Preparation of Molecularly Imprinted Polymers: Diethyl(3-methylureido)(phenyl)methylphosphonate as a Dummy Template for the Recognition of Its Organophosphate Pesticide Analogs

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Received 13 June 2011; accepted 27 July 2011
DOI 10.1002/app.35373
Published online 23 November 2011 in Wiley Online Library (wileyonlinelibrary.com).

ABSTRACT: A novel compound, diethyl(3-methylureido)(phenyl)methylphosphonate (DEP), possessing an organophosphate skeleton, was synthesized and used as a dummy template to prepare molecularly imprinted polymers (MIPs) for the recognition of organophosphate pesticide analogs. Computational modeling was used to study the primary intermolecular interactions in the prepolymerization mixture. It was found that the interaction force between DEP and the monomers was hydrogen bonding. A series of MIPs were synthesized with different monomers and were evaluated by adsorption experiments, which showed that methacrylic acid was used as an appropriate monomer and a molar ratio of DEP to MAA of 1 : 9 was optimal. Scatchard analysis showed that there might have been two types of binding sites in the MIPs. DEP and several pesticides were used in molecular recognition specificity tests of DEP–MIP, which exhibited better selectivity and reservation ability for organophosphate pesticides, such as methamidophos and orthene, possessing amino or imino groups and a smaller steric hindrance. On the basis of the use of a dummy molecule as template, the problem of template leakage could be avoided; this, thereby, improved the specificity of analysis. © 2011 Wiley Periodicals, Inc. J Appl Polym Sci 124: 3737–3743, 2012

Key words: computer modeling; molecular imprinting; molecular recognition; templates

INTRODUCTION

Organophosphate pesticides (OPs) are one of the most widely used pesticides used to control diseases and increase agricultural productivity.[1–3] However, scientific research has shown that residual OPs in food can poison humans and animals through bioaccumulation in the food chain and affect the metabolic balance of intravital hormones,[4,5] especially in the normal development of children's nervous systems.[6] To guarantee food safety and protect the environment, governments in many countries have claimed stricter requests for OP residue limits. For example, the European Union has set maximum admissible concentrations of 0.1 μg/L for individual pesticides and their related compounds in drinking water and 0.05 mg/kg for foods of plant origin for most OPs;[7] this increases the difficulties of detection. Residual analysis of trace pesticide pollutions in food and the environment depends on sensitive instrument analysis, rapid, simple, and reliable sample preparation, and cleanup techniques. Solid-phase extraction[8] and liquid–liquid extraction[9–13] are some typical sample preparation techniques. Detection methods for organophosphate pesticides residues in fruits and vegetables are mainly based on gas chromatography or high-performance liquid chromatography (HPLC) coupled with various detections[14–16] or liquid chromatography (LC)/mass spectrum (MS)/MS or gas chromatography/MS/MS.[17] The analysis of trace materials makes sample pretreatment the key link of the whole analysis process because of the variety and complexity of the matrix. Sample pretreatment is not only time consuming, but it also influences the accuracy and precision of the analysis results. Consequently, the development of a simple, rapid, inexpensive, and sensitive analytical method for routine organophosphate (OP) screening in various complicated matrices is of particular significance and necessity.

In recent years, on the basis of molecular recognition theory, the molecular imprinting technique has been rapidly developed and has become a research hotspot in the field of chemistry. Molecularly imprinted polymers (MIPs) are stable synthetic polymers that possess selective molecular recognition sites, which are fixed in the polymer matrix during polymerization process in the presence of a template molecule. The removal of the template from the polymer matrix leaves cavities of complementary size, shape, and chemical

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Contract grant sponsor: 863 Project from the Ministry of Science, China; contract grant number: 2007AA10Z433.
Contract grant sponsor: Modern Agro-Industry Technology Research System; contract grant number: nycytx-18.

