## Title

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# Sources of experimental variation in calibration curves for enzyme-linked immunosorbent assay

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**Abstract**

Enzyme-linked immunosorbent assays are usually performed by running standard and unknown concentrations together on the same microtiter plate, because the standard curve is known to vary considerably from one assay to the next. Here we examine experimentally the sources and nature of this variation, and discuss the possibility of reducing the cost of the assay by using a batch of plates, only one of which is used to generate the calibration curve. We present a method for doing this, and test it empirically.

*Keywords:* Immunoassay; Calibration; Enzymatic methods; ELISA

## 1. Introduction

Enzyme-linked immunosorbent assays (ELISAs) are rapid and sensitive methods for quantitating clinical or environmental analytes in trace amounts [1–7]. The assay is typically run on a 96-well microtiter plate, with some cells reserved for the generation of a calibration curve using known standard concentrations, the remaining cells being used for the unknown samples (Fig. 1). Responses (in the form of optical densities) from the unknowns are then transformed via the calibration curve into estimated concentrations.

Typically, the calibration curve obtained is sigmoidal in shape, and a number of different curve-fitting and estimation procedures have been developed: from non-parametric smoothing techniques, through the use of empirical mathematical models, to theoretical models based on the mass-action law [8].

The four-parameter log-logistic model:

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

where $y$ is the ELISA response (optical density), $x$ the analyte concentration, $A$ and $D$ the responses at zero and infinite dose, $C$ the IC50 (the concentration

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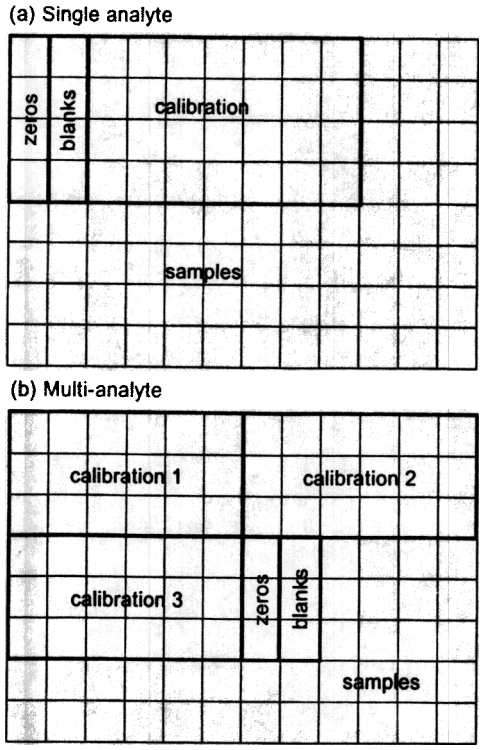


Fig. 1. Typical ELISA templates.

giving 50% inhibition) and $B$ a slope parameter, has been shown to be a useful and flexible tool in assaying the concentration of a single analyte for a variety of ELISA formats [9–11], and to be preferable to the mass-action model in many situations [12,13].

The parameters of the log-logistic model are known to be subject to considerable experimental variation, so it is usually deemed necessary to evaluate separate calibration curves for each microplate. Since space on each plate has therefore to be allocated to standard known concentrations, this limits the number of unknown samples to be included and increases the cost of the assay, a consideration of particular importance in the environmental field where the speed, low cost and parallel nature of the assay make it especially suitable for large monitoring programs. In the case of multi-analyte determination for cross-reacting analytes [14–16] this limitation can be severe (see Fig. 1b).

The question naturally arises, therefore, as to whether some or all of the parameters could be 'borrowed' from previously generated standard curves, or from curves generated on the same day in the same laboratory, or from curves generated on other plates treated simultaneously with plates of unknowns. Clearly the important criterion, as ever, will be the accuracy of calibration of unknown samples: the parameter values themselves are only intermediaries in the calibration process. Nevertheless a study of the way in which the curves vary from day to day, from lab to lab, and from plate to plate, should give some indication as to which of the above suggestions, if any, is reasonable. It is known that the relationship between response and analyte concentration can vary significantly from one location to another on the same plate [17]: if the variation between curves on different plates is no greater than this, there is nothing to be lost by borrowing the curve, or some of its parameters, from another plate.

