

## **A Membrane-based ELISA Assay for the Herbicide Isoproturon in Soil Samples**

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## **Abstract**

We describe within this paper a membrane based enzyme linked immunosorbent assay (MELISA) for the detection of a common herbicide, isoproturon. A heterogeneous competitive ELISA was the format chosen for isoproturon detection. An immunoassay system with a horseradish peroxidase (HRP) labeled polyclonal antibody preparation was developed and characterised before suitable sensitivity and selectivity for isoproturon was attained. After development as a microtitre plate immunoassay the system was transferred to an affinity membrane sorbent based ELISA where the isoproturon/ovalbumin conjugate was immobilised on commercial membranes. Different porosities and immobilisation conditions were utilised to optimise the MELISA, including sensitivity, selectivity and stability studies. This enabled detection of isoproturon in the range  $0.5 \text{ ng ml}^{-1}$  -  $20 \text{ } \mu\text{g ml}^{-1}$ , with an LLD90 of  $0.5 \text{ ng ml}^{-1}$ . The use of acetonitrile extracts from soil samples was found to not overly impair the performance of the MELISA. Good correlation between ELISA and HPLC could be obtained for extracts from spiked soil samples.

**Keywords:** Immunoassay, Isoproturon, herbicides, Soil analysis

## Introduction

There is widespread public concern regarding the effects of pesticides upon ecosystems and the environment, along with hazards posed to human health through contamination of drinking water supplies and foodstuffs. Stringent legislation has been introduced to monitor and control the release of such contaminants. The European Union passed a Drinking Water Directive (No. 80/778/EC) (Gardiner and Mance 1984) stating that maximum concentrations in water sources of individual pesticides should not exceed  $0.1 \mu\text{g l}^{-1}$  and total pesticides and related compounds should not exceed  $0.5 \mu\text{g l}^{-1}$ . Further legislation such as EU Water Framework Directive 2000/60/EC (European Commission, 2000), the Drinking Water Directive 98/83/EC (European Commission, 1998) and EU Water Framework Directive 2008/105/EC (European Commission, 2008) were later brought into force to safeguard the environment, foodstuffs and drinking water supplies.

Due to previous widespread use and disposal of pesticides, there is a need for analysis of large batches of water and soil samples. ‘Off site’ analysis requires sample transportation to a laboratory for testing by gas chromatography, liquid chromatography, thin film chromatography and various spectroscopic techniques (Aprea *et al.*, 2002). This allows accurate quantification, high recovery rates and low detection limits but requires availability of dedicated and sophisticated equipment and personnel. In contrast, the more favourable and ideal method is ‘on site’ analysis (Tothill, 2001). With this approach the number of samples required for further analysis at the laboratory is reduced.

Isoproturon (IPU) is one of the phenylurea series of herbicidal compounds which include some of the most commercially important herbicides in the UK. IPU has been classified as very toxic to aquatic organisms and is on the priority list of substances included in EU Water Framework Directive 2008/105/EC (European Commission, 2008) which are of major concern for European Waters. A variety of methods have been utilised to determine of isoproturon. ELISA tests demonstrated detection of  $20\text{--}250 \mu\text{g l}^{-1}$  of IPU extracted from soil with negligible cross-reactivity using a monoclonal antibody (Liegeois *et al.*, 1992). An indirect enzyme immunoassay found that  $0.64 \mu\text{g l}^{-1}$  IPU inhibited 50% of antibody-antigen binding with minimal cross-reactivity with other pesticides (Rejeb *et al.*, 1998). An indirect ELISA assay (Kramer *et al.*, 2004a) using a polyclonal antibody

had a test-midpoint at an IPU concentration of  $1.06 \mu\text{g l}^{-1}$  and a detection limit of about  $0.1 \mu\text{g l}^{-1}$  but assays using a monoclonal antibody gave higher sensitivity, test midpoint of  $0.07 \mu\text{g l}^{-1}$  and detection limit of  $0.003 \mu\text{g l}^{-1}$ . Another monoclonal antibody gave a test-midpoint of  $5.5 \mu\text{g l}^{-1}$  and this and the previous antibody could be combined to give an extended working range (Kramer *et al.*, 2004b). ELISA tests for common herbicides have also been developed (Kramer *et al.*, 2007) and commercial ELISA's for IPU are available.

We utilised the previously published ELISA system (Mouvet *et al.*, 1997) to develop a membrane-based ELISA protocol for the determination of isoproturon, in aqueous solution and extracts from soil samples. We adapted this protocol to give an ELISA which demonstrated high correlation with HPLC and commercial ELISA test analyses. A conjugate of an isoproturon derivative with ovalbumin (OA) was covalently immobilised onto commercial membranes. Enzyme labeled polyclonal antibodies to the herbicide were then used along with the immobilised conjugate in both competitive and displacement assays. The resultant assay is relatively fast and inexpensive to perform and is capable of detecting  $\text{ng ml}^{-1}$  levels of the herbicide and could potentially be utilised within the fabrication of a screen-printed sensor.

