ELSEVIER

Journal of Chromatography A, 786 (1997) 23–29

JOURNAL OF CHROMATOGRAPHY A

Chromatographic characterization of a molecularly imprinted polymer binding theophylline in aqueous buffers

C. Baggiani[a,*], F. Trotta[b], G. Giraudi[a], G. Moraglio[b], A. Vanni[a]

[a]*Dipartimento di Chimica Analitica, Università di Torino, Via P. Giuria 5, 10125 Turin, Italy*

[b]*Dipartimento di Chimica Inorganica, Fisica e dei Materiali, Università di Torino, Via P. Giuria 7, 10125 Turin, Italy*

Received 13 December 1996; received in revised form 6 May 1997; accepted 6 May 1997

Abstract

A theophylline-binding polymer was prepared utilizing the technique of non-covalent molecular imprinting polymerization. The polymer obtained was packed in a high-performance liquid chromatography (HPLC) column, and the molecular recognition mechanism regulating the binding behavior was studied by frontal and zonal chromatography, determining the effects of pH and methanol on the affinity constant and concentration of the binding sites. The values of binding capacity found show the ability of the imprinted polymer to bind theophylline in aqueous buffer, even if with reduced affinity with respect to the values reported in literature for the same polymer in an organic medium. Hydrogen bonding was confirmed as leading interaction in the recognition mechanism. © 1997 Elsevier Science B.V.

Keywords: Molecular imprinting; Molecular recognition; Stationary phases, LC; Binding sites; Affinity constants; Theophylline

1. Introduction

Polymers obtained with the innovative technique of non-covalent molecular imprinting polymerization have molecular recognition properties with selectivity towards the template molecule, analogously with natural occurring binding polymers such as antibodies, enzymes or receptors [1–3]. Many practical applications have been proposed in the field of analytical chemistry: stationary phases for chiral resolution [4,5], selective sorbents [6,7], components for solid state sensing devices [8] and antibody mimics in immunoassays [9–11].

The molecular recognition effect is a consequence of the presence in the polymerization mixture of template molecules capable of establishing non-covalent interactions with monomers and cross-linkers. The growth of a three-dimensional polymeric structure around the template produces binding sites with proper shape and charge distribution. It has been demonstrated that a significant contribution in the interaction between template molecules and functional groups on the surface of the binding sites is due to the hydrogen bond and ion-exchange phenomena, and the environment around the binding site, i.e., whether the mobile phase in a molecular imprinted chromatographic column is able to influence binding capacity and behavior [12,13].

In this work we have considered the influence of an aqueous environment on the binding capacity for the template molecule of a theophylline-imprinted polymer. The effect of buffers with different pH

*Corresponding author.

PII S0021-9673(97)00537-2

levels was studied by frontal chromatography, determining the affinity equilibrium constant and the concentration of accessible binding sites, while the effect of increasing amounts of a polar organic solvent was studied by zonal chromatography, determining the capacity factor of the column.

2. Experimental

2.1. Materials

All reagents, organic solvents and salts for buffers were from Merck (Darmstadt, Germany). Theophylline hydrate (3,7-dihydro-1,3-dimethyl-1H-purine-2,6-dione hydrate), pH standards and 0.1 *M* sodium hydroxide were from Carlo Erba (Milan, Italy). Theophylline solutions were prepared dissolving 1 mmol of theophylline hydrate, recrystallized from water, in 1 l of 50 m*M* phosphate buffer, pH 5 and diluted to proper concentration with the same buffer immediately before use. Chloroform was dried by distillation on 4.5 Å molecular traps. Methacrylic acid and ethylene glycol dimethacrylate were distilled at reduced pressure immediately before the use. High-performance liquid chromatography (HPLC) apparatus (pump L-6200, UV–Vis detector L-4200 and integrator D-2500) was from Hitachi–Merck (Darmstadt, Germany). Glass electrode and pH meter were from Radiometer (Copenhagen, Denmark).

