Available online at www.sciencedirect.com
ScienceDirect
Dyes and Pigments 74 (2007) 572–577
DYES and PIGMENTS
www.elsevier.com/locate/dyepig

Characterization and quality assessment of binding properties of malachite green molecularly imprinted polymers prepared by precipitation polymerization in acetonitrile

Shoulei Yan [a,b], Zhixian Gao [a,*], Yanjun Fang [a], Yiyong Cheng [a], Huanying Zhou [a], Hongyong Wang [a]

[a] *Institute of Hygienic and Environmental Medicine, Tianjin 300050, PR China*
[b] *College of Food Science, Huazhong Agricultural University, Wuhan 430070, PR China*

Received 9 November 2005; received in revised form 12 January 2006; accepted 29 March 2006
Available online 5 June 2006

Abstract

Molecular imprinting technology was employed to produce one kind of malachite green (MG) molecularly imprinted polymers by precipitation polymerization using MG, methacrylic acid, ethylene glycol dimethacrylate, azobisisobutyronitrile, and acetonitrile as template, functional monomer, cross-linker, initiator and porogen, respectively. The binding properties of MG on imprinted polymers were evaluated in acetonitrile by equilibrium rebinding experiments. Scatchard plot's analysis revealed that there was one class of binding sites populated in the imprinted polymers with an apparent maximum number of MG (2.33 μmol/g). The specificity and selectivity of the imprinted polymers were investigated by binding analysis using MG and structurally related compounds. The results indicated that the imprinted polymers showed a good selectivity and specificity for MG. So the polymers can be used to separate MG from seafood, water and other matrices.

Keywords: Molecular imprinting; Precipitation polymerization; Malachite green

1. Introduction

Malachite green is an *N*-methylated diaminotriphenylmethane dye widely used in the fish and dye industries country because of its relatively low cost, availability, and efficacy. The powerful antimicrobial activity of malachite green has been attributed to the inhibition of intracellular enzymes intercalation into DNA, and/or interaction with cellular membranes [1,2]. Concern over the use of malachite green is due to the potential for significant worker and consumer exposure, and evidence of tumor promotion in rodent liver was suggested [3]. Alderman and Clifton-Hadley [4] studied the uptake, distribution, and elimination of the dye after exposing trout to a 1.6 ppm of dye bath treatment for 40 min. The maximum concentrations of malachite green in the serum, liver, and kidney (ranging from 7.8 to 34.0 ppm) occurred immediately after exposure, while a peak concentration (10.8 ppm) in the muscle was reached only after 90–120 min of exposure. Other studies have showed that malachite green is reduced to and persists as leucomalachite green in the tissues of fish. The malachite green in the whole fish homogenate decreased with time, while leucomalachite green increased up to 24 h after exposure and remained steady for the next 7 days. At present there are many determination methods, HPLC is the one mostly used among them, connected to mass spectroscopy. But the sample pretreatment is laborious and time-consuming. Strong cation-exchange solid-phase extraction column (SCX cartridge (500 mg/3 mL)) is used for solid-phase extraction; the recovery

* Corresponding author. Fax: +86 22 84655403.
E-mail address: gaozhx@163.com (Z. Gao).

0143-7208/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.dyepig.2006.03.021

rate is in the range of 42–51% [5]. There are many chemical materials and biomaterials that have been employed to adsorb MG from contaminated water, like rice husks produced by chemical–thermal process [6], 'de-oiled soya'[7], the thermal power plant waste material, 'bottom ash'[8] and *Prosopis cineraria* sawdust [9], etc. But the specificity and selectivity of the materials mentioned above are limited.

During the last years, molecularly imprinted polymers (MIP) have been used as synthetic materials that are able to rebind the target analytes. MIP have been utilized in solid-phase extraction, sensor and chromatography for their potential absorptive selectivity to the target molecules and the related compounds [10]. The origin of molecular imprinting of inorganic and organic polymers goes back to Pauling's production of antibodies in vitro and Fischer's lock & key principle, which was for the first time described by Wuff et al. in 1972.

