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An immunoarray for the simultaneous determination of multiple triazine herbicides

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Abstract

An immunochemical method for simultaneous analysis of cross-reacting analytes is presented. We demonstrate the general principle using triazine herbicides as the model system. The analysis is based on a combination of individual enzyme immunoassays (immunoarray) for triazine herbicides using antibodies with different cross-reactivity patterns towards the selected analytes. The assay signals obtained can be mathematically evaluated to estimate concentrations of each analyte out of a ternary or quaternary mixture. The mathematical model utilizes an extension of the empirical four parameter log-logistic fit. Using mono- and polyclonal antibodies it was possible to quantify the four analytes atrazine, simazine, cyanazine, and prometon in the low to sub-ppb range simultaneously.

Keywords: Immunoassay; Triazine herbicides

1. Introduction

In analytical techniques based on antibodies it is frequently observed that "specific" antibodies bind to a number of structurally similar compounds, rather than being monospecific for one analyte. This phenomenon, which occurs with both mono- and polyclonal antibodies, is named cross-reactivity. It is the immunochemical analogue of the general analytical term interference. A suitable example for non-immunochemical cross-reactivity is an ion-selective electrode, which usually also responds to interfering ions with a radius of the same size as the measuring ion.

The problem of multiple cross-reacting analytes in a sample is a very general one, occurring in clinical, environmental, agricultural, and other branches of analytical chemistry. If the presence of cross-reacting compounds in a sample is unknown, false data will be obtained during immunoanalysis when assuming a single analyte. On the other hand, cross-reactivity enables the use of antibodies as a screening tool for multiple analytes or for a whole class of analytes. Usually this approach yields a sum signal which is not weighted for a specific compound, but rather indicates whether a certain class of analytes is present or absent. Sometimes this yes–no type sum–answer is sufficient, but in other cases structurally

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very similar compounds need to be differentiated, as for example in dioxin analysis. Determination of the mere sum of dioxins is followed in most cases with quantitation of individual congeners since the toxicity of the different species is extremely dependent on their actual structure.

The approach to correcting for interferences depends strongly on the type of dose–response curve generated by a method. Since immunoassays generate sigmoidal curves, the approach has to be more complex than an approach for a linear dose–response curve. Models developed for sigmoidal curves are not restricted to immunoassays but can be transferred to other receptors, biological or even physical methods that also produce sigmoidal curves.

The idea of simultaneous immunochemical analysis of analytes which do not cross-react has previously been demonstrated. One approach is to use dual labels for two analyte analysis, thereby performing two independent assays on the same solid phase. It can be based on the use of two different enzymes [1], radioactive markers [2], fluorophores [3,4], metal-labels [5] or others. If only a single label is used but no cross-reactivity occurs, spatial resolution allows more complex multianalyte analysis as was described for the multispot immunoassay based on fluorescence detection [6–8]. The dual label approach as well as the spatial resolution approach, however, depend on the use of "monospecific" antibodies or on the presence of analytes that do not interfere with each other at their actual concentration levels. Especially in clinical chemistry even minute cross-reactivities can be undesired when these interfering compounds are present at a much higher level than the analyte(s) of interest.

Few groups have addressed the multianalyte immunoassay problem for truly cross-reacting analytes. Muldoon et al. [9] quantified ternary mixtures of the triazine herbicides atrazine, simazine and cyanazine in pesticide rinsate. The mathematical approach used a linear extension of the four-parameter curve fit. The limit of detection for triazines in this assay system was 200 ppb, which is feasible for rinsate analysis but not for trace analysis in drinking water. A more complex mathematical approach to determine only a single but previously unidentified triazine herbicide in a sample was described by Cheung et al. [10]. The authors' approach comprised principal component analysis, minimum estimates of variance and K nearest neighbors cluster analysis. Karu et al. [11] investigated four alternative methods of multivariate analysis: discriminant analysis, maximum likelihood analysis, classification and regression trees and computational neuronal networks. The all-monoclonal assay system was applied to triazine herbicides, phenyl urea herbicides and avermectins.

We present an approach to quantify structurally similar triazine herbicides present at low to sub ppb levels in aqueous solutions. We chose the class of triazine herbicides as model analytes for several reasons. Since they are a heavily used class of agrochemicals worldwide, triazines have already been found in ground water in some areas [12]. Although of lower toxicological interest concerning human consumption, triazine herbicides are a valuable indicator of water pollution. Immunoassays for triazine herbicides have been described by many groups in recent years [13–20]. Since more than just one triazine herbicide may be used, especially in areas with intensive and diverse agriculture, there is a need to screen for multiple (triazine) herbicides. In the present study we focus on the three heavily used selective triazine herbicides atrazine, simazine, cyanazine and the non-selective herbicide prometon as a fourth analyte.

The principle is to use a set of data produced by an array of triazine antibodies which exhibit different cross-reactivity patterns. The combined assay responses are evaluated by modifying the four parameter log-logistic equation to yield estimated concentrations of the individual herbicides as well as the total herbicide concentration. The mathematical principle of this approach to multianalyte enzyme-linked immunosorbent assay (MELISA) was recently described by Jones et al. [21]. In contrast to Cheung et al. and Muldoon et al., we included polyclonal antibodies into our assay system instead of only using monoclonal antibodies. Our immediate goal was to develop and test a multianalyte immunoarray for triazine herbicides. Using the large library of triazine immunoassays we want to determine what influence the properties of an individual assay will have on a multianalyte problem. The long term goal is to develop general mathematical approaches which will facilitate solving future multianalyte problems based on cross-reacting antibodies.

