Preparation and Characterization of a Sol−Gel Immunosorbent Doped with 2,4-D Antibodies

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An optimized sol−gel technique was used for the entrapment of 2,4-dichlorophenoxyacetic acid (2,4-D) antibodies in a silica matrix derived from tetraethoxysilane (TEOS). Immunoextraction cartridges with reproducible binding capacity for 2,4-D methyl ester (test solute) were prepared from the doped dry gels and wet hydrogels. Although the surface of these biomaterials was characterized by wide macropores, there was no evidence of antibody leakage, as demonstrated by the good precision of the results obtained from repeated extractions performed in the same cartridge. The 2,4-D antibody (commercially obtained), free or encapsulated, was highly selective toward the esterified 2,4-D molecule. Optimal extraction was achieved from samples containing phosphate buffer 0.01−0.15 M (pH ∼7), which were loaded at a maximal flow rate of 1 mL min^{-1} in cartridges left under buffer for at least 30 min between consecutive experiments. Recoveries of 100%, for loaded ester amounts lower than the cartridge capacity, were obtained under these conditions. A binding capacity of 130 ng of 2,4-D ester per mg of immobilized antibody, corresponding to 42% of the free antibody activity, was obtained with the best gels. The capacity of these immunosorbent cartridges remained practically constant during eight weeks or 50−60 adsorption−desorption cycles.

Introduction

The development of new adsorbent materials and better methodologies for the solid phase extraction (SPE) of trace pollutants from environmental matrixes has been the subject of much research work during recent years. Because of their high selectivity, immunosorbents are becoming one of the most interesting new SPE phases for environmental analysis. Indeed, immunosorbents for some pesticides (phenylureas, triazines) and mycotoxins (aflatoxins) have already been successfully commercialized, and many others are actually under development (benzidines, polyaromatic hydrocarbons, and imidazolinones).[1−4]

The antecedent of these biomaterials can be found since the late 1960s in the works of Porath et al.,[5−6] and many others, that gave rise to a new form of chromatography based on biological recognition. Affinity (or more properly bioaffinity) chromatography was initially devoted to the isolation and purification of proteins, nucleic acids, and in general big biomolecules or their corresponding substrates, from tissue extracts or biological fluids. Thus, the applications of this separation technique were restricted for a long time to the medical and biological fields (drug therapy, clinical diagnosis, biotechnology, etc.). The great progress in immunology during the past decade has rendered it theoretically possible to generate antibodies against almost any compound. This has permitted extension of application of affinity chromatography to the environmental field.

Immunosorbents are prepared by the physical or chemical immobilization of an antibody (or sometimes an antigen) in a solid support. The production of antibodies against pesticides is based on the conjugation of the small target molecule (the hapten) to a large immunogenic carrier molecule (typically bovine serum albumin, BSA, or keyhole limpet hemocyanin, KLH). Subsequent immunization of a suitable vertebrate (sheep or rabbit) followed by collection of the antiserum after several months and isolation of the G-type immunoglobulin (IgG) fraction, finally renders the prime material for the immunosorbent preparation.[7]

The most usual way to immobilize antibodies in a solid support is by covalent linkage. To present, this has been the only approach used in the production of sorbents for the commercialized immunoextraction cartridges. However, some undesirable effects, such as

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distortion or loss of the three-dimensional structure of the antibody or steric hindrance of its binding site,[8] can occur during the coupling reaction. An alternative approach for the immobilization of sensitive molecules is their encapsulation in the pores of a solid support by means of sol−gel techniques.[9−16] This process is carried out under mild conditions and at ambient temperature, which lowers the risks of conformational changes in the guest molecule. Besides, because the molecules are not attached to the support, there are no orientation problems, and the access of the target solute to the active site is less hindered. It has also been claimed that the physical entrapment prevents leakage of the biomolecule and increases its resistance to thermal and chemical denaturation.[13−15,17] These facts have encouraged the development of sol−gel sorbents for applications in the environmental field. Thus, the preparation and evaluation of immunosensors or immunoaffinity columns for isoproturon,[18] atrazine,[19] polyaromatic hydrocarbons,[20] and parathion[21] have already been reported.

However, some problems still have to be solved to obtain a highly active, stable, and rugged material by sol−gel methods. For example, the production of high levels of alcohol during the hydrolysis and polymerization of the precursor can result in significant denaturation or inactivation of the biomolecule.[11−13] Shrinkage and pore collapse effects during the drying of the gel need to be reduced to prevent excessive loss of porosity, and also because matrix compression can disable conformational sensitive proteins.[11,17]

The phenoxyalkyl acids, usually formulated in the form of salt or alkyl esters, are an important group of selective growth-stimulating herbicides. They have been extensively used in many countries since the 1940s for the control of weeds in dams, cereal lawns, and pasturelands. In particular, 2,4-D and 4-(2,4-dichlorophenoxy)butyric acid (2,4-DB) are among the most commonly used herbicides in Mexico. Although these compounds are considered only moderately toxic, their widescale production and use, and their relatively high solubility and persistence in water[22] demand the development of selective, sensitive, and yet simple methods for their regular monitoring in natural and potable waters.

The aim of this work was to optimize the sol−gel conditions for the encapsulation of 2,4-D antibodies, to evaluate the performance, selectivity, and stability of cartridges packed with the obtained material, and to propose an immunoextraction protocol derived from this evaluation. 2,4-D Antibodies are commercially available, but, to our knowledge, an immunosorbent for 2,4-D has not yet been commercialized or reported.

