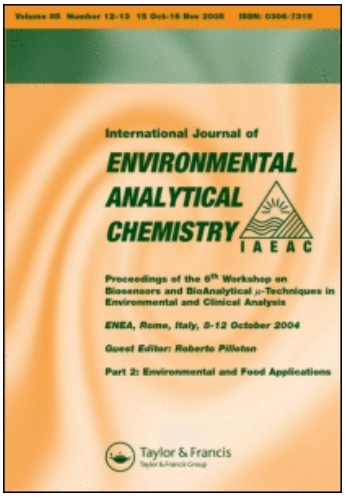

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MOLECULARLY IMPRINTED POLYMERS FOR NITROPHENOLS – AN ADVANCED SEPARATION MATERIAL FOR ENVIRONMENTAL ANALYSIS

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Molecularly imprinted polymers (MIPs) for 4-nitrophenol have been successfully prepared by a thermal polymerization method using 4-vinylpyridine (4-VP) and ethylene glycol dimethacrylate (EDMA) as functional monomer and cross-linker, respectively. The obtained materials were evaluated with respect to their selective recognition properties for 4-nitrophenol by HPLC using organic and aqueous eluents. Furthermore, the specific binding sites that have been created during the polymerization process were analyzed via radioligand binding assays. The successful imprinting against 4-nitrophenol provides a new opportunity for advanced separation materials used in environmental analysis.

Keywords: 4-Nitrophenol; molecular imprinting; HPLC separation

INTRODUCTION

As is generally known phenolic components are present in many industrial processes and are consequently released in industrial effluents and waste water. Apart from their influence on taste and odor of fish and water, some of them reveal toxic effects [1,2] to aquatic biota. Particularly, 4-nitrophenol (4-NP) is considered as a substance with a high environmental impact due to its toxicity and per-

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sistence. 4-NP enters the environment as a breakdown product of chemicals or pesticides, as a result of industrial discharges or from car exhausts and is therefore listed as priority pollutant by the US Environmental Protection Agency (EPA) [3] and the European Union [4]. Hence, the determination of nitrophenols in water has become increasingly important and there is a strong demand for efficient extraction and characterization methods of these compounds from water matrices. As described in the literature solid-phase extraction (SPE) [5,6] and high-performance liquid chromatography (HPLC) [7] have been applied for the analysis of phenols in water as well as an on-line method using solid-phase extraction coupled to supercritical fluid chromatography [8]. In addition, a surface plasmon resonance based optical sensing system with specific receptors entrapped in sol-gel and polymer matrices [9], an optical fluorescence sensor [10] and an amperometric biosensor [11,12] have been reported for the detection of phenolic pollutants.

The selective imprinting of polymers has developed into a powerful technique for the creation of recognition elements in highly cross-linked polymeric matrices. Generally, the methodology of molecular imprinting is based on the principle of using the functionality of a target molecule (called template or print molecule) to assemble its own recognition site by forming interactions with complementary functional groups of appropriate polymerizable monomers. These interactions are then "frozen" by polymerization carried out in a solution with high concentration of cross-linker. Subsequent removal of the template by extraction creates binding sites with the precise spatial arrangement of functional groups ensuring reversible binding and highly selective recognition of the target molecule in the optimal case (see Figure 1). As some of these polymers have high selectivity and affinity constants, this feature makes them particularly suitable as separation material for HPLC [13,14,15] or for enhancing the selectivity in solid-phase extraction [16]. Besides, a novel chemical sensor principle based on molecularly imprinted polymers as selective recognition layers and infrared evanescent wave spectroscopy (IR-EWS) has recently been reported [17] presenting a new perspective in the field of on-line environmental monitoring.

In the present paper we demonstrate the feasibility of preparing 4-nitrophenol imprinted polymers and discuss their application as stationary phase for HPLC measurements. Different mobile phases have been tested with 4-nitrophenol and structurally related molecules as analytes to characterize the recognition abilities of this novel separation material. Furthermore, radiolabelled binding assays have been performed in order to retrieve information on the specific binding sites of the polymer. These results may yield novel approaches for the development of enrichment and clean-up materials for 4-NP and possible sensor coatings for IR-EWS analysis.

