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An approach to the construction of an immunoarray for differentiating and quantitating cross reacting analytes

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bstract

We demonstrate the construction of an immunoarray to categorize, identify and quantitate different triazine herbicides or their environmental metabolites in water. In a first step cluster analysis is used to examine the performance of a small subset of antibodies out of a larger library, and hence to select a small array that is capable of categorizing the triazine herbicides into different groups. At the 1 ppb level it is possible to categorize an analyte as a chloro-*s*-triazine, a hydroxy metabolite, or as a methoxy/methylthio substituted triazine when using only 2–4 antibodies. At higher concentrations even the identity of the triazine can be determined with the same number of antibodies. A selected combination of antibodies is then used to identify and quantify unknowns by comparing their immunoassay responses to an array of calibration curves and using the maximum likelihood criterion.

Keywords: Immunoarray construction; Triazine herbicides; Herbicides; Environmental analysis

1. Introduction

Triazines are a heavily used class of herbicides throughout the world, and atrazine is the most commonly applied single herbicide in the US alone. Although it is most likely to find atrazine when one is analyzing soil or water samples for presence of triazines, chances are good that one will find instead or in addition to atrazine one of a number of compounds such as simazine, cyanazine or their metabolites. Gas chromatography (GC) [1,2], liquid chromatography (LC) [3–5] or capillary electrophoresis (CE) [7,8] are suitable methods to identify and quantitate different analytes within a class of compounds (here: triazines). For screening large numbers of samples, screening a wide range of analytes within a class of compounds and for on-site analysis, immunoassays sometimes are a more powerful and simpler tool than instrumental analytical methods. Immunoassays can either be independent methods or complement instrumental analytical techniques by sorting out contaminated samples.

Many groups have developed immunoassays for triazine herbicides such as atrazine [9–12], cyanazine [13], terbutylazine [14], terbutryn [15], hydroxyatrazine [16,17], or *N*-dealkylated triazines [18–20]. Cross reactivity to any other triazine herbicide besides the one an antibody has been developed for is

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disadvantageous if the triazine present in a sample is not the target analyte. The data obtained will be incorrect, because one immunoassay alone may lack the discriminatory power to distinguish among cross reacting compounds. On the other hand, cross reactivity is a very useful feature for the quantitation of multiple analytes of the same chemical class since structurally similar compounds will most likely cross react. Especially in the case of small analytes such as pesticides, many cross reacting compounds may exist for a given antibody. With many compounds it is possible to design immunizing haptens that will give assays that are specific for a single molecule or will detect a range of related structures.

Recently, several groups have used cross reactivity of antibodies to perform multianalyte immunoassays within a class of compounds [21–23]. Muldoon et al. [21] assayed concentrated ternary mixtures of atrazine, simazine and cyanazine in pesticide waste and rinsate using multiple regression. They assumed that the log standard curves of different analytes are parallel. In their multianalyte ELISA (MELISA) Jones et al. [22] extended the four parameter log-logistic curve to mixtures where log parallelity was not assumed. Wortberg et al. [23] applied the MELISA-methodology of [22] for analysis of ternary and quaternary mixtures of triazines at low to sub-ppb levels.

Multivariate statistical analysis as a means of identifying (and sometimes quantitating) analytes is a well-established method. An example for the identification of triazine herbicides by pattern recognition has been described in [24]. The underlying assumption is that the characteristic pattern generated by each analyte is consistent over a certain range of its concentration. A good overview over different mathematical approaches to multivariate analysis is given in [25].

The MELISA-approach to mixture analysis is equivalent to maximum likelihood estimation (MLE) if the number of antibodies used exceeds the number of analytes. Assaying a mixture requires at least as many antibodies as there are components in the mixture. Thus, if there is a large set of possible analytes, a full mixture analysis becomes costly and difficult. The simultaneous quantification of four analytes seems to be the limit, both in terms of accuracy and cost due to the number of antibodies and calibration curves involved. It would be advantageous to have a method that allows categorizing analytes into certain subgroups and thus narrows down the number of antibodies needed for MELISA. A subgroup would comprise analytes with similar substitution patterns or the same functional group, thus highly cross reacting analytes.

Very importantly, categorization and identification of an analyte by immunoassay would be a novel confirmation method for conventional single analyte immunoassays. The immunoarray could replace existing confirmation strategies which now are based on GC, GC–MS or HPLC.

The categorization would also help select the appropriate method in case instrumental analysis is still needed. For example, one would use different HPLC methods for chromatographic analysis of metabolites (hydrophilic) than for parent compounds (more lipophilic). If one were using an immunochemical detection system for HPLC, prior categorization would facilitate selection of the optimum assay for postcolumn detection.

It is desirable to find a small subset of antibodies from a larger pool which will differentiate sufficiently between analytes. In practice one may have prior knowledge of likely candidates for inclusion, i.e., the cross reactivity patterns may indicate the potential usefulness or uniqueness of a certain antibody. Cheung et al. [24] suggest principal component analysis (PCA) as a means of eliminating antibodies with little to contribute. Our approach is to use cluster analysis to examine the performance of selected antibody subsets. Since the cluster method enables immediate visual assessment of the results, it is a useful and user friendly tool, allowing many possible combinations to be compared. As Karu et al. [25] point out, cluster analysis is not suitable for quantitation of unknowns. We suggest that it is useful for the above task of selecting antibodies, and with an appropriate metric, which we derive below, can be interpreted in terms of statistical likelihood. This gives the advantage of a firm basis for deciding whether two samples are different or identical, based on the actual assay responses. We demonstrate our methodology and the application of the resulting assay to triazine herbicides. Although we focus on triazine herbicides, they are meant to serve as an example representing a more general problem.

