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Development of an immunoassay for terbutryn: Study of the influence of the immunization protocol

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ABSTRACT

Heterologous immunization has proven to be useful to enhance the selectivity and specificity of catalytic antibodies. However, in the field of immunoassays, few studies have been done to establish how the immunization protocol influences the antibody characteristics. In the present study, we have developed an enzyme-linked immunosorbent assay (ELISA) for the detection of the pesticide terbutryn following a homologous and a heterologous immunization strategy. No significant differences have been observed between the immunization procedures regarding immunoassay sensitivity and selectivity. Thus, immunoassays with a limit of detection below the 25 ng/l established by current European regulations have been obtained with both immunization protocols. Initial studies have been performed to assess the applicability of these ELISAs to the analysis of real water matrixes.

1. Introduction

Terbutryn (*N-tert*-butyl-*N*-ethyl-6-methylthio-1,3,5-triazine-2,4-diamine) is a selective herbicide that belongs to the s-triazine group. Triazine herbicides are absorbed by the roots and foliage and act as inhibitors of the photosynthesis. Terbutryn is a preemergent and postemergent control agent for most grasses and many annual broadleaf weeds. It is also used as an aquatic herbicide for control of submerged and free-floating weeds and algae in water courses, reservoirs and fish ponds [1]. Although terbutryn water solubility (22 mg/l) is higher than that of other s-triazine herbicides, its partition coefficient ($P = 3.65$) favors its association to sediments and suspended particulate matter [2]. Despite of this fact, terbutryn has been detected in sea waters [3], river waters [4,5] and urban wastewater [6,7]. Additionally, terbutryn shows a low degradation rate in static aerobic systems [8]. Due to these characteristics terbutryn could imply a risk to the environment and to human health.

The Environmental Protection Agency (EPA) has classified terbutryn as slightly toxic (Toxicity Class III), possible carcinogen, potential ground water contaminant and suspected endocrine disruptor [9–11]. On top of that, it has been recently shown that terbutryn reacts with hypochlorite during the disinfection process of drinking water, leading to the formation of by-products of higher toxicity [12]. As a result, the EPA has proposed the revocation of all tolerances for residues of terbutryn and does not plan to recommend action levels to replace the tolerances (Federal register 59(138): 37019). In Europe, terbutryn is regulated by the European Council Directive 91/414/EEC and the European Commission Regulation (EC) 2076/2002. Under these regulations, authorization of plant protection products containing terbutryn has been withdrawn for all members of the EU.

Detection methods for terbutryn are common to other triazines. The techniques most frequently described in the literature are based on liquid and gas chromatography coupled with mass spectrometry [6,12–14]. However, these methods involve the laborious extraction of the herbicide from samples prior to the analysis. For example, the EPA uses solid-phase extraction followed by gas chromatography with a nitrogen–phosphorus detector for the analysis of triazines (EPA Method 821/R-93-010-A) [9]. On top of that, large sample volumes have to be collected and processed in order to reach the 25 ng/l limit of detection (LOD) in drinking water, required by the European legislation for the analytical methods applied. Alternatively, immunochemical techniques have demonstrated their capacity to simultaneously process numerous samples without performing clean-up or preconcentration steps. Moreover,

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immunoassays have shown to provide satisfactory detectability to achieve international regulations regarding pesticide residues in environmental samples.

Numerous immunoassays have been developed for the determination of triazine herbicides, which include enzyme-linked immunosorbent assays (ELISAs) [15,16], fluoroimmunoassays [17,18], chemiluminescent assays [19] and immunochromatography [20,21], among others. More recently, traditional immunotechniques have led to novel approaches such as immunosensors [22,23]. These techniques detected or do not detect terbutryn depending on the immunization hapten used to produce antibodies. In this context, two specific immunoassays for terbutryn can be found in the literature [24,25]. However, none of them achieved the LOD required by the European legislation.