Experimental Section

Reagents. HPLC-grade acetonitrile (AcCN) was from Prolabo (Paris, France). Reagent water was obtained from a Nanopure deionizer (Barnstead Thermolyne, Dubuque, IA). Other analytical grade reagents used for the preparation of buffers (NaCl, KCl, H_3PO_4, NaOH, acetic acid, and formic acid) or as additives ($HClO_4$) in some HPLC mobile phases, were purchased from Baker (Phillipsburg, NJ). Tetraethoxysilane 99% (TEOS) from Fluka (Buchs, Switzerland) was used to prepare the sol−gel glasses. Phosphate-buffered saline solutions (PBS) of pH 7.2 were 0.02 M phosphate, 0.157 M NaCl, and 0.0027 M KCl (unless otherwise indicated). They were prepared in reagent water by dilution of the appropriate aliquot of H_3PO_4, neutralization with NaOH, and addition of the alkaline salts. The PBS solutions of different concentration used in some experiments had the same phosphate/NaCl/KCl molar ratio.

2,4-D, 2,4-DB, and the related compounds (2,4,5-trichlorophenoxy)acetic acid (2,4,5-T), 2-(2,4-dichlorophenoxy)propionic acid (2,4-DP), 2,4-dichlorophenol (2,4-DPh), 2,4-D methyl ester (2,4-D ester), 2,4-DB methyl ester (2,4-DB ester), and 2,4,5-T methyl ester (2,4,5-T ester) were purchased from Chem Service (West Chester, PA) or Polyscience (Niles, IL). The acidity constants (pK_a) of the phenoxyalkyl acids are between 2.6 (2,4-D) and 4.8 (2,4-DB). Stock solutions of these compounds (1000 mg/L) were prepared in AcCN. Working solutions (single compounds or mixed compounds) were prepared from the stock solutions in AcCN/water 50:50 (v/v) for HPLC analysis, or in PBS containing 1−10% (v/v) AcCN for the immunoextraction experiments. The solutions of the esters in PBS were always prepared just before use.

Polyclonal 2,4-D antibodies were purchased from Abkem (Ottawa, ON) in the format of 2-mg portions of lyophilized 95% pure IgG fraction. The lyophilized material was stored at −20 °C, and only one 2-mg portion at a time was dissolved in PBS to obtain a protein concentration of 1 mg mL^{-1} (antibody solution). When not in use, this solution was kept at 4 °C. After a period of not more than 2 months, the remaining antibody solution was discarded and a new one was prepared. Two different lots of polyclonal 2,4-D antibodies were used during this work.

HPLC Analysis. The chromatographic system consisted of a model LC-1150 quaternary pump from Polymer Laboratories (Amherst, MA) equipped with a model 7600 mobile phase degasser from Jones Chromatography (Glamorgan, UK) and a Spectromonitor 3200 (Thermo Separation Products, Riviera Beach, FL) UV detector, set at a wavelength of 230 nm. Chromatograms were recorded and integrated by a Hewlett-Packard (Avondale, PA) 3396 Series II integrator. A 7125 Rheodyne valve with a 100-μL loop was used to inject the standards and the samples (eluates from the SPE experiments) in the analytical column (150 × 4.6 mm i.d.), packed with 5 μm Hypersil ODS (Cheshire, UK). To increase the sensitivity of detection, some SPE eluates containing very low solute amounts were preconcentrated by solvent evaporation under a gentle N_2 stream and reconstituted in 200 μL of mobile phase prior to analysis. Mobile phases were AcCN/water mixtures in different proportions, depending on the compound(s) to be determined. For the phenoxyalkyl acids, the mobile phase was acidified with $HClO_4$ (pH 1.5 in the aqueous portion). A flow rate of 1 mL min^{-1} was used throughout.

Preparation of the Sol−Gel Immunosorbent. The sol−gel entrapment of the antibodies was carried out by a

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procedure in which thorough hydrolysis of the precursor was achieved in the presence of a relatively high acid concentration, but, prior to the addition of the antibody, the excess acid was buffered with a concentrated PBS solution. Briefly, 0.1 mL of 0.1 M aqueous HCl, 0.4 mL of reagent water, and 2.5 mL of TEOS were mixed with stirring. The mixture was sonicated under ice cooling for 30 min and left at ambient temperature until it acquired an elastic consistency. A 2-mL aliquot of 0.2 M PBS was then added to the sol, immediately followed by addition of antibody solution (300−700 μL) and vigorous stirring; gelation occurred within 1 min. After 5 min, the gel was superficially rinsed with PBS, thoroughly crushed with a spatula, and transferred to a vacuum filter unit where it was then abundantly washed, first with water and finally with PBS. The Bradford test[23] applied to the liquid fractions collected during the washing gave a negative result, indicating that there was no antibody leakage from the gel. Therefore, it was assumed that all the added IgG was permanently encapsulated in the silica matrix. The amount of gel obtained by this procedure (wet basis) was 3.6±0.2 g (n = 6).

The crushed gels (wet hydrogel) suspended in PBS were carefully poured into inverted 3-mL (1 cm i.d.) plastic syringes (Nipro, Miami, FL), using stainless steel frits at both ends of the column to retain the packing. Then, the gel bed was washed with PBS and stored in this buffer at 4 °C until use. To settle down the packing, three or four blank runs consisting of the sequential passage of PBS, water, and AcCN/water 50:50 (v/v) through the cartridges were initially performed. The bed was progressively compacted to about half its initial size, arriving at a final height of 1.4−1.6 cm (bed volume 1.10−1.25 mL).