Our approach to studying the variation in the curves clearly depends on the particular parameterization used, which could be regarded as arbitrary. We would argue, however, that the parameters have a natural interpretation which makes them worthy of study: $A$ and $D$ relate to the maximum and minimum amount of bound antibody, which in turn depend on the adsorptive properties of the plate; $B$ and $C$ should be characteristic of the bonding reaction itself, and so might not be expected to vary significantly from plate to plate.

The aim of this paper then is to examine experimentally some possible factors which could cause variation in the parameters $A$, $B$, $C$ and $D$, and to estimate their relative importance. In particular we are interested in the stability of the parameters from plate to plate when other factors are held constant. Our results suggest a method for running additional plates without the need to re-evaluate complete standard curves: since plate-to-plate variation in $B$ and $C$ is found to be non-significant compared to variation within plates, these parameters can be estimated on one plate per batch; the $A$ and $D$ parameters, which do exhibit significant plate-to-plate variation, can then be estimated for each additional plate using zeros and blanks only.

We then test the method empirically by examining the accuracy of calibration of unknowns, for both single-analyte and multi-analyte ELISA, and compare our suggested method with a more simplistic approach in which $A$ and $D$ are not re-estimated.

## 2. Experimental

### 2.1. Design of study

There are many possible factors which might affect the complex immunochemical reaction taking place in each cell of the microtiter plate, and thereby the estimated curve parameters: external physical conditions, particularly temperature, variations in experimental procedure, differences in the adsorptive properties of different plates, or of different locations on the same plate, variations between batches of stock solutions. Some of these are beyond the normal control of the experimenter. In choosing the factors for our experiment, we need a partition of the sources of variation which will have practical relevance for the technician. The following factors were identified and used in the study:

**DAY**, assays were run on two different days one week apart;

**LAB**, assays were performed each day in two different laboratories by different technicians;

**TIME**, two incubation times were used for the substrate conversion step: 20 min and 40 min;

**PLATE**, two plates were run for each possible combination of the above factors;

**LOCATION**, each plate was divided into four equal sections, each containing a set of standards for the estimation of a calibration curve.

In addition two different antibodies were used, both reactive to the analyte but one monoclonal and the other polyclonal; the data for each antibody were analyzed separately and compared. Thus a total of 32 plates was run over two days, giving 64 sets of estimated $A$, $B$, $C$ and $D$ parameters for each antibody. The same stock solutions were used throughout, and a common dilution series, to be used by both laboratories, was prepared on each day. All microtiter plates used were from the same manufacturer.

It was decided to exclude data where the curves were suspect or where there were outlying points: from a practical viewpoint such assays would presumably be rejected by a technician, and theoretically they would lead to an inflated error variance and cause problems in the analysis of our experiment.

All factors except TIME are regarded as random

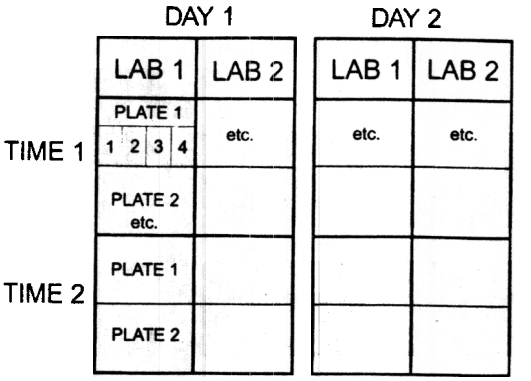


Fig. 2. Design of components of variance experiment.

effects: an incubation time of, say, 20 min can be chosen by the technician, and might be supposed to give a consistent effect, whereas conditions vary from lab to lab and from day to day. The design uses a nesting structure as shown in Fig. 2; thus LAB is nested in DAY since the LAB effect may vary from day to day, whereas TIME is crossed with LAB since the same incubation time is used in each laboratory. Since LOCATION effects cannot be separated from pure replication error, LOCATION is not included as a factor in the analysis; thus the residual variation between curves is attributable in part to location effects on the plates.

Analysis of the results from this experiment suggested a methodology which was tested in two further assay systems as described below.

### 2.2. Materials

The monoclonal antibody AM7B2.1 was kindly donated by A. Karu (University of California, Berkeley, CA) [18], K1F4 was provided by B. Hock and T. Giersch (TU Muenchen-Weihenstephan, Germany) [19]. The polyclonal antibody 842 was produced by Harrison et al. [20], the polyclonal 2266 by Lucas et al. [21]. The triazine herbicide derivatives were synthesized by 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, and tetramethylbenzidine (TMB) were purchased from Sigma. Dimethylformamide (DMF) of LC grade and *N*-hydroxysuc-

cinimide (NHS) were obtained from Aldrich (Milwaukee, WI). Buffer reagents of analytical grade were purchased from Fisher (Fair Lawn, NJ). For purification of ovalbumin–hapten conjugates we used 10-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 equipped with standard ELISA software.

