Focusing Review

Development and Applications of Fragment Imprinting Technique

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Abstract

In analyses of chemicals, selective separation is strongly desired for high accuracy and sensitivity. In order to separate selectively targeting compounds from complex matrices, molecular imprinting (MI) technique is believed to be one of the most promising techniques. The polymer-based materials prepared by MI have the selective recognition ability for targeting compounds like as "lock-and-key model". However, the technique is hard to apply for toxic compounds and rare compounds because the targeting compounds are required directly for the template molecules in the process of preparation of the molecularly imprinted materials. Therefore, we developed alternative technique by the name of Fragment Imprinting Technique (FIT) in which the targeting compounds are not necessary. In FIT, the pseudo-template molecules are utilized, and the imprinted materials have selective recognition ability for a part of characteristic chemical structure on the targeting compounds. Additionally, FIT was applied for the selective separation of the environmental pollutants and natural toxins.

Keywords: molecular imprinting, fragment imprinting, selective separation

1. Introduction

Selective separation technologies are very important for the purification and/or quantitative analysis of toxins, drugs, biological materials, and chemicals. In general, it is difficult to achieve selective separation of target compounds from environmental or biological samples due to the complex nature of the sample matrices that interfere with the separation and detection of targeting compounds in these samples. Therefore, we urgently require a separation methodology with built-in selectivity for the targeting compounds.

Molecular imprinting (MI) technique is one of the most attractive methods to achieve the selective recognition abilities [1, 2]. In this technique, the crosslinking agent, polymerization initiator,template molecule, and functional monomer which can be interacted with the template molecule are simultaneously polymerized. Then, the template molecules are removed from the prepared polymer by washing with organic solvent as well as buffered solution. As a result, selective recognition based on the imprinting effect can be achieved. Usually, non-covalent bonding such as hydrogen bonding, ionic interactions, and hydrophobic interactions are utilized in the preparation of molecularly imprinted polymers (MIPs). This technique is relatively easy so that several approaches have been reported such as artificial antibodies, biosensors, and stationary solid phases in liquid chromatography (LC) [1-4].

However, usual MI technique has some problems for applications involving environmental samples and/or biological samples. For example, highly toxic or rare compounds cannot be utilized for target as template molecules since the targeting molecule itself is used in the process of the preparation of MIPs. Another serious problem is the leakage of template molecules. In the general procedure, the template molecule cannot be removed completely from the MIPs even after exhaustive repeated washing with organic solvents as well as buffered solutions. This is because the imprinted sites can be formed not only on the surface but also deeply inside the crosslinked polymer network where it is difficult for the solvent reaching [5]. Therefore, contrary to expectations, the template molecules may leak from the inside of the polymer: this becomes a serious problem and interferes with the correct quantitative determination and purification.

In this paper, author presents their recent studies focusing on the alternative MI techniques (Fragment Imprinting Technique: FIT) which can overcome the above-mentioned problems. In FIT, we can utilize alternative template molecules which have similar

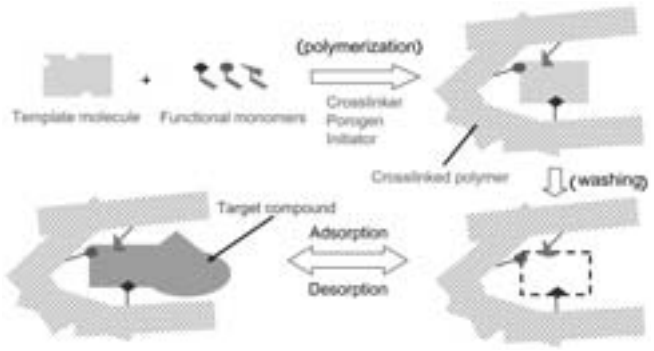


Figure 1. Basic concept of the Fragment Imprinting Technique

moiety of targeting compounds (Figure 1). The separation media prepared by FIT have selective recognition ability for targeting compounds and their homologues. We applied this technique to several type compounds such as halogenated aromatic compounds, water-soluble compounds, flexible compounds and so on. Also, we hope to describe each strategy in this paper.

Basically, the recognition sites on MIPs lead to decrease the homogeneousness of separation media and broader peaks on liquid chromatographic evaluations. However, the MIP media has higher selectivity for targeting compounds than the commonly used separation media. Therefore, the MIPs are well suited to the solid-phase extraction (SPE) and batch separations. In this study, liquid chromatography was utilized for the evaluation of prepared MIPs to clarify the selective adsorption strength. But, we hope to show the-possibility of practical application such as separation media of SPE and others.