functionality to the template. The imprinting process is known for its synthetic efficiency and versatility. A wide range of molecules, for example, pharmaceuticals,[18] amino acids,[19] pesticides,[20] carbohydrates,[21] and nucleic acids,[22] can be used as templates. Because of their high selectivity, along with their chemical and physical stabilities, MIPs have become popular solid adsorbents for the separation of structurally related substrates or enantiomers.[23–25]

The application of MIPs in pesticide residue analysis is mainly represented in the use of MIPs as simulation biology antibodies in attempts at single pesticide residue detection and the exploitation of the molecularly imprinted sorbent assay method or as solid extractants in sample cleanup and the exploitation of molecularly imprinted solid-phase extraction techniques. Works in the literature related to OP–MIPs for separation and adsorption in food have continued to appear in recent years.[25,26–29] However, there is a potential risk of leakage of molecular templates during adsorption and elution, which has been reported and has remained as a problem in the imprinting process. During the preparation of MIPs, a relative excess of template is needed. However, the removal of all templates molecules from the obtained polymer matrix is very difficult. Usually, about 5% template molecular remains.[30] Sometimes, the tested sample only contains picogram - nanogram (pg–ng) trace or ultratrace analyte when 1% template molecules are not removed and leak during extraction; this can cause more disturbance for detection. A better solution for the problem is to select a structural analog of the analyte as a template molecule; this can effectively solve the leakage of templates.[31,32] At present, only a few analytes have appropriate structural analogs. The fundamental resolution of the residue or leakage of template molecules is yet to be found. In this study, we designed and synthesized a new compound, diethyl(3-methylureido)(phenyl)methylphosphonate (DEP), which possessed an organophosphate skeleton, and used this compound as a dummy template to prepare MIPs for the recognition of organophosphate pesticide analogs to prevent template leakage. Selective adsorption and chromatographic evaluation experiments of DEP and several pesticides in MIPs were conducted to determine its recognition capability; this provided academic and practical experience for the separation and adsorption of residual pesticides with dummy templates to improve the veracity of analysis and detection.

EXPERIMENTAL

Chemicals and apparatus

The pesticides methamidophos, orthene, chlorfenvenphos, chlorpyrifos, fenitrothion, diflubenzuron, hexaflumuron, and azmethiphos were supplied by Pesticide Analysis Laboratory of China Agricultural University (Beijing, China). Methanol (99.9%), chloroform (99.9%), and acetonitrile (99.9%) were HPLC grade. Methacrylic acid (MAA; 98%), ethylene glycol dimethacrylate (95%), and chromatograph solvents were purchased from J&K Chemical, Ltd. (Beijing, China), and all other reagents were from Beijing Chemical Reagents Co. (Beijing, China). Purified water was from Wahaha Group Co., Ltd. (Beijing, China).

IR spectra were recorded in a Tensor 27 spectrophotometer equipped with Quick IR software (Bruker, Germany). NMR spectra were recorded on a Bruker DPX 300-MHz NMR spectrometer (Bruker Biospin, UK) at 25°C. HPLC analyses were performed on an Agilent model 1100 series LC. The surface morphology of the MIPs was assessed by a Hitachi S-4800 scanning electron microscope.

Synthesis of DEP

A solution of benzaldehyde (20 g) in toluene (35 mL) was added to a round-bottom flask containing 10 g of methyl urea and 28 g of diethyl phosphate, and then, a solution of BF_3Et_2O (0.8 mL, prepared according to the literature[33]) in toluene (6 mL) was dropped in with stirring. After 30 min of stirring, the resultant mixture was refluxed for 15 h, then the formed precipitate was removed by filtration, and the filtrate was evaporated under reduced pressure to dryness. The crude product was purified by silica gel column chromatography with ethyl acetate and petroleum ether (ethyl acetate/petroleum ether = 2 : 1 v/v) as eluents to give pure DEP at a 65% yield.

