Binding assays with molecularly imprinted polymers—why do they work?

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Abstract

The design of homologous displacement ligand binding assays based on molecularly imprinted polymers (MIP) is discussed in terms of the MIP adsorption isotherm. It is shown that only MIPs having a binding isotherm with varying slope are suitable for the assay, but there is no need to interpret the isotherm in terms of site affinity and population. One can calculate the calibration plot of the binding assay from the isotherm and vice versa.
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1. Introduction

Molecularly imprinted polymers (MIP) are a novel class of selective sorbents [1]. They are usually custom made for every particular substrate that should be selectively bound. Their unique selectivity is due to the manufacturing procedure. A rigid, usually heavily crosslinked polymer is produced in the presence of a relatively large quantity of the substrate. When the process has been finished the substrate is removed by thorough washing of the polymer with a suitable strong solvent. When exposed next to a solution of the substrate in a weaker solvent, the polymer will adsorb ("rebind") the substrate with notable selectivity over other, even closely related, substances. This remarkable memory effect has been attributed to interactions between the substrate and functional groups of the polymer during production. These interactions appear to arrange the polymer into a structure, which is a chemical and steric imprint of the substrate. As the substrate had been used in the process as a molecular template, it is alternatively called the template. There are two main types of imprinting: covalent [2,3] and noncovalent. In this paper we consider only noncovalent imprints.

In an ideal case the MIP binding sites should all be chemically equal, i.e. they should bind the substrate in exactly the same way. If this were so one might expect that the adsorption equilibrium with solutions of the substrate in a particular solvent can be described with a Langmuir isotherm characterized by a single equilibrium constant. With some covalently imprinted polymers this is approximately true but with most noncovalent imprints the situation is more complex. Many investigators have established the binding isotherms of their novel MIPs. These binding isotherms are generally quite featureless, monotonously increasing curves with gradually decreasing slope. Based on the visual appearance of the respective Scatchard plots, several investigators fitted a bi-Langmuir isotherm [4–8] to the curves with reasonable success. This is equivalent to the assumption that there are two distinct types of binding sites on the polymer.

In recent years, several investigators expressed doubts concerning this two-site model. Umpleby et al. [9] assumed a continuous distribution of binding site strength (equilibrium constant) and obtained good fit to measured isotherms. Guiochon and coworkers [10] concluded from a large series of accurate isotherm measurements that the Freundlich isotherm and the bi-Langmuir isotherm gave equally good fits, while the simple Langmuir isotherm did not fit well. Umpleby et al. also noticed that the

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Freundlich isotherm gave good fits to their data as well. What these studies show from the point of view of the present discussion is, that binding isotherms do not reliably support any specific idea about the site distribution of MIPs.

These newer results raise some doubts about the concepts underlying a particularly fascinating application of MIPs, i.e. their use as artificial antibodies. The selective and strong binding of various substrates to their MIPs resembles the similar features of natural antibodies. Indeed, Haupt and Mosbach [12], and Andersson [11,13] have shown in many papers that MIPs can be used in essentially the same assay formats as antibodies, with strikingly similar results. In the course of years better and better MIPs ("plastic antibodies", "artificial antibodies") have been developed for such assays (molecularly imprinted sorbent assay, MIA).

MIP binding assays have been generally based on homologous competition, i.e. competition between the substrate and its radiolabeled version, for the limited number of binding sites. This is the point where questions are raised by the difficulties of isotherm interpretation. If we cannot be sure that there is only one type of site present, which is limited in its quantity, why should there be any competition then? One answer lies immediately at hand and has been used by researchers quite often. In classical immunoassays polyclonal antibodies were used. These are mixtures of antibodies of varying binding strength (equilibrium constant). This is obvious from the calibration curves of the respective immunoassays as the curve extends over a considerably larger concentration range than with a monoclonal antibody population. Similar extended calibration curves have been observed in MIP binding assays. This has led to the conclusion that site heterogeneity is not a problem in understanding MIP binding assays.