As the cross reactivity of antibodies among compound classes or groups of compounds becomes less, the power of this technique increases. We have selected assays in common use and/or assays which we feel are typical of those generated in the field.

2. Methods

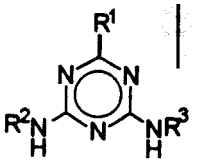
2.1. Materials

The monoclonal K1F4 antibody [15] was kindly provided by B. Hock and T. Giersch (TU Weihenstephan, Germany), AM7B2.1 [29] was donated by A. Karu (University of California, Berkeley, CA). The polyclonal antibodies 194, 841 and 842 were produced by Harrison et al. [9]. The polyclonal antibody 2652 was produced by Lucas [18], the polyclonal 4653 is described in [30]. Five of the triazine herbicide derivatives were synthesized by Goodrow et al. [31], one by Muldoon et al. [20]. Triazine herbicide standards were from Ciba-Geigy (Greensboro, NC). Horseradish peroxidase (HRP) conjugates of anti-mouse IgG and anti-rabbit IgG as well as ovalbumin grade VI, crude ovalbumin, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), and tetramethylbenzidine (TMB) were purchased from Sigma (St. Louis, MO). Dimethylformamide (DMF) and dimethylsulfoxide (DMSO) of LC grade and *N*-hydroxysuccinimide (NHS) were obtained from Aldrich (Milwaukee, WI). Buffer reagents of analytical grade were purchased from Fisher Scientific (Fair Lawn, NJ). For purification of ovalbumin–hapten conjugates we used 5 ml Presto desalting columns (Pierce, Rockford, IL). Microtiter plates were obtained from Nunc (Denmark). For reading the optical densities we used a Molecular Devices UVMax Reader (Sunnyvale, CA) equipped with the standard ELISA software Softmax. Cluster analysis and MLE were performed with the S Plus software package.

2.2. Analytes

Triazine herbicides are derived from cyanuric chloride by substitution of 2 of the chlorine atoms with alkylamino groups. Additionally the third chlorine may be substituted with a methoxy or methylthio group. The general structure of triazine herbicides as well as the structures of the eight triazines used in this study are shown in Table 1. Atrazine,

Table 1
Structure of the triazine herbicides used in this study



Analyte	R^1	R^2	R^3
Group 1: "azines"			
Atrazine	Cl	ethyl	isopropyl
Cyanazine	Cl	ethyl	(cyano)isopropyl
Simazine	Cl	ethyl	ethyl
Group 2: hydroxymetabolites of "azines"			
Hydroxyatrazine	OH	ethyl	isopropyl
Group 3: dealkylated "azines"			
Deethylatrazine	Cl	H	Isopropyl
Group 4: "tryns/tons"			
Prometon	OCH_3	isopropyl	isopropyl
Prometryn	SCH_3	isopropyl	isopropyl
Terbutryn	SCH_3	ethyl	tert. butyl

The classification into 4 groups was done according to similarities and differences of the substitution patterns. The group "names" were derived from the compounds' common names.

simazine, deethylatrazine and cyanazine all have one Cl-atom as R^1. In hydroxyatrazine the third chlorine is substituted with a hydroxy group. Prometon bears a methoxy group in the R^1 position, terbutryn and prometryn a methylthio group. The differences in positions $R^{2/3}$ are less distinct.

Abiotic degradation of chloro-*s*-triazines leads to the loss of the third chlorine and formation of a hydroxy metabolite [26]. Bacterial or fungal degradation in soil leads to *N*-dealkylation and therefore to the loss of one or both alkyl side chains [27,28]. Roughly, triazine herbicides can be grouped into four different structural "classes": (1) one chlorine left ("azines"), (2) hydroxy metabolites of (1), (3) *N*-dealkylation products of (1) or (2), and (4) methylthio/methoxy substituted ("tryns/tons").

2.3. Antibodies and haptens

We utilized several different anti-triazine herbicide antibodies, both monoclonals (mAb) and polyclonals (pAb) which have been characterized elsewhere. Table 2 shows the cross reactivities of the antibody-hapten systems used here. The pAb 194 prefers atrazine and prometryn [9], 841 and 842 show the highest cross reactivity for atrazine and simazine [9], whereas the mAb AM7B2.1 [31] prefers cyanazine and atrazine. MAb K1F4 prefers analytes substituted in the R^1-position (terbutryn, prometryn, prometon) but cross reacts with some other analytes as well [15]. Pab 4653 reacts almost exclusively with hydroxyatrazine, but at higher concentrations prometon can be also detected [30]. Pab 2652 detects only *N*-monodealkylated triazines, to a lower extent also didealkylated ones [18]. The last two antibodies are almost specific, so that their incorporation should largely facilitate the pattern recognition process and improve the cluster analysis. However, the antibody for the *N*-dealkylated compounds is insensitive compared to the other antibodies in terms of limit of detection. Therefore, quantitation of low concentrations of deethylatrazine will be difficult without better reagents.