### 2.3. ELISA format

For both single analyte and multianalyte ELISA we used a coating hapten format. The competitive type assay comprised 3 steps: competitive incubation of standard or spiked sample together with the specific antibody, introduction of a secondary HRP labeled antibody and conversion of the enzyme substrate TMB into a colored product. The three assay systems used (for the original investigation and two confirmatory experiments) were:

System I: atrazine calibration curves using the two antibodies AM7B2.1 and 842;

System II: atrazine calibration curves with spiked samples on the same and on separate plates, demonstrated with AM7B2.1;

System III: ternary triazine mixture analysis using atrazine, hydroxyatrazine and prometryne calibration curves with spiked samples on the same and on separate plates, and three antibodies: AM7B2.1, K1F4 and 2266 (following Kreissig et al. [16]).

For the two different triazine derivatives to be used as coating haptens with the antibodies K1F4, AM7B2.1 and 2266 the coupling technique chosen was the active ester method [23]. Coupling comprised transforming the acid functional group on the triazine derivatives in an *N*-hydroxy succinimide ester using EDC and subsequent reaction with the

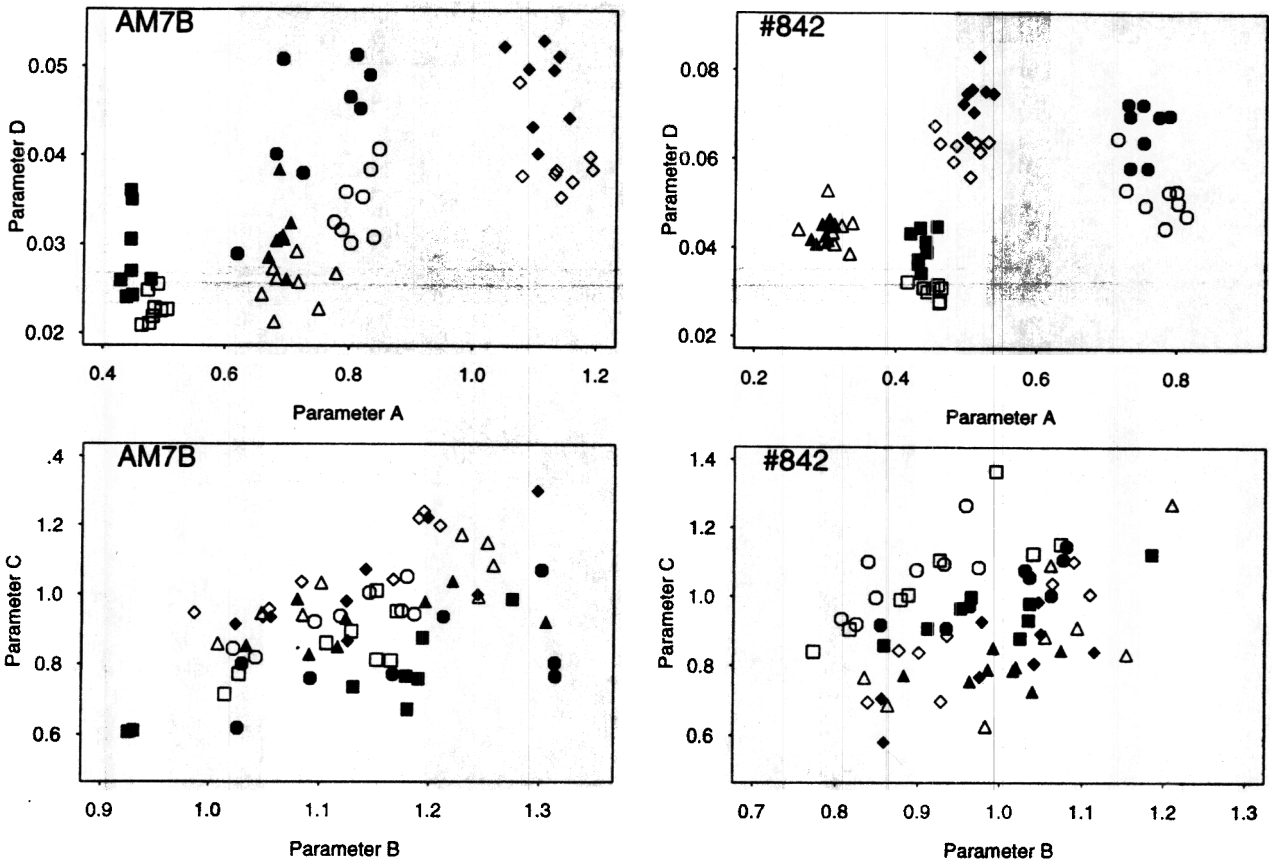


Fig. 3. Effect of DAY, TIME and LAB on curve parameters, shown separately for each antibody. ■, Day1, Time1, Lab1; □, Day1, Time1, Lab2; ●, Day1, Time2, Lab1; ○, Day1, Time2, Lab2; ♦, Day2, Time1, Lab1; ◇, Day2, Time1, Lab2; ▲, Day2, Time2, Lab1; △, Day2, Time2, Lab2.

protein ovalbumin. A more reactive sulfoxide functionalized triazine derivative which was used in combination with antibody 842 was coupled to the protein without addition of activating agents. The coupling is described in more detail in [15].