Production of antibodies against low-weight molecules requires the preparation of a hapten to allow covalent linkage to a carrier protein. This immunization hapten is designed to maximize the exposure of the characteristic groups of the analyte to the immune system of the animal. Numerous studies demonstrate the importance of the design of the most suitable immunization hapten to favor the rising of specific and selective antibodies. Indeed, computer-assisted molecular modeling is now being used to help with hapten designing [26–28]. However, not so much effort has been put into investigating how the immunization protocol influences the characteristics of antibodies raised against low molecular weight analytes. Homologous immunization implies inoculating a single substance into the host animal to produce antibodies against the target compound. Alternatively, heterologous immunization uses a combination of different immunization haptens, each of them maximizing the recognition of a single epitope. Heterologous immunization was first devised to generate more efficient catalytic antibodies [29,30]. This strategy was also used in our group to develop antibodies with the ability to detect, to a similar extent, different congeners of structurally related families of substances [31,32]. Two different protocols have been described for heterologous immunization. In the first one, an equimolar mixture of the immunogens is inoculated into the animal in the initial and in the following booster injections. This strategy has proven efficient to obtain antibodies with a broad recognition spectrum [31,32]. In the other procedure, the immunization is done by alternating the different immunogens. So far, this second method has only been used to produce catalytic antibodies [33,34].

In this study, we have developed an ELISA for the detection of terbutryn with a LOD below the 25 ng/l obliged by current regulations. For such a purpose, we have evaluated how different immunization protocols affect the sensibility and the specificity of the immunoassay. Moreover, we have compared homologous vs heterologous immunization strategies for the production of antibodies against terbutryn.

2. Experimental

2.1. Chemicals and immunochemicals

Chemical reagents were purchased from Aldrich Chemical Co. (Milwaukee, WI). Pesticide standards used for cross reactivity studies and as standards were purchased from Riedel De Häen (Seelze, Germany) and Dr. Ehrenstorfer (Augs burg, Germany). Atrazine and irgarol were obtained as a gift from Ciba-Geigy (Barcelona, Spain). Enzymes and immunochemicals were acquired from Sigma (St. Louis, MO).

2.2. Buffers

Phosphate-buffered saline (PBS) is 0.01 M phosphate buffer and 0.8% saline solution and the pH is 7.5. PBST is PBS with 0.05% Tween 20. Borate buffer is 0.2 M boric acid-sodium borate, pH 8.7. The coating buffer is 0.05 M carbonate-bicarbonate buffer, pH 9.6. Citrate buffer is a 0.04 M solution of sodium citrate, pH 5.5. The substrate solution contains 0.01% tetramethylbenzidine (TMB) and 0.004% H_2O_2 in citrate buffer.

2.3. Instrumentation

The pH and the conductivity of all buffers were measured with a pH 540 GLP pHmeter (WTW, Weilheim, Germany). Polystyrene microtiters plates were purchased from Nunc (Maxisorb, Roskilde, DK). Washing steps were carried out using a 96PW-TECAN CE microplate washer (Tecan GmbH, Grödig, Austria). Absorbances were read with a Spectra Max Plus microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). The competitive curves were analyzed with a four parameter logistic equation using the software Softmax v2.6 (Molecular Devices) and GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA)

2.4. Synthesis of haptens

Hapten **4c** was synthesized following the procedure described by Ballesteros et al. [16] (see Table 1 for the chemical structures of the triazine haptens). The preparation of haptens **2e** and **4d** has been previously described [35]. The synthesis of the carboxylic acid derivatives of atrazine (**2b** and **4b**) and simazine (**4a**) has been reported, as well [36,37]. The chemical structures of the s-triazine haptens are shown in Table 1.

