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Cross-Reactivity Analysis Using a Four-Parameter Model Applied to Environmental Immunoassays

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Immunoassays with a high degree of specificity for pesticides have been developed for a variety of analytes (Itak *et al.,* 1993; Lawruk *et al.,* 1992, 1993; Rubio *et al.,* 1991; Van Emon *et al.,* 1989). Even with highly-specific antibodies, other compounds in a sample can cross react with antibody binding sites, affecting the quantitation of target analyte concentration. To estimate cross-reactivity, the levels at which compounds significantly affect the assay are determined. Information about the levels of cross-reactivity allows an analyst to judge the usefulness of an assay for a sample with known contaminants. For cases of high cross-reactivity, it may be necessary to use more than one immunoassay to determine the concentration of a target analyte. Under these circumstances, a method is needed to decouple the effects of cross-reacting analytes on the respective assays.

To separate the effects of two or more analytes present in an assay, reactivities of the analytes in the competition for antibody binding sites must be taken into account. In practice, this means that concentrations cannot generally be determined from linear approximations of an immunoassay standard curve and, conversely, the assay response cannot be related directly to the sum of the individual analytes' concentrations. The contribution of a given analyte to a sample's assay response must be weighted by the analyte's ED50 (concentration at the midpoint of the assay's response range) and reaction coefficient (see Equations 1, 2, and 3 in MATERIALS AND METHODS) in the given assay; these values are needed for all crossreacting analytes in the immunoassays to be run.

As an added complication, immunoassay analyses may also have to account for non-specific binding contributions that might otherwise be attributed to cross-reactive analytes. Based on these considerations, a modified four-parameter model was developed to calculate concentrations for atrazine and terbutryn in reciprocally cross-reacting assays (Jones *et* a*l*., 1994). In the method described in the following section, a four-parameter model is modified using the law of mass action to include two analytes. This method is used to calculate the recovery of alachlor from samples fortified with metolachlor; the analyte pair was chosen because metolachlor is a known cross-reactant in a commercially-available alachlor assay. Alachlor concentrations recovered from the derived method are also compared with values obtained by the method of Jones *et al.* The objective of this paper is to show that alachlor concentrations can be adequately recovered with relative independence of metolachlor concentration. Although only alachlor concentrations are calculated here, the method is

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generally applicable to determine several analyte concentrations in a sample by simultaneous solution of a system of nonlinear equations.

MATERIALS AND METHODS

The protocol for the magnetic particle-based Alachlor RaPID Assay® has been described elsewhere (Lawruk *et al.,* 1992) and is summarized briefly here. Antibodies raised to an alachlor-BSA (bovine serum albumin) immunogen were coupled to paramagnetic particles, which serve as the solid phase for the immunoassay system. Sample or calibrator, enzyme conjugate, and magnetic particles are added to a polystyrene tube for a fixed incubation period. Following the incubation period, the tubes are placed in a magnetic rack to draw the particles to the side of the tubes and the reaction solution is decanted. After washing and decanting two times, a color reagent is added to each tube; the enzyme conjugate generates a color from hydrogen peroxide and tetramethyl(benzidine) in proportion to the amount of conjugate bound. A 0.5% sulfuric acid solution is added after a set reaction period to stop the color reaction. Absorbance values of calibrators and samples are then read at 450 nm with a spectrophotometer.

Standard curves were obtained using anti-alachlor antibody-coupled magnetic particles for alachlor (zero standard, 0.1 parts per billion (ppb), 1.0 ppb, and 5 ppb) and metolachlor (zero standard, 12.5 ppb, 25.0 ppb, 50.0 ppb, and 75.0 ppb) calibrators (ChemService, Inc., West Chester PA). Alachlor and metolachlor concentrations were chosen based on previously obtained cross-reactivity data. A set of 16 mixtures of alachlor and metolachlor were made from paired combinations of 0.5 ppb, 1.0 ppb, 2.0 ppb, 3.0 ppb alachlor with 12.5 ppb, 25.0 ppb, 50.0 ppb, and 75.0 ppb metolachlor. Analyte concentrations were selected to span the dynamic response range of the assay. Mixtures were run in duplicate and the average absorbance value was evaluated to recover a sample's alachlor concentration.