To test the effect of drying on the immunosorbent performance, some hydrogels (doped with 500 μg of antibody) that had been previously crushed and washed were transferred to open glass beakers and stored in refrigeration until a weight loss of 50% was achieved (about 2−3 days). The obtained sol−gel glasses (dried gel) were ground in a mortar, and the powder was suspended in PBS to withdraw the finest particles; the particle sizes in the remaining material (measured with an Olympus CE-0849 optical microscope) were between 10 and 25 μm. Finally, the immunosorbents were packed and stored in refrigeration under buffer. In this case, the packed bed (∼1.4 cm; bed volume ∼1.10 mL) remained practically constant during the initial blank runs performed in the cartridges and also during their further use.

Scanning electron micrographs (SEM) of the doped gels and a blank (undoped) gel were obtained on a JEOL JSM 5900 LB instrument.

Immunoextraction. To control the nonspecific adsorption of the analytes, preliminary experiments were carried out in a cartridge packed with a dried blank gel. The latter was prepared in exactly the same way as the immunosorbent but without addition of the antibody. Solutions of the different compounds (100 ng) in 10 mL of PBS containing 0−10% (v/v) AcCN were separately percolated through the cartridge; after rinsing with water (2 mL), an AcCN/water 50:50 (v/v) eluent was used to dislodge the eventually retained compounds. From the HPLC analysis of the eluates, the amount of AcCN required in the sample to avoid or limit the adsorption of each compound on the silica support or the cartridge walls was deduced.

The binding of the test solute (2,4-D ester) to sol−gel cartridges doped with anti 2,4-D was studied as a function of several experimental parameters. From the results of this study, the following general procedure was finally adopted for the immunoextraction experiments.

The immunosorbent cartridge (kept in refrigeration) is first equilibrated to room temperature for 1 h and rinsed with 15 mL of fresh PBS. The analyte solution (in PBS with the appropriate AcCN content) is passed through the cartridge, using the syringe plunger to push the solution at a flow rate of ∼1 mL min^{-1}. Then, the cartridge is rinsed with 2 mL of reagent water and eluted with 2 mL of AcCN/water 50:50 (v/v) at the same flow rate as before. The eluate is analyzed by HPLC to determine the amount of bound compound. Finally, the cartridge is rinsed with an extra 4-mL volume of the AcCN/water mixture, 10 mL of water, and 15 mL of PBS. After at least 30 min under buffer, the cartridge is ready for a new SPE experiment.

The immunoextraction procedure was used to determine the capacity of cartridges packed with wet hydrogels doped with 300, 500. and 700 μg of 2,4-D antibody and dried gels doped with 500 μg; the test solute for these experiments was 2,4-D ester. The selectivity of the immunosorbent was studied in the dried gel cartridge; 10-mL solutions containing 100 ng of the analyte (the acid herbicides, the esters, or the phenol) were processed in this cartridge according to the established procedure. Finally, some extraction experiments of 2,4-D solutions in various buffers (acetic acid/acetate pH 4.7, formic acid/formate pH 3.8, and perchloric acid pH 1.5) were also carried out in the dried gel.

Stability and Reusability. A cartridge packed with wet hydrogel doped with 300 μg of antibody was used in numerous SPE experiments. From time to time its capacity was evaluated by processing a 10-mL solution containing 120 ng of 2,4-D ester. Another test was carried out in a cartridge packed with dried gel doped with 500 μg of antibody. This cartridge was stored in refrigeration for 14 weeks and its capacity was measured several times during this period.

Activity of the Free Antibody. Two solutes, 2,4-D and 2,4-D ester, were used to measure the binding capacity of the antibody in solution. Briefly, 1 mL of PBS/AcCN 99:1 (v/v) containing 60 or 120 ng of the solute and 50 μL (50 μg) of the antibody was placed into a Centricon Y-30 tube; the mixture was slightly sonicated and then incubated for 15 min at room temperature. At the end of the incubation, the solution with the unbound compound was separated from the solute−antibody complex by centrifugation at 2000g for 16 min. The complex was washed twice with 350 μL of PBS−AcCN 99:1 (v/v), centrifuging each time at the same speed as before for 7 min. The flow-through was analyzed by HPLC to determine the amount of unbound compound.

Results and Discussion

Sol−Gel Immunosorbent. In accordance with some reported techniques,[18,21] the first doped gels were dried by different methods (vacuum, lyophilization, and air-dry at room temperature, or in refrigeration) until constant weight was acheived. The results were disappointing, as neither the target antigen 2,4-D, nor the closely related compound 2,4-D ester, were retained to any extent in these gels. Considering the good results reported by Bronshtein et al.[19] using a nondried anti-atrazine gel, a freshly prepared wet gel doped with 300 μg of 2,4-D antibody was crushed and packed, keeping it under buffer at all times. The successful extraction of 2,4-D ester indicated the persistence of the antibody activity in the gel. As will be discussed later, the lack of retention of 2,4-D was not due to activity loss during the encapsulation of the antibody but was a problem of the antibody itself. Therefore, 2,4-D ester was used as the "antigen" to evaluate the obtained gels and to optimize the immunoextraction conditions.

The wet hydrogels doped with different antibody amounts presented interesting retention properties and higher binding capacities than other (dry or wet) sol−gel immunosorbents previously reported.[19,24,25] However,

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an important drawback of these gels was the progressive decrease of the bed permeability at each SPE experiment. The latter provoked a high pressure drop and rendered the cartridge operationally useless after some adsorption−desorption cycles. This effect was small for gels containing the lowest antibody amount (300 μg), which could be reused in more than 50 cycles, but became critical at higher antibody density. To understand the origin of this phenomenon, a cartridge was packed with a crushed blank hydrogel and was submitted to the same solvent changes as in the SPE experiments. In this case, the packed bed also suffered an initial compacting (the same as the doped hydrogels) but the permeability of the cartridge did not change thereafter, even after multiple assay cycles. Therefore, the permeability loss in the doped gel cartridges is effectively related to the presence and concentration of the antibody, although its exact cause could not be discerned.

