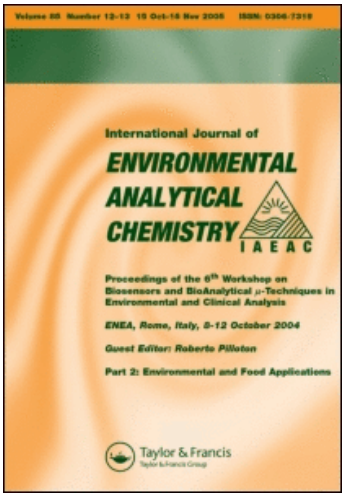


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# Synthesis of derivatives and production of antiserum for class specific detection of pyrethroids by indirect ELISA

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Two series of haptens including 3-phenoxybenzoic acid (PBA) and 3-(2-chloro-3,3,3-trifluoroprop-1-enyl)-2,2-dimethylcyclo-propanecarboxylic acid ($CF_3$MPA) were used to prepare immunogens through attachment of 4-C or 6-C handles. Class selective antibodies were produced by immunising rabbits. Ab502 showed the highest reactivity towards tau-fluvalinate ($IC_{50}$ 1.3 ng mL$^{-1}$), λ-cyhalothrin ($IC_{50}$ 2.3 ng mL$^{-1}$), cyfluthrin ($IC_{50}$ 2.2 ng mL$^{-1}$) and fenpropathrin ($IC_{50}$ 18.5 ng mL$^{-1}$) among the antibodies in a competitive ELISA. The effects of methanol, pH and salt concentration were optimised for maximum efficiency of the ELISA (Enzyme-Linked ImmunoSorbent Assay). Ab502 (1:80000)/2-OVA-1(0.2 μg mL$^{-1}$) was chosen for ELISA optimisation. Finally, 0.05 M phosphate buffered saline (PBS) at pH 6.5 containing 30% methanol (v/v) was used to dilute the standards. Target analytes in honey samples were extracted with ethyl acetate by sonication. The samples were spiked with three different concentrations of each compound (tau-fluvalinate, 0.5 ng g$^{-1}$, 3 ng g$^{-1}$, 12 ng g$^{-1}$; λ-cyhalothrin and cyfluthrin 1 ng g$^{-1}$, 5 ng g$^{-1}$, 65 ng g$^{-1}$). The recoveries were 36–59% at the lowest spiking concentration and 61–81% at the higher concentration. This assay might be useful to screen pyrethroid residues in honey or other matrix.

**Keywords:** pyrethroid; ELISA; class selective antibody; tau-fluvalinate

## 1. Introduction

Pyrethroids are synthetic analogues of pyrethrins, which are widely used in agricultural, veterinary and domestic applications because of their efficient insecticidal properties and relatively low mammalian toxicity. The structures of some typical pyrethroids are shown in Figure 1. An increasing number of toxicological tests carried out on pyrethroids have shown that some pyrethroid pesticides can impair the immune system, endocrine balance and lymph node [1,2]. Non-target invertebrates such as honeybees and aquatic organisms are very sensitive to these compounds [3]. In recent years, as strains of pyrethroid-resistant insects have increased in China, application of combined insecticides has been increasing,

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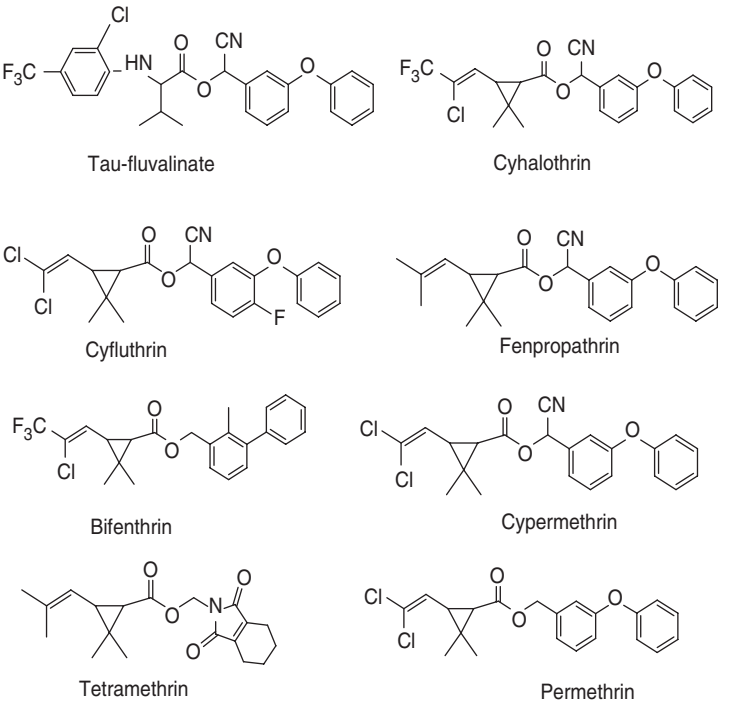



Figure 1. Chemical structure of some typical pyrethroids.



especially preparations of organophosphorous pesticides with pyrethroids. More than 916 kinds of pesticide mixture preparations have been marketed in China [4].

Pyrethroids are very potent lipophilic compounds with high octanol–water partition coefficients [5]. Pyrethroid contamination of foods and ecosystems may occur as a result of their abuse in agricultural practices and they are often detected as contaminants in surface water and residues in tea, vegetables, milk, meat or other fatty tissues [6–8], and this has caused increasing concern throughout the world. Fenpropathrin, tetramethrin, allethrin and flucythrinate have been banned by European Commission. Acrinathrin, tau-fluvalinate and tefluthrin have been withdrawn from EU market for lack of data on safety evaluation [9].