The general principle of molecular imprinting is in detail. (1) specific complex formed based on (non)-covalent bonding interactions between template molecule and polymerizable functional monomer in an apolar and aprotic solvent before polymerization by assembling the functional monomers around the template molecule; (2) yield of a rigid and porous copolymer in the presence of cross-linker and initiator; and (3) the distinct cavities remain in the copolymer with the removal of the target template, which is tailor-made complementary to the template molecule in size, shape and functionality.

At present, there are many methods for the preparation of MIP, such as bulk polymerization [11], suspension polymerization [12], emulsion polymerization, two-step polymerization and precipitation polymerization [13]. Among them, precipitation polymerization is the easiest one, and can overcome the drawbacks of the polymers obtained in other methods. A kind of desired copolymer particles with a narrow particle size and more homogeneous binding sites can be obtained by this method.

In a precipitation polymerization process, the polymerization takes place in a medium, which is a solvent for the functional monomer, template molecule, cross-linker and initiator but is not a solvent for the obtained polymer. The polymer particles are not stabilized and tend to agglomerate and precipitate from the solvent to form a narrow sized particle. In the process there is no emulsifier or suspending reagent being added, so the copolymer is at high purity.

So in this work, precipitation polymerization was employed to prepare MG molecularly imprinted polymers in acetonitrile using MG, methacrylic acid (MAA), ethylene glycol dimethacrylate (EGDMA), and azobisisobutyronitrile (AIBN) as template, functional monomer, cross-linker, and initiator, respectively. The characteristics of the obtained polymers were analyzed through environmental scanning electron microscope (ESEM); the rebinding properties were demonstrated by equilibrium rebinding experiments. The selectivity of the obtained particles was elucidated by the different rebinding capabilities of the MG and the structurally related compounds. Desired MG imprinted polymers can be used for the separation, enrichment and analysis of trace MG in seafood, water and other matrices.

2. Experimental

2.1. Chemicals

MG was purchased from Tianjin North Chemical and Glass Purchase and Sale centre, leucomalachite green (LMG) was purchased from Shanghai Reagent Co., China Medicine Group, crystal violet (CV) from Tianjin Guangfu Fine Chemical Institute, and methyl violet (MV) from Tianjin Reagent Institute. MAA, EGDMA and AIBN were obtained from Sigma–Aldrich. Acetonitrile, acetone, methanol, and acetic acid (analytical grade) were purchased from Tianjin No. 1 Chemical Reagent Factory.

2.2. Preparation of molecularly imprinted polymers and non-molecularly imprinted polymers

In a 60 mL glass flask, functional monomer MAA, cross-linker EGDMA, and reaction initiator AIBN were dissolved in acetonitrile. The ratio of them is given in Table 1. The mixture solution was poured into a sonication bath for 10 min to dissolve the chemicals well, under this condition the complex of the template and the functional monomer formed by hydrogen bonding, ionic bonding or by other interactions. Then the solution was sparged with nitrogen gas for about 5 min to remove oxygen which inhibits the polymerization, and the flask was sealed under nitrogen gas. Polymerization was performed in a water bath with the temperature being maintained at 60 ° C for 12 h. After polymerization, the polymers were collected by centrifugation at 5000 rpm for 5 min, and then the template molecule was removed by washing with acetone first and then with a methanol:acetic acid (9:1) mixture until no template molecules were detected, then the polymers were placed in an oven at 40 °C under vacuum for drying. Blank microspheres were made with the same procedure just in the absence of the template molecules; the obtained polymers were non-imprinted polymers (N-MIP).

2.3. Characterization of the size and shape of the obtained polymers

The surface, size and the shape of the polymers prepared by precipitation polymerization were studied using an environmental scanning electron microscope (ESEM) (Philips XL30 ESEM).