2. Experimental

2.1. Materials

Monoclonal K1F4 [17] and K4E7 [18] antibodies were kindly provided by B. Hock and T. Giersch (TU Weihenstephan, Germany), AM7B2.1 [16] was donated by A. Karu (University of California, Berkeley, CA). The polyclonal antibodies 194, 355, 357, 841 and 842 were produced by Harrison et al. [13]. The triazine herbicide derivatives were synthesized by M. Goodrow [22]. 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 standard ELISA software.

2.2. Analytes, haptens and antibodies

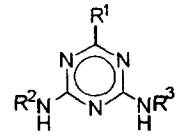
Triazine herbicides are derived from cyanuric chloride by stepwise nucleophilic substitution of the chlorine atoms. The general structure as well as the structures of the four triazine herbicides to be determined simultaneously are shown in Table 1.

Atrazine, simazine and cyanazine all have one Cl-atom as R^1 and an ethyl group as ring substituents in position R^3. The only difference arises from the third ring substituent in position R^2. Prometon bears a methoxy group in the R^1 position and thus has a different substitution pattern compared to the other analytes. In addition it has a second isopropyl group instead of an ethyl group.

For the mixture analysis immunoassay, we utilized several different anti-triazine herbicide antibodies, three monoclonals (mAb) and five polyclonals (pAb) which have been described elsewhere. The mAb K4E7 [18] and pAbs 194, 357 and 842 [13] showed the highest cross-reactivity for atrazine, whereas the mAb AM7B2.1 [16] preferred cyanazine. PAb 841 [13] is somewhat more specific for simazine than for atrazine but not reactive towards prometon and cyanazine. MAb K1F4 prefers analytes substituted in the R^1-position like prometon, but cross-reacts with all other analytes as well [17]. PAb 355 [13] is very sensitive to all four triazines, preferring prometon over the other analytes. Thus, the response patterns of these antibodies towards the analytes atrazine, cyanazine, simazine and prometon are different. Cross-reactivity data for these antibodies will be discussed below.

The curve midpoint obtained in a competitive immunoassay is a function of the hapten derivative used as coating hapten or enzyme tracer. The curve midpoint indicates the relative affinities of the antibody to the analyte and the hapten derivative, respectively. For a multianalyte assay it is necessary to obtain the same range of curve midpoints for at least the main analyte for each antibody and thus comparable limits of detection. Therefore the coating hapten has to be selected carefully to match the curve midpoints. Simply diluting a chosen coating hapten decreases the optical density and therefore the signal to noise ratio. The selected combinations of triazine derivatives and antibodies as they were used throughout this study are given in Table 2. The table

Table 1
Structures of selected triazine herbicides



Triazine herbicide	R^1	R^2	R^3
Atrazine	Cl	isopropyl	ethyl
Simazine	Cl	ethyl	ethyl
Cyanazine	Cl	cyanoisopropyl	ethyl
Prometon	OCH_3	isopropyl	isopropy

Table 2
List of the antibodies used for the experiments with their corresponding "best" coating haptens and the immunogens. The best coating hapten yields a curve with a low IC_{50} and sufficient optical density

Antibody	"Best" coating hapten	Immunogen
AM7B2.1	2-carboxypentylamino-4-chloro-6-isopropylamino-1,3,5-triazine	2-carboxyethylthio-4-ethyl-6-isopropyl-1,3,5-triazine
K1F4	2-carboxypentylamino-4-chloro-6-isopropylamino-1,3,5-triazine	2-ethylamino-4-isopropylamino-6-sulfoxomethyl-1,3,5-triazine
K4E7	2-amino-4-carboxypentylamino-6-chloro-1,3,5-triazine	2-carboxypentylamino-4-chloro-6-ethylamino-1,3,5-triazine
194	2-carboxypentylamino-4-chloro-6-ethylamino-1,3,5-triazine	2-carboxyethylthio-4-ethyl-6-isopropyl-1,3,5-triazine
355	2-ethylamino-4-isopropylamino-6-sulfoxomethyl-1,3,5-triazine	2-carboxyethylthio-4,6-diethyl-1,3,5-triazine
357	2-carboxypentylamino-4-chloro-6-ethylamino-1,3,5-triazine	2-carboxypentylamino-4-chloro-6-isopropylamino-1,3,5-triazine
841	N-acetyl-S-[4-ethylamino-6-(1-isopropyl-amino)-1,3,5-triazin-2-yl]-L-cysteine	2-carboxypentylamino-4-chloro-6-ethylamino-1,3,5-triazine
842	2-ethylamino-4-isopropylamino-6-sulfoxomethyl-1,3,5-triazine	2-carboxyethylthio-4,6-diethyl-1,3,5-triazine

also includes the immunogens used for antibody productions as indicated in the respective literature.

2.3. Preparation of the triazine–ovalbumin conjugates ("coating haptens")

Since most of the triazine derivatives used in this study have a carboxylic acid functional group the coupling technique of choice was the active ester method [23]. This comprised transforming the acid in an *N*-hydroxysuccinimide ester using EDC as coupling agent in a water miscible organic solvent and subsequent incubation with the protein in the aqueous phase. The more reactive sulfoxide functionalized triazine derivative was coupled to the protein without addition of activating agents.