Immunoassays as rapid screening methods, offer many potential advantages in residue monitoring such as sensitivity, specificity, speed of analysis, cost and time effectiveness. Stanker *et al.* [10] established an immunoassay to detect permethrin in meat. Miyake *et al.* [11] used chrysanthemic acid (CAA) conjugated with ovalbumin (OVA) as antigens to prepare polyclonal and monoclonal antibodies specific to allethrin, bioallethrin, pyrethrins and tetramethrin. Queffelec *et al.* [12] used the acid moiety of the deltamethrin structure as a hapten to prepare a specific monoclonal antibody against deltamethrin. Pullen *et al.* [13,14] used 1R-*trans*-permethric acid (PMA)-bovine serum albumin (BSA) as the antigen to prepare an antibody specific to allethrin. Nakata *et al.* [15] established an ELISA (Enzyme-Linked ImmunoSorbent Assay) based on the monoclonal antibody against flucythrinate to detect the residue in environmental and grain samples. Gao *et al.* [16] developed an antibody against cyhalothrin to detect residue in water with a low limit of detection of $4.7\,\mathrm{ng\,L^{-1}}$.

Bruce D. Hammock and John H. Skerritt have undertaken a large number of studies relating to the development of various immunoassays for pyrethroid insecticides over a period of 20 years. Hammock's laboratory has developed antibodies specific to S-bioresmethrin, permethrin, esfenvalerate, deltamethrin, cypermethrin, fenpropathrin, permethrin metabolites and esfenvalerate metabolites [17–27] while Skerritt's laboratory has prepared specific antibodies for permethrin and bioresmethrin [28,29].

A more broad-specificity antibody is very useful for screening the presence of a class of compounds or their metabolites. Skerritt's laboratory developed a class selective antibody against deltamethrin, cypermethrin and λ-cyhalothrin [30]. Hammock's laboratory developed a class-specific immunoassay for the type I pyrethroid insecticides permethrin ($IC_{50}$ 30 μg $L^{-1}$), phenothrin ($IC_{50}$ 20 μg $L^{-1}$) [31] and a class selective immunoassay for the type II pyrethroid insecticides: cypermethrin ($IC_{50}$ 78 μg $L^{-1}$), cyfluthrin ($IC_{50}$ 205 μg $L^{-1}$), cyhalothrin ($IC_{50}$ 120 μg $L^{-1}$), deltamethrin ($IC_{50}$ 13 μg $L^{-1}$), esfenvalerate ($IC_{50}$ 6 μg $L^{-1}$), fenvalerate ($IC_{50}$ 8 μg $L^{-1}$) and fluvalinate ($IC_{50}$ 123 μg $L^{-1}$) [32]. Based on the antibodies above, various immunoassays have been developed for pyrethroid pesticide residue screening in different matrices. In addition, Kaware *et al.* [33] used an immunoaffinity column to purify bioallethrin from water.

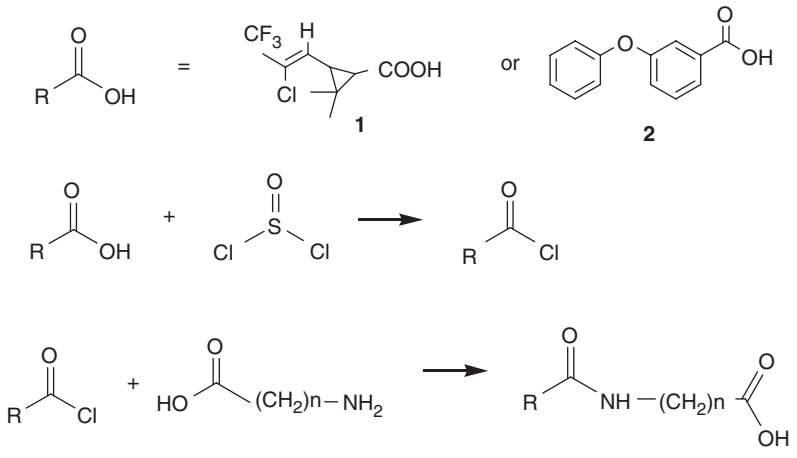
Tau-fluvalinate is a synthetic pyrethroid which is a highly effective acaricide and the active ingredient of commercial formulations such as Aspitan, Mavrik or Klartan [34]. China is one of the world's major honey exporters. Recently, there have been frequent reports of the presence of tau-fluvalinate or fenpropathrin residues in honey products from China. Many chromatographic methods have been developed to tackle this problem [35–41]. Different national regulations have established maximum residual levels (MRLs) of tau-fluvalinate in honey. In the United States and the Netherlands, the MRL is 0.05 mg $kg^{-1}$ while it was 0.01 mg $kg^{-1}$ in Germany and Italy [40]. It was our intention to produce a class-specific antibody for screening pyrethroid residues, especially tau-fluvalinate residues in honey products.

The most remote attachment site of the linker to the carrier protein should have the least effect on the geometry at the distinctive electrostatic binding sites of the analyte [42]. Modification of the haptens by altering handle length and position was used in this study. We developed haptens by attachment of 4-C or 6-C handles to the acid and alcohol moieties of the pyrethroid cyhalothrin. A class-specific antibody against tau-fluvalinate, λ-cyhalothrin, cyfluthrin and fenpropathrin was prepared by immunising rabbits. Honey samples spiked with tau-fluvalinate, λ-cyhalothrin and cyfluthrin were assayed using indirect competitive ELISA.

## 2. Experimental

### 2.1 *Chemicals and immunoreagents*

1-Cyclohexyl-3-(2-morpholinoethyl)-carbodiimide-metho-*p*-toluenesulphonate (morpho-CDI) and γ-aminobutyric acid was purchased from Sigma (St. Louis, MO, USA). ε-Aminocaproic acid and 3-(2-chloro-3, 3, 3-trifluoroprop-1-enyl)-2,2-dimethylcyclo-propanecarboxylic acid ($CF_3$MPA, structure **1** in Scheme 1) were gifts from Prof. Xu ChuanLai. Isobutyl chloroformate and 3,3′,5,5′-tetramethylbenzidine (TMB) were obtained from Fluka (Buchs, Switzerland). 3-Phenoxybenzoic acid (PBA, 99%, structure **2**) was obtained from Alfa Aesar China (Tianjin) Co., Ltd.