There are, however, some arguments against this opinion. Biochemists can—at least in principle—separate mixtures of antibodies and determine the quantities and binding strengths of individual protein fractions. This is not possible at the moment with MIPs. Also, biochemists have developed refined immunization strategies that lead to practically useful antibody mixtures. Similarly, MIPs have been optimized for better performance in MIP based binding assays. These results have solved the practical problem, but the question still remains: Are all site distributions suitable for competitive binding assays? Or, to ask a more practical question: can we judge the usefulness of a MIP for competitive assays by measuring its adsorption isotherm but without attempting to interpret it in terms of site distribution? The goal of this paper is to answer these questions. We shall show that knowledge of the isotherm is sufficient to determine the MIP binding assay calibration curve and thus also the expectable detection limit and useful concentration range of the measurement. If the isotherm is linear, the assay is not possible. Our results will hopefully also contribute to improved MIP designs.

2. Theory

2.1. Assay format

Immunoassays exist in many different formats. Some of these are making use of antibody properties, which are unlikely to be imitated by MIPs. In a sandwich immunoassay, e.g. the solubility of the antibody in the assay solvent is important. MIPs are by their nature insoluble. For such reasons the practically demonstrated MIP binding assays have been limited to competitive assays. Competitive binding assays exist in two main types: homologous and non-homologous. In both cases two species compete for the binding sites. In a homologous assay these are chemically identical or almost identical, the only difference being that one of them is radiolabeled. In MIP binding assays the radiolabel has been generally tritium, so that chemical identity of the competitors was nearly perfect. In non-homologous binding assays the competing species are chemically different. In a typical case the competitor is a fluorescently labeled antigen. Non-homologous assays have also been developed with MIPs. We do not consider them here for reasons to be explained later.

2.2. Assay design

When a suitable MIP has been produced the homologous competitive binding assay [11,12,14,15] is typically developed in the following steps:

1. The type and amount of radiotracer is selected. The choice of amount is directed by sensitivity considerations: the lowest amount is taken which would give sufficient sensitivity. We shall denote this fixed amount of radiolabeled substrate by n^* (moles).
2. The assay solvent and volume (V) is chosen. The volume has been typically 1 ml, apparently for practical considerations. The choice of solvent is at the heart of the analytical problem. Aqueous or nonaqueous solvents may be preferred for different reasons. This choice is usually made before the MIP is synthesized because synthesis needs to be optimized accordingly. Additives to the solvent (e.g. buffering) are selected during test development.
3. An optimum quantity of MIP to be used in the test is determined by the following experiment. Increasing amounts of polymer are added to a solution of n^* moles of radiolabeled substance dissolved in V milliliters assay solvent. The distribution of the radiotracer between the two phases is determined in each case. This occurs by equilibrating the phases for some time (typically hours, "incubation") and subsequently measuring the tracer concentration in the solution phase (typically after phase separation, by scintillation). The amount of polymer that gives an approximately equal distribution between the phases (i.e. $n^*/2$ moles in each phase) is determined and used in subsequent steps. We denote this particular

polymer mass by m (grams). (This choice is dictated by precision considerations and is generally adopted also in biochemical immunoassays of this kind.)

4. The next step is calibration of the assay. A series of solutions of the unlabeled substrate is prepared at different concentrations. To V milliliters of each solution m grams of polymer and n^* moles of radiolabeled substrate are added. After incubation the tracer concentration of the solution phase is measured. The calibration plot usually shows the amount of radiotracer bound to the polymer from each calibrating solution, divided by the amount bound at zero analyte concentration. (Note that at zero analyte concentration the radiolabeled substrate distributes about equally between the phases, as explained in step 3.) The abscissa of the concentration plot shows the logarithm of the unlabeled analyte concentration in the original sample (not after equilibration).

5. The final step is the actual analysis of some unknown sample. This is carried out like the calibration assays. The result is read from the calibration plot.

2.3. The displacement process

The essential idea of such an assay is as follows. To a system consisting of n^* moles of radiolabeled substrate distributed between m grams of polymer and V milliliters of solution there are added n moles of unlabeled substrate. Thus the total quantity of substrate in the system increases from n^* to $n^* + n$ moles. After re-equilibration the distribution of the radiolabeled and unlabeled substrate, respectively, between the phases will be equal to each other, since they are chemically identical species. This distribution will generally differ, however, from the distribution (typically 1:1) that existed when only n^* moles of (radiolabeled) substrate were present. In typical assays, after addition of the unlabeled substrate some radiolabeled substrate is released from the polymer to the solution. In other words the polymer-bound fraction of the radiolabeled substrate decreases, and at the same time some unlabeled substrate becomes bound. This process is called displacement. One should note, however, that the amount of unlabeled substrate bound is generally not equal to the displaced amount of radiolabeled substrate. This is so because the total amount bound (radiolabeled and unlabeled together) will be higher than before. As we have seen the success of the assay depends on whether and how the distribution of the radiolabeled substrate shifts upon addition of n moles of unlabeled substrate. If there were no shift, there would be no measurable effect since the tracer concentration in the solution would be the same as in the absence of unlabeled substrate.

