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Immuno-arrays for multianalyte analysis of chlorotriazines

Sunil Bhand[a,1], Ioana Surugiu[a], Anatoly Dzgoev[a], Kumaran Ramanathan[a], P.V. Sundaram[b], Bengt Danielsson[a,*]

[a] *Pure and Applied Biochemistry, Lund University, Box 124, S-22100 Lund, Sweden*
[b] *PROMED, The Voluntary Health Services, Adyar, Chennai 600113, India*

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Abstract

In this paper, a novel strategy for multicomponent analysis of two classes of pesticides such as triazines (atrazine and simazine) and phenoxyalkanoic acids (2,4-dichlorophenoxy acetic acid (2,4-D), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), 4-chlorophenoxyacetic acid (CPOAc), phenoxyacetic acid (POAc)) employing immuno-arrays is demonstrated. The approach is based on cross-reactive arrays of specific antibody pairs coupled to chemometric pattern recognition. The monoclonal antibody pairs employed in this work (atrazine–simazine and 2,4-D) are specific towards a set of analytes and preclude a particular set of others present in the sample matrix. Antibody pairs of atrazine, simazine, and 2,4-D are used to discriminate and quantify analyte of interest. Atrazine was quantified in presence of trace concentration of simazine and that of 2,4-D. The combinatorial cross-reactivity of antibody pairs towards simazine, atrazine and 2,4-D is used to distinguish among different classes of analytes and their influence on the signal suppression in immuno-techniques. These sensors exclude recognition by carbamates such as carbaryl and carbofuran.

Keywords: Multicomponent analysis; Immunosensor; Cross-reactive arrays; Triazines; Phenoxyalkanoic acids; Discriminative detection

1. Introduction

The adverse environmental health effects of pesticides and herbicides (viz. atrazine, simazine, 2,4-D, 2,4,5-T), propazine, organophosphates and carbamates (such as carbaryl and carbofuran) results from exposure to either a single or a mixture of analytes present in the matrix. Low-level detection of pesticides is important in many process control, environmental and food quality analyses. Several immunoassays have been developed for the analysis of pesticides [1–3]. Microformat imaging (96-flat well, slide or 384-well plate) chemiluminescent immunoassays based on charge coupled device (CCD) camera have been developed for high throughput analysis [4–6]. In order to facilitate field screening, a portable module based on a photomultiplier tube (PMT) has also been developed for pesticides [7]. However, the need for a highly selective multianalyte detection system is acute. Cross-reactivity of antibodies is exploited in the construction of some multianalyte assays based on chemiluminescent and optical fluorescence detection [8,9]. Few attempts have been made towards development of immunosensors capable of both discriminative detection and class-selective analysis. Recent approaches, applied to multianalyte sensing, utilize either the exquisite specificity found in the ligand–receptor interactions or use non-specific sensors in a cross-reactive array format often dubbed as 'Electronic Noses' [10]. Schauer et al. [11] utilized a combination of the above approaches to facilitate detection of class-specific molecules. Ohmura et al. [12] proposed coordinated use of two antibodies to extend dynamic range and detect multiple analytes. Reder et al. [13] have presented a fluorescent immunosensor

* Corresponding author. Tel.: +46 462228257; fax: +46 462224611.
E-mail address: bengt.danielsson@tbiokem.lth.se (B. Danielsson).
[1] Present address: Chemistry Group, Birla Institute of Technology and Science, Pilani 333031, India.

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utilizing cross-reactivity of polyclonal antibodies for the analysis of atrazine and simazine. Herein, we report the use of triazine-ab pairs in combination with phenoxyalkanoic acid-ab for extended class-specific and class-selective analysis of simazine, atrazine, 2,4-D, 2,4,5-T and CPOAc. Thus, three different classes of analytes are measured. Moreover, the signal suppression by cross-reacting analytes such as simazine and 2,4-D is characterized in presence of the analyte of interest. Using the chemometric cross-reactivity patterns, the atrazine, simazine, 2,4-D, 2,4,5-T and CPOAc are detected in the sample mixture.

