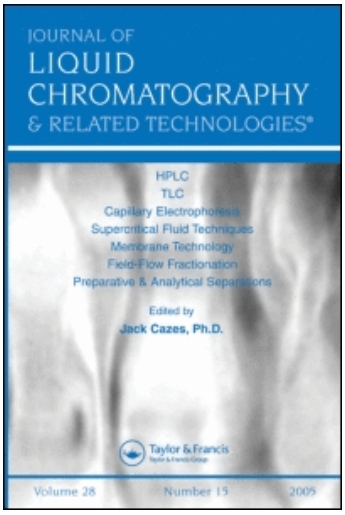


## Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

# The Use of Molecularly Imprinted Polymer for Selective Extraction of (+)-Catechin

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Online publication date: 09 July 2004

JOURNAL OF LIQUID CHROMATOGRAPHY & RELATED TECHNOLOGIES®
Vol. 27, No. 17, pp. 2715–2731, 2004

# The Use of Molecularly Imprinted Polymer for Selective Extraction of (+)-Catechin

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## ABSTRACT

Molecularly imprinted polymer (MIP) was prepared using acrylamide and ethylene glycol dimethacrylate (EDMA) as functional and cross-linking monomers, respectively. (+)-Catechin (C) was chosen as the template molecule and solvent acetonitrile as porogen. The polymer was investigated as solid-phase extraction (SPE) sorbent for the clean-up of organic extracts of green tea. Specific binding capacity of prepared MIP was evaluated by comparison of specifically and nonspecifically [on blank polymer (BP)] bound amounts of C. Different media were tested for this purpose: acetonitrile, methanol, and water and the results were compared. The best specific capacity (3.84 μg/100 mg of MIP) was obtained in acetonitrile, which was used as porogen. This solvent was chosen for the next evaluation of the binding capacity of polymer

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DOI: 10.1081/JLC-200029276 1082-6076 (Print); 1520-572X (Online)

for the other six catechins that occur in green tea. The MIP showed good selectivity towards C in the presence of structurally related compounds. Next, several washing solvents were applied to the developed SPE procedure with the aim to obtain maximum recovery for C. Acetonitrile, water, and methanol, with different additions of acetic acid, were used for this purpose. It was observed that water and acetonitrile are suitable for washing out of interferences, and the best elution solvent was methanol without any presence of acid. Very good recovery (95%) for C was obtained using the developed molecularly imprinted SPE (MISPE) procedure. The results of the presented work show that prepared MIP can be used as SPE sorbent for the clean-up of methanol extract of green tea.

*Key Words:* MISPE; Catechins; Tea; HPLC.



## INTRODUCTION

Molecular imprinting (MI) is a technique used for the preparation of polymers with synthetic recognition sites having a predetermined selectivity for a specified analyte. The imprint is obtained by the polymerization of functional and crosslinking monomers in the presence of a template (target) molecule. The resultant imprints possess a steric (size and shape) and chemical (spatial arrangement of complementary functional groups) memory for the template molecule. Removal of the template from the polymer matrix creates vacant recognition sites that enable the polymer to selectively rebind the imprint molecule from a mixture of closely related compounds.[1]

In general, MI can be achieved in two ways. The first approach is covalent MI, in which polymerizable derivates of the template molecule are copolymerized with a crosslinking monomer. These derivates are obtained by forming covalent bonds between the template and suitable polymerizable monomers. To remove the template from the polymer, these covalent bonds have to be chemically cleaved. The second, noncovalent, approach is based on the formation of a prepolymerization complex between monomers carrying suitable functional groups and the template, through noncovalent bonds such as ionic interactions or hydrogen-bonding. The template can be removed after the polymerization simply by solvent extraction.[2]

Since molecularly imprinted polymer (MIPs), as separation materials, provide good selectivity, which is their most widely investigated use, their use as preconcentration and clean-up sorbents in molecularly imprinted SPE (MISPE) has recently been evaluated. MISPE can be based on selective adsorption, as can be expected in the extraction of analytes in media of low polarity, or selective desorption, as in the extraction of hydrophobic analytes

from aqueous media. In the aqueous media, analytes are nonspecifically adsorbed, and a selective washing is needed before the elution to disrupt nonspecific interactions between the analytes and the polymeric matrix.[3]

MIPs were used as SPE sorbents in many applications, including clinical samples,[4–10] extracts of plants,[11–13] and chewing gum,[14] or in beverage samples.[15] The authors[10–12] prepared MIP using quercetin as a template molecule. Quercetin is a member of the family of flavonoids and its chemical structure and properties are similar to catechins, which are studied in the presented paper.