2.2. Polymer preparation

The polymer was prepared according to the literature with minor modifications [9]. In a 500 ml round-bottom three-neck flask provided with a nitrogen inlet, a Liebig condenser and a dry trap, a solution was prepared by dissolving 2.35 g of theophylline hydrate in 125 ml of chloroform under a continuous stream of dry nitrogen. Then, 4.5 ml of methacrylic acid, 95 ml of ethylene glycol dimethacrylate and 0.6 g of 2,2′-azobis(2-methylpropionitrile) were added under rapid stirring. The mixture was purged with nitrogen and sonicated in a water-bath for 5 min, then was left to polymerize overnight at 60°C.

This polymerized product, a colorless solid mass, was broken with a steel spatula and manually ground in a mortar; then, theophylline was extracted in a Soxhlet apparatus by repeated extraction with methanol, acetonitrile and water. No efforts were made to measure the amount of template molecule recovered. The polymer was mechanically wet-sieved to 20–30 μm particle size and dried overnight at 50°C. A blank polymer was prepared and treated in the same manner, omitting the theophylline.

2.3. Polymer titration

About 1.2 g of the imprinted polymer were suspended in deionized water, filtered on a sintered glass G4 funnel and converted in the acid form by several washings in 1 *M* hydrogen chloride. The acidified polymer was washed again with anhydrous acetone to remove interstitial water and dried at 80°C overnight. Then, 1.0 g was suspended in 100 ml of 0.1 *M* sodium chloride, accurately degassed immediately before use, and titrated by progressive addition of 200 μl of 0.100 *M* sodium hydroxide, allowing 20 min between additions. The changing of pH was monitored with a glass electrode, calibrated between pH 3 and 9 with standard buffers and pH steps of 2.

2.4. Column packing

An adequate amount of polymer with 20–30 μm particle size was suspended in a methanol–water (50:50, v/v) mixture and slurry packed in a 250 mm stainless-steel HPLC column (I.D. 4 mm, geometrical volume 3.14 ml). The packing of the stationary phase was performed by gradually adding the slurry of the polymer to the column and eluting it with the mobile phase [methanol–water (50:50, v/v)] at a constant pressure of 20 MPa. Finally, the column was equilibrated at 1 ml/min with water, and washed extensively with methanol until a stable baseline was reached (270 nm). After equilibration, the pressure in the column was 7–10 MPa with aqueous buffers and a flow-rate of 1 ml/min.

2.5. Chromatography

Frontal chromatography was performed by eluting the imprinted and the blank columns at room temperature with buffers containing variable amounts of

theophylline. The effect of pH was evaluated with 50 m*M* buffers (citrate pH 3–5, phosphate pH 6–7, Tris pH 8 and glycine pH 9) containing 50 m*M* of sodium chloride. For each elution experiment the columns were equilibrated at a flow-rate of 1 ml/min with 30 ml of the proper buffer; then, the pumps and the eluent pipes of the chromatographic apparatus were filled with buffer containing theophylline, purging out the excess eluent. The elutions were performed at 1 ml/min, recording the absorbance at 270 nm. The breakthrough volume was measured by elaboration of the digital signal: this is the maximum numerical value of the first derivative of the curve corresponding to the frontal chromatogram. The breakthrough volume for a non-interacting molecule was measured by eluting the columns with buffers containing 10 m*M* of sodium azide, and recording the absorbance at 230 nm.

Zonal chromatography was performed eluting the imprinted and the blank columns at room temperature with buffers containing increasing amounts of methanol, ranging from 0 to 90% (v/v), with steps of 10%, as organic modifier. Columns were equilibrated at an flow-rate of 1 ml/min with 30 ml of 50 m*M* buffers (citrate pH 3–5, phosphate pH 6–7, Tris pH 8 and glycine pH 9) containing 50 m*M* of sodium chloride and methanol; then, 20 μl of 1 m*M* theophylline were injected and eluted at 1 ml/min, recording the absorbance at 270 nm. Elutions with only methanol were also performed. The void volumes of both columns were measured for each mobile phase formulation by eluting 20 μl of sodium azide 10 m*M*, and recording the absorbance at 230 nm.

