**Immunoassay and amperometric biosensor approaches for the detection of deltamethrin in seawater**

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# ABSTRACT

The study of an Enzyme-Linked Immunosorbent Assay (ELISA) and an amperometric biosensor for the detection of the pyrethroid deltamethrin in seawater is reported. The preparation of specific polyclonal antibodies is addressed using two immunizing haptens based on deltamethrin and cypermethrin compounds, with a spacer arm placed at the cyano residue in the pyrethroids structure. Different conjugates based on bovine serum albumin and aminodextran are prepared depending on the lipophilic profile of the competitors haptens studied. A reproducible and sensitive indirect competitive ELISA is developed, reaching a limit of detection of 1.2 ± 0.04 µg L-1 and an IC50 value of 21.4 ± 0.3 µg L-1 (both n = 3). For validation of the assays described, artificial seawater samples fortified with deltamethrin are analyzed. For the ELISA assay, these accuracy studies reported a slope of 0.904. An amperometric immunosensor is developed using the same immunoreagents and achieving a comparable detectability in terms of LOD of 4.7 µg L-1, measuring seawater without any pretreatment. These results suggested that both techniques can be used as rapid and simple analytical methods for deltamethrin quantification in seawater samples, being great candidates for initial environmental screening programs.

# KEYWORDS

Immunoassay, amperometric biosensor, seawater, deltamethrin

# INTRODUCTION

Nowadays α-cyano pyrethroids are being extensively used as insecticides in domestic, agriculture, horticulture, public health, and veterinary applications, mainly due to its low mammalian toxicity and its activity against a broad range of pests [[1-3](#_ENREF_1)]. Cyphenothrin, λ-cyhalothrin, cypermethrin and deltamethrin (Figure 1) among other synthetic pyrethroids have reported improved physicochemical properties and biological actions compared to their natural analogues. Amongst them, deltamethrin have been presented as one of the more lipophilic and stable insecticide-permethrins, and in consequence its residues have been detected in agricultural products [[4](#_ENREF_4)], food [[5](#_ENREF_5),[6](#_ENREF_6)], and surface waters [[7](#_ENREF_7),[8](#_ENREF_8)]. This pyrethroid has been classified as highly toxic to aquatic life [[9](#_ENREF_9)], and although α-cyano pyrethroids are thought to be safe for human health, some negative related-effects have been reported , encouraging the need of creation of control platforms. During the last decades, a wide range of global and regional environmental monitoring programs have been developed [[10](#_ENREF_10),[11](#_ENREF_11)], aiming for early warning systems that can provide extreme sensitivity and selectivity information about contaminants. Together with the REACH (Registration, Evaluation, Authorization and Restriction of Chemicals) law (EC 1907/2006) and the Drinking Water Directive (DVD, the Council Directive 98/83/EC) new initiatives for the regulation of the pollutants presence in aquatic environments have been developing during the last years.

Current analytical methods for deltamethrin detection are usually based on chromatographic techniques usually coupled to universal detectors such as UV or mass spectrometry [[12-21](#_ENREF_12)], achieving good levels of sensitivity and accuracy but at the expense of being time-consuming, expensive, not suitable for the analysis of large numbers of samples and incapable of being easily field-deployable.

Immunoassays have become great candidates to complement chromatographic techniques in environmental studies because of its attractive features, including rapid detection, low cost, the possibility of analysis of complex matrices without extensive pretreatment, variety of platforms and high-throughput capabilities. Detection of pyrethroids by immunochemical procedures have been widely explored [[22](#_ENREF_22)]. Antibodies addressing specific detection of deltamethrin or cypermethrin [[23](#_ENREF_23),[24](#_ENREF_24),[3](#_ENREF_3),[25](#_ENREF_25)] or the pyrethroid family [[7](#_ENREF_7),[26](#_ENREF_26)] have been produced and used to develop a broad variety of immunochemical techniques [[27](#_ENREF_27),[28](#_ENREF_28)]. However, few examples of immunosensors for the detection of deltamethrin have been reported. A Surface Plasmon Resonance (SPR) based on antibody-antigen recognition with enhancement with nanoparticles has been described for the detection of deltamethrin, achieving a LOD of 0.01 µg L-1[[29](#_ENREF_29)], however the need of operate in flow conditions and using magnetic nanoparticles to enhance the final signal can be a limitation in terms of cost and usability in field assays. On the other hand, an electrochemical impedance spectroscopy (EIS) assay has also been described for the detection of deltamethrin, and although it showed a great LOD of 0.303 µg L-1, the authorsstated that their technique exhibited large dispersion in most of the calibration points and real samples, decreasing the accuracy and precision of the insecticide measuring [[30](#_ENREF_30)].

Considering all mentioned points, we therefore set out to develop an immunosensor with electrochemical readout for the detection of deltamethrin in different water environments. Electrochemical biosensors are based on antigen-antibody assays, which eventually produce a change in an electrical parameter that can be measured by the transducer. They are considered attractive options due to its advantages in rapidness, reliability, low costs and efficiency for monitoring (organic) contaminants in aquatic environments [[26](#_ENREF_26),[31](#_ENREF_31)]. In comparison to optical sensors, which an example has been described for deltamethrin detection, the electrochemical variants usually are considered to be cheaper alternatives in addition to being more suitable for "on site" measures [[22](#_ENREF_22),[31](#_ENREF_31)]. In addition to this and to the best of our knowledge, there is no immunosensor with *amperometric* readout described for deltamethrin in the literature, which makes such a sensor highly desirable.