To quantify the simultaneous competition of two analytes, alachlor and metolachlor, for binding sites raised against the alachlor-BSA immunogen the following derivation was used. The binding of alachlor to its specific antibody is given by

$$b1 \cdot Alachlor + S \rightarrow Alachlor - S \qquad (1)$$

where *S* represents the total number of available antibody binding sites, *Alachlor-S* is the alachlor-antibody complex, and *b1* is the reaction coefficient. The value *b1* is equivalent to the slope of a ln-logit transform (Itak *et al.,* 1993; Lawruk *et al.,* 1992, 1993; Rubio *et al.,* 1991). If metolachlor is present in the sample, it will also compete for the same antibody binding site; this requires an additional equation given by

$$b2 \cdot Metolachlor + S \rightarrow Metolachlor - S \qquad (2)$$

where *Metolachlor-S* represents the metolachlor-antibody complex and *b2* is the reaction coefficient.

To use this analysis in an assay, the competing enzyme conjugate, Alachlor*, needed to generate the signal must also be taken into account. This adds yet a third reaction given by

$$b^* \cdot Alachlor^* + S \;\text{®}\; Alachlor^* - S \qquad (3)$$

The law of mass action for this system is complicated by the presence of the additional analyte. To simplify this analysis, it is assumed that the respective analytes take up binding sites *without* interacting with each other, so that the net effect is only on the number of available binding sites of the reaction. In the case of the three analytes, the number of available binding sites is now {*S*-[*Alachlor-S*]-[*Metolachlor-S*]-[*Alachlor*-S*]} and the relationships for the law of mass action can be written then as

$$\frac{[Alachlor - S]}{[Alachlor]^{b1}(S - [Alachlor - S] - [Metolachlor - S] - [Alachlor^* - S])} = k_1 \qquad (4)$$

$$\frac{[Metolachlor - S]}{[Metolachlor]^{b2}(S - [Alachlor - S] - [Metolachlor - S] - [Alachlor^* - S])} = k_2 \qquad (5)$$

$$\frac{[Alachlor^* - S]}{[Alachlor^*]^{b^*}(S - [Alachlor - S] - [Metolachlor - S] - [Alachlor^* - S])} = k^* \qquad (6)$$

where k_1, k_2, and k^* are the binding constants for alachlor, metolachlor, and the enzyme conjugate, respectively.

Non-interaction assumes no pairing or segregation of the analyte of interest and the conjugate, implying that the equations describing the analyte-antibody and the enzyme conjugate-antibody could be written as separate reactions. Non-interaction also assumes that all antibody binding sites are essentially equivalent and that binding at one site of the antibody is not affected by binding at its other site. This assumption does not rule out exchange reactions; for example, an analyte could displace a conjugate bound to an antibody site. Another assumption is that the system has sufficiently equilibrated so that the relative binding of analytes (including conjugate) determines their respective binding affinities, however, the method should be applicable to any system whose responses are proportional to the equilibrium responses. Finally, it is also assumed that equilibrium constants (k_1, k_2, k^*) and reaction coefficients (b_1, b_2, b^*) can effectively describe analyte and conjugate binding to polyclonal antibodies. Although these assumptions are not strictly valid, the behavior of this and other systems suggests they are good approximations.

These equations can be manipulated to calculate the relative binding of the conjugate to the antibody, [*Alachlor* -S*]/*S,* for the three-analyte competition as

$$\frac{[Alachlor^{*} - S]}{S} = \frac{k^{*}[Alachlor^{*}]^{b^{*}}}{1 + k_1[Alachlor]^{b1} + k_2[Metolachlor]^{b2} + k^{*}[Alachlor^{*}]^{b^{*}}} \quad (7)$$

The relative conjugate binding derived in Equation 7 is proportional to the signal generated in the assay color development. The [*Alachlor**] term in the numerator on the right-hand side of Equation 7 represents the identical amount of conjugate added to every sample in an assay. By setting both [*Alachlor*] and [*Metolachlor*] concentration: equal to zero, Equation 7 yields the constant term *k**[Alachlor*]/(1+*k**[*Alachlor**]), which will be written simply as *k.* Factoring out *k* from the right-hand side of equation 7 yields the following general formula for the absorbance, *B,* generated by the bound conjugate

$$B = \alpha\frac{[Alachlor^{*} - S]}{S} = \frac{A}{1 + \left(\frac{[Alachlor]}{ED50_{ALA}}\right)^{b1} + \left(\frac{[Metolachlor]}{ED50_{MET}}\right)^{b2}} \quad (8)$$

