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Determination of carbofuran in surface water and biological tissue by sol–gel immunoaffinity extraction and on-line preconcentration/HPLC/UV analysis

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ABSTRACT

A selective and simple analytical method for the trace level determination of carbofuran in complex environmental and biological samples was developed based on immunoaffinity extraction (IAE) followed by on-line preconcentration and HPLC/UV analysis of the purified extract. The immunosorbent for IAE was prepared by sol–gel encapsulation of monoclonal anti-carbofuran antibodies, and was fully characterized for capacity, repeatability, binding strength, binding kinetics and cross-reactivity. Method performance was evaluated with two different types of difficult samples: dam water and methanolic extracts of epithelial cervical-uterine tissue. Linear behavior and quantitative recoveries were obtained from the analysis of samples spiked with carbofuran at 0.2–4 ng/mL (dam water, 50 mL samples) and 10–40 ng/mL (biological tissue extract, 2 mL samples). RSD ($n = 7$) and detection limits were, respectively, 10.1% (spike 0.40 ng/mL) and 0.13 ng/mL for dam water; 8.5% (spike 20 ng/mL) and 5 ng/mL for the biological tissue extract. The excellent sample purification achieved with the IAE column allows precise and accurate determination of carbofuran in complex matrices, even when using non-selective UV detection in the chromatographic analysis.

1. Introduction

The trace level determination of pesticides in complex environmental or biological matrices often requires long and laborious sample pretreatment prior to chromatographic analysis. Solid-phase extraction (SPE), in a variety of formats (cartridges, precolumns, fibers, disks) and operation modes, is actually the technique of choice for sample preconcentration and cleanup. Indeed, on-line coupling of SPE with high-performance liquid chromatography (SPE-HPLC) has been the basis for the development of highly sensitive methods, allowing determination of different pollutants at ppb (parts per billion) or sub-ppb levels in aqueous samples [1]. However, the reversed phase sorbents commonly used in these methods (C18 and polymeric phases) are non-selective; so, important amounts of other matrix components are co-extracted during the SPE of more complex samples, and may severely interfere in the determination of the compounds of interest [2]. Although selectivity is less of a problem when MS (mass spectrometry) is coupled to HPLC, high amounts of co-extracted material can affect the detector response or decrease the capacity of the SPE precolumn to quantitatively retain the analytes [3].

In the last decade, several papers have described the preparation of selective sorbents based on molecular recognition. Immunosorbents (IS) and molecularly imprinted polymers (MIP) have been successfully applied in environmental analysis for the extraction, preconcentration and cleanup of specific contaminants from water matrices, soil extracts and food extracts [3–6]. Immunosorbents are prepared by physical entrapment or chemical immobilization of an antibody in a porous solid support; as this antibody has been raised against the compound of interest, the selectivity and binding properties of the IS column derive from the strong affinity and specificity of antigen–antibody interactions. MIP are synthetic materials possessing specific cavities designed for a template molecule; the structure and functionalities of this molecule, as well as the reagents and solvent used for the synthesis, define the properties of the binding sites [6]. Incidentally, no MIP for pesticides of the N-methylcarbamate family has been yet reported, whereas, antibodies against various members of the family have already been produced and used for analytical purposes, mostly in immunoassays or immunosensors, but also in immunoaffinity extraction (IAE) columns [3,7–12].

Carbofuran (2,3-dihydro-2,2-dimethyl-7-benzofuranyl methylcarbamate) is a systemic N-methylcarbamate pesticide, widely used in a variety of agricultural crops as insecticide nematicide and

Abbreviations: IAE, immunoaffinity extraction; IS, immunosorbent; SG-IAE, sol–gel immunoaffinity extraction; MIP, molecularly imprinted polymer; SPE, solid phase extraction; ELISA, enzyme-linked immunoassay; ACN, acetonitrile; MeOH, methanol; PBS, phosphate buffer saline solution; TEOS, tetraethoxysilane; Mab, monoclonal antibody; SD, standard deviation; MDL, method detection limit.

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acaricide. In Mexico, carbofuran is intensively applied for pest control in citric culture fields. Carbofuran is a potent cholinesterase inhibitor and, therefore, it is also highly toxic to human and wildlife (oral LD_{50} in rats is 8 mg/Kg) [13]. In recent years, an increasing number of cases of cervical-uterine anomalies, often turning to cancer, have been detected in young countrywomen from regions dedicated to citric culture in Mexico. Local clinicians suspect a direct contamination of the epithelial tissue with a toxic substance (in fact carbofuran) because of insufficient protection and deficient hygienic conditions. A regular screening of small cervical-uterine biopsies from exposed countrywomen with detected lesions would be necessary to determine if carbofuran is at the origin of the grave health problem in these regions. Moreover, monitoring of carbofuran in local water sources would also be necessary to evaluate the risks for the population and the extent of contamination.

Numerous publications have reported on the trace level determination of N-methylcarbamates, mainly in surface water and groundwater [14], but also in biological matrices such as fruits, vegetables and other foodstuffs [12]. However, studies for the detection of these pesticides or their metabolites in animal or human tissue are scarce [15–17]. In general, the preferred analytical technique has been HPLC because some N-methylcarbamates are thermally labile and not directly amenable to gas chromatography [12,18]. Indeed, analytical procedures reported in many works are variations of EPA method 531.1 (determination of N-methylcarbamates in aqueous samples), which is based on direct injection of the sample in a reversed phase column, followed by a two-step post-column reaction (hydrolysis and derivatization) and fluorescence detection [12,19,20]. Despite the high selectivity and sensitivity of the detection mode, application to biological extracts often requires laborious sample cleanup prior to chromatography to avoid severe interference from matrix components. Besides, the method requires sophisticated and relatively expensive equipment that is not commonly available in analytical laboratories of developing countries. The research group of Dr. Montoya [8–10], proposed an interesting alternative for the determination of carbofuran and other carbamates in water, fruits and vegetables, requiring practically no sample cleanup. This group produced monoclonal antibodies for the target pesticides and developed enzyme-linked immunoassays (ELISA), demonstrating that analytical results were comparable to those obtained with the HPLC-fluorescence method.

Although ELISAs are gaining reputation for the quantitative determination of pollutants in different matrices, most environmental and clinical laboratories still prefer the classical chromatographic techniques with robust detectors for routine analyses. Therefore, the aim of this work was to prepare an immunoaffinity extraction column by sol–gel encapsulation of a monoclonal anti-carbofuran antibody, and use it for the selective extraction of the pesticide from epithelial cervical-uterine extracts and surface water samples. In order to achieve a high sensitivity using a simple UV detector, the whole purified extract from the IAE column was re-concentrated and analyzed by on-line SPE-HPLC.