# MATERIALS AND METHODS

## *Chemicals and Biochemicals*

The chemical reagents used were obtained from Aldrich Chemical Co. (Milwaukee, WI, USA) and from Sigma Chemical Co. (St. Louis, MO, USA). The preparation of the described bioconjugates and antibodies has been performed with the support of the U2 of the ICTS “NANBIOSIS”, more specifically by the Custom Antibody Service (CAbS, CIBER-BBN, IQAC-CSIC).The synthesis of the immunizing haptens D133 and C134 and competitors used in this paper are described in the Supporting Information. The immunogens and bioconjugates prepared are also described in the same section. Deltamethrin standard was purchased from Sigma Chemical Co. (St. Louis, MO, USA). For the matrix studies, artificial seawater (aSW) was purchased from Sigma Chemical Co. (St. Louis, MO, USA), prepared at 40 mg mL-1 in MilliQ water; its pH and conductivity were monitored being 8.14 ± 0.27 and 49.69 ± 1.51 respectively (n=20).

## *Instruments and general methods*

The pH and the conductivity of all buffers and solutions were measured with a pH-meter pH 540 GLP and a conductimeter LF 340, respectively (WTW, Weilheim, Germany). Polystyrene microtiter plates were purchased from Nunc (Maxisorp, Roskilde, Denmark). Dilution plates were purchased from Nirco (Barberà del Vallés, Spain). Washing steps were performed on a Biotek ELx465 (Biotek Inc.). Absorbances were read on a SpectramaxPlus (Molecular Devices, Sunnyvale, CA, USA). The competitive curves were analyzed with a four-parameter logistic equation using the software SoftmaxPro v4.7 (Molecular Devices) and GraphPad Prism 5.03 (GraphPad Software Inc., San Diego, CA, USA).

All amperometric measurements were performed with an Autolab PGSTAT302N equipped with an ECD module (Utrecht, NL) and recorded using Nova software. Screen printed electrodes (SPE, AT220) consisting of a 4-mm smooth Au working electrode, Au counter electrode and Ag pseudo-reference electrode were bought from a commercial source (Dropsens, Spain) and functionalized prior to their use. For the flow cell setup, the corresponding flow-cell (DRP-FLWCL, DropSens, Spain) were used. Initial cleaning of the electrodes was performed via a UV/Ozone Procleaner™ unit (Bioforce nanoscience, Ames, IA, USA) and further functionalization was done using different functionalized PEG chains from Polypure (Oslo, Norway). Within the setup a peristaltic pump from Ismatec (ISM597D) (Wertheim, Germany) and Tygon tubes (inner diameter = 0.75 mm) were used. The connection between the peristaltic pump and the flow cell was made with PTFE tubes with an inner diameter of 0.5 mm. The calibration curves were fitted to a four-parameter logistic equation using using OriginPro software (Northampton, MA, USA).

## *Buffers.*

Unless otherwise indicated, phosphate buffer saline (PBS) is 0.01 M phosphate buffer in a 0.8% saline solution, pH 7.5. Coating buffer is a 0.05 M carbonate-bicarbonate buffer, pH 9.6. PBST consists of PBS with 0.05% Tween 20 with a pH of 7.5. PBST optimized was PBS with 0.005% Tween 20, 1.6% saline solution with a pH of 7.5. PBT optimized was PB (no saline solution) with 0.01% Tween 20 with a pH of 7.5. Citrate buffer was 0.04 M sodium citrate, pH 5.5. The substrate solution contains 0.01% 3,3’,5,5’-tetramethylbenzidine (TMB) and 0.004% H2O2 in citrate buffer. Borate buffer is 0.2 M boric acid/sodium borate, pH 8.7.

## Polyclonal antisera.

The antisera (As) obtained by immunizing female white New Zealand rabbits with C134-HCH (cypermethrin immunogen; As358, As359 and As360) and with D133-HCH (deltamethrin immunogen; As361, As362 and As363) following the protocol usually used in our group [[32](#_ENREF_32)]. After 6 boosting injections, the animals were exsanguinated, and the blood was collected in vacutainer tubes provided with a serum separation gel. Antisera were obtained by centrifugation at 4ºC for 10 min. at 10000 rpm and stored at -80 °C in the presence of 0.02% NaN3. Unless otherwise indicated, working aliquots were stored at 4 °C. Evolution of the antibody titer was assessed on a non-competitive indirect ELISA, by measuring the binding of serial dilutions of the different antisera to microtiter plates coated with a fixed concentration of C134-AD and D133-AD (1 µg mL-1) for their respective antisera.