where *a* is a proportionality constant for the measurement set-up (optics, absorptivity, etc.) and $A = a\,k$ is the maximum assay signal, which is also referred to as B_o. To simplify the expression, the terms $(1/ED50_{ALA})^{b1}$ and $(1/ED50_{MET})^{b2}$ are derived by factoring $k_1/(1+k^{*}[Alachlor^{*}]^{b^{*}})$ and $k_2/(1+k^{*}[Alachlor^{*}]^{b^{*}})$, respectively, from the denominator. The respective ED50 terms represent the concentrations at which the measured signal is one-half the assay dynamic range when only the respective analyte is present. It should be noted that the ratio, $ED50_{ALA}^{b1}/ED50_{MET}^{b2}$ is simply k_2/k_1 or the inverse ratio of the binding constants.

To compensate for non-specific binding and nonuniformities in data variance, the four-parameter model was adapted for immunoassay applications (Rodbard *et al.,* 1978). In this case, the four-parameter model for Equation 8 would take the form

$$B = \frac{A - D}{1 + \left(\frac{[Alachlor]}{ED50_{ALA}}\right)^{b1} + \left(\frac{[Metolachlor]}{ED50_{MET}}\right)^{b2}} + D \quad (9)$$

where *D* is the response at infinite concentration and the other terms retain their previous definitions. By way of comparison, a recent model using a four-parameter derivation given by

$$B = \frac{A - D}{1 + \left(\frac{[Alachlor]}{ED50_{ALA}} + \left(\frac{[Metolachlor]}{ED50_{MET}} \right)^{b2/b1} \right)^{b1}} + D \qquad (10)$$

was used to calculate concentrations from atrazine-terbutryn data (Jones, 1994). In the RESULTS AND DISCUSSION section, these two models are used to calculate the alachlor concentrations in samples containing metolachlor. Calculations for standard curve fits and alachlor concentrations using Equation 9 are referred to as Method 1; calculations using Equation 10 are referred to as Method 2. A logistic model from the SigmaPlot® Curve Fit option was used to obtain the fitting parameters for Equations 9 and 10 for the standard curves. In the SigmaPlot package, the Marquardt-Levenberg algorithm is used to obtain a convergent result for the parameter fit (Kuo, 1994).

RESULTS AND DISCUSSION

Standards curves were measured for the alachlor and metolachlor calibrators; logisitic parameters derived from the alachlor and metolachlor standard curves are shown in Table 1. Because the same zero standard, antibody-coupled magnetic particles, and conjugate were used with the alachlor and metolachlor calibrators, the A and *D* parameters for the metolachlor fit were constrained to be equal to those derived for alachlor. Correlation coefficients, *r,* for the standard curve fits were greater than 0.99 for both alachlor and metolachlor. The *b1, b2* values in Table 1 are reaction coefficients for alachlor and metolachlor, respectively, in the alachlor assay (Equations 1 and 2).

Concentration recoveries for alachlor in the alachlor-metolachlor samples calculated with the parameters in Table 1 using Method 1 and Method 2 are given in Table 2. The average alachlor concentration recovery was calculated using the four metolachlor concentrations (12.5 ppb, 25.0 ppb, 50.0 ppb, and 75.0 ppb); recovery and standard deviation for each alachlor level are listed under the corresponding method used for the calculation. When viewed as a function of the expected alachlor recovery level, Method 1 over-recovered at lower concentrations and under-recovered at higher concentrations; Method 2 over-recovered for all concentrations tested. Recovered concentrations for the two methods were normalized to expected values to obtain the average percent recovery at each alachlor level (average of 100% x calculated concentration divided by expected concentration) and an average percent recovery over the 16 alachlor-metolachlor mixtures (Table 2). For the 16 samples normalized to the respective concentrations, Method 2 had a group-average percent recovery of 122% *versus* 104% for Method 1.

Table 1. Values for the four-parameter model obtained for alachlor and metolachlor.

Parameter	Alachlor	Metolachlor
A	1.1705	1.1705
b1, *b2*	0.827	0.567
ED50 (ppb)	0.526	57.33
D	0.1023	0.1023
r	> 0.999	0.998

Table 2. Average concentration (in ppb) and standard deviation (SD) of alachlor recovered from alachlor-metolachlor mixtures for methods 1 and 2. The average percent recovery is also given for each level of alachlor.