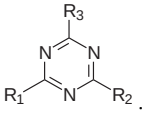
2.5. Preparation of the immunogens, enzyme trazers (ETs) and the coating antigen *4d*-BSA

The immunizing haptens **4c** and **4d** were covalently attached through their carboxylic acid moiety to the lysine residues of the protein keyhole limpet hemocyanin (KLH) following the mixed anhydre method described elsewhere [36]. Conjugates were stored freeze-dried at −80 °C. Following described procedures, competitor haptens **2b**, **2c**, **4a**–**4d**, were coupled to horseradish peroxidase (HRP) using the active ester method [35,38]. Working aliquots were stored at 4 °C in 0.01 M PBS at 1 mg/ml. The coating antigen **4d**-BSA used for evolution titer evaluation was prepared as indicated by Ballesteros et al. [16].

2.6. Polyclonal antisera

Production of polyclonal antisera As13–18 following a homologous immunization protocol has been previously described [16].

Table 1
Chemical structure of the s-triazine haptens.



	R_1	R_2	R_3
Terbutryn	$NHBu^t$	NHEt	SCH_3
2b	$NHPr^i$	$NH(CH_2)_3COOH$	Cl
2e	$NHBu^t$	$NH(CH_2)_3COOH$	Cl
4a	NHEt	NHEt	$S(CH_2)_2COOH$
4b	NHEt	$NHEr^i$	$S(CH_2)_2COOH$
4c	$NHBu^t$	$NHPr^c$	$S(CH_2)_2COOH$
4d	$NHBu^t$	$NH(CH_2)_3COOH$	SCH_3

Table 2
Heterologous inmunization protocols being A = **4c**-KLH and B = **4d**-KLH.

Inoculation	Protocol 1 Rabbits 80–81	Protocol 2 Rabbits 82–83	Protocol 3 Rabbits 84–85	Protocol 4 Rabbits 86–87
1	AB	B	A	B
2	AB	A	A	B
3	AB	B	A	B
4	AB	A	B	A
5	AB	B	B	A
6	AB	A	B	A

Briefly, immunogen **4c**-KLH was used to raise antisera As13–15, while As16–18 was obtained from immunization with **4d**-KLH. For the production of antisera using a heterologous strategy, eight female New Zealand white rabbits (80–87) weighing 1–2 kg were immunized with **4c-** and **4d**-KLH and a mixture of both (1:1) following the immunization protocols indicated in Table 2. The immunizing antigen (100 μg) was dissolved in PBS (0.5 ml) and emulsified with Freund's complete adjuvant (0.5 ml) and injected intradermally at multiple sites in the back. After one month, the animals were boosted with additional 100 μg of the antigen emulsified with Freund's incomplete adjuvant according to the heterologous strategies shown in Table 2. The corresponding antisera (As) obtained were named with the rabbit numbers. Evolution of the antibody titer was assessed by measuring the binding of serial dilutions of the different As to microtiter plates coated with **4d**-BSA. After and acceptable antibody titer was observed, the animals were exsanguinated and the blood was collected on vacutainer tubes provided with a serum separation gel. Antisera were obtained by centrifugation and stored at −80 °C with 0.02% NaN_3.

2.7. ELISA general protocol

The plates were coated with the antisera (100 μl/well, in coating buffer) overnight at 4 °C covered with adhesive plate sealers. The day after, the plates were washed four times with PBST (300 μl/well) and the solutions of the analyte (100 nM to 0.0013 nM; 50 μl/well in PBST; zero analyte is only PBST) and/or the ETs (50 μl/well in PBST; 100 μl/well for the noncompetitive assays) were added and incubated for 30 min at room temperature (RT). The plates were washed again as before, and the substrate solution was added (100 μl/well). Color development was stopped after 30 min a RT with 4 N H_2SO_4 (50 μl/well), and the absorbance were read at 450 nm. The standard curve was fitted to a four-parameter logistic equation according to the following formula: $y = \{(A - B\}/[1 + (x/C)^D] + B$, where A is the maximal absorbance, B is the minimum absorbance, C is the concentration producing 50% of the maximal absorbance and D is the slope at the inflexion point of the sigmoid curve.