3. Results and discussion

3.1. Frontal chromatography

In a frontal chromatographic experiment, the breakthrough volume, *V*, can be related to the affinity equilibrium constant of the ligand for the stationary phase, K_{eq}, and the molar concentration of accessible binding sites, *S*, according to the following equation [14]:

$$V = V_0 + v \cdot \frac{K_{eq} S}{1 + K_{eq} C} \quad (1)$$

where *V* is the breakthrough volume of the interacting molecule, V_0 the breakthrough volume of a non-interacting molecule, *v* the bed volume of the column, K_{eq} the affinity equilibrium constant for the reaction between the interacting molecule and the stationary phase, *S* the molar concentration of accessible binding sites on the stationary phase, and *C* the molar concentration of the interacting molecule. From a set of elutions in which the molar concentration of the interacting molecule was gradually changed, it was possible to calculate the numerical values of K_{eq} and *S* by fitting Eq. (1) with the experimental data. To achieve reliable values and reduce the influence of experimental errors which affected the numerical results, it was also necessary to perform no less than 8–10 elutions for each buffer formulation. The isotherms for blank and imprinted polymers on which the numerical values of K_{eq} and *S* were calculated are reported in Figs. 1 and 2.

As regards the affinity constant values, reported in Table 1, the imprinted column differs from the blank column not only for the higher values of affinity along the entire pH range considered, but also for the behavior in the region of pH 5–7. Both columns show higher affinity to theophylline in acidic conditions, but in the presence of more basic buffers the

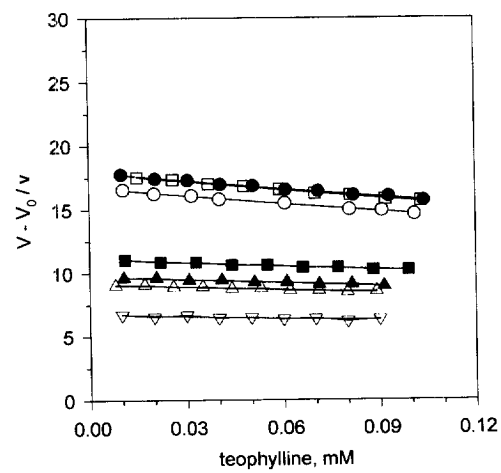

Fig. 1. Isotherms for blank polymer. ○: pH 3, ●: pH 4, □: pH 5, ■: pH 6; △: pH 7, ▲: pH 8, ▽: pH 9.

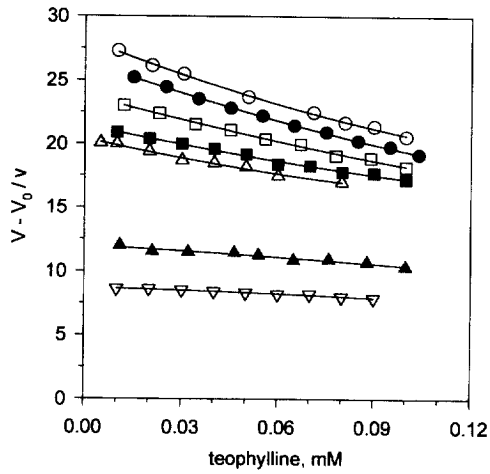

Fig. 2. Isotherms for imprinted polymer. Symbols as in Fig. 1.

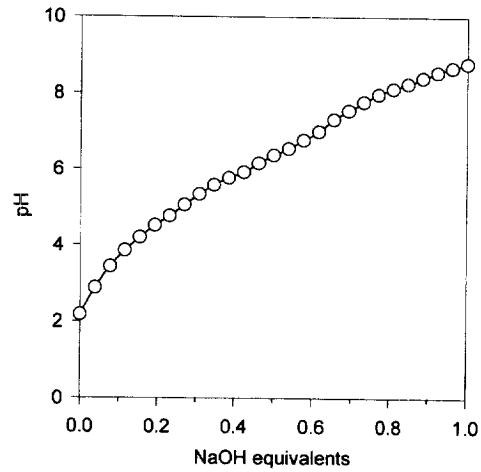

Fig. 3. Titration curve for imprinted polymer. The sodium hydroxide equivalents are calculated on the basis of the theoretical amount of carboxylic groups present in the polymer. The same titration curve was obtained for blank polymer.

imprinted column shows a progressive decrease in affinity, while the blank column shows a sharp decrease in vicinity of pH 6, with substantially stable values of affinity for more basic conditions.