2. Experimental

2.1. Materials

Atrazine, simazine, 2,4-D, 2,4,5-T, CPOAc and POAc, bovine serum albumin (BSA) were obtained from Sigma (St. Louis, MO). Carbaryl and carbofuran were obtained from Riedel-de Haën (Seelze, Germany). Colorimetric substrate *o*-phenylenediamine dihydrochloride, ImmunoPure OPD, was procured from Pierce (Rockford, IL) and hydrogen peroxide 30% from J.T. Baker Inc. (USA). Monoclonal anti-atrazine, anti-simazine, anti-2,4-D antibodies, and horseradish peroxidase HRP-labelled antigen (atrazine-HRP, simazine-HRP and 2,4-D-HRP) were obtained from Dr. Milan Franek, Veterinary Research Institute (Brno, Czech Republic). Antibody stock solutions of 1 mg ml^{-1} were prepared in 50 mM carbonate buffer (CB) (pH 9.6) and refrigerated for further use. Buffers and standards were prepared using ultrapure water (Maxima, Elga, UK). Washing buffer solution (PBST) contained phosphate-buffered saline (PBS) 0.01 M, pH 7.4, containing 0.0027 M potassium chloride and 0.137 M sodium chloride with 0.1% Tween 20. A stock solution of 1 mg ml^{-1} BSA in PBST was used as blocking solution. Stock solutions (1 mg ml^{-1}) of 2,4-D and 2,4,5-T were prepared in methanol. Simazine, atrazine, carbaryl, and carbofuran stock solutions (1 mg ml^{-1}) were prepared in liquid chromatography grade dimethylsulphoxide (DMSO). Competitive assays were carried out in collapsible ELISA 96-well plates (MaxiSorp, Nunc Roskilde, Denmark). An optical plate reader, Multiskan MCC/340 (Lab systems Helsinki, Finland) was used to measure the absorbance.

2.2. Experimental design and assay protocol

Colorimetric ELISA in competitive mode was performed sequentially as follows:

(i) Calibration for single analytes such as atrazine, simazine and 2,4-D employing respective mAbs.
(ii) Measurement of cross-reactivity of mAbs atrazine, simazine and 2,4-D towards different classes (triazines, phenoxyalkanoic acids, and carbamates) and types of pesticides.
(iii) Analysis of atrazine in presence of varying concentrations of simazine and 2,4-D at concentration [1/10 of IC_{50}].
(iv) Analysis of atrazine in presence of simazine (study and analysis of signal suppression) and their discrimination with 2,4-D and 2,4,5-T.
(v) Calibration for 2,4-D and measurement of cross-reactivity of mAb 2,4-D with 2,4,5-T and CPOAc and POAc.

Employing different classes of monoclonal antibodies, the direct competitive ELISA was performed as described. Antibodies were adsorbed in the 96-well plates. To each well 200 μl of a predetermined concentration of antibodies in CB was added and incubated overnight at 4 °C. For all the three antibodies, a dilution of 1:1000 was found to be optimal. After removing the solution, the wells were washed thrice with PBST. Non-specific binding sites were blocked using BSA (1 mg ml^{-1}) solution. After a subsequent washing step, a premixed solution of standard and the labelled antigen was incubated for 2 h at 4 °C. The labelled antigen concentrations, atrazine-HRP 1:5000, simazine-HRP 1:3000 and 2,4-D-HRP 1:10,000, were used throughout the experiments. The assay buffer used was PBST. The unbound analyte and the labelled antigen were removed by washing (thrice) with PBST. Colorimetric substrate solution was prepared by adding 25 mg OPD and 3.13 μl of H_2O_2 to 8.33 ml phosphate citrate buffer PCB (44 mM, pH 5.5). Two-hundred microlitres colorimetric substrate solution was added to each well. The plates were covered with aluminium foil and kept in dark at room temperature for 40 min. The resulting colorimetric absorption signal from the specifically bound enzyme conjugate reaction was measured at 492 nm. Cross-reactivity of different analytes was evaluated by incubating a premixed solution of HRP-labelled antigen and competing analyte at various dilutions. In multicomponent analysis of mixtures, such as atrazine and 2,4-D, the cross-reactant, standard and the labelled antigen were added before measurement step.

