Chapter 27

Immunoassay of Cross-Reacting Analytes

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Cross-reactivity is often seen as a drawback in the use of immunoassay for environmental analysis. We consider here the problem of identifying and quantitating single compounds and mixtures from within a large class of cross-reacting analytes, combining work by earlier authors on pattern recognition and mixture analysis. Careful choice of antibodies together with appropriate statistical analysis has the potential not only to overcome the cross-reactivity problem but to turn it into an advantage.

A great deal of effort goes into the development of antibodies which are monospecific for a particular target analyte. Often there is still some cross-reactivity with other compounds of similar structure, although this may be slight and may be discounted in the analysis of field samples if the cross-reactants can reasonably be assumed to be absent. There are many classes of environmental pollutants, however, which can sometimes occur together in samples and which are so similar in structure that 100% monospecificty is difficult to obtain. Furthermore, the number of members in the class may be so large that the development of specific antibodies to each one is extremely costly, and the subsequent monitoring of each member of the group by a different assay may be inefficient and slow.

One such class of compounds is the triazine herbicides. Triazines are used in large quantities throughout wide areas of the world. In 1991, eight different triazines were in use in the State of California alone; the total amount being nearly 1.4 million pounds (*1*). Some triazines have been found in groundwater samples from California, Iowa, Maryland, Nebraska, Pennsylvania and Wisconsin at concentrations above health advisory levels (*2*). There is a need therefore for a general groundwater monitoring program for pesticide contamination. This will involve analysis of a large

number of samples for many different pesticides, and will therefore require a simple, inexpensive and rapid method that can identify and quantitate a range of analytes. Enzyme-linked immunosorbent assay (ELISA) has been shown to be a sensitive analytical tool for such pesticide analysis (*3*), although it is generally thought to be a single-analyte method. By generalizing the format to the use of an array of antibodies with different patterns of affinity for the target class of compounds, it is possible to retain many of the advantages of low cost and rapid process time of single-antibody immunoassay, without the need for complete monospecificity. Moreover the number of antibodies required will typically be less than the number of distinct analytes in the class, so that the procedure is potentially more efficient than the use of many monospecific antibodies.

Investigation of multiple immunoanalysis using an immunoarray, a panel of less selective antibodies with differing affinity patterns, has been proceeding in two slightly different directions. One is the identification and quantitation of single-analyte samples, where the sample submitted for analysis is assumed to contain one unknown analyte from a large class of possible candidates. The reponses to the immunoarray are used first to identify the analyte and then to estimate the concentration. The general approach is discussed by Kauvar (*4*). Cheung *et al.* (*5*) give some experimental results, using a number of different multivariate statistical techniques to select the identity of test samples. Karu *et al.* (*6*) present an overview and evaluation of various alternative methods of analysis. Wortberg *et al.* (*7*) describe the construction and application of an immunoarray for the triazine herbicides, concentrating on the problem of selecting a suitable small set of antibodies and giving an approximate statistical criterion on which to base decisions.

An approach with a slightly different emphasis has been that of mixture analysis. Here, samples are assumed to contain possible mixtures of analytes, with the components of the mixture coming from a known small set of cross-reacting analytes (usually no more than four). Mixture analysis requires at least as many antibodies as there are components in the mixture. The approach was proposed by Rocke (*8*) and implemented by Muldoon *et al.* (*9*) for mixtures of three triazines at the high levels typical of pesticide waste rinsate. A model for mixture analysis was given by Jones *et al.* (*10*), and implemented successfully for mixtures of up to four analytes at low ppb levels (*11*).

Either one of these approaches may prove useful in a particular setting, depending on the assumptions one is able to make about the possible identity of the sample and the likely complexity of a mixture. It is helpful, however, to have a unified approach to the modeling and analysis of immunoarray responses, so that appropriate decisions can be made and tested. The situation is of sufficient complexity as to require the close cooperation of immunologists and statisticians to obtain the best possible assay design and a reliable method of data analysis.

The assays used in our description are competitive ELISAs with a coating hapten format conducted on a 96-well microtiter plate, with the response, an optical density, being read by an automatic photometric plate-reader. Details of equipment, reagents and experimental procedures may be found in the literature cited (*7*). The general ideas we present for modeling and analyzing multiple immunoassay response

are not necessarily limited to this format, but may be extended to other assay types with appropriate modification.