The effect of pH level in the mobile phase on the binding properties of a molecular imprinted stationary phase is known from literature [12]. This behavior is explained by authors as direct consequence of a ligand-specific ion-exchange interaction with the stationary phase, strictly related to the degree of ionization of both the ligand and the polymer. As a result, it was possible for the authors to accurately describe the interaction in terms of a model based only on cation-exchange mechanism.

To verify if the imprinted and blank polymers are subjected to the same mechanism, we have titrated the polymers and calculated the dissociation constant and the degrees of ionization (α) between pH 3 and 9, employing a modified Henderson–Hasselblach equation suitable for a polymeric acid [15]. From the titration curves, reported in Fig. 3, we have found pK values of about 6.4 for both the imprinted and the blank polymer, in satisfactory agreement with the value of 6.5 reported in literature for the polymethacrylic acid [15]. We have also calculated the degree of ionization for the theophylline, directly from the values of pK ($pK_1 = 0.28$, $pK_2 = 8.77$) reported in literature [16].

Considering an ion-exchange mechanism of binding, the affinity constant for the reaction between the ligand and the stationary phase should be proportional to the degree of ionization of all the species

Table 1
Effect of mobile phase pH on the affinity equilibrium constant and concentration of binding sites of the blank and theophylline-imprinted polymers

pH	Blank polymer			Imprinted polymer		
	K_{eq} ($\cdot 10^{-3}$), M^{-1}	S, mM	$K_{eq}S$	K_{eq} ($\cdot 10^{-3}$), M^{-1}	S, mM	$K_{eq}S$
3	1.42±0.122	11.8±0.96	16.8±1.99	3.60±0.340	7.83±0.375	28.2±2.98
4	1.36±0.169	13.2±1.53	18.0±3.05	3.67±0.263	7.26±0.728	26.6±3.28
5	1.46±0.198	12.3±1.55	17.9±3.32	2.98±0.376	8.00±1.11	23.8±4.48
6	0.926±0.210	12.0±2.56	16.7±4.34	2.48±0.234	8.66±.774	21.5±2.79
7	0.917±0.211	9.98±2.17	13.7±3.65	2.39±0.185	8.49±0.526	20.3±2.01
8	0.976±0.152	10.0±1.48	9.76±2.10	1.39±0.298	8.68±1.81	12.1±3.61
9	0.845±0.163	7.97±1.37	6.73±1.74	1.14±0.121	7.68±1.66	8.76±2.11

involved in this interaction, i.e., the dissociated form of the polymer (αP) and the protonated form (αTH2) of the theophylline.

The various degrees of ionization for the species involved in the ion-exchange model and the affinity constant are reported in Fig. 4. It is clear that experimental values of affinity show a dependency on pH inconsistent with the ion-exchange model; consequently, it is necessary to invoke others types of interaction to explain the affinity behavior of the polymer. A good candidate is hydrogen bonding interaction: molecular imprinted polymers based on methacrylic acid are known to be able to form these types of bonds easily [13,17,18].

Firstly we should consider that the more significant interaction could be between the carbonyl groups of theophylline and the non-dissociated carboxyl of the polymer. In this interaction the affinity constant for the reaction between the ligand and the stationary phase should be inversely proportional to the degree of ionization of all the species involved in this interaction, i.e., the undissociated form of the polymer (αPH) and the neutral form (αTH) of the theophylline. From Fig. 4, it is clear that experimental values of affinity match this model better than the ion-exchange mechanism.