2. Selective separation of halogenated aromatic compounds

2.1. Selective separation of halogenated bisphenol A

Endocrine disrupters in the environment have caused an increasing concern for their possible impact on wildlife and human health. Also, chlorinated (bisphenol A) BPAs are produced during bleaching processes of recycled paper in paper factories [6] and brominated BPAs are primarily used as flame retardants in epoxy resins used in printed circuit boards [7, 8]. Especially, tetrabromo-BPA (3,3',5,5'-Tetra-Br-BPA) has produced the largest size among brominated flame-retardants in the world. During incomplete combustion such as spontaneous fires at waste disposal sites, polymers containing 3,3',5,5'-Tetra-Br-BPA converted to polybrominated dibenzofurans and polybrominated dibenzo-p-dioxins [9]. Also, 3,3',5,5'-Tetra-Br-BPA has been reported to exhibit some estrogen -like properties in vitro [10], and to bind to the thyroid hormone transporting protein transthyretin in vitro [11] and the thyroid hormone receptor [12]. The chlorinated homologues, tetrachlorinated BPA (3,3',5,5'-Tetra-Cl-BPA) has been also reported to bind to transthyretin in vitro although the affinity was lower than 3,3',5,5'-Tetra-Br-BPA. It is very important to know the accurate concentration of these BPAs'derivatives in the environment. Additionally, the derivatives should be separated by the differences of number, halogen species and position of substituent because the activity of each derivative is much varied.

To achieve the selective separation of these halogenated BPAs, MI is one of the most attractive techniques. However, general MI method cannot be used for toxic compounds because the method has above-mentioned problems. Therefore, some alternative molecular imprinting procedures have been reported [13-15] and we have also previously reported some alternative molecular imprinting procedures that the template molecule has been selected from analogues of the target compound or partially resemble compounds of the target compound, but not used the target compound itself [16-18]. In this study, we developed the separation media for selective separation and concentration of halogenated BPA derivatives. FIT (Figure 1) was utilized for preparation of polymer-based separation media.

Uniform-sized MIPs particles prepared by two-step swelling and polymerization method [19, 20] were packed into stainless columns and evaluated with liquid chromatography (LC). We investigated several template molecules for preparation of MIPs and all MIPs were evaluated by LC. As result, BTFB-MIP which was prepared with 2,6-bis-(trifluoromethyl)-benzoic acid as template molecule indicated the most selective separation ability for a few halogenated BPAs suchas 3,5- and/or 3',5'- brominated or chlorinated BPA derivatives. The chromatograms of the brominated BPA using BTFB-MIP and Blank (non-imprinted polymer) were shown in Figure 2. The results showed that 3,3',5,5'-Tetra-Br-BPA was retained selectively on BTFB-MIP whereas the retention strength for 3-Br-BPA was almost the same on both the polymers. These results suggested that the selective recognition for halogenated BPAs could be achieve by the appropriate template based on the molecular structure and/or structural size [21].

2.2. Selective separation of hydroxy polychlorinated biphenyls (HO-PCBs)

Mono hydroxy polychlorinated biphenyls (HO-PCBs) have been shown to inhibit mitochondrial oxidative phosphorylation, thyroid hormone sulfation, estrogen sulfotranferase and the sulfation and glucuronidation of 3-hydroxybenzo[a]pyrene, to affect thyroxine (T 4) levels and to exhibit estrogenic or antiestrogenic activity [22-24]. HO-PCBs are increasing attention as potentially endocrinologically active metabolites of PCBs. In association with these risks, the effective separation method has not been developed and the selective separation is strongly required since targeting compounds are extracted together with a lot of biological compounds which interfere with the analyses.