Table 1
Composition of the polymerization mixture for MIP and N-MIP

Function	Compound	Composition of the polymer (mass or volume)	
		MIP	N-MIP
Template	MG	0.5 mmol	0 mmol
Monomer	MAA	2 mmol	2 mmol
Cross-linker	EGDMA	10 mmol	10 mmol
Initiator	AIBN	10 mg	10 mg
Porogen	Acetonitrile	30 mL	30 mL

2.4. Spectrophotometric analysis

A series of solution was prepared with a fixed concentration of MG and varying amounts of MAA in acetonitrile. The changes in absorbance and difference absorption spectra of their solutions were determined with a Beckman DU530 Spectrophotometer using pure acetonitrile as reference.

2.5. Equilibrium rebinding experiments and Scatchard analysis

About 20 mg MIP and N-MIP were added into 2 mL Eppendorf tubes, respectively. Then 1.5 mL MG acetonitrile solution with concentration varying from 4 μmol/L to 2.2 μmol/L was added. The mixture was incubated under room temperature for 12 h and then was centrifuged for 10 min at 5000 rpm; the free MG in acetonitrile was determined using spectrometer at 620 nm. Bound MG to the polymers was obtained by the difference between the initial concentration and the free MG in supernatant.

The selectivity of the MIP was investigated using MG and the structurally related compounds' adsorption on the MIP and N-MIP.

All data obtained in the experiments were processed by Origin 7.0.

3. Results and discussion

3.1. Preparation of MIP and N-MIP

Functional monomers are responsible for the binding interactions in the imprinted binding sites, and for non-covalent molecular imprinting protocols, and are normally used in excess relative to the number of moles of template to favor the formation of template and functional monomer assemblies. The molar ratio of template, monomer, cross-linker and initiator was applied from a reported literature [14]. At present, the most commonly used monomer is MAA, which can form hydrogen bond with template monomer in porogen prior to polymerization; in this work the carboxylic group in MAA can form ionic bond with the amine in MG [15], the specific and positioned interactions would contribute to the MIP's selective affinity. EGDMA and AIBN were used as cross-linker and reaction initiator, respectively. Cross-linker fulfills three functions in an imprinted polymer. First of all, it is important in controlling the morphology of the polymer matrix; secondly, it serves to stabilize the imprinted binding sites; and finally, it imparts mechanical stability to the polymer matrix. The high cross-linker ratio is generally preferred in order to access permanently porous materials and to generate materials with adequate mechanical stability, polymers with cross-linker ratio in excess of 80% are often used. The volume of acetonitrile in the polymerization experiment was found out by a series of small-scale format polymerization experiments. The obtained polymer is shown in Fig. 1. Fig. 1a shows that a kind of globular polymer was obtained, while Fig. 1b shows a kind of bulk polymer, which is like pieces of clouds formed, the possible reason is that the MG affected the procedure in the polymerization.

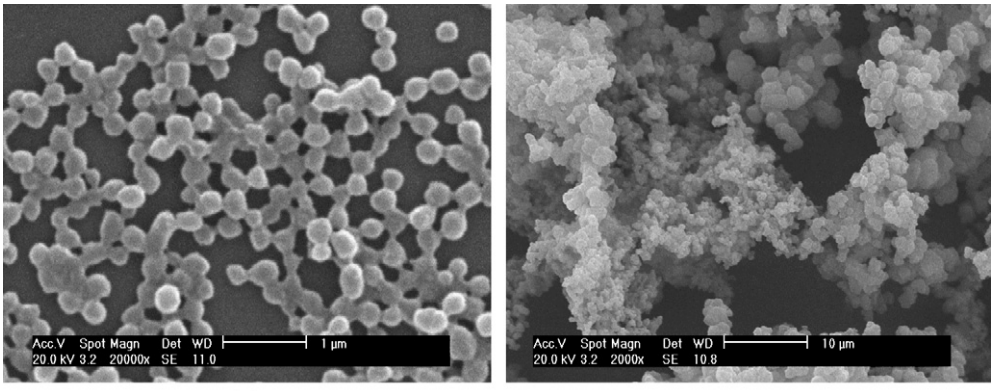
3.2. Spectrophotometric analysis

The principle of molecular imprinted technology is the fixation of the host–guest structure formed by the interaction between template and monomer through hydrogen bond, ionic bond or other interaction forces. In the MG imprinting process, spectrophotometric analysis was employed to elucidate the recognition mechanism on a molecular level. The complexes formed between the template and monomer can be expressed as follows:

$$\mathrm{T} + n\mathrm{M} \overset{K}{\leftrightarrow} \mathrm{TM}_n \quad (1)$$

where T is MG, and M is MAA, $n = 1, 2, 3\ldots q$. K refers to association constant.

In order to elucidate the ratio between the template and the functional monomer in the complex in acetonitrile, spectrophotometric method was employed. In this study the difference spectra of MG (0.008 mM) in acetonitrile are sensitive to the presence of various amounts of MAA (the concentration of MAA is 0.04 mM, 0.08 mM, 0.12 mM, 0.20 mM, and



(a) (b)

Fig. 1. SEM photograph of N-MIP (a) and MIP (b).

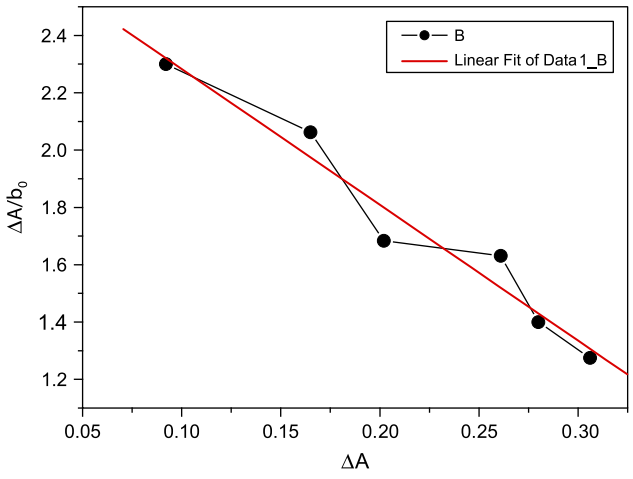


Fig. 2. Plots of $\Delta A/b_0$ versus ΔA at 202 nm.

0.24 mM). In theory, at the interaction equilibrium, the complex AB_n will be formed; Eq. (3) will be obtained as follows.

$$A + nB = AB_n \tag{2}$$

$$k = \frac{[AB_n]}{[A][B]^n} \tag{3}$$

As the initial concentration of MAA (b_0) is greater than that of MG (a_0), the concentration of the complex (c) can be calculated according to:

$$c = \frac{a_0 b_0^n k}{1 + b_0^n k} \tag{4}$$

and the absorbance can be measured at the maximum wavelength:

$$A = [(a_0 - c)\varepsilon_A + (b_0 - nc)\varepsilon_A + c\varepsilon_C]l \tag{5}$$

where ε_A, ε_B and ε_C are the molar absorptivities of A, B and C, respectively. The absorbance is

$$A_0 = (a_0\varepsilon_A + b_0\varepsilon_A)l \tag{6}$$

and the absorbance difference measured is

$$\Delta A = A - A_0 = c\Delta\varepsilon l \tag{7}$$

where $\Delta\varepsilon = \varepsilon_C - \varepsilon_A - n\varepsilon_B$. Substitution of Eq. (7) in Eq. (4) yields:

$$\frac{\Delta A}{b_0^n} = -K\Delta A + K\Delta\varepsilon_C a_0 l \tag{8}$$

where n refers to the composition of complex, $n = 1, 2, 3, \ldots, q$. K refers to association constant; the value can be calculated by plotting $\Delta A/b_0^n$ versus ΔA.