On the contrary, the binding site concentration does not seem to correlate with a hydrogen bonding model. In the range of pH 3–7 the imprinted

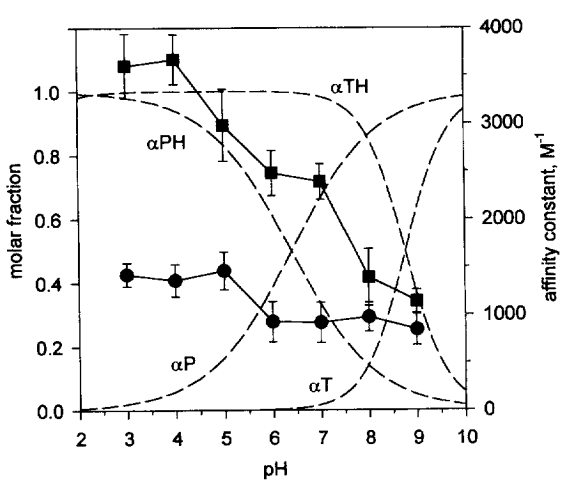

Fig. 4. Degrees of protonation and deprotonation for theophylline and polymers, and effect of pH on the affinity constants. ■: Imprinted polymer, ●: blank polymer.

polymer has values of sites concentration smaller than blank polymer, while between pH 7 and 9 these values are comparable. Even if the experimental data give no sufficient information on the nature of this difference, it is possible that the reduced number of binding sites for the imprinted polymer should be attributed to the presence of several carboxyl functions in each binding site: on the imprinted polymer a single molecule of theophylline can interact simultaneously with more than one carboxyl, while on the blank polymer each carboxyl can interact with a molecule of theophylline, causing an increased number of binding sites. The hypothetical presence of more than one carboxyl in the binding sites of the imprinted polymer can also explain the higher affinity for theophylline, as result of a multiple hydrogen bonding interaction between the molecule and the solid-phase.

The values of affinity ($K_1 = 2.86 \pm 0.40 \cdot 10^6\ M^{-1}$, $K_2 = 1.54 \pm 0.40 \cdot 10^4\ M^{-1}$) and binding site concentration ($S_1 = 16 \cdot 10^{-6}$ mol/g, $S_2 = 1.3 \cdot 10^{-3}$ mol/g) found by a radioimmunoassay-mimicking method and reported in literature [9] for this type of imprinted polymer are completely different from those founded by frontal chromatography in this work. In any case, several data obtained by frontal chromatography and reported in literature [13,19–21] with different imprinting molecules or mobile phase formulations, shows values of the same order of magnitude, or slightly greater than those found here.

We suppose that the magnitude of affinity values measured by equilibrium competition should be a consequence of this technique. It is reasonable that an imprinted polymer, resembling a polyclonal antiserum, has many classes of binding sites, each of them characterized by different affinities and molar concentration. By the nature of the radioimmunoassay-mimicking method the analysis of binding data performed with the Scatchard plot orderly show only the best binding sites, characterized by higher affinity constants and low molar concentration, while low affinity–high molar concentration sites are ignored [22]. On the other hand, the frontal chromatography is known as to be substantially not sensitive to the presence of different classes of binding sites in the stationary phase or to effects related to slow dissociation/association kinetics [23]; this technique shows a general picture of the binding properties of

the imprinted polymer, and consequently the low affinity–high molar concentration sites predominate and qualify for the most part the experimental values for affinity and binding site concentration.

3.2. Zonal chromatography

In a zonal chromatographic experiment the retention volume of an interacting solute can be quantitatively related to the affinity equilibrium constant of the ligand for the stationary phase, K_{eq} and the molar concentration of accessible binding sites, S, according to the following equation [24]:

$$\frac{V - V_{col}}{V_{col} - V_0} = K_{eq}S \quad (2)$$

where V is the retention volume of the eluted molecule, V_0 the retention volume of a non-interacting molecule, V_{col} the geometrical volume of the column, and other symbols are the same as those reported for Eq. (1) (frontal chromatography). As regards the numerical value of the product $K_{eq}S$, for a set of elutions obtained without methanol in the elution buffer, it is possible to observe in Fig. 5 that there is a good correlation between the values obtained by zonal chromatography, and the values obtained by frontal chromatography, thus confirming the validity of these methods to measure the binding capacity of an imprinted polymer.