To see why MIP binding assays work we need to discuss the principles of distribution of the substrate between the two phases. MIPs are essentially solid sorbents; this means that the distribution of substrate between the solution and MIP is an adsorption equilibrium. Adsorption equilibria can be quantitatively characterized by isotherms. An isotherm typ-
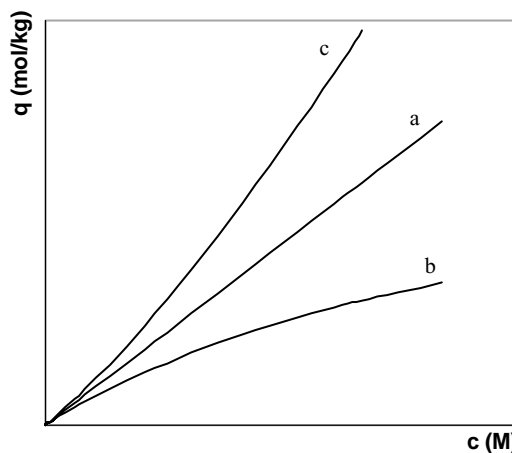

Fig. 1. Typical isotherms: linear (a), decreasing slope (b), increasing slope (c).

ically shows the dependence of adsorbed substance amount (per unit weight of sorbent) as a function of the equilibrium concentration of substrate in solution. Fig. 1 shows typical isotherms: one is linear (a), one shows decreasing slope (b) and the third has an increasing slope (c). The apparent shape of an isotherm depends very much on the width of the solution concentration range studied. A good HPLC packing, e.g. is expected to have a linear isotherm over several orders of magnitude of solution concentration, so that chromatographic calibration lines can be linear. Even these phases, however, have a bent isotherm at very high solution concentrations, which is important in preparative chromatography.

3. Quantitative analysis

Let us consider a typical assay experiment. The total molar amount of radiolabeled analyte (n^*) distributes into adsorbed ("bound") amount (n_b^*) and dissolved ("free") amount (n_f^*). The molar amount of unlabeled analyte in the whole system is n, which distributes as n_b and n_f between the two phases. The concentrations in the solution phase shall be denoted by c and expressed in M, whereas in the adsorbed phase by q and expressed in mol/kg. To avoid complications we shall use c and q, respectively, only to denote total concentrations, i.e. the sum of radiolabeled and unlabeled species. If we need other concentrations, we shall express them as amount over volume or mass, e.g. n_f/V is the concentration of unlabeled substance in solution. Note that all these concentrations are equilibrium concentrations, i.e. concentrations after equilibration of phases. We shall need only one concentration, which is non-equilibrium, this is the unlabeled analyte concentration before equilibration: $c_{anal} = n/V$. This is the concentration of the calibration solutions or the analytical sample. Note also that the assay volume V is essentially the volume of the calibrating solution or the sample.

The isotherm is the relationship between q and c. We do not assume here any particular adsorption model and

therefore we consider the isotherm only as a graphically given curve (which may have arisen by fitting an equation to some measured points, but it is not important if this equation has any physical meaning). The directly measured quantity in the assays is typically the concentration of radiolabeled species in the solution phase after equilibration: n_f^*/V. From this one can easily calculate other quantities, like n_f^*, $n_b^* = n^* - n_f^*$, n_b^*/n_f^* or $n_b^*/[n_b^*$ (at $n = 0$)]. Any of these quantities may be considered as the output of the measurement.

Let us consider n_f^* as the result of the measurement. (Since this is essentially the measured quantity it is possible to base error considerations directly on this quantity.)