To obtain a 5 fold hapten over protein excess, per 7.5 mg (1.67×10^{-7} mol) of ovalbumin 8.3×10^{-7} mol triazine derivative dissolved in DMF were used. We also modified the reagent ratios to obtain a 10 fold or a 20 fold excess, respectively. Per mol triazine derivative 1.6 mol NHS and 1.2 mol EDC were used. Stock solutions of the coupling agents were prepared in dry DMF: 20 mg/ml NHS and 5 mg/ml EDC, both stored in tightly sealed Eppendorff vials at −20° C. The coupling agents were active for several weeks under these conditions.

The triazine derivative and the respective amount of EDC and NHS solution were stirred in a small plastic vial for 6-16 hours at room temperature. After the urea had precipitated (only occasionally observed) the mixture was centrifuged. The supernatant was pipetted in small (10–20 μl) aliquots on the stirred protein solution which was prepared by dissolving 7.5 mg ovalbumin grade VI per 1.5 ml in a 1:1 mixture of PBS buffer and 0.1 M $NaHCO_3$ solution. Prior to coupling the protein solution was cooled to 4° C and then stirred overnight either at 4° C or at room temperature. The sulfoxide triazine derivative required overnight stirring at room temperature or 2 days at 4° C to yield the same coupling efficiency. Coupling efficiency was determined from dilution factors obtained in two-dimensional titrations of the coating haptens.

After coupling, precipitated protein was removed by centrifugation and the supernatant purified on 5 ml gel filtration columns equilibrated in PBS buffer. For storage, the solution was brought to 0.02% (w/v) with NaN_3 and the conjugate was frozen. According to two-dimensional titrations against antibody dilutions for most of the haptens a dilution of 1:10,000 was preferred.

The resulting hapten density on the hapten–ovalbumin conjugate was not determined, since the conventional method of measuring the absorbance of the triazine ring overlaps with the absorption band of the protein at 280 nm. Exact determination of hapten loading is the subject of further work.

2.4. Coating hapten format enzyme-linked immunosorbent assays

The competitive type assay 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.

For plate coating, hapten–ovalbumin conjugates were diluted 1:10,000 in 0.1 M PBS buffer. Per well, 175 μl were incubated over night at 4° C. Subsequently the wells were emptied and incubated with 175 μl of a 0.5% (w/v) solution of crude ovalbumin in PBS for 1 h. After washing the wells 4 times with 0.01 M PBS containing 0.05% Tween 20 (washing buffer) the plates were ready to use.

For analysis, triazine standards were assayed together with the triazine mixtures on the same plate. On each plate we used a different combination of coating hapten and antibody, employing at least as many antibodies as analytes to be determined.

For competition, 100 μl triazine standard or sample in PBS were pipetted into the wells. Standards were prepared from 1 mg/ml stock solutions in DMF which were stored at room temperature. DMF concentrations in the wells containing standards were lower than 0.01%. The 10,000 μg/l standard used to determine the blank contained 1% DMF. The respective antibody was added in 50 μl. The dilution factors of the antibodies were as follows: 194, 1:3000; 842, 841 and 355, 1:2000; 357, 1:4000; K1F4 (ascites), 1:8000; AM7B2.1 (cell culture), 1:500; and K4E7 (cell culture), 1:250. After 1 h of competition the wells were rinsed 4 times with washing buffer. The secondary antibody HRP conjugates were diluted 1:8000 (anti-mouse) and 1:15,000 (anti-rabbit)

respectively. The labeled antibody solutions (100 μl) were incubated for 1 h, then the wells were again rinsed 4 times. The substrate solution was prepared by mixing 400 μl of a 6 mg/ml TMB stock solution (in DMSO) and 100 μl 1% H_2O_2 per 25 ml 0.1 M sodium acetate buffer, pH 5.5. Per well, 100 μl of this substrate solution were allowed to react for 15–45 min, then the reaction was stopped by adding 50 μl of 2 M H_2SO_4.

Plates were read at 450 nm in the ELISA reader, using a 650 nm background correction. The raw data were processed with a custom written software based on the extended four parameter fit. The mathematical model is described below in more detail.

3. The mathematical model

3.1. Curve analysis

Receptor based analytical methods such as immunoassays or immunosensors often generate sigmoidal dose-response curves for a single analyte. This type of curve can be described using various mathematical approaches [24–29]. One can either try to base the curve on a mass action model, which purports to describe the thermodynamic aspects of equilibrium binding, or use an empirical method which will fit the shape of the data but without an immunochemical justification. Such methods may be parametric, using a specified mathematical form such as a hyperbolic or polynomial function, or non-parametric such as linear interpolation or spline fitting. The four-parameter log-logistic model, a parametric method introduced by Rodbard in the context of radioimmunoassay [24], is widely used because of its flexibility in fitting sigmoidal curves [25], and has been shown to be superior in performance to the mass-action model in many cases [29]. Eq. 1 is included in many ELISA reader software packages to automatically fit standard curves:

$$y = \frac{A - D}{1 + \left(\frac{x}{C}\right)^{B}} + D \qquad (1)$$

The assay response y, namely the optical density in ELISA, for any analyte concentration is a function of the readings at "zero" concentration (parameter A), infinite concentration (parameter D), the actual analyte concentration (x), the curve midpoint at 50% inhibition (parameter C) and a slope parameter B. For a second analyte which cross-reacts with the same antibody, the ELISA response will be equal to some "equivalent concentration" of the first analyte and can be described with the log-logistic function as was demonstrated by Muldoon et al. [9].