Scheme 1. Synthesis pathway of hapten 3–6. Compounds **1** and **2** were modified using 4-C and 6-C spacer arm through amidation. Final products were: hapten 3 ($R = CF_3$ MPA, $n = 3$), hapten 4 ($R = CF_3$ MPA, $n = 5$), hapten 5 ($R =$ PBA, $n = 3$), hapten 6 ($R =$ PBA, $n = 5$).



The following proteins: BSA, OVA, thyroglobulin (TYR), human serum albumin (HSA) and rabbit serum albumin (RSA) were purchased from Wanger Co., China. Goat anti-rabbit immunoglobulin conjugated to horseradish peroxidase was obtained from Hua Mei, Co., China. The pyrethroid standards were purchased from Dr Ehrenstorfer GmbH (Augsburg, Germany). All other chemicals and reagents were analytical grade or better purchased from the chemical reagent company Beijing Company Ltd.

### 2.2 *Chemical analyses*

Analysis of hapten structure was carried out using an LTQ linear ion trap mass spectrometer (Thermo, USA). ESI–MS was operated in negative ion mode and the collision energy was optimised for each compound.

### 2.3 *Hapten synthesis and verification*

The first series of haptens was obtained with a butyl or hexylic functional group attachment at the aromatic moiety of PBA [28], and the second series of haptens was prepared by reaction of $CF_3$MPA with $\gamma$-aminobutyric acid or $\varepsilon$-aminocaproic acid. The spacer arm was prepared using the method of Shan [27] and the process is shown in Scheme 1. All the reactions were straightforward and the yields were good. The mass spectra of compounds **3**–**6** confirmed that the modifications were successful. Compound **3**: [M−1] $m/z$ 326, main fragmental ions are [M-1-Cl] $m/z$ 289.9, [M-1-HF] $m/z$ 270, [M-1-$(CH_2)_3$COO] $m/z$ 183. Compound **4**: [M−1] $m/z$ 354.1, main fragmental ions are [M-1-Cl] $m/z$ 318.2, [M–HF] $m/z$ 334, [M-1-$(CH_2)_5$-COO] $m/z$ 204, [M-$(CH_2)_5$COO-HF] $m/z$ 184. Compound **5**: $[M-1]^-$ $m/z$ 298.1, main fragmental ions are [M–$H_2O$] $m/z$ 280.1, [M–COO] $m/z$ 254, [M–$(CH_2)_3$COO] $m/z$ 212. Compound **6**: [M−1] $m/z$ 326.1, main fragmental ions are [M–COOH] $m/z$ 280, [M–$H_2O$] $m/z$ 308.2, [M–$(CH_2)_5$COO] $m/z$ 212.

### 2.4 *Protein conjugation*

Conjugates were synthesised using the mixed anhydride and carbodiimide methods [19]. To obtain the immunogens, hapten **3**–**6** were conjugated to BSA using the mixed anhydride method and the carbodiimide method. Coating antigens were prepared by coupling compounds **1**–**6** to OVA and TYR using the mixed anhydride method.

#### 2.4.1 *Mixed anhydride method*

The hapten (0.05 mM) was dissolved in 2 mL dioxane and treated with tributylamine (0.15 mM). The solution was stirred in an ice–salt bath at 12–14°C. Following this procedure, isobutyl chloroformate (0.1 mM) was slowly added dropwise and the reaction mixture continued to be stirred for 45 min. A carrier protein, BSA (50 mg), TYR (100 mg) or OVA (100 mg) was dissolved in 3 mL cold aqueous solution of dioxane (80%, v/v, pH 7.4). Aliquots of the activated hapten solution were added slowly with stirring. The resulting solution was stirred in an ice bath for 5 h and the pH was maintained at a constant value during the procedure. The conjugates were then purified by dialysis in PBS (0.02 M, pH 7.2) for at least 72 h and the dialysis solution was changed three times per day. The purified conjugates were stored in aliquots at −20°C.



#### 2.4.2 *Carbodiimide method*

The procedure was similar to that previously described by Shan [19]: morpho-CDI (0.2 mM) was added to the solution of hapten (0.1 mM) in 1 mL DMF (N, N-dimethylformamide), a further volume of DMF was then added until the final volume of the reaction mixture was 3 mL and finally water was added dropwise to the point of oil-out. BSA (80 mg), TYR (150 mg) or OVA (150 mg) were dissolved in 20 mL cold water and adjusted to pH 6.5 using diluted HCl. DMF (1 mL) was then added to the protein solution, which was stirred in an ice bath. The hapten solution was added slowly to the protein solution. The mixture was stirred for 2 h at room temperature and purified as described above.

### 2.5 *Analysis of conjugations*

Conjugates were analysed for protein content using an ultraviolet spectrophotometer and the degree of hapten substitution was measured by calculation of the loss of free amino groups on the carrier protein using the method described by Plapp [43]. The substitution ratios are as follows: (1) **3**-BSA-1(Mixed anhydride) 9.4, **3**-BSA-2 (CDI) 12.7, **4**-BSA-1 8.9, **4**-BSA-2 6.7, **5**-BSA-1 12.1, **5**-BSA-2 11.3, **6**-BSA-1 15.4, **6**-BSA-2 13.8; (2) **1**-OVA-1 5.8, **2**-OVA-1 6.1, **3**-OVA-1 7.1, **4**-OVA-1 4.9, **5**-OVA-1 8.1,**6**-OVA-1 5.1; (3) **1**-TYR-1 4.2, **2**-TYR-1 5.6, **3**-TYR-1 4.8, **4**-TYR-1 5.2, **5**-TYR-1 3.9, **6**-TYR-1 4.1.