In this study the plot of $\Delta A/b_0^n$ versus ΔA in the studied range is linear as shown in Fig. 2, K is calculated to be 4740 $(\text{mol/L})^{-1}$ from its slope. This indicated that the 1:1

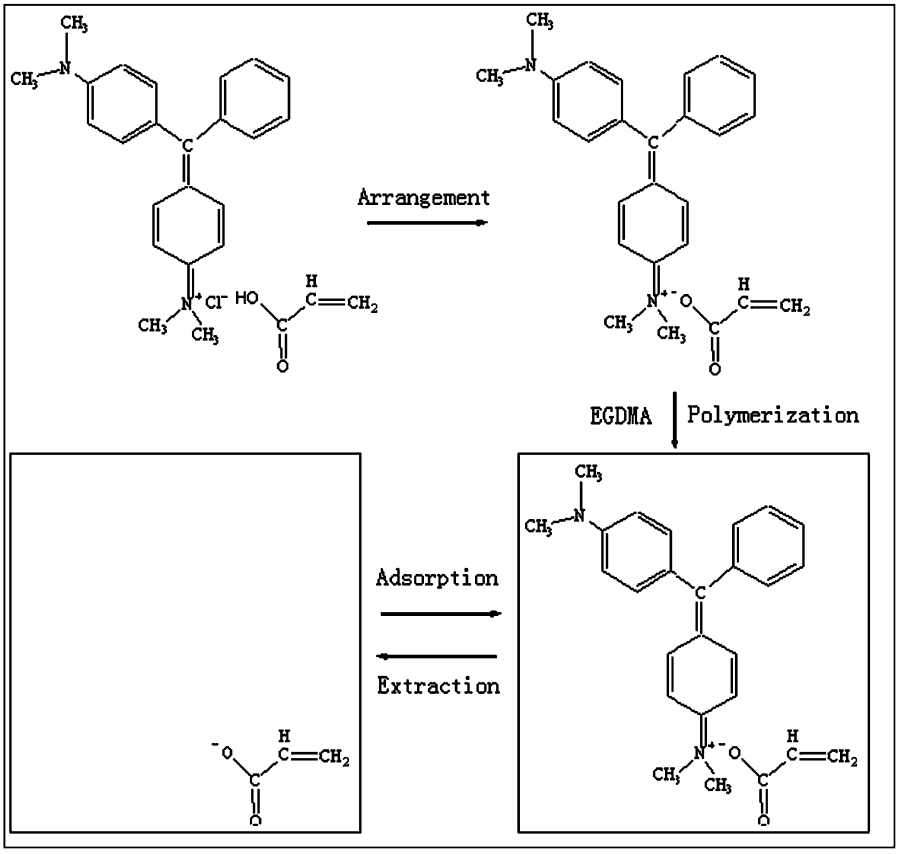


Fig. 3. The procedure for MG MIP preparation.

complexes may be formed in the solution prior to polymerization. So the procedure of MG MIP preparation is possible as follows (shown in Fig. 3).

3.3. Equilibrium rebinding experiments and Scatchard analysis

In order to investigate the MG binding characteristics on the imprinted polymers, the binding isotherm was determined in the range of 4.0–22.0 μmol/L as the initial concentrations of MG, the results are shown in Fig. 4. In the studied concentration range, the MG adsorption amount increases with the increasing of the MG's initial concentration, and the MG adsorption amount on MIP is larger than that on N-MIP, which indicates that there are specific rebinding sites on imprinting polymers for the template.

The data obtained in the batch binding experiment were fitted by Langmuir model (shown in Fig. 5).

$$B = \frac{B_{\max} \times C}{K_D + C} \tag{9}$$

where K_D is an equilibrium dissociation constant, C is the free equilibrium concentration of MG, and $B_{\max}$ is an apparent maximum number of MG binding on the polymers. The $B_{\max}$ of MIP (2.33 μmol/g) is larger than that of N-MIP (0.51 μmol/g).