Catechins (flavanols) also rank among flavonoids and form the major tea components. They exhibit strong antioxidant activity, antibacterial, antiviral, and antiallergenic properties.[16] Seven of them [(+)-catechin (C), (−)-gallocatechin (GC), (−)-epigallocatechin (EGGC), (−)-epicatechin (EC), (−)-epigallocatechin gallate (ECG), (−)-gallocatechin gallate (GCG), (−)-epicatechin gallate (ECG)], were investigated in the presented work. These phenolic antioxidants contain hydroxyl groups, which enable the creation of hydrogen-bonding. In this study, MIP was prepared by noncovalent approach in solvent acetonitrile using (+)-catechin as template. MIP was evaluated in the sense of its specific binding capacity towards all catechins, and was applied for extraction of target molecule from methanolic tea extracts.



## EXPERIMENTAL

### Chemicals and Solutions

Acrylamide, ethylene glycol dimethacrylate (EDMA), and azobisisobutyronitrile (AIBN) were bought from Merck (Slovakia). A Standard of C was supplied by Fluka (Switzerland) and standards of EC, GC, EGC, GCG, ECG, and EGCG were purchased from MGP (Czech Republic). Acetonitrile and methanol (both HPLC grade) and triethylamine (TEA) were from Merck (Slovakia). Formic acid (p.a.), acetic acid (p.a.), and acetone (p.a.) were supplied by Lachema (Czech Republic).

Stock solutions of standards were prepared in methanol and stored in a freezer at $-20^{\circ}$C. Working solutions were prepared by diluting the stock solutions with formic acid (0.28%).

### Equipment

The HPLC analyses were performed using an HP 1100 system (Hewlett-Packard, Waldbronn, Germany), which includes a pump with a degasser,

a diode-array detector (DAD), and an HP ChemStation. Alltima-Rocket C18 ($53 \times 7\,mm^2$, 3 μm) (Alltech, Belgium) with guard column Separon SGX C18 ($10 \times 4\,mm^2$, 7 μm), Watrex (Slovakia) were used for chromatographic separation of compounds. Analytes were separated by means of gradient elution with mobile phase consisting of acetonitrile and 0.28% formic acid. The mobile phase composition during analysis is in Table 1. The injection volume was 20 μL and the analyses were carried out at laboratory temperature (20°C). DAD worked in the range of 200–400 nm and the chromatograms were acquired at wavelengths of 270 and 280 nm.

Solid-phase extraction (SPE) was performed by means of Vacuum Manifold from Alltech (Belgium), and solvent extraction was realized in a ultrasonic bath Sonorex, Bandelin electronic (Germany).



## Preparation of the MIP

The monomer acrylamide (6 mmol), the template molecule catechin (1.2 mmol), and the porogen acetonitrile (10 mL) were placed into glass tube. Then crosslinker EDMA (30 mmol) and initiator AIBN (30.0 mg) were added. The ratios of reagents for the preparation of polymers are taken.[10] The polymerization was carried out in a water bath at 60°C for 24 hr. The prepared polymer was passed through a 80-μm sieve, fine particles were removed by flotation in acetone, and the final product was dried under vacuum. Non-imprinted, control blank polymer (BP) was prepared and treated in the same manner as in the case of MIP, but in the absence of template molecule during polymerization. The template was removed from the imprinted polymer by using ultrasonication in methanol/acetic acid (9 : 1) for 3 hr. A fresh extraction mixture was added every 30 min. Before using, 100 mg of MIP placed into cartridge was washed with 20 mL of the same mixture in the polypropylene cartridge for sufficient removing of template, and then acetic acid was washed out with 5 mL of methanol. After washing, sorbent was dried by means of vacuum and thereafter it was ready for the use.