Noncompetitive direct ELISA was used for the screening of the avidity of the antisera As80–87 obtained by heterologous immunization vs the 6 ETs. For this purpose, the binding of serial dilutions (1/1000 to 1/64,000) of each ET to the microtiter plates coated with different dilutions (1/1000 to 1/256,000) of each antisera was measured. From these experiments, optimum concentration for antisera and ETs were chosen to produce around 0.7–1 units of absorbance. Avidity of the antisera As13–18 vs the 6 ETs was already assessed [16].

For the combination As87/**4a**-HRP, microtiter plates were coated with **As87** (1/32,000), overnight at 4 °C (100 μl/well in coating buffer) covered with adhesive plate sealers. The following day the plates were washed 4 times with PBST (300 μl/well). Next, terbutryn standards (100–0.0013 nM in PBST), and the **4a**-HRP (1/6000) was added to the plates (50 μl/well each) and incubated for 30 min at RT. After this step, the plates were processed as described above.

2.8. Cross-reactivity determinations

Stock solutions of different structurally related triazine pesticides were prepared in DMSO at a concentration of 2.5 mM and stored at 4 °C. Standard curves were prepared in PBST (100–0.0013 nM) as indicated before. Each IC_{50} value was determined in the competitive experiments following the protocol described above. The cross-reactivity values were calculated according to the following equation: (IC_{50} terbutryn/IC_{50} triazine derivative) × 100.

2.9. Matrix effect studies

A terbutryn standard curve was prepared in water containing 3.5% of seasalts and was used for studying the effect of the matrix in the immunoassay. In these studies the enzyme tracer was added to the plates diluted in PBS with 0.1% Tween 20. The competitive immunoassay was carried out as described above.

3. Results and discussion

3.1. Antibody production. Heterologous immunization

In the present work, we have investigated different immunization tactics to produce antibodies with the best characteristics for terbutryn detection. Antisera raised following a homologous immunization strategy, using **4c**- and **4d**-KLH as immunogens, was previously produced in our group [16]. Regarding the heterologous approach, four different protocols were assessed with the goal of enriching the antisera with antibodies that recognized the methylthio and tert-butyl moieties of terbutryn. In this study, the immunogens **4c-** and **4d**-KLH were administrated combined or alternatively (see Tables 1 and 2 for chemical structures and immunization protocols).

3.2. Screening of the antiserum avidity for the enzyme tracers

A battery of 6 ETs was screened vs antisera As80–87, obtained by heterologous immunization, with the aim of finding the competitor that provided the immunoassay with the best features. ETs could be divided into three groups of haptens with an increasing degree of structural homology with terbutryn (see Table 1): (i) haptens **2b** and **4b**, which only had the triazine ring in common with the terbutryn molecule; (ii) haptens **2e**, **4a** and **4c** that shared with the analyte one of the substituents of the triazine ring and finally; (iii) hapten **4d**, which had the highest degree of homology with the target analyte, as it retained the methylthio and *tert*-butyl substituents of terbutryn. The avidity of antisera As80–87 for the battery of ETs was evaluated using noncompetitive direct ELISAs (see Table 3). As expected, the highest avidity was observed for the immunization haptens **4c** and **4d**. Although both hapten **4a** and **2e** shared a substituent with terbutryn, a remarkable difference in recognition was noticed between them. While hapten **4a** was recognized by most antisera, hapten **2e**, which had a chlorine atom replacing the methylthio group, showed lower titers. This result suggested the high influence of the sulfur atom of the immunization haptens in the immune response. Haptens **2b** and **4b**, featuring only in common with terbutryn the triazine ring, were recognized to a lesser extent and only by few antisera. Overall, these results indicated that all antisera showed the same pattern of recognition of the chemical structures tested as competitors. Therefore, it seemed that the heterologous immunization protocol used did not influence the recognition pattern of the antibodies for the competitors. Regarding antisera As13–18 produced by

Table 3
Relative avidities[a] of the antisera raised against terbutryn vs the battery of competitive haptens.