2.4. Coating hapten format enzyme-linked immunosorbent assays for cluster analysis

The assay and the synthesis of ovalbumin–hapten conjugates (coating haptens) has been described previously [23]. Plates were coated with ovalbumin–hapten conjugates and the surface of the wells was subsequently blocked with ovalbumin.

The competitive ELISA comprised 3 steps: competitive incubation of sample together with the specific antibody, introduction of a secondary HRP-labeled antibody and conversion of the enzyme substrate hydrogen peroxide into a colored product by using TMB. Optical densities (ODs) were read at 450 nm with a 650 nm background correction. For each antibody the assay was optimized in a way that the curve midpoints (IC_{50}s) for the main analytes were as similar as possible to achieve comparable limits of detection for all analytes. As mentioned above this was not possible with pAb 2652 which had a 10 times higher limit of detection than the other antibodies.

Table 2
Cross reactivities of the antibodies AM7B, K1F4, 194, 841, 842, 2652 and 4653 used in the study towards the chosen 8 triazine herbicides

Analyte	Antibody cross reactivity (%)						
	AM7B [32]	K1F4 [16]	194 *	841 [9]	842 [9]	2652 [19]	4563 *
Atrazine							
Cyanazine							
Simazine							
Hydroxyatrazine							
Deethylatrazine							
Prometon							
Prometryn							
Terbutryn							

The percentages quoted from the literature were converted by setting the most cross reacting of the 8 analytes to 100%. Data indicated with a * are original data. Some cross reactivities vary from literature data, because there are slight variations depending on the coating hapten.

Initially we only compared antibody responses to identical concentrations (in ppb) of the eight analytes. Standards of 1, 2 and 10 ppb of each triazine were pipetted in quadruplicate on the same plate and incubated with just one antibody. The same samples were run on several other plates, using a different antibody for each. It was important to have all samples on one plate because the raw optical densities were used for clustering, and plate to plate variation would have an effect on the absolute readings. Using four replicates of each sample allowed to split them into two sets of duplicates and treat them as independent samples in clustering. Details about the clustering procedure are described in Section 3.

2.5. ELISA for the identification of unknowns by maximum likelihood estimation (MLE)

The clustering assay helped select the final combination of antibodies for an assay that is capable of identifying and possibly quantifying unknown analytes within a class of compounds. We used the Abs AM7B2.1, K1F4, 2652 and 4653 for the following experiments.

For the MLE assays we generated standard curves for all triazines with 4 antibodies. On each plate standards of 0, 0.5, 1, 2, 10 and 10,000 ppb (= blank) were assayed in duplicate. The remaining wells were used for the samples, a dilution series of each of the 8 different triazines (5, 1.5 and 0.75 ppb). Eight wells were dedicated to negative controls. The sample concentrations were chosen to fall between calibration standard concentrations. Using a dilution series ensures that an unknown falls within the useful range of the assay. We were also interested in whether assaying a dilution series as opposed to unrelated samples would improve the performance of the assay. The "unknowns" in each dilution series were classified separately and quantitated using a maximum likelihood routine (see Section 3).

3. Statistical methods

3.1. Cluster analysis

The ODs for an unknown assayed with n different antibodies can be represented by a point $y = (y_1, y_2, \ldots, y_n)$ in n-dimensional space. Here y_i is the OD for the unknown (or an average of replicates of the unknown) assayed with antibody i. Given a number of unknowns, the method of cluster analysis can then be used to group them according to the distances between the corresponding points. It is important however to use an appropriate metric for measuring this distance.

We assume here that the ODs have constant coefficient of variation (C.V.), in which case the log transform is variance-stabilizing, i.e., log y has constant variance. If we assume further that log y is approximately normally distributed then we can write:

$$\log y = \log \mu + \varepsilon$$

where μ is the mean OD for the unknown and ε is a Gaussian error term.

The Euclidean distance d between two points y_1 and y_2 for two identical unknowns can now be expressed as:

$$d^2 = \sum_{(i=1)}^{n} (\varepsilon_{1i} - \varepsilon_{2i})^2$$

If the C.V.s are the same for assays with different antibodies, it can be shown that for the means of two k-replicates of the same unknown, $kd^2/2s^2$ will have approximately a chi-square distribution with n degrees of freedom. Here s^2 is the variance of log y estimated from the replicates on the plates. (If the variances are found to vary significantly from plate to plate, the components of the distance could be weighted appropriately; this was not found to be necessary for our data.)

By using upper percentage points of the chi-squared distribution we can now find a distance d^* within which points from unknowns which are really the same would be expected to lie with a high probability. On our cluster diagrams we show two lines representing 95% and 99.9% probability. If however the unknowns are different (either different analytes or different concentrations), the μs will be different and thus the distance between points will tend to be larger. Thus if two points on the cluster diagram are joined at a distance larger than d^*, this can be regarded as an indication that they are different.

Clearly the power of this method depends on the μs being different enough, which in turn depends on the discriminatory power of the antibodies used. By examining cluster diagrams we get a clear picture of whether a particular panel of antibodies achieves sufficient discrimination, as well as a grouping of our analytes according to their similarities. Most statistical packages which perform cluster analysis will enable the selected antibodies to be changed easily, so many different selections can be examined quickly in this way.