Figure 1

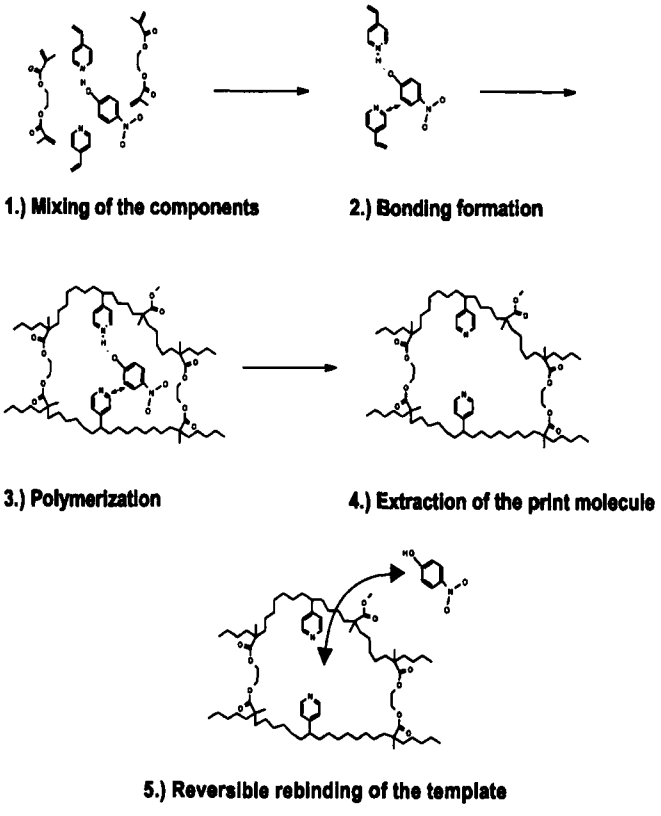

FIGURE 1 Principles of non-covalent molecular imprinting: 4-NP is the template molecule, 4-vinylpyridine the functional monomer and EDMA used as cross-linking agent

EXPERIMENTAL

Chemicals

Ethylene glycol dimethacrylate (EDMA), 2,2′-azo-bisisobutyronitrile (AIBN), 4-vinylpyridine (4-VP), 3-nitrophenol (3-NP), and 4-nitrophenol (4-NP) were purchased from Merck (Darmstadt, Germany). [^{14}C] 4-nitrophenol and 2-nitrophenol (2-NP) were obtained from Sigma (St. Louis, MO, USA), phenol, *p*-cresol and 4-nitrobenzyl alcohol were provided by Aldrich (Gillingham-Dorset, USA). 4-VP was distilled prior to use. All other chemicals were of analytical grade, the solvent were of HPLC quality.

Preparation of molecularly imprinted polymers

2 mmol of the template, either 4-NP, 3-NP or 2-NP, was dissolved in 11.2 mL dry acetonitrile (AcN) together with 8 mmol of the functional monomer 4-vinylpyridine (4-VP). 40 mmol of the crosslinker, ethylene glycol dimethacrylate (EDMA), and 0.48 mmol of the polymerization initiator, azo-bisisobutyronitrile (AIBN), were added and the solution was purged thoroughly with nitrogen on an ice bath for 5 minutes. The degassed pre-polymerization mixture was subsequently polymerized by thermal initiation in a 60°C waterbath overnight. The bulk polymer was grinded in a mechanical mortar (Retsch, Haan, FRG) and wet-sieved by hand with acetone through a 25 μm sieve (Retsch, FRG). The resulting suspension was sedimented several times until all the fines were removed. Finally, the polymer particles were filtrated, washed with methanol and dried at 45 °C for 24 hours. As reference, a non-imprinted polymer (control polymer) was prepared in the same way without the addition of template.

Preparation of the column

3 g of the sieved polymer particles were sonicated in acetone and were packed into stainless-steel HPLC columns (250x4.6 mm) with acetone at 200 bar using an air driven fluid pump (Haskel Engineering Supply Co., Burbank, USA). In order to extract the template molecule from the polymer material, the column was washed on-line with methanol/acetic acid (AcOH) (7:1, v/v) at 1 mL min^{-1} until a stable baseline was obtained.

HPLC analysis

The HPLC-analysis was performed using a Beckman HPLC (Brommola, Sweden) including a 126 binary gradient system, high pressure mixing and a 168 diode array detector. After complete extraction of the template, each column was equilibrated with different mobile phases, either AcN/Heptan/AcOH (94:5:1,v/v/v) or phosphate buffer (40 mmol)/AcN/AcOH (490:500:10, v/v/v); the pH of the aqueous part was adjusted to pH 7 with 1M NaOH prior to mixing with AcN. The elutions were performed at ambient temperature and monitored spectrophotometrically at 190 – 350 nm. The flow rate was kept constant at 1 mL min^{-1} throughout the whole study.