ETs	Antisera							
	As80	As81	As82	As83	As84	As85	As86	As87
2b-HRP								
2e-HRP								
4a-HRP								
4b-HRP								
4c-HRP								
4d-HRP								

Absorbance: >1.5 1 – 1.5 0.5 – 1 < 0.5

[a] The relative avidities have been expressed as the absorbance obtained when measuring the binding of the ET 1/1000 times diluted in PBST to microplates coated with the antisera at 1/8000 dilution in coating buffer.

homologous immunization, the antiserum avidity followed the same pattern as As80–87 (data not shown).

3.3. Competitive direct Elisa. Effect of the immunization protocol on the immunoassay

Those As/ET combinations showing reasonable titers were tested on a competitive ELISA format to determine the antiserum's ability to recognize terbutryn. The appropriate concentrations of each immunoreagent were established by two-dimensional checkerboard titration experiments. The results of the competitive assays are reported in Table 4. Within the heterologous immunization, significant differences were observed depending on the protocol used. Thus, protocol 1, in which animals were immunized with equimolar concentrations of the immunogens **4c**- and **4d**-KLH, rendered the highest number of competitive assays with acceptable features (i.e. slope and maximal absorbance vs noise ratio). In contrast, protocol 4, in which rabbits were first immunized with **4d**-KLH and then with **4c**-KLH, provided only two competitive assays with adequate characteristics. However, the best IC_{50} values were obtained with As87 from protocol 4. It was noticeable that none of the As86/ETs combinations provided a competitive assay. This fact could be attributed to the individual differences in antibody production.

In a competitive immunoassay the sensitivity is highly dependent on the ratio of the affinity constants K_a (analyte-Ab) and K'_a (HRP tracer-Ab). In order to favor the binding of the antibodies to the analyte, competitors with slightly different chemical structure from the immunizing hapten are normally used. Nevertheless, good assays have been obtained under homologous conditions, in which the immunization and the competitor haptens have the same chemical structure [36,39]. In this study, a noteworthy fact was that the homologous **4d**-HRP competitor provided the highest number of useful assays; being combination As87/**4d**-HRP one of the most sensitive.

Regarding the homologous immunization, it was also observed a difference in the number of competitive assays obtained depending of the hapten used for immunization. As13–15 raised using **4c**-KLH as immunogen recognized terbutryn in most cases. On the contrary, As16–18 obtained by inoculation with **4d**-KLH rendered a significant lower number of useful assays. Nevertheless, the immunoassays with the best features were obtained within this last group. Thus, As17 provided the most sensitive assays. In the same group, As16 rendered no competitive assays, therefore emphasizing how the immune response is conditioned by individuality. Opposite to the heterologous immunization, the best ELISAs (As17/**2b**-HRP and As17/**4a**-HRP) were obtained using a competitor hapten with a chemical structure different from the immunization one. Altogether, these results highlighted that it could not be established a clear correlation between the detectability of an assay and the degree of analogy between the chemical structure of the competitor and the analyte.

A comparison between the homologous and the heterologous immunization results did not allow us to conclude which of the two strategies was the best from the sensitivity point of view. Good immunoassays were obtained with the two tactics (i.e. with As17 and with As87) with no significant differences in the IC_{50} value between them. At present, there is no other study in the field of immunoassay development in which homologous and heterologous immunization protocols have been compared. The only referent found is the work by Tsumuraya et al. [34]. This study, performed in the area of catalytic antibodies, showed how the heterologous immunization improved the catalytic activity of the antibodies in relation with the homologous strategy. Nevertheless, these results cannot be extrapolated for comparison to the immunoassay field.