The saturation binding data were further processed with Scatchard equation to estimate the binding sites' properties of MG on the imprinted and non-imprinted polymers. The Scatchard equation is as follows:

$$B/C = (B_{\max} - B)/K_D \tag{10}$$

Fig. 6 shows that the Scatchard plots are only a single straight line (line 1), which indicates that there is only a class of binding sites populated in the non-imprinted polymers. The Scatchard plots are also a single straight line (line 2), which indicated that there exists one kind of binding sites populated in the imprinted polymers.

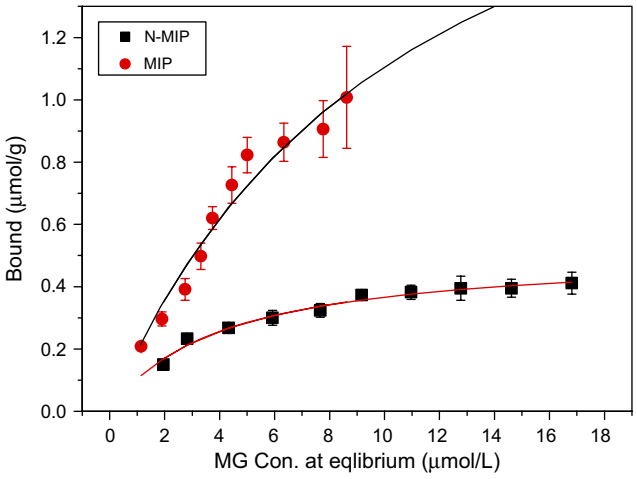


Fig. 5. Fit results of MG adsorption isotherm on MIP and N-MIP ($n = 3$).

3.4. Binding selectivity of MIP

In order to verify that the imprinted polymers are selective to MG, the binding of MG and some structurally related compounds on the polymers was investigated. The different compounds and their structures are shown in Fig. 7. The imprinted polymers obviously exhibited high binding affinity for MG, while the structurally related compounds used showed less binding capacity. As for the non-imprinted polymer microspheres, they showed considerably less binding for most of the analytes although some of them seem to show slight binding to the non-imprinted polymers, but the binding on the non-imprinted polymers is lower than that on the imprinted polymers.

The selectivity of the MIP may also give some insights into the molecular recognition mechanism. Fig. 8 shows that LMG, CV and MV have relatively high affinity for the MIP, but the binding amount of them on MIP is obviously lower than that of MG on the MIP. This could be easily explained by their close homology to MG, but there is still a difference among

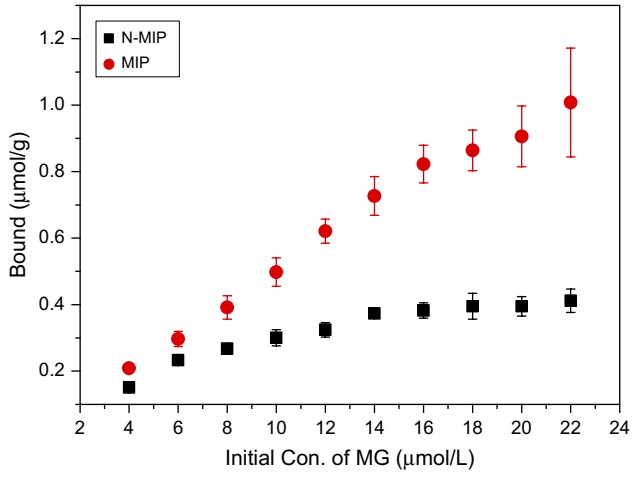


Fig. 4. Isotherm of MG adsorption on MIP and N-MIP ($n = 3$).