We have three quantitative relationships:

(a) the mass balance for the substance:

$$mq + Vc = n^* + n \tag{1}$$

(b) the isotherm as a q versus c function given graphically or in some functional form

(c) an expression for the measured quantity, n_f^*:

$$n_f^* = \frac{n^*}{n^* + n} cV \tag{2}$$

The last equation says that after equilibration there are cV moles of total substance in solution, and the radiolabeled fraction of this is the same as the overall fraction of radiolabeled substance in the system. This equation uses the assumption about the chemical equivalence of radiolabeled and unlabeled substance.

Using these relationships we can construct the calibration plot of the assay, i.e. the relationship between the measured quantity, n_f^*, and the analytical concentration of the sample (or calibrating solutions), which is $c_{anal} = n/V$. (For convenience one can alternatively plot $\log c_{anal}$ on the abscissa.) The calculation of a point of the curve goes as follows. We take a point (q,c) on the isotherm and put it in the mass balance Eq. (1). As m, V and n^* are known, we obtain n. From this we get c_{anal} as n/V, and n_f^* by insertion into Eq. (2).

The reverse procedure is also possible, i.e. we can calculate the binding assay calibration plot. For this reverse calculation one has to use, however, also the parameters V, m and n^* of the particular assay. Thus the isotherm is more general and hence more useful way of characterizing the binding system, than the calibration curve of a particular assay.

Fig. 2 shows two isotherms based on curves fitted to the experimental binding assay data obtained by Andersson [13] and Karlsson et al. [16], respectively. Fig. 2A shows the isotherms as q versus c, Fig. 2B as $D = q/c$ versus $\log c$. The latter plot shows the details of the isotherm at low concentrations more clearly. Fig. 3A shows the n_f^* versus $\log c_{anal}$ type calibration plot calculated from the (S)-propranolol isotherm in Fig. 2 and from the corresponding V, m and n^* values [13]. In the cited work the calibration plot was shown as relative binding $= n_b^*/[n_b^*$ (at $n = 0$)] versus $\log c_{anal}$. We have also calculated this curve and show it in Fig. 3B. This agrees with the measured curve except that the calculated
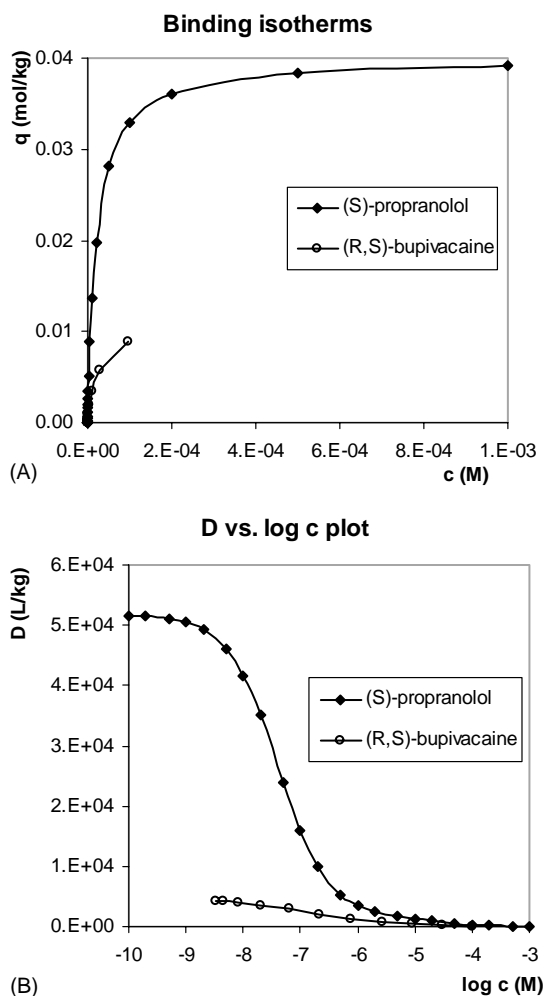

Fig. 2. (A) Binding isotherms of (S)-propranolol (calculated from the equation fitted in [13] to the measured binding data MIP "A" in toluene containing 0.5% acetic acid) and of (R,S)-bupivacaine (calculated from the measured $\log B/B_0$ vs. $\log c_{anal}$ plot [16] and the corresponding m, V and n^* values of the measurements in toluene with 0.5% acetic acid). (B) D vs. $\log c$ plot based on the same results as (A).

curve does not reproduce a small kink of the experimental values at low analyte concentrations. This is due to using isotherm data obtained by curve fitting.