2. Experimental

2.1. Reagents and solutions

HPLC-grade acetonitrile (ACN) and methanol (MeOH) were from Prolabo and Fisher Scientific. Type 1 reagent water was obtained from a Nanopure deionizer (Barnstead Thermolyne). Pesticide and metabolite standards were from Chem Service (certified purity >99%), other common reagents were analytical grade from Baker. Stock solutions of carbofuran, four carbofuran metabolites and 2,4-dichlorophenol were prepared in ACN and stored at −20 °C Working standards at various concentrations were prepared from the stock solutions in MeOH–water 20:80 (v/v) or in phosphate buffer saline solution (PBS) and kept in refrigeration (4 °C) when not in use; these standards were frequently renewed. Unless otherwise indicated, PBS was 0.02 M NaH_2PO_4, 0.137 M NaCl and 0.0027 M KCl, adjusted to pH 7 with concentrated NaOH solution. PBS of higher concentration, required for some experiments, was prepared maintaining the same salt molar ratio and pH. Tetraethoxysilane (TEOS) from Fluka (99%) was the precursor for the preparation of sol–gel materials. Monoclonal antibody (Mab) LIB-BFNB67 raised against carbofuran, was purchased from I3BH – Universitat Politècnica de València, Spain. The original Mab suspension (in 50% saturated $(NH_4)_2SO_4$) was placed in a Centricon YM-30 tube (cutoff 30,000 Da, Amicon Bioseparations), and desalted by repeated washing with water and centrifugation; the retained portion was re-suspended in PBS, carefully transferred to a vial and kept at 4 °C until use. Concentration of the final Mab solution was 1 mg/mL.

2.2. Evaluation of the free Mab activity

The capacity of native Mab LIB-BFNB67 to form a complex with carbofuran was evaluated according to previously reported procedures with minimal modification [21–23]. Aliquots of 1 mg/mL Mab solution (50 μL, equiv. to 0.333 nmol) and 1 μg/mL carbofuran solution in PBS (70 or 140 μL, equiv. to 0.316 or 0.633 nmol) were diluted with 1 mL of PBS-ACN 98:2 (v/v) into a Centricon YM-30 tube and incubated for 15 min at room temperature. Then, the solution with unbound pesticide was separated from the carbofuran–Mab complex and the free Mab by centrifugation (2500 × *g*, 15 min); the retained portion was washed twice with 350 μL of the PBS-ACN mixture, centrifuging after each washing (same speed, 10 min). The flow-through fractions containing the unbound carbofuran were collected in the same beaker, brought to a volume of 2 mL, and analyzed by the developed on-line SPE-HPLC procedure described later (Section 2.8).

2.3. Sol–gel immunoaffinity extraction column (SG-IAE column)

The procedure used in previous works for the sol–gel entrapment of different polyclonal antibodies [22,23] was first assayed for the encapsulation of four anticarbofuran Mabs: LIB-BFNB67, LIB-BFNB62, LIB-BFNB52 and LIB-BFN21 (kindly provided for preliminary tests by Dr. A. Montoya from I3BH – Universitat Politècnica de València, Spain). From results obtained with the prepared immunosorbents, the Mab LIB-BFNB67 was chosen for further studies. However, these preliminary assays also showed that our sol–gel procedure was not adequate for the encapsulation of monoclonal antibodies as the maximum binding of carbofuran was 18 ng, which was too low compared to the capacity of previously prepared biomaterials.

Optimization of the most critical steps finally led to the following procedure: 2.5 mL of TEOS, 0.1 mL of 0.1 M HCl and 0.4 mL of water were chilled on ice for 30 min; the mixture was then submitted to continuous and gentle shaking (Burrell shaker, model 75) at ambient temperature until the sol became quite elastic, completely transparent and no TEOS odor was perceived (~3.5–4 h). A 2-mL volume of concentrated PBS (0.1 M), immediately followed by a 500 μL aliquot of the Mab solution (500 μg of antibody) were added to the sol under vigorous stirring. Gelation occurred within 1 min. The formed hydrogel was separated from the walls of the recipient, completely covered with PBS and left at rest for 10 min; afterwards it was thoroughly crushed with a spatula and rinsed under vacuum with 25 mL each of the following: water, MeOH–water 50:50 (v/v), 5% glycerol in water and PBS. The drained particles were weighted and allowed to age and dry very slowly in refrigeration (4 °C) until loss of 50% weight. The obtained xerogel (3.6–3.9 g) was ground

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in a mortar, the powder was suspended in PBS to withdraw the finest particles and it was finally slurry packed into inverted plastic syringes (10 mm I.D.), using stainless steel frits at both ends to retain the packed bed (~3 mL wet basis). The SG-IAE column was rinsed with fresh PBS and stored in this buffer at 4 °C until use. Blank gel columns were prepared in exactly the same way but without addition of antibody.

With respect to our previously reported procedure for encapsulation of polyclonal antibodies, the actual modifications included: (1) a more thorough hydrolysis of the precursor promoted by continuous shaking of the sol during the hydrolysis time, (2) an abundant rinsing of the hydrogel with water and MeOH–water to eliminate harmful species (partially hydrolyzed TEOS), and with glycerol solution and PBS to restore and preserve the folded configuration of the biomolecule, (3) a much slower and homogeneous drying of the hydrogel, which was achieved by maintaining the beaker with the biogel particles covered with a piece of open-meshed cotton (tulle) in refrigeration and turning the particles up-down at least once a day; in these conditions, drying to 50% weight loss takes about 6 days.

2.4. Non-specific retention

A blank gel column was loaded with 25 mL of carbofuran solutions (200 ng/mL) in PBS with varying MeOH content, in the range 0–10% (v/v). After rinsing with 5 mL of water, elution of the eventually retained analyte was performed with 2 mL of ACN–water 65:35 (v/v) and the eluate was directly injected in the HPLC. From these experiments, appropriate loading and rinsing conditions were deduced to avoid non-specific interaction of carbofuran with the silica matrix or the walls of plastic syringes in SG-IAE columns.