This simplistic linear approach assumes that calibration curves generated by the same antibody for different analytes have the same slope parameter B and thus only differ in their curve midpoints C. However, a linear combination of the "equivalent concentrations" can lead to false results in multianalyte analysis when the slope parameters B of the dose–response curves for different analytes are not the same as is usually the case.

3.2. The extended log-logistic model

To deal with the shortcomings of the basic four parameter fit for multiple analytes, a modified version of Eq. 1 uses different slope parameters B for each analyte, and a hypothetical "average" reference curve with slope parameter B^*. Eq. 2 gives an example for multiple analytes with different parameters B [21]:

$$y = \frac{A - D}{1 + \left(\left(\frac{x_1}{C_1}\right)^{B_1/B^*} + \left(\frac{x_2}{C_2}\right)^{B_2/B^*} + \ldots\right)^{B^*}} + D \qquad (2)$$

$B_{1,2,\ldots}$ are the slope parameters for analytes 1, 2, etc., B^* is the "average" slope. Since B values for non-reacting analytes (i.e., having high IC_{50}s) are poorly estimated, we use a weighted mean of the individual B values, weighting each by $1/IC_{50}$ before averaging. An alternative would be to completely exclude the B values of non-reacting analytes. But since there is no justification to define a certain threshold $1/IC_{50}$ for rejection of a particular B, the idea of smoothly reducing the weight of B values with increasing IC_{50} seemed more reasonable.

It can be noted that if only one analyte is present,

the equation turns into the single analyte Eq. 1, and if the B values for different analytes are identical, the equation turns into the simplistic approach using the same B for all analytes.

4. Results and discussion

4.1. Selecting the coating haptens

Suitable coating haptens were selected by running a panel of hapten conjugates versus each of the antibodies, utilizing only the major analyte of interest. The target IC_{50} range was 1 ppb for the main analyte. The triazine haptens that were selected for each antibody are listed in Table 2 together with the immunogens given in the literature.

In general, the lower the hapten density on the protein-conjugate the lower the curve IC_{50}. This can be explained with the concept of avidity or the density of available epitopes for antibody binding [23]. Thus, it was observed that a relatively high hapten to protein ratio of 10–20:1 during coupling had negative effects on the assay sensitivity. While a 10 fold hapten over protein excess resulted only in a slight increase of the curve midpoint, a 20 fold hapten excess additionally caused Hook effects with some antibodies, indicating that affinity of the antibodies to the conjugates relatively increases with hapten density. A suitable hapten to protein ratio during coupling was 5–10:1.

It is widely believed that coating hapten format assays per se yield a less sensitive assay than tracer format assays do. But since hapten densities on hapten-protein conjugates (with e.g. albumin) are usually higher than on enzymes (e.g. HRP) which have fewer sites available for hapten attachment, it seems likely that the effect is a function of the hapten density rather than of the format. To prove this, quantitative analysis of the conjugates with suitable tools like matrix assisted laser desorption / ionization mass spectrometry MALDI is necessary [30,31].

4.2. Calculation of the cross-reactivities

After the most suitable hapten was chosen for each antibody, cross-reactivities of the target analytes atrazine, simazine, cyanazine and prometon were determined using the particular coating hapten. The analyte yielding the lowest IC_{50} is referred to as the main analyte and is the basis for the calculation of cross-reactivities of the other analytes according to Eq. 3:

$$\%\text{cross-reactivity} = IC_{50}\ \text{main analyte} / IC_{50}\ \text{cross-reacting analyte} \times 100 \quad (3)$$

Since IC_{50}s vary from assay to assay, cross-reactivities calculated based on the lowest IC_{50} are also subject to variation. Very low cross-reactivities correspond with curves having a very high IC_{50} compared to the main analyte. The estimation of a high IC_{50} is associated with a huge error because curves cannot be fit well when the IC_{50} is close to the

Table 3
Cross reactivities of the mono- and polyclonal antibodies towards the four selected analytes atrazine, simazine, cyanazine and prometon. They were determined using the "best" selected coating hapten for each antibody. 100% indicates the analyte with the highest cross reactivity (lowest IC_{50})

Antibody	Atrazine		Simazine		Cyanazine		Prometon	
	IC_{50} [ppb]	xrct [%]	IC_{50} [ppb]	xrct [%]	IC_{50} [ppb]	xrct [%]	IC_{50} [ppb]	xrct [%]
194	1.40	**100**	24	6	36	4	6.47	22
355	0.23	65	0.57	26	0.74	20	0.15	**100**
357	0.64	**100**	10.4	6	630	$\ll 1$	39	2
841	1.39	94	1.31	**100**	230	$\ll 1$	53	2
842	0.56	**100**	0.91	62	48	1	2.94	19
AM7B2.1	0.83	83	7.84	9	0.69	**100**	18	4
K1F4	1.23	41	7.84	6	3.50	14	0.50	**100**
K4E7	1.06	**100**	5.44	19	3.02	35	–	$\ll 1$

Table 4
Parameters A, B, C and D calculated in the ternary mixture analysis of atrazine, simazine and cyanazine using the extended four-parameter fit. A and D values are the same for a particular antibody with all analytes: they were derived from the same microtiter plate by using a zero standard for A and a 10,000 ppb standard (excess) of the most reactive analyte for D