The statistical interpretation of the distances de-

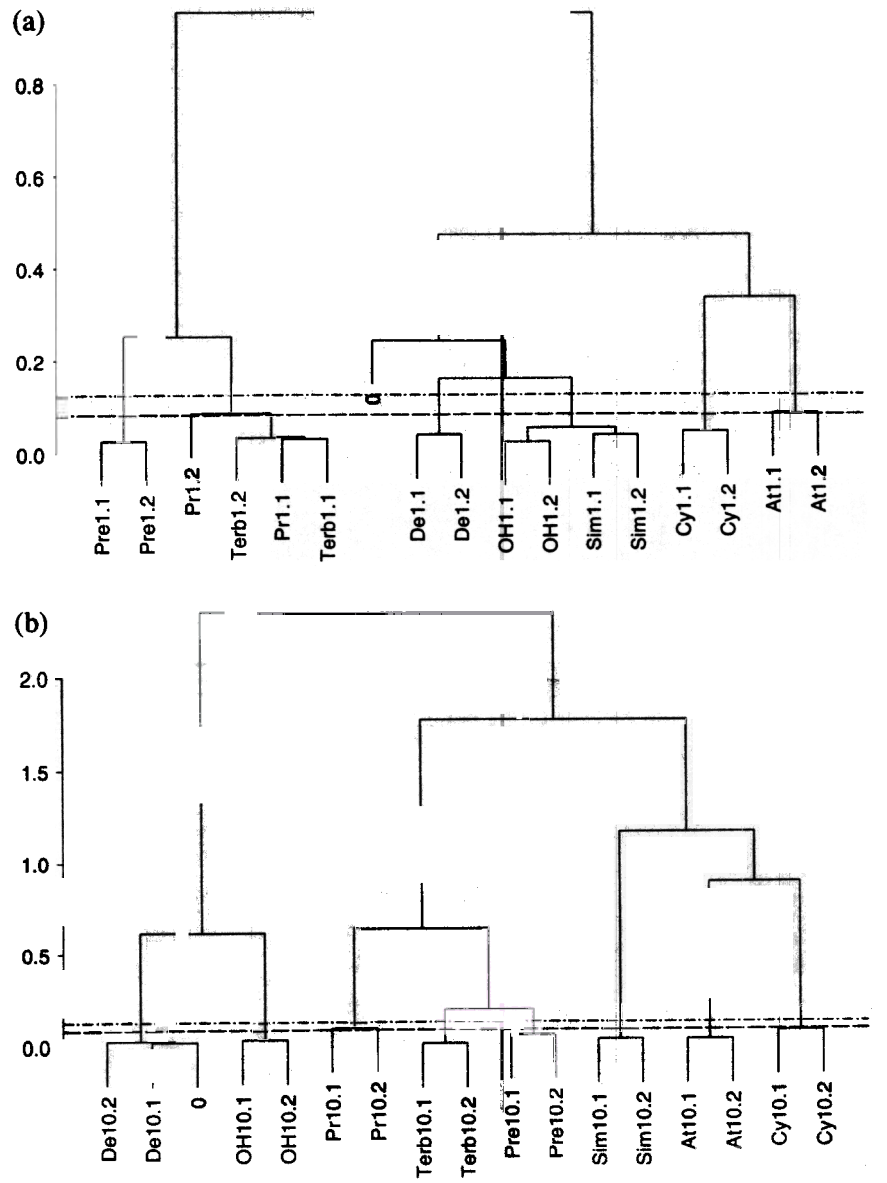


Fig. 1. Clustering diagrams of 8 triazines using the 2 antibodies K1F4 and AM7B2.1. (a) At 1 ppb and (b) at 10 ppb. The dotted lines represent distances which, with probabilities of 95% and 99.9% respectively, would not be exceeded by two samples of the same identity. Two samples are regarded (with 95% or 99.9% confidence) as identical if joined underneath the line or as different if joined above the line. The distances were derived from a chi-square-distribution. Four replicates of each sample were split in two sets of duplicates. At1.1: atrazine 1 ppb, set 1; At1.2: atrazine 1 ppb, set 2; Cy: cyanazine; De: deethylatrazine; OH: hydroxyatrazine; Pr: prometon; Pre: prometryn, Sim: simazine; Terb: terbutryn.

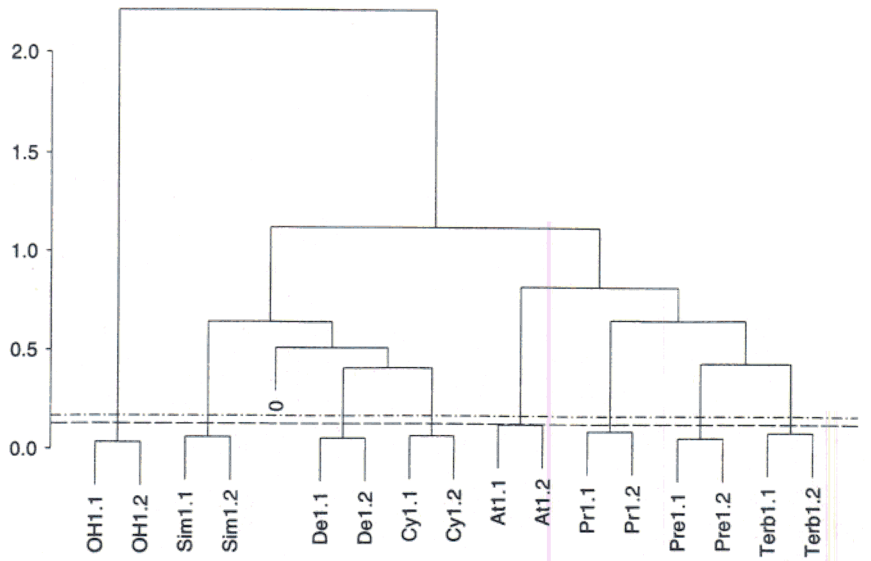


Fig. 2. Clustering diagram of the same 1 ppb samples as in Fig. 1A but using all 7 antibodies instead of 2.