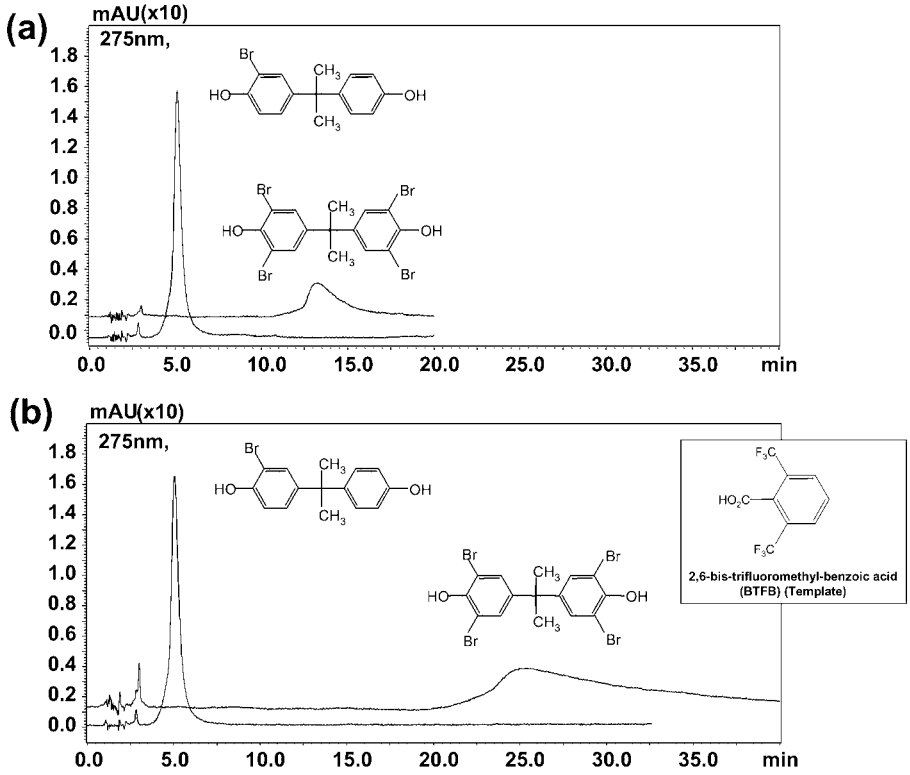


Figure 2. Chromatograms of brominated BPAs on Blank and BTFB-MIP
(a): Blank polymer packed column, (b): BTFB-MIP packed column
LC conditions: Mobile phase; 90% aqueous MeOH, Column size; 150 mm×4.6 mm i.d., Flow rate; 1.0 ml min^{-1}, Temperature; 40°C, Detection; Photo Diode Array (PDA) and MS (selected ion monitoring)

In this study, we developed new separation media by the fragment imprinted polymers having selective recognition ability for specific HO-PCBs. The MIPs were also prepared as uniform-sized polymer particles. The separation abilities for HO-PCBs on MIPs were evaluated by LC and considered the relationship between retention ability and pKa value dependent on the chemical structure of HO-PCBs. Moreover, the direct separation of a few HO-PCB analogues, which may have a possibility of thyroid hormone activity, from 19 kinds of HO-PCBs was carried out by LC using MIP packed column.

As results of the detail LC evaluation, the prepared polymers had selective separation ability due to pKa value dependent on chemical structure of HO-PCBs. Additionally, the pKa and molecular volume of template molecules were related on the construction of recognition sites [25]. Finally, direct selective separation of three HO-PCB analogues having a possibility of thyroid hormone activity from the mixture sample was completely achieved on BCA -MIP (template: Biphenyl-4-carboxylic acid) (Figure 3). We anticipate that the method using in this study will contribute for the selective separation of thyroid hormone activate compound and the breakthrough of structure activity relationship of HO-PCBs.

3. Interval immobilization technique for water-soluble compounds

In general MI technique, organic solvent is utilized to dissolve the template molecules and construct the porous structure. Therefore, the water-soluble compounds having ionic groups can be hardly used for targeting compounds. In order to accomplish the selective separation for water-soluble compounds such as alkaloids, we proposed the novel separation media prepared through "interval immobilization technique". In this technique, the interval between the two ionic functional groups can be immobilized on crosslinked polymer using molecular assembly with appropriate pseudo-template molecule coupled with some ionic functional monomers. The concept of this technique for cyanobacterial toxin, cylindrospermopsin (CYN) is illustrated in Figure 4.

The bulk MIP was crushed and classified at the range of 25 μm ~ 45 μm to be packed into LC column. Results of LC analysis suggested that the MIP using tributyl-(4-carboxybenzyl)-ammonium chloride (TCBA) as pseudo-template had selective recognition ability for CYN. Additionally, scatchard analysis [15, 26] also supported that the associate constant between CYN and MIP was higher than that of Blank polymer (Figure 5, Table 1) [27].