^{1}H-NMR (300 MHz, $CDCl_3$, trimethylsilyl (TMS), δ, ppm): 1.05–1.11 (CH_3, 3H), 1.34–1.38 (CH_3, 3H), 2.68(CH_3, 3H), 2.76 (CH, 1H), 3.61–3.89 (CH_2, 2H), 4.15–4.27 (CH_2, 2H), 5.38–5.49 (NH, 1H), 6.95–6.96 (NH, 1H), 7.27–7.31 (C_6H_5, 3H), 7.44–7.47 (C_6H_5, 2H). MS (Na^+): 323.2. IR (KBr, ν_{max}, cm^{-1}): 1020.06 (P—O—C of PO_3), 3000–3100 (C—H of C_6H_5—), 1575.7 (N—H).

Preparation of imprinted microspheres and nonimprinted microspheres

The DEP–MIPs were prepared by a precipitation polymerization method. With this method, MIP nanometer microspheres can be obtained. In a typical process, the template DEP and functional monomer were dissolved in chloroform in an ampule and fully mixed under ultrasonic conditions, and then, the crosslinking agent and initiator were dissolved in this solution. The mixtures were sonicated, sparged with nitrogen for 5 min, sealed, and placed in a water bath at 60°C for 24 h. The obtained polymer microsphere was removed from the ampule,

extracted with acetic acid/methanol (1 : 9 v/v) until no template was detected in HPLC, and then dried. The corresponding nonimprinted control polymers were synthesized and treated under the same conditions, except the template DEP was omitted.

Equilibrium binding experiments

To investigate the binding capacity of the imprinted polymers, an adsorption experiment was employed in this work. A portion of 20 mg of the imprinted particles and 3.00 mL of DEP solution (concentration range = 0.0066–1.65 mmol/L) was placed in a 10-mL flask. After they were wobbled at room temperature for 24 h, the solutions were centrifuged and filtered, and then, the supernatant concentrations were determined by HPLC. This method can also be used to study selectivity adsorption.

Column experiments

In this study, the DEP–MIPs or DEP–non-imprinted polymer (NIPs) column were prepared and applied to HPLC for the evaluation of the retention capabilities of DEP or pesticides on polymers.

The polymer particles (2.5 g) were suspended in isopropyl alcohol solution and mechanically slurry-packed into HPLC stainless-steel columns (length = 15.0 cm and i.d. = 4.6 mm) with isopropyl alcohol at a constant pressure of 40 MPa.

The HPLC chromatography operating parameters were as follows: the mobile phase was 70 : 30 (v/v) acetonitrile/water, the flow rate was 1 mL/min, the diode array detection wavelength was 254 nm, and the injection volume was 10 μL; The standard solutions were prepared by the dissolution of 10 mg of selected pesticides into 10 mL of methanol to obtain concentrations of 1.0 g/L.

Molecular simulation

In this part, the intermolecular interactions between the template DEP and different functional monomers were investigated. The total potential energy of each of these compound systems was calculated. All computations were performed by Material Studio software.

With the Discover module of Material Studio, the template and monomers were conducted to energy-minimize, respectively, to determine the optimal configurations from which the template–monomer complex formation could continue to minimize energy to obtain the total energies in the most stable states; this could be used to compare the stability of the compound systems.

TABLE I
Adsorption and Imprinting Effects of MIPs Prepared with Different Monomers and Different DEP-to-Monomer Molar Ratios

	DEP/monomer molar ratio				
Polymer	DEP/MAA	DEP/Acrylamide (AA)	DEP/Itaconic acid (IA)	Q (μmol/g)	I_e (Q_{MIP}/Q_{NIP})
MIP1	1 : 4			0.74	1.27
NIP1	—			0.58	
MIP2	1 : 6			1.04	1.70
NIP2	—			0.61	
MIP3	1 : 9			1.62	3.30
NIP3	—			0.49	
MIP4	1 : 12			0.73	1.22
NIP4	—			0.60	
MIP5		1 : 6		1.43	0.95
NIP5		—		1.51	
MIP6		1 : 9		0.70	1.32
NIP6		—		0.53	
MIP7		1 : 12		0.19	0.95
NIP7		—		0.20	
MIP8			1 : 6	1.80	1.40
NIP8			—	1.29	
MIP9			1 : 9	0.80	1.19
NIP9			—	0.67	
MIP10			1 : 12	0.15	1.00
NIP10			—	0.15	