For plate coating, hapten–ovalbumin conjugates were diluted 1:10 000 in 0.1 M phosphate buffered saline (PBS) buffer. 175 $\mu$l per well was incubated overnight at 4°C. Subsequently, the wells were emptied and incubated with 175 $\mu$l of a 0.5% (w/v) solution of crude ovalbumin in PBS for 1 h. After washing the wells four times with 0.01 M PBS containing 0.05% Tween 20 (washing buffer) the plates were ready to use for the assay systems I–III. For single and multianalyte analysis, triazine standards were either assayed together with the triazine mixtures on the same plate or on separate plates. For multianalyte analysis, the same standards and/or samples were run three times on three different plates, each using a different antibody-coating hapten combination.

In the competitive step, 100 $\mu$l triazine standard or sample were pipetted into the wells. Standards were diluted in PBS from 1 mg/ml DMF stock

Table 1
Analysis of variance of log-transformed curve parameters using antibody AM7B

| | SS [a] | df [b] | MS [c] | MSE [d] | $F$ [e] | $p$ [f] |
|---|---|---|---|---|---|---|
| *Log A* | | | | | | |
| DAY | 2.40833 | 1 | 2.40833 | 0.03114 | 77.34 | 0.013 |
| LAB | 0.06228 | 2 | 0.03114 | 0.00943 | 3.30 | 0.075 |
| TIME | 3.90958 | 1 | 3.90958 | 0.00943 | 414.6 | 0.0001 |
| PLATE | 0.10372 | 11 | 0.00943 | 0.00092 | 10.20 | 0.0001 |
| ERROR | 0.04438 | 48 | 0.00092 | | | |
| TOTAL | 6.52829 | 63 | 0.10362 | | | |
| *Log B* | | | | | | |
| DAY | 0.00060 | 1 | 0.00006 | 0.00371 | 0.16 | 0.73 |
| LAB | 0.00742 | 2 | 0.00371 | 0.00350 | 1.06 | 0.38 |
| TIME | 0.00084 | 1 | 0.00084 | 0.00350 | 0.24 | 0.63 |
| PLATE | 0.03846 | 11 | 0.00350 | 0.00794 | 0.44 | 0.93 |
| ERROR | 0.38126 | 48 | 0.00794 | | | |
| TOTAL | 0.42858 | 63 | 0.00680 | | | |
| *Log C* | | | | | | |
| DAY | 0.59760 | 1 | 0.59760 | 0.09571 | 6.24 | 0.13 |
| LAB | 0.19142 | 2 | 0.09571 | 0.01367 | 7.00 | 0.011 |
| TIME | 0.11036 | 1 | 0.11036 | 0.01367 | 8.07 | 0.016 |
| PLATE | 0.15037 | 11 | 0.01367 | 0.01548 | 0.88 | 0.56 |
| ERROR | 0.74318 | 48 | 0.01548 | | | |
| TOTAL | 1.79292 | 63 | 0.02846 | | | |
| *Log D* | | | | | | |
| DAY | 0.18419 | 1 | 0.18419 | 0.36102 | 0.51 | 0.55 |
| LAB | 0.72204 | 2 | 0.36102 | 0.01707 | 21.15 | 0.0002 |
| TIME | 2.89297 | | 2.89297 | 0.01707 | 169.5 | 0.0001 |
| PLATE | 0.18777 | 11 | 0.01707 | 0.01378 | 1.24 | 0.29 |
| ERROR | 0.66161 | 48 | 0.01378 | | | |
| TOTAL | 4.64858 | 63 | 0.07379 | | | |

