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# Characterisation and quality assessment of binding sites on a propazine-imprinted polymer prepared by precipitation polymerisation

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**Abstract**

In this paper, the Langmuir–Freundlich isotherm (LF) is used to characterise a propazine-imprinted polymer obtained by precipitation polymerisation (MIP-P). Different rebinding studies were carried out allowing to explain the different interactions taking place between the molecularly imprinted polymer and six triazinic herbicides (desisopropylatrazine, desethylatrazine, simazine, atrazine, propazine and prometryn). The LF fitting parameters obtained (total number of binding sites, heterogeneity index and mean binding affinity) were compared to those obtained in a previous work for a propazine-imprinted polymer prepared by bulk polymerisation (MIP-B). From that study, it was concluded that precipitation polymerisation yielded polymers with a more homogeneous binding site distribution and higher affinity constants.


*Keywords:* Precipitation polymerisation; Quality assessment; Molecular imprinting; Propazine

## 1. Introduction

During the last years, molecularly imprinted polymers (MIPs) have been widely used as synthetic materials able to rebind the target analyte (template) for which they have been prepared. From those studies, it has been possible to demonstrate their potential to be used in the analytical chemistry field, especially in those areas where a high degree of selectivity is required such as solid-phase extraction [1], sensors [2,3], chromatography [4] and catalysis [5,6].

MIPs are synthesised by the polymerisation of an appropriate monomer and cross-linker in the presence of the target analyte (the template molecule). Once the polymer is obtained, the template molecule is removed leaving cavities complementary in size and shape to the analyte able to selectively rebind this molecule. However, the typical bulk polymerisation method used is far from ideal as a random shape and size distribution of particles is obtained. In addition, some authors have reported that the process of crushing and sieving the polymer after polymerisation can break the imprinted sites [7]. Also, a substantial number of the cavities may shrink after the template removal with polar organic solvents [7]. On the other hand, and especially when the non-covalent approach is used, the pre-polymerisation step in which template and monomer have to form an stable complex, is a non-well defined process. As a consequence, complexes with different template:monomer stoichiometry can be formed [8,9] and thus, the obtained MIPs present a heterogeneous binding site distribution limiting their applicability range (i.e. broad peaks in chromatography, non-linear response in sensors) and their selectivity.

In order to overcome these drawbacks, several polymerisation strategies allowing the preparation of spherical particles with a narrow particle size and a more homogeneous binding site distribution have been proposed in the literature [10]. From our point of view, precipitation polymerisation [11] is one of the most easy and well-suited proposed methods to obtain MIP micro-spheres with the desired characteristics. This methodology consists basically in the polymerisation of the mixture (template, monomer and

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cross-linker) in the presence of a higher amount of porogen than the typically used in bulk polymerisation method.

Recently, this polymerisation strategy was used to prepare a non-covalent fenuron-imprinted polymer [12], which leaded to the synthesis of spherical particles (∼1 μm) with a homogeneous binding site distribution. This result was remarkable as up to now only covalent MIPs had shown this kind of binding site distribution but not those synthesised using the non-covalent approach. In order to confirm the suitability of this strategy to prepare high quality molecularly imprinted polymers with homogenous binding site distribution, a new polymer using a different template (propazine) was prepared (MIP-P) and evaluated in the present work. Thus, the main aim of this paper is the characterisation and evaluation of the homogeneity of the binding sites of this polymer and to compare the results with those obtained for another non-covalent propazine-imprinted polymer prepared by bulk polymerisation (MIP-B) in a previous work [13]. This study was carried out by equilibrium rebinding experiments of not only propazine (template molecule) but also of other structurally related compounds able to interact with the obtained polymers.