## ELISA C134-AD/As360 protocol

Microtiter plates were coated with the antigen C134-AD (0.45 μg mL−1 in coating buffer, 100μL/well), overnight at 4 °C and covered with adhesive plate sealers. The next day, the plates were washed four times with PBST (300 μL well-1), and the deltamethrin standards (from 10000 to 0 µM in DMSO, and diluted 200 times in PBST or aSW just prior de assay) or the samples were added (50 µL well-1), followed by the solution of antisera As360 (1/8000 both in PBST, and aSW, 50 µL well-1). After 30 min at RT, the plates were washed as mentioned before, and a solution of anti-IgG-HRP (1/6000 in PBST) was added to the wells (100 μL/well) and incubated for 30 minutes at RT. The plates were washed again, and the substrate solution was added (100 μL/well). Color development was stopped after 30 min at RT with 4 N H2SO4 (50 μL/well), and the absorbance was read at 450 nm. The standard curves were fitted to a four-parameter 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 inflection point of the sigmoid curve. Unless otherwise indicated, data presented correspond to the average of at least two wells replicates.

*Specificity studies.*

Solutions of different structurally-related compounds such as cypermethrin, cypermethrin fractions (F1 and F2), λ-cyhalothrin, esfenvalerate, permethrin, 3-phenoxybenzaldehyde, (see *Figure S4*). The standard curves were performed following the protocol described before. The cross-reactivity (CR) values were calculated according to the equation: IC50 [nM] (Deltamethrin)/ IC50 [nM] (cross-reactant) × 100.

## Biofunctionalized SPEs

The gold SPEs were rinsed with water, ethanol and cleaned in a UV/Ozone chamber for 15 minutes prior to their functionalization. A mixed self-assembling monolayer (m-SAM) was prepared by immersing the electrodes on an ethanolic solution consisting on a mixture of 2.5 mM O-(2-carboxyethyl)-O′-(2-mercaptoethyl)-heptaethylene glycol (acid-PEG-thiol) and 7.5 mM O-(methyl)-O′-(2-mercaptoethyl)-hexaethylene glycol (m-PEG-thiol) for 2 h at R.T. Afterwards, the electrodes were washed with ethanol, dried and biofunctionalized placing on the surface of the electrode a solution of 25 L of the competitor C134-AD (200 g mL-1) and 25 L of a mixture of EDC/NHS (200 mM ) in PBS. After 3 h at RT, the electrodes were rinsed with PBS and the remaining reactive carboxylic acids were capped by immersing the electrodes in a 1 M ethanolamine solution in PBS for 1 h at RT. After this treatment, the functionalized electrodes were washed with water, dried and stored in a desiccator until use.

## Immunosensor protocol

The used immunosensor protocol was similar to a recently developed [[33](#_ENREF_33)] using a flow rate of 100 µL min-1 during the whole procedure. The electrodes were placed in a flow cell and flushed for 10 minutes with PBST buffer. Deltamethrin standards (prepared in aSW or PBS) or samples (600 L) were mixed with a solution of As360 (1/500 in PBST, 600 L) and the mixture (1mL) flushed over the sensor and subsequently washed with PBST buffer (5 min). Afterwards, a solution of antiIgG-HRP, 1/1000 in PBST, 1 mL) was flushed through the cell, followed by another washing step with PBST (5 min). After this time, a solution of citrate buffer was passed (5 min) and the amperogram (C) was recorded (60 s) followed by the substrate solution (0.01% TMB and 0.004% H2O2 in citrate buffer, 3 min) and another amperogram was recorded (S). The amperograms were acquired applying a potential of -0.10 *vs.* the Ag pseudo reference electrode and recording the signal for a period of 60 seconds. The chip was regenerated by passing a solution of citrate buffer (5 min), 0.3 M NaOH solution (10 min) and PBST (5 min) buffer, and used for the next measurement (see *Table 1* for a graphical summary of the protocol)

# RESULTS AND DISCUSSION

## *Immunoreagents preparation and characterization*

The immunizing haptens D133 (for Deltamethrin) and C134 (for Cypermethrin) were selected and synthesized according to the Skerrit *et.al [*[*34*](#_ENREF_34)*]* work with slight modifications (see Figure S1). HCH conjugates were prepared for raising antibodies As358-360 (C134) and As361-363 (D133) while BSA and AD bioconjugates were prepared for their use as competitors. The highly hydrophilic polysaccharide AD was proposed instead of BSA in some cases, to minimize the potential risk of the haptens folding. Deltamethrin and cypermethrin are highly lipophilic (see *Table S2*) and have a tendency to be buried into the hydrophobic core of proteins (data not shown), therefore the hydrophilic character of AD could prevent the hapten folding, being an alternative option to the ones based on the use of short or rigid spacer arms [[35](#_ENREF_35),[3](#_ENREF_3)]. Furthermore, the amino groups allow covalent coupling to the carboxylic groups on the haptens. Confirming our hypothesis, the antibodies evaluated during the first titer screening did not show any signal when exposed to C134 and D133 coupled to BSA, but higher responses at higher antisera dilutions were obtained when the non-competitive assay were performed against aminodextran conjugates (data not showed).