pends on the many assumptions listed above. To test the appropriateness of our d^* values, we included in our diagrams pairs of unknowns which were really the same. It can be seen that in most cases these were joined at a distance less than d^*, but there are a few glaring inconsistencies. The assumptions might be violated in a number of ways; in particular we believe that outliers and spatial effects on the plate might cause problems. Nevertheless the statistical interpretation does seem to hold reasonably well for our data.

3.2. MLE analysis

Suppose we have an unknown which might be any one of m possible analytes (we assume here that it is not a mixture). We obtain the ODs y of the unknown and the $m \times n$ calibration curves, with n being the number of antibodies. As above, we assume that for each component log y_i is approximately normally distributed, its mean being given by the appropriate points on the correct calibration curves.

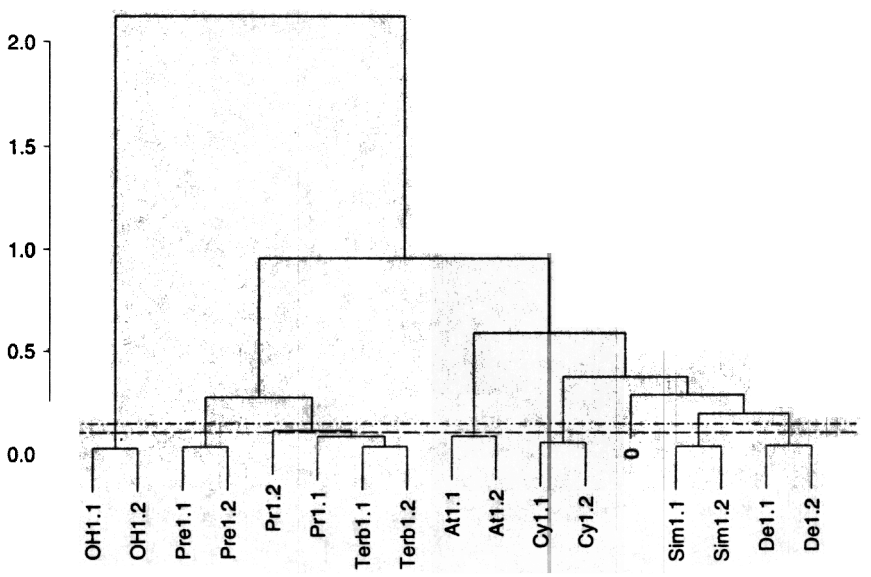


Fig. 3. Clustering diagram of the 1 ppb samples as in Fig. 1 and 2, using the 4 antibodies AM7B2.1, K1F4, 2652 and 4653.