## 2. Experimental

### 2.1. Reagents

Methacrylic acid (MMA), ethylene glycol dimethacrylate (EDMA) and azobisisobutyronitrile (AIBN) were purchased from Sigma–Aldrich Química S.A. (Madrid, Spain). Atrazine (A), simazine (SIM), propazine (PPZ), prometryn (PMT), desisopropylatrazine (DIA) and desethylatrazine (DEA) were purchased from Riedel de Haen (Seelze, Germany). Stock standard solutions (1 g $l^{-1}$) were prepared in toluene and stored at −18 °C.

Purified water was obtained from a MilliQ water system purchased from Millipore Ibérica S.A. (Madrid, Spain) and HPLC grade solvents (acetonitrile, toluene and methanol) were obtained from Scharlab S.L. (Barcelona, Spain).

### 2.2. Polymer preparation

The preparation of the propazine-imprinted polymers by bulk and precipitation polymerisation was described elsewhere [13,14]. Briefly, the template molecule (propazine, 1 mmol) and the monomer (MMA, 4 mmol) were added to a 25 ml glass tube and left in contact for 5 min. Then, the cross-linker (EDMA, 20 mmol), the initiator (AIBN, 2 mmol) and the porogen (toluene, 5 or 12 ml for bulk or precipitation polymerisation, respectively) were added. The mixture was purged with $N_2$ for 5 min and the glass tube was sealed under this atmosphere. Polymerisation was carried out in a thermostated water bath at 60 °C for 24 h, after which the template molecule was removed by Soxhlet extraction with a methanol:acetic acid (1:1) mixture for about 12 h. The recovery of the template after Soxhlet extraction was around 99% in both imprinted polymers. Non-imprinted polymers were obtained following the same procedure without the addition of the template molecule. Bulk polymer was crushed and sieved before Soxhlet extraction and particles within the 50–105 μm range were selected.

### 2.3. Rebinding experiments

About 100 mg of the polymer particles were placed in an empty solid-phase extraction cartridge. After conditioning with 50 ml of methanol, 50 ml of acetonitrile and 25 ml of toluene, 1 ml of standard solution of each herbicide independently or a mixture of all of them in toluene at a concentration range from 0.05 to 500 mg $l^{-1}$ was loaded onto the cartridge. Non-specifically bound triazines were removed by washing with 5 × 1 ml of a toluene:acetonitrile (4:1) mixture, and the analytes were quantitatively eluted with 1 ml of acetonitrile and 7 × 1 ml of methanol. Then, the extract was evaporated to dryness and redissolved in 1 ml of MilliQ water. Loading, washing and elution steps were carried out at a flow-rate of 1 ml $min^{-1}$.

Analyte concentrations in the final solution, representing the amount of analyte bound to the polymer (B), were determined by HPLC-UV as described below. The amount of unbound analyte to the polymer (F) was obtained by subtracting B from that of initial analyte loaded to the polymer.

### 2.4. Chromatographic analysis

All measurements were performed in an HPLC system from Thermo Separation Products consisting of a ConstaMetric 4100 Series high pressure pump, a Spectro Monitor 5000 photo diode-array detector and a Rheodyne 7725i injection valve equipped with a 100 μl loop. The analytes were separated on a Symmetry® Waters C18 column (150 mm × 3.0 mm i.d., 3.5 μm) using a linear gradient elution as follows: from 70% A (purified water) and 30% B (acetonitrile) to 30% A and 70% B in 25 min, and returning to initial conditions in 5 min. In those experiments where each triazine was analysed independently, isocratic elution using two different mobile phases (70% A:30% B for DIA and DEA, and 50% A:50% B for A, SIM, PPZ and PMT) was used. Triazinic herbicides were monitored at 220 nm and quantified by external calibration using peak area measurements.

### 2.5. Data analysis

Langmuir–Freundlich adsorption isotherms were fitted to the log–log plot of the experimental adsorption isotherms using the solver function in Microsoft Excel 98 by varying the fitting parameters to reach a value of 1 for $R^2$ as described by Umpleby et al. [15].