2.5. Characterization of specific retention in SG-IAE columns

First, the conditions for quantitative elution of carbofuran from the SG-IAE column were determined, then, the binding properties of encapsulated Mab were studied by loading the SG-IAE column with carbofuran solutions at different conditions. Examined parameters were: flowrate (0.6–2.9 mL/min), loaded mass of carbofuran (50–300 ng) and loaded volume (25–200 mL). The eluate obtained from each experiment was analyzed according to the on-line SPE-HPLC procedure described in Section 2.8. In some cases, the flow-through of loading and rinsing steps (hereafter called "the effluents") was also collected and analyzed to corroborate results.

Four carbofuran metabolites (3-hydroxycarbofuran, 3-ketocarbofuran, 7-phenolcarbofuran and 3-keto-7-phenolcarbofuran) and a compound from a different family (2,4-dichlorophenol) were used to study the selectivity of the SG-IAE column. Each compound (100 ng) was dissolved in 50 mL of PBS–2% MeOH and individually tested according to the protocol described in Section 2.8. For these experiments, the effluents and eluate of the SG-IAE column were analyzed.

To avoid compacting of the packed bed and to better control the flowrate, all solutions were percolated from bottom to top of the SG-IAE column by means of an isocratic pump connected to the tip of the syringe. Emerging liquids were sucked by a piece of Teflon tube (1/16″ O.D.) attached to the upper end of the syringe (just over the stainless steel frit), and sent to a gauged vessel placed at convenient height with respect to the column for controlling the transfer rate.

2.6. Instrumentation and chromatographic conditions

The chromatographic system consisted of a quaternary HPLC pump (LC-1150 form Polymer Laboratories), an on-line mobile phase degasser (model 7600 from Jones Chromatography), a UV detector (Spectromonitor 3200 or UV-DAD Spectromonitor 5000, both from Thermo Separation Products) set at 280 nm, and a manual injection valve (Rheodyne 7125i) with a home-calibrated loop (26 μL). Data acquisition and processing were performed using Eurochrom software v. 3.05 and a Knauer 76019 interface connecting the detector to the computer. An on-line solid-phase extraction section was coupled to the chromatographic system (SPE-HPLC) for re-concentration of fractions collected from the SG-IAE column. This section consisted of a six port switching valve (Rheodyne 7000) with a small precolumn placed between two ports and an isocratic auxiliary pump connected to a third port for the delivery of samples and other solutions (Eldex model SC-100). The switching valve was inserted between the injector and the analytical column of the chromatograph.

The HPLC column (150 mm × 4.6 mm I.D.) was a Hypersil ODS (Thermo-Hypersil-Keystone) with 5 μm particles. The concentration precolumn was home-packed with polymeric reversed phase PLRP-S, 10 μm (Polymer Laboratories). Samples loaded in the precolumn or standards injected in the coupled precolumn–column system were eluted and separated with isocratic ACN–water mobile phases at flowrate of 1 mL/min.

2.7. Samples

Surface water was collected from Tenango Dam, located in an agricultural zone of the state of Puebla (Mexico). Upon arrival to the laboratory, the sample volume was estimated and appropriate aliquots of methanol and concentrated PBS (0.4 M) were added in the same bottle to obtain final concentrations of ~2% (v/v) organic solvent and ~0.02 M buffer. The sample was filtered through nylon membranes (0.45 μm pore diameter) and stored in amber glass bottles at 4 °C until analysis. Validation of the analytical method was performed with 50-mL aliquots of this water spiked with carbofuran at different concentrations.

Epithelial cervical-uterine tissue samples were provided by Dr. José Velázquez Ramírez from Hospital de subzona 4 del IMSS in Tecomán, Colima (Mexico). These samples were of different sizes and weights (100–500 mg, wet basis) and had been stored at −20 °C for several months. Samples from non-exposed women were used as blank samples for method development and validation, other 4 samples coming from countrywomen highly exposed to carbofuran were used as target samples to verify the applicability of the method. Conditions for sample extraction were taken from a previous work with minimal modification [24]. Briefly, the biological tissue was thoroughly chopped and extracted with 5 mL of MeOH in an ultrasonic bath (Sonicor, model SC-100) for 45 min; after decantation of the liquid portion, the solid residue was washed twice with 2-mL portions of fresh methanol. All liquids were pooled in a small beaker, evaporated to almost dryness under a gentle N_2 stream and reconstituted with 1 mL of MeOH–water 1:1 (v/v), adding 100 μL of formic acid. The suspension was filtered through a nylon membrane (0.2 μm pore diameter), rinsing the retained particles and membrane with 1 mL of the MeOH–water mixture and ~1.9 mL of water. The filtrate (4 mL, containing 1 mL of MeOH) was collected in a vial and kept at −20 °C until analysis. Because of sample limitations, assays for method development were made with 1-mL aliquots of the final extract, whereas method validation and target samples analyses were carried out with half the extract (2 mL). Prior to analysis, the extract aliquot was diluted to 25 mL with PBS; the methanol content in this solution was 2% (v/v) (or adjusted to this value in the case of 1-mL extract aliquots).

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Table 1
Binding capacity of free and encapsulated Mab LIB-BFNB67.[a,b].

Mab_0 (μg)	Cf_0 (ng)	Cf_B (ng)	Binding capacity[c]	
			(ng/mg)	(mol/mol)
Mab in solution				
50	70	53.2	1064	0.72
50	140	67.9	1358	0.92
Mab in SG-IAE column[d]				
500	300	285 (±7)	570 (±13)	0.39 (±0.01)

[a] Mab_0: initial Mab; Cf_0: initial carbofuran; Cf_B: bound carbofuran.
[b] Mab MW = 150,000 g/mol, carbofuran MW = 221.26 g/mol.
[c] ng (or mol) of bound carbofuran per mg (or mol) of initial Mab.
[d] Mean (±SD) from determinations in 3 independently prepared columns.

2.8. Analytical method

The following analytical protocol was finally established:

2.8.1. Immunoextraction

The SG-IAE column is first equilibrated to room temperature and conditioned with 10 mL of fresh PBS. Then, a 50-mL volume of the aqueous sample (containing 0.02 M PBS and 2% MeOH), or a 25-mL volume of the diluted tissue extract, is loaded in the column followed by a 5-mL rinsing with MeOH–PBS 4:96 (v/v). Next, the retained analyte is eluted with 8 mL of MeOH–water 64:36 (v/v) and the column is immediately rinsed with 10 mL water and regenerated with 15 mL PBS. All steps are carried out at flowrate of 1 mL/min. After each adsorption–desorption cycle, the SG-IAE column must be left at rest in PBS for at least 3 h before the next use.