Methods

Quantitative immunoassay is a controlled calibration experiment in which standard laboratory-prepared samples of known concentration are assayed together with unknown samples. The responses from the standards are used to estimate a relationship between sample concentration and assay response. This is then inverted to estimate the concentrations of the unknowns from their responses. In the case of multiple immunoassay, the response from each sample is a vector $Y= (Y_1, Y_2,...,Y_n)$ in which each component is the optical density when the sample is assayed with a different antibody.

Taking the simplest case of two antibodies, the responses (Y_1, Y_2) for each sample can be represented on a two-dimensional plot as in Figure 1. Suppose we have standards for two analytes. Provided the antibodies used have different affinity patterns for the analytes, the responses will tend to occur in different regions of the two-dimensional space. If we now add the response from an unknown sample, its position in this space should inform us as to its identity: it will either "look like" Analyte 1, or like Analyte 2. If it looks like neither, we might conclude that it is a mixture (assuming no other cross-reactants). We need a reasonable method or algorithm for deciding among these alternatives.

This is both a calibration and a discrimination problem, and a number of methods have been suggested for tackling it (*6*). Some, such as discriminant analysis, have been taken from standard statistical practice, and inevitably ignore some aspects of the problem. In fact much is known already about the nature of immunoassay response. Our approach is to use this knowledge to form an explicit statistical model, with explicitly-stated assumptions, so that coherent rules can be derived regarding the decision of identity of the unknown sample and the estimation of its concentration. More "automatic" methods can also achieve good results, and are attractive because of the apparent lack of need for assumptions or an algorithm. In fact, there is always an algorithm, and there are always assumptions, although these may be hidden from the user and may, if made explicit, be found to be inappropriate.

Response Paths. One obvious fact concerning the multivariate response Y from a single analyte is that, as the concentration increases, the expected response will trace out a path in n-dimensional space, starting from a point representing maximal response at zero concentration and finishing at minimal response (corresponding to non-specific binding). It seems sensible to use these response paths, rather than the points corresponding to a few particular concentrations, to make our decisions. The paths are clearly nonlinear, so that the pattern of responses to the immunoarray changes with analyte concentration; any attempt to use pattern-matching techniques borrowed from the analysis of spectra, as for example in spectroscopy, must take account of this. One could employ some simple smoothing technique, such as smoothing splines, to estimate the paths; again, the use of this automatic method does not necessarily treat errors or departures from the true path caused by experimental

variation in an appropriate way. We know that the errors in the individual responses $Y_1,...Y_n$ are independent since they are derived from independent assays, and it is reasonable to assume that the likely size of the error depends on the response. It is often assumed that enzyme immunoassay errors have constant coefficient of variation (cv). The expected response for each individual assay is S-shaped, and can usually be represented reasonably well by a parametric curve (*12*). We next consider how to build this prior knowledge into our analysis.

Statistical Model. As with any fully-specified statistical model, two components need to be considered in modeling immunoassay response. The first is the deterministic part, which specifies the expected or typical response level Y for a given concentration of the analyte; it is this part which is used to produce the characteristic response path of the analyte. It is usually given as a mathematical function of analyte concentration and some other parameters which depend on the affinity of the antibody for the analyte and other immunochemical conditions. Some practitioners rely on a linear function, restricting the range to "the linear part of the curve". This is not appropriate for multiple immnoassay since even a null response for one component may be informative about the identity of the analyte. A common choice of function for modeling the whole curve is the four-parameter log-logistic model (*12*):

$$Y = \frac{A - D}{1 + (x / C)^B} + D$$

where x is the analyte concentration, A and D are the maximum and minimum responses (corresponding to zero and infinite concentration respectively), C is the concentration which gives 50% inhibition of the signal (commonly the IC_{50}) and B is a slope parameter. The parameters A, B, C and D have to be estimated from the responses of the standard concentrations, to give the standard or calibration curve. It is common practice to re-estimate the curve for each experiment, although some preliminary work has been done on "borrowing" parameters from another plate (*13*).

