whatman, Piscataway, NJ, USA) before being injected into the HPLC system.

HPLC analysis: The HPLC system comprised a M930 solvent delivery pump (Young Lin Co. Korea), a UV detector (M 720 Absorbance Detector, Young-In Scientific Co. Korea) and an integrated data system (Autochrowin. Ver. 1.42, Young Lin Co., Korea). Injection valves with 20 μL sample loops were used. The HPLC analysis was performed with a commercial C_{18} column (4.6 mm × 150 mm, 5 μm) purchased from RStech Co. (Daejeon, Korea). The mobile phase was methanol/water with different ratios used as isocratic elution in room temperature, the flow-rate was set at 0.5 mL/min, the UV wavelength was set at 280 nm and the injection volume was 10 μL. Each sample was repeatedly injected 5 times for evaluating the precision and accuracy of analysis.

A mixed methanol and water mobile phase separated the mixture of standard catechins by HPLC under the conditions described above. Methanol content of 20, 30 and 40 % were tested to optimize the mobile phase. 30 % methanol was considered suitable for the mobile phase as the four catechins could be separated within 20 min (c) (Fig. 2).

Preparation of catechin-MIPs and NIP: Sorbents were synthesized by a similar method to that described by Jin[24]: 5 mmol monomer (MAA), 30 mmol crosslinker (EGDMA), 0.12 g initiator (AIBN), 9 mL porogen (ACN) and 1 mmol template ((+) catechin) were added to a 250 mL two-neck glass flask. The reaction mixture was supersonicated for 10 min, sparged with helium for 10 min to remove oxygen, vacuumed for 10 min and then sealed under vacuum. Polymerization was performed in a water bath at 60 ºC for 24 h. C-MIP resulted. Two other MIPs were synthesized by similar processes, though with different templates (EC and EGCG). The NIP was synthesized without a template. After polymerization, the bulk polymer was removed from the reaction flask and dried in an oven. The dried polymer was ground into particles and passed through a 35 μm sieve; small particles were removed by repeated sedimentation in water. Particles of 25-35 μm were collected.

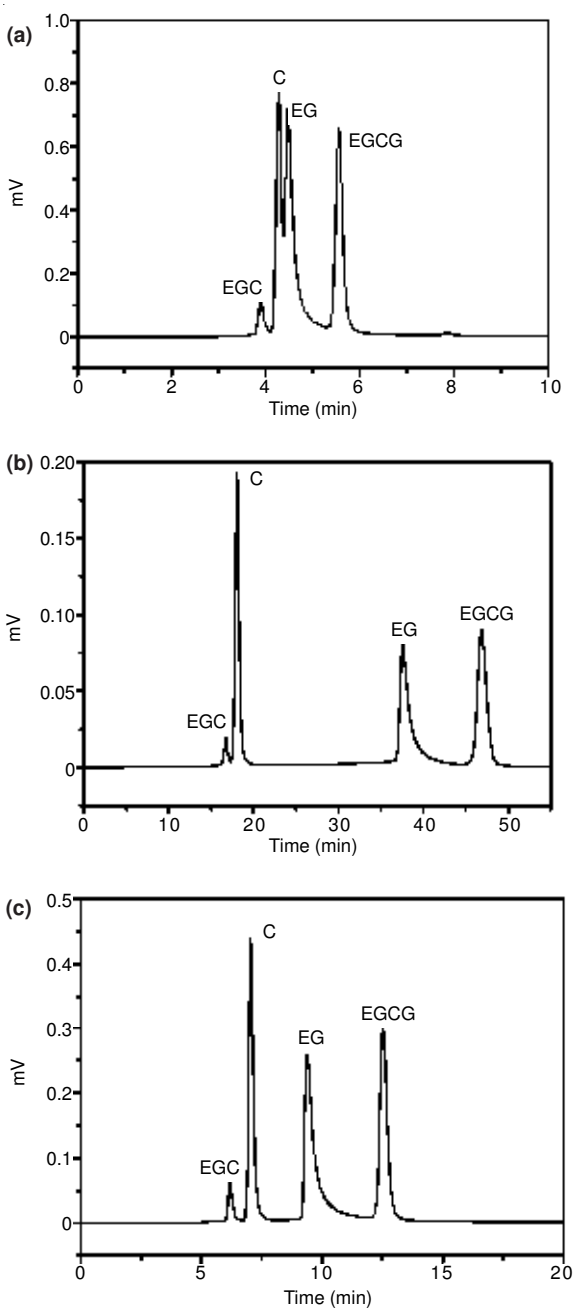

Fig. 2. Chromatographic separation of 4 catechins (column: 0.46 cm × 15 cm, C_{18}, 5 μm; flow rate: 0.5 mL/min; UV: 280 nm; injection volume: 10 μL; mobile phase: (a) 40 % methanol; (b) 20 % methanol; (c) 30 % methanol)

Adsorption isotherm: Mixtures of all the four catechins (+C, EC, EGC and EGCG) in the mobile phase were used as stock standard solutions at 0.25, 0.50, 0.75, 1.00, 1.25, 2.00,

2.50 mg/mL concentrations of each catechin. They were stored in vials at 4 ºC. The total catechin concentrations in these stock solutions were 1.0, 2.0, 3.0, 4.0, 6.0, 8.0, 10.0 mg/mL. To prevent oxidation, the standard solutions were used within 5.0 h of preparation.