2.8.2. On-line SPE-HPLC analysis

The eluate of the SG-IAE column is first diluted to 50 mL with reagent water (final methanol content is 10.2%, v/v), and then loaded in the SPE precolumn by means of the auxiliary pump. For the analysis of surface water, the precolumn is only rinsed with 5 mL of reagent water; in the case of biological tissue, the precolumn is rinsed with 5 mL ACN–water 5:95 (v/v), 2 mL of 0.01 M NH_3 solution adjusted to pH 11, and 5 mL of reagent water. During loading and washing steps (flowrate 2 mL/min), the switching valve is maintained in the "load" position and liquids emerging from the precolumn are sent to waste; meanwhile, the chromatographic pump is turned-on to condition the analytical column with the mobile phase (flowrate 1 mL/min). Then, the valve is switched to "inject" position for the on-line elution and HPLC determination of carbofuran.

The same procedure was used for the SPE-HPLC analysis of flow-through fractions collected from the YM-30 Centricon tube (determination of free antibody activity) or the SG-IAE column (loading and rinsing effluents), except that these fractions were directly loaded in the SPE precolumn without any dilution.

3. Results and discussion

3.1. Activity of free and encapsulated antibody

Results of experiments for estimation of the Mab activity in solution (free) and encapsulated in sol–gel glasses are presented in Table 1. For a better comparison of results, a relative binding capacity (ng of bound carbofuran per mg Mab) and the molar ratio of bound carbofuran to initial Mab are reported in this table. In the case of the immunosorbent, it was supposed that all antibody added to the sol was encapsulated because previous works have demonstrated that leakage of biomolecules entrapped in sol–gel glasses is negligible [5,22].

As reported in Table 1, the binding capacity of free Mab (0.333 nmol) increased with pesticide dose but the molar ratio of bound antigen to initial antibody only changed from 0.72 to 0.92 when the carbofuran dose was doubled. Theoretically, a molar ratio approaching the value of 2 was expected for the higher dose (0.633 nmol) because antibodies have two binding sites and association constants for antigen–antibody complexes normally are quite high. Although only two carbofuran doses were examined for the assessment of free Mab activity, the saturation capacity (Cf_{max}) and the association constant (K_{ass}) for carbofuran were roughly estimated from results in Table 1 using the Lineweaver relation (Eq. (1)):

$$(Cf_B)^{-1} = (Cf_{\max})^{-1} + ([Cf] \times Cf_{\max} \times K_{ass})^{-1}, \quad (1)$$

where Cf_B is the amount of bound carbofuran (nmol) and $[Cf]$ is the molar concentration of unbound carbofuran at equilibrium. The estimated values for our initial Mab amount were: $Cf_{max} = 0.338$ nmol, and $K_{ass} = 3.6 \times 10^7\ M^{-1}$. It is thus confirmed that the saturation capacity of the tested LIB-BFNB67 only corresponded to one mol of carbofuran per mol of Mab. Either a large fraction of antibody (~50%) was already denaturated due to inadequate managing and very long delays before arrival to the laboratory, or the binding to the second antibody site was of much lower energy and required higher carbofuran doses to be appreciated. On the other hand, the value of K_{ass} is somewhat low as compared with constants reported for other antigen–antibody complexes, which are about 2–3 orders of magnitude higher [4,25]. Because K_{ass} is independent of the number of actual active sites in the biomolecule, it is believed that the addition of 2% ACN to PBS, for avoiding non-specific interactions of carbofuran with the walls or membrane of the Centricon tube, was responsible for decreasing the antibody affinity to carbofuran.

The activity of encapsulated antibody was determined using the optimal immunoextraction conditions described in Section 2.8. Initially, an SG-IAE column was loaded with increasing amounts of carbofuran until the pesticide was detected in the flow-through of the loading solution. From these assays it was estimated that the binding capacity of the column was between 250 and 300 ng of carbofuran. More precise capacity measurements were made with three new columns that were loaded with 300 ng of carbofuran. These columns were prepared and tested at different times during the course of this study, and one of them was prepared with a different lot of Mab LIB-BFNB67. Analysis of the eluted fractions showed that the mean binding capacity of immunosorbents doped with 500 μg (3.33 nmol) of Mab was 285 ng (1.29 nmol) of carbofuran. From the standard deviation (SD), reported in Table 1, it is clear that the binding capacity of all columns was quite similar, confirming that our sol–gel entrapment procedure provides a biomaterial with repeatable characteristics. Indeed, it is also confirmed that monoclonal antibodies present identical properties from lot to lot. By comparison with the binding capacity of the free Mab, the preservation of antibody activity upon encapsulation was about

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50%. This is a very good performance of our optimized immobilization technique, considering that reported activity losses during the sol–gel entrapment of antibodies commonly are higher [5,25]. Moreover, the initial binding capacity of immunosorbents doped with this Mab is 4–5 times higher than the capacity of previously prepared sol–gel immunosorbents doped with the same amount of polyclonal anti-malathion, anti-atrazine or anti-2,4-D antibodies; maximum binding of the corresponding antigen for those materials was 0.18–0.27 nmol (50–65 ng) [22,23]. An antecedent of an IAE column for carbofuran was reported several years ago by Rule et al. [3]. That column was prepared by chemical bonding of ~700 μg of pre-purified anti-carbofuran polyclonal antibody to aldehyde-activated silica particles; the saturation capacity of the column was 0.12 nmol of carbofuran (~27 ng). It is obvious that the much lower binding capacity of that column as compared to our carbofuran SG-IAE column is due, in part, to the antibody type, because only a limited fraction of polyclonal antisera is analyte specific. However, the immobilization technique probably also accounts for differences in binding capacity; sol–gel entrapment of antibodies does not involve chemical manipulation and the risk of denaturation is, thus, reduced. All the previous data confirm that immunosorbents with the highest activity are obtained using monoclonal antibodies and physical entrapment instead of chemical immobilization techniques.

3.2. Binding characteristics of encapsulated Mab

The kinetics of formation of the carbofuran–Mab complex was evaluated by loading the SG-IAE column with 25 mL of carbofuran solutions (150 ng) at different flowrates. Quantitative analyte binding was only achieved when the loading flowrate was ≤1 mL/min. The effect of flowrate on the binding of small molecules to sol–gel encapsulated antibodies is quite variable. In previous works we found that anti-atrazine and anti-malathion antibodies (polyclonal) could bind their corresponding antigen without any losses up to loading flowrates of 5 mL/min, whereas anti-2,4-D antibody (polyclonal) required a lower flowrate (≤1 mL/min) to completely bind 2,4-dichlorophenoxy methyl ester [22,23]. It is unlikely that the effect of flowrate could be ascribed to a more or less restricted diffusion of the analyte through the porous structure of sol–gel matrices because the preparation of all biomaterials was quite similar, the size of the antigen molecules is comparable and neither of them is ionic. Therefore, the different flowrate effects must reflect different kinetics of formation of antigen–antibody complexes in the pores of the gel matrix.