Finally, a more delicate drying of the gels was tried, which was stopped when a weight loss of 50% was achieved. In these conditions the material could be easily ground in a mortar without agglomeration of the gel particles, and the antibody activity was not lost. During the slow drying of the material, the liquid phase (aqueous buffer) was progressively expelled from the pores, leading to consolidation of the aggregates and densification of the solid part of the gel but apparently maintaining a highly porous three-dimensional network. Indeed, compared to the wet hydrogels containing the same antibody amount, the dried gels were fairly superior: the binding capacity of the encapsulated antibodies was still higher and the permeability of the cartridges remained constant during their use in multiple SPE experiments. It has been mentioned that the way the gelation is conducted is crucial to the final properties of the dried solid. For example, the formation of large micelles in the first stage of the sol−gel process, combined with a high degree of interconnection during their progressive aggregation, conduct to highly porous silica xerogels. The micelle size increases with longer hydrolysis times, whereas the type of aggregation depends on the relative importance of the hydrolysis and condensation reactions.[26,27] It is known that acid media promote rapid hydrolysis and slow condensation, but basic or neutral conditions accelerate the condensation reactions; in addition, it has been well established that the latter continue to occur long past the gelation point.[17,28] Even in an apparently dry sol−gel, evolution of the dopant microenvironment has been observed over a time period of one month.[27] In our sol−gel procedure, the low pH of the initial mixture and the relatively long hydrolysis time probably produced large elementary particles that very slowly condensed to form branched aggregates. Upon addition of PBS followed by the antibody solution, also in PBS, condensation was greatly accelerated, and gelation rapidly occurred. However, further evolution of the gels maintained under buffer and those slowly dried in refrigeration led to different properties and behavior for each gel type.

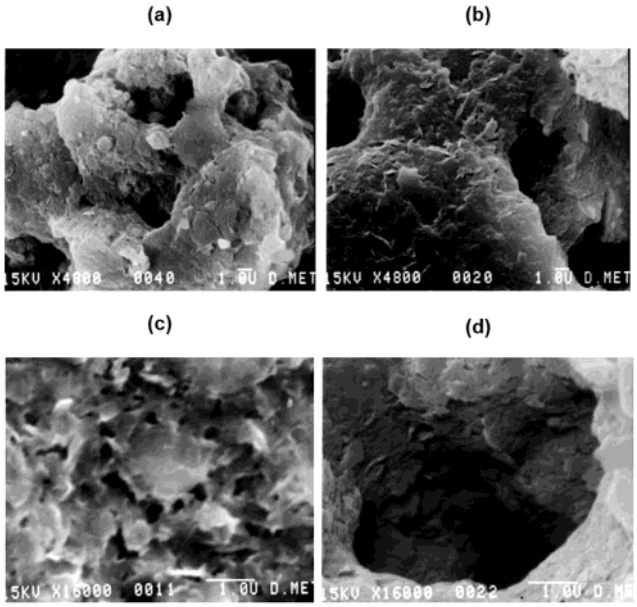

Figure 1. Scanning electron micrographs of silica gels derived from TEOS: (a) wet hydrogel doped with 500 μg of anti 2,4-D that was previously used in several immunoextraction experiments (4800-fold magnification); (b) freshly prepared dried gel doped with 500 μg of anti 2,4-D (4800-fold magnification); (c) freshly prepared dried blank gel (16 000-fold magnification); (d) focusing of one pore in the freshly prepared dried gel doped with 500 μg of antibody (16 000-fold magnification). All the gels were thoroughly vacuum-dried before the SEM study. The scale bar in the figure panels represents 1 μm.

Figure 1 shows the reproduction of the SEM images obtained from a wet hydrogel that was previously used in several SPE experiments doped with 500 μg of antibody (Figure 1a), and two freshly prepared dried gels, one was doped with 500 μg of antibody (Figure 1b and d) and the other was a blank gel (Figure 1c). The used hydrogel (recovered from a cartridge with a high pressure drop) was first slowly dried in refrigeration (to ∼50% weight loss) and ground to a fine powder; then, the three gels were thoroughly vacuum-dried in closed vessels for 2 days at ambient temperature. Although this treatment could have altered the porosity of the materials, a reduction of their volume during the vacuum-drying was not perceived. For comparison purposes, it is important to keep in mind that all the gels were prepared using the same procedure, and prior to the SEM study were submitted to identical drying conditions. Figure 1 panels c and d (same magnification) clearly show the astonishing difference in pore size between the antibody-doped and undoped glasses. The surface of the latter presents multiple nanopores with maximal diameter of about 200−400 nm, whereas the former is characterized by much larger cavities with external diameters of 3−5 μm. Indeed, if the antibody was trapped inside those wide pores it is really surprising that leakage did not occur. This reinforces some hypotheses[17,28] about the existence of interactions and/or interpenetration of some parts of the guest biomolecule and the silica matrix; probably, the antibody is not so free inside the pore. On the other hand, there is no remarkable difference between the used hydrogel and the freshly prepared dried gel (Figure 1a−b), indicating that the repeated SPE experiments performed in the former did not affect the apparent structure. Thus, the

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Table 1. Required Acetonitrile Content in the Aqueous Samples to Avoid Non-Specific Adsorption of the Compounds in a Blank Sol−Gel Cartridge[a]

compound	retained amount with plain aqueous samples (ng)	acetonitrile required for no retention (%)
2,4-D	n.d.[b]	0
2,4-DB	25	7
2,4,5-T	10	1
2,4-DP	7	1
2,4-DPh	n.d.[b]	0
2,4-D ester	8	1
2,4-DB ester	60	>10[c]
2,4,5-T ester	50	10

[a] 10-mL Samples with 100 ng of solute were loaded in the cartridge. [b] n.d., Compound not detected in the eluting solvent. [c] With this acetonitrile content in the sample, the solute peak in the chromatogram of the eluate corresponded to 10 ng.

permeability loss in the wet hydrogel cartridges cannot be ascribed to structural changes of the silica matrix.