## 3. Results and discussion

As was stated in the Introduction, precipitation polymerisation seems to be one of the most suitable methods to prepare imprinted polymers with a homogeneous binding site distribution. In order to confirm this statement, a propazine-imprinted polymer was prepared by precipitation polymerization (MIP-P) and different rebinding experiments were carried out in order to assess the characteristics of this polymer.

Several mathematical models (Langmuir, bi-Langmuir, Freundlich, Toth, Langmuir–Freundlich, etc.) [16] have been used to address the heterogeneity observed in MIPs. From our point of view, Langmuir–Freundlich (LF) isotherm is the more appropriated mathematical model as it is able to describe how adsorption processes take place both in the subsaturated and saturated zones. In addition, this model allows the direct measurement of the fitting parameters, which can be used either for the comparison of different MIPs [15] or to study the adsorption of different compounds to the same molecularly imprinted polymer [13].

The LF isotherm describes a relationship between the concentration of bound (B) and free (F) guest in heterogeneous systems with three different coefficients according to the following equation:

$$B = \frac{N_t a F^m}{1 + a F^m} \quad (1)$$

where $N_t$ is the total number of binding sites, $a$ is related to the median binding affinity constant $K_o$ ($K_o = a^{1/m}$), and $m$ is the heterogeneity index, which will be equal to 1 for a homogeneous material, or will take values within 0 and 1 if the material is heterogeneous.

It is important to point out that conclusions derived from a rebinding experiment may only be accurate if the system studied is under equilibrium conditions and if analytes are only specifically bound to the polymer. Therefore, both equilibrium conditions and non-specific interactions have to be studied. Non-specific interactions were firstly evaluated in previous papers [13,14] by loading onto the corresponding non-imprinted polymer 1 ml toluene solution containing all the triazines under study (Fig. 1) and testing different toluene:acetonitrile mixtures as washing solutions. It was concluded that 20% of acetonitrile in toluene was enough to quantitatively remove non-specifically bound compounds from both non-imprinted polymers prepared by bulk (NIP-B) and precipitation (NIP-P) polymerisation, respectively.

In order to assess equilibrium, mixtures of polymer particles (about 100 mg) and 1 ml toluene solution containing 10 mg $l^{-1}$ of each triazine were kept in contact for 15, 30, 45, 60 and 120 min, in independent experiments, at room temperature in solid-phase extraction cartridges. After this incubation, the polymer was washed with 5 × 1 ml toluene:acetonitrile (4:1) and the analytes were quantitatively eluted with 1 ml acetonitrile and 7 × 1 ml methanol and B calculated as described in Section 2. A parallel