[a] Sum of squares.
[b] Degrees of freedom.
[c] Mean square.
[d] Appropriate error mean square for testing if the effect is zero.
[e] $F$-ratio.
[f] $P$-value.

solutions. Then 50 μl of the respective antibody diluted in PBS was added. The dilution factors of the antibodies were: 842, 1:2000; 2266, 1:5000; K1F4 (ascites), 1:8000; and AM7B2.1 (cell culture), 1:500. After 1 h of competition the wells were rinsed four times with washing buffer. The secondary antibody HRP conjugates were diluted 1:8000 (anti-mouse) and 1:15000 (anti-rabbit) respectively. A 100-μl aliquot of the labeled antibody was 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. A 100-μl aliquot of this substrate solution was allowed to react for exactly 20 or 40 min for the assay system I or 15–45 min for the systems I and III, 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. Curves were fitted using a non-linear minimization routine [24], assuming a constant coefficient of variation for the response–error relationship [25].

### 2.4. Assay system I (32 plates)

For this assay the plates were divided into four columns of 3 × 8 wells each. Four standard curves were run on the same plate using concentrations of 0, 0.1, 0.3, 1, 3, 10, 100 and 10000 ppb atrazine in triplicates. The template was the same for all plates, each being divided into four 8 × 4 sections. Half of the plates were run by one operator in one lab, the other half by a second operator in another lab (as described in Section 2.1). Each operator dedicated

Table 2
Analysis of variance of log-transformed curve parameters using antibody #842

| | SS | df | MS | MSE | F | p |
|---|---|---|---|---|---|---|
| *Log A* | | | | | | |
| DAY | 2.55350 | | 2.55350 | 0.00307 | 832.6 | 0.001 |
| LAB | 0.00613 | 2 | 0.00307 | 0.00424 | 0.72 | 0.51 |
| TIME | 4.38206 | 1 | 4.38206 | 0.00424 | 1034.4 | 0.0001 |
| PLATE | 0.04660 | 11 | 0.00424 | 0.00184 | 2.30 | 0.023 |
| ERROR | 0.08827 | 48 | 0.00184 | | | |
| TOTAL | 7.07656 | 63 | 0.11233 | | | |
| *Log B* | | | | | | |
| DAY | 0.03400 | 1 | 0.03400 | 0.04132 | 0.82 | 0.46 |
| LAB | 0.08265 | 2 | 0.04132 | 0.00304 | 13.59 | 0.001 |
| TIME | 0.01004 | 1 | 0.01004 | 0.00304 | 3.30 | 0.097 |
| PLATE | 0.03345 | 11 | 0.00304 | 0.01068 | 0.28 | 0.99 |
| ERROR | 0.51252 | 48 | 0.01068 | | | |
| TOTAL | 0.67266 | 63 | 0.01068 | | | |
| *Log C* | | | | | | |
| DAY | 0.65243 | | 0.65243 | 0.04850 | 13.45 | 0.067 |
| LAB | 0.09700 | 2 | 0.04850 | 0.00642 | 7.55 | 0.009 |
| TIME | 0.01129 | 1 | 0.01129 | 0.00642 | 1.76 | 0.21 |
| PLATE | 0.07067 | 11 | 0.00642 | 0.02264 | 0.28 | 0.99 |
| ERROR | 1.08650 | 48 | 0.02264 | | | |
| TOTAL | 1.91788 | 63 | 0.03044 | | | |
| *Log D* | | | | | | |
| DAY | 0.56016 | 1 | 0.56016 | 0.31185 | 1.80 | 0.31 |
| LAB | 0.62370 | 2 | 0.31185 | 0.01629 | 19.14 | 0.0003 |
| TIME | 3.70235 | 1 | 3.70235 | 0.01629 | 227.2 | 0.0001 |
| PLATE | 0.17922 | 11 | 0.01629 | 0.00588 | 2.77 | 0.007 |
| ERROR | 0.28246 | 48 | 0.00588 | | | |
| TOTAL | 5.34788 | 63 | 0.08489 | | | |

half the total number of plates to each antibody. The coating hapten solution, antibody dilutions and atrazine standards were prepared from the same stock solution prior to each experiment and then split. Thus, both operators used the same solutions. Incubation times were monitored strictly using a stop watch, with particular emphasis put on the timing of the substrate conversion step.