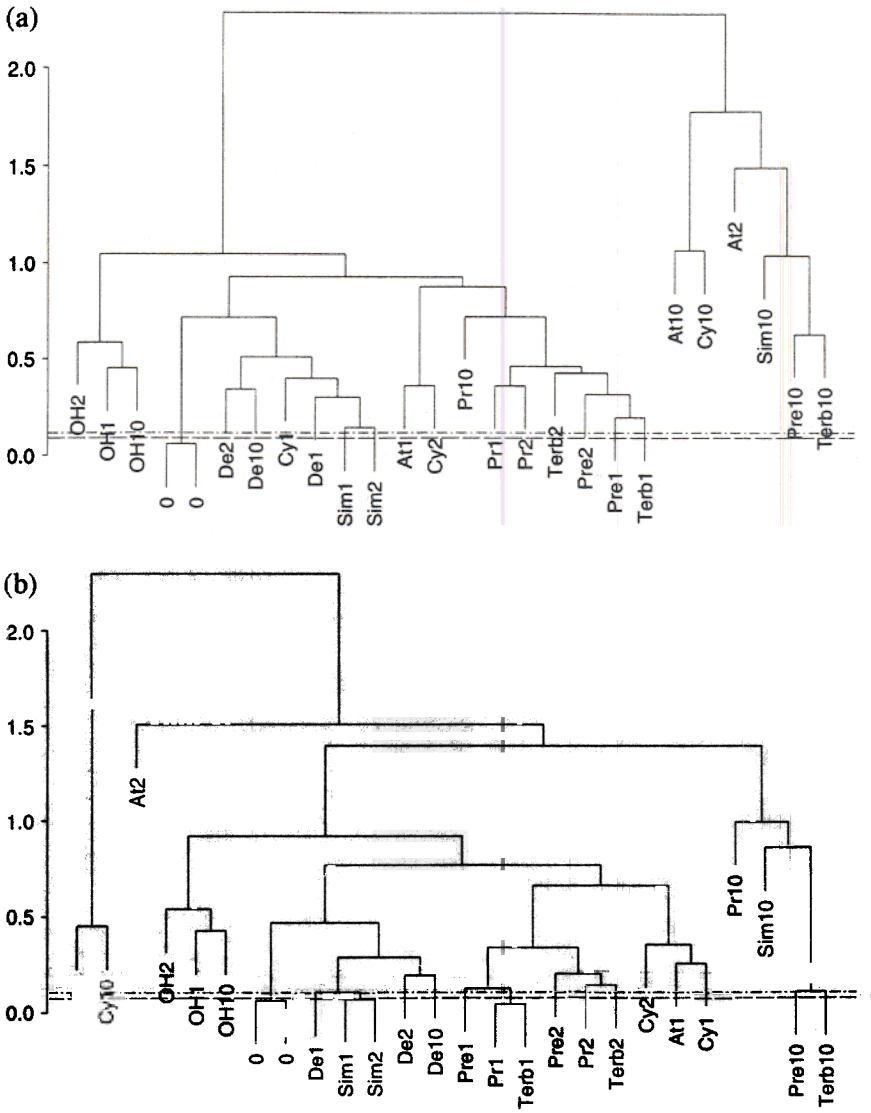


Fig. 4. Clustering of 8 triazines at 3 different concentrations (1, 2, and 10 ppb) when (a) using all 7 antibodies and (b) using only the 4 antibodies AM7B2.1, K1F4, 2652 and 4653. At1: atrazine 1 ppb; At2: atrazine 2 ppb, etc.

Identification and quantitation of an analyte is performed according to the following algorithm: for an analyte j from 1 to m, we first assume that the unknown is analyte j and then minimize the sum of squares of the distances from log y to the mean point given by the calibration curves for analyte j. This gives a least squares estimate x_j of the concentration and a minimized sum of squares F_j. Then we repeat the minimizing procedure by assuming the analyte now is a different one and repeat this for all possible analytes. Now we choose the minimum among the F_js: this gives the analyte and concentration which minimizes the sum of squares of the errors overall. This is also, by the assumption of normality of the errors, the MLE. Although we have used here a constant C.V. model, the method is easily adapted for other assumptions about the variance structure.

4. Results and discussion

4.1. Cluster analysis

In the clustering experiment 8 triazines were assayed at 1, 2 and 10 ppb using 7 different antibodies.

A zero sample was included to serve as a negative control. We subsequently performed cluster analysis on the ODs from selected subsets of antibodies. Likely antibody combinations were chosen according to their individual cross reactivity patterns. If one uses two antibodies with very different cross reactivity patterns one can gain more information about the analytes than when one uses two very similar antibodies. Also, if none of the antibodies chosen cross reacts with an analyte to be identified, categorization might be impossible or just work by an exclusion principle. Considering this, certain less useful combinations can be excluded right away.

As an example Fig. 1a shows a clustering diagram obtained with combining only the ODs of the two monoclonal antibodies K1F4 and AM7B2.1. AM7B2.1 is essentially an antibody for "azines", whereas K1F4 preferably binds to the "tryns/tons". For the plot the four replicates of each sample were split into two sets of duplicates, which were treated as independent samples. Replicates of the same analyte and the same concentration (here: 1 ppb) should cluster with themselves, which was observed. The dotted lines represent probabilities of 95% (and 99.9%, respectively) that two samples are joined below the lines if they are the same. All samples joined above these lines are considered different. The location of the lines has been derived from the chi-square distribution as described in Section 3. At the 1 ppb level the analytes fall nicely into two categories, the "azines" and all metabolites vs. the "tryns/tons". The group of the "azines" is divided in a subgroup comprising cyanazine and atrazine and a second subgroup containing simazine and the metabolites deethylatrazine and hydroxyatrazine. The zero is sorted out clearly. By the exclusion principle the two antibodies can categorize an analyte as a member of two groups or as a non-member, then forming a third category. At 10 ppb the atrazine metabolites form a separate cluster together with the zero (Fig. 1b), and all analytes can even be discriminated from each other.

When the ODs of all seven antibodies are used for clustering, all analytes can clearly be distinguished at the 1 ppb level already (Fig. 2), i.e., different analytes are joined above the lines (dissimilarity) and the replicates of the same analytes are joined below (similarity). The discriminative power of this antibody combination is superior to the one of just 2 antibodies as was to be expected. Hydroxyatrazine is sorted out very distinctly because of the presence of a highly selective antibody for hydroxyatrazine. Simazine is sorted out clearly from the other "azines", and terbutryn and prometryn also can be distinguished.

To reduce the number of antibodies but to still be able to categorize the compounds we chose antibodies K1F4, AM7B2.1, 4653 and 2652. The monoclonals K1F4 and AM7B2.1 already ensured a classification into "azines" and "tryns/tons". Including 4653 and 2652 should help categorize hydroxyatrazine and deethylatrazine, respectively. Results are shown in Fig. 3. Due to the lack of a sufficiently sensitive antibody for deethylatrazine and because of excluding the most cross reacting antibody for simazine, 842, these analytes cluster closely together at 1 ppb. Since the IC_{50} for deethylatrazine was found to be 10 ppb with 2652, identifying deethylatrazine at all at these concentrations is difficult. Terbutryn and prometon are indistinguishable now, but are separated from prometryn. Overall there is little loss in discriminative power with the four antibodies compared to all 7 antibodies. This suggests that the 3 antibodies that were left out did not contribute much valuable additional information.