The second component of the model is the stochastic part which represents the part played by experimental error in producing an observed response, and is important in constructing an appropriate estimation procedure and valid decision criteria. For definiteness we will assume a constant cv model in which errors are multiplicative in effect, and use a logarithmic transformation to transform to additive errors with constant variance. Specifically, we model the response Y_i of the ith antibody to a concentration x of analyte j as:

$$\log Y_i = \log\left(\frac{A_i - D_i}{1 + \left(x / C_{ij}\right)^{B_{ij}}} + D_i \right) + \varepsilon_i$$

and assume that the error term ε_i follows a zero-mean gaussian distribution with constant variance σ_i^2. The parameters A_i, B_{ij}, C_{ij}, D_i and σ_j^2 are estimated from the

standards of analyte j assayed with antibody i. We assume that the maximum and minimum binding constants A and D will be the same for each analyte, but will vary with different antibodies.

Given the response vector Y of an unknown sample, we want to determine its identity and then estimate the concentration of that particular analyte. Figure 1 suggests an intuitive approach based on the distance of the sample point from the response paths, namely to choose the analyte whose response path comes closest to the observed sample response, and take the concentration corresponding to the point on that response path where that distance is a minimum. It can be shown that, with a suitably chosen scaling, this intuitive approach corresponds to a standard statistical procedure. In the case of our model above, we would calculate the distance for each component on a log scale (because of the constant cv assumption) and divide by the estimated σ_i so that the difference in precision of different component assays is allowed for. It may also be necessary to adjust for uncertainty in the estimated standard curves. Details of the calculation, along with statistical arguments and simulation studies, are given by Jones and Rocke (Jones, G.; Rocke, D.M. *J. Am. Stat. Ass.*, submitted). With these adjustments, the squared distance (d^2) from the sample point to the correct response path will follow a known statistical distribution, whereas the distance to an incorrect path will be too large. Tabulated values of the appropriate chi-squared distribution can thus be used to decide whether a particular analyte identity is reasonable or not. Either the distance to the response path will be acceptably small or it will be implausibly large.

It is clear from Figure 1 that the method will not reliably differentiate between analytes when the response paths are close together. This occurs inevitably at large and small concentrations, but can also be caused by having similar patterns of cross-reactivity to the antibodies used, so that the response paths of the analytes are always close together. Thus, there will be a range of concentrations, as in single immunoassay, within which analysis is feasible. For this range to be useful in practice, a good combination of antibodies in the immunoarray which can look at the analytes in different ways is required.

Mixture Analysis. If all the single-analyte identities for a given sample are found to be inadequate (i.e. the sample response is too far away from all the individual response paths) the obvious conclusion would be that the sample contains a mixture of analytes. One possible next step would be to try a separation technique such as liquid chromatography (*14*). However, it is also feasible to investigate the possibility of simple mixtures using the immunoassay responses already obtained. The extended four-parameter log-logistic model (*10*) enables us to estimate the response paths (now response surfaces) for mixtures using only the standard curves for the individual analytes. Thus, using the existing data we can now examine possible mixtures to see if they give a plausible identity for our sample. For any given mixture we find the concentrations of the components in the mixture which minimize the distance from the sample point to the response surface for that mixture, and refer the minimum distance to the appropriate statistical distribution.

In practice there will be a very large number of possible mixtures (e.g. with eight individual analytes there will be 2^8-9=247 different mixtures), so it seems

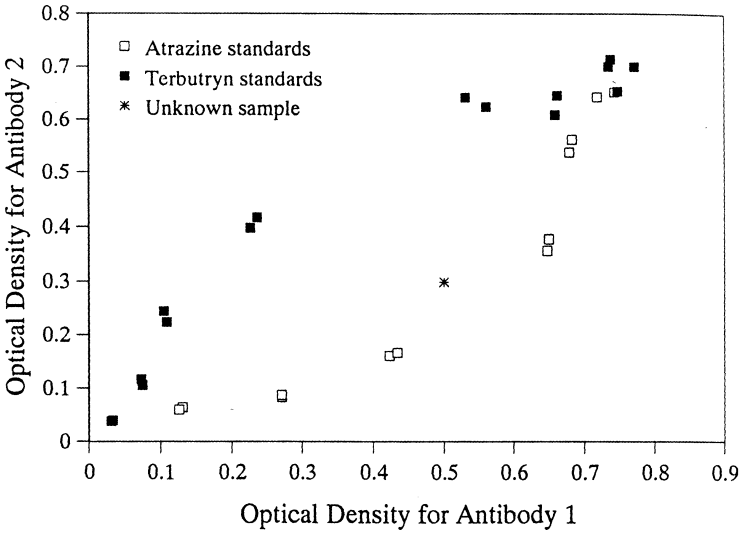


Figure 1. Response paths for atrazine and terbutryn with two antibodies (standard concentrations were a dilution series of each analyte in duplicates, with zeros and blanks).