For each chromatographic run 0.002 mg of the compounds in 20 μl of the mobile phase were injected, using 0.2 μl acetone as void marker. Capacity factors were calculated as $k'=(t-t_0)/t_0$ where t is the retention time for the compound and t_0 corresponds to the retention time for the void marker. Separation factors

were calculated as $\alpha=k'_{PM}/k'_{TS}$, with PM indicating the print molecule and TS the respective test substance. The retention index (RI) was calculated as $RI=\alpha_{CP}/\alpha_{MIP}$, where MIP and CP indicate the molecularly imprinted polymer and control polymer, respectively.

Radioligand binding assays

Saturation studies

The binding capacity of the polymers was measured by saturation studies as described previously [18]. Varying amounts of polymer were incubated overnight and at room temperature with 4.5 pmol (1,10Bq) "hot" [^{14}C] 4-nitrophenol in 1 mL AcN. A rocking table ensured gentle mixing. The polymer particles were then separated by centrifugation at 14.000 rpm for 10 min, 500 µL of the supernatant was mixed with 10 mL scintillation liquid (Ecoscint O, National Diagnostics, Atlanta, GA) and the radioactivity was then measured using a model 2119 Rackbeta β-radiation counter from LKB Wallac (Sollentuna, Sweden).

Competition analysis

The imprinted polymer particles were suspended in AcN (72 mg mL^{-1}) and sonicated to form polymer stock suspensions, from which 200 µL were transferred into microcentrifuge tubes. Varying amounts of non-radiolabelled ligand and the same amount of radioligand as used in saturation studies were added and the final volume adjusted to 1 mL with AcN. The competition binding was allowed to proceed overnight by incubation at ambient temperature using a rocking table. The amount of bound radioligand was estimated by measuring the radioactivity from 500 µL supernatant following centrifugation at 14.000 rpm for 10 min. Affinity constants and binding site populations were calculated using the software EBDA/LIGAND from Elsevier-Biosoft (Amsterdam, NL).

RESULTS AND DISCUSSION

HPLC measurements

Primarily, imprinted and non-imprinted HPLC columns were compared for the retention time of nitrophenols and structurally related molecules. Two different mobile phases were used for characterization, AcN/Heptan/AcOH as organic mobile phase and phosphate buffer (25 mmol, pH 7)/AcN/AcOH as aqueous mobile phase. The results for the capacity factors, separation factors and reten-

tion indices, which are usually used to characterize the extent of the imprinting effect [13], are presented in Table I.

TABLE I Capacity factors, separation factors and retention indices of different analytes eluted on 4-nitrophenol imprinted polymers and on blank polymers

	Organic Mobile Phase					*Aqueous Mobile Phase*				
	k'_{CP}	k'_{MIP}	α_{CP}	α_{MIP}	*RI*	k'_{CP}	k'_{MIP}	α_{CP}	α_{MIP}	*RI*
4-nitrophenol	0.84	1.76	1	1	1	1.82	2.42	1	1	1
2-nitrophenol	0.19	0.22	4.39	8.07	0.54	1.82	1.83	1.00	1.32	0.76
3-nitrophenol	0.66	0.91	1.28	1.94	0.66	1.80	1.98	1.01	1.22	0.82
p-cresol	0.31	0.41	2.70	4.03	0.62	1.16	1.28	1.56	1.89	0.83
phenol	0.36	0.44	2.34	4.03	0.58	0.99	1.07	1.82	2.26	0.80
4-nitrobenzyl alcohol	0.21	0.26	3.93	6.86	0.57	0.70	0.76	2.58	3.17	0.81

As the capacity factors of the analytes are higher for the MIPs than for the control polymer, the 4-NP imprinted polymer shows a markedly stronger affinity for each analyte. This effect is most pronounced for 4-NP, (k'_{MIP}=1.76, k'_{CP}=0.84 in organic mobile phase, k'_{MIP}=2.42, k'_{CP}=1.82 in aqueous mobile phase). As the retention indices for the other analytes are less than one, the polymer is considered in both mobile phases to be selective to the original print molecule.

It is proposed that an ionic interaction takes place between 4-VP and the nitro-group of 4-NP as well as hydrophobic interaction with the aromatic ring system of 4-NP. An H-bonding interaction, which is possible in organic eluent, is disturbed in the presence of water explaining the lower selectivity in aqueous eluent than in organic eluent [19].