In contrast with the previous result, no effect was observed when different sample volumes containing 50 ng of carbofuran were loaded in the SG-IAE column at flowrate of 1 mL/min. Analyte was quantitatively bound up to the largest assayed volume (200 mL). This is indicative of the high stability of carbofuran–Mab complex, although the formation of this complex is somewhat slow. From a practical point of view, this result is interesting because large volumes of sample can be preconcentrated in the biogel column without breakthrough of the analyte. Thus, a high sensitivity can be achieved for the determination of carbofuran in environmental waters, even if a simple UV detector is used for analysis.

A counterpart to the high stability of carbofuran–Mab complex was the extreme difficulty to dissociate this complex during the elution step. Initially an acidic buffer was assayed (0.01 M citric acid adjusted to pH 2) but even with 50 mL of eluting buffer, carbofuran was only partially recovered. Then, the buffer was modified with increasing contents of methanol. The analyte could only be totally recovered in <10 mL of eluent when the methanol content was >60% (v/v). At this point it was realized that the acidic buffer was not playing any role, thus, final elution conditions were 8 mL of MeOH–water 64:36 (v/v). It must be mentioned that acetonitrile–water mixtures could not be used for elution because Mab activity was completely and rapidly lost in the presence of >10% acetonitrile. In our previous works with sol–gel encapsulated polyclonal antibodies, elution of bound pesticides was carried out with acetonitrile–water mixtures containing 50–65% organic solvent, with minimal damage to the antibody [22,23]. Apparently, monoclonal antibodies are notably more fragile than polyclonal antibodies. The observed Mab behavior is in accordance with a report from Abad et al. [9], who developed ELISA methods for the determination of carbofuran using Mab LIBBFNB67. They found that Mab tolerance to methanol was quite good (up to 30% in the sample) but a rapid activity loss occurred in the presence of other organic solvents, such as acetone or isopropanol.

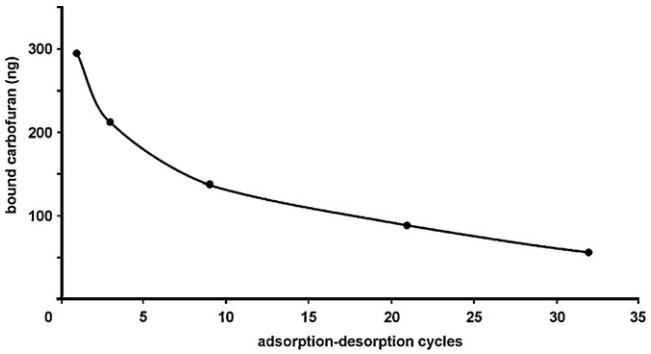

Fig. 1. Binding capacity of encapsulated anticarbofuran Mabs. Variation of binding properties with the number of adsorption–desorption cycles carried out in the same SG-IAE column.

During the course of this study, different immunoextraction experiments were carried out in the same column for characterization of binding behavior. In all cases, the established elution conditions were used, followed by rapid rinsing of the column with water and regeneration with PBS. From time to time, the binding capacity was measured as described in the previous section. Fig. 1 shows the decay of antibody activity as a function of the number of adsorption–desorption cycles performed in the column during a time period of 70 days. As observed, the binding capacity decreased very rapidly with the first uses of the column but after some cycles the activity loss became less pronounced. Probably, the high content of methanol in the eluent was responsible for irreversible denaturation of some antibody molecules at each cycle. From the shape of the decay curve, two populations of encapsulated Mab molecules can be distinguished. Those molecules that remained trapped in narrow pores were more resistant to harmful agents because the restricted space prevented their free motion. On the contrary, molecules located in wide pores could easily unfold and were thus less protected against the denaturating effect of an adverse environment. After 32 adsorption–desorption cycles, the binding capacity of the SG-IAE column had dropped to 52 ng of carbofuran (~18% of the initial capacity). By comparison, our previous sol–gel immunosorbents doped with polyclonal antibodies maintained their initial binding capacity for the same or larger number of repeated uses [22,23]. However, as mentioned before, the capacity of those immunosorbents was about the same as the final capacity of the Mab column. Thus, both types of antibodies have advantages and disadvantages for their application in IAE columns. Main advantages of monoclonal antibodies are their identical properties from lot to lot and their much higher native activity. Main disadvantages are their lower tolerance to harmful agents (especially organic solvents) and their high cost.

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3.3. Cross-reactivity

Retention of carbofuran or other compounds in the SG-IAE column occurs either by specific interaction with the antibody active sites or by non-specific interaction with surface silanols of the solid matrix, as well as by hydrophobicity because of the aqueous nature of the loaded sample. To eliminate unwanted non-specific retention, a blank gel column was loaded with carbofuran solutions in PBS modified with small portions of methanol, and was then eluted with a strong solvent. From these experiments it was determined that a 4% MeOH content in the sample was sufficient to avoid non-specific retention of the target analyte in the blank gel column. The anti-carbofuran Mab, however, could not adequately bind the antigen under these conditions; therefore, the organic modifier content in the loaded sample was lowered to 2%, and instead a stronger solvent with 4% MeOH was used for rinsing. Apparently, when the antigen–antibody complex is already formed, it can stand stronger conditions in the following steps without being dissociated [23]. Indeed, as previously mentioned, elution of the bound carbofuran was quite difficult.