So far we compared only identical concentrations for all analytes, except for a "zero" as a negative control. The actual clustering not only depends on the analyte but also on its concentration level: one analyte at one concentration might look like another at a different one. At a low concentration cross reactivity of an analyte may not yet be evident, whereas it will be obvious at higher concentrations. Thus, clustering analytes at different concentration levels will lead to different pictures, since at higher concentrations classification of an analyte becomes easier, unless the upper limit of the linear dynamic range of the calibration curve is exceeded. In our case cross reactivity of an analyte may not be apparent at the 1 ppb level but be obvious when its concentration reaches 10 ppb. To visualize the situation where different concentrations of each triazine are allowed, Fig. 4a shows clustering of 8 analytes at the concentration levels 1, 2 and 10 ppb, using all 7 antibodies. For reasons of clarity we clustered means of quadruplicates instead of two sets of duplicates.

The diagram indicates that the only obvious mix-up occurs between 1 and 2 ppb simazine. Two zero samples are identified as being the same and fall within a single cluster. When the same clustering analysis is done with only four antibodies, some discriminative power is lost (Fig. 4b). Especially the methoxy/methylthio-substituted triazines prometon, prometryn and terbutryn cannot be distinguished well, but the different concentration levels itself form individual clusters. Low simazine concentrations cannot be distinguished from low deethylatrazine concentrations. Hydroxyatrazine on the other hand forms a very distinct cluster containing all possible concentrations.

4.2. Maximum likelihood analysis (MLE)

The immunoarray was then evaluated in another experiment which was based on maximum likelihood estimation utilizing the standard curve parameters A, B, C and D. These four parameters are routinely used for immunoassays to fit the sigmoidal dose–response curves. The idea was to identify and quantitate one unknown analyte at an unknown concentra-

Table 3
Identification and quantitation of an analyte, demonstrated for three different herbicide samples

c (ppb)	Analyte chosen	F (AM7B)	F (K1F4)	F (2652)	F (4653)	ΣF^2
		−0.142	0.034	0.018	−1.566	2.473
		−0.128	0.068	0.018	−1.566	2.472
		−0.079	0.090	0.018	−1.565	2.465
		−0.027	0.079	0.018	−1.565	2.458
		−0.076	0.048	0.018	0.004	0.008
		−0.142	0.034	0.018	−1.566	2.473
		−0.142	0.034	0.018	−1.566	2.473
		−0.142	0.034	0.019	−1.566	2.473
(B) Atrazine 0.75 ppb						
0.00	Prometon	−0.182	−0.279	−0.044	0.011	0.133
0.68	Atrazine	0.002	−0.001	−0.044	0.011	0.002
2.66	Simazine	0.052	−0.058	−0.042	0.013	0.008
0.73	Cyanazine	0.076	−0.175	−0.043	0.011	0.039
0.00	Hydroxyatrazine	−0.182	−0.279	−0.044	0.011	0.113
0.37	Prometryn	−0.176	0.004	−0.044	0.011	0.033
0.34	Terbutryn	−0.175	0.004	−0.044	0.011	0.033
0.00	Deethylatrazine	−0.182	−0.279	0.000	0.011	0.111
(C) Negative control						
0.00	Prometon	−0.123	0.054	0.004	0.085	0.025
0.00	Atrazine	−0.123	0.054	0.004	0.085	0.025
0.61	Simazine	−0.091	0.090	0.005	0.085	0.024
0.31	Cyanazine	−0.035	0.089	0.005	0.085	0.016
0.00	Hydroxyatrazine	−0.123	0.054	0.004	0.085	0.025
0.00	Prometryn	−0.123	0.054	0.004	0.085	0.025
0.00	Terbutryn	−0.123	0.054	0.004	0.085	0.025
0.00	Deethylatrazine	−0.123	0.054	0.005	0.085	0.025

The maximum likelihood routine first estimates a concentration for each possible analyte and subsequently gives the corresponding lack of fit (F) for each of the four antibodies as well as an overall lack of fit (ΣF^2). The combination of an analyte and a corresponding concentration with the lowest lack of fit is the one chosen as the estimate. Sometimes 2 or 3 analytes have a very similar lack of fit. The final choice is made by picking the minimal lack of fit: the lowest overall lack of fit of a combination analyte/concentration means the combination is the most likely one. As an example the data for 0.75 ppb hydroxyatrazine (A), 0.75 ppb atrazine (B) and a negative control (C) are given. For hydroxyatrazine the decision is very distinct, whereas for atrazine there are two similarly likely choices. For the negative control all choices are almost equally likely, therefore the choice is interpreted as "zero".

tion level. Since for a maximum likelihood analysis knowledge of the calibration curve parameters is necessary we had to assay several standard concentrations of all analytes. The concentrations of the unknowns were chosen to fall in-between the standard concentrations of 0.5, 1, 2 and 2 ppb.