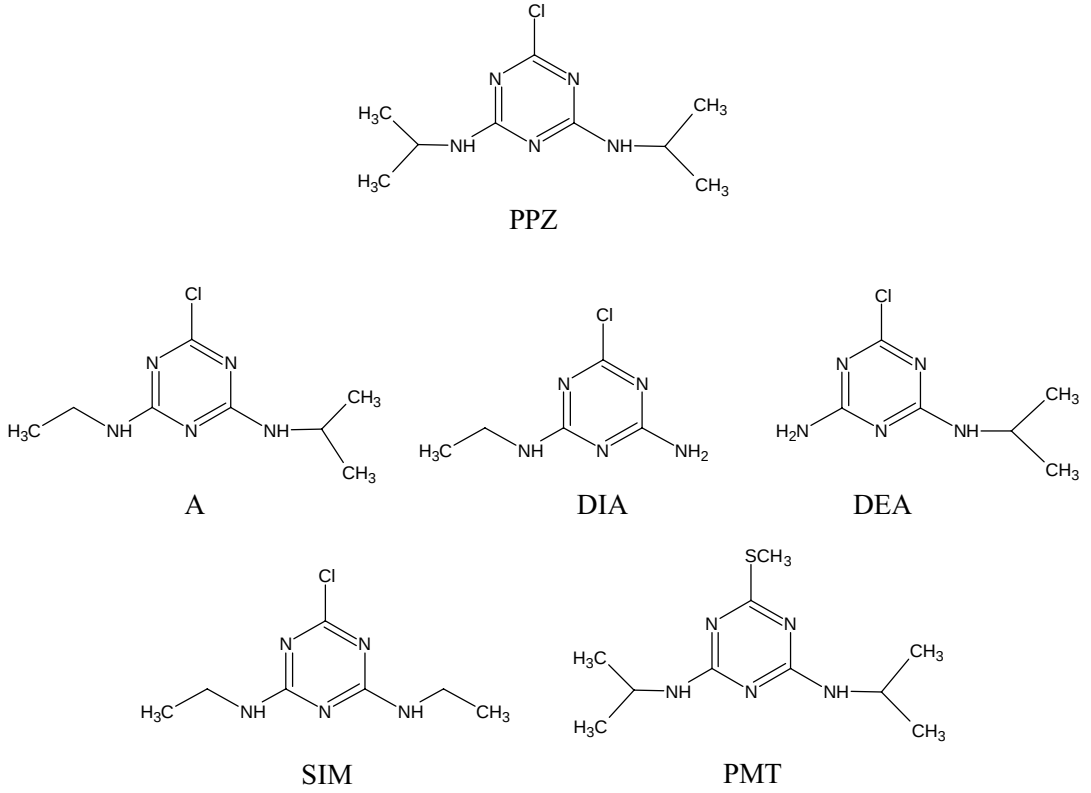


Fig. 1. Chemical structures of the selected triazines.

experiment loading the toluene solution containing analytes at $1\,ml\,min^{-1}$ (incubation time = 0) was also performed. The obtained results were compared each other and not significant differences were found between the B values obtained for the different periods of incubation, which means that equilibrium was immediately reached. This result clearly indicates that diffusion mass transfer is very fast, which, in principle, would allow the use of these polymers as a chromatographic stationary phase. However, it is important to stress that the behaviour of the polymers studied herein cannot be extended to other imprinted polymers as it is known that the template used during polymerisation affects some of the physical properties (i.e. porosity) of the obtained polymers.

### 3.1. Rebinding experiments

To perform this study, solutions of each herbicide at concentration ranging between 0.05 and $75\,mg\,l^{-1}$ were loaded independently onto the cartridge filled with the imprinted-polymer (MIP-P) and B and F were calculated as described in Section 2. The obtained data were initially compared to those obtained using a propazine-imprinted polymer prepared by bulk polymerisation (MIP-B), in order to evaluate the influence of the polymerisation strategy on the quality of the obtained binding sites.

#### 3.1.1. MIP-P versus MIP-B

Fig. 2 shows the experimental adsorption isotherms of each triazine in the propazine-imprinted polymer prepared by precipitation polymerisation (MIP-P) and the corresponding fitting LF isotherms. Table 1 shows the corresponding fitting coefficients together to those obtained in the previous study [13] using a propazine-imprinted polymer prepared by bulk polymerisation (MIP-B). The accuracy of these fitting parameters is assessed by the low relative standard error obtained for the fitting analysis (around 5% in all cases), and by the wide concentration range in which measurements were made, including both saturated and subsaturated zone. The validity of this range is demonstrated by the fact that the