3. Results and discussion

3.1. ELISA

Calibrations were obtained for atrazine, simazine and 2,4-D employing anti-atrazine, anti-simazine and anti-2,4-D mAb. In ELISA, the signal is inversely proportional to the concentration of the analyte. Absorption curves were converted to the absorption signal (B/B_0 (%)), where B_0 is the maximum signal obtained in absence of analyte, and B is the signal obtained in presence of analyte concentration. Typical calibration obtained for atrazine is presented in Fig. 1. A linear range 0.1–48 ng ml^{-1} and lower limit of detection 0.05 ng ml^{-1} was obtained. Following the calibration procedure similar to atrazine, a linear range 0.48–51 ng ml^{-1} for simazine was obtained (Fig. 2).

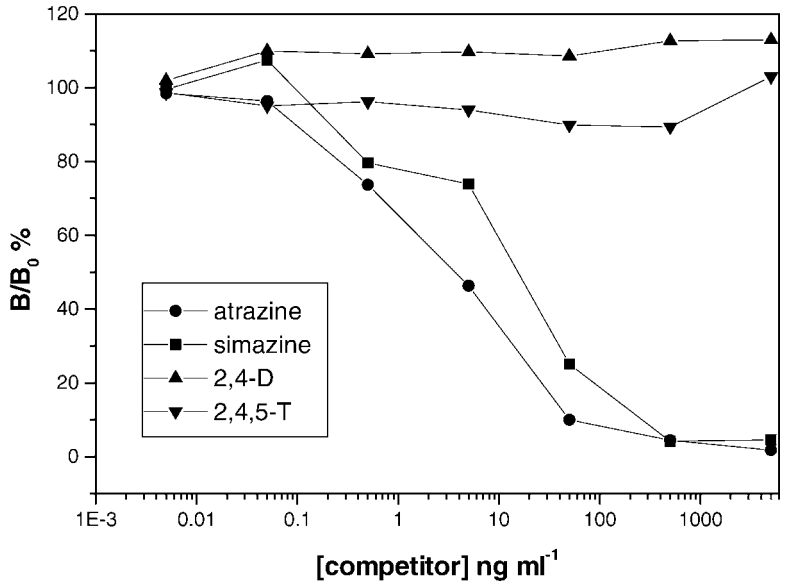

Fig. 1. Calibration curve for (●) atrazine against mAb-atrazine and measurement of cross-reactivities with (■) simazine, (▲) 2,4-D and (▼) 2,4,5-T in competition with tracer. The concentration of cross-reactant was identical in all the measurements.

3.2. Cross-reactivity measurement and discriminant analysis

The antibodies have specificity to a set of analytes known as cross-reactivity. Therefore, a single antibody will respond to different analytes with varying specificity. The cross-reactivity of mAb pairs was determined in competition with tracer. For standard analyte (for which CR is 100%), concentrations that result in 50% inhibition (IC_{50}) of the signal were obtained form calibrations and were used to compute the CR using the formula

$$\text{CR}\,(\%) = \frac{\text{IC}_{50}\text{ value of standard analyte}}{\text{IC}_{50}\text{ value of cross-reacting analyte}} \times 100$$

Cross-reactivity of mAb-atrazine towards three classes, namely triazines (atrazine and simazine), 2,4-D and 2,4,5-T (phenoxyalkanoic acids) and carbamates (carbaryl and carbofuran), was studied in the concentration range of