RESULTS AND DISCUSSION

Preparation of molecularly imprinted microspheres

The imprinted polymers prepared by different monomers and different molar ratios of DEP to monomer are shown in Table I. Q is the adsorption capacity of MIPs of NIPs and was calculated as follows:

$$Q = (C_0 - C_r) \times V/nr \quad (1)$$

where C_0 is the initial concentration of DEP solution (μmol/L), C_r is the free concentration of DEP at equilibrium (μmol/L), V is the volume of the initial solution (mL), and m is the mass of the polymer particles (g).

In addition, the imprinting efficiency (I_e) was introduced to evaluate the special adsorption ability. I_e was obtained from the ratio of Q_{MIP} to Q_{NIP} according to the following formula:

$$I_e = Q_{MIP}/Q_{NIP} \quad (2)$$

From these results, MIP3 in the ratio of DEP to MAA of 1 : 9 showed the highest adsorption capacity and the best I_e. So we selected MIP3 as a research object in the remainder of the study (MIP3 is hereinafter referred to as MIP).

The scanning electron microscopy images of the MIPs and NIPs are shown in Figure 1. As observed,

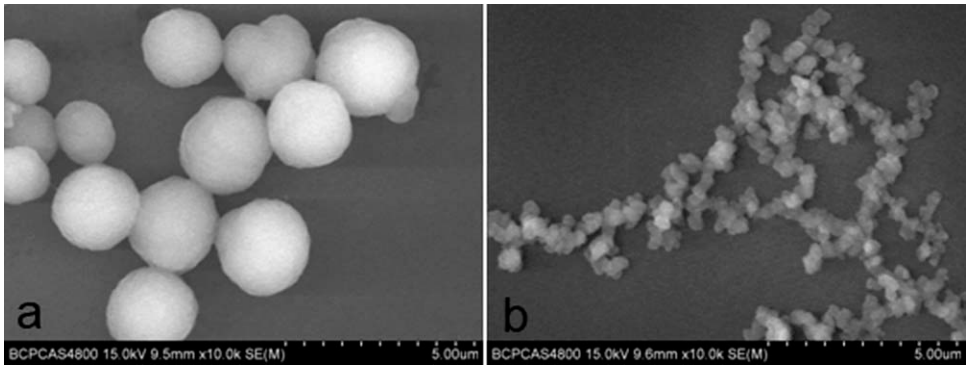

Figure 1 Scanning electron microscopy images of (a) MIP and (b) NIP.

the MIPs showed uniformly sized microspheres, and their mean particle sizes of 1.8–2.5 μm were larger than that of the NIPs (200–400 nm). The MIP microspheres could be used as absorption materials to apply in HPLC for quick separation.

Binding isotherms of DEP on the MIPs and Scatchard analysis

Binding isotherms were determined in the DEP concentration range 0.0066–1.65 mmol/L. It was found that in the studied concentration range, compared with the little changed Q_{NIP}, Q_{MIP} increased with increasing initial concentration of DEP and was inclined to be saturated; it finally reached a stable value [Fig. 2(a)]. The higher affinity of the MIPs was ascribed to their imprinting effect for DEP, which suggested that there existed specific rebinding sites or matching space structures for DEP on the MIPs.

The data obtained from the binding isotherms were further processed with Scatchard analysis with the Scatchard equation:

$$Q/C = (Q_{max} - Q)/k_d \quad (3)$$

where Q_{max} is the maximum binding capacity, C is the free concentration of analyte at equilibrium, and k_d is the dissociation constant.