Molecular imprinted polymers were packed in the cartridges and washed by methanol to remove the catechins template. Then 0.5 g MIP or NIP sorbent was added to 0.5 mL standard catechin mixture in a vial and shaken at room temperature for 6 h. The concentration of unabsorbed catechins was measured by HPLC until equilibrium adsorption was obtained. Total catechin adsorption on the sorbents was calculated by subtracting the concentration of unabsorbed catechins. Adsorption tests were conducted in the absence of light and oxygen.

Sample preparation: 5 g dry green tea was immersed for 24 h in 100 mL distilled water maintained at 80 ºC by an electric-heated thermostatic water bath. The extraction solutions were then evaporated to 20 mL to concentrate the solution. The mixture of solid leaves and extract solution was then separated by centrifugation at 10,000 × g for 5 min. The pigment extracts were filtered (0.2 μm), evaporated in nitrogen, redissolved in 30 % methanol mobile phase and stored at 0 ºC for further separation and analysis. All samples were protected from air and light to prevent oxidation or degradation. Repeatability was assessed by extracting green tea three times over a 5- day period. Two-sided *t*-tests assessed the data from independent samples[25].

Solid-phase extraction procedure: MIP and NIP sorbents (0.4 g) were separately packed into empty cartridges and sequentially preconditioned with 4.0 mL each of distilled water, ethanol and methanol. They were then dried at 50 ºC.

0.2 mL standard catechin solution (the total catechin concentration was slightly higher than the extract solution) was loaded into the SPE cartridges for 3 h and allowed to achieve adsorption equilibration. Subsequent washing and elution were carried out with water, ethanol, acetonitrile and *n*-hexane (1.0-4.0 mL) to optimize the separation conditions. The preconditioned green tea extract solution was then similarly extracted.

RESULTS AND DISCUSSION

Adsorption isotherms: A suitable SPE sorbent for separating catechins from green tea extract was found by measuring adsorption isotherms of the interactions between the target compounds and the sorbents (Fig. 3). Total catechin adsorption (Q/mg/g) on the polymer particles was determined as follows:

$$Q = \frac{(C_0 - C)V}{m} \quad (1)$$

where C_0 (mg/mL) is the initial concentration, C (mg/mL) the unabsorbed concentration, V (mL) the volume of the sample solvent and m (g) the mass of the sorbent particles.

The results of the adsorbed amounts shown in Fig. 3. According to the data, the adsorbed amounts of the total catechin compounds on catechin - MIPs and NIP decreased in the order of EGCG-MIP > EC-MIP > C-MIP > NIP, respectively. These results could be attributed to the multiple interactions between MIP or NIP particles and the catechins that might include hydrogen bonding, as well as dipole, H-p, electrostatic and inductive interactions.

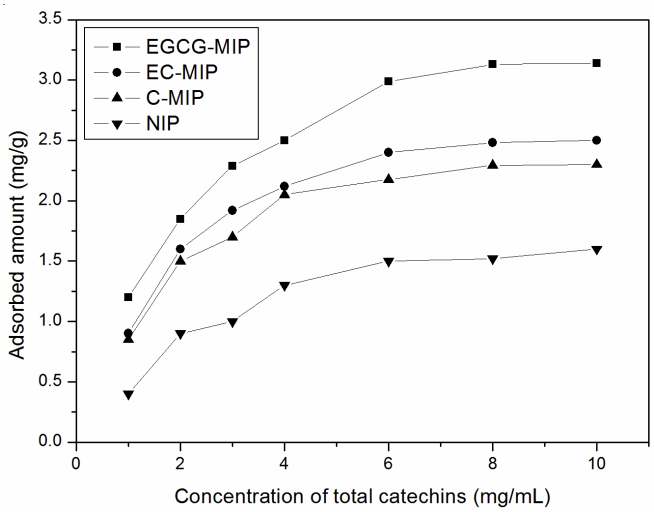

Fig. 3. Effect of the concentration of catechins on the adsorbed amount by 3-MIPs and 1-NIP

Firstly, between the MIPs and NIP, all the adsorbed amounts of the total catechins on catechin-MIPs were higher than that of NIP. The reason is that there are two main kinds of adsorptions between the target compounds and MIP or NIP particles: special selective adsorption proceeded from the "template core" and non-selective adsorption from "surface" of the particles. As known that hydrogen bonding or electrostatic force during self-assembly between the template and monomer mainly forms the selective binding sites, while the other part of MIP surface remains non-selective, *i.e.,* hydrophobic, the same as that of NIP. So, for MIPs, not only their "template core" but also "surface" could adsorb the catechins, while for NIP, it has only "surface" adsorption, so the adsorbed amount was lower than that of MIPs.