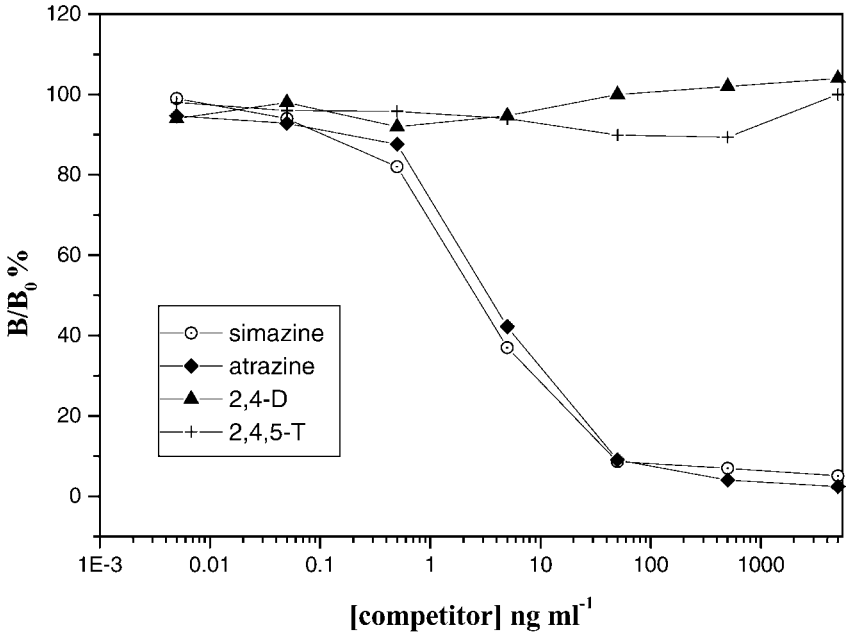

Fig. 2. Calibration curve for (⊙) simazine against mAb-simazine and measurement of cross-reactivities with (♦) atrazine, (▲) 2,4-D and (+) 2,4,5-T in competition with tracer. The concentration of cross reactant was identical in all the measurements.

Table 1
Inhibition of mAb-atrazine for different triazines, phenoxyalkanoic acids and carbamate

Sample no.	Concentration (ng ml^{-1})	Inhibition level, B/B_0 (%)				
		Atrazine	Simazine	2,4-D	2,4,5-T	Carbaryl
1	5000	1.79	4.59	113.0	103	92.2
2	500	4.45	4.2	112.7	89.4	94.8
3	50	10.00	25.1	108.6	89.9	99.56
4	5	46.30	73.9	109.7	94.0	99.13
5	0.5	73.60	79.6	109.2	96.2	89.0
6	0.05	96.40	107.5	110.0	95.1	84.84
7	0.005	98.50	99.6	102.0	98.7	89.17

0.005–5000 ng ml^{-1} and the resulting signals are presented in Table 1. The variations in B/B_0 (%) with concentration for the cross-reactants are presented along with atrazine calibration in Fig. 1. It is evident that simazine cross-reacts (CR = 25.4%) being structurally similar to atrazine. The other tested compounds, 2,4-D and 2,4,5-T (phenoxyalkanoic acids), are not recognised by the sensor. The carbamates tested from practical point of view were also excluded from recognition. In the second set, mAb-simazine is used as sensor and its cross-reactivity was tested towards atrazine, 2,4,5-T and 2,4-D. The results are presented along with simazine calibration in Fig. 2. In contrast to the cross-reactivity of simazine towards mAb-Atrazine, mAb-simazine exhibited a higher affinity towards atrazine (CR = 75.4%). Thus, immunosensors for such coexisting analytes need signal resolution. The sensor also excluded recognition of phenoxyalkanoic acids. To enhance the capability of multicomponent analysis, mAb-2,4-D (sensor from phenoxyalkanoic acid class) was deployed. This sensor is specific for 2,4-D, it cross-reacts with 2,4,5-T, CPOAc and excludes POAc.

3.3. Chemometric analysis

Inspection of the cross-reactivity data from the first two sensors provided some clue towards the possibility of using them as sensing pairs to be able to simultaneously detect or quantify more than one component in ELISA. As discussed earlier, mAb atrazine can be used to quantitatively estimate atrazine and discriminate from 2,4-D and 2,4,5-T. The second sensor will recognize 2,4-D and 2,4,5-T as well as CPOAc excluding POAc. Wortberg et al. [14] reported on an immuno-array for differentiating cross-reacting analytes based on cluster analysis. Herein, we have studied the qualitative and quantitative relationship among analytes, cross-reactants, and sensors employing chemometric analysis. The data set from Table 1 and those obtained for the other two sensors were subjected to multivariate treatment employing *Statgraphics plus* Professional version 5.0 evaluation package (Manguistic Inc., USA). Using factor analysis, the response patterns were obtained at different concentrations of analyte and cross-reactants for three types of antibodies.