In the next step, a mixture of the print molecule and structurally related substances such as 2-NP and 3-NP were applied to the HPLC system to study the separation properties of the polymers. The chromatograms in Figure 2 show the ability of the imprinted polymer to achieve a significantly improved separation for the print molecule 4-NP, which becomes even more obvious when applied in aqueous phase. While the control polymer has no ability of resolving the mixture, the imprinted stationary phase separates 4-NP from the other substances. The considerably pronounced tailing of the 4-NP peak is a hint for a heterogeneous binding site distribution with specific and non-specific sites within the polymer. The print molecule can interact differently with these binding sites; it is retained for a longer time in the column due to these interactions and, consequently, produces a broader peak with tailing. In some cases of molecular

imprinting this tailing effect creates extensively long peaks and consequently makes the column unsuitable for HPLC analysis but in the case of 4-NP this feature is not too pronounced. Moreover, the obtained data proof that such imprinted polymers will be highly suitable for solid phase extraction (SPE) and selective enrichment layers for sensor applications, which are favored as commercial application for MIPs [20].

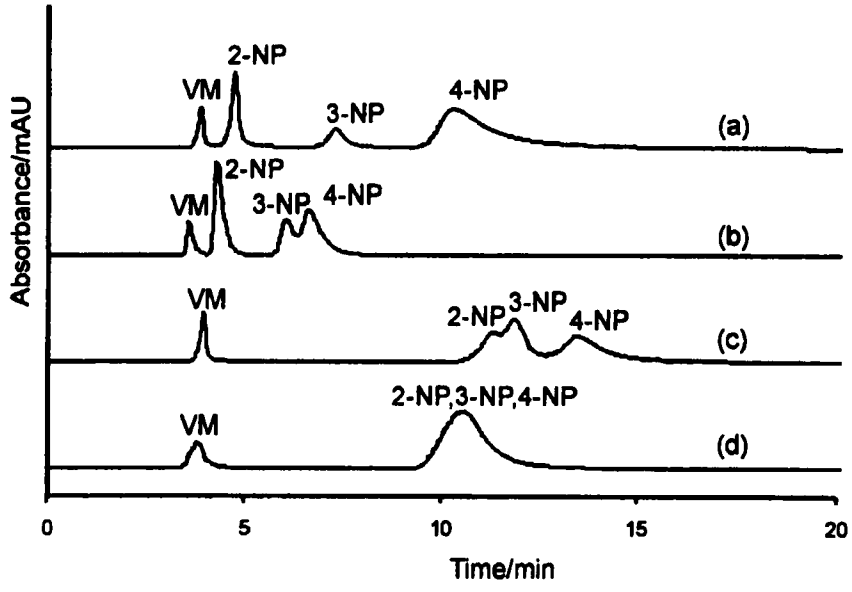

FIGURE 2 Chromatograms of a mixture of void marker (VM), 4-NP, 3-NP, 2-NP applying: (a) the 4-NP imprinted polymer as stationary phase and an organic mobile phase, (b) control polymer as stationary phase and organic mobile phase, (c) 4-NP imprinted polymer as stationary phase and aqueous mobile phase, (d) control polymer as stationary phase and aqueous mobile phase

The selectivity for the print molecule can be gained because of the different shapes of analyte molecules and the various strengths of interaction between the analytes and the sites of the imprinted polymers. Besides, the different steric structures, the change of the hydrogen bond acidity of the hydroxy-group, the electronic structure of the phenyl ring caused by a different position of the nitro-group (ortho or meta), or a different or even no functional group (*p*-cresol, phenol, 4-nitrobenzyl alcohol), may also be responsible for less binding to the polymer. As the RI values of nitrobenzyl alcohol, phenol and *p*-cresol are very similar to the ones of 2-NP and 3-NP (see Table I) only HPLC separations of 2-, 3- and 4-nitrophenol as a mixture have been performed. Since the template mainly interacts with the 4-VP via hydrogen bonds and ionic interactions, it can be assumed that the more separated the hydroxy-group and the nitro-group are

within the molecule, the stronger it will bind, hence, resulting in a more pronounced imprinting effect.