### 2.5. Assay system II (2 plates)

This assay was designed to compare the accuracy of analysis of samples on the same plate as the standard curves with that of samples on additional plates with borrowed curve parameters. Thus one plate contained a set of standard atrazine concentrations for calibration and two sets of 6 spiked samples: the second plate contained three sets of the samples together with zeros and blanks. All samples and standards were in replicates of four. The concentrations of the standards were 0, 0.01, 0.1, 0.3, 1, 3, 10, and 100 ppb. The atrazine concentrations in the samples were 0.25, 0.5, 1, 1.5, 2, and 3 ppb, thus covering the dynamic range of the calibration curve.

Table 3
Estimated variance components (VC) and associated coefficients of variation (cv) of the random effects for each curve parameter

| | AM7B | | 842 | |
|---|---|---|---|---|
| | | cv (%) [b] | | cv (%) |
| *A* | | | | |
| DAY | 0.074287 | 31.33 | 0.079701 | 32.62 |
| LAB | 0.001357 | 3.75 | 0 | 0.00 |
| PLATE | 0.002126 | 4.72 | 0.000599 | 2.48 |
| ERROR | 0.000925 | 3.09 | 0.001839 | 4.38 |
| TOTAL | 0.078695 | 32.38 | 0.082139 | 33.19 |
| *B* | | | | |
| DAY | 0 | 0.00 | 0 | 0.00 |
| LAB | 0.000013 | 0.36 | 0.002393 | 5.01 |
| PLATE | 0 | 0.00 | 0 | 0.00 |
| ERROR | 0.007943 | 9.32 | 0.010677 | 10.89 |
| TOTAL | 0.007956 | 9.33 | 0.013070 | 12.11 |
| *C* | | | | |
| DAY | 0.015684 | 13.34 | 0.018873 | 14.73 |
| LAB | 0.005127 | 7.42 | 0.002630 | 5.26 |
| PLATE | 0 | 0.00 | 0 | 0.00 |
| ERROR | 0.015483 | 13.25 | 0.022635 | 16.24 |
| TOTAL | 0.036294 | 20.99 | 0.044138 | 23.38 |
| *D* | | | | |
| DAY | 0 | 0.00 | 0.007760 | 9.21 |
| LAB | 0.021497 | 15.79 | 0.018472 | 14.56 |
| PLATE | 0.000822 | 2.91 | 0.002602 | 5.23 |
| ERROR | 0.013783 | 12.46 | 0.005885 | 7.97 |
| TOTAL | 0.036102 | 20.93 | 0.034719 | 20.48 |

Table 4
Estimates of incubation time effect on each curve parameter

| | AM7B | 842 | |
|---|---|---|---|
| | 40 min | 20 min | 40 min |
| A | | | |
| B | | | |
| C | | | |
| D | | | |

### 2.6. Assay system III (6 plates)

This assay was used to compare accuracy of analysis in the case of multi-analyte samples. We prepared 27 different herbicide mixtures, which contained each of the three triazines atrazine, hydroxyatrazine and prometryne at 0, 0.5 ppb or 3 ppb, thereby allowing all possible combinations of these three concentrations with the three analytes. Three microplates, each assayed using a different antibody, are needed for this mixture analysis. One set of three plates contained standards for three different calibration curves, using concentrations of 0.01, 0.1, 0.3, 1, 3, 10, 100 ppb for each analyte, a zero sample and a 10 000 ppb sample of the main analyte, all measured in triplicate. Thus, only 27 wells per plate were available and these were used for analysis of nine samples in triplicates. The other set of three plates dedicated nine wells to a zero standard and six wells to a 10 000 ppb sample, thus leaving 81 wells for samples. Samples 1–9 were assayed on both sets of plates, samples 10–27 only on the plates without standard curves on them.