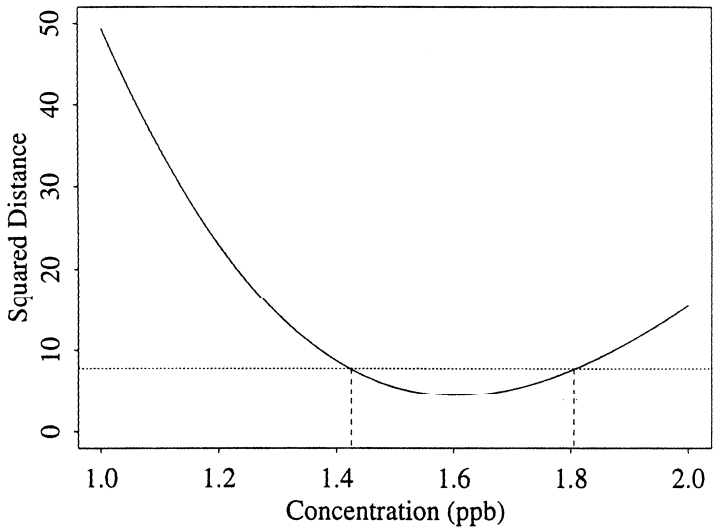


Figure 2. 90% confidence interval calculation for an estimated concentration (the dotted line represents the cutoff value, which is the upper 10% point of a chi-squared distribution with four degrees of freedom).

sensible to order them in some way. If it is believed that simple mixtures, with few components, are more likely than complex ones, the following iterative procedure could be followed:

(1) Assay unknown samples together with standards for all analytes under consideration using each antibody in turn, with separate microplates being used for each antibody assay.

(2) Estimate the standard curve parameters A_i, B_{ij}, C_{ij}, D_i and σ_i^2 for each antibody.

(3) Calculate the minimum distance from the sample point to each single-analyte response path, together with the analyte concentration which gives this minimum. This requires the use of a nonlinear minimization computer routine, many of which are available in standard statistical packages and subroutine libraries.

(4) By comparing d^2 with the appropriate statistical distribution, find which, if any, of these distances is plausible (see examples below). Report all plausible analyte identities together with the estimated concentration.

(5) If no single analyte is found to be plausible in (4), return to (3) but substituting binary mixtures for single analytes.

(6) Repeat until the number of components in the mixture equals the number of antibodies in the immunoarray or the total number of analytes, whichever is smaller.

Results

We have applied the above methodology to single-analyte samples and binary mixtures in the class of triazine herbicides and their derivatives, using immunoarrays of four or five mono- and polyclonal antibodies and up to eight candidate analytes with concentrations in the range 0.5-5.0 ppb. Experimental details are given in a previous work (*7*). To test our procedure we selected from our library of assays those which were more general in their cross-reactivity for a variety of triazines. In the case of single-analyte samples identification was usually successful and the resulting concentration quite well-estimated, although there was sometimes confusion of identity within subgroups, particularly between prometon, prometryne and terbutryn or atrazine and simazine. This occurs when subgroups of analytes exhibit similar patterns of affinity for the antibodies in the immunoarray, and is perhaps indicative of the need for a better choice of antibodies. Based on our results improved assays have been developed to distinguish among some of these subgroups. For illustrative purposes we show in Table I the complete results of single-analyte analysis (i.e. up to step (4)) for two "unknowns".

For the first sample, containing 1.5 ppb prometryne, the assay procedure is unable to decide between prometon, prometryne and terbutryn: all three have response paths which come reasonably close to the sample response. We can see however that the estimated concentration of prometryne is quite accurate. The second sample has a higher concentration of the analyte, and now the identification is unambiguous, with again a reasonably accurate estimate of concentration. The statistical analysis can be carried a stage further by evaluating the distance over a range of possible concentrations and using a 90% cutoff point to give a confidence interval for the estimate. The procedure is illustrated graphically in Figure 2, the cutoff value (the

upper 10% point of a chi-squared distribution with four degrees of freedom) being shown as a dotted line.