The recognition of other compounds by the encapsulated antibody was evaluated under the optimized loading and rinsing conditions. Four closely related compounds (carbofuran metabolites) and a structurally different compound (2,4-dichlorophenol) were individually loaded in the column to avoid competition for the active antibody sites. A total of 100 ng of compound in 50 mL of solution was passed through the column, collecting the effluents (from loading and rinsing) and the eluted fraction for analysis. Despite the similarity of carbofuran metabolites to the parent compound, only 3-hydroxycarbofuran was partially recognized by the antibody. For this metabolite, 30 ng were determined in the eluted fraction and the complement was found in the flow-through of loading and rinsing. All other metabolites (3-ketocarbofuran, 7-phenolcarbofuran, 3-keto-7-phenolcarbofuran) and the dichlorophenol, passed through the biogel column without being retained. Under the same conditions, carbofuran (100 ng) was totally retained, so, it is estimated that the cross-reactivity of Mab LIB-BFNB67 for 3-hydroxycarbofuran is about 30%. Abad et al. [9] studied the specificity of the same Mab by performing competitive immunoassays using several N-methylcarbamates and carbofuran metabolites as competitors. Their estimation of the antibody cross-reactivity for the three metabolites assayed in the present work was: 25.2% for 3-hydroxycarbofuran, 0.02% for 7-phenolcarbofuran and 0.4% for 3-ketocarbofuran. Taking into account the great difference between the two techniques, results can be considered as quite similar. Basically, the antibody only recognizes the metabolite 3-hydroxycarbofuran to a significant extent.

3.4. Chromatographic determination

To achieve a high sensitivity in the determination of carbofuran, our strategy was to analyze the whole extract obtained from the SG-IAE column (8 mL), rather than injecting only some μL in the chromatographic system. Therefore, an on-line setup, consisting of a small precolumn packed with a highly retentive polymeric reversed phase adsorbent and coupled to the analytical C18 column, was used for trapping, separation and quantitation of all carbofuran present in the extract. However, direct loading of the extract in the precolumn was not feasible due to the high methanol content (64%). From preliminary experiments, it was determined that the breakthrough volume of carbofuran from the precolumn was larger than 50 mL for samples containing 10% MeOH, and larger than 100 mL for samples with 2% MeOH. So, the extract was diluted to 50 mL with reagent water before re-concentration in the precolumn, whereas the effluents from loading and rinsing of the biogel column were directly loaded without any dilution. A weak rinsing of the precolumn with 5 mL of water was sufficient in the case of environmental water samples because most interferences had been previously eliminated during immunoextraction. However, very complex samples, such as cervical-uterine epithelial tissue extracts, required additional cleanup to lower the background noise in chromatograms. This cleanup was performed in the precolumn because the polymeric adsorbent can resist harsh conditions that could otherwise seriously damage the antibody. A rinsing consisting of 5% acetonitrile in water, followed by an alkaline solution of pH 11 and finally pure water was a good compromise for decreasing matrix absorbance without losing the retained analyte.

Table 2
Method performance for the determination of carbofuran in surface water and biological tissue ($n = 7$).[a]

Concentration (ng/mL)		SD (ng/mL)	%RSD	MDL (ng/mL)
Spiked	Found			
Surface water				
0.40	0.42	0.042	10.1	0.13
Biological tissue extract				
20	20.2	1.71	8.5	5.1

[a] SD: standard deviation, RSD: relative standard deviation, MDL: method detection limit (defined as 3× SD).

3.5. Method performance with real samples

3.5.1. Surface water

The sample collected at Tenango Dam was analyzed according to the developed method. Carbofuran was not detected in the blank sample. Then, 5 sample aliquots (50 mL) were spiked with the carbamate at different concentrations in the range 0.2–4 ng/mL. The determined relation for recovered carbofuran (Cf_R, ng) as function of spiked pesticide (Cf_S, ng) was linear in the studied concentration range (Eq. (2)):

$$Cf_R = 0.993\, Cf_S + 0.089 \quad (r^2 = 0.999,\ n = 5). \tag{2}$$

Representation of results as in Eq. (2) was preferred because the slope and ordinate are indicators of method performance. From statistical analysis, the slope was equivalent to unity and the ordinate equivalent to zero; this indicates a complete analyte recovery and the absence of systematic errors in the method, respectively. Precision was evaluated from the analysis of 7 replicate surface water samples spiked with carbofuran at sub-ppb level (20 ng in 50 mL). From results, the standard deviation (SD), relative standard deviation (%RSD) and detection limit (3× SD) were calculated and are reported in Table 2. Analyte recovery was 104 ± 9.7% (confidence level $\alpha = 0.05$), confirming the good accuracy and precision of the method for the trace level determination of carbofuran in surface water. The method detection limit (MDL) for 50-mL surface water samples was 0.13 ng/mL, well below the CFR regulatory level of 40 ng/mL for carbofuran in drinking water [26]. By comparison with other reported methods, this MDL is lower than the detection limit of 1.5 ng/mL estimated for carbofuran in ground water using the official EPA method 531.1 [26]. On the contrary, the immunoextraction-HPLC–MS method, developed by Rule et al. [3], was more sensitive, with a reported detection limit of 0.04 ng/mL for carbofuran in surface water. The present method has, however, the potential for increased sensitivity, either by increasing the sample volume (at least 4 times without analyte losses), or using MS detection instead of UV.

The specificity of the method for carbofuran determination in surface water is illustrated in Fig. 2. Tenango Dam water (50 mL) was spiked with carbofuran (1.12 ng/mL), 7-phenolcarbofuran (2.24 ng/mL), 3-keto-7-phenolcarbofuran (2.24 ng/mL) and 2,4-dichlorophenol (2.27 ng/mL), and loaded in the SG-IAE column,