One can read from the constructed plots some typical quantities used to characterize MIP binding assays. The lower limit of detection (LLD) is usually characterized by displacement of 10% of the radiolabeled substance, which had been bound in the absence of unlabeled substance. In the present example this displacement (from the solid phase into the solution) increases n_f^* from 3.22×10^{-13} mol (at $c_{anal} = 0$) to 4.05×10^{-13} mol. This gives from Fig. 3A an LLD of 4.78×10^{-8} M. Similarly the midpoint of the assay, IC$_{50}$, which is characterized by 50% displacement, can be found as 2.6×10^{-7} M. The respective values found by Andersson [13] were LLD $= 8.6 \times 10^{-9}$ M and IC$_{50} = 2.9 \times 10^{-7}$ M. The difference of LLD is apparently due to the kink of the experimental calibration plot mentioned above.
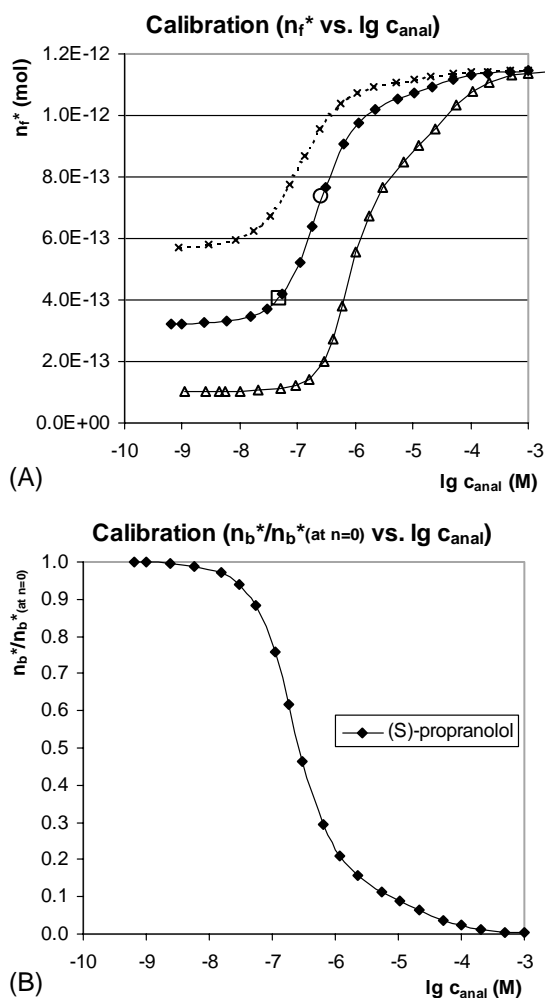
Calibration (n_f^* vs. lg c_{anal})

(A)

Calibration (n_b^*/n_b^*(at n=0) vs. lg c_{anal})

(B)

Fig. 3. (A) Calibration plot of the (*S*)-propranolol binding assay calculated from the isotherm shown in Fig. 2 using different polymer masses in the assay (dotted line 20 μg, filled rhombus 50 μg, open triangles 200 μg). In the original experiment the polymer mass was 50 μg. Characteristic points of the assay with 50 μg mass: LLD (open square) and IC50 (open circle). (B) The recalculated displacement plot of the (*S*)-propranolol assay in ref. [13].

4. The role of isotherm shape

The MIP isotherm has been sometimes found to start with a linear section at low concentrations [17,18]or it was described by the Langmuir or bi-Langmuir equations [19,20] which become also linear at low concentrations. Occasionally, an approximate linearity may extend to high substance concentrations. This is actually a goal in developing MIPs for HPLC stationary phases. We show now that in this linear range of the isotherm the displacement assay does not work.

The measured quantity in the assay is n_f^* which is expressed by Eq. (2). Inserting Eq. (1) into Eq. (2) gives:

$$n_f^* = \frac{n^*}{n^* + n}cV = \frac{n^*}{mq + cV}cV = \frac{n^*}{(m/V)D + 1} \qquad (3)$$

If the isotherm begins with a linear range, the distribution coefficient $D = q/c$ is constant and thus n_f^* is also con-

stant, independently from n, the amount of unlabeled analyte added to the system. In other words the addition of unlabeled analyte does not cause any displacement of the radiolabeled substance. The above consideration shows that the q versus c isotherm needs to begin to curve at some concentration for a useful binding assay.