Figure 2(b,c) shows the Scatchard plots of the binding of DEP to the MIPs and NIPs, respectively. It was clear that the Scatchard plot for the MIPs contained two distinct linear sections, which indicated that there existed two types of binding sites in the MIPs. From the slope and intercept of the Scatchard plot, k_d and Q_{max} for the higher affinity binding sites were calculated to be 4.08 μmol/L and 1.23 μmol/g, whereas k_d and Q_{max} for the lower affinity binding sites were 314.28 μmol/L and 2.75 μmol/g, respectively. For the NIPs, the nonlinearity indicated that there were not selective adsorption sites for DEP on them. From the Scatchard analysis, we could assume that the two kinds of binding sites in the MIPs may have been produced in the following way: during the prepolymerization, DEP and MAA formed complexes by two kinds of hydrogen binding, which were fixed in the MIP matrix. After the removal of template DEP, the two binding sites were reserved

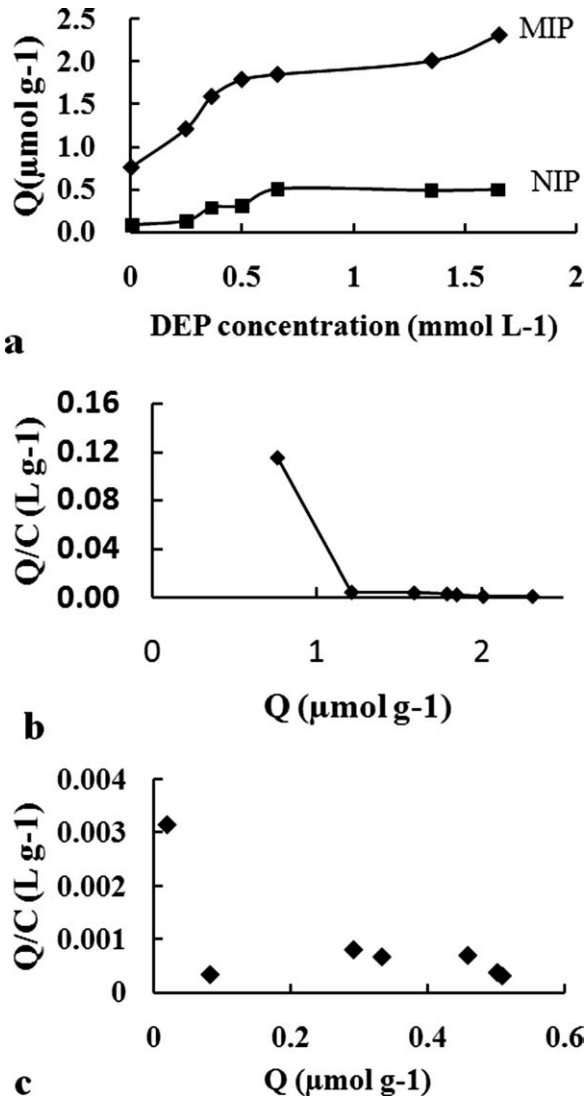

Figure 2 (a) Binding isotherms of DEP on MIP and NIP and Scatchard analysis of (b) DEP–MIP and (c) DEP–NIP.