The avidity of antisera raised towards homologous and heterologous bioconjugate competitors was tested aiming to obtain the best analytical performance of the competitive immunochemical assay, since it has been reported that the nature of the coating antigen can modulate such parameters of an assay [[36](#_ENREF_36)]. Conjugates based on cypermethrin and deltamethrin moieties (F1 and F2 haptens), for exploring the importance of the halogen atoms on the pyrethroids structure over the antibody recognition profile, and F3 and 3-phenozybenzaldehyde (3-PBA) for the double-ring structure with the phenoxy benzene fragment) were proposed as heterologous competitors. Moreover, bioconjugates with different hapten densities were also prepared (see *Table S1*), because lower densities have been often related to better sensitivities [[37](#_ENREF_37)]. The appropriate dilutions of the antisera (As358-363) and the coated antigens (C134-AD, D133-AD, F1-BSA, F2-BSA, F3-BSA and 3-PBA-BSA) were established after two-dimensional checkerboard titration assays performed as described in [[38](#_ENREF_38)] . It was found some good combinations that allowed the detection of deltamethrin (see *Table S3*), however, despite of the precedents, the best As/bioconjugate combination consisted on As360 (C134-HCH) with the homologous competitor C134-AD. The ELISA developed using these immunoreagents detected deltamethrin directly in seawater with an IC50 value of 21.4 ± 0.3 µg L-1 and a limit of detection of 1.21 ± 0.04 µg L-1. Under these conditions cypermethrin was detected in this assay with an IC50 of 78.6 ± 0.7 µg L-1 and a LOD of 4.56 ± 0.05 µg L-1. The fact that the antiserum raised against a chlorinated compound (cypermethrin, X=Cl, see *Figure S1*) recognize better the brominated derivative (deltamethrin, X=Br) has been previously reported [[37](#_ENREF_37),[39](#_ENREF_39)]. Between the hypotheses to explain this fact there is the size of the bromine atoms, bigger than the chlorine atoms, and therefore, fitting better into the antibody binding cavities, favoring the antibody recognition of the deltamethrin. Another explanation relies in the differences of the electronic distribution between chlorinated and brominated analogues, which could create differences in dipole-dipole interactions in the antigen-antibody complex.

The effects of different physicochemical parameters related to the final matrix and the buffer selected for the development of the assays were tested introducing variations in the initial conditions of the assay. The concentration of Tween 20 in the assay buffer, the pH, the ionic strength, as well as the incubation time of the competitive step or the option of add a preincubation step were investigated in order to try to improve the features of the final ELISA (see Figure S3). From all the parameters studied, only slightly decreasing the concentration of Tween20 and increasing the conductivity of the buffer produced a positive effect in the assay increasing the detectability. The calibration curve of the deltamethrin assay after optimization is shown in *Figure 2* and the final features of the assay are summarized in *Table 2*. The parameters shown correspond to the average of 3 assays performed on different days using three-well replicates and as it can be observed, the assay is very reproducible. In these experiments the intra-day and interday variability at the IC50 value was found to be below than a 5% in all the cases.

The specificity of the immunoreagents was assessed by testing structurally-related compounds such as cypermethrin, cypermethrin fractions (F1 and F2), λ-cyhalothrin, esfenvalerate, permethrin and 3-phenoxybenzaldehyde (see *Figure S4*). *Table 3* summarizes the IC50 and cross-reactivity results for each compound, showing that the antibody raised and selected for this study was specific for deltamethrin, although a slight recognition (CR< 25%) of pyrethroids sharing the 2,2-dimethyl-ciclopropane group or terminal halogen atoms was also observed. Additionally, other contaminants usually found in environmental samples such as antibiotics (sulfapyridine, chloramphenicol), pesticides (Irgarol 1051®), polybrominated flame retardants (BDE-47), hormones (17β-estradiol)or toxins (domoic acid), did not interfere with the assay (data not shown).

Potential non-specific interferences caused by the sample matrix were studied by preparing a standard curve directly in seawater, however, as it can be seen in *Figure S5*, the assay performed very well in seawater, observing only a mild signal loss in respect to the assay carried out in PBST, while the detectability was in the same order.

Accuracy studies performed using blind spiked samples of artificial seawater demonstrated that the results obtained match the spiked values (see *Figure 3*). The sample concentrations were calculated interpolating the results to deltamethrin standard curves prepared in aSW, showing in the linear regression analysis a very good correlation value (*R2=0.995)* with a slope of 0.904, close to the perfect correlation.

## *Amperometric flow detection*

With the immunoreagents completely characterized we proceeded to their implementation on an amperometric sensor platform. For this purpose, we used gold SPE since the objective of this work was to develop a cost-effective device. The biofunctionalization of the electrodes was accomplished through the formation of a stable mixed-self-assembled monolayer with a ratio of 1:3 between acid-PEG-thiol/m-PEG-thiol as described in [[40](#_ENREF_40)] and based on previous studies [[41](#_ENREF_41),[33](#_ENREF_33)]. The biofunctionalization of the SPE was monitored by measuring CV. The experiments were carried out in order to determinate the effect on the rate of the electron transfer in the surface of the coated SPE. The CVs obtained for the SPE in different stages in Fe(CN)63-/4- solution are shown in Figure 4. As it can be seen, a substantial effect on the electron transfer was observed after the formation of the pegylated SAM, according to the fully passivation of the surface. The immobilization of the coating antigen C134-AD on the functionalized surface has also affected the electron transfer, however the highest impact was observed in the SAM formation. According to these results, the biofunctionalization took place successfully and the electrodes were ready to test the immunochemical assay.