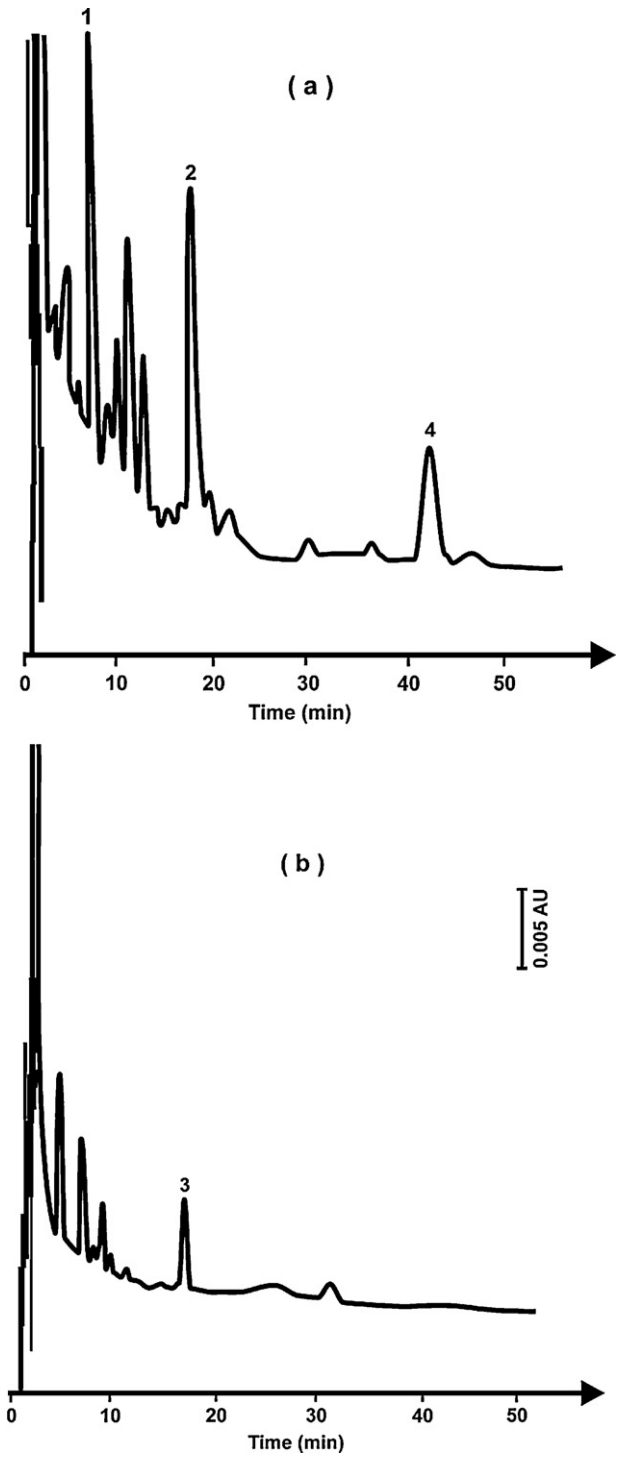

Fig. 2. Analysis of a spiked Tenango Dam water sample (50 mL). Chromatograms of: (a) effluents, and (b) eluate from the immunoaffinity column, analyzed by on-line SPE-HPLC/UV (280 nm). Mobile phase: ACN–water 23:77 (v/v), flowrate 1 mL/min. Solutes: (1) 3-keto-7-phenolcarbofuran (spike 2.24 ng/mL), (2) 7-phenolcarbofuran (spike 2.24 ng/mL), (3) carbofuran (spike 1.12 ng/mL), (4) 2,4-dichlorophenol (spike 2.27 ng/mL).

collecting the effluents and the eluted fraction for analysis. The chromatogram obtained from analysis of effluents showed a large matrix peak with several signals and 3 distinct peaks, corresponding to carbofuran metabolites and dichlorophenol. The chromatogram of eluted fraction was cleaner, with a reduced matrix peak and a well isolated carbofuran peak. Under the chromatographic conditions used in Fig. 2, carbofuran and

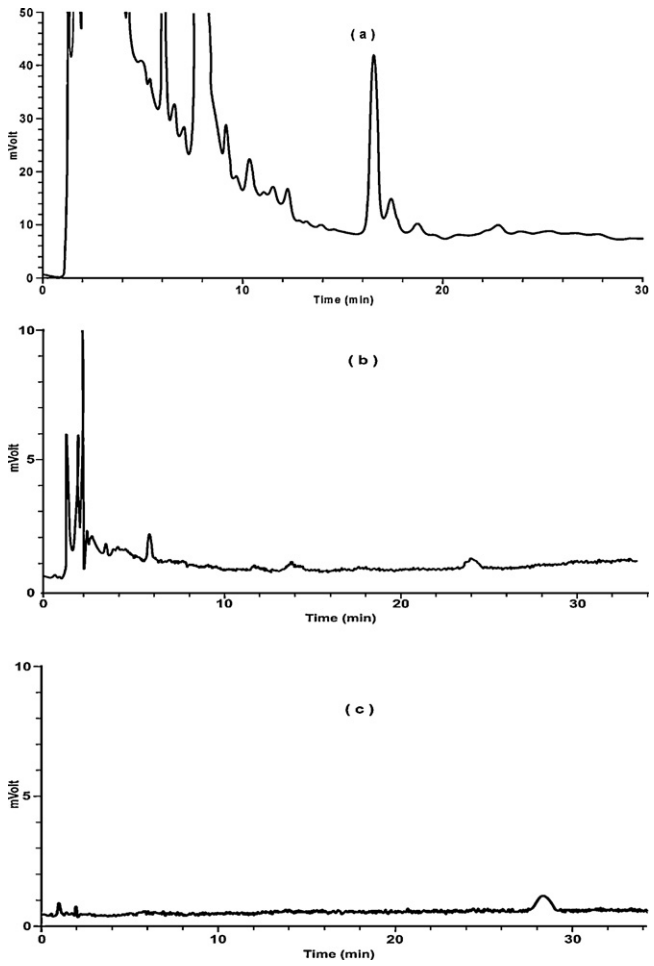

Fig. 3. Analysis of methanolic extracts of cervical-uterine tissue. Chromatograms of: (a) 1 mL of non-purified extract (effluents), (b) 2 mL of purified extract (eluate), and (c) injected carbofuran standard. Aliquots of tissue extract diluted to 25 mL with PBS were loaded in the IAE column, collecting effluents and eluate for analysis by on-line SPE-HPLC/UV (280 nm). An additional cleanup of the eluate fraction was performed in the concentration precolumn with ACN–water 5:95 (v/v) + NH_3 solution at pH 11 + water (5 mL each). Mobile phase: ACN–water 21:79 (v/v), flowrate 1 mL/min.

7-phenolcarbofuran were poorly resolved when a standard mixture was directly injected in the HPLC system; however, with the use of the SG-IAE column for sample pretreatment, carbofuran was already separated from all other compounds and optimization of the mobile phase composition was not necessary.

3.5.2. Biological tissue

A 2-mL portion of the methanolic extract obtained from each cervical-uterine tissue sample (blank samples from non-exposed women) was spiked at 10–40 ng/mL of carbofuran, diluted to 25 mL with PBS and analyzed according to the developed method. From linear regression of the data, the following relation for recovered carbofuran (Cf_R, ng) as function of spiked carbofuran (Cf_S, ng) was found (Eq. (3)):

$$Cf_R = 0.990\,Cf_S + 0.955 \quad (r^2 = 0.998,\ n = 4). \tag{3}$$

Statistical analysis demonstrated that the slope was equivalent to 1 and the intercept equivalent to zero, so, the method for determination of carbofuran in the biological tissue extract is accurate and presents no systematic errors in the range of studied pesticide amounts. Precision was evaluated from the analysis of 7 extract portions (2 mL) spiked with 40 ng of carbofuran. Results reported in Table 2, show the good repeatability and excellent accuracy of the method for the determination of this carbofuran amount in tissue

extracts; indeed, the calculated analyte recovery was 100 ± 7.9% (for $\alpha = 0.05$). Estimated detection limit (3× SD) was 5.1 ng/mL of carbofuran in the extract portion, which is equivalent to 20 ng in the biological tissue sample because the whole extract volume was 4 mL.