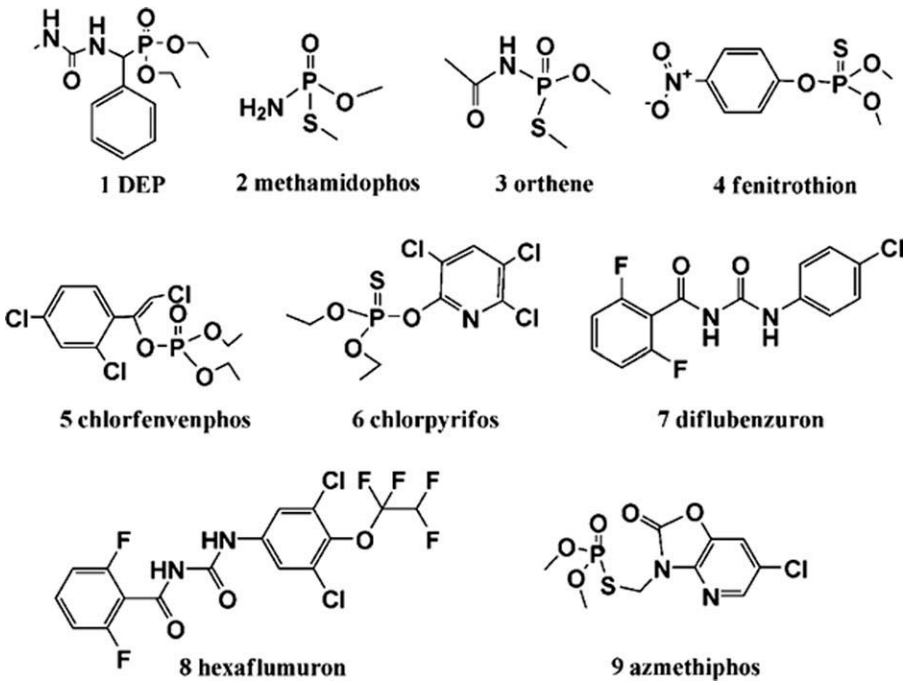

Figure 3 Structures of template DEP and a series of pesticides (**2–9**).

in the MIPs and possessed recognition memory ones, although the interaction between the NIPs and DEP was mainly from nonspecific adsorption.

Recognition selectivity studied by HPLC

The selectivity test of the MIPs was carried out with a series of pesticides, whose structures are shown in Figure 3. The results are listed in Figure 4. It was obvious that the MIPs exhibited the highest binding affinity for DEP, methamidophos, and orthene in the next place. Beyond that, hexaflumuron and diflubenxuron exhibited weak absorption abilities, and other pesticides showed no obvious absorption effects. These results suggest that there were sure specific recognition sites and space structure matching for the template molecule in the process of imprinting. Molecular simulation results can better describe the interaction between template and monomers. With the DEP–MAA system taken for illustration (as shown in Fig. 5), the main interaction forces were hydrogen bonds between the imino groups or phosphate oxygen groups of DEP and the carboxyl groups of MAA. It was the key factor in the characterization of alkali groups of the analyte and the matching degree of the structure of analyte for the cavity of the MIPs. For methamidophos or orthene, which possess amino or imino groups and P=O groups, the basic sites could interact with the acidic site of MAA, and its smaller space volume easily made methamidophos or orthene absorbed in the MIPs. Methamidophos, with lesser steric hindrance than orthene, could be better captured by the acidic

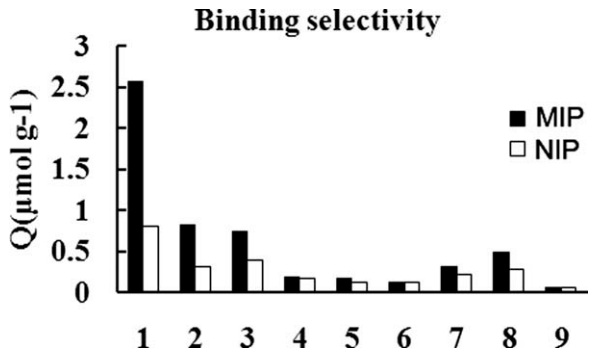

Figure 4 Binding selectivity of DEP and a series of pesticides (**2–9**) on MIP and NIP.