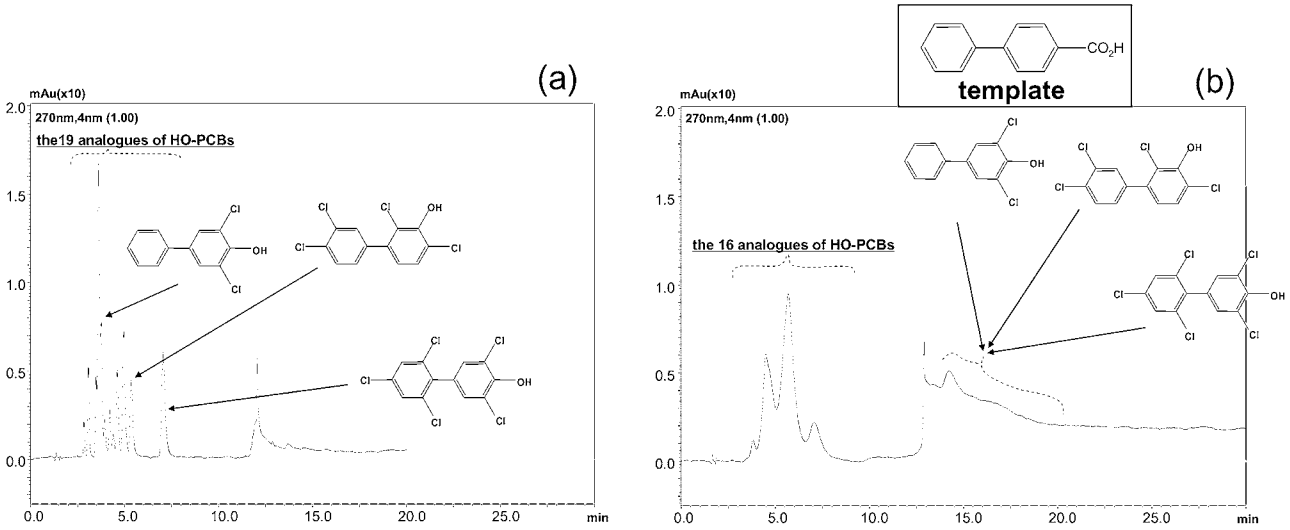


Figure 3. Chromatograms of HO-PCBs (19 analogues) on ODS and BCA-MIP
(a): InertSil ODS-3 (150 mm*4.6 mm i.d.), (b): BCA-MIP packed column (150 mm*4.6 mm i.d.)
LC conditions: Mobile phase; A: AN/H_2O=8/2, B: AN/0.1% aqueous formic acid = 8/2, 0-10 min; 100% A, 10-20 min; 100% B, stepwise gradient, Flow rate; 1.0 mL min^{-1}, Temperature; 40°C; Detection; Photo Diode Array