## Experimental Methods

### *Materials and equipment*

General laboratory chemicals were from Sigma-Aldrich (Poole, UK) or BDH (Poole, UK) and were of the highest purity available. Water used within this work had been purified using Elga (High Wycombe, UK) reverse osmosis equipment. The 96-well microtitre plates were high binding capacity polystyrene purchased from Corning Costar (High Wycombe, UK). These were flat-bottomed wells with a  $300 \mu\text{l}$  volume. Pesticide samples were purchased from the Laboratory of the Government Chemist. Commercial EnviroGuard<sup>®</sup>. Isoproturon ELISA Kits were supplied by SDI Europe Ltd (Four Marks, Hampshire, UK).

### *Antibody production and conjugation with HRP*

The polyclonal antibody to isoproturon was a gift from Dr. Ramadan Abuknesha (Kings College London, UK) and was an affinity purified sheep IgG preparation produced

with a BSA carrier protein (Mouvet *et al* 1997). Conjugation to horseradish peroxidase (HRP) was achieved via a procedure, modified from previous work (Hermanson *et al* 1992, Hermanson, 1996). HRP, 20 mg in 1 ml phosphate buffer (0.1 M  $\text{Na}_3\text{PO}_4$ , 0.15 M NaCl, pH 7.2) and 100  $\mu\text{l}$  of 88 mM sodium periodate solution were combined and incubated in the dark at room temperature for 20 minutes. The oxidised enzyme was immediately purified using a Sephadex<sup>®</sup> G-25 gel filtration column. Antibody and enzyme solutions (10 mg  $\text{ml}^{-1}$ ) were mixed at room temperature for 2 hours at a 1:1 (v/v) ratio (an equal mass of antibody to enzyme in the final solution results in a 3.75 molar excess of HRP over IgG). This was followed by addition of 10  $\mu\text{l}$  of 5 M  $\text{NaCNBH}_3$  in 1 M NaOH per millilitre of reaction volume and this mixture was allowed to react for 30 minutes at room temperature. Unreacted aldehyde sites were blocked with the addition of 50  $\mu\text{l}$  of 1.0 M ethanolamine in carbonate buffer for 30 minutes at room temperature. The conjugates were then doubled in volume with 20 mM sodium phosphate pH 7.0 buffer. To concentrate IgG preparations, purified IgG was applied to a 100 kD molecular cut-off centrifugal concentrator. The sample was spun for 1½–3 hours at 3000g and resuspended in 3.5 ml 20 mM sodium phosphate pH 7.0 buffer with 0.01% thimerosal added to complete the final reagent, which was stored at 4°C.

#### *Synthesis of isoproturon derivatives*

A 3.07 g of N-methylamino acetic acid in 50 ml saturated  $\text{NaHCO}_3$  was cooled in ice and 0.322 ml of 4-isopropylphenyl isocyanate added dropwise over 30 minutes, then stirred overnight at ambient temperature. Precipitated by-product was removed, the filtrate acidified to pH1 and cooled to 4°C for two hours to precipitate the product (Figure 1,  $\text{R} = -(\text{CH}_2)_3\text{COOH}$ ) which was filtered, washed with acidified water and dried. Structure and purity of the compounds was verified by TLC, NMR and FAB-MS. It was conjugated to ovalbumin (OA) (Fraction V) at a 1:40 molar ratio of protein:derivative (Katmeh *et al.*, 1994) by combining 1 mmol of product (8 mg  $\text{ml}^{-1}$  solution in dry dioxane), 227 mg of N,N'-dicyclohexyl- carbodiimide and 127 mg of N-hydroxysuccinimide and reacting for 2 days at room temperature. A 50 ml of a 20 mg  $\text{ml}^{-1}$  OA solution in 3%  $\text{NaHCO}_3$  + 20% DMF was added, the mixture stirred for 2 hours at room temperature then dialysed first against 0.1%  $\text{NaHCO}_3$  followed by PBS. A

derivative with a shorter linker ( $R = -CH_2COOH$ ) was synthesised but showed a high background reactivity and we also synthesised a longer chain ( $R = -(CH_2)_5COOH$ ) derivative but this showed only a minimal binding response.