[*Alachlor*] expected	Method 1 [*Alachlor*] recovered/SD	Method 1 Average %recovery	Method 2 [*Alachlor*] recovered/SD	Method 2 Average %recovery
0.5	0.56 (0.05)	112	0.70 (0.09)	140
1.0	1.13 (0.13)	113	1.33 (0.17)	133
2.0	1.92 (0.08)	96	2.16 (0.13)	108
3.0	2.89 (0.13)	96	3.18 (0.13)	106
	Group-average % recovery	104	Group-average % recovery	122

Table 3. Average percent recovery of the four alachlor levels averaged for a given metolachlor concentration for Methods 1 and 2.

	Method 1	Method 2
[*Metolachlor*]	Average percent alachlor recovery	Average percent alachlor recovery
12.5 ppb	94	105
25.0 ppb	108	123
50.0 ppb	110	130
75.0 ppb	106	129

To determine whether the recoveries were correlated to the metolachlor levels, the average percent recovery for alachlor was calculated for the four alachlor levels at a given metolachlor level (Table 3). Both methods show the lowest average percent recovery at 12.5 ppb metolachlor (94% vs. 105%). At 25, 50, and 75 ppb metolachlor, both methods over-recover, however, Method 2 over-recovered compared to Method 1 at all metolachlor concentrations. It should be noted that experimental error did not appear to be a significant factor in the over-recovery or under-recovery trends.

Based on the results in Tables 2 and 3, Method 1 provides relatively accurate alachlor recoveries for the samples tested. Recoveries determined by Method 1 are more accurate than those using Method 2; the main reason is apparently due to the weight that Method 2 gives metolachlor in the alachlor assay. One consequence of the assumptions made in Method 2 is that metolachlor is weighted in the alachlor assay with an equivalent concentration given by $ED50_{ALA}([Metolachlor]/ED50_{MET})^{b2/b1}$ (Jones *et al.,* 1994). Conditions in the alachlor assay *([Metolachlor]/ED50* $_{MET}$ < 1 for 12.5, 25, and 50 ppb, and *b2/b1* < 1) cause the metolachlor contribution to be overestimated in the response, which results in an over-recovery of alachlor. Method 1 does not suffer from this problem because the exponents, *b1* and *b2,* are associated only with their respective analytes. An alternative formulation of Method 2 may alleviate this weighting problem (Jones et al., 1994).

For the application described here, an assay with very low alachlor cross-reactivity (Metolachlor RaPID Assay) is available to determine a sample's metolachlor concentration; once the metolachlor concentration is known, Method 1 can be used to determine the sample's alachlor concentration from the Alachlor RaPID Assay. For general application to other analyte pairs, however, it may be necessary to determine both analyte concentrations simultaneously. To use Method 1 for a sample with two unknown analyte concentrations requires two assays with different reactivities for the respective analytes and assay parameters for both analytes in the respective assays (see Table 1). Concentrations for two analytes in a sample can then be found by simultaneous solution of two equations (see Equation 8) using the assay parameters and the sample's absorbance values measured in both assays.

Extending Method 1 to determine the concentration of *k* analytes in a sample requires having *k* antibodies and assays optimized for the respective analytes. With reaction coefficients and ED50s determined for each analyte for a given antibody, concentrations of the *k* analytes in the sample could be calculated from the responses of the *k* assays. Sample response in the i^{th} assay would be given by

$$B_i = \frac{A_i - D_i}{1 + \sum_{n=1}^{k} \left(\frac{[Analyte_n]}{ED50_{n,i}} \right)^{b_{n,i}}} + D_i \qquad (11)$$

where B_i is the response of the i^{th} assay, i = 1, ..., *k* and *k* is the number of antibodies; [*Analyte*]n is the n^{th} analyte (n = 1, ..., *k*) with corresponding $ED50_{n,i}$ and slope, $b_{n,i,}$ for that analyte in the ith assay; and A_i, D_i are the zero and infinite concentration response for the i^{th} assay. The *k* analyte concentrations would be calculated from this system of *k* nonlinear equations.

With appropriate experimental design, the method described in this paper should provide adequate analyte concentration recovery (well within ±20%) in two-analyte systems. There are two practical limitations to this method for the number of analytes that can be accurately determined: first, the number of assay parameters needed for *k* analytes increases as $4k^2$, which requires substantial effort even for 3 to 4 analytes; and, second, because the number of parameters to be determined increases with the square of the number of analytes, the likelihood of error and error propagation increases significantly. Careful choice of antibodies, calibrators, and measurement technique, however, should reduce the effort and potential for error in this method and provide satisfactory results for multi-analyte concentration recovery.

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