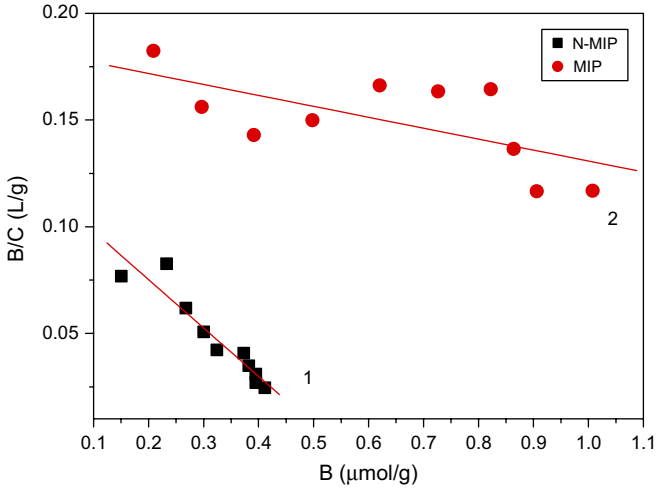


Fig. 6. Scatchard plots of MG adsorption on MIP and N-MIP.

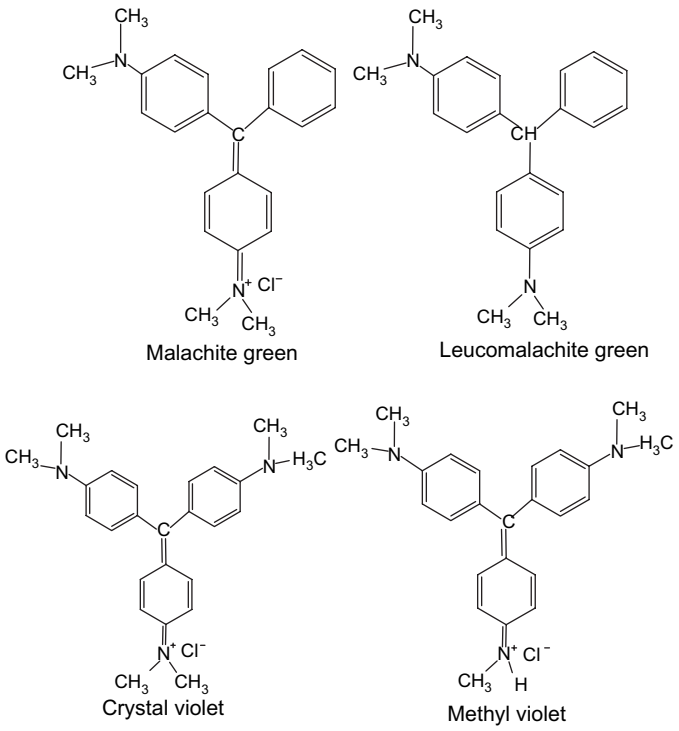


Fig. 7. Structures of the compounds used in this study. MG was used as template in the molecularly imprinted polymers' preparation.

them, the spatial diameter of MV or CV is larger than that of MG, for there are two $-N(CH_3)_2$ functional group in MV and CV, but there is only one $-N(CH_3)_2$ functional group in MG. LMG is the metabolite of MG in vivo, the spatial structure of the former has changed. So the binding amount of LMG on the imprinted polymers is lower than that of MG on the imprinted polymers. The binding amount of MG and the structurally related compounds on the non-imprinted polymer is lower than that on the imprinted polymer. The results indicated that there are binding sites in imprinted polymers and the binding sites show good specific and selective capability to the template molecule MG.

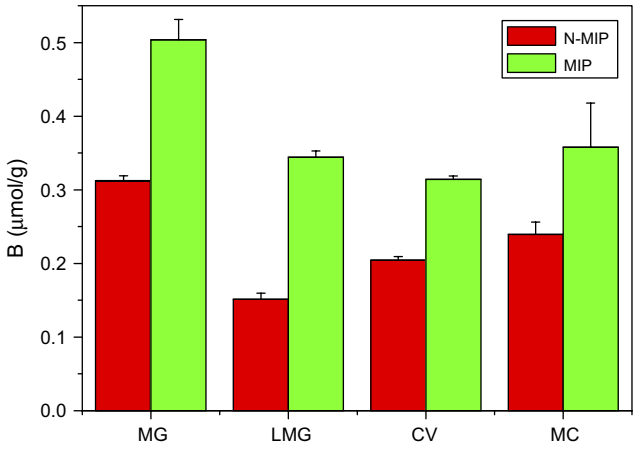


Fig. 8. Binding selectivity test of MG and the structurally related compounds on MIP and N-MIP.