Antibody	Analyte	A (OD_{450})	B	C (ppb)	D (OD_{450})
194	Atrazine	1.307	1.09	1.49	0.039
	Cyanazine	1.307	0.863	32.0	0.039
	Simazine	1.307	1.09	19.2	0.039
K4E7	Atrazine	0.692	1.03	1.45	0.038
	Cyanazine	0.692	1.11	3.88	0.038
	Simazine	0.692	1.28	11.3	0.038
AM7B2.1	Atrazine	0.815	1.21	1.39	0.038
	Cyanazine	0.815	1.22	1.37	0.038
	Simazine	0.815	1.11	5.18	0.038
842	Atrazine	0.665	0.89	0.83	0.076
	Cyanazine	0.665	0.96	22.4	0.076
	Simazine	0.665	0.79	1.00	0.076

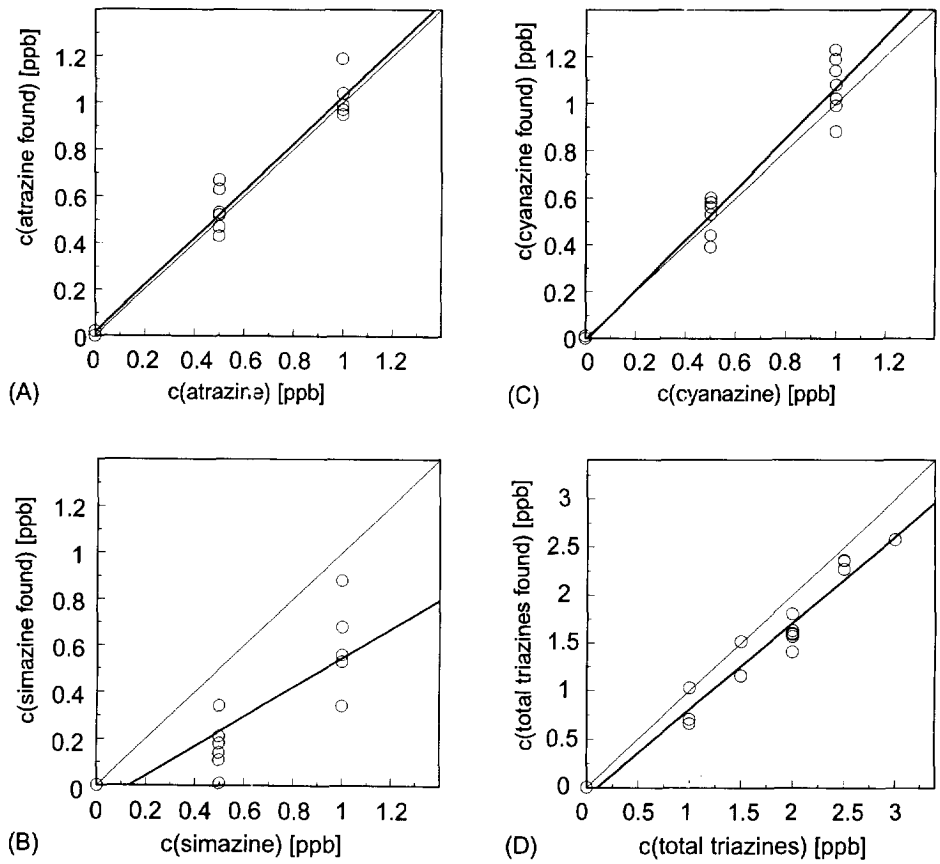


Fig. 1. Correlation plots of the actual amount of triazine herbicide on the amount found in ternary mixtures. Regression based on 14 data points each. (A) Atrazine, (B) simazine, (C) cyanazine, (D) total triazines. The bold line is the actual regression line, the thin line indicates the ideal regression with a slope of 1.00.

highest standard concentration used. As a result, the ratio of a well defined IC_{50} and a poorly estimated one cannot be precisely determined. Cross-reactivities therefore can only be calculated approximately. For example, an estimated cross-reactivity of 5% is not significantly different from one estimated at 1%. Therefore, we see little justification in indicating misleadingly "accurate" numbers. Table 3 lists the IC_{50} and the cross-reactivities in percentage determined for all eight antibodies using the four analytes atrazine, simazine, cyanazine and prometon.

Contrary to common perception it was observed that some mAbs tend to exhibit more cross-reactivity than some of the pAbs. Also, the range of IC_{50}s for the four analytes was broader for polyclonals than for monoclonals. Antibodies should be evaluated based on their properties not on their source.

The two analytes that are picked up by all antibodies are atrazine and simazine, the structurally most related triazines. Still, the cross-reactivity numbers for atrazine and simazine are sufficiently different to be significant.

4.3. Ternary triazine mixtures

Our first goal was to simultaneously analyze a ternary triazine mixture consisting of atrazine, simazine and cyanazine, all of which have the same pattern of substitution (see Table 1). Therefore, we assayed mixtures together with the three standard curves four times, using a different antibody each time (K4E7, AM7B2.1, 194 and 842). The reason for using four instead of only three antibodies is discussed below.

The calibration curves consisted of 7 triazine standards in duplicates and a "zero" in triplicates. The calibration curve parameters A, B, C, and D for each analyte/antibody are given in Table 4.

Sixteen samples, containing "0", 0.5 ppb or 1 ppb of each atrazine, simazine and cyanazine were assayed on four plates. Seven samples contained only two analytes, 8 contained all three, and one sample was negative. The plots in Figs. 1A–C show the estimated concentrations of the three triazines versus the actual concentration in the mixture based on the curve parameters. Fig. 1D gives the total triazine concentration determined by combining the individual estimates.