#### *Development of an ELISA test for isoproturon*

An ELISA test protocol was developed based on previous work (Mouvet *et al.*, 1997). This ELISA was optimised and shown to be capable of detecting isoproturon in buffer and in soil extracts. Comparison tests to the ELISA analyses were made by HPLC or utilisation of a commercial SDI Isoproturon ELISA Test Kit. HPLC analyses were carried out upon a Kontron Series 300 chromatograph using a Lichrosorb RP-18 column (25 cm x 4 mm ID, Merck) run with an acetonitrile:water:orthophosphoric acid (70:30:0.25 v/v) solvent system at a 1 ml min<sup>-1</sup> flow rate (Walker *et al.*, 2000). On-line detection was by UV absorbance at 240 nm and calibration standards for these analyses were prepared from a 10 µg ml<sup>-1</sup> (10 ppm) isoproturon standard in acetonitrile. Minimal cross-reactivity was observed for several herbicides of the same family with relative sensitivities being <1% for diuron, chlortoluron, monuron, fenuron, linuron and monolinuron.

#### *Membrane Preparation and Immobilisation*

Membrane discs (6 mm diameter) were cut from UltraBind™ US-450 and SV-450, 0.45 µm pore size and US-800 0.8 µm pore size covalent attachment membranes (Pall Gelman Sciences Ltd (Portsmouth, UK). Ovalbumin or ovalbumin-1.3 antigen conjugate was immobilised using 4 µl of 10 mg ml<sup>-1</sup> antigen in 10 mM PBS pH7.5 immobilisation buffer (which allowed complete saturation of the disc). This solution was added to each membrane disc and allowed to air dry at room temperature for up to 45 minutes. The discs were then placed into separate 1% OA blocked microtitre wells and 200 µl/well 1% OA blocker in 10 mM PBS pH 7.5 added and agitated for 1 hour at 37°C.

#### *Competitive Indirect MELISA*

After blocking, 50 µl/well of pesticide standard/sample concentrations followed by 50 µl/well anti-IPU-/HRP, both in 15 mM PBS pH 7.0 with 0.05% (v/v) Tween 20

(PBST) containing 0.1% of the blocking protein, were added to four wells and incubated for 30 minutes at 37°C. The anti-IPU IgG/HRP and antibody buffer were mixed at double concentration to give the final [IgG] required in pesticide free PBST+0.1% blocking protein. Washing was applied after every reaction. Finally, 100 µl/well of 1 mg ml<sup>-1</sup> o-phenylenediamine dihydrochloride in 0.1 M sodium citrate, pH 5.0 buffer with 1 µl ml<sup>-1</sup> 30% H<sub>2</sub>O<sub>2</sub> was added to each well and after 3–20 minutes of colour development the reaction was stopped with 100 µl/well of 1.0 M H<sub>2</sub>SO<sub>4</sub>. The absorbencies were immediately read spectrophotometrically at 495 nm using a computer controlled Dynex Technologies Ltd (Billingshurst, UK) MRX plate reader. All assay permutations investigated were normally conducted in quadruplicate wells.

#### *Displacement Indirect MELISA*

The displacement assay format was essentially the same as for an indirect non-competitive assay. However, after the antibody reactions a displacement reaction was included. This consisted of 200 µl/well of various pesticide standard/sample concentrations in quadruplicate incubated for 30 minutes at 37°C. After washing the substrate was developed and the absorbance measured as before.

#### *Soil Extract Matrix Effect*

A series of 33 soil samples contaminated with isoproturon along with 10 blank soil samples (pesticide free) and soils contaminated with the pesticides chlortoluron or linuron (10 of each), all containing about 2-3% organic matter content were supplied by Prof. Alan Walker (HRI, Wellesbourne, UK). The herbicide residues were extracted from the soils by mixing 25 g soil with 30 ml 9:1 (v/v) acetonitrile:water for 1 hour. A pesticide free soil sample was utilised as a blank. The pesticide levels were quantified at the HRI using HPLC.

### **Results and Discussion**

Before determining the calibration profile of the MELISA, attempts were made to optimise its performance. If there is no pesticide in the added sample, the anti-IPU-HRP will bind to the IPU-ovalbumin-1.3 conjugate immobilised onto the membrane and by the addition of the substrate (hydrogen peroxide and o-phenylenediamine), high colour will

be developed and this is recorded at 495 nm. Conversely with high levels of pesticide, minimal binding will occur and lower colour will develop. To obtain maximum sensitivity it is necessary to optimise the ratio of the absorptions of the saturated membranes (i.e. maximum signal) to the background absorption due to non-specific effects.