Optimization of the Immunoextraction Conditions. Table 1 shows the results obtained from the preliminary experiments carried out in a blank gel cartridge. It is observed that all the analytes, with the exception of 2,4-D and 2,4-DPh, were retained to some extent in the cartridge when the applied samples were plainly aqueous. The AcCN amount required in the different analyte solutions to avoid their nonspecific adsorption was highly dependent on the compound hydrophobicity, indicating that a reverse phase mechanism was responsible for this adsorption. The most hydrophobic compound, 2,4-DB ester, was still slightly retained (about 10% of the loaded amount) with the highest acetonitrile content assayed in these experiments. To discern between adsorption on the cartridge walls or on the silica support, a simulated extraction of each analyte (in PBS with 1% of AcCN) was performed in an empty syringe with only the two frits inside. The absence of solute peaks in the analyzed eluates demonstrated that the cartridge material did not adsorb any of the compounds in these conditions. Therefore, nonspecific adsorption was mainly due to the silica matrix.

To establish the immunoextraction protocol, the optimal elution conditions were first determined. A 300-μg anti-2,4-D cartridge was loaded with a fixed excess of 2,4-D ester (120 ng in PBS with 1% of AcCN) and eluted with different AcCN/water mixtures. Figure 2a shows that the maximal amount of recovered compound was reached with 2 mL of an eluent containing 50% (v/v) AcCN. Higher acetonitrile contents did not increase the solute recovery, and on the contrary may increase the risk of antibody damage. During these experiments, it was observed that a water rinsing of the cartridge was absolutely necessary before the elution step. This was because of the PBS that remained in the interstitial volume of the packing after loading the sample; in the presence of rich acetonitrile mixtures, the phosphate salts form a very fine precipitate that may provoke frit-clogging problems, and also undesirable perturbations in the chromatogram when the eluate is injected in the HPLC system. It was verified that the solute recovery was not affected by water rinsing volumes of up to 5 mL, but a 2-mL rinsing was enough to avoid all the mentioned problems.

The ionic strength and the pH of the sample are important parameters that commonly affect the performance of immunosorbents. Therefore, the extraction of 2,4-D ester from PBS (pH 7.2) at different concentrations (0.005−0.15 M) and from other buffers at different pH levels (1.5, 3.8, and 4.7) was studied. Maximal solute binding was obtained with samples containing PBS at concentrations 0.01−0.15 M (Figure 2b), which approximately correspond to a range of 0.097−1.45 M in ionic strength. This result is not surprising considering that the natural media for antibodies (i.e., biological fluids) have a pH close to neutrality and a relatively high saline concentration of about 0.1−0.15 M. Indeed, a null 2,4-D ester recovery was observed in the experiments with acid buffers, probably because in this particular case the pH of the solution affects not only the solute−antibody interaction but also the solute stability. As the pH decreases, the ester hydrolysis is accelerated.

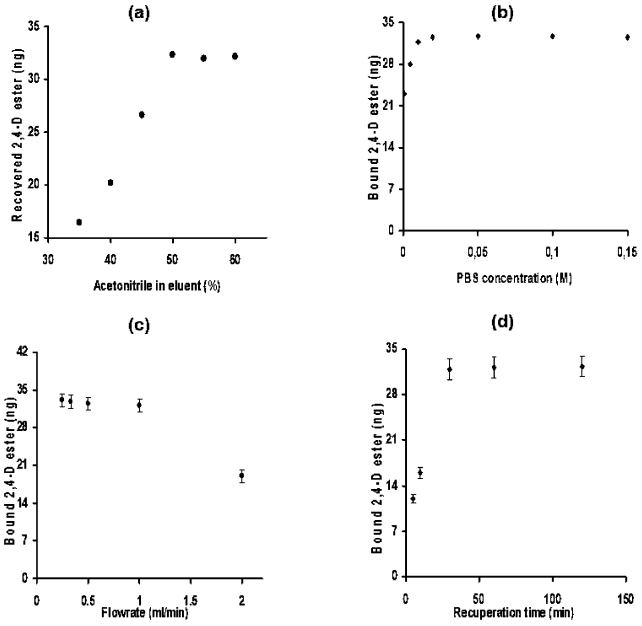

Figure 2. Optimization of immunoextraction conditions in a cartridge packed with wet hydrogel containing 300 μg of anti 2,4-D. Panel (a) amount of recovered 2,4-D methyl ester as a function of the AcCN content in the eluting solvent. Panels (b−d) effect of other experimental parameters on the solute binding: (b) PBS concentration in the applied sample, (c) flow rate used during the percolation of the sample, and (d) recuperation time between consecutive immunoextraction experiments. 10-mL Samples containing 120 ng of the ester in 0.02 M PBS (variable in b) with 1% (v/v) of AcCN were loaded in the cartridge at a flow rate of 1 mL min^{-1} (variable in c). Elution was performed with 2 mL of 50% (v/v) AcCN in water (variable in a). The recuperation time between experiments was at least 1 h (except in d).