The regression data of the correlation plots are given in Table 5. There was a high correlation between amount found and amount added of atrazine and cyanazine, and the slope of the correlation line was close to 1.0. Simazine was significantly underestimated (lower slope) and also yielded a lower correlation coefficient. This bias is reflected in the estimation of total triazines, which showed a slope of 0.90. Since all four antibodies used have only different cross-reactivity ratios for atrazine vs. simazine but none really favors simazine over atrazine, some bias in the simazine determination was to be expected.

The overall quality of the estimations was improved by using four antibodies rather than three (data not shown). "Zero concentrations" of a single or all analytes were correctly identified, thus no false positives occurred. One false negative was observed for simazine, but none for the estimation of the total triazine concentration.

4.4. Simulation of random variation in binary triazine mixtures

To focus more closely on the problem of detecting simazine in the presence of atrazine, we studied a binary mixture system. These two analytes are the structurally most similar ones, therefore their dis-

Table 5
Linear regression analysis of the amount found on the amount added for the ternary system atrazine, simazine and cyanazine, based on the four antibodies 194, K4E7, AM7B2.1 and 842. Each regression is calculated from 14 points. Also included is the regression for the estimated total triazine content (sum of the individual estimates) on the actual one. The numbers in brackets are the standard errors

	Atrazine	Simazine	Cyanazine	Total triazines
Slope	1.009 (0.049)	0.589 (0.092)	1.090 (0.062)	0.908 (0.063)
Intercept	0.017 (0.038)	−0.076 (0.064)	−0.005 (0.045)	−0.083 (0.122)
R^2	0.968	0.747	0.957	0.936

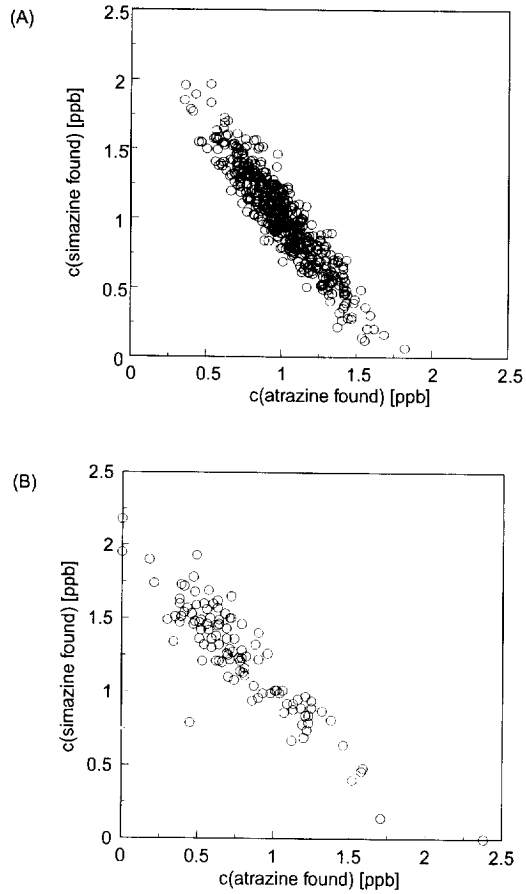


Fig. 2. Simulation of random variation in a binary mixture of 1 ppb atrazine and 1 ppb simazine. (A) Simulated distribution, 500 data points. (B) Experimental data, 132 data points.

crimination was expected to be crucial. The idea was to compare simulation with experimental data.

To simulate the effect of experimental variation in optical density on the triazine estimation we used the curve parameters to calculate 500 expected optical density readings for the same mixture (1 ppb atrazine and 1 ppb simazine). Random variation was added by using a log normal distribution to give constant coefficient of variation. The standard errors used in the simulation were derived from the curve-fitting, thus including the lack-of-fit of the standard curves as well as the replication error. Fig. 2A shows the simulated distribution. For comparison with real data, we experimentally determined the optical densities of 132 replicates of a sample containing 1 ppb atrazine and 1 ppb simazine (Fig. 2B).

Both correlation plots do not yield simple clusters centered at the "true" concentrations but form an elongated ellipse, indicating that underestimation of one analyte causes an overestimation of the other analyte. The sum of both analytes is accurately determined as is indicated by a hypothetical "sum line" of 2 ppb in both plots. Although the simulated and experimental data gave similar results, there are suggestions of possible systematic bias in the results, with simazine being overestimated more often than atrazine. This result is in contradistinction to the findings for the ternary mixtures, where simazine tends to be underestimated in the presence of two other analytes. Since we used only two antibodies for the binary mixture analysis, but four for the ternary mixture, conclusions derived from one particular combination cannot be transferred to another combination using more (fewer) antibodies and/or more (fewer) analytes.

4.5. *Quaternary triazine mixtures*

To extend the analysis, prometon was included as a fourth analyte. To find suitable combinations of antibodies we used all eight antibodies in this assay. However, the goal was to accomplish the analysis with as few antibodies as possible.

Our methodology allows the use of more antibodies than analytes to be quantitated. If two antibodies have similar patterns of cross-reactivities with respect to the analytes, the use of both will clearly lead to some redundancy: conversely antibodies with different cross-reactivity patterns will give further information which should help in assaying the mixture. Thus it is to be expected that some subsets of the antibodies used will be inferior to others since they do not contain sufficient information to solve the equation system. By using a number of different antibodies we were able to evaluate the suitability of various combinations.