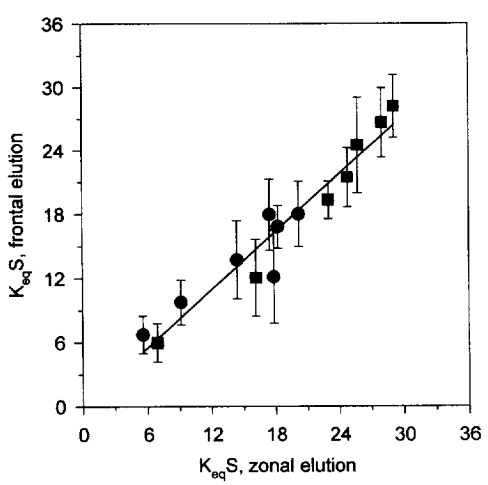

Fig. 5. Correlation between values of binding capacity calculated by zonal and frontal chromatography for imprinted (■) and blank (●) polymers.

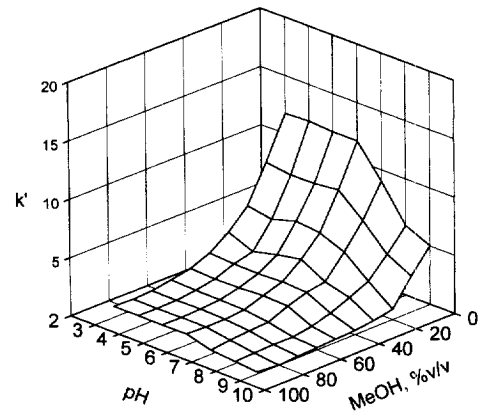

Fig. 6. Retention surface for theophylline on blank polymer.

The effect of methanol in the elution buffers is reported as retention surfaces in Fig. 6 (blank column) and Fig. 7 (imprinted column). Increasing amounts of this solvent very rapidly and without significative difference diminish the capacity factor both for blank and imprinted polymers, even if imprinted polymers maintains its higher binding capacity. This effect is not influenced by pH level, and the same behavior for both polymers indicates an effect which is not strictly dependent on specific recognition of the binding sites. It is not easy to explain this behavior, that could not be merely due to the increasing presence of a less polar eluent in column; from experimental data reported in litera-

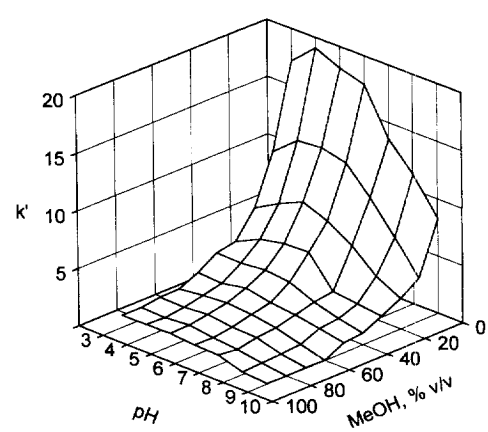

Fig. 7. Retention surface for theophylline on imprinted polymer.

ture, this type of imprinted polymer explains high binding capacity when a more hydrophobic solvent (acetonitrile) is used to elute theophylline [25]. Even if it is not possible to draw a clear picture of the mechanism, it is possible that the effect of methanol depends largely on its ability to substitute for the protonated carboxyl of the polymer in forming hydrogen bonding with theophylline, interfering directly with the binding of theophylline on the surface of stationary phase, while hydrophilic solvents with a lower ability to form hydrogen bonds, like acetonitrile, have less influence on the binding.