In order to verify the results of a possible imprinting effect against 4-NP, imprinted polymers against 3-NP have been prepared following the same procedure. If in this case the print molecule would also be eluted last, the existence of an imprinting effect could be confirmed superiorly. In comparison to the 4-NP imprinted polymers, these imprinted polymers show different separation capabilities. Despite the fact that the polymers still have the ability to separate structurally related molecules, the differences, concerning the retention indices, between imprinted or non-imprinted polymers are much smaller (see Table II).

TABLE II Capacity factors, separation factors and retention indices of different analytes eluted on 3-nitrophenol imprinted polymers and control polymers, in organic mobile phase

	k'_{CP}	k'_{MIP}	α_{CP}	α_{MIP}	*RI*
3-nitrophenol	0.84	0.95	1	1	1
2-nitrophenol	0.19	0.20	4.47	4.72	0.95
4-nitrophenol	0.84	0.99	1	0.96	1.04
p-cresol	0.31	0.34	2.76	2.84	0.97
phenol	0.37	0.39	2.26	2.43	0.93
4-nitrobenzyl alcohol	0.21	0.23	4.05	4.21	0.96

The 3-NP imprinted polymer still shows a remarkable change in the separation properties in comparison to the non-imprinted counterpart (see Figure 3). The analyte 3-NP yields a longer retention time due to interactions with the specific cavities.

In addition, HPLC runs using either a 3-NP imprinted stationary phase in combination with an aqueous mobile phase or a 2-NP imprinted stationary phase with organic and mobile phase have been also performed. In contrast to the ability of 4-NP imprinted polymers to resolve different phenol types, these imprints show no separation of the applied mixture and in particular no affinity to the print molecule. The decreased imprinting effect for 3-NP and 2-NP respectively corroborates the theory of varying binding properties of the three nitrophenol isomers due to the different position of the nitro-group.

Binding capacity and specificity of 4-NP imprinted polymers

In order to obtain more detailed information on the imprinted polymer, binding properties have been determined by saturation studies of these polymers with "hot" [^{14}C] 4-nitrophenol and by a competitive binding assay. Primarily, the

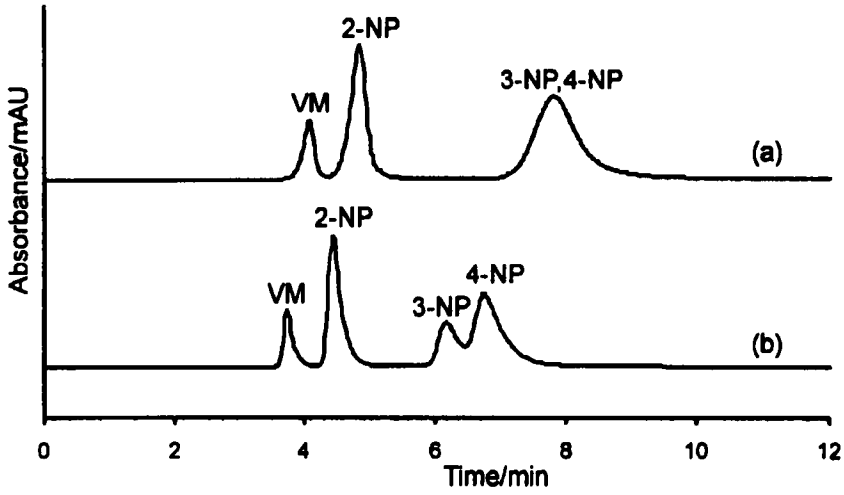

FIGURE 3 Chromatograms of a mixture of 4-NP, 3-NP, 2-NP applying: (a) the 3-NP imprinted polymer as stationary phase and an organic mobile phase, (b) control polymer as stationary phase and organic mobile phase

uptake of "hot" [^{14}C] 4-nitrophenol at various polymer concentrations by the MIP and the control-polymer was quantified by a binding assay in AcN (water-free). It was determined that there is a significant difference between the uptake of the MIP and the control polymer. The uptake of 25% initiate "hot" [^{14}C] 4-nitrophenol and a difference of about 15% to the control polymer at 15 mg mL^{-1} was found suitable for applying this polymer concentration in a competitive assay in order to obtain the displacement binding curve as shown in Figure 4.