Table I. Multiple Immunoassay Results for Two Single-Analyte Samples

	Sample 1 (1.5 ppb Prometryne)			Sample 2 (5.0 ppb Prometryne)		
Analyte:	*Conc.*	d^2	*P-value*	*Conc.*	d^2	*P-value*
Prometon	1.05	0.94	0.816	2.69	68.52	0.000
Atrazine	1.07	214.35	0.000	2.30	414.81	0.000
Simazine	4.10	161.50	0.000	1.91	830.34	0.000
Cyanazine	0.56	415.84	0.000	1.60	848.93	0.000
OH-atrazine	0.01	396.26	0.000	0.02	1197.84	0.000
Prometryne	1.60	4.49	0.213	4.12	2.05	0.562
Terbutryn	1.31	1.41	0.703	3.29	12.75	0.005
DEatrazine	0.01	395.74	0.000	0.05	1197.40	0.000

Samples containing binary mixtures of analytes are, as expected, more difficult to identify. Often there were a number of possible identities for the samples, with the problem of confusion within subgroups being compounded. Thus for example a mixture of atrazine and prometon might look like simazine and terbutryn, or atrazine and prometryne. In most cases, mixtures were clearly identified as such, i.e. not as single analytes, although mixtures of prometon and terbutryn, or prometryne and terbutryn, were incorrectly classified as containing terbutryn only. Table II shows the acceptable results for a mixture of 1 ppb atrazine with 1 ppb cynazine. There were eight candidate analytes, so 28 possible binary mixtures. The computer searches through all 28, looking for acceptable solutions. There were three acceptable solutions found, one being the correct identity.

Table II. Multiple Immunoassay Results for 1 ppb Atrazine + 1 ppb Cyanazine

Analyte 1:	*Analyte 2:*	*Conc. 1*	*Conc. 2*	d^2	*P-value*
Atrazine	Cyanazine	0.35	1.61	1.41	0.494
Simazine	Cyanazine	0.38	1.84	1.28	0.527
Cyanazine	DIatrazine	2.03	2.46	1.16	0.560

As with single-analyte solutions, we can evaluate the distance over a grid of values to produce a confidence region for the concentration estimates. If we add contour lines corresponding to different distances, we can see how the estimation of one analyte concentration affects the other (see Figure 3). In the case of atrazine and cyanazine the estimates are correlated so that over-estimation of one component causes under-estimation of the other component. We can see that the point estimates

of the atrazine and cyanazine concentrations are not very accurate, but that the total amount is well-estimated.

Discussion

We have demonstrated an approach to the immunoanalysis of samples containing one or more members of a group of cross-reacting analytes, using a parametric model for the assay responses and making some explicit assumptions about the way in which experimental error affects them. Other approaches could also be taken. For example, neural networks have also been applied to the analysis, with the inputs being either the untransformed responses (*6*) or an estimated concentration of a chosen reference analyte (*15*). The results so far have been similar to ours in terms of correct identification and concentration accuracy, although with neural networks the algorithm and assumptions are hidden from the user so that there is no way of telling whether they are appropriate, and no way of applying statistical tests or calculating confidence intervals. It seems that the most important limiting factor on the reliability of these methods at present is the discriminatory power of the immunoarray used. We now consider some aspects of this.

Since mixture analysis requires at least as many antibodies in the immunoarray as there are components in the mixture, it might be supposed that our antibody array should be as large as the class of candidate analytes. If this were the case, we would clearly be better off using the same number of monospecific antibodies, and this multiple immunoanalysis would be useful only for those cases where specific antibodies were unavailable. However, there may be situations in which one might want to perform the kind of analysis described above, in which a small immunoarray is capable of differentiating between subgroups of the analyte class. If necessary more specific antibodies could be used at a second stage for any samples not clearly identified at the first stage, or else the ambiguous cases could be submitted to a different form of analysis. Alternatively, if one is prepared to believe that simple mixtures, with only two or three components at the most, are more likely than complex mixtures, then a small immunoarray would again suffice, with any unresolved samples being submitted to a second stage. Such a procedure could be much more efficient than many single immunoassays while still providing an effective analysis for the majority of samples.