### 2.6 *Antibody production*

Two New Zealand white rabbits (1.5 kg–2.0 kg, female) were immunised with 1 mg $kg^{-1}$ of each immunogen using Freund's complete adjuvant for the first intradermal injection and Freund's incomplete adjuvant for the booster endermic injection. The antigen was dissolved in 0.9% normal saline then emulsified with adjuvant (1:1, v/v). Booster injections

were given at 20-day intervals. Blood was sampled 10 days after each boost. Rabbits were bled from the heart 10 days after final injection and the sera were isolated by centrifugation. The antibodies were purified by ammonium sulphate precipitation, dialysis against PBS (0.02 M, pH 7.2) at 4°C for 24 h and concentration to the original volume using PEG20000. Antibodies were stored in aliquots at −20°C. Animal experiments were carried out in accordance with the guidelines of the Animal Welfare Association of the China Agricultural University.

### 2.7 *ELISA*

A competitive indirect ELISA was developed. The microtitre plates were coated at 37°C for 2 h with 100 μL well$^{-1}$ of coating antigen in carbonate–bicarbonate buffer (pH 9.6, $Na_2CO_3$ 1.59 g, $NaHCO_3$ 2.93 g in 1 L $H_2O$). After one washing step using 200 μL PBST (0.05% Tween 20 in 0.02 M PBS pH 7.2), the plates were incubated with 150 μL well$^{-1}$ of 0.5% OVA in PBS for another 2 h at 37°C. The solutions in the wells were then discarded and the plates dried by tapping on bibulous paper. The standard analytes were prepared in concentrations ranging from 0.01 ng mL$^{-1}$ to 1000 ng mL$^{-1}$ in PBS buffer (pH 6.5, 0.05 M). A total of 50 μL well$^{-1}$ of standard solution was added and 50 μL well$^{-1}$ of antiserum diluted in PBS (0.02 M, pH 7.2) was then added and the plates were incubated at 37°C for 30 min. After four washing steps, 100 μL well$^{-1}$ goat-anti-rabbit-IgG labelled with horseradish peroxidase (1:5000 in PBST) was added and the plates incubated at 37°C for 30 min. The plates were washed and 50 μL well$^{-1}$ substrate solution A (1 mg mL$^{-1}$ TMB in 4 mM citrate–acetate buffer, pH 5.5) together with 50 μL well$^{-1}$ of B (0.1% $H_2O_2$ in 10 mM disodium hydrogen phosphate) were added. After incubation at 37°C for 15 min, the reaction was stopped with 2 M sulphuric acid. The absorbance was recorded at dual wavelengths of 450 nm and 650 nm. The optical density was calculated by subtracting the absorbance value at 650 nm from that at 450 nm.



### 2.8 *Assay optimisation*

To analyse the non-specific binding between antibody and carrier protein and to reduce the noise of the ELISA format, the microtitre plates were coated with a solution of protein, BSA, OVA, TYR, HSA or RSA in PBS (pH 7.2, 0.02 M, 1 μg mL$^{-1}$). The following procedure was similar to the process described above without the addition of the pyrethroid standards. Optimal dilutions for coating antigen and antisera were determined by a two-dimensional screening method. Based on previous reports [17–21], the methanol content in the PBS buffer (0, 15, 30, 60 or 90%, v/v) used for dilution of the pyrethroid standards was tested to investigate the effects of solvent on the assay. Effects of pH (5.3, 5.6, 5.9, 6.2, 6.5, 6.8, 7.0, 7.2, 7.4, 7.7, 8.0) and ion strength (0.01, 0.02, 0.05, 0.1, 0.2 M) of the PBS buffer were also tested. These solutions were used for dissolving the standards. The $IC_{50}$ for the various solutions was calculated.

### 2.9 *Antibody specificity*

Antibodies were coded and cross-reactivity (CR) studies were carried out using the haptens and pyrethroid standards under the optimised conditions. The CR was calculated as the ratio of the $IC_{50}$ of the target analyte and the $IC_{50}$ of the compounds tested.

### 2.10 *Analysis of recovery from spiked honey samples*

Blank honey samples which had never been exposed to pyrethroid pesticides were obtained from China Center for Veterinary Medicine Evaluation, and their residue-free status had been confirmed by GC/MS. Samples were treated using the method reported by Jin *et al*. [44].

Briefly, 1 g of honey was weighed and spiked to give the final concentrations, then diluted with 1 mL water. Samples were heated at 60°C in a water bath for 10 min. Then 1 mL methanol was added and vortexed for 1 min to reduce the viscosity. The honey sample was extracted with 10.0 mL ethyl acetate for 10 min in an ultrasonic water bath at 60°C and the upper phase was collected after centrifugation at 5000 rpm $min^{-1}$ for 5 min. The residue was again treated with 5.0 mL ethyl acetate. The combined extracts were concentrated to dryness under nitrogen and the residues were dissolved with 2 mL buffer solution (0.05 M PBS, pH 6.5, 30% methanol content, v/v).

Standard curves were constructed as a plot of the absorbance *versus* pyrethroid concentration for each compound. The recoveries were evaluated by extracting replicated spiked samples ($n = 8$). Individual honey samples were spiked with tau-fluvalinate ($0.5\,ng\,g^{-1}$, $3\,ng\,g^{-1}$, $12\,ng\,g^{-1}$), cyfluthrin ($1\,ng\,g^{-1}$, $5\,ng\,g^{-1}$, $65\,ng\,g^{-1}$) or λ-cyhalothrin ($1\,ng\,g^{-1}$, $5\,ng\,g^{-1}$, $65\,ng\,g^{-1}$). Inter- and intra-day coefficients of variation were calculated based on determinations on three consecutive days.