3.4. Influence of the type of immunization in the specificity of the immunoassay

To evaluate how the immunization protocol affected the specificity, we determined the cross-reactivity of several triazines in the ELISAs for terbutryn. In this case, the enzyme trazer **4d**-HRP was used as competitor, since it was the only enzyme tracer that rendered a competitive assay with antisera obtained from all the protocols (see Tables 4 and 5 for specificity study results). As described above, the methylthio and the *tert*-butyl epitopes were the most relevant regarding the avidity of the antisera. Therefore, it was expected that irgarol, which has both substituents in its chemical structure, showed a high cross-reactivity value in all the assays. Accordingly, it has been described that terbutryn interferes in an immunoassay developed for irgarol [16]. Terbuthylazine and terbumeton share the *tert*-butyl moiety with terbutryn, but the methylthio epitope is changed for a chlorine atom and a methyloxy group, respectively. This substitution modifies the net charge and the electronic distribution of the triazine ring. Therefore, the cross-reactivity of these triazines was diminished compared with that of irgarol. Atrazine has an ethyl group in common with terbutryn. However, since the immunogens **4c**- and **4d**-KLH lacked this epitope in their structure, it was plausible that atrazine was not recognized in the immunoassays.

In relation to the influence that the immunization method had in the specificity, it was remarkable that the same pattern of cross-reactivities was obtained for both the heterologous and homologous immunization. Although cross-reactivity values for a specific compound varied between the different As/ET combinations, it could be established with a few exceptions that irgarol was the triazine that showed the highest interference followed by terbuthylazine and terbumetron. In the homologous protocol, irgarol cross-reactivity was higher for As15 than for As17. This result was logical since the immunization hapten **4c**-KLH (As15) had in its structure the *tert*-butyl and the isopropyl epitopes of irgarol plus an alkylthio moiety that resembled the methylthio group. On the other hand, in the immunization hapten **4d**-KLH (As17) the isopropyl epitope was absent. This argument also justifies why in the heterologous immunization the combination As87/**4d**-HRP had the lowest cross-reactivity for irgarol. Immunization with protocol 4 was initiated with 3 inoculations of hapten **4d**-KLH, therefore rendering antibodies with higher recognition for this hapten than for hapten **4c**. Regarding hapten design, it has been hypothesized that

Table 4
IC_{50} values[a] obtained when screening As13–18 (homologous immunization) and As80–87 (heterologous immunization) against **2b**-, **2e**-, **4a**-**4d**-HRP tracers for competitive inmunoassays.[b]

Immunization Protocol	Immunogen	Antisera	Enzyme tracers					
			2b	**2e**	**4a**	**4b**	**4c**	**4d**
Homologous	**4c**-KLH	13	290	282	118	444		210
		14	333		309	603		497
		15	222	265	108	652	234	265
		16						
	4d-KLH	17	77		36	92		89
		18			386	603		442
Heterologous	**4c**- and **4d**-KLH	80		362	434	516		262
	Protocol 1	81	681		821			203
	4c- and **4d**-KLH	82				755	995	
	Protocol 2	83	226		498			270
	4c- and **4d**-KLH	84	157	375				239
	Protocol 3	85						
	4c- and **4d**-KLH	86						
	Protocol 4	87			54			67

[a] IC_{50} values are expressed as ng/l.
[b] Squares in blank indicate no competence or assays with an IC_{50} higher than 1000 ng/l.

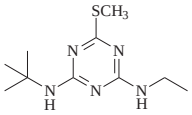
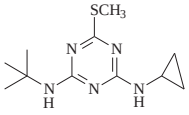
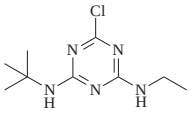
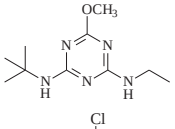
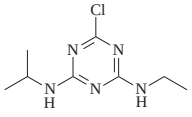
a chlorine atom may mimic a sulfur atom [35]. According with this basis, it would have been expected that terbuthylazine showed a higher interference than terbumeton. However, the cross-reactivity of these compounds in the different assays did not follow a specific pattern. Thus, we could not find a line of reason that explained why these triazines did not interfere in some of the combinations while cross-reacted in others.