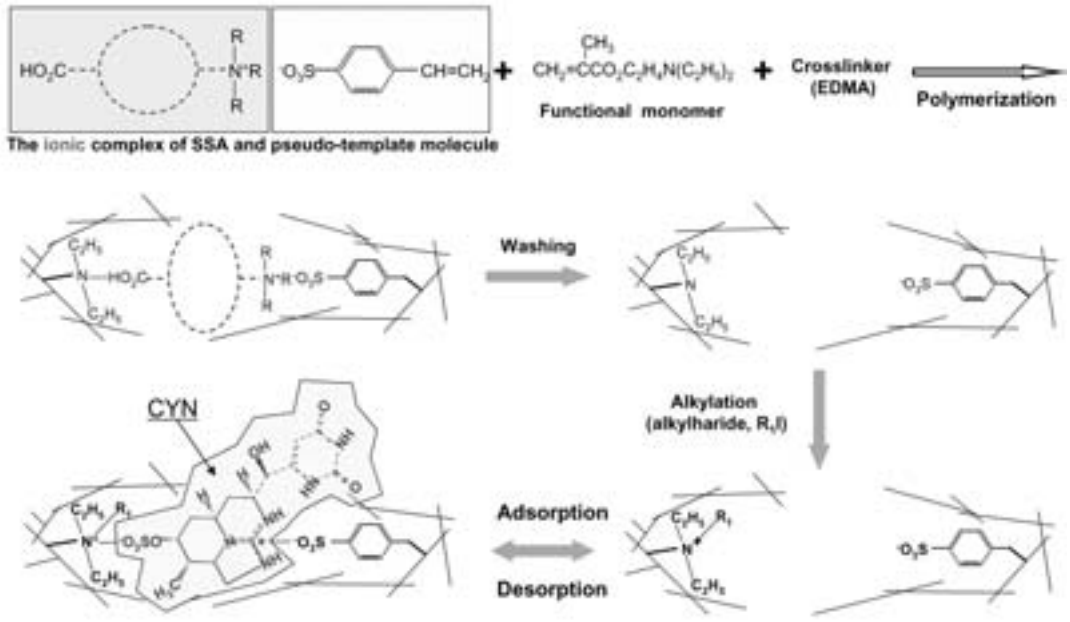


Figure 4. Concept of Interval Immobilization Technique for CYN
SSA: *p*-styrene sulfonic acid, EDMA: Ethyleneglycol dimethacrylate

According to these results, this technique can be expected as novel selective separation media for water-soluble compounds.

Furthermore, as another problem of MI technique, non-selective adsorption was observed on MIP-based SPE, originating from hydrophobic interactions on the surface of the polymer matrix. Consequently, a novel preparation method, which can be performed in a 100% water system and has much lower hydrophobic characterization, is needed. Recently, several researchers have reported new MIPs for oligopeptides, that are prepared in water using poly (ethylene glycol) diacrylate [28, 29] or acryloyl-cyclodextrins with bisacrylamide [30]. These polymers exhibited slight selective recognition. However, the recognition sites in the polymers were mainly controlled through hydrogen bonding, and they appeared to be heterogeneously dispersed because excess amounts of functional monomers were used in these cases. Additionally, ionic compounds such as guanidyl group-containing alkaloids cannot be utilized in these preparation methods. For these reasons, we developed a new MIP preparation system using water as the porogenic solvent

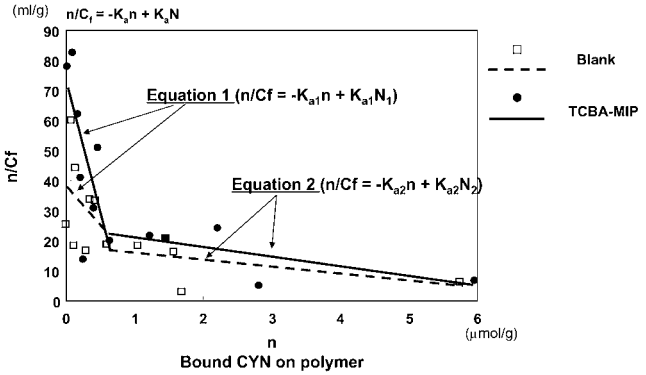


Figure 5. Scatchard plot for CYN on Blank and TCBA-MIP
The association constant (**K_a**) and number of binding sites (**N**) was determined from the slope and intercept, respectively, of fitted line (**$n/Cf = -K_a n + K_a N$**) obtained by least squares regression.

Table 1. Binding parameter of polymer and CYN determined by scatchard analysis

	K_{a1} (M^{-1})	K_{a2} (M^{-1})	N_1 (μ mol/g)	N_2 (μ mol/g)
Blank	2.7×10^4	2.3×10^3	1.4	8.1
TCBA-MIP	8.9×10^4	3.2×10^3	0.82	7.6

The association constant (**K_a**) and number of binding sites (**N**) was determined from the slope and intercept, respectively, of fitted line (**$n/Cf = -K_a n + K_a N$**) obtained by least squares regression.