## 3. Results and discussion

### 3.1 *Hapten design and synthesis*

Hapten design is the most important factor in eliciting useful antibodies. Our objective was to maximise the recognition class of molecules with the lowest limit of detectability (LLD). Attachment of handles enables the potent epitopes to react fully to generate selective antibodies. With regard to the acidic moiety of some pyrethroids (chysanthemic acid and its halo analogues) and the corresponding alcohol moiety (3-phenoxybenzyl and α-cyano alcohol), arms of different length (4-C and 6-C) were used to retain the identity of each determinant group.

### 3.2 *Antisera screening*

Rabbits were bled 6 months after immunisation and used for subsequent screening. During the immunisation, two rabbits died. One died of serious sarcoptic mange (*Sarcoptes scabiei*) and the other died of foot canker. The titration tests were carried out using the final bleeds (Table 1). Antibodies (Ab301–303, Ab401–403) elicited by the series of $CF_3$MPA immunogens gave lower titers which indicated that the acidic moiety of pyrethroid pesticides only weakly stimulates the production of antibodies. The possible reason for this is that the long spacer arm can be folded and the potential determinants from the cyclopropyl moiety may have been embedded in the carrier protein and were therefore unable to act as effective epitopes. For a given antibody, the LLD is observed when the affinity of the antibody for the target molecule is greater than the affinity of the antibody for coating antigen [42]. In all formats, the homologous assay in which the same hapten was used in the coating antigen and immunogen showed higher titres than the heterologous assay and the results were consistent with previous reports [21].

Table 1. Reactivity of the antisera with different hapten-carrier conjugates.

| | | Coating antigen | | | | | |
|---|---|---|---|---|---|---|---|
| Immunogen | Antiserum | 1-OVA-1 | 1-TYR-1 | 3-OVA-1 | 3-TYR-1 | 4-OVA-1 | 4-TYR-1 |
| 3-BSA-1 | Ab301 | + | + | + | + | − | − |
| 3-BSA-2 | Ab302 | + | + | ++ | ++ | + | + |
| | Ab303 | − | − | − | − | − | − |
| 4-BSA-1 | Ab401 | ++ | ++ | + | + | +++ | +++ |
| | Ab402 | + | + | + | + | ++ | ++ |
| 4-BSA-2 | Ab403 | − | − | + | + | ++ | ++ |
| | | 2-OVA-1 | 2-TYR-1 | 5-OVA-1 | 5-TYR-1 | 6-OVA-1 | 6-TYR-1 |
| 5-BSA-1 | Ab501 | +++ | +++ | +++ | +++ | ++ | ++ |
| | Ab502 | ++++ | ++++ | ++++ | ++++ | ++ | ++ |
| 5-BSA-2 | Ab503 | +++ | +++ | +++ | +++ | +++ | ++ |
| | Ab504 | + | + | + | + | − | − |
| 6-BSA-1 | Ab601 | ++ | ++ | ++ | + | +++ | ++ |
| | Ab602 | + | + | + | + | ++ | + |
| 6-BSA-2 | Ab603 | ++++ | ++++ | ++++ | ++++ | ++++ | +++ |
| | Ab604 | ++++ | ++++ | ++++ | ++++ | ++++ | +++ |

Note: Symbols refer to the absorbance determined at an antibody dilution of 1:20000, concentration of coated antigen of $0.6\,\mu g\,mL^{-1}$ and 15 min reaction time from substrate addition: (−) absorbance < 0.5, (+) absorbance = 0.5–1.0, (++) absorbance = 1.0–1.5, (+++) absorbance = 1.5–2.0, (++++) absorbance > 2.00.



Coating antigen and antiserum systems that yielded absorbance values >1.0 were selected to further optimise the experimental conditions. Competitive inhibition experiments were performed to screen for the appropriate combinations. Ab401 (1:10000)/4-OVA-1 ($0.3\,\mu g\,mL^{-1}$), Ab501 (1:20000)/2-OVA-1 ($0.5\,\mu g\,mL^{-1}$), Ab502 (1:80000)/2-OVA-1 ($0.2\,\mu g\,mL^{-1}$), Ab503 (1:50000)/2-OVA-1 ($0.8\,\mu g\,mL^{-1}$), Ab603 (1:100000)/2-OVA-1 ($0.2\,\mu g\,mL^{-1}$) and Ab604 (1:50000)/6-OVA-1 ($0.5\,\mu g\,mL^{-1}$) were chosen for further experiments.

The assays described above were used to identify the system which was able to detect tau-fluvalinate with the greatest sensitivity since this is the major pyrethroid residue found in honey products [38]. Ab502 with the lowest $IC_{50}$ (Table 2) for tau-fluvalinate ($1.3\,ng\,mL^{-1}$), λ-cyhalothrin ($2.3\,ng\,mL^{-1}$), cyfluthrin ($2.2\,ng\,mL^{-1}$) and fenpropathrin ($18.5\,ng\,mL^{-1}$) was chosen for further optimisation.

### 3.3 *Optimisation*

Analysis of the non-specific binding between proteins and antibodies was carried out so that an appropriate carrier protein for coating the antigen could be identified in order to achieve a significant reduction in background noise. The optical density was evaluated based on Ab502 (1:20000) and the results showed the lowest level of binding occurred with OVA followed by TYR, HSA, BSA and RSA. It is known that B cell clones targeted to self proteins are almost completely eliminated or suppressed due to B cell clone abortion, clone depletion, the effects of suppressor T cells (Ts) and

Table 2. Average 50% inhibition concentration ($IC_{50}$) in ng mL$^{-1}$ listed for Ab401 Ab501, Ab502, Ab503, Ab603 and Ab604 ($n = 8$, eight replicates on four different experimental days).