Initial experiments were addressed to assess if the biofunctionalized electrode was able to detect deltamethrin. Thus, a single concentration of the analyte was passed throw the flow cell to assess inhibition of the binding of the antibodies to the surface of the electrode. In absence of the analyte, an average absolute maximum signal of 256.0 ± 46.4 nA (N= 3) was obtained, while in the presence of deltamethrin (125000 nM) the signal was reduced to 94.7 ± 23.1 nA (N= 3). The reproducibility of the electrodes was deeply studied by the analysis of two different SPE coated with the C134-AD, being an average absolute maximum signal of 285.0 ± 33.5 nA (N=4) and 245.5 ± 62.33 nA (N=6). According to these results, it can be stated that the regeneration of the surface can take place using the same SPE, and it can be reproduced in different SPE. When the measurements were performed using seawater, the maximum signal was 201.3 ± 60.9 nA ((N= 7), slightly lower than in buffer, and in the presence of deltamethrin the amperometric signal was 103.7 ± 19.9 nA (N= 7), which is in the same range to the results of our PBST measurements. With these precedents we proceeded to build standard curves and measure them with the biofunctionalized electrodes. The results obtained are shown in *Figure 5* and the parameters of the assay in *Table 4*. The data shown corresponds to the measuring of the standard curve for at least three consecutive days. The amperometric signal within the recorded cycles showed to be very similar, and the standard deviation for the different normalized measurement points was also satisfying with 3 to 10 % for buffer and 5 to 15 % for artificial sea water. The IC50 obtained resulted 272 and 230 nM (116 and 137 µg L-1) and a LOD of 14.9 and 9.3 nM (7.5 and 4.7 µg L-1) in buffer and in sea water, respectively.

# CONCLUSIONS

An ELISA platform for the direct determination and quantification of a potential pollutant of the ocean, deltamethrin, has been developed and its suitability to analyze seawater samples has been evaluated, allowing a quick and cheap first step in the field of pyrethroids’ monitoring. The antiserum 360 raised against C134, demonstrated a great specificity for deltamethrin detection, with mild cross-reactivity to other members of the α-cyano pyrethroids, and the assay developed didn’t show any matrix-related effect when analyzing seawater spiked samples directly. Good recoveries from seawater spiked samples were obtained, demonstrating its suitability to analysis environmental samples. Finally, a proof-of-concept using an amperometric sensor was demonstrated, detecting deltamethrin with good detectabilities compared with the ELISA. Although the accuracy and the limit of detection of our device is lower than within chromatographic measurements, the developed sensor is cheaper and could be used for autonomous measurements within a prolonged time period. In addition to this, the simplicity of the device itself and the possibility for remote control is an advantage, since chromatographic methods also requires skilled personnel. Another advantage of this system is, that the sensor could be directly placed in the sea water, enabling it to detect rapidly occurring high contamination levels at any time. For this reason, the electrode could be used as alarm system for water quality in harbors, fish farms and coastal areas.

# COMPLIANCE AND ETHICAL STANDARDS

# Rabbit immunizations included in this study were carried out in the animal facility of the Research and Development Center (CID) from the Spanish Research Council (CSIC) -Registration Number: B9900083. All efforts were made to minimize suffering of the animals. The protocol used for the production of antibodies was conducted in accordance with the institutional guidelines under a license from the local government (DAAM 7463) and were approved by the Institutional Animal Care and Use Committee at the CID-CSIC.

# ACKNOWLEDGMENT

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# TABLES

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| *Table 1.* Measurement protocol for the developed immunosensor. | |
| *[min]* | *Sensor’s Channel* |
| 5 | PBST |
| 10 | Sample & As360 |
| 5 | PBST |
| 10 | Anti-IgG-HRP |
| 5 | PBST |
| 5 | Citrate (measure) |
| 3 | Substrate (measure) |
| 5 | Citrate |
| 10 | Regeneration |
| The different buffers for the development of the deltamethrin assay in the sensor’s format have been listed, along with the flow times of each step. | |

|  |  |  |
| --- | --- | --- |
| *Table 3.* Cross-reactivity of related compounds in the As360/C134-AD | | |
|  | IC50 (nM) | % CR |
| Deltamethrin | 42 | 100 |
| Permethrin | 307.1 | 13 |
| Cypermethrin | 189.1 | 22 |
| Esfenvalerate | 218.1 | 19 |
| λ-Cyhalothrin | 185.8 | 23 |
| 3-Phenoxybenzoic acid | > 10000 | ˂0,05 |
| F1 | > 10000 | ˂0,05 |
| F2 | > 10000 | ˂0,05 |
| aCross-reactivity is expressed as a percentage of the relation between the IC50 (nM) of deltamethrin and the IC50 (nM) of the other compounds tested. Data obtained using ELISA | | |

# FIGURES

*Figure 1.*Chemical **s**tructures of some of the most used synthetic pyrethroids.