### *Membrane Optimisation*

The UltraBind™ membranes were available in three types with codes; US-450, US-800 and SV-450, where ‘US’ denotes an unsupported polyethersulfone membrane and ‘SV’ denotes a supported polyethersulfone membrane cast on a polypropylene backing. These membranes were compared by an indirect noncompetitive assay. Results showed that the US-800 membrane was superior for this application, giving a higher adsorption (1.2) with a background level of almost half that of the 0.45 µm membranes and a signal/background ratio of 7.44. The larger 0.8 µm pore size would allow better perfusion of the reagents into the inner matrix of the membrane enabling more efficient covalent coating/blocking of membrane and diffusion of anti-IPU-HRP for binding and washing. This was also facilitated by the noticeably faster flow-rate of the US-800 membrane of 3-9 secs 100 ml<sup>-1</sup> (9.62 cm<sup>2</sup>)<sup>-1</sup> at 24" Hg (from product data). The presence of the SV-450 polypropylene support resulted in a higher response and better S/N ration (3.70) than the US-450 membrane (3.44). The MELISA was therefore developed with the US-800 membrane. Because the S/B ratio was low (< 10) it was important to optimise the membrane coating and blocking fully to minimise the background and maximise the signal achieved. Due to the porous nature of the membrane more thorough washing steps were required.

### *IPU-Ovalbumin-1.3 coating optimisation*

Various IPU-ovalbumin-1.3 coating conjugate concentrations (10 mg ml<sup>-1</sup> stock concentration in 10 mM PBS, pH 7.5 buffer) were cast and allowed to air dry at room temperature to give the titration profile shown in Figure 1. Coating reproducibility was high (CV < 4.2%) with steady background levels. Suitable coating concentrations were from 5-10 mg ml<sup>-1</sup>. Saturation was achieved reproducibly at 10 mg ml<sup>-1</sup> (40 ng in 4 µl)

per membrane. Coating at 37°C compared to room temperature led to lower background levels whilst maintaining the overall response as reflected by increased S/B ratios obtained from 5.52 for room temperature to 7.22 for 37°C.

#### *Comparing Membrane Blocker Efficiency*

A variety of different blocking proteins were investigated at pH 7.5. 1% Casein and nonfat dry milk were not suitable with casein reducing the assay response by 50% when compared to 1% OA blocker. Due to the porous nature of the membrane low molecular weight amine containing blocking compounds were investigated that could penetrate the membrane matrix. These were glycine and ethanolamine which were used both individually and in combination with 1% OA. These results are presented in Figure 2a. Glycine and ethanolamine containing blockers showed an overall increase in variability and background of the assay responses compared to the OA protein blocker. OA at 1% was shown to be the most effective blocking protein and concentration to maximise signal and reproducibility whilst maintaining background levels. Blocking at 37°C led to lower background levels with minimal loss in overall response as reflected by increased S/B ratios (5.52 ) compared to 3.45 for room temperature blocking.

#### *Investigating Coating/Blocking Duration*

The length of the coating and blocking incubations were also investigated. These were tested in combination with up to an hour for each incubation. From the data shown in Figure 2b the signal and S/B ratio increased (background decreased) with increasing coating duration up to 45 minutes, beyond which no further increase in signal was achieved. Signal reduction and increased background was observed when blocking was reduced from 1 hour incubation. The most effective incubation combination was therefore 45 minutes coating with 1 hour blocking giving a CV of 6.35% and S/B ratio of 8.32.

#### *IPU Detection by Competitive/Displacement MELISA*

Both assay systems were able to detect IPU as shown in Figure 3. The competitive IPU assay demonstrated the lowest limit of detection, determined at 90% of the maximum response ( $LLD_{90}$ ), of  $0.5 \text{ ng ml}^{-1}$  with a 7.8% mean assay coefficient of variation. The displacement assay was less sensitive with an IPU  $LLD_{90}$  of  $7.5 \text{ ng ml}^{-1}$  and a CV of

6.5%, with the response reaching 25% of the maximum response at the maximum solubility of IPU in water. A higher stringency buffer of 5X PBS + 0.1% Tween 20 was used to assist displacement. The S/B ratios were also seen to increase, resulting in a value of 12 for the competitive and 16 for the displacement assay, reflecting lower background levels. Table 1 shows some of the figures of merit for the MELISA compared to a commercial SDI ELISA. A dynamic range of  $0.5 \text{ ng ml}^{-1}$  -  $20 \text{ } \mu\text{g ml}^{-1}$  was observed for the competitive assay, with a linear relationship between the absorbance and the log of the isoproturon concentration ( $R^2 = 0.985$ ). A similar linear relationship between the absorbance and the log of the isoproturon concentration was observed for the displacement assay except that this occurred in the range  $8.0 \text{ ng ml}^{-1}$  –  $300 \text{ } \mu\text{g ml}^{-1}$  ( $R^2 = 0.994$ ). It was noticeable that the ranges for these assays were much larger than for the commercial ELISA which is an advantage for soil analysis, but sensitivities were lower.