3.5. Additional studies with the As87/4a-HRP ELISA

From all the immunoassays obtained the As17/**4a**-HRP combination had the best IC_{50} value. Nevertheless, for further studies we chose combination As87/**4a**-HRP since it showed better slope value, lower background noise and higher reproducibility than combination As17/**4a**-HRP. The features and the calibration curve of the As87/**4a**-HRP immunoassay are shown in Fig. 1 and Table 6, respectively. It is worth noting that this assay has a LOD of 5.02 ± 0.17 ng/l, which is below the 25 ng/l obliged by European current regulations.

Cross Reactivity Studies: The potential interference of structurally related triazines in the As87/**4a**-HRP assay was evaluated (see Table 7). As described above for other immunoassays, irgarol showed the highest degree of recognition. Terbuthylazine was also recognized but in a lesser extent, while atrazine was hardly detected in the assay. The same was observed for simazine, which has the ethyl substituent in common with terbutryn. These results strengthen the fact that the *tert*-butyl and the methylthio

Table 5
Comparison of the cross-reactivity of several triazines on competitive direct ELISAs for terbutryn that use the enzyme tracer **4d**-HRP as competitor.[a]

Compound	Structure	Heterologous immunization								Homologous immunization			
		Protocol 1 As80/**4d**-HRP		Protocol 2 As83/**4d**-HRP		Protocol 3 As84/**4d**-HRP		Protocol 4 As87/**4d**-HRP		**4c**-KLH As15/**4d**-HRP		**4d**-KLH As17/**4d**-HRP	
		IC_{50}	% CR	IC_{50}	% CR	IC_{50}	% CR	IC_{50}	% CR	IC_{50}	% CR	IC_{50}	% CR
Terbutryn		222	100	241	100	229	100	79.7	100	203	100	50.7	100
Irgarol		166	134	182	115	177	129	152	52	144	141	70.9	71
Terbuthylazine		>10^3	<1	655	37	717	32	117	68	827	25	62.0	82
Terbumeton		254	87	>10^3	<1	>10^3	<1	167	48	237	86	507	10
Atrazine		>10^3	<1	>10^3	<1	>10^3	<1	>10^3	<1	>10^3	<1	>10^3	<1

[a] Cross-reactivity is expressed as a percent of the IC_{50} value (ng/l) of terbutryn divided by the IC_{50} of the compound.

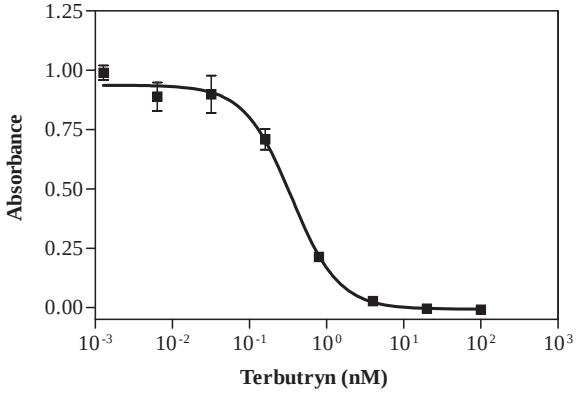


Fig. 1. Calibration curve of the immunoassay As87/**4a**-HRP for terbutryn. The data presented correspond to the average and the standard deviations of five assays run on five different days. The curves were run using well duplicates. See Table 5 for the features of the immunoassay.

Table 6
Features of the immunoassay As87/**4a**-HRP for terbutryn.[a]

A_{min}	−0.02 ± 0.03
A_{max}	1.01 ± 0.04
Slope	−1.05 ± 0.17
Ic_{50} (ng/l)	62.50 ± 4.02
LOD (ng/l)	5.02 ± 0.17
r^2	0.94 ± 0.01

[a] The parameters are extracted from the four-parameter equation used to fit the standard curve. The data presented correspond to the average of five calibration curves run on five different days. Each curve was built using two-well replicates.

substituents constitute the stronger antigenic determinants. The specificity of the assay was also assayed with other non-related chemical pesticides. Table 7 shows that none of these compounds interfered in the immunoassay.