***Table 1.*** Gradient elution system.

| Time (min) | 0.28% Formic acid (%) | Acetonitrile (%) |
|---|---|---|
| 0 | 88 | 12 |
| 5 | 70 | 30 |

## Evaluation of MIP and BP Towards C

### Determination of the Cartridge Capacity

A 100 mg of dried polymer was placed into polypropylene cartridge. The capacity was tested in different solvents: methanol, acetonitrile, acetonitrile with addition of formic acid (3 μL/1 mL of acetonitrile), acetonitrile with addition of TEA (2 μL/1 mL of acetonitrile), and water. Firstly, the polymer was washed with 5 mL of methanol and then with 5 mL of solvent in which the capacity was studied. Then, aliquots (0.5 mL) of solution of catechin with different concentration levels for particular tested solvents were applied gradually on MIP until release was detected. For methanolic solution, 0.25 μg/mL concentration of catechin was used, in acetonitrile concentration 0.5 μg/mL was applied, for the mixtures acetonitrile/formic acid and acetonitrile/TEA concentration was 0.25 μg/mL, and concentration of catechin in aqueous solution was 2.0 μg/mL. The same solvents and concentration levels, except acetonitrile/TEA mixture, were applied on BP in the same manner.

### Washing Solvents

The choice of washing solution was realized as follows: 100 mg of MIP or BP were packed into polypropylene cartridge. Sorbent was washed with 5 mL of solvent, which was used in the washing step, and then with 5 mL of acetonitrile. Next, acetonitrile solution of catechin (volume of 1 mL with concentration of 2 μg/mL) was applied onto the polymer. The same procedure was used for the BP and concentration of catechin in acetonitrile was 0.5 μg/mL. The cartridge was washed with following solvents: methanol, acetonitrile, water, and methanol with different addition of acetic acid (5%, 10%, 15%). Each effluent was collected and analyzed by HPLC.

## Evaluation of MIP and BP Towards all Catechins

### Determination of the Cartridge Capacity

The capacity of MIP towards all studied catechins was determined in acetonitrile. The concentration of C was 1 μg/mL and of the other catechins were 0.5 μg/mL. The volume of aliquots and procedure of application were the same as described in Section 2.4.1. The concentration of all catechins in acetonitrile was 0.2 μg/mL when they were applied on BP and the procedure was the same as for the MIP.

Washing Solvents

The choice of washing solvent was conducted as described in evaluation towards C section. Concentrations of compounds in acetonitrile were: GC, EGC, and EC 0.75 μg/mL, EGCG, GCG, and ECG 0.2 μg/mL, and for C it was 1 μg/mL. The concentration levels were chosen with regard to cartridge capacity towards particular compounds. There were applied 2 mL of this solution onto the cartridge and acetonitrile, methanol, and water were tested as the washing solvents.

## Preparation of Green Tea



A sample of tea (1 g) was ground to powder and extracted with 10 mL of methanol in an ultrasonic bath Sonorex (Bandelin electronic, Germany). There were six extraction steps, and each step took 30 min. The extraction was performed at the temperature of 25°C. The extracts were filtered through nylon microfilters Tessek (Czech Republic) prior to injection into the HPLC column.

The MIP (200 mg) was placed in a polypropylene cartridge and conditioned with 10 mL of methanol, 10 mL of water, and finally with 10 mL acetonitrile. A 20 μL of the methanolic extract was evaporated to dryness and redissolved in 10 mL of acetonitrile. Each diluted extract (5 mL) was slowly applied onto the conditioned MIP. The cartridge was washed with 3 mL of water and then with 3 mL of acetonitrile. Elution was performed with 5 mL of methanol. All effluents were collected, evaporated to dryness, reconstituted in the mixture of acetonitrile/0.28% formic acid (1 : 9), and analyzed by HPLC.

# RESULTS AND DISCUSSION

## Evaluation of MIP and BP Towards Catechin

Determination of the Cartridge Capacity

As it was described above, the capacity of MIP was evaluated in different solvents. The same attempts as on MIP were performed on BP and results are shown in Table 2. As is obvious, the best specific recognition of analyte was in acetonitrile, which was used as porogen.

***Table 2.*** Capacity of the MIP, BP and specific bonding capacity for C in different solvents.

| | Capacity (μg C/100 mg sorbent) | | |
|---|---|---|---|
| Solvent | MIP | BP | Specific[a] |
| Acetonitrile | 4.35 | 0.51 | 3.84 |
| Methanol | 0.10 | 0.05 | 0.05 |
| ACN/formic acid (1 mL/3 μL) | 2.84 | 0.48 | 2.36 |
| ACN/triethylamine (1 mL/2 μL) | 0 | 0 | 0 |
| Water | 93.9 | 93.9 | 0 |

*Note*: RSD values for capacity were 2.0–5.1% ($n = 3$).
[a]The specific capacity was calculated by the deduction of the amount nonspecifically adsorbed on BP from the amount adsorbed on the MIP.