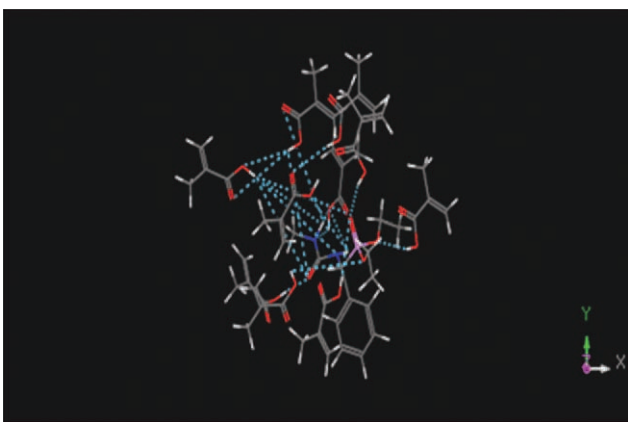

Figure 5 Optimal configuration of DEP–MAA in molar ratio of 1 : 9. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

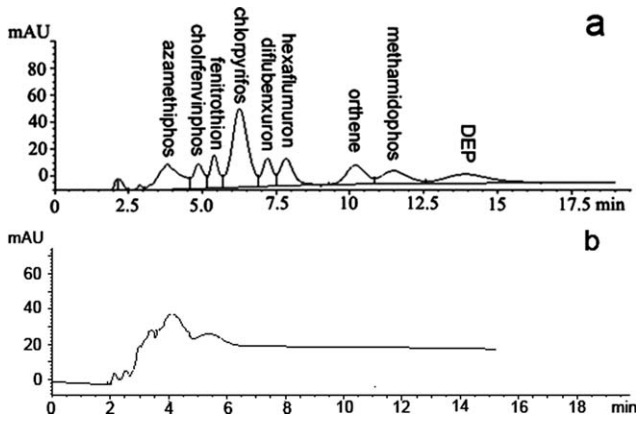

Figure 6 Chromatograms of DEP and a series of pesticides on the (a) DEP–MIP and (b) NIP columns.

site on the surface of the MIPs, so the amount of methamidophos bound to the MIPs was larger than that of orthene. For other analytes, chlorpyrifos, fenitrothion, cholrfenvinphos, and azamethiphos, there was no obvious absorption effect on the MIPs, which possessed orientated benzene rings and P=O(S) groups. This was possibly due to the fact that there were no alkaline groups in these compounds. In addition, the greater steric hindrance also baffled the analytes into and out the cavities of the MIPs. In comparison, hexaflumuron and diflubenxuron exhibited weak absorption abilities on the MIPs, although these were not OPs. The main reason may have been the same as discussed previously; that is, the alkali functional groups of hexaflumuron and diflubenxuron had a significant effect on the formation of the complex between the template and MAA.

From these results, we concluded that both noncovalent interaction and spatial structure matching for the template were very important in the imprinting process.

Chromatographic evaluation of the MIPs

One of the most extensive research fields of MIPs is the separation of mixtures with their specific recognition. As shown in Figure 6 [DEP–MIP, Fig. 6(a), and NIP, Fig. 6(b)], DEP–MIP columns showed a better separation effect for the selected pesticides, in which DEP showed the strongest retention ability, with methamidophos and orthene in the next place. Azamethiphos showed the weakest retention ability. However, all of these compounds did not show obvious retention behavior in the DEP–NIP column in the same conditions. The results indicate that the DEP–MIP column could be used to separate or enrich the structural analog. Moreover, the retention behavior abilities of DEP and the analytes on the MIP column were coincident with their selective adsorption abilities on the forenamed MIP; this also indicated that the advantage of using MIP as a chromatographic stationary phase was predictable selectivity, that is, predictable peak sequence.

CONCLUSIONS

In this study, a new compound, DEP, possessing an organophosphate skeleton was synthesized and used as a dummy template to prepare DEP–MIPs for the recognition of organophosphate pesticide analogs. Computer-aided simulation was applied to illuminate the host–guest recognition mechanism. DEP–MIPs showed the specific recognition ability for DEP and OPs possessing alkaline groups through selective binding experiments and chromatographic experiments. Moreover, these revealed that the specific recognition mainly depended on the fitting efficiency of the size, shape, and functional group of analyte with the DEP–MIPs.

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