3.4. Factor analysis

Factor analysis helps to identify a set of dimensions, not easily observable in a large set of variables. For instance, it is difficult to resolve the signals obtained in a 96- or 384-well plate ELISA employing multiple analytes and multiple antibody pairs. Moreover the technique helps to devise means for condensing large number of observations into distinct groups. The factor loading plots obtained employing mAb-atrazine is presented in Fig. 3(a). Overall four factors contribute to variability of data. The negative values reveal that 2,4-D and 2,4,5-T have no significant contribution towards the cross-reactivity while atrazine and simazine form a close group due to cross-reactivity. Thus the factor plot can be treated as signature for discrimination. Further refinement of the data is achieved by rotating the factor (viz. one of the factors 2,4-D)

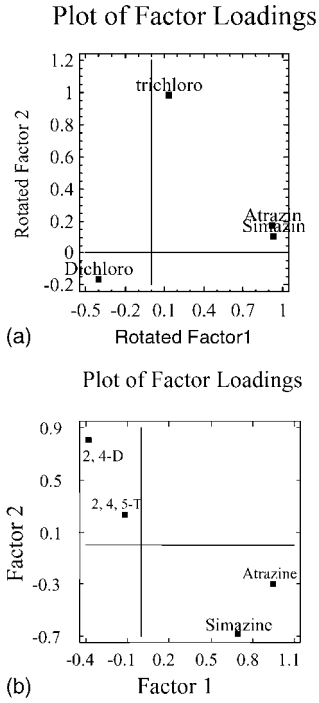

Fig. 3. (a) Factor analysis for cross-reactivity data using mAb-atrazine sensor: trichloro (2,4,5-T) and dichloro (2,4-D). (b) Cross-reactivity pattern (factor plot) for mAb-simazine sensor towards simazine, atrazine, 2,4-D and 2,4,5-T.

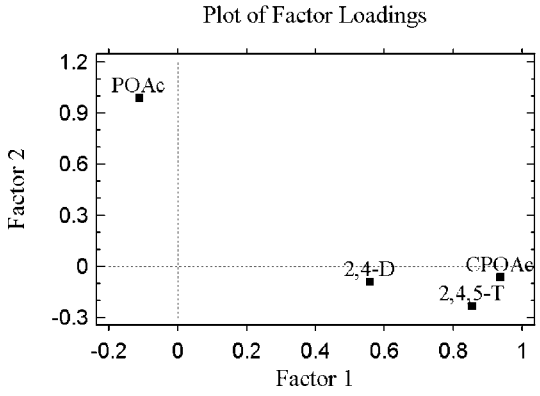

Fig. 4. Cross-reactivity pattern (factor plot) obtained using mAb-2,4-D sensor towards 2,4-D, 2,4,5-T, CPOAc and POAc.

to a new position to obtain exact influence of individual compound on the sensor used. To resolve simazine–atrazine overlap as observed in Fig. 3(a), the cross-reactivity of simazine mAb was tested for the same set of compounds in parallel with atrazine mAb. The statistical treatment generated a new response pattern presented in Fig. 3(b). As evident, mAb-simazine also excludes 2,4-D and 2,4,5-T recognition. However, it has a resolved signal for atrazine (positive contribution in signal variability). Comparison of the two factor plots also reveals that by switching between the sensors, influence of the major cross-reactant significantly differs in magnitude. Thus using the antibody pair mAb (atrazine/simazine) two components can be discriminated within the same class of pesticides. In addition to triazine antibodies, anti-2,4-D and 2,4,5-T were also tested to provide increased class selectivity of the arrays. Factor plot obtained for mAb-2,4-D sensor is presented in Fig. 4. The sensor exhibits high affinity towards 2,4-D, and shows cross-reactivity towards 2,4,5-T and POAc. However, it excludes recognition (no contribution in variability of the signal) for POAc, a non-chlorinated phenoxyalkanoic acid, among the cross-reactants tested.

3.5. Signal suppression in multicomponent immunoassays

In immunoassays, the presence of cross-reacting analytes in a mixture causes signal suppression or positive bias (i.e. concentrations higher than expected values are detected). However, the presence of a cross-reactant in the sample might result in suppression of recovery as observed in clinical immunoassays [15]. Such reports in pesticide mixtures are not available to date. Hence, we have attempted to quantify the negative bias contribution by simazine and 2,4-D in immuno-analysis of atrazine. Signals obtained from assays conducted with mixtures of analyte and cross-reactant in the sample are presented in Fig. 5. The signal for atrazine (when present up to 0.5 ng ml^{-1}) is suppressed in presence of 1 and 4 ng ml^{-1} 2,4-D, as observed in extreme left portion of the curves. Atrazine can still be quantified in the range 0.5–5 ng ml^{-1} with a compromise in sensitivity and reduced detection limit. Since simazine is found to cross-react significantly with mAb-atrazine, the effect of bias on atrazine in presence of low concentrations of simazine was investigated. The factor plot obtained for addition of 0.5, 1, 2 and 4 ng ml^{-1} simazine during atrazine analysis (0.01–1000 ng ml^{-1}) is presented in Fig. 6(a). The variability in signal is significant at 4 ng ml^{-1} simazine. Using the cluster analysis, a dendrogram is obtained and is presented in Fig. 6(b). The dendrogram shows a linear effect of negative bias over analyte. The most significant information obtained from these experiments is the possibility of identifying the tolerance limits within which both simazine and atrazine can be quantitatively evaluated.