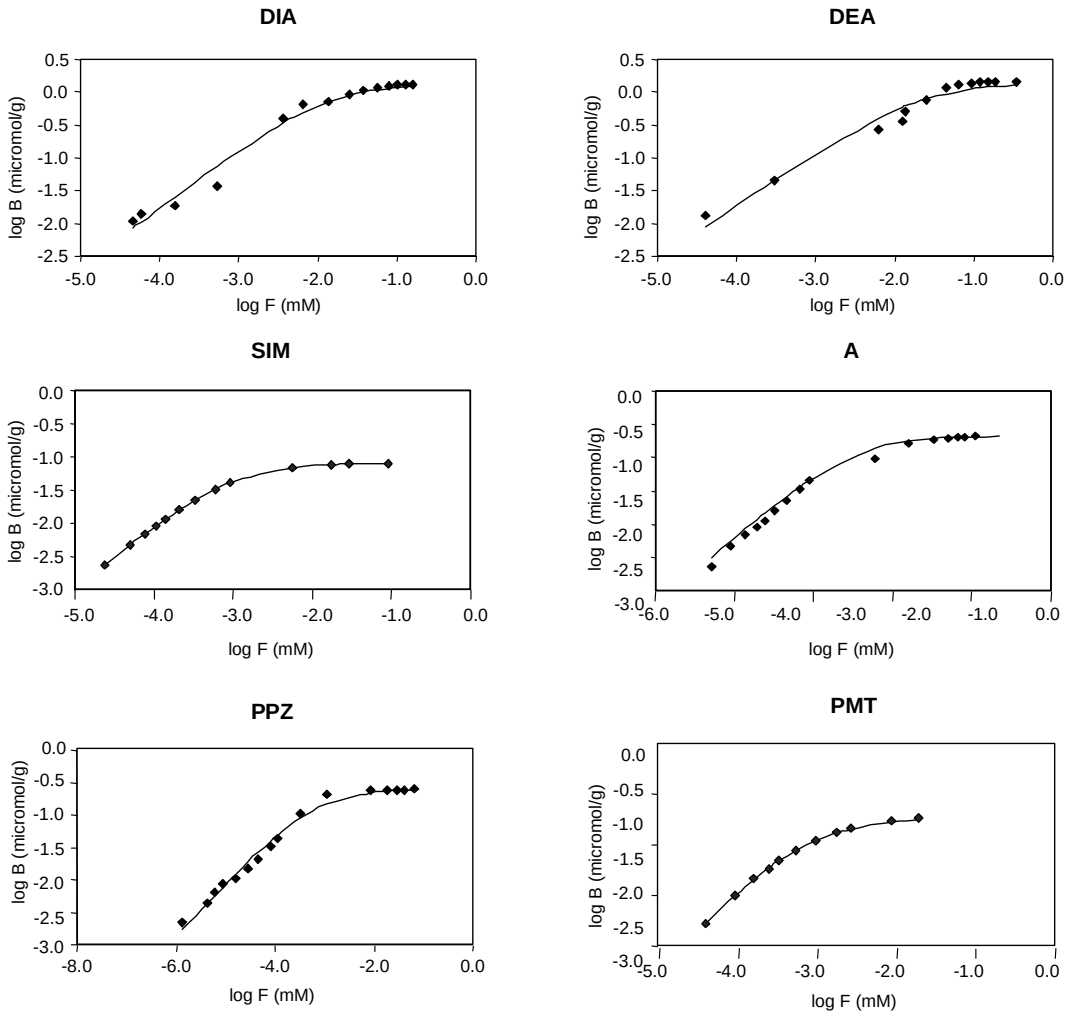


Fig. 2. Log plots of the adsorption isotherms for studied analytes in MIP-P. The experimental data (◆) were fit to Langmuir–Freundlich isotherm (solid line).

Table 1
Binding parameters obtained for LF fit to the experimental adsorption isotherms of studied triazines in two propazine-imprinted polymers obtained by precipitation (MIP-P) and bulk (MIP-B) polymerisation[a]