Secondly, among the four kinds of catechin-MIPs, EGCG-MIP had the highest adsorbed amount of the total catechins. The reason comes out of the differences of catechins' chemical structures, which formed different shape and size of "template core" in MIPs. (-) Epigallocatechin gallate has the larger spatial structure and more -OH than the other three (Fig. 1), which could form larger "template cores" during preparation of MIPs. These "template cores" have the ability of special selective adsorption. Furthermore, the main part of the four catechins is almost the same, so the EGCG-MIP could adsorb not only EGCG but also the other three catechins (C, EC and EGC). While C-MIP or EC-MIP could not adsorb EGCG, because EGCG has a larger space structure than the "template core" in C-MIP or EC-MIP.

Optimum SPE conditions: Comparing the adsorption isotherms of total catechins on the MIP and NIP particles, EGCG-MIP, with the highest adsorbed capacity, was selected for the SPE of catechins from green tea extract solution and then the SPE conditions were optimized.

Determination of loading volume by the adsorption isotherm: Total catechin adsorptions on the sorbents were investigated by plotting adsorption isotherms for the standard catechins. Experimental data were fitted to the following adsorption isotherms[26,27]:

$$Q = aC + b \quad (2)$$

$$Q = \frac{dC}{1+eC} \quad (3)$$

$$Q = fC^{1/g} \quad (4)$$

where, C (mg/mL) is the equilibrium concentration of the solute in the liquid phase and Q (mg/g) is the adsorbed amount of the solute in the solid phase. a, b, d, e, f and g are parameters. Linear (2), Langmuir (3) and Freundlich (4) adsorption isotherm models were used.

The coefficients of determination (r^2) in Table-1 show that the Langmuir equation was most suitable.

The theoretical loading volume was determined from the following equations:

$$Q_{max} = mQ \quad (5)$$

$$V_{max} = \frac{Q_{max}}{C} \quad (6)$$

In this experiment, 0.4 g SilprEMIm was packed into the SPE cartridge (m = 0.4 g). The data in Table-1 (d = 1.77, e = 0.45) were fitted to the Langmuir eqn. 3 of EGCG-MIP, giving a maximum theoretical loading volume of:

$$V_{max} = \frac{0.4 \times 1.77}{1+1.45C} \quad (7)$$

where, Q_{max} (g) is the total mass of adsorbed target compounds, C (mg/mL) the concentration of catechins in the operating solution and V_{max} (mL) the SPE cartridges' maximum theoretical loading volume of extraction solution.

Eqn. 7 and the total concentration of catechins in the operating solution (0.5 mg/mL) give an optimal theoretical loading volume of 0.58 mL. 0.5 mL green tea extract solution was therefore loaded into SPE cartridges containing EGCG-MIP without leakages. The system was then allowed to equilibrate for 6 h.

Washing and elution with different solvents: Washing and elution were optimized. First, washing solvents of different polarities (water, ethanol, acetonitrile and *n*-hexane) were investigated. Water could not wash out the catechins from catechin-MIP but NIP, making it suitable to remove the water-soluble interferences from the green tea extract solutions. 0.2 mL catechin standard solution was loaded into the cartridges and then eluted by 1 mL methanol. Catechins were diluted by the eluting but their total mass was not reduced, indicating that methanol almost completely eluted the catechins absorbed by the MIPs and NIP (Fig. 4).

Therefore, the following procedure was considered optimal to separate catechins from green tea extract. First, the pretreated green tea extract should be loaded into a cartridge containing EGCG-MIP particles. Interferences should be washed out with water, leaving the catechins able to be eluted by 1.0 mL methanol (Fig. 5). The recovery of EGC, +C, EC and EGCG were 73.2, 91.7, 80.5 and 86.9 %, respectively.