One might suppose that more antibodies would always give better resolution, but this is not necessarily the case. Each additional antibody adds both signal and noise to the system, and if the cross-reactivity pattern of the new antibody is not sufficiently different from those already present, the additional information it provides will be swamped by the increase in noise, leading to a deterioration in performance. The most important consideration then is to choose antibodies which contribute independent or near-independent information; geometrically this means that their cross-reactivity patterns, measured perhaps by their IC_{50} "spectra", should be as close to orthogonal as possible. It would be relatively straightforward to construct a mathematical optimality criterion along these lines, and by searching through all possible choices to construct an "optimal" immunoarray, but the nonlinearity complicates this. In practice, each assay has a limited workable range, and the

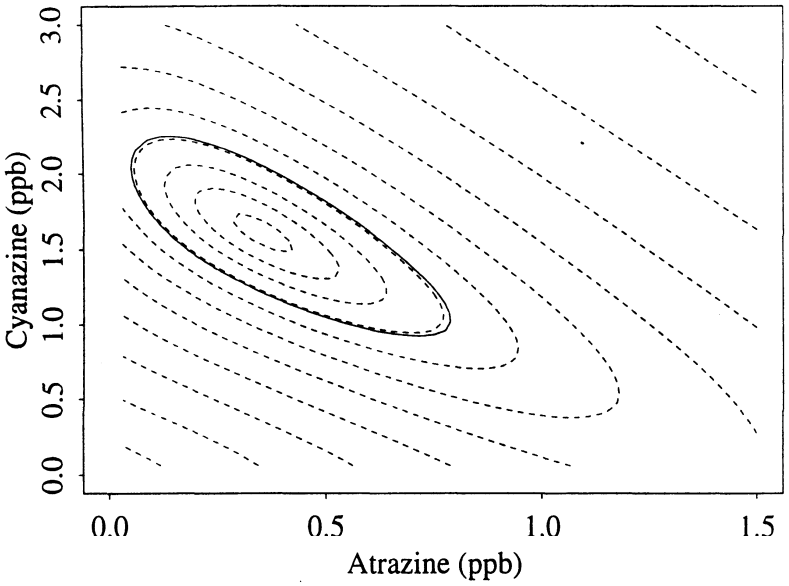
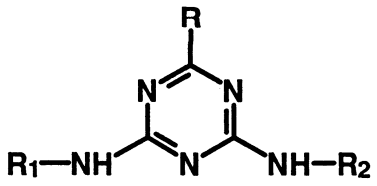


Figure 3. 90% confidence region for a binary concentration estimate (the true value of (1,1) is inside the 99% region).



triazine	R	R_1	R_2
atrazine	Cl	isopropyl-	ethyl-
propazine	Cl	isopropyl-	isopropyl-
simazine	Cl	ethyl-	ethyl-
cyanazine	Cl	ethyl-	$NHCCN(CH_3)_2$
prometryn	$S\text{-}CH_3$	isopropyl-	isopropyl-
prometon	$O\text{-}CH_3$	isopropyl-	isopropyl-
terbutryn	$S\text{-}CH_3$	tert. butyl-	ethyl-

Figure 4. Molecular structure of triazine herbicides.

overlapping or intersection of these ranges is also important. It would probably be advisable to weaken the sensitivity of some of the assays in order to have a suitable range of accurate identification and quantitation for each analyte.

The performance of an immunoarray itself provides information on where the resolution is weakest, for example in the existence of indistinguishable subgroups, thus helping to direct research toward the development of reagents most valuable for multianalyte determination. In practice the immunoarray might be tailored to fit the requirements of a particular geographical location based on known triazine usage. Basing our procedure on a fully-specified statistical model enables decisions to be made concerning the performance of a particular hypothetical immunoarray, including whether it would be desirable to add more antibodies to an existing array.

A complementary approach to the development of immunoarrays is to consider the molecular structures of the analytes; antibodies can perhaps be chosen or developed to bind to particular molecular moieties or substituents, so that classification uses recognition of details of the molecular structure of the analytes. This allows the possibility of designing an immunoarray with deliberately-created less-specific antibodies so that the cross-reactivities are used positively in an efficient assay system. The concept can be well illustrated by using the triazine example (see Figure 4): antibodies capable of distinguishing among the common substituents R at C-2 on the triazine heterocycle will define several major classes while other antibodies selective for R1NH or R2NH groups at C-4 and C-6 will define subclasses. Such work, requiring the close collaboration of immunologists, synthetic chemists and statisticians, could provide another useful immunochemical tool to assist in the efficient and cost-effective analysis of complex environmental samples.

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