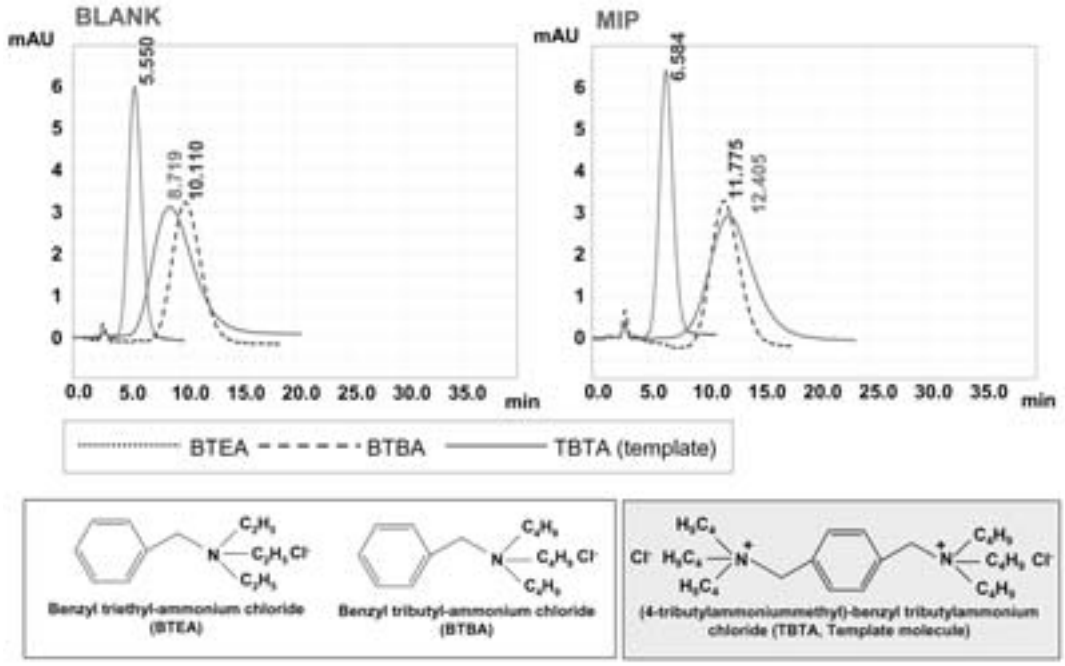


Figure 6. Chromatograms of the ionic solutes on Blank and MIP
LC conditions: Mobile phase; 0.1 M NaCl aqueous, Flow rate; 0.5 ml min^{-1}, Columnsize; 100 mm*4.6 mm i. d., Temperature; 30°C, Detection; Photo diode array

by the interval immobilization technique.

The MIP was prepared by the interval immobilization technique with water and water-soluble crosslinking agent. The MIP showed improved hydrophobicity and the selective recognition for the template in 100% aqueous solution (Figure 6) [31]. This technique should therefore be used in selective SPE of environmental and/or the biological aqueous samples.

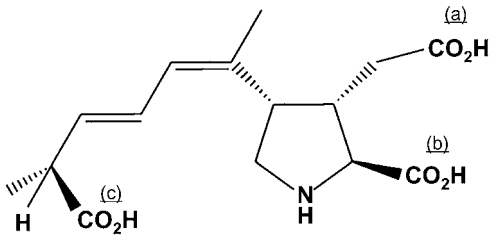
4. 3 D recognition for domoic acid having flexible structure

In typically, targeting compounds of MI technique have inflexible and relatively rigid chemical structures. Therefore, in order to apply the FIT to a larger number of compounds, we have to establish a novel concept to obtain the selective recognition ability for compounds having flexible chemical structures.

In this study, we reported 3 D recognition for the flexible structural compound domoic acid, DA (Figure 7) by the fragment imprinted polymers. DA is a water-soluble tricarboxylic amino acid with a molecular weight of 311: it acts an analogue of the neurotransmitter glutamate and is a potent glutamate receptor agonist. Its first identification was reported from the rhodophyta Chondria armata as an insecticide [32]. Finally, the cell died and symptoms such as memory loss (which is also a typical feature of ASP) were

observed [33-35]. Additionally, some biological researchers have reported that DA is assembled with a kainate-type subunit of the glutamate receptor. According to that study, the author has described that multipoint recognition was worked effectively toward selective holding and three COOH groups were selectively recognized. In our previous study, we have reported "two-point" recognition of COOH groups for DA by MIPs [36]. In this study, we investigated "three-point" recognition. We prepared MIPs using many simple and commercially used template molecules and the selectivity of MIPs for DA was evaluated using LC. Additionally, in order to verify the selective recognition results, we also investigated the conformational alternation of DA by computer modeling.