Whereas at low competitive "cold" 4-NP concentrations, no "hot" [^{14}C]-4-nitrophenol is displaced, starting at a "cold" 4-NP concentration of about 1 μg mL^{-1} the "hot" 4-NP is displaced down to a bound/free ratio of about 34%. This displacement effect is the usual indication for specific binding sites within the polymer although a high content of unspecific binding can be expected due to the slow displacement curve and a maximum displacement down to 34% [18]. The following formula was used to obtain a Scatchard-plot (not shown) ([L] and [B] are the concentrations of free ligand and bound ligand; all binding sites can be categorized into n different types, and non-specific binding is taken into account):

$$[B] = \sum_{i=1}^{i=n} \left(\frac{B^i_{\max} \cdot [L]}{\frac{1}{K^i_a} + [L]} \right) + N \cdot [L]$$

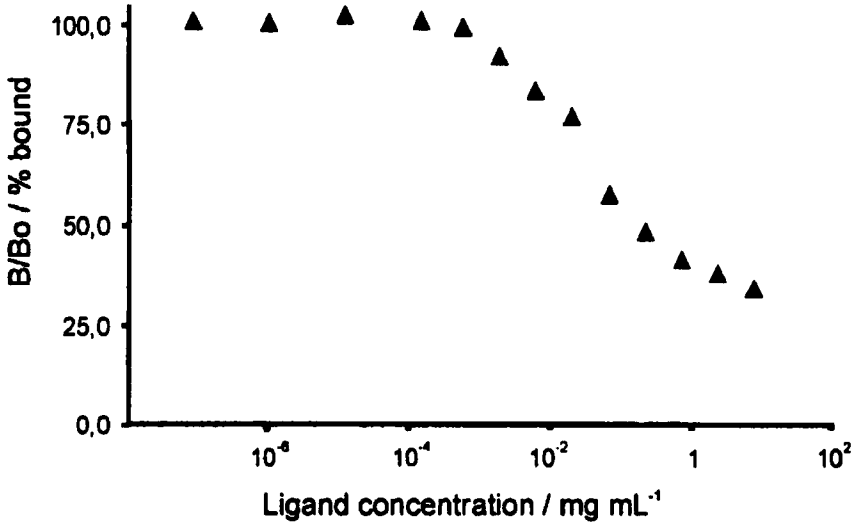

FIGURE 4 Displacement of radioligand binding to a 4-NP imprinted polymer under equilibrium condition. B/B_0 is the ratio of the amount (B) of radioligand [^{14}C] 4-nitrophenol bound in the presence of displacing ligand 4-nitrophenol to the amount (B_0) bound in the absence of displacing ligand

B^i_{max} and K^i_a are the population and equilibrium association constant for ligand binding to site i. N is the ratio of bound/free at infinite free concentration [21]. Using a two binding site model, 2 tangents can be drawn to gain the affinity constants (K_a) and the binding site populations (B_{max}) and non-linear curve fitting with a two-site model was performed using the computer program EBDA/LIGAND. While in reality the molecularly imprinted polymers carry various numbers of different binding sites each with its own population and association constant, the two-site model assumes only two sites: the high specific and the low specific ones. In this experiment the two-site model was found to satisfy quantitative evaluation of the imprinted polymers prepared via the non-covalent approach. As expected, the amount of low specific binding sites (K_a: 197 M^{-1}, B_{max}: 8.24 $\mu molg^{-1}$) in comparison to high specific binding sites (K_a: $2{,}2x10^4$ M^{-1}, B_{max}: 0.44 $\mu molg^{-1}$) is rather high but, nevertheless, the heterogeneous distribution of various shaped binding sites, as the commonly known characteristics of imprinted polymers, could be demonstrated. Compared with affinity constants for anti-nitrophenol monoclonal antibodies in literature [22], ranging from 10^7 to 10^8 M^{-1}, the K_a values for the 4-NP imprinted polymer are notably lower. Nevertheless they are in the range of previously obtained results of equilibrium association constants as well as binding site populations as reported in literature

[23,24]. Thus, they represent the first successful step towards the direction of a 4-NP "plastic antibody".

CONCLUSION

In the present study we have prepared an imprinted polymer against 4-nitrophenol and evaluated its characteristics by HPLC and radio-ligand binding assays. It was possible to demonstrate the selective recognition ability of the polymer for the print molecule in both, organic and aqueous media. Even though the amount of high specific binding sites, determined by the radioligand binding assay, is rather low, we proved the creation of MIPs with selective binding cavities for 4-nitrophenol. In particular, the behavior in aqueous media is of significant importance with respect to a potential application in SPE and for sensing purposes. Thus, research is currently ongoing in our laboratory aiming at developing chemically selective layers for 4-nitrophenol as recognition element for optical sensor systems.

Acknowledgements

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