| | $IC_{50}$ng mL$^{-1}$ | | | | | |
|---|---|---|---|---|---|---|
| Analyte/Antibody | Ab401 | Ab501 | Ab502 | Ab503 | Ab603 | Ab604 |
| Tau-fluvalinate | – | 21.7 ± 2.5 | 1.3 ± 0.54 | 34.8 ± 3.8 | 42.1 ± 4.1 | 32.3 ± 3.6 |
| Fenpropathrin | – | 67.3 ± 5.7 | 18.5 ± 2.3 | 193.2 ± 12.1 | >1000 | >1000 |
| λ-Cyhalothrin | >1000 | 31.6 ± 3.4 | 2.3 ± 0.47 | 45.7 ± 5.5 | 89.7 ± 9.8 | 62.8 ± 5.3 |
| Cyfluthrin | 83.8 ± 6.21 | 45.3 ± 4.4 | 2.2 ± 0.32 | 88.8 ± 9.7 | 153.1 ± 19.1 | 94.2 ± 10.5 |
| Bifenthrin | 120.2 ± 13.2 | – | – | – | – | – |
| Cypermethrin | >1000 | >1000 | >1000 | >1000 | >1000 | >1000 |
| Permethrin | 117.8 ± 10.6 | >1000 | >1000 | >1000 | >1000 | >1000 |
| Fenvalerate | >1000 | >1000 | >1000 | >1000 | 68.1 ± 7.5 | 129.2 ± 12.2 |
| Deltamethrin | >1000 | 212.5 ± 12.5 | 135.6 ± 16.1 | >1000 | >1000 | >1000 |
| PBA | – | 86.5 ± 9.5 | 31.7 ± 4.3 | >1000 | >1000 | >1000 |
| Hapten 5 | – | 9.1 ± 2.1 | 0.7 ± 0.16 | 14.2 ± 1.6 | 21.2 ± 2.3 | 16.1 ± 2.3 |
| Hapten 6 | – | 12.1 ± 1.8 | 1.6 ± 0.4 | 62.1 ± 8.1 | 15.9 ± 1.7 | 14.2 ± 1.5 |
| $CF_3$MPA | 70.3 ± 8.4 | – | – | – | – | – |
| Allethrin | >1000 | – | – | – | – | – |
| Resmethrin | >1000 | – | – | – | – | – |
| Tetramethrin | 36.9 ± 4.1 | – | – | – | – | – |
| Hapten 3 | 22.5 ± 3.2 | – | – | – | – | – |
| Hapten 4 | 15.4 ± 1.7 | – | – | – | – | – |

Note: symbols of '–'mean no determination.



blocking factors [45]. However, there are various components in sera and further non-specific binding between RSA and certain proteins in sera may boost the background noise. BSA was used as the carrier protein for immunisations and there were antibodies in the sera that bound BSA. Our results indicated that haptens conjugated with OVA or TYR were suitable for our experiment.

Ab502 (1:80000)/2-OVA-1 (0.2 μg mL$^{-1}$) was used to test for matrix effects. Pyrethroids are lipophilic and therefore a water-miscible organic co-solvent is necessary in standard solutions. As observed in previous studies [16–19], methanol is the common co-solvent used in ELISA. The methanol content significantly influenced the absorbance and sensitivity of the assay. As the methanol concentration was increased, the maximum absorbance and background tended to decrease (Figure 2). The lowest $IC_{50}$ of tau-fluavinate was found for Ab502 at 30% methanol in dilution.

The potential influence of ionic strength and pH on ELISA was tested in this study. In system Ab502/2-OVA-1, no significant effects on the $IC_{50}$ of tau-fluvalinate were observed in the pH range 6.2–7.4 (Figure 3). There are small changes in maximum absorbance (OD ranged from 1.9 to 2.4) at different salt concentrations (0.01–0.2 M). The background values tended to increase when ionic strength ranged from 0.01 to 0.05 M (Figure 4). In the optimised ELISA PBS (0.05 M, pH 6.5, 30% methanol content, v/v) was used to prepare the standard solution. Selected competitive ELISA screening against pyrethroid standards and haptens under optimised assay condition were used to determine the $IC_{50}$ (Table 2).

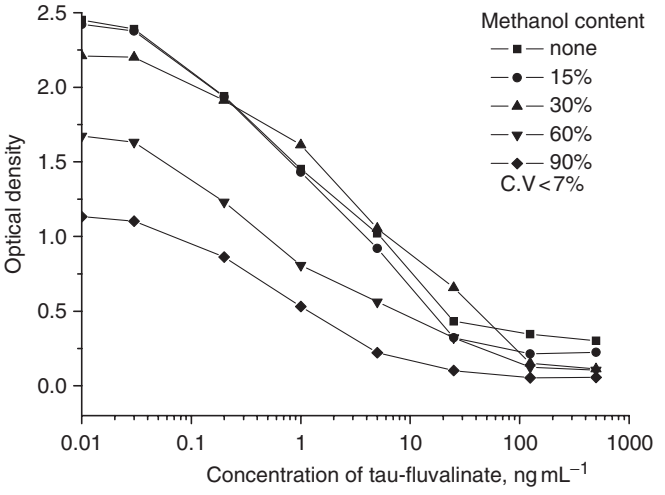



Figure 2. Effects of methanol content (0, 15%, 30%, 60%, 90%) in PBS dilution for standards on $IC_{50}$ of tau-fluvalinate. Each point on curve represents the average of six replicates on one plate and coefficient of variation on each point was <7%.