As results of LC evaluations, simple aliphatic tricarboxylic acid (pentane-1,3,5-tricarboxylic acid: 1,3,5-PeTA-MIP) was worked as a template molecule for selective recognition of DA. Moreover, the results of the Gibbs free energies in the chromatographic analysis [37] were compared with the estimated isomers and the alteration of the Gibbs free energies calculated by computer modeling (Table 2 and Figure 8). After all, the recognition sites constructed on 1,3,5-PeTA-MIP could recognize DA by the flexible conformational changes of DA: these conformational changes were incorporated into the recognition sites. In other words, these results suggested that three-point recognition was achieved by the conformation change of DA onto the recognition site constructed by the template molecule (the recognition image is illustrated in Figure 9) [38]. Additionally, 1,3,5-PeTA-MIP could be used for purification of DA as SPE media from blue mussel extracts (Figure 10).



Domoic acid (DA)

Figure 7. Structure of domoic acid
Where (a)-(c) shows each COOH group to compare the distances on computer modeling.

Here, we proposed a new concept of the selective 3 D recognition for the flexible compounds by the FIT. We expect that this concept will be applied to a greater number of compounds having several functional groups and flexible conformation changes.

Conclusion

Author has been studying the modified molecular imprinting technique. It is expected that the several methodological procedures described in this review will become practical tools for the selective separation of a lot of toxic compoundsand rare natural compounds. In the future works, we should develop the novel separation media for the selective separation of biomolecules such as protein and nucleic acids, and the additional sensor techniques for detection.

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Table 2. Gibbs free energies of DA onchromatographic evaluation

Polymers	k'of DA	ΔG-ΔG (on BASE) (kJ/mol)	ΔG-ΔG (on BLAMK) (kJ/mol)
BASE	0.10	———	———
BLANK	1.10	6.04	———
OPA-MIP	9.62	11.5	5.46
2,3-NDA-MIP	13.7	12.4	6.35
2,6-NDA-MIP	5.94	10.3	4.25
EDA-MIP	12.6	12.2	6.14
FDA-MIP	11.6	12.0	5.93
KDA-MIP	14.5	12.6	6.51
1,3,5-PeTA-MIP	53.5	15.8	9.79

The Gibbs free energies in the chromatographic analysis were estimated by the equation $\Delta G=-RT\ln K$, where $k'=\beta K$ (k' is the retention factor and β is a chemical equilibrium constant). Moreover, ΔΔG between two stationary phases can also be estimated by the equation $\Delta\Delta G=-RT\ln\alpha$ (α is the separation factor).