#### *Membrane Temperature Tolerance*

With a view to fabricating screen-printed immunosensors where the US-800 disc can potentially be held in place with an integrated mesh, it was necessary to determine if the membrane could withstand potential insulation ink curing conditions of  $130^\circ\text{C}$  for 2 hours and still remain active for covalent binding of the IPU- ovalbumin conjugate. Only minimal changes were observed upon heat treatment, suggesting that the US-800 membrane was stable and retained activity, indicating the MELISA could operate when incorporated into a fabricated device and exposed to the fabrication conditions.

#### *Membrane Immunosorbent Storage*

The initial stability of the ovalbumin-1.3 conjugate bound US-800 after desiccated  $+4^\circ\text{C}$  storage for up to 2 weeks was investigated based upon the maximum asymptotic response. The maximum response was seen to remain stable for at least 8 days with increased signal and background levels. After this time however the response declined.

Whereas the stability of the prepared immunosorbent is applicable to the storage ability of the assay in a competitive format, the displacement assay must be stored with the anti-IPU-HRP already bound. Complexed enzyme conjugate activity however is generally not stable during periods of storage, which makes this less robust as an assay

format. Stability of the membrane bound complex was determined after storage (+4°C, 24 hours in wet (buffer) or dry conditions). Instability was demonstrated with both storage conditions at +4°C after 24 hours. Dry storage showed an increase in S/B ratio but with a drop in overall response. With buffer storage an increase in response was obtained, but the background level also increased, giving a lower S/B value. An increase in CV of nearly 4% was observed for both storage conditions.

#### *Analysis of soil extracts*

Samples of pesticide free soil were extracted as described and the resultant extracts utilised within the MELISA assay. The soil extracts were diluted and added along with anti-IPU-HRP to IUP-ovalbumin-1.3 and control ovalbumin grafted membranes. Samples of anti-IPU-HRP with added acetonitrile were also utilised. This was to determine whether any effects were due to the soil matrix or the acetonitrile solvent. Interestingly the incorporation of 20% acetonitrile into the solution led to an increase in both the sample and background signals, perhaps due to increased non-specific binding. When a solution containing 2% acetonitrile was used, no solvent effects could be seen. When soil extracts were used, again there was some increase in the background, perhaps due to the organic matter present in the soils being extracted and interfering with the MELISA results by increasing non-specific binding. However, these increases are not of a magnitude to render the protocol unusable. A more stringent and rigorous washing steps can assist in reducing the matrix effects.

Figure 4 shows comparison of the MELISA assays for isoproturon of the soil extracts compared with the values for HPLC. As can be seen there is a reasonable correlation between the two methods (a slope of 1 with  $R^2=0.85$ ), however there are still some non-specific effects which means the MELISA overestimates isoproturon concentration. This could be mitigated by further sample clean-up and better blocking. Blank samples and those contaminated with chlorotoluron or linuron both consistently gave isoproturon levels of  $<0.1 \text{ mg l}^{-1}$ .

## Conclusions

The anti-IPU-HRP ELISA was transferred successfully to a membrane bound format demonstrating a strong response. Similar competitive detection limits to the ELISA ( $0.19 \text{ ng ml}^{-1}$ ), suitably low for soil extract analysis, were observed. A competitive IPU LLD<sub>90</sub> of  $0.5 \text{ ng ml}^{-1}$  was achieved. The displacement assay format was less sensitive with an IPU LLD<sub>90</sub> of  $7.5 \text{ ng ml}^{-1}$ . The MELISA indicated a high potential for continued development into an immunosensor device with good temperature stability and possibility of utilisation with samples extracted from soils. These membranes have been successfully incorporated into an electrochemical immunosensor for isoproturon and details of this will be published in a future paper.

## Acknowledgements

The authors would like to express their gratitude to Dr Ramadan Abuknesha (Kings College London, UK) for providing the antibodies and the late Prof. Allen Walker (HRI, Wellesbourne, UK) for the soil samples, also the BBSRC (UK) for the financial support through a BBSRC-Case studentship scheme.

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## Captions

Table 1: Comparison of competitive and displacement anti-IPU-HRP assays with a commercial ELISA test for isoproturon.

Figure 1: IPU-Ovalbumin-1.3 coating antigen titration. US-800 membranes were coated at RT with IPU-ovalbumin-1.3 (signal) or ovalbumin (background) in concentrations from 10 - 0.625 mg ml<sup>-1</sup> and blocked with 1% ovalbumin. They were developed using a 1/10000 dilution of anti-IPU-HRP with an OPD substrate for 7 mins, mean assay %CV < 4.2%. Error bars = ±SD, n = 4.. Inset: Structure of isoproturon (R=CH<sub>3</sub>) and substituted derivatives for conjugation to ovalbumin (2-4) and 4-isopropylphenyl isocyanate (5).