Matrix effect studies. Terbutryn has been found in aquatic media of different salinity such as sea [3], river [5], sludge [40] and waste waters [7]. This fact prompted us to evaluate the performance of the immunoassay As87/**4a**-HRP in waters of high salinity values. For this purpose, standard curves done in PBS buffer and in water of the highest encountered salinity value (35U) were compared. Fig. 2 shows the parallelism of both standards curves. No significant difference existed between the slope and the IC_{50} values of both calibration curves. Only a small decrease in the maximal absorbance was observed at low concentration values of terbutryn. Therefore, it was concluded that salinity does not significantly affect the immunoassay performance.

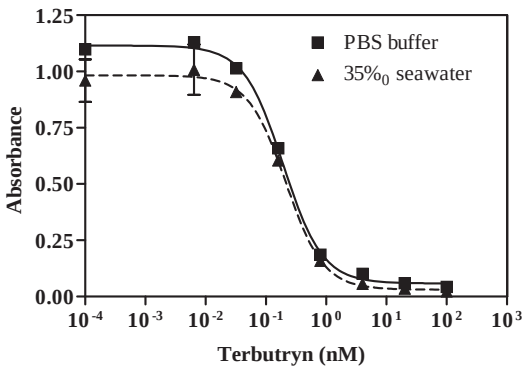
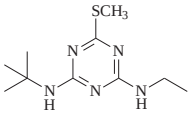
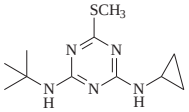
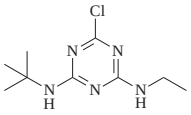
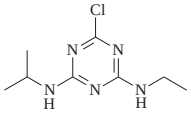
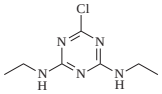
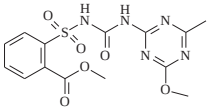
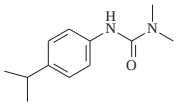
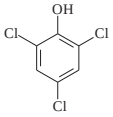
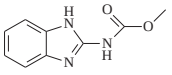
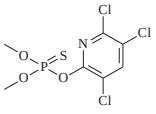


Fig. 2. Calibration graphs showing the effect of 35‰ sea salt water in the ELISA As87/**4a**-HRP.

Table 7
Interference caused by structurally related and non-related chemical herbicides, insecticides and fungicides on the As87/**4a**-HRP immunoassay.[a]

Compound	Structure	IC_{50} (ng/l)	% CR
Terbutryn		62.7	100
Irgarol		73.5	85
Terbuthylazine		85.0	74
Atrazine		3257	2
Simazine		>5000	<0.1
Metsulfuron methyl		>5000	<0.1
Isoproturon		>5000	<0.1
TCP[b]		>5000	<0.1
Carbendazim		>5000	<0.1
Chlorpyrifos		>5000	<0.1

[a] Cross-reactivity is expressed as a percent of the IC_{50} value of terbutryn divided by the IC_{50} of the compound.
[b] TCP, 2,4,6-trichlorophenol.

4. Conclusion

In the present work, we have analyzed how the homologous and the heterologous immunization strategies affect the features of an immunoassay for terbutryn. Comparison between different heterologous immunization protocols showed that inoculation with an equimolar mixture of immunogens rendered a higher number of competitive assays than an alternate inoculation. Nevertheless, this last strategy provided the most sensitive assays. No differences were observed between the homologous and heterologous immunization regarding immunoassay sensitivity and selectivity. ELISAs for terbutryn with LODs below the minimal risk levels permitted in drinking water by the European Union were obtained using both strategies. Additionally, the same pattern of recognition of other triazine pesticides was observed independently of the immunization protocol used. Summarising, further studies are necessary to provide enough information to achieve conclusions in relation to

the role that the immunization protocol plays in immunoassay features.

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