Acetonitrile has weak hydrogen-bonding capacity and limited ability to compete for the hydrogen-bonding sites on the template or the binding sites. That is why it enables catechin to be specifically retained by hydrogen-bonding in the cavities created during polymerization process. However, we suppose that low capacity in methanol is because of hydrogen-bonding of this solvent with catechin, and then such medium does not allow analytes to bind on the specific sites. The situation is different in the aqueous medium, where the retention is due to the hydrophobic effect. Hydrogen-bonding, which is formed in water, causes catechin to be molecules complexes that are larger than single catechin molecules. They are not present in such form, as the case of acetonitrile, in which the polymerization and creation of cavities was conducted and where no hydrogen-bonding was formed. In aqueous environment, analytes and all hydrophobic compounds are non-specifically adsorbed. The addition of formic acid into acetonitrile caused a decrease of capacity of MIP. Added acid forms hydrogen-bonding with catechin and causes its release. In the medium of acetonitrile/TEA, it is not possible to retain any amount of catechin because of its instability in the alkaline solution and it decomposes in a few minutes.[17]

## Washing Solvents

Three solvents were tested with different polarities: acetonitrile, water, and methanol. Water was tested because of the washing-out of matrix components hydrophilic properties. Acetonitrile was applied as washing solvent because the specific binding of catechin on MIP is the highest in its

environment. Methanol was chosen as solvent, which was supposed to wash out retained catechin with high recovery, because in this medium almost nothing was specifically bound. After acetonitrile and water application, no amount of catechin was washed out from the cartridge filled with MIP. This means that recovery is nought for these two solvents, and it is possible to use them to remove interferences; but they are not suitable for the final elution of retained compound. However, when acetonitrile was applied onto BP, the release of catechin from the cartridge was observed, as is obvious from Fig. 1. It proves that catechin was nonspecifically adsorbed on the BP because acetonitrile which does not form hydrogen-bonding, is able to wash out catechin from polymer sorbents. In the case of water application on the BP, the situation was similar as for acetonitrile, and approximately 50 % of catechin was washed out with 5 mL of water.

Almost the total elution of catechin was observed after methanol washing. Different additions of acetic acid in methanol were tested (5%, 10%, 15%) and the influence of acid on the recovery was investigated. As is obvious from Fig. 2, the amount of catechin eluted decreased to ca. 73% by the addition of 5% of acid, and each increase of acid content caused other decreases of recovery. When elution solvent contained 15% of acid the recovery was only 65%. This trend can be explained by suppressed dissociation of hydroxyl groups of catechin after addition of acid. Catechin was presented in the same