*Figure 2.* Calibration curve of the As360/C134-AD ELISA. The data shown correspond to the average of three assays performed on three different days. Each assay was built using three well replicates. LOD corresponds to limit of detection, calculated as the concentration given at 90% of the maximum signal. Includes the information of the different parameters of the assay in *Table 2.*

*Figure 3.* Correlation studies performed to assess accuracy of the ELISA in artificial seawater. The graph shows the linear regression analysis. The dotted line corresponds to a perfect correlation (m = 1). The data correspond to the average of at least three-well replicates from 3 different days.

*Figure 4*. Cyclic voltammograms of 10 mM Fe(CN)63-/4- in PBS 10 mM pH 7.5 in different stages. Scan rate 100 mV·s-1

*Figure 5.* Calibration curve obtained in the immunosensor for deltamethrin detection in PBST and in artificial seawater. The data correspond to the average of at least 3 different days.

|  |  |  |
| --- | --- | --- |
|  | | |
| Pyrethroid | R1 | R2 |
| Cyphenothrin | CH3 | CH3 |
| λ-Cyhalothrin | CF3 | Cl |
| Cypermethrin | Cl | Cl |
| Deltamethrin | Br | Br |

Fruhmann*et al. Figure 1*

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | |  |  |  |  | | --- | --- | --- | --- | | *Table 2.* ELISA As360/C134-AD | | | | | [As] = 1/8000, [Competitor]= 0.45 µg mL-1 | | | | | *Absorbancemin* | 0.162 ± 0.024 | *Tween 20* | 0.005% | | *Absorbancemax* | 0.944 ± 0.020 | *pH* | 7.5 | | *Slope* | -0.714 ± 0.022 | *Ionic strength* | 28.6 mS/cm | | *IC50, nM*  *(µg L-1)* | 42.4 ± 0.59 (21.4 ± 0.30) | *Competition time* | 30’ | | *LOD, nM*  *(µg L-1)* | 2.39 ± 0.85 (1.21 ± 0.04) | *Preincubation time* | No | | *R2* | 0.998 ± 0.001 |  |  | |

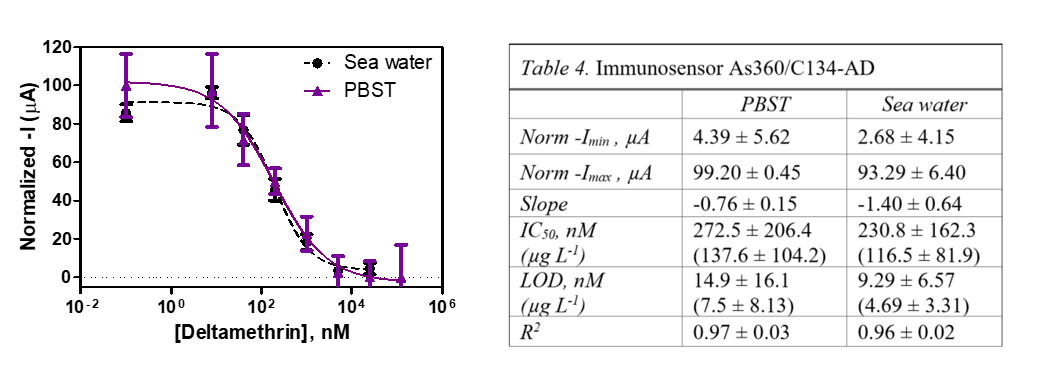
Fruhmann*et al. Figure 2*

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Fruhmann*et al. Figure 3*

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Fruhmann*et al. Figure 4.*



Fruhmann*et al. Figure 4*

# REFERENCES

1. Mak SK, Shan G, Lee H-J, Watanabe T, Stoutamire DW, Gee SJ, Hammock BD (2005) Development of a class selective immunoassay for the type II pyrethroid insecticides. Analytica chimica acta 534 (1):109-120. doi:10.1016/j.aca.2004.11.021

2. Shan G, Wengatz I, Stoutamire DW, Gee SJ, Hammock BD (1999) An enzyme-linked immunosorbent assay for the detection of esfenvalerate metabolites in human urine. Chem Res Toxicol 12:1033-1041

3. Lee H-J, Shan G, Watanabe T, Stoutamire DW, Gee SJ, Hammock BD (2002) Enzyme-Linked Immunosorbent Assay for the Pyrethroid Deltamethrin. J Agric Food Chem 50:5526-5532

4. Taheri N, Lan M, Wei P, Liu R, Gui W, Guo Y, Zhu G (2016) Chemiluminescent Enzyme Immunoassay for Rapid Detection of Three α-Cyano Pyrethroid Residues in Agricultural Products. Food Analytical Methods 9 (10):2896-2905. doi:10.1007/s12161-016-0482-x

5. Hernandes T, Dores EFGC, Ribeiro ML, Rossignoli PA, Malm O (2014) Simple Method to Determine Residual Cypermethrin and Deltamethrin in Bovine Milk. Journal of the Brazilian Chemical Society 25 (9):1656-1661. doi:10.5935/0103-5053.20140154

6. Palma DCA, Lourencetti C, Uecker ME, Mello PRB, Pignati WA, Dores EFGC (2014) Simultaneous Determination of Different Classes of Pesticides in Breast Milk by Solid-Phase Dispersion and GC/ECD. Journal of the Brazilian Chemical Society 25 (8):1419-1430. doi:10.5935/0103-5053.20140124