## 3. Results

A graphical summary of the variations in parameter values is shown in Fig. 3. It can be seen that the *A* and *D* parameters form clearly differentiated clusters, suggesting that DAY, TIME and LAB have an important effect on these two, i.e. that these three

Table 5
Recovery of atrazine in spiked samples. Amounts S1, S2 are from samples on the same plate as the standard curve, A1–A3 on an additional plate with the A and D parameters re-estimated from zeros and blanks, A1*–A3* on the additional plate but without adjustment

| True conc. (ppb) | Standard | | Additional (adj.) | | | Additional (unadj.) | | |
|---|---|---|---|---|---|---|---|---|
| | | S2 | | A2 | A3 | A1* | A2* | A3* |
| 0.25 | 0.43 | 0.35 | 0.39 | 0.47 | 0.47 | 0.34 | 0.41 | 0.42 |
| 0.50 | 0.58 | 0.55 | 0.70 | 0.71 | 0.69 | 0.63 | 0.62 | 0.62 |
| 1.00 | 1.09 | 1.10 | 1.07 | 1.25 | 1.20 | 0.96 | 1.07 | 1.07 |
| 1.50 | 1.42 | 1.53 | 1.56 | 1.75 | 1.66 | 1.38 | 1.46 | 1.46 |
| 2.00 | 2.00 | 1.94 | 1.93 | 2.02 | 2.04 | 1.68 | 1.77 | 1.77 |
| 3.00 | 2.84 | 2.65 | 2.77 | 2.79 | 2.63 | 2.33 | 2.23 | 2.23 |

factors account for much of the observed variation in $A$ and $D$. Conversely there is no such pattern discernible from inspection of the $B$ and $C$ plots.

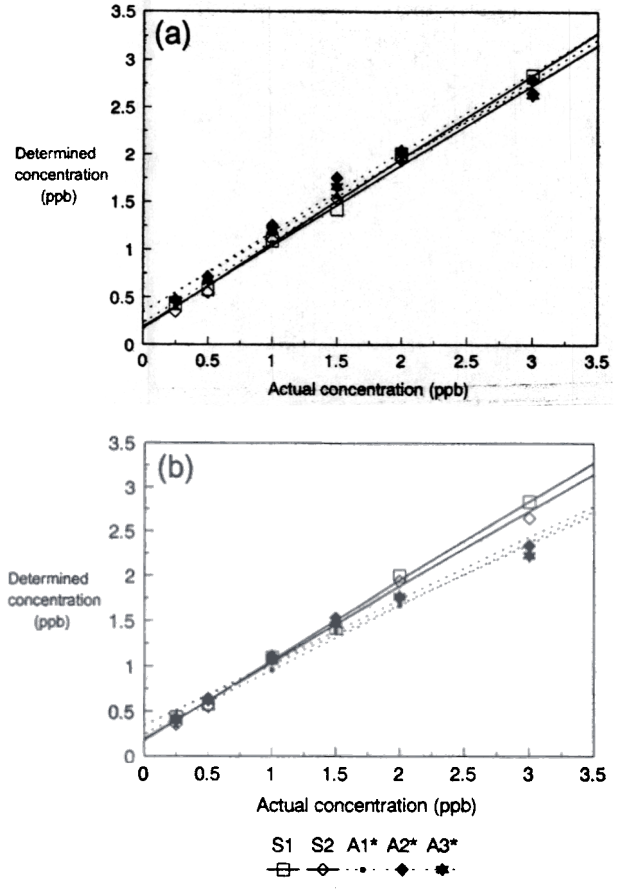


Fig. 4. Correlation plot of amount of atrazine recovered against amount added in the single-analyte confirmatory experiment, with (a) and without (b) adjustment of the $A$ and $D$ parameters. Samples S1, S2 were on the same plate as the standard curve; A1–A3 were on an additional plate without a standard curve. The lines show the best linear fit for each set of points.

However, $B$ and $C$ do appear to be approximately linearly related, with the linear relationship changing slightly for each different combination of factors. We now proceed to a statistical analysis of the variations.

### 3.1. Statistical analysis

Mixed model analysis of variance [26] for each of the four parameters was carried out using the GLM procedure in SAS [27]. The results are given in Tables 1 and 2. The raw parameter values were transformed by taking logarithms: as well as being intuitively reasonable (corresponding to constant cv, or proportional errors in the parameter estimates), this gave residuals which showed no obvious departure from the assumptions of Gaussian errors and homoscedasticity required for the use of $F$-tests. The DAY–TIME and LAB–TIME interactions were originally included in the analysis, but were subsequently omitted as these effects were found to be small and mostly non-significant.