We tested 14 mixtures of the four herbicides, each containing 0, 0.5 or 2 ppb of each analyte. Five samples contained all 4 analytes, the remaining 9 samples contained 3 analytes. Since the discrimination of atrazine and simazine is difficult, as the evaluation of the ternary mixture as well as the simulation showed, we defined "success intervals"

for each triazine concentration. Zero was claimed to be correctly identified if the estimate was < 0.099 ppb, since the limit of detection for the individual assays was usually higher than 0.1 ppb. Estimates between 0.1 and 0.99 ppb were accounted to represent samples containing 0.5 ppb. Samples containing 2 ppb were claimed as correctly identified if the estimate was > 1.00 and < 3.00 ppb. Thus, the samples were categorized into "none", "small", and "large".

Of the possible combinations of antibodies we picked either all eight antibodies or some combinations of six antibodies for the determination of four analytes. Since AM7B2.1 is the only antibody with preference for cyanazine we always included this antibody. Also, K1F4 always was included because the other "prometon" antibody 355 is very sensitive to all analytes, thereby possibly not generating a distinctive pattern. This left 15 possible combinations of six antibodies.

The usefulness of a combination was judged according to the following criteria: (i) number of samples, where all analytes were correctly determined; (ii) number of individual analytes correctly identified; (iii) number of false negatives; (iv) number of false positives; and (v) strong overestimation, underestimation.

Besides the most desirable criterion, a high number of correct estimates, special emphasis was put on the avoidance of false negatives. To be practically useful, false negatives should not occur, but false positives are also undesirable. Therefore, a combination yielding the same number of correct answers as another one, but yielding false negatives for any of the analytes was judged to be inferior.

The regression data for the four analytes, obtained with four selected antibody-combinations are given in Table 6A–D. Regression data for the total triazine content are only given for the combinations A–C, which perform reasonably well, while combination D gives poor estimations for simazine and is therefore not suitable for determination of the total triazine content. Since these regression data are estimated for each analyte individually, the influence of one analyte on the determination of the others is not obvious. To circumvent this we also plotted the results for 9 selected mixtures (Fig. 3). The results were obtained with all eight antibodies. By comparing actual with estimated "patterns" it is visible that the determination of simazine in the presence of atrazine

Table 6
Linear regression analysis of a ternary/quaternary mixture system consisting of atrazine, simazine, cyanazine and prometon. Each regression is calculated from 14 points, the standard errors are indicated in brackets. 6A–D were obtained by including different antibody combinations in the equation system which give results of varying quality. (A) All 8 antibodies. (B) All but K4E7 and 357. (C) All but K4E7 and 355. (D) All but 842 and 841. The regression data for the total triazine content are derived using the sum of the individual estimates

(A)	Atrazine	Simazine	Cyanazine	Prometon	Total Triazines
Slope	1.036 (0.089)	0.780 (0.140)	0.730 (0.124)	0.850 (0.064)	0.777 (0.047)
Intercept	−0.007 (0.111)	0.046 (0.140)	0.219 (0.141)	−0.069 (0.073)	0.456 (0.181)
R^2	0.918	0.721	0.743	0.936	0.958
(B)					
Slope	1.025 (0.129)	0.721 (0.163)	0.813 (0.136)	0.804 (0.066)	0.794 (0.053)
Intercept	0.091 (0.160)	−0.011 (0.163)	0.188 (0.154)	0.063 (0.075)	0.392 (0.203)
R^2	0.841	0.620	0.748	0.925	0.950
(C)					
Slope	1.018 (0.119)	0.552 (0.130)	0.797 (0.135)	0.780 (0.060)	0.775 (0.041)
Intercept	0.067 (0.148)	0.001 (0.130)	0.251 (0.153)	−0.078 (0.068)	0.330 (0.157)
R^2	0.859	0.599	0.744	0.934	0.968
(D)					
Slope	0.940 (0.107)	0.564 (0.367)	0.600 (0.062)	0.838 (0.047)	–
Intercept	−0.053 (0.132)	1.454 (0.367)	0.187 (0.070)	−0.064 (0.054)	–
R^2	0.866	0.164	0.886	0.963	–

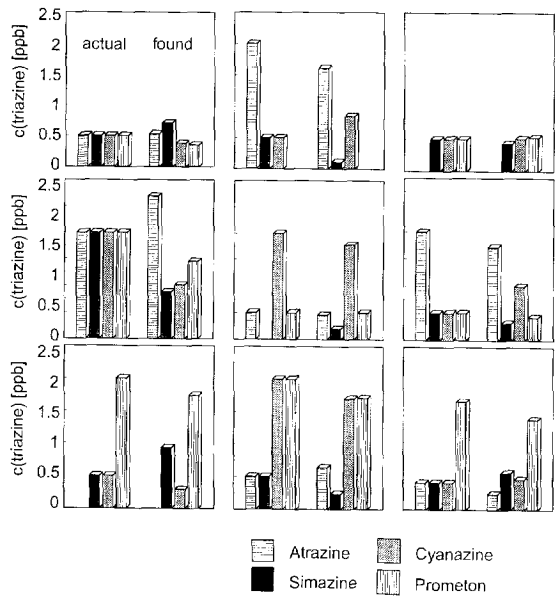


Fig. 3. Quaternary mixture analysis of atrazine, simazine, cyanazine and prometon. Each bar diagram represents an individual sample. The "bar pattern" on the left side of each plot indicates the actual concentrations in the spiked samples, the "bar pattern" on the right gives the estimated concentration.

(and vice versa) can be biased, but the overall concentration patterns are sufficiently well recognized.