Loading of the sample in the cartridge (300 μg of antibody, wet hydrogel) was carried out at different flow rates, and the effect of this variable on the amount of bound 2,4-D ester was determined. Figure 2c shows that maximal binding is obtained at flow rates lower than 2 mL min^{-1} (<1.8 bed vols min^{-1}). From these results, it is evident that the solute−antibody complex is not instantaneously formed. Considering the enormous pores in the doped gels (Figure 1), it is tempting to conclude that the rate-controlling factor is not solute diffusion but the complexing reaction itself. Complicated phenomena, including position or conformational local changes occur as the antigen or the substrate ap-

proaches the active site of an antibody or an enzyme, respectively. Although there are many cases where protein−ligand binding in solution is extremely fast, the same is probably not true for an entrapped biomolecule because the matrix can more or less hinder its reorientational motion. Shabat et al.[29] studied the kinetics of hydrolysis of some esters catalyzed by antibodies encapsulated in a silica hydrogel; from the obtained results they concluded that the diffusion rates of substrates and products within the gel matrix did not inhibit the catalytic reaction to any significant extent. However, it has also been pointed out[30] that the presence of small pores or bottlenecks even in large pores can reduce the diffusion coefficients of solutes in the interior of a silica matrix. Additionally, solute partitioning between the support and the bulk solution may also contribute to decreasing the rate of transport of species to the encapsulated biomolecule. Whether the observed flow rate effect on the binding of 2,4-D ester is mostly due to matrix restricting conformational transitions near the active site of the antibody, or to the solute diffusion rate through the porous network, cannot be resolved with the current data. Nevertheless, from a practical point of view, a flow rate of 0.5−1 mL min^{-1} (0.45−0.90 bed vols min^{-1}), where the binding of 2,4-D ester is maximal, is sufficiently good for immunoextraction purposes.

Finally, using the previously found optimal conditions, a series of adsorption−desorption cycles was carried out in the same cartridge. At the end of each cycle, the cartridge was left in PBS for some variable time (recuperation time). Figure 2d shows the dependence of the extracted 2,4-D ester amount on the recuperation time. For maximal binding, the cartridge must be left under buffer for 30 min or more between consecutive cycles. This kinetical effect is undoubtedly related to the conditions used during the elution step and the phenomena occurring at that moment. The rich acetonitrile mixture probably induces a profound conformational change in the entrapped biomolecule, resulting in the dissociation of the solute−antibody complex. After washing the cartridge with water (10 mL) and PBS (15 mL) to dislodge the organic solvent from the void volume of the support, the antibody still needs a relatively long time in the favorable PBS environment to recover its original (active) conformation.

Conformational changes in sol−gel entrapped biomolecules have been reported in some works.[11,13−15] These studies are based on the comparison of optical properties (absorbance and fluorescence spectra) between the encapsulated and the native proteins. Variations in the intensity of the optical response and/or wavelength shifts are indicative of the extent of conformational change or denaturation due to, for example, the presence of organic solvent in the pores of doped gels or resulting from the aging/drying of the biomaterial. Drastic drying conditions led to irreversible denaturation as shown by Wang et al.[11] by comparing the fluorescence spectra of sol−gels doped with fluorescein alone and with the fluorescein−antifluorescein complex. On the contrary, reversible conformational changes of the entrapped cytochrome *c* have been well established by Dunn et al.[14−15] Of special relevance to our own work is their observation of the partial denaturation of the protein after soaking the gel in pure methanol, which was indicated by the wavelength shift of the heme soret band in the absorption spectrum; however, when the same gel was immersed in buffer for 48 h, the recovered spectrum was identical to that of the native protein. The authors suggested that the confinement of cytochrome *c* within the silica matrix constrained its mobility so that aggregation in alcohol was prevented, yet the molecule remained sensitive to changes in the local environment. Accordingly, the previously described behavior of the encapsulated 2,4-D antibody indicates that this molecule can also switch between different conformations without permanently losing its activity.

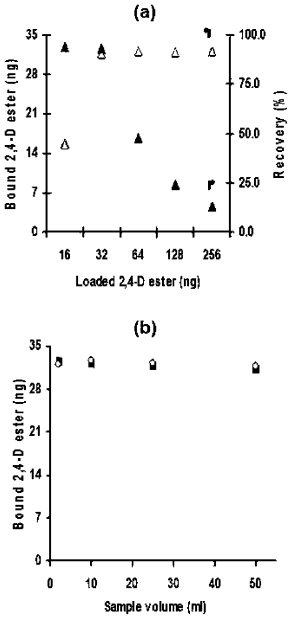

Figure 3. Binding capacity of a sol−gel cartridge (300 μg of anti 2,4-D, wet hydrogel). The cartridge was loaded (flow rate 1 mL min^{-1}; recuperation time at least 1 h) with (a) 10-mL sample volumes containing different 2,4-D ester amounts: (△) bound analyte (ng), (▲) % recovery; (b) different sample volumes containing a fixed dose (120 ng) of 2,4-D ester: (○) alone, (■) in the presence of 2,4-D and 2,4-DPh (120 ng each).

Binding Capacity of the Encapsulated Antibodies. The extraction of 10-mL solutions of 2,4-D ester at different concentrations was performed in a cartridge packed with wet hydrogel (300 μg of antibody). Figure 3a shows the variation of the extracted compound amount (expressed as bound 2,4-D ester and as % recovery) as a function of the total loaded amount. A break point is observed in the curves. Before this point, the analyte binding increases linearly with the loaded amount and all the applied ester is extracted (100% recovery); afterward, the amount of retained compound remains constant, at about 32 ng, which means that the excess solute breaks through from the cartridge during the loading step.