Figure 2: (a) US-800 membranes were coated with 5 mg ml<sup>-1</sup> IPU-ovalbumin-1.3 (signal) or ovalbumin (background) and blocked with 1% ovalbumin, 1 M glycine and 1 M ethanolamine combinations. (b) blocked with 1% OA at 37°C in different combinations of exposure/blocking to up to 1 hour. 1/6000 dilution of anti-IPU-HRP, OPD substrate was developed for 5 mins. Error bars = ±SD, n = 3.

Figure 3: Comparing competitive and displacement anti-IPU-HRP assays. Each assay was coated with 10 mg ml<sup>-1</sup> of IPU-ovalbumin-1.3 and 1% ovalbumin blocked. IPU standards were then either; competed without pre-mixing, with a dilution of 1/6000 anti-IPU-HRP or incubated to displace bound 1/6000 dilution anti-IPU-HRP. OPD substrate developed for 7 mins, respective assay means %CVs of 7.76% and 6.46%. Error bars = ±SD, n = 4.

Figure 4: Comparison of isoproturon concentrations in soil extracts using ELISA and HPLC.

Table 1: Comparison of competitive and displacement anti-IPU-HRP assays with a commercial ELISA test for isoproturon.

|                                                     | Competitive anti-IPU-HRP assay                   | Displacement anti-IPU-HRP assay                   | Commercial ELISA test        |
|-----------------------------------------------------|--------------------------------------------------|---------------------------------------------------|------------------------------|
| Limit of detection<br>(3 X Std. Dev. of zero value) | 1.2 ng ml <sup>-1</sup>                          | 37.5 ng ml <sup>-1</sup>                          | 0.02 ng ml <sup>-1</sup>     |
| Mean Coefficient of Variance (n=4)                  | 7.8%                                             | 6.5%                                              | 2.3%                         |
| Dynamic range                                       | 0.5 ng ml <sup>-1</sup> - 20 µg ml <sup>-1</sup> | 8.0 ng ml <sup>-1</sup> – 300 µg ml <sup>-1</sup> | 0.02 – 2 ng ml <sup>-1</sup> |
| R <sup>2</sup>                                      | 0.985                                            | 0.994                                             | 0.997                        |
| LLD90                                               | 0.5 ng ml <sup>-1</sup>                          | 7.5 ng ml <sup>-1</sup>                           | 0.023 ng ml <sup>-1</sup>    |