7. Hao XL, Kuang H, Li YL, Yuan Y, Peng CF, Chen W, Wang LB, Xu CL (2009) Development of an Enzyme-Linked Immunosorbent Assay for the alpha-Cyano Pyrethroids Multiresidue in Tai Lake Water. J Agric Food Chem 57:3033-3039

8. Moschet C, Vermeirssen EL, Seiz R, Pfefferli H, Hollender J (2014) Picogram per liter detections of pyrethroids and organophosphates in surface waters using passive sampling. Water research 66:411-422. doi:10.1016/j.watres.2014.08.032

9. Wolansky MJ, Harrill JA (2008) Neurobehavioral toxicology of pyrethroid insecticides in adult animals: a critical review. Neurotoxicology and teratology 30 (2):55-78. doi:10.1016/j.ntt.2007.10.005

10. Laranjeiro F, Sánchez-Marín P, Galante-Oliveira S, Barroso C (2015) Tributyltin pollution biomonitoring under the Water Framework Directive: Proposal of a multi-species tool to assess the ecological quality status of EU water bodies. Ecological Indicators 57:525-535. doi:10.1016/j.ecolind.2015.04.039

11. Shephard S, Greenstreet SPR, Piet GJ, Rindorf A, Dickey-Collas M (2015) Surveillance indicators and their use in implementation of the Marine Strategy Framework Directive. ICES Journal of Marine Science: Journal du Conseil 72 (8):2269-2277. doi:10.1093/icesjms/fsv131

12. Esteve-Turrillas FA, Pastor A, Guardia Mdl (2005) Determination of pyrethroid insecticide residues in vegetable oils by using combined solid-phases extraction and tandem mass spectrometry detection. Analytica chimica acta 553 (1-2):50-57. doi:10.1016/j.aca.2005.08.004

13. Ferrer C, Gómez MJ, García-Reyes JF, Ferrer I, Thurman EM, Fernández-Alba AR (2005) Determination of pesticide residues in olives and olive oil by matrix solid-phase dispersion followed by gas chromatography/mass spectrometry and liquid chromatography/tandem mass spectrometry. Journal of Chromatography A 1069 (2):183-194. doi:10.1016/j.chroma.2005.02.015

14. Pavan FA, Dallago RM, Zanella R, Martins AF (1999) Determination of deltamethrin in cattle dipping baths by high-performance liquid chromatography. J Agric Food Chem 47:174-176

15. Boonchiangma S, Ngeontae W, Srijaranai S (2012) Determination of six pyrethroid insecticides in fruit juice samples using dispersive liquid-liquid microextraction combined with high performance liquid chromatography. Talanta 88:209-215. doi:10.1016/j.talanta.2011.10.033

16. Hu L, Wang X, Qian H, Wang H, Lu R, Zhang S, Zhou W, Gao H (2016) In-syringe low-density ionic liquid dispersive liquid–liquid microextraction for the fast determination of pyrethroid insecticides in environmental water samples by HPLC-DAD. RSC Adv 6 (73):69218-69225. doi:10.1039/c6ra09668a

17. Mukdasai S, Thomas C, Srijaranai S (2014) Two-step microextraction combined with high performance liquid chromatographic analysis of pyrethroids in water and vegetable samples. Talanta 120:289-296. doi:10.1016/j.talanta.2013.12.005

18. López-López T, Gil-Garcia MD, Martínez-Vidal JL, Martínez-Galera M (2001) Determination of pyrethroids in vegetables by HPLC using continuous on-line post-elution photoirradiation with fluorescence detection. Analytica chimica acta 447:101-111

19. Vazquez PP, Mughari AR, Galera MM (2008) Solid-phase microextraction (SPME) for the determination of pyrethroids in cucumber and watermelon using liquid chromatography combined with post-column photochemically induced fluorimetry derivatization and fluorescence detection. Analytica chimica acta 607 (1):74-82. doi:10.1016/j.aca.2007.11.027

20. Feo ML, Ginebreda A, Eljarrat E, Barceló D (2010) Presence of pyrethroid pesticides in water and sediments of Ebro River Delta. Journal of Hydrology 393 (3-4):156-162. doi:10.1016/j.jhydrol.2010.08.012

21. Shirani M, Haddadi H, Rezaee M, Semnani A, Habibollahi S (2016) Solid-Phase Extraction Combined with Dispersive Liquid–Liquid Microextraction for the Simultaneous Determination of Deltamethrin and Permethrin in Honey by Gas Chromatography–Mass Spectrometry. Food Analytical Methods 9 (9):2613-2620. doi:10.1007/s12161-016-0455-0

22. Ahn KC, Kim HJ, McCoy MR, Gee SJ, Hammock BD (2011) Immunoassays and biosensors for monitoring environmental and human exposure to pyrethroid insecticides. J Agric Food Chem 59 (7):2792-2802. doi:10.1021/jf1033569

23. Kong Y, Zhang Q, Zhang W, Gee SJ, Li P (2010) Development of a monoclonal antibody-based enzyme immunoassay for the pyrethroid insecticide deltamethrin. J Agric Food Chem 58 (14):8189-8195. doi:10.1021/jf101483w