One may ask how the direction of the isotherm curvature (see Fig. 1b and c) influences the assay. In the case of Fig. 1b, D decreases as c increases. If n is increased, i.e. more analyte is added, c also increases and thus D decreases so that by Eq. (3) n_f^* increases, i.e. radiolabeled substance is displaced from the polymer. In the case of Fig. 1c the situation is just the opposite, n_f^* decreases when more analyte is added. This latter effect, i.e. cooperative adsorption, has apparently not been used yet for MIP binding assays although isotherms of the type of Fig. 1c are possible [21,22].

A further discussion of the influence of isotherm shape on the assay parameters would need a longer discussion. This discussion would have to include considerations about the specific radioactivity of the label and about the signal-to-noise (S/N) ratio as a function of the measured radioactivity. It is clear, however, from the foregoing discussion, that the concentration where D begins to change appreciably and the rate of change of D with concentration will have important effects on the detection limit, the sensitivity and the measurement range. To avoid complicated calculations one can simply use the isotherm to calculate the assay calibration plot at different possible values of V, m and n^*, just as it was shown earlier in this paper. In this way one gets a series of n_f^* versus log c_{anal} plots. An example is shown in Fig. 3A for the (*S*)-propranolol case [13] with three different values of m (V and n^* being kept constant). The noise of n_f^* as a function of n_f^* can be measured without using any polymer, simply with standard solutions with different activities. Thus one can apply error bars on n_f^* in the simulated calibration plots and compare the different parameter options for the corresponding concentration ranges and detection limits. In the comparison one can use exact S/N arguments instead of the rules of thumb quoted above for finding LLD and IC50.

The above discussion shows that if the isotherm is known one can find optimal values of m and n^* (and V if it is not kept constant) satisfying particular needs. The isotherm itself can be obtained from a preliminary assay calibration plot and measurements made with varying m at constant n^*.

5. Conclusion

Among the analytical applications of MIPs chromatographic type applications (HPLC, SPE, CEC, etc.) and binding assay type applications are probably the most important. Researchers working in these two fields of applications have been using the traditional terminologies of chromatography and immunoanalysis, respectively. In particular, chromatographers look at MIPs as solid sorbents,

which can be characterized by their adsorption isotherms, kinetics and morphology. In immunoanalysis the MIP is considered as a selective chemical reagent or as a mixture of selective reagents with varying affinity (equilibrium constant) towards the analyte. The reagents are the chemically and sterically different binding sites of the polymer.

In this paper, we gave a quantitative analysis of MIP binding assays in terms of the adsorption isotherm. Since MIP binding assays include a sufficiently long incubation time, kinetic and morphological aspects are of less relevance. It has been shown that from the adsorption isotherm one can easily construct the calibration plot(s) of the binding assay and can read important quantities, like LLD and IC_{50}.

We have also shown that one can reconstruct the MIPs isotherm from the calibration plot of a MIP binding assay.

A further important conclusion is that a linear equilibrium isotherm, or in other terms a horizontal D versus $\log c$ plot, does not allow a binding assay to be made. This observation shows that MIP optimization for chromatography and for binding assay may need different strategies. In analytical chromatography a linear adsorption isotherm is usually considered advantageous. In MIP binding assay it would be detrimental.

The discussion of this paper relates to homologous binding assays. Non-homologous binding assays rely on competition between two chemically different species for the adsorption sites. In this latter case adsorption isotherms are more complex but the principles of binding assay analysis, in terms of the role of isotherms would be the same.

Last but not least the discussion given in this paper shows that the quantitative description of homologous MIP binding assays does not depend on the chemical interpretation of the adsorption isotherm. In other words it is not necessary to know the distribution of sites of varying strength. This is important because the determination of site distributions from isotherms is usually not sufficiently reliable. On the other hand, researchers developing improved MIPs for binding assays usually need to have some ideas about the site distribution. The final design of the assay, however, does no more depend on such considerations. It is worth to note in this respect the enormous difference in the D values of MIPs used in binding assay ($D > 1000\,l/kg$) and in chromatography (D around $20\,l/kg$) when both values are considered at the lowest concentration values where they have been measured [7,10,23]. Until now this remarkable difference has

always been expressed in terms of site strengths and concentrations, which is not quite reliable or useful given the difficulties of identifying the different sites.

Acknowledgements

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