The high degree of sample purification that can be achieved using immunoextraction, combined with optimized rinsing of the SPE precolumn, is illustrated in Fig. 3. Chromatograms (a) and (b) correspond, respectively, to the analysis of effluents (flow-through of loading and rinsing), and eluate from the SG-IAE column; chromatogram (c) comes from a direct injection of carbofuran standard (52 ng), included as reference. The complexity and high absorbance of the biological matrix is appreciated in the first chromatogram (Fig. 3a); in this case, only 1 mL of tissue extract was diluted and loaded in the biogel column, collected effluents were preconcentrated in the SPE precolumn, rinsed with water and analyzed. It is obvious that trace amounts of carbofuran would not be detectable in the unpurified sample. A dramatic difference is observed in the chromatogram of tissue extract (2 mL) treated according to the optimized protocol (Fig. 3b). Here, immunoextraction plus some additional cleaning in the preconcentration precolumn eliminated most matrix interferences, minimizing background absorption. Indeed, this chromatogram corresponds to the analysis of epithelial cervical-uterine tissue from a countrywoman that was highly exposed to pesticide contamination; as observed, no carbofuran was detected in this sample. Other 3 target samples were analyzed obtaining similarly clean chromatograms and the same negative result. Carbofuran was not really expected in these samples because they had been stored for several months before extraction and analysis. Even in frozen samples, degradation of the pesticide was highly probable during this long time. However, the main objective of this study was fulfilled because using the developed analytical method, no matrix interferences were observed at the retention time of carbofuran in any sample.

4. Conclusions

The sol–gel encapsulation of fragile anti-carbofuran monoclonal antibodies was optimized, allowing the preparation of immunosorbents with reproducible characteristics and high activity. Columns packed with this biomaterial showed a remarkable efficiency for the recovery, preconcentration and cleanup of carbofuran from environmental water and biological tissue samples. This mode of sample preparation followed by on-line SPE-HPLC/UV analysis of the whole extract resulted in a selective and sensitive analytical method for the determination of the pesticide in complex samples. The proposed method only requires of common analytical instrumentation and is therefore well adapted for use in most analytical laboratories of developing countries. Indeed, the procedure for preparation of the SG-IAE column is relatively simple and only requires of a small amount of antibody per column (500 μg). Besides, the possibility of column reuse in several analyses (about 30 for water samples) easily compensates the cost of monoclonal antibodies.

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References

[1] M.-C. Hennion, V. Coquart, J. Chromatogr. 642 (1993) 211–224.
[2] M.-C. Hennion, C. Cau-Dit-Coumes, V. Pichon, J. Chromatogr. A 825 (1998) 147–161.
[3] G.S. Rule, A.V. Mordehal, J. Henion, Anal. Chem. 66 (1994) 230–235.
[4] N. Delaunay, V. Pichon, M.-C. Hennion, J. Chromatogr. A 745 (2000) 15–37.
[5] M. Cichna-Markl, J. Chromatogr. A 1124 (2006) 167–180.
[6] V. Pichon, J. Chromatogr. A 1152 (2007) 41–53.
[7] G.S. Nunes, M.P. Marco, M.L. Ribeiro, D. Barceló, J. Chromatogr. A 823 (1998) 109–120.
[8] A. Abad, M.J. Moreno, R. Pelegri, M.I. Martinez, A. Saez, M. Gamon, A. Montoya, J. Chromatogr. A 833 (1999) 3–12.
[9] A. Abad, M.J. Moreno, A. Montoya, J. Agric. Food Chem. 47 (1999) 2475–2485.
[10] M.J. Moreno, A. Abad, R. Pelegri, M-I. Martínez, A. Sáez, M. Gamón, A. Montoya, J. Agric. Food Chem. 49 (2001) 1713–1719.
[11] G. Yi-Rong, L. Shao-Ying, G. Wen-Jun, Z. Guo-Nian, Anal. Biochem. 389 (2009) 32–39.
[12] G.S. Nunes, D. Barceló, Trends Anal. Chem. 18 (1999) 99–107.
[13] R.C. Gupta, J. Toxicol. Environ. Health 43 (1994) 383–418.
[14] J.M. Soriano, B. Jiménez, G. Font, J.C. Moltó, Crit. Rev. Anal. Chem. 31 (2001) 19–52.
[15] K. Takeshi, S. Osamu, J. Chromatogr. B 747 (2000) 241–254.
[16] K.H. Liu, J.Y. Byoun, H.J. Sung, H.K. Lee, K. Kim, H.S. Lee, J.H. Kim, Chromatographia 53 (2001) 687–690.
[17] S. Tennakoon, B. Perera, L. Haturusinghe, Legal Med. 11 (2009) S500–S502.
[18] R. Carabias-Martínez, C. García-Hermida, E. Rodríguez-Gonzalo, L. Ruano-Miguel, J. Sep. Sci. 28 (2005) 2130–2138.
[19] A. de Kok, M. Hiemstra, C.P. Vreeker, J. Chromatogr. 507 (1990) 459–472.
[20] M.V. Bassett, S.C. Wendelken, B.V. Pepich, D.J. Munch, J. Chromatogr. Sci. 41 (2003) 100–106.
[21] A. Bronshtein, N. Aharonson, D. Avnir, A. Turniansky, M. Altstein, Chem. Mater. 9 (1997) 2632–2639.
[22] J.C. Vázquez-Lira, E. Camacho-Frías, A. Peña-Alvarez, L.E. Vera-Avila, Chem. Mater. 15 (2003) 154–161.
[23] L.E. Vera-Avila, J.C. Vázquez-Lira, M. García de Llasera, R. Covarrubias, Environ. Sci. Technol. 39 (2005) 5421–5426.
[24] L.E. Vera-Avila, D. Macías-Mendoza, R. Covarrubias-Herrera, J.G. Velázquez-Ramírez, J. Mex. Chem. Soc. 49 (2005) 32–38.
[25] R. Wang, U. Narang, P.N. Prasad, F.V. Bright, Anal. Chem. 65 (1993) 2671–2675.
[26] D. Barceló, J. Chromatogr. 643 (1993) 117–143.