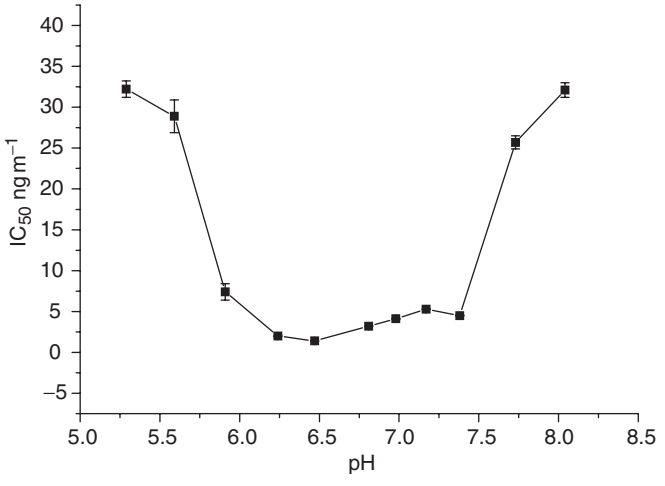



Figure 3. Effects of pH on $IC_{50}$ of tau-fluvalinate ($n = 6$, six replicates on one plate).

### 3.4 *Cross-reactivity*

All CRs were determined on the basis of the results in Table 2. There is a higher affinity between antibodies and their corresponding immunising haptens. All antibodies produced by the immunisation haptens of PBA demonstrated similar CR spectra (Table 3). These results provide information regarding the nature of the epitopes recognised by antibodies. Hapten PBA which does not contain an $\alpha$-cyano group could be easily synthesised, and would still contain those important determinants in an exposed position thus facilitating detection of pyrethroids containing this group. Nevertheless, Ab603 and Ab604 from

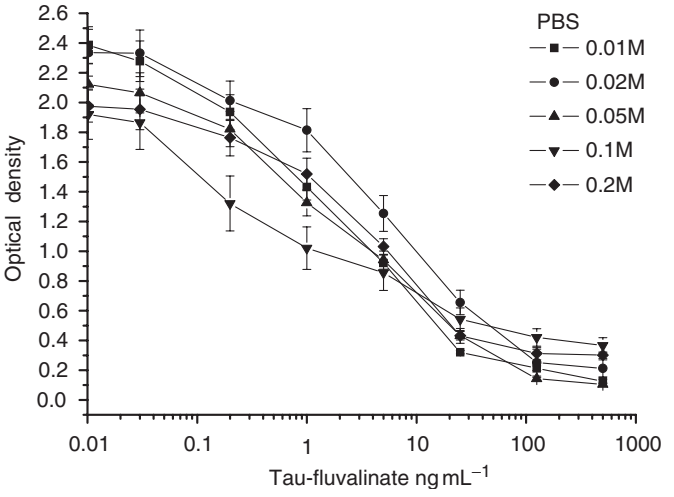



Figure 4. Effects of different ionic strength on tau-fluvalinate determination by competitive ELISA. The curve represents the average of six curves ± SD (six replicates were from six plates).



Table 3. Cross-reactivity of structurally related pyrethroids in direct ELISA[a].

| | CR | | | | | |
|---|---|---|---|---|---|---|
| Analyte | Ab401 | Ab501 | Ab502 | Ab503 | Ab603 | Ab604 |
| Tau-fluvalinate | –[b] | 100 | 100 | 100 | 100 | 100 |
| λ-cyhalothrin | – | 68.7 | 56.5 | 76.1 | 46.9 | 51.4 |
| Fenpropathrin | – | 34.5 | – | 18.0 | – | – |
| Tetramethrin | 100 | – | – | – | – | – |
| Permethrin, | 68.4 | – | – | – | – | – |
| Deltamethrin | – | 10 | – | – | – | |
| Fenvalerate | – | – | – | – | 61.3 | 25.0 |
| Cyfluthrin | 44.0 | 47.9 | 59.1 | 39.2 | 27.5 | 34.3 |
| Bifenthrin | 30.7 | – | – | – | – | – |

[a]All assay conditions were as described in Section 2. [b]<10% inhibition was measured at highest concentration (1000 ng mL$^{-1}$).

hapten 6 are able to recognise fenvalerate while Ab501–Ab503 from hapten 5 do not. Molecular recognition relies on molecular shape as defined by the geometry and on low-energy interactions such as hydrogen bonding, hydrophobic interactions, electrostatic and dipole–dipole forces together with $\pi$–$\pi$ complementary ring bonding [46]. An antibody recognises the steric shape rather than single atoms in a molecule. Different pyrethroids have up to eight isomers. We used mixed isomers for the standards and the ratios of isomers were unclear. Here, the spacer arm length could play some role in molecular recognition by changing the electrostatic distribution and further affecting the binding between antibody and pyrethroids.

In addition, it seemed unusual that Ab401 was able to recognise tetramethrin (Table 2) which contains a CAA (Figure 5) moiety, more readily than λ-cyhalothrin

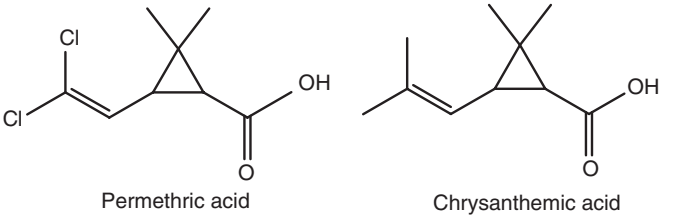



Figure 5. Chemical structure of PMA and CAA.

and bifenthrin (Figure 1) both of which contain a CF3MPA moiety but did not show any obvious affinity for resmethrin or allethrin both of which also contain the CAA moiety. In previous research, Pullen *et al.* [14] used PMA (Figure 5) as the hapten to produce a monoclonal antibody but found that the antibody cross-reacted with allethrin, bioallethrin and S-bioallethrin which all contain the CAA moiety. However, pyrethroids containing PMA as well as the free acid moiety PMA, were not recognised. PMA contains a dichlorovinyl group instead of dimethylvinyl group. Pullen deduced that the immunogen might have been modified in the animal body during the immune response. In our study, antibodies reacted more specifically with the modified structure rather than with $CF_3$MPA itself (Table 2). This is consistent with the evaluation that the $CF_3$ and Cl groups of $CF_3$MPA may have been degraded or modified in the rabbits. However, this does not explain the lack of binding to allethrin and resmethrin. There is some evidence that the $\alpha$-cyano group on the pyrethroid may have some effect on the binding of Ab401 because permethrin which contains a dichlorovinyl group binds to Ab401, but cypermethrin which differs only in the presence of the $\alpha$-cyano group does not. Similarly, bifenthrin that contains a $CF_3$/Cl substitution binds, but $\lambda$-cyhalothrin which contains the same group, and an $\alpha$-cyano group does not. The exception is cyfluthrin that contains a dichlorovinyl group and an $\alpha$-cyano group, yet still binds well. Molecular modelling of the structure of these pyrethroids may clarify the three-dimensional representations for antigen–antibody recognition.