Breakthrough can be due to elution of the compound (weak retention) or to saturation of the immunosorbent (limited adsorption sites). To determine which effect was responsible for the observed behavior, the binding of 2,4-D ester was studied as a function of the sample volume applied to the cartridge. First, the immunoex-

(29) Shabat, D.; Grynszpan, F.; Saphier, S.; Turniansky, A.; Avnir, D.; Keinan, E. *Chem. Mater.* **1997**, *9*, 2258.
(30) Bhatia, R. B.; Brinker, C. J.; Gupta, A. K.; Singh, A. K. *Chem. Mater.* **2000**, *12*, 2434.

Table 2. Immunoextraction of a Fixed Dose (120 ng) of 2,4-D Ester in Sol−Gel Cartridges Doped with Different Anti 2,4-D Amounts

antibody amount (μg)	IgG lot[a]	gel type	n	extracted solute (ng)	RSD (%)	binding capacity (ng/mg)[b]
300	1	wet hydrogel	9	31.4	1.4	105
500	1	wet hydrogel	9	46.7	2.8	93
500	2	wet hydrogel	5	47.0	2.9	94
700	1	wet hydrogel	9	58.8	1.8	84
500	2	dried gel	5	62.9	1.1	126
500	2	dried gel	5	65.2	1.4	130

[a] Two different lots of immunoglobulin (IgG) were used. [b] Binding capacity of the entrapped antibody expressed as ng of extracted solute per mg of entrapped antibody (commercially purchased IgG).

traction of samples containing 120 ng of the ester in volumes varying from 5 to 50 mL was carried out. In a second study, the same experiment was repeated but the samples also contained 120 ng of 2,4-D and 2,4-DPh. From the results shown in Figure 3b, it can be deduced that the immunosorbent strongly retains a certain ester amount (∼32 ng) by interaction with the active antibody sites and not by other nonspecific interactions (i.e., hydrophobic), because neither the higher sample volumes, nor the presence of possible competitors provoked a decrease in the amount of bound compound. However, the number of available (active) adsorption sites in the gel limits the cartridge capacity.

Increasing the amount of encapsulated antibody can, in principle, increase the cartridge capacity. Therefore, a new set of experiments was carried out in cartridges with different antibody contents. Four cartridges packed with wet hydrogels and two others packed with dried gels were loaded with 10 mL of a 12 ng mL^{-1} 2,4-D ester solution. Several replicates were performed in each cartridge. The amount of extracted solute and the corresponding relative standard deviation (RSD) are presented in Table 2. Considering first the results from the wet hydrogels; it is observed that, as expected, the amount of bound solute increases with the amount of entrapped antibody. The calculated RSD, lower than 3%, demonstrates the excellent repeatability obtained with the optimized immunoextraction procedure and also gives an indication of the good immunosorbent stability. It is also interesting to note the similarity of results obtained with the two gels doped with antibody (500 μg) from different lots; apparently, the purchased IgG had the same or similar activity in the two lots. This fact is extremely important for the future development and more widespread use of immunosorbents and immunoextraction protocols, which require ensuring the availability of reproducible antibodies. On the other hand, the last column in Table 2 shows the normalized binding capacity of the encapsulated antibody in each cartridge. This parameter decreases as the concentration of IgG in the gel increases. A similar effect was observed by Pulido-Tofiño et al.[18] in (dried) gels doped with isoproturon antibody. As proposed in that work, the decrease of the binding capacity with the antibody density could be due to reduced solute accessibility to the active sites (or to other perturbations of the solute−antibody interaction) provoked by the nearest neighbor protein molecules in the gel. Therefore, the preparation of immunosorbents with high antibody load is not cost-effective, and the question of how much capacity is really needed in the SPE cartridge should be carefully analyzed.

Table 3. Immunoextraction of Phenoxyalkyl Methyl Esters in a Sol−Gel Cartridge Doped with 500 μg of Anti 2,4-D[a]

compound	total retained amount (ng)	specific retention (ng)[b]
2,4-D ester	63	63
2,4,5-T ester	20	20
2,4-DB ester	45	35

[a] 100 ng of the compound in 10 mL of 0.02 M PBS containing 1% (2,4-D ester) or 10% (2,4,5-T ester and 2,4-DB ester) of acetonitrile were loaded in the cartridge. [b] Specific retention = total retention − retention in a blank cartridge under the same conditions.

Concerning the experiments carried out in the dried gel cartridges; unexpectedly it was found that the extracted solute amount was about 30% higher than in equivalent wet hydrogel cartridges (Table 2). Indeed, the normalized binding capacity of the antibody was the highest one, even higher than that of the wet hydrogel with the lowest antibody density. The reason for this is not clear because the external appearance and porosity of dried and nondried gels are practically identical (Figure 1). It could be possible that a certain evolution of the internal pore structure occurred during the slow drying of the gel which favored the positioning of some antibody molecules rendering their active site more accessible. Alternatively, this evolution could have created well-separated cages for the entrapped molecules, thus reducing harmful perturbations between neighbors during the solute binding.

Finally, comparison of the results obtained in equivalent cartridges (the two dried gels or the two wet hydrogels with 500 μg of antibody each) proves that the immunosorbents prepared by the optimized sol−gel technique proposed in this work are fairly reproducible.