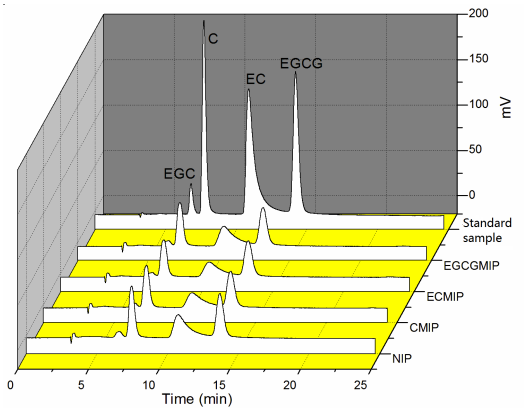

Fig. 4. Chromatographic of 4 catechins after loading to cartridges and elution with methanol (loading volume: 0.2 mL, washing volume: 1 mL)

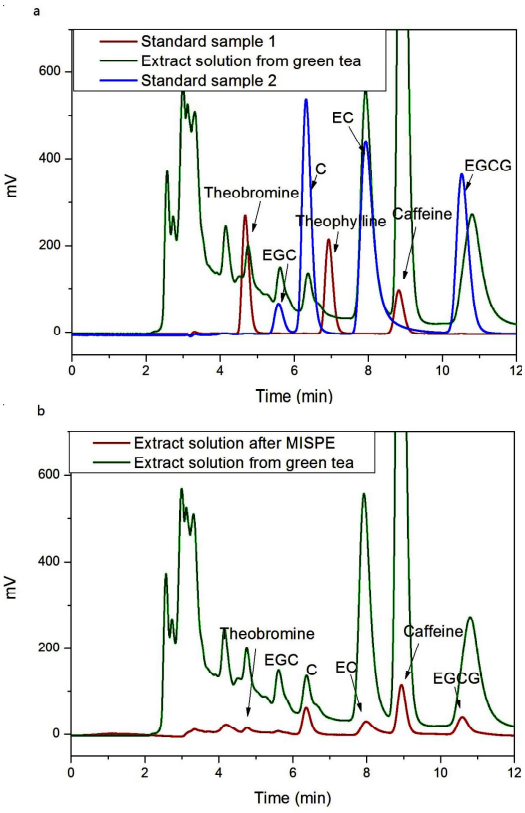

Fig. 5. Chromatograms: (a) extract solution from green tea; standard sample 1: mixture of theobromine, theophylline and caffeine; standard sample 2: mixture EGC, C, EC and EGCG; (b) SPE of catechins by EGCG-MIP. (Column: 0.46 cm × 15 cm, C_{18}, 5 μm; flow rate: 0.5 mL/min; UV: 280 nm; injection volume: 10 μL; mobile phase: 30 % methanol)

TABLE-1
PARAMETERS IN ADSORPTION ISOTHERMS OF CATECHINS ON MIPS AND NIP

	Linear eqn. 2			Langmuir eqn. 3			Freundlich eqn. 4		
	a	b	R^2	d	e	R^2	f	1/g	R^2
EGCG-MIP	0.20	1.46	0.831	1.77	0.45	0.992	1.44	0.37	0.944
EC-MIP	0.15	1.24	0.755	1.56	0.50	0.981	1.21	0.35	0.899
C-MIP	0.14	1.16	0.747	1.47	0.52	0.974	1.13	0.34	0.892
NIP	0.12	0.60	0.797	0.68	0.31	0.965	0.62	0.44	0.904

Validation of proposed analytical method: Calibration curves were constructed using duplicate injections of seven concentrations in the range of 0.92-42.0 μg/mL for the standard and had coefficients of determination greater than 0.97, with regression equations (Table-2). The precision of the method, like repeatability, was evaluated using the relative standard deviation (RSD) of catechins. Twelve replicates of the same sample prepared on the same day were determined. The result was 0.32 %, sufficient for the routine analysis of catechins. The detection limit for catechins at three times the basis of signal-to-noise ratio was 32.0 ng/mL, confirmed that these values were of acceptable precision and accuracy.

TABLE-2
CALIBRATION EQUATIONS OF CATECHIN COMPOUNDS

Compounds	Equation	R^2
EGC	$Y = 1.170 \times 10^{-3}$ X-0.0283	0.9852
C	$Y = 1.767 \times 10^{-4}$ X-0.0643	0.9756
EC	$Y = 1.135 \times 10^{-4}$ X-0.0109	0.9728
EGCG	$Y = 1.584 \times 10^{-4}$ X-0.0511	0.9876

X: Peak area (mAU × s); Y: concentration of catechin compounds (mg/mL).

Conclusion

In this study, some catechin compounds, (+) catechin (+C), epicatechin (EC), (-) epigallocatechin (EGC) and (-) epigallocatechin gallate (EGCG) were extracted from green tea by hot water and then separated by molecular imprinted polymers (MIP) as sorbent materials in a solid-phase extraction (SPE) process. For synthesis of MIP, catechins were employed as the template, methacrylic acid as the monomer, ethylene glycol dimethacrylate as the cross-linker and α,α′-azobis(isobutyronitrile) as the initiator. A comparison of the adsorption isotherms of total catechins between catechin-MIPs and non-template imprinted polymer (NIP) were also investigated. According to the adsorption isotherms, a suitable catechin-MIP sorbent was selected and successfully applied for separation of catechin compounds from green tea extract solutions using optimized SPE procedure.

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