24. Lee H-J, Shan G, Ahn KC, Park E-K, Watanabe T, Gee SJ, Hammock BD (2004) Development of an Enzyme-Linked Immunosorbent Assay for the Pyrethroid Cypermethrin. Journal of Agricultural and Food Chemistry 52 (5):1039-1043. doi:10.1021/jf030519p

25. Queffelec A-L, Nodet P, Haelters J-P, Thouvenot D, Corbel B (1998) Hapten Synthesis for a Monoclonal Antibody Based ELISA for Deltamethrin. Journal of Agricultural and Food Chemistry 46 (4):1670-1676. doi:10.1021/jf9703922

26. Zhang Z, Zeng K, Liu J (2017) Immunochemical detection of emerging organic contaminants in environmental waters. TrAC Trends in Analytical Chemistry 87:49-57. doi:10.1016/j.trac.2016.12.002

27. Ge S, Zhang C, Yu F, Yan M, Yu J (2011) Layer-by-layer self-assembly CdTe quantum dots and molecularly imprinted polymers modified chemiluminescence sensor for deltamethrin detection. Sensors and Actuators B: Chemical 156 (1):222-227. doi:10.1016/j.snb.2011.04.024

28. Abegao LM, Ribeiro JH, Ribeiro PA, Raposo M (2013) Nano-molar deltamethrin sensor based on electrical impedance of PAH/PAZO layer-by-layer sensing films. Sensors (Basel) 13 (8):10167-10176. doi:10.3390/s130810167

29. Liu X, Li L, Liu YQ, Shi XB, Li WJ, Yang Y, Mao LG (2014) Ultrasensitive detection of deltamethrin by immune magnetic nanoparticles separation coupled with surface plasmon resonance sensor. Biosens Bioelectron 59:328-334. doi:10.1016/j.bios.2014.03.020

30. Castellarnau M, Ramon-Azcon J, Gonzalez-Quinteiro Y, Lopez JF, Grimalt JO, Marco MP, Nieuwenhuijsen M, Picado A (2017) Assessment of analytical methods to determine pyrethroids content of bednets. Trop Med Int Health 22 (1):41-51. doi:10.1111/tmi.12794

31. Jiang X, Li D, Xu X, Ying Y, Li Y, Ye Z, Wang J (2008) Immunosensors for detection of pesticide residues. Biosens Bioelectron 23 (11):1577-1587. doi:10.1016/j.bios.2008.01.035

32. Broto M, Matas S, Babington R, Marco MP, Galve R (2015) Immunochemical detection of penicillins by using biohybrid magnetic particles. Food Control 51:381-389. doi:10.1016/j.foodcont.2014.11.043

33. Salvador JP, Marco MP (2016) Amperometric Biosensor for Continuous Monitoring Irgarol 1051 in Sea Water. Electroanalysis 28 (8):1833-1838. doi:10.1002/elan.201600172

34. Lee N, McAdam DP, Skerrit JH (1998) Development of Immunoassays for Type II Synthetic Pyrethroids. 1. Hapten Design and Application to Heterologous and Homologous Assays. J Agric Food Chem 46:520-534

35. Sanborn JR, Gee SJ, Gilman SD, Sugawara Y, Jones AD, Rogers J, Szurdoki F, Stanker LH, Stoutamire DW, Hammock BD (1998) Hapten Synthesis and Antibody Development for Polychlorinated Dibenzo-p-dioxin Immunoassays. J Agric Food Chem 46:2407-2416

36. Oubiña A, Ballesteros B, Carrasco PB, Galve R, Gascón J, Iglesias F, Sanvicens N, Marco MP (2000) Immunoassays for environmental analysis. In: Barceló D (ed) Sample Handling and Trace Analysis of Pollutants: Techniques, Applications and Quality Assurance, vol 21. Elsevier Science B.V., Techniques and Instrumentation in Analytical Chemistry, pp 287-339

37. Galve R, Nichkova M, Camps F, Sanchez-Baeza F, Marco MP (2002) Development and Evaluation of an Immunoassay for Biological Monitoring Chlorophenols in Urine as Potential Indicators of Occupational Exposure. Anal Chem 74:468-478

38. Sanvicens N, Varela B, Ballesteros B, Marco MP (2012) Development of an immunoassay for terbutryn: study of the influence of the immunization protocol. Talanta 89:310-316. doi:10.1016/j.talanta.2011.12.033

39. Nichkova M, Gemani M, Marco MP (2008) <Immunochemical Analysis of 2,4,6-Tribromophenol for Assessment of Wood Contamination. J Agric Food Chem 56:29-34

40. Fernández F, Sánchez-Baeza F, Marco MP (2012) Nanogold probe enhanced Surface Plasmon Resonance immunosensor for improved detection of antibiotic residues. Biosensors and Bioelectronics 34 (1):151-158. doi:<https://doi.org/10.1016/j.bios.2012.01.036>

41. Fernandez F, Hegnerova K, Piliarik M, Sanchez-Baeza F, Homola J, Marco MP (2010) A label-free and portable multichannel surface plasmon resonance immunosensor for on site analysis of antibiotics in milk samples. Biosens Bioelectron 26 (4):1231-1238. doi:10.1016/j.bios.2010.06.012