Specificity of the Immunosorbent. A cartridge packed with dried gel (500 μg of antibody) was used to test the binding of four phenoxyalkyl acids (2,4-D, 2,4-DB, 2,4,5-T, and 2,4-DP), three phenoxyalkyl methyl esters (2,4-D ester, 2,4-DB ester, and 2,4,5-T ester) and a structurally related phenol (2,4-DPh). Each solute dissolved in PBS with the appropriate AcCN content was individually tested. Analysis of the eluates, and in some cases of the flow-through loading solution, demonstrated that the phenoxy acids and the phenol were not retained at all. Only 2,4-D ester was significantly bound to the antibody and although the two other esters were also extracted, their specific binding was much lower, as shown in Table 3. It is possible that the higher AcCN content in the percolated samples of these esters provoked a weaker binding to the antibody; however, in the absence of organic solvent these hydrophobic esters are excessively adsorbed in a nonspecific manner to the silica matrix (Table 1). Cichna et al.[20] used a surfactant to decrease the nonspecific adsorption of polyaromatic hydrocarbons in an anti-pyrene sol−gel cartridge; by adjusting the surfactant concentration, they arrived at a compromise between the extent of specific binding and the extent of hydrophobic adsorption.

The null retention of the target antigen 2,4-D was surprising; therefore, additional extraction experiments were carried out with more acidic solutions of this

Table 4. Binding of 2,4-D and 2,4-D Methyl Ester to a Fixed Dose of Free Antibody

compound	added amount (ng)	complexed amount (ng)	binding capacity (ng/mg)[a]
2,4-D	60	0	0
2,4-D	120	0	0
2,4-D ester	60	15	300
2,4-D ester	120	15.2	304

[a] ng of complexed solute per mg of antibody. 50 μg of the purchased IgG fraction and the indicated solute in 1 mL of 0.02 M PBS containing 1% (v/v) of acetonitrile were incubated at room temperature for 15 min in Centricon tubes. The solution was separated from the solute−antibody complex by centrifugation and the unbound compound was determined in the flowthrough by HPLC.

analyte (pH 4.7, 3.8, and 1.5) but the initial result was confirmed: the entrapped antibody did not recognize 2,4-D. To know whether the encapsulation of the antibody or the antibody itself were responsible for the nonretention of the nominal antigen, the binding of 2,4-D and 2,4-D ester to the free antibody (in solution) was studied. A small amount of acetonitrile (1%, v/v) was added to the PBS solution to prevent adsorption of the studied solutes on the walls or the membrane of the Centricon tubes. The results presented in Table 4 confirm that there is absolutely no binding of the acid herbicide to the antibody. It must be remarked that in the conditions used for these experiments (pH 7.2), 2,4-D was totally ionized ($pK_a = 2.6$). As the antibodies were commercially obtained, it was not possible to know how they were produced, but from the observed behavior it can be speculated that the hapten was conjugated to the carrier protein by its carboxylic moiety. In that case, the antibodies generated by the immunized animal would not be capable of recognizing an ionized antigen. Indeed, the good complexation of the 2,4-D methyl ester supports the hypothesis of an antibody raised against a un-ionized and esterified 2,4-D molecule.

Comparison of the experimentally determined activities for the native protein (~302 ng of bound 2,4-D ester per mg of IgG; Table 4) and the dried sol−gel doped with 500 μg of IgG (~128 ng of bound ester per mg of IgG; Table 2) leads to the conclusion that an activity loss of about 58% occurred upon encapsulation. This indicates that the hindrance of some active sites and/or the permanent denaturation of some antibody molecules could not be avoided during the sol−gel process. However, the remaining antibody activity still is quite interesting for the preparation of selective and reusable immunoextraction cartridges with application to the analysis of trace compounds in aqueous matrixes.

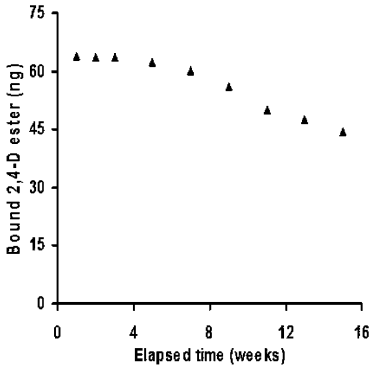

Figure 4. Stability of the immunosorbent. Modification of the binding capacity of a sol−gel cartridge (500 μg of anti 2,4-D, dried gel) during a 14 week period. Sample volume 10 mL, flow rate 1 mL min^{-1}, loaded 2,4-D ester 120 ng.

Stability and Reusability of the Immunosorbent. Most of the optimization and characterization work previously described was carried out in the same cartridge (wet hydrogel) which was thus used in about 50−60 SPE experiments. Finally, it was discarded because the excessive pressure drop rendered it impossible to percolate samples at reasonable flow rates. However, until the last use, the encapsulated antibody was still capable of binding the same amount of 2,4-D ester. Another cartridge packed with dried gel was kept in refrigeration under buffer for several weeks in order to test the long-term stability of the encapsulated antibodies. Figure 4 shows the evolution of the cartridge capacity. For at least 8 weeks, the entrapped antibody retained more than 90% of its initial activity and after 14 weeks the remaining activity still was about 47% of the initial one.

These results confirm the good stability of sensitive biomolecules encapsulated in sol−gel matrixes, which has been previously observed and reported for some enzymes and other antibodies. But, from a practical point of view, the most interesting fact is the excellent reusability of the biomaterial. Although it was subjected to continuous regeneration-loading-elution cycles, implicating drastic changes and harsh environments, the entrapped antibody maintained the same activity during the repeated uses of the cartridge.

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