| Triazine | MIP-P | | | | | MIP-B | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | $N_t$ ($\mu$mol g$^{-1}$) | $a$ (mM$^{-1}$) | $m$ | $K_o$ (mM$^{-1}$) | $K$ limits (mM$^{-1}$)[b] | $N_t$ ($\mu$mol g$^{-1}$) | $a$ (mM$^{-1}$) | $m$ | $K_o$ (mM$^{-1}$) | $K$ limits (mM$^{-1}$)[b] |
| DIA | 1.350 | 58.2 | 0.92 | 84.6 | 6–21687 | 0.530 | 8.8 | 0.65 | 27.9 | 11.2–34750 |
| DEA | 1.450 | 17.3 | 0.81 | 48.2 | 3–25333 | 0.511 | 8.4 | 0.65 | 26.4 | 3.4–8934 |
| Simazine | 0.079 | 1144.5 | 0.99 | 1201.9 | 10–41666 | 0.185 | 6.7 | 0.51 | 41.6 | 4.7–134400 |
| Atrazine | 0.214 | 325.1 | 0.84 | 978.6 | 14–143678 | 0.188 | 32.0 | 0.81 | 69.3 | 5.2–30812 |
| Propazine | 0.249 | 344.6 | 0.80 | 1484.1 | 14–758333 | 0.181 | 42.9 | 0.84 | 86.2 | 4.4–17271 |
| Prometryn | 0.019 | 545.1 | 0.85 | 1656.4 | 53–25368 | 0.018 | 85.6 | 1.00 | 85.6 | 26.5–6630 |

[a] The correlation coefficient $R^2$ was higher than 0.980 in all cases.
[b] Calculated from the experimental maximum and minimum free analyte concentration ($F_{max}$ and $F_{min}$) by the relationships $K_{max} = 1/F_{min}$ and $K_{min} = 1/F_{max}$.

obtained $K_o$ values fall within the limits $1/F_{max}$ and $1/F_{min}$ (calculated form the experimental maximum and minimum free analyte concentration $F_{max}$ and $F_{min}$) as it is required for the LF model.

Several conclusions may be derived from the comparison of the parameters obtained for both polymers. Firstly, it is clear that the capacity of the MIP-P is higher than that of the MIP-B. These results can be attributed to the breakage of some of the binding sites present in the polymeric matrix during the crushing and sieving steps necessaries in the preparation of MIP-B. However, the capacity obtained for both polymers is much lower than the theoretic maximum capacity (~400 $\mu$mol g$^{-1}$), which can be calculated taking into account the amount of template used during polymerisation and the amount of polymer obtained. This fact suggests that the main responsible step of imprinted sites destruction is the Soxhlet extraction of the template with polar organic solvents, since this procedure has been used in both cases.

On the other hand, it is remarkable how the MIP-P presents much higher affinity constants for all the triazines tested than those obtained when bulk polymerisation was carried out. This result can only be attributed to the presence of better-defined binding sites that would be able to strongly interact with the triazines loaded on MIP-P. In addition, as it was expected, MIP-P presents a much more homogeneous binding site distribution than that shown by MIP-B. Thus, apparently, precipitation polymerisation may prevent the formation of complexes of different template:monomer stoichiometry (one of the reported reasons for the observed heterogeneity of non-covalent MIPs) during the pre-polymerisation step, as this step is carried out in a very dilute system.

However, the heterogeneity indexes obtained in MIP-P are slightly lower than 1, whereas a perfect homogeneous binding site distribution ($m = 1$) was observed in a previous study with a fenuron-imprinted polymer. This polymer was prepared using the same experimental conditions than MIP-P [12] but with the only difference of the template employed. Fig. 3 shows the scanning electronic micrographs of both imprinted polymers using fenuron (Fig. 3A) or propazine (Fig. 3B) as templates obtained by precipitation polymerisation. It is clear that for the fenuron imprinted polymer, discrete uniformly sized micro-spheres (~1 $\mu$m) were obtained, whereas MIP-P consisted of agglomerates of different sizes formed by those particles (~1 $\mu$m). The different polymer morphology, which can only be attributed to the different template used, could explain the different degree of binding site homogeneity presents in both polymers. In addition, this result makes questionable the suitability of precipitation polymerisation strategy for the direct synthesis of uniformly sized micro-spheres since clearly the template used has a direct influence on the final morphology of the obtained polymer. Thus, further research on precipitation polymerisation should be done using other template molecules (with differences in size, shape and chemical structure) in order to establish a link between the template used and the final polymer morphology obtained.