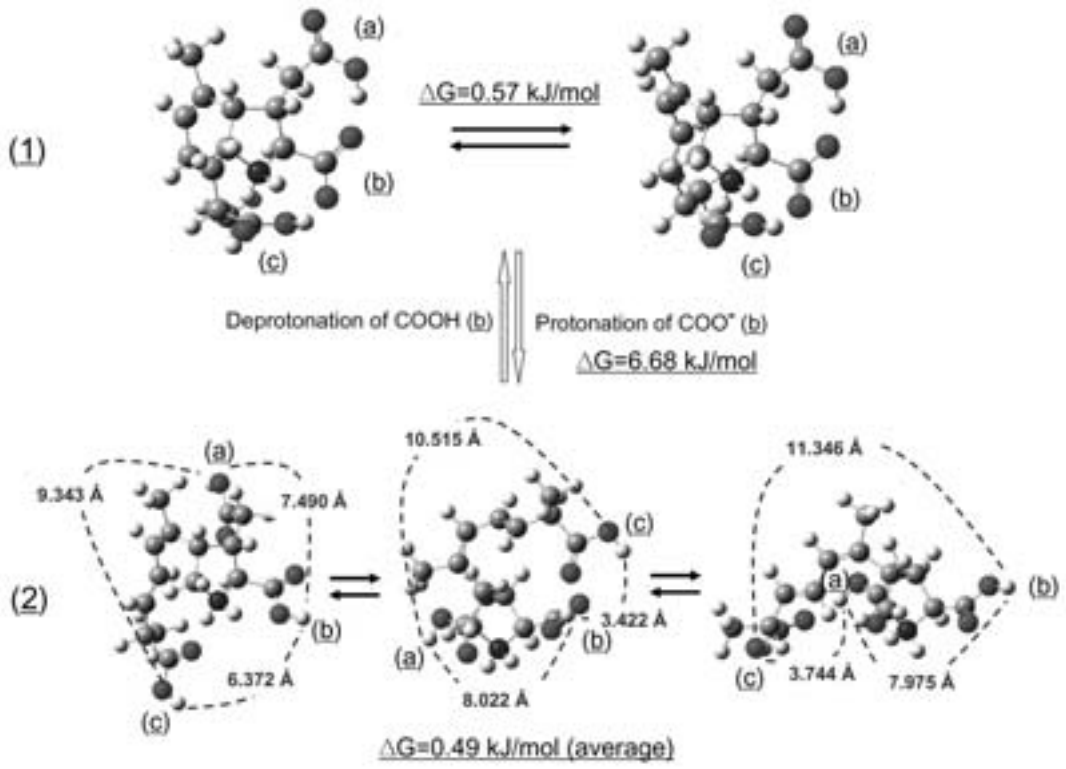

Figure 8. Alteration of ΔG by conformation changes in DA
Stable conformation of DA in the mobile phase condition was estimated by RHF/6-31G (d), SCI-PCM in Gaussian 03 W. (1): most stable conformation of DA and (2): conformation of DA after protonation of COOH (b)

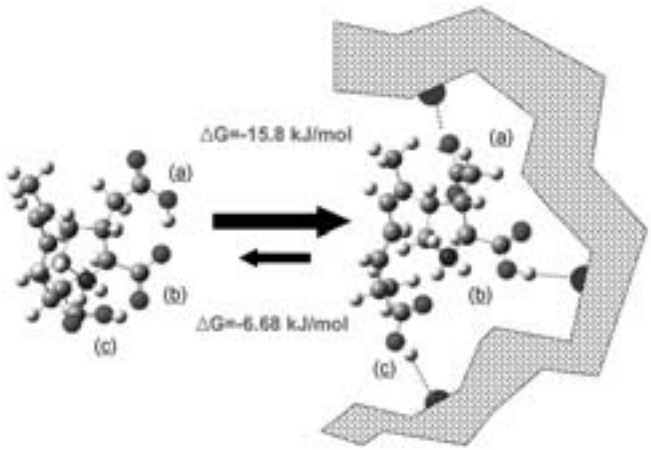

Figure 9. Recognition image for DA on the constructed imprinting site

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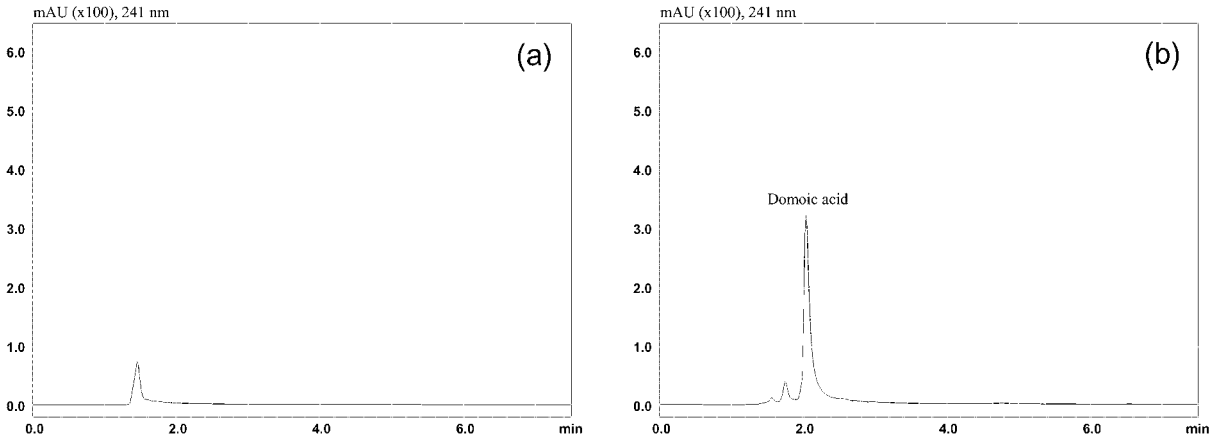

Figure 10. Chromatograms of blue mussel extracts containing DA
(a): passed-through fraction from 1,3,5-PeTA-MIP and (b): collected fraction from 1,3,5-PeTA-MIP
Chromatographic conditions for determination of DA
Column: Mightysil (Kanto Chemical, Japan, 150 mm×4.6 mm i.d.); Mobile phase: AN/0.05% AcOH aqueous=3/7; Flow rate: 1.0 ml min^{-1}; Temperature: 30°C; Detection: Photodiode array; Injection volume: 1 μl

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