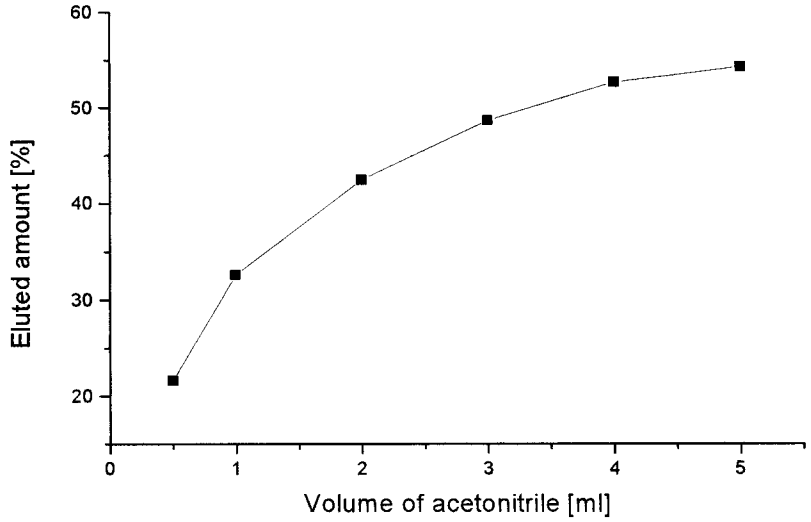


***Figure 1.*** The relationship between volume of acetonitrile and percentage of eluted amount of catechin.

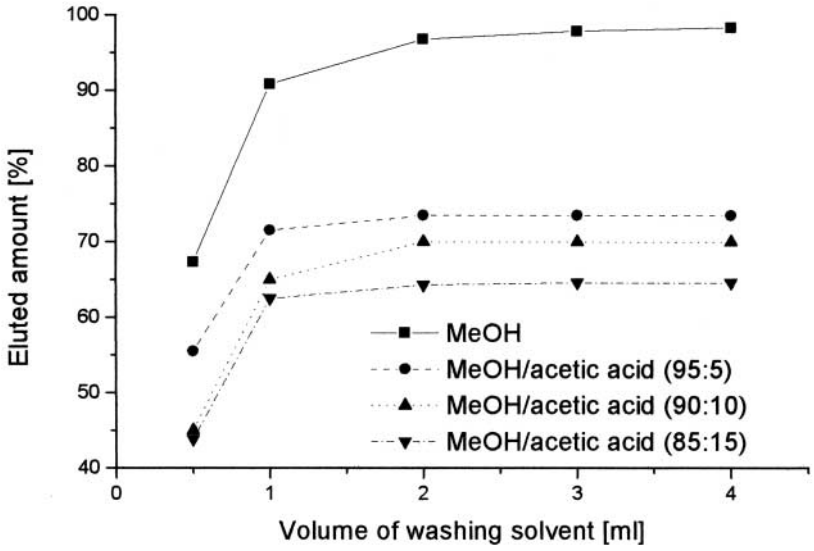


*Figure 2.* The relationship between volume of elution agent with different addition of acetic acid and percentage of eluted amount of catechin.



form as in acetonitrile and part of it remained specifically bound in the cavities of the MIP. However, the influence of acid on the recovery was not observed in the case of the BP.

## Evaluation of MIP and BP Towards all Catechins

### Determination of the Cartridge Capacity

Selectivity of prepared MIP was studied by investigation of its ability to selectively adsorb C in the presence of structurally related compounds. For this purpose, the other six catechins, which occur in green tea were chosen. Capacity of MIP and BP were determined for each studied compound. Acetonitrile was chosen as medium for the adsorption because the amount of specifically adsorbed catechin was the highest in this solvent. The results are summarized in Table 3.

As is obvious from the presented results, the best specific capacity was achieved for C. The value was lower when the mixture was applied than in the case of application of C without presence of other structurally related compounds. These compounds occupied part of active sites and that is why this

***Table 3.*** Capacity of MIP, BP and specific bonding capacity for the mixture of all compounds in acetonitrile.

| | Capacity (μg compound/100 mg sorbent) | | |
|---|---|---|---|
| Compound | MIP | BP | Specific[a] |
| GC | 1.95 | 1.93 | 0.02 |
| EGC | 1.95 | 1.91 | 0.04 |
| C | 3.97 | 0.35 | 3.62 |
| EC | 1.45 | 0.40 | 1.05 |
| EGCG | 0.35 | 0.25 | 0.10 |
| GCG | 0.38 | 0.38 | 0 |
| ECG | 0.35 | 0.35 | 0 |

*Note*: RSD values for capacity were 2.1–5.6% ($n = 3$).
[a]Calculated as in the previous case.



decrease was observed. The MIP showed relatively high capacity also for EC, because it is the epimer of template molecules (See structures, Fig. 3). Very low specific binding was observed in the case of GC and EGC, and this means that most amounts of them are sorbed nonspecifically. They contain one more hydroxyl group than C, and therefore, their low capacity can be explained by the lack of functional groups in the cavity providing possible interaction with molecule. Very low or no ability of MIP to bind specifically gallates (EGCG, GCG, ECG) is due to their molecule size. They are formed with "catechin" part and "gallate" partly because they contain remainders of gallic acid. So, they have larger size than template molecules, and size-exclusion effect contributes to the selective binding of C. Results from above indicate that MIP showed good selectivity towards C, which was used as template.

## Washing Solvents

Likewise as above, different washing solvents (acetonitrile, water, and methanol) were tested also for catechins mixture. Volume of 5 mL of each solvent was applied. The influence of solvent volumes on recovery of compounds was investigated. Figures 4–6 show the relationship between percentage cumulative recovery of catechins amount sorbed onto MIP and the volume of washing solvent. It is obvious from Fig. 4 that 5 mL of acetonitrile washed out less than 5% C, GC, and EGC, whereas the same amount of solvent washed out approximately 10% of EC and GCG. Higher recovery (about 50%) with acetonitrile as washing solvent was obtained for EGCG.