4. Conclusions

Desired uniform MG imprinted polymers were synthesized with good yield by precipitation polymerization, the polymers exhibited highly specific and selective affinity for MG in acetonitrile, which was used as porogen in polymerization. From above it can be concluded that the imprinted polymers could be used as a good material for analytical purposes, such as for separation, enrichment, purification and analysis of trace MG in seafood, water or soil matrices.

Acknowledgements

The authors gratefully acknowledge the financial support by the National Natural Science Foundation of China (No. 30371218) and High Technology Research and Development Program of China (863 Project No. 2004AA649110).

References

[1] Elferink JGR, Booij HL. The action of some triphenylmethane dyes on yeast and erythrocyte membranes. Drug Res 1975;25:1248–52.
[2] Culp SJ, Beland FA. Malachite green: a toxicological review. J Am Coll Toxicol 1996;15:219–38.
[3] Fernandes C, Lalitha VS, Rao KVK. Enhancing effect of malachite green on the development of hepatic pre-neoplastic lesions induced by *N*-nitrosodiethylamine in rats. Carcinogenesis 1991;12:839–45.
[4] Alderman DJ, Clifton-Hadley RS. Malachite green: a pharmacokinetic study in rainbow trout, *Oncorhynchus mykiss* (Walbaum). J Fish Dis 1993;16:297–311.
[5] Stubbings G, Tarbin J, Cooper A, Sharman M, Bigwood T, Robb P. A multi-residue cation-exchange clean up procedure for basic drugs in produce of animal origin. Anal Chim Acta 2005;547:262–8.
[6] Rahman IA, Saad B, Shaidan S, Sya Rizal ES. Adsorption characteristics of malachite green on activated carbon derived from rice husks produced by chemical–thermal process. Bioresour Technol 2005;96:1578–83.
[7] Mittal A, Krishnan L, Gupta VK. Removal and recovery of malachite green from wastewater using an agricultural waste material, de-oiled soya. Sep Purif Technol 2005;43:125–33.
[8] Gupta VK, Mittal A, Krishnan L, Gajbe V. Adsorption kinetics and column operations for the removal and recovery of malachite green from wastewater using bottom ash. Sep Purif Technol 2004;40:87–96.
[9] Garg VK, Kumar R, Gupta R. Removal of malachite green dye from aqueous solution by adsorption using agro-industry waste: a case study of *Prosopis cineraria*. Dyes Pigments 2004;62:1–10.
[10] Lavignac N, Allender CJ, Brain KR. Current status of molecularly imprinted polymers as alternatives to antibodies in sorbent assays. Anal Chim Acta 2004;510:139–45.
[11] Milojkovic SS, Dusan K, Comor JJ, Nedeljkovic JM. Radiation induced synthesis of molecularly imprinted polymers. Polymer 1997;38(11): 2853–5.
[12] Zhang LY, Chend GX, Fu C. Synthesis and characteristics of tyrosine imprinted beads via suspension polymerization. React Funct Polym 2003;56:167–73.
[13] Pérez-Moral N, Mayes AG. Comparative study of imprinted polymer particles prepared by different polymerization methods. Anal Chim Acta 2004;504:15–21.
[14] Tamayo FG, Casillas JL, Martin-Esteban A. Highly selective fenuron-imprinted polymer with a homogeneous binding site distribution prepared by precipitation polymerization and its application to the clean-up of fenuron in plant samples. Anal Chim Acta 2003;482:165–73.
[15] Attardi ME, Porcu G, Taddei M. Malachite green, a valuable reagent to monitor the presence of free COOH on the solid-phase. Tetrahedron Lett 2000;41:7391–4.