### 3.1.2. Characterisation of the binding sites present in MIP-P

Apart from these considerations on the polymer morphology, it is still crucial to understand how molecular

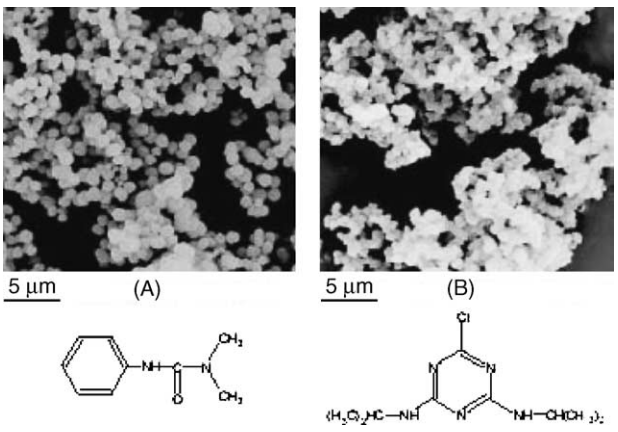


Fig. 3. Scanning electron micrographs of fenuron- (A) and propazine- (B) imprinted polymers obtained by precipitation polymerisation and the corresponding chemical structures of the templates used.

recognition takes place in MIPs in order to improve their quality in the future. In the previous study on the performance of the MIP-B carried out by our group [13], it was demonstrated that the LF isotherm fitting coefficients obtained can be used to establish the kind of binding sites present in MIPs. In such study, it was concluded that the recognition mechanism in MIP-B was mainly governed by the molecular size but apparently slight structural differences (presence of an aminoethyl or an aminoisopropyl group) did not play an important role. However, and according to the fitting coefficients obtained in the MIP-P for the selected triazines, an identical behaviour cannot be assigned in this case, although some similarities can be found. For instance, it is clear that molecular size has also a direct influence in the recognition mechanism of MIP-P, since bigger molecules are unable to access all the binding sites present in the polymer. In this sense, prometryn shows the lower capacity of the compounds tested, due to the bigger size of the thiomethyl group present in its structure compared to that of the chlorine atom, preventing its access to certain binding sites (see triazinic structure in Fig. 1). Similarly, molecular size can also justify the higher capacity obtained for the smaller compounds (DIA and DEA) that would be able to rebind those sites shrinked during the Soxhlet extraction of the template molecule, not accessible for the other triazines.

However, the clearest difference in the recognition of triazines by both polymers was found in the case of simazine. Firstly, and according to the value obtained for the coefficient *m* of simazine in MIP-P, it can be concluded that this analyte can only interact with one kind of binding site. On the other hand, the capacity obtained for simazine in this polymer is rather lower than those of atrazine and propazine. This did not occur in the case of MIP-B, suggesting that structural differences relating to the presence of an aminoisopropyl or aminoethyl group in the triazine molecule are more important in the recognition mechanism in MIP-P. According to results obtained for SIM, A and PPZ, different binding sites with different degrees of structural selectivity can be defined in MIP-P. One kind of triazinic-structure selective sites able to interact with any of the triazines tested, regardless of whether an aminoethyl or an aminoisopropyl group were present; and a second kind of more selective sites in which only the triazines with at least one aminoisopropyl group in their structure (therefore excluding simazine) could interact. Additionally, the slightly higher capacity observed for propazine compared to atrazine suggests the presence of a small fraction of specific binding sites where only those analytes with two aminoisopropyl groups in their structure could interact (PPZ and PMT in some extent).

To summarise, according to both steric effects and the selectivity observed, four different kinds of binding sites can be distinguished in MIP-P. On the one hand, there would be a large amount of small binding sites where only the smallest triazines (DIA and DEA) would be able to interact. The three other kinds of binding sites present in the polymeric matrix would be a consequence of the difference in MIP-P selectivity for aminoethyl or aminoisopropyl groups in the triazinic structure.