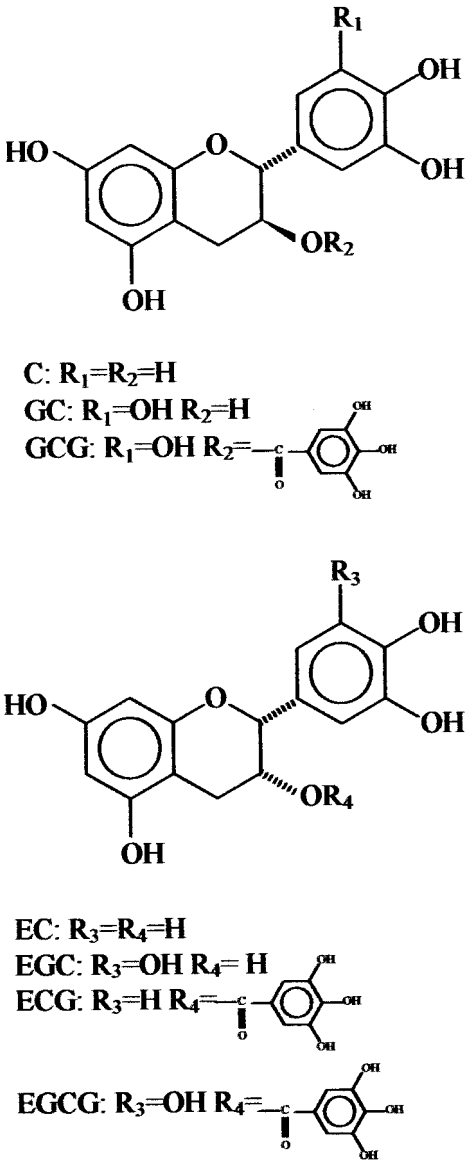


*Figure 3.* Chemical structures of studied catechins.

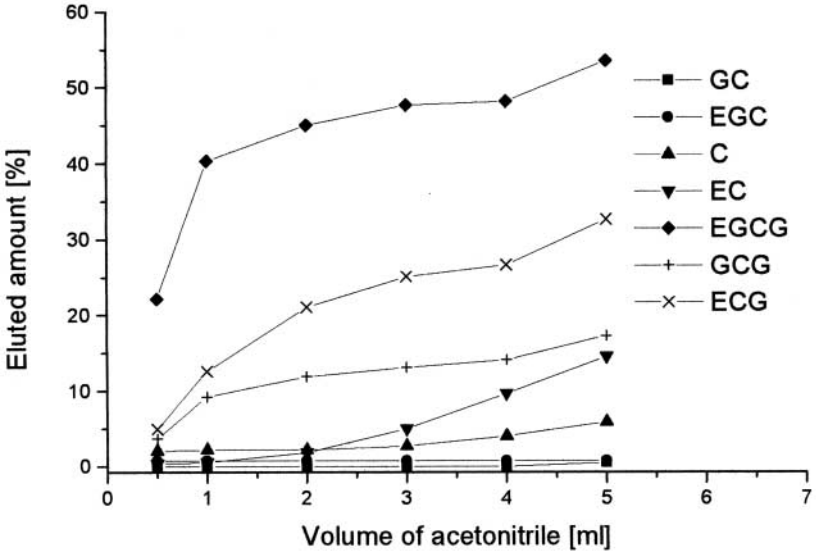


*Figure 4.* The relationship between volume of acetonitrile and percentage of eluted amount for all catechins.

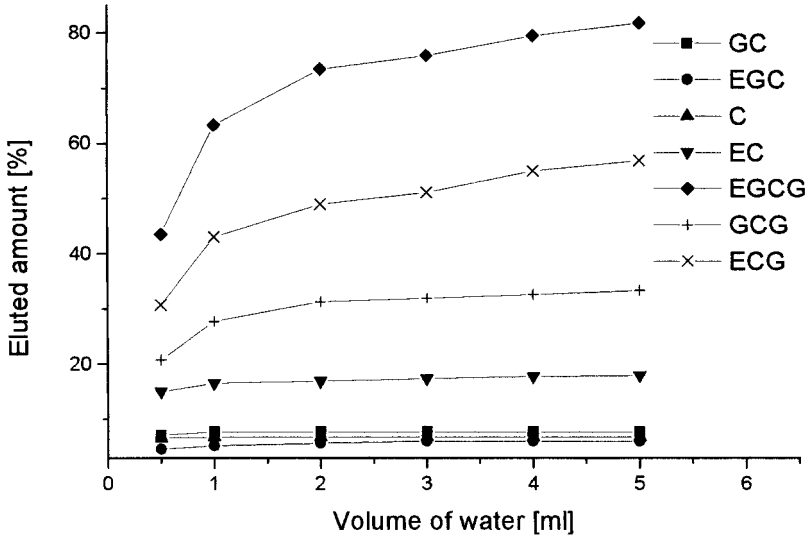


*Figure 5.* The relationship between volume of water and percentage of eluted amount for all catechins.