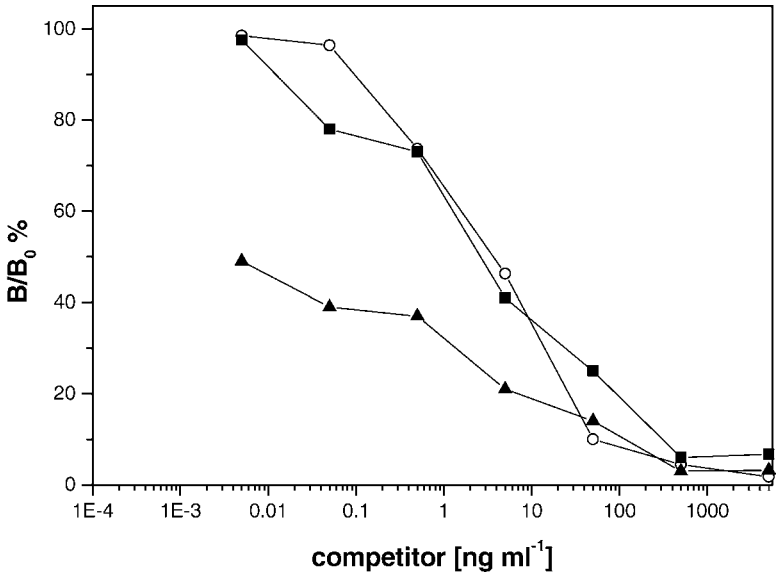

Fig. 5. Signal suppression due to trace 2,4-D in atrazine analysis using mAb-atrazine. (◯) Atrazine standard, (▲) atrazine + 1 ng ml^{-1} 2,4-D, (■) atrazine + 4 ng ml^{-1} 2,4-D.

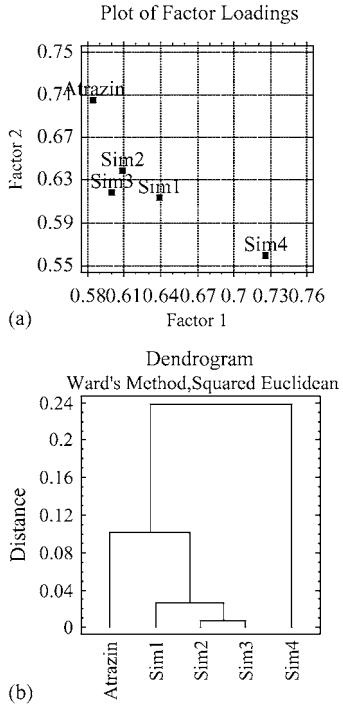

Fig. 6. (a) Factor plot obtained from analysis of the mixture of atrazine and cross-reactant simazine (Sim1, Sim2, Sim3 and Sim4) at various concentrations. Sim1: 0.5 ng ml^{-1} simazine; Sim2: 1 ng ml^{-1}; Sim3: 2 ng ml^{-1}; Sim4: 4 ng ml^{-1} simazine. (b) Dendrogram obtained from analysis of the mixture of analyte (atrazine) and cross-reactant (simazine).

4. Conclusion

The potential of cross-reacting antibody pairs such as mAb-(atrazine/simazine) and mAb-2,4-D in multianalyte, multicomponent analysis of two classes of pesticides with three components is demonstrated. The proposed technique can be extended to discriminate among different classes of pesticides such as organophosphates and organochlorines. The signal suppression reported in pesticide analysis might be a useful tool for resolution of mixtures of cross-reacting analytes. The work on integration of such assays to a field portable module is in progress.

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