Figure 1

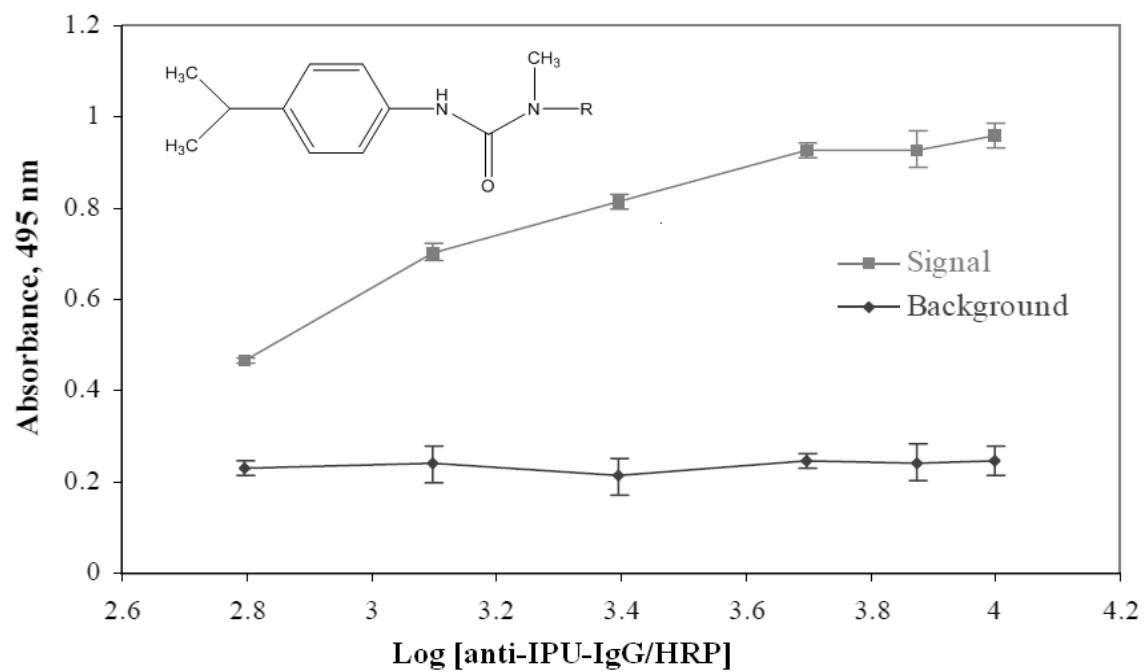


Figure 2

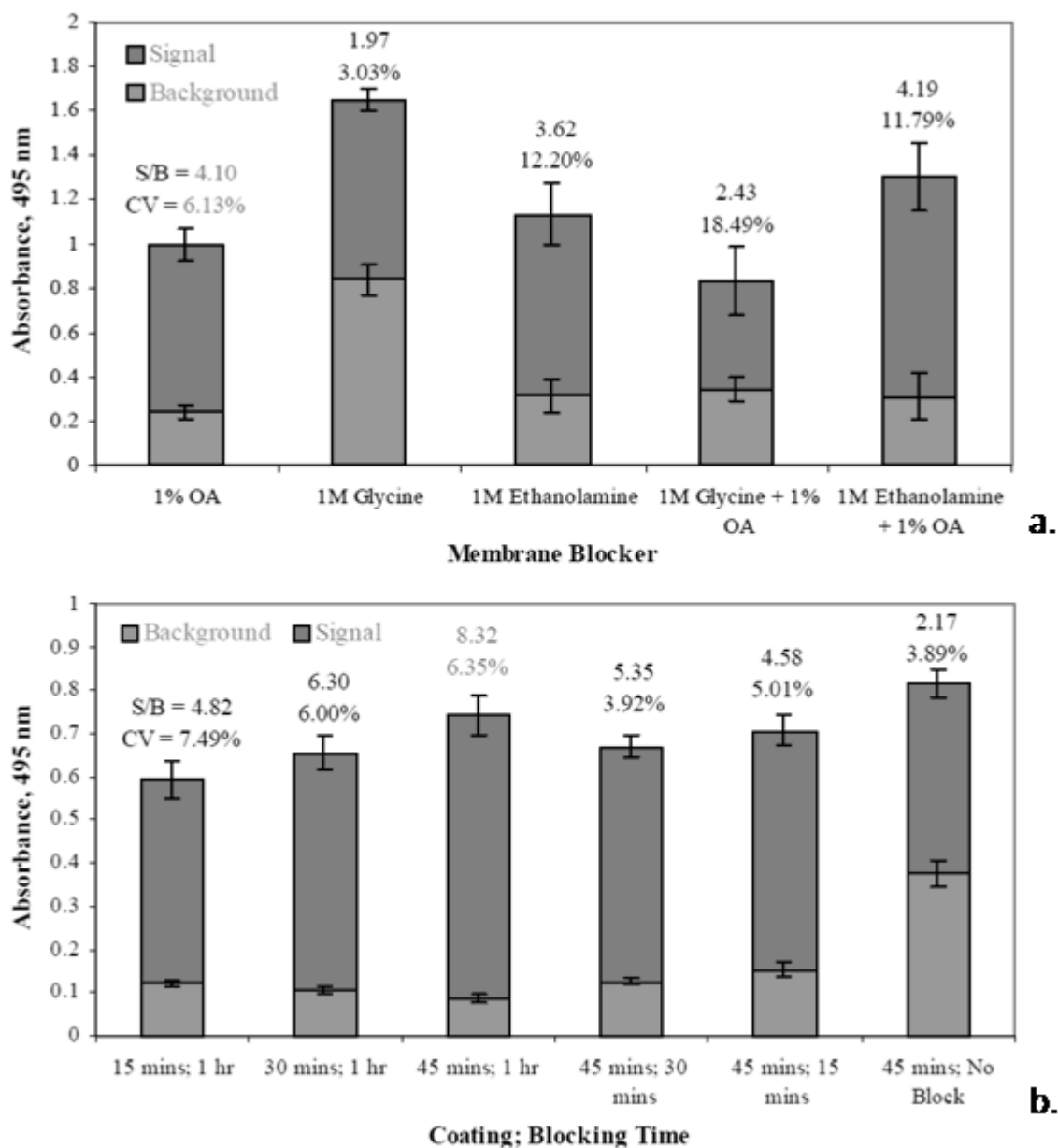


Figure 3

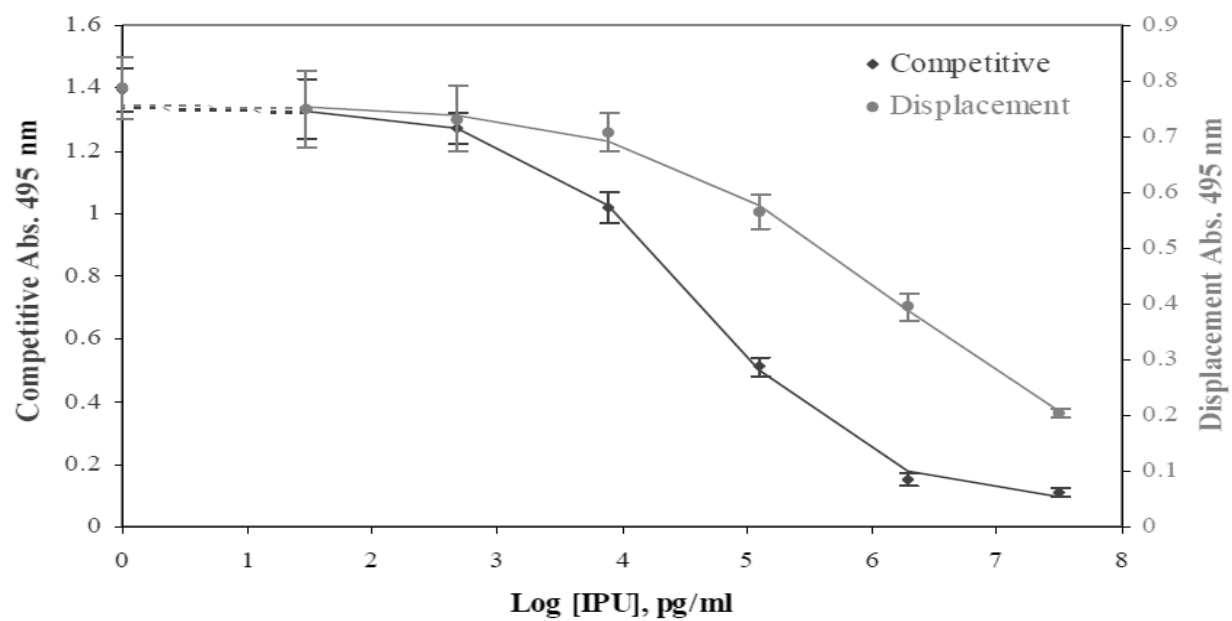
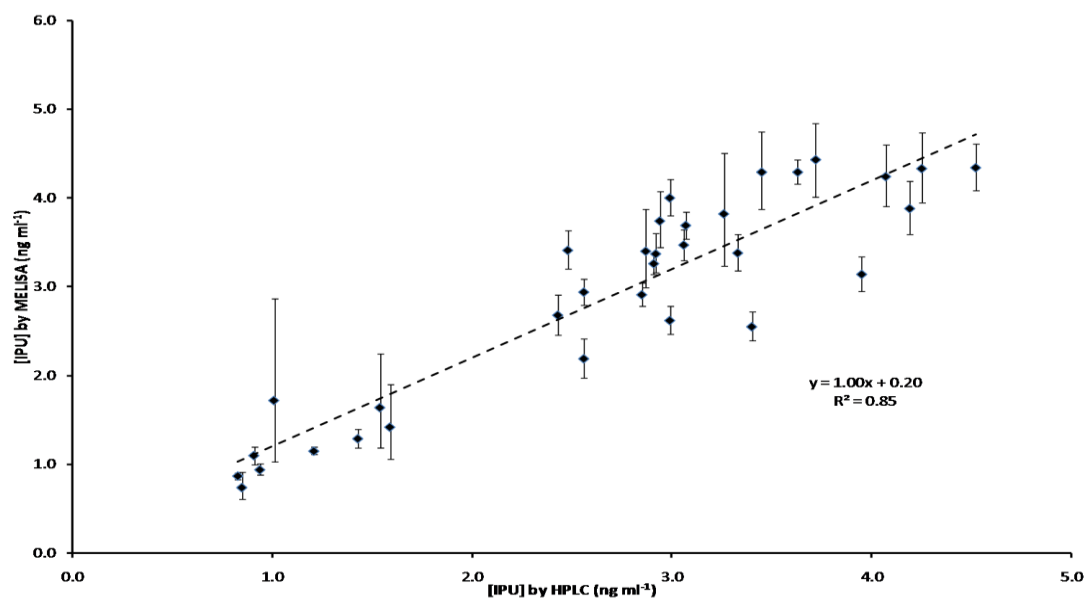


Figure 4





**DSPACE**

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**2012-01-31T00:00:00Z**

Taylor & Francis

<http://dx.doi.org/10.1080/00032719.2011.582555>

Damian E. H. Baskeyfield, Frank Davis, Naresh Magan, Ibtisam E. Tothill. A membrane-based ELISA assay for the herbicide Isoproturon in soil samples. *Analytical Letters*, 45: 99–109, 2012