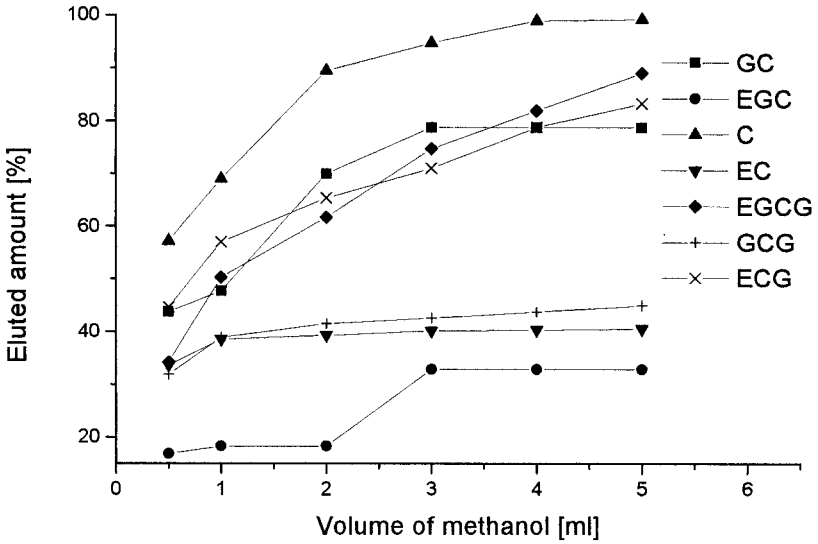


*Figure 6.* The relationship between volume of methanol and percentage of eluted amount for all catechins.

The part of C washed out with acetonitrile was nonspecifically adsorbed because of the presence of structurally related compounds, which occupied part of active sites. Similar results were observed with water as a washing solvent, and volumes of 5 mL washed out ca. 7% of C, GC, and EGC, and the best washing (30–80%) were again obtained for gallates (EGCG, GCG, ECG) (Fig. 5).

However, methanol was the most effective elution agent because the highest recoveries were obtained for all catechins in comparison with previous solvents. Application of 5 mL of methanol was sufficient for elution of almost the whole adsorbed amount of C. Relatively low recovery (30–40%) was obtained in the case of EGC, EC, and GCG (Fig. 6). So, it is suitable to use acetonitrile and water as washing solvents for removal of structurally related compounds and for selective extraction of C. However, amount of GC and EGC washed out with these two solvents was low (similar to the amount of C), but it is not a problem as their recovery in the elution step with methanol was lower than for C, which was eluted almost in 100%.

Mentioned solvents were tested on the BP and the recoveries for GC, EGC, and all gallates were similar as on the MIP after application of 5 mL of agents. More than 50% of the adsorbed amount of catechin was washed

out with 5 mL of acetonitrile and ca. 30% with 5 mL of water. Behaviour of all catechins, except C, was very similar on imprinted and non-imprinted polymer. It means that these compounds are to a large extent bound nonspecifically on the polymer matrix.

## Analysis of Green Tea Extract

Samples of green tea were prepared according to preparation of green tea section. At the beginning, the contents of catechins in diluted methanolic extract (see Experimental) were determined and the results are summarized in Table 4 as amounts of catechins applied on MIP. Then, extract was cleaned up using SPE on MIP. Preparation of cartridge, application of sample, washing, and elution solvents is described in Experimental. After sample application, the cartridge was washed with water to remove hydrophilic components of tea sample. Next, acetonitrile was applied to wash out compounds, which were not removed sufficiently with water. Each effluent was analyzed and amounts of washed catechins were calculated. Elution was performed by means of methanol. Amounts of catechins applied onto cartridge, sorbed amounts, amounts which were washed out with water and acetonitrile