In general, most six-antibody combinations yielded good atrazine estimates. This was to be expected, since four of the eight antibodies detect atrazine most sensitively, thereby providing a certain degree of redundancy when using six antibodies for the analysis. Simazine, however, tended to be underestimated as previously in the ternary mixture with some antibody combinations. Combinations without the "simazine antibodies" 841 and 842 resulted in extreme overestimation of simazine, generating also false positives. One would assume that leaving out the more specific simazine antibodies would result in a strong underestimation of simazine. Instead, the opposite was observed. Cross-reactivities make it difficult to predict the direction of a bias. Prometon tended to be more precisely determined than cyanazine, which can be explained by the fact that only one antibody (AM7B2.1) was most specific for cyanazine, whereas 355 and K1F4 both exhibited a preference for prometon. Some combinations generated many false negatives for simazine, thus not identifying simazine at all. The choice of appropriate antibodies was much more critical for simazine than for the other analytes. One combination totally failed on simazine, but yielded good results for all three remaining analytes (6D). Combination 6B was superior in terms of almost equally distributing false estimates on the four analytes. This combination of six antibodies was not substantially inferior to the calculation based on all eight antibodies (6A). The regression data obtained with all eight antibodies produced results compatible to those obtained with two of the 15 combinations of six antibodies. The overall trend is a slight underestimation of cyanazine and prometon, resulting in an underestimation of the total triazine content in ternary or quaternary mixtures.

Additionally we performed an assay using the best combination of six antibodies but only allowed the presence of one or two of four possible analytes in the same sample at either 0.5 or 3 ppb. In this case, reducing the number of antibodies to only five by also leaving out No. 842 gave slightly better results than using six antibodies. The regression data obtained with the best combination of five antibodies is given in Table 7. Reduction of this combination of five to only four antibodies did not result in further improvement. Since for this assay correlation plots consisted of 28 points per analyte with only 11 concentrations being non zero, the intercepts found

Table 7
Linear regression for a single analyte or mixtures of two analytes out of a pool of the four possible triazine herbicides atrazine, simazine, cyanazine and prometon (28 data points each, standard errors in brackets). For each triazine 17 of the 28 samples are negative and 11 are positive. The analysis was based on the five antibodies K1F4, AM7B2.1, 841, 194 and 355

	Atrazine	Simazine	Cyanazine	Prometon	Total Triazines
Slope	1.005 (0.043)	1.105 (0.069)	0.864 (0.050)	0.901 (0.021)	0.958 (0.061)
Intercept	0.045 (0.050)	−0.018 (0.081)	0.075 (0.058)	0.025 (0.025)	0.150 (0.159)
R^2	0.954	0.907	0.920	0.986	0.904

were lower than in the previous mixture analysis. Also, we found better correlations and higher slopes for all analytes, since the absence of an analyte was in most cases correctly identified, thereby improving the assay quality. In this assay the total triazine content is determined most accurately of all experiments. The tendency of underestimating individual triazines and thus the total triazine content seems less pronounced when only one or two analytes are present.

5. Conclusions

Using an extended four-parameter log-logistic fit as a mathematical model it is possible to quantify up to four highly cross-reacting analytes simultaneously by performing multianalyte ELISAs which give combined responses to all analytes. Both mono- and polyclonal antibodies were used in the immunoarray. The method is feasible for individual concentrations of lower than 1 ppb. Discrimination of analytes with identical or similar substitution patterns is feasible as was demonstrated for the four triazine herbicides atrazine, simazine, cyanazine, and prometon.

Quantitation of an individual analyte in the presence of another analyte can be biased, since overestimation of one analyte can cause underestimation of another analyte. On the other hand, the total content of triazines can be determined with sufficient accuracy. When more antibodies are used than required by the number of analytes to be determined, the results can become more accurate. A certain degree of apparent redundancy in information can still improve the estimation.

The model is applicable to any multianalyte problem being addressed by methods which yield sigmoidal dose–response curves. Although we used triazine herbicides as model analytes, it should be emphasized that this type of analysis should be feasible for clinical analysis. The model presented here can possibly be implemented with other multianalyte approaches that previously relied on absence of cross-reactivity as e.g. the multispot immunoassay [6].

General guidelines for the selection of appropriate antibodies for the analysis of complex analyte mixtures are under investigation. Also, we will examine the possibility of evaluating mixtures which contain analytes of largely varying concentrations and very different concentration ratios. This reflects the situations often found in clinical analysis. Another important issue is to reduce the number of standard curves by allowing them to be on separate plates, which would make the assay more attractive for screening of large numbers of samples by saving resources and labor. This involves a closer analysis of the statistical variation of the curve parameters which is currently under investigation.

Our long term goal is to be able to identify and quantify a limited number of analytes from a larger pool of possible compounds. It seems more likely to find various different combinations of only few triazine herbicides (analytes) in a sample than many different triazine herbicides (analytes) at the same time.

One possible approach to extend the analysis to more compounds might be to identify different substituents rather than individual compounds, thereby indicating the presence of e.g. hydroxymetabolites, parent triazines with a Cl-atom, parent triazines with an S-methyl or O-methyl group or an N-dealkylated compound. Since atrazine and simazine are often used in combination but are sometimes hard to distinguish it may be sufficient to define and determine a sum parameter for these particular compounds rather than speciating them. For the purpose of identifying substituents instead of individual compounds the use of few highly cross-reacting antibodies seems to be more promising than the approach of using very specific antibodies that recognize only one compound.

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