4. Conclusions

This work shows that a theophylline-imprinted polymer can interact with the template molecule also in an aqueous environment, and the resulting interaction is strongly influenced by the buffer pH. The thermodynamic parameters measured by frontal chromatography, the equilibrium affinity constant and the binding sites concentration, were confirmed by zonal chromatography, and were found to be smaller than those found by equilibrium competition with [^{3}H]theophylline reported in the literature. The mechanism of this interaction seems to be related to a hydrogen bonding between the protonated carboxyls of the stationary phase and the molecule of theophylline, while ionic interaction appears not to be significant. This behavior is in apparent contrast with a previously reported mechanism of interaction between templates and imprinted polymers based on an ion-exchange mechanism [12], while is in accordance with any other study which reports an hydrogen bonding mechanism [13]. We believe that our results confirm the fact that the recognition based on molecular imprinting is not subjected to a single kind of molecular interaction, but that different imprinted polymers in different environments could show recognition properties based on different molecular interactions.

The observed behavior of the polymer make feasible for practical purposes, as a stationary phase for an analytical HPLC to separate, preconcentrate and evaluate theophylline from complex aqueous samples. On this point further studies are in progress, to evaluate the real selectivity of this polymer in discriminating between the imprinting molecule and others analogous substances.

References

[1] G. Wulff, Angew. Chem., Int. Ed. Engl. 34 (1995) 1812.
[2] J. Steinke, D.C. Sherrington, I.R. Dunkin, Adv. Polym. Sci. 123 (1995) 81.
[3] K. Mosbach, O. Ramström, Biotechnology 14 (1996) 163.
[4] I.A. Nicholls, L.I. Andersson, K. Mosbach, B. Ekberg, Trends Biotechnol. 13 (1995) 47.
[5] M. Kempe, K. Mosbach, J. Chromatogr. A 694 (1995) 3.
[6] B. Sellergren, Anal. Chem. 66 (1994) 1578.
[7] M. Kempe, K. Mosbach, J. Chromatogr. A 691 (1995) 317.
[8] E. Hedborg, F. Winquist, I. Lundström, L.I. Andersson, K. Mosbach, Sens. Act. A 37/38 (1993) 796.
[9] G. Vlatakis, L.I. Andersson, R. Müller, K. Mosbach, Nature (London) 361 (1993) 645.
[10] M.T. Muldoon, L.H. Stanker, J. Agric. Food Chem. 43 (1995) 1424.
[11] L.I. Andersson, Anal. Chem. 68 (1996) 111.
[12] B. Sellergren, K.J. Shea, J. Chromatogr. A 654 (1993) 17.
[13] I.A. Nicholls, O. Ramström, K. Mosbach, J. Chromatogr. A 691 (1995) 349.
[14] K. Kasai, Y. Oda, M. Nishikata, S. Ishii, J. Chromatogr. 376 (1986) 33.
[15] A. Katchalsky, P. Spitnik, J. Polym. Sci. 2 (1947) 432.
[16] The Merck Index, 11th ed., Merck, Rahway, NJ, 1989.
[17] L.I. Andresson, K. Mosbach, J. Chromatogr. 516 (1990) 313.
[18] K.J. Shea, D.A. Spivak, B. Sellergren, J. Am. Chem. Soc. 115 (1993) 3368.
[19] M. Kempe, K. Mosbach, Anal. Lett. 24 (1991) 1137.
[20] M. Kempe, Anal. Chem. 68 (1996) 1948.
[21] M. Siemann, L.I. Andersson, K. Mosbach, J. Agric. Food Chem. 44 (1996) 141.
[22] P. Tjissen, in R.H. Burdon and P.H. Knippenberg (Editors), Practice and Theory of Enzyme Immunoassay, Elsevier, Amsterdam, 1985, p. 126.
[23] A. Jaulmes, C. Vidal-Madjar, Adv. Chromatogr. 28 (1989) 1.
[24] D.M. Abercrombie and I.M. Chaiken, in P.D.G. Dean, W.S. Johnson and F.A. Middle (Editors), Affinity Chromatography: a Practical Approach, IRL Press, Oxford, 1985, Ch. 7, p. 173.
[25] J. Matsui, Y. Miyoshi, R. Matsui, T. Takeuchi, Anal. Sci. 11 (1995) 1017.