***Table 4.*** Applied, sorbed, washed, and eluted amount and recovery of all catechins from tea extract on the MIP.

| | Amount of compound (μg) | | | | | |
|---|---|---|---|---|---|---|
| | | | Washed out | | | |
| Compound | Applied on MIP[a] | Adsorbed on MIP[b] | Water | Acetonitrile | Eluted with methanol | Recovery (%) |
| GC | 1.35 | 1.34 | 0 | 0.17 | 0.11 | 8.2 |
| EGC | 4.26 | 3.90 | 0.33 | 0.12 | 0.35 | 8.2 |
| C | 1.26 | 1.26 | 0.06 | 0.01 | 1.20 | 95.2 |
| EC | 2.84 | 2.84 | 0.35 | 0.31 | 2.20 | 77.5 |
| EGCG | 18.21 | 0.65 | 0.29 | 0.26 | 0.10 | 0.6 |
| GCG | 0.28 | 0.28 | 0.02 | 0.06 | 0.03 | 10.7 |
| ECG | 5.52 | 0.80 | 0.20 | 0.30 | 0.25 | 4.5 |

*Note*: RSD values for recovery were 1.9–4.5% ($n = 3$).

[a]Determined in diluted methanol extract before SPE.

[b]Calculated as the difference between applied amount and amount determined in the effluent after sample application.

in the washing step, and amounts of catechins eluted with methanol in the final step, are summarized in Table 4. The recovery was calculated as the ratio of amount of compound eluted by methanol and amount applied on the MIP.

It can be seen from the presented results that the best recovery was obtained for C, which was used as the template molecule; EC also had relatively high recovery. These two molecules are structurally the most related compounds, and therefore, EC was extracted with recovery $>77\%$. Low values for EGCG and ECG are due to low selectivity of MIP towards these two catechins. However, the recovery of the third gallate (GCG) is slightly higher than of the other two. Capacity of MIP for this compound is also low but its content in tea extract was much lower than in the case of EGCG and ECG, and therefore, the calculated recovery was higher. Figure 7 demonstrates chromatogram of methanolic extract before SPE (a) and after SPE (b). It is obvious that most of interfering compounds were removed



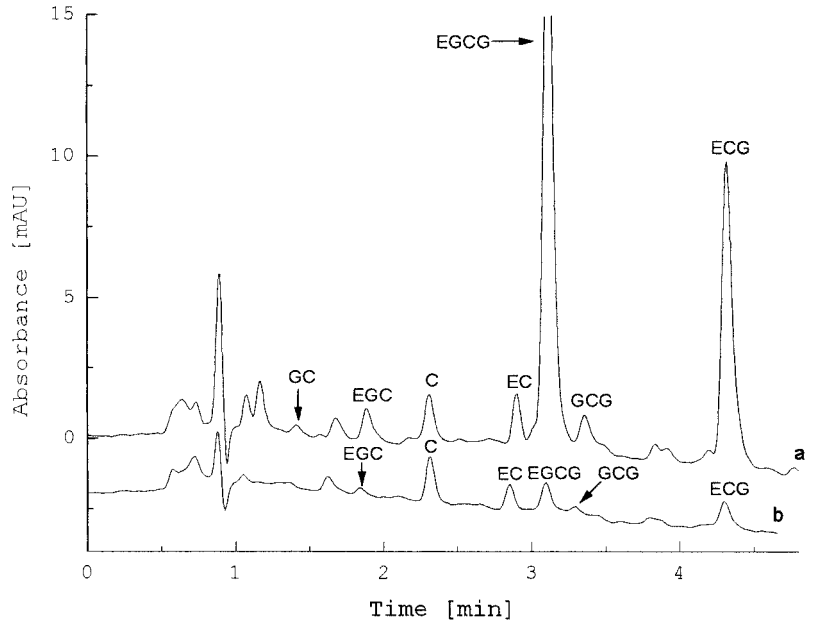


***Figure 7.*** HPLC chromatogram of methanolic extract of green tea before (a) and after (b) MISPE. HPLC column: Alltima-Rocket C18. Mobile phase: acetonitrile : formic acid (0.28%), gradient elution, $F = 2\,\text{mL/min}$. Detection: DAD, 280 nm. Injected volume: 20 μL.

from the tea extract during SPE procedure and so, it is effective clean-up and preconcentration technique for such matrices.

It can be concluded that prepared MIP shows good selectivity for target molecule C. MIP is able to retain interferences, which are structurally related to the template, but most of them are sorbed nonspecifically on polymer matrix, and washing with water and acetonitrile allows their removal. During washing step, the loss of catechin was $<6\%$ and the recovery of the whole SPE procedure was $95.2\% \pm 1.8\%$.

## ACKNOWLEDGMENT

This research was supplied by the Slovak Grant Agency for Science, VEGA, Grant No. 1/9128/02.



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Received May 24, 2004
Accepted June 30, 2004
Manuscript 6405