### 3.5 *Recovery studies*

Three concentrations of each pesticide were used to spike the honey samples in order to give 20, 50 and 80% inhibition of antibody binding after dilution in 2 mL buffer solution. Initially, we diluted the honey samples with buffer solution to determine the concentration of analyte directly, however, absorbance values were low due to the matrix effect of the honey sample. Furthermore, direct excessive dilution potentially increases the false positive rate due to dilution errors. Some authors have used calibration curves constructed in the presence of a representative matrix to eliminate disturbance of the matrix [24–28]. Calibration curves should be prepared before detection and different samples should be represented by separate curves. This procedure can decrease the accuracy and precision of the method. Ultrasonic extraction was fast and effective in our experiment and considerably decreased the matrix effect. Standard curves (Figure 6) showed good linearity for tau-fluvalinate (0.06–8.5 ng $g^{-1}$), $\lambda$-cyhalothrin and cyfluthrin (0.2–11 ng $g^{-1}$). These lower recoveries (Table 4) confirm that the method used in this study is currently suitable for screening of pyrethroid

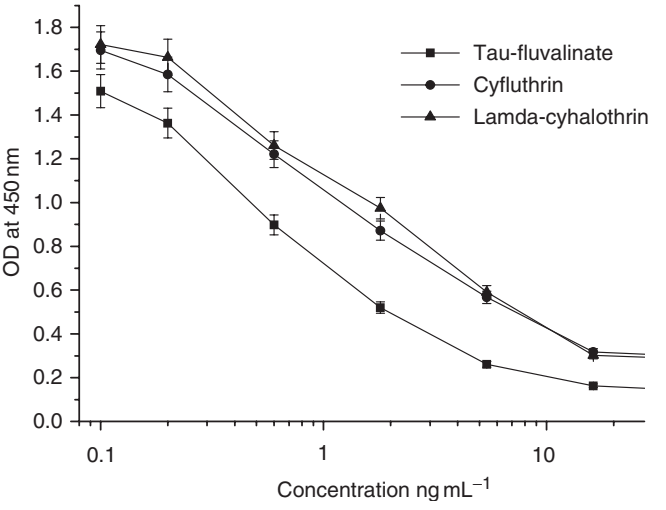



Figure 6. Standard curves for tau-fluvalinate, cyfluthrin and λ-cyhalothrin ($n = 6$, six replicates on one plate).



Table 4. Recoveries and coefficient variation of tau-Fluvalinate, λ-cyhalothrin and cyfluthrin in fortified honey samples by ELISA.

| Compound | Spiked level (ng) | Recoveries% | Coefficient of variation % | |
|---|---|---|---|---|
| | | | Inter-day ($n = 8$) | Intra-day ($n = 3$) |
| Tau-fluvalinate | 0.5 | 36–54 | 22.5 | 27.3 |
| | 3 | 49–65 | 18.2 | 22.4 |
| | 12 | 66–78 | 16.7 | 19.8 |
| λ-Cyhalothrin | 1 | 41–59 | 21.2 | 25.4 |
| | 5 | 52–62 | 18.4 | 18.7 |
| | 65 | 64–81 | 16.2 | 20.3 |
| Cyfluthrin | 1 | 39–52 | 19.2 | 19.8 |
| | 5 | 56–67 | 17.1 | 17.9 |
| | 65 | 61–79 | 16.5 | 15.2 |

residues in honey at maximum residue limits. Further development in the experimental method could result in a more quantitative assay.

## 4. Conclusion

Pyrethroids are not planar molecules and in most molecules of this type there are up to three chiral C-atoms which determine their steric configuration. Class-specific antibodies can be developed by leaving a common moiety exposed. In this study, we used a much simpler modification on the racemic CF3MPA and PBA molecules by inserting spacer arms (γ-aminobutyric acid and ε-aminocaproic acid) at the aromatic ring or cyclopropyl group.

One of the best antisera (Ab502) produced by an immunogen (5-BSA) was selected and it recognised tau-fluvalinate, λ-cyhalothrin, fenpropathrin and cyfluthrin very well with a low $IC_{50}$. Overall, the antibody produced by the hapten of $CF_3$MPA (hapten **3** and **4**) gave poor titres and it appeared that the hapten had undergone some structural changes within the animal's body. In addition, with smaller molecules the retention of the identity of each determinant becomes more important [42]. Introduction of the carbon chain on CF3MPA possibly resulted in some geometry or electronic changes of the intrinsic structure. Another possible explanation is that the antigenic determinants on CF3MPA were not fully exposed and could not therefore, elicit a strong immune response. These results again emphasised that the chemical structure and electronic distribution of the target analyte play an important role in antibody recognition since it is based on the establishment of non-covalent interactions [43].

An indirect competitive ELISA was developed based on Ab502 (1:80000)/2-OVA-1 ($0.2\,\mu g\,mL^{-1}$) and was optimised in terms of pH, solvent and salt. Fortification studies in honey showed that the assay was suitable for the detection of tau-fluvalinate, λ-cyhalothrin and cyfluthrin in honey samples at the Method Detection Limit (MDL). Further work targeted at decreasing the matrix effect should be carried out to detect pyrethroids residue in liquid matrix by simple dilutions and to improve recoveries.



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