The maximum likelihood routine first estimates a concentration for each possible analyte and subsequently gives the corresponding lack of fit for each of the four antibodies as well as an overall lack of fit. The combination of an analyte and a corresponding concentration with the lowest lack of fit is the one chosen as the estimate. However, sometimes 2 or 3 analytes have a very similar lack of fit. Table 3 shows some original lack of fit data obtained for each possible pair of analyte and concentration demonstrated for 0.75 ppb atrazine, 0.75 ppb hydroxyatrazine and a negative control. The lack of fit shows a distinct minimum for the two positive samples. Interestingly, if the sample is a negative control, a uniform and relatively low lack of fit for each possible combination of analyte and concentration is observed, thus making a decision impossible. A uniform and very low lack of fit seems to indicate that the sample is a negative. Low concentrations of simazine or deethylatrazine show the same uniformly low lack of fit (data not shown). This corresponds with the antibodies' low sensitivity or selectivity for these analytes. At this point it is not possible to define a threshold level for a lack of fit value which identifies a negative sample. In our experiments, however, a correctly identified analyte had a lack of fit much lower than the maximum lack of fit for the "worst" choice. For making a choice the absolute

Table 4
Maximum likelihood estimations for 3 different concentrations of single analytes

Actual conc. (ppb)	Actual analyte	Found conc. (ppb)	Identified as
0.75		0.70	
1.5		1.08	
5		3.54	
0.75	At	0.68	
1.5		1.34	
5		3.63	
0.75	Sim	–	
1.5		–	–
5		4.61	Sim
0.75	Cy	**0.75**/2.29	Cy/Sim
1.5		1.64	Cy
5		4.57	Cy
0.75	Pre	**0.72**/0.64/0.44	Pre/Terb/Pr
1.5		1.31/1.05/**1.62**	Terb/Pr/Pre
5		4.06	Pre
0.75	Pr	1.10/0.92/**0.69**	Pre/Terb/Pr
1.5		**1.79**/2.04	Pr/Terb
5		6.09	Pr
0.75	Terb	0.84/**0.73**/0.52	Pre/Terb/Pr
1.5		1.41/**1.67**	Pr/Terb
5		4.75	Terb
0.75	De		
1.5			
5			

Sometimes the estimates are ambiguous, i.e., there are two or three possibilities for the identity of the analytes. Depending on the choice of the actual analyte the concentration estimate also varies. The combination of the "right" analyte with its concentration is printed in bold.

numbers for highest and lowest lack of fit are less important, but their ratio may be useful in interpreting the resulting choice. In the present system we observed that the highest lack of fit is between 10 and 1000 times higher than the minimum. In ambiguous cases, i.e., when the analyte concentration is below the limit of detection or when the sample is a true negative, the ratio between the two numbers drops to 4:1 or lower. Ambiguous lack of fit data therefore seems to indicate that each analyte is as likely as any other to be present, which is only possible when the concentration is zero. Further work is being done on the interpretation of these ambiguous cases.

The estimated concentrations and the respective identified analytes are listed in Table 4.

From Table 3 the discriminatory importance of an antibody can be derived. The strongest variations in lack of fit occur with K1F4 and AM7B2.1 with most analytes. For most analytes the polyclonal antibodies do not contribute much to the decision process. However, when hydroxyatrazine is present, antibody 4653 shows a very distinct minimum of the lack of fit. Again, since antibody 2652 is relatively insensitive to deethylatrazine the difference of lack of fit when deethylatrazine is present is small (data not shown). It can be concluded that 2652 contributes very little to the overall estimation and can therefore be regarded as having little weight in terms of discriminative power. The lack of fit data of the MLE can be used to assess discriminatory power of different antibodies and can thereby help to decide which antibodies are useful and which are not.

5. Conclusions

We have shown that it is possible to categorize the structural identity of an unidentified analyte out of a class of related compounds (triazine herbicides) by using an array of antibodies and cluster analysis. In this particular case categorization meant identifying the substituent in the R^1-position of the triazine ring as a chlorine (''azine''), a methylthio (''tryn'') or methoxy (''ton'') group or as a hydroxy group. The absence of an alkyl side chain (*N*-dealkylated metabolite) was less easily categorized due to the relatively high limit of detection of all antibodies available for analysis. At 1 ppb a categorization was possible with just two antibodies. At 10 ppb even the identity of the analyte could be determined, with deethylatrazine being regarded as a zero sample. The same was accomplished at 1 ppb when using more antibodies. The possibility to even identify an analyte with immunoassay has the potential to replace instrumental analytical confirmation methods, which generally accompany single analyte immunoassays once positive samples are sorted out.

Using a combination of four antibodies that showed good discriminative power in cluster analysis we utilized maximum likelihood analysis to additionally quantify an unknown analyte. This was accomplished by using calibration data for each analyte–antibody combination.

Sometimes the presence or absence of different functional groups in structurally related molecules is an indicator for toxicity. In dioxin analysis it is necessary to know the structure of a congener to assess its toxicity. In cases where, e.g., a metabolite and the parent compound possess different physiological or environmental effects, it is also critical to distinguish among them. Thus, knowing the substitution pattern or identifying certain groups of a compound can in some cases reveal sufficient analytical information and further specification is not necessary.

We want to extend the analysis to binary and possibly more complex mixtures and study the clustering behavior. In the future we hope to avoid the necessity of running calibration standards for the maximum likelihood analysis every time the assay is performed.

We anticipate that various approaches to pattern recognition in environmental immunoassay will become increasingly important over the next few years. This trend will improve the value of immunoassay in addressing multianalyte problems. One driver for this trend will be the improvement in mathematical approaches to the interpretation of immunoassay data from multiple assays [21–25], another driver will be the decrease in cost and increase in computing power and more sophisticated software associated with microtiter plate readers. A third aspect is the increased availability of many antibodies recognizing environmental contaminants. Finally, for a number of reasons we also can anticipate that immunoassays will

become smaller and faster [32,33]. This will allow large number of samples to be processed in tiny, convenient arrays employing a variety of mathematical paradigms.

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