### 3.1.3. Rebinding experiments of mixtures of triazines

In order to evaluate how analytes compete for the binding sites present in this polymer, rebinding experiments using mixtures containing all the triazines studied were carried out according to the procedure described in Section 2. The B and F experimental values were fitted to the LF isotherm and the binding coefficients obtained are shown in Table 2. As it was expected, the obtained capacities in this second study are lower than those obtained when the triazines were loaded independently, due to competition between the different triazines for the binding sites present in the polymer. However, this diminishment on the capacities did not occur in the same rate for all the triazines. As can be observed in Table 2, DIA and DEA suffered the lower diminishment, suggesting that these analytes were less displaced during the competition.

These results can be explained taking into account that DIA and DEA compete only with each other for the smaller binding sites, to which the other triazines cannot access. But also, the huge decrease on the capacities of the other triazines, especially in the case of atrazine and propazine, suggest that DIA and DEA are able to displace them from the other binding sites, despite that lower $K_o$ values were obtained for these analytes (Table 1). However, it is important to stress that $K_o$ represents an average of the strength of the interaction between a given analyte and the polymer. Thus, this behaviour may suggest that the strength of interaction of DIA and DEA with some binding sites may be higher than those of the other triazines for the same binding sites. Obviously, the amount of this kind of binding sites would be very low and thus the *K* values associated to them would have a minor influence in the calculation of $K_o$.

Table 2
Binding parameters obtained for LF fit to the experimental adsorption isotherms of studied triazines loaded simultaneously in a propazine-imprinted polymer obtained by precipitation polymerisation (MIP-P)[a]

| Triazine | $N_t$ ($\mu$mol g$^{-1}$) | $a$ (mM$^{-1}$) | $m$ | $K_o$ (mM$^{-1}$) | $K$ limits (mM$^{-1}$)[b] |
|---|---|---|---|---|---|
| DIA | 0.901 | 64.05 | 0.95 | 80.4 | 0.2–43300 |
| DEA | 0.867 | 21.06 | 0.81 | 42.3 | 0.3–62500 |
| Simazine | 0.028 | 1140 | 0.97 | 1442 | 0.2–22400 |
| Atrazine | 0.028 | 1135 | 0.97 | 1445 | 0.2–25200 |
| Propazine | 0.026 | 479 | 0.82 | 1856 | 0.2–50000 |
| Prometryn | 0.006 | 553 | 0.81 | 2430 | 0.2–12600 |

[a] The correlation coefficient $R^2$ was higher than 0.980 in all cases.
[b] Calculated from the experimental maximum and minimum free analyte concentration ($F_{max}$ and $F_{min}$) by the relationships $K_{max} = 1/F_{min}$ and $K_{min} = 1/F_{max}$.

## 4. Conclusions

In this paper, it has been demonstrated that a careful observation of the binding parameters obtained from Langmuir–Freundlich fitted isotherms allows to characterise the binding sites present in imprinted polymers as well as to asses the observed cross-reactivity. Based on that methodology, it has been possible to confirm that precipitation polymerisation yields polymers with a more homogeneous binding site distribution and higher affinity constants compared to those obtained by bulk polymerisation using two different propazine-imprinted methacrylic-based polymers as models. However, unexpected morphology of the polymer prepared by precipitation polymerisation was observed making questionable the ability of this polymerisation strategy for the synthesis of molecularly imprinted uniformly-sized micro-spheres.

On the other hand, taking into account all the considerations commented along this paper, MIP-P could be an appropriated material to be used as stationary phase in HPLC because of its highly homogeneous binding sites distribution and the fast diffusion mass transfer. Thus, further research should be done in order to confirm the ability of MIPs to be used as highly selective stationary phases in chromatography.

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