Estimated variance components and associated cv's for the random effects were calculated from the ANOVA output (see Table 3). When the appropriate error mean square exceeded the effect mean square, the corresponding variance component was taken as zero. The cv's here represent estimates of the resulting cv of the actual parameter value if the corresponding factor could be allowed to vary with all other factors kept fixed. The estimated TIME effects are given in Table 4.

### 3.2. Conclusions

It can be seen that all four parameters vary considerably between different locations on the same

Table 6
Linear regression analysis of amount of atrazine found on amount added, showing the coefficient of determination ($R^2$), intercept ($a$) and slope ($b$) of the least squares line, with standard errors in brackets

| | Standard | | Additional (adj.) | | | Additional (unadj.) | | |
|---|---|---|---|---|---|---|---|---|
| | S1 | S2 | A1 | A2 | A3 | A1* | A2* | A3* |
| $R^2$ (%) | 99.7 | 99.3 | 99.8 | 98.9 | 98.6 | 99.4 | 98.1 | |
| $a$ | 0.17 (0.04) | 0.19 (0.06) | 0.23 (0.03) | 0.34 (0.07) | 0.35 (0.08) | 0.24 (0.04) | 0.34 (0.08) | |
| $b$ | 0.89 (0.03) | 0.85 (0.03) | 0.85 (0.02) | 0.84 (0.04) | 0.80 (0.05) | 0.71 (0.02) | 0.70 (0.05) | |

plate: in fact this seems to be the most important source of variation for the $B$ and $C$ values. Of particular importance is the finding that $B$ and $C$ do not vary significantly from plate to plate, so that observed variation in these parameters between plates is not greater than between different locations on the same plate. Thus it would seem that putting the standard calibration concentrations on the same plate as the unknowns might not lead to significantly better estimates than having them on a separate plate, provided that both plates receive identical treatment under the same conditions.

The $A$ and $D$ parameters, representing the upper and lower horizontal asymptotes to the calibration curve, do however seem to vary significantly from plate to plate, but these can be estimated from zero concentrations and blanks (infinite concentrations), respectively. We therefore propose running additional plates devoted almost entirely to unknowns, with a few cells used for the re-estimation of the $A$ and $D$ parameters; the additional plates should be run at the same time as the standard plate, under the same experimental conditions.

### 3.3. Confirmatory experiment — single analyte

Table 5 shows the true atrazine concentrations in the six spiked samples together with the amounts recovered: S1 and S2 are the estimates from the two sets of samples on the standards plate; A1, A2, A3 were estimated from the three sets of samples on the additional plate using the $B$ and $C$ parameters from the standards plate but $A$ and $D$ re-estimated from the zeros and blanks on the additional plate; A1*, A2*, A3* are as for A1, A2, A3 but using all the standards plate parameters (i.e. without adjusting for a different $A$ and $D$).

Regression of amount found on amount added is shown in Fig. 4 and Table 6. It appears from these results that reasonable estimations can be achieved

Table 7
Simultaneous recovery of atrazine, prometryne and OH–atrazine in multi-analyte analysis, for samples either on the standard plate or on an additional plate with and without adjusting for re-estimated A and D

| True conc. (ppb) | | | Standard | | | Additional (adj.) | | | Additional (unadj.) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Atrazine | Prometryne | OH–atrazine | Atrazine | Prometryne | OH–atrazine | | Atrazine | Prometryne | OH–atrazine | | |
| 0 | 0 | 0 | 0.00 | 0.00 | 0.00 | 0.01 | 0.00 | 0.00 | 0.01 | | |
| 0 | 0.5 | 3 | 0.00 | 0.41 | 2.15 | 1.99 | 0.00 | 0.49 | 1.83 | | |
| 0 | 3 | 0.5 | 0.00 | 3.43 | 0.29 | 0.27 | 0.00 | 3.39 | 0.27 | | |
| 0.5 | 0 | 3 | 0.38 | 0.16 | 2.58 | 1.94 | 0.34 | 0.19 | 1.90 | | |
| 0.5 | 0.5 | 0.5 | 0.60 | 0.41 | 0.40 | 0.33 | 0.41 | 0.41 | 0.33 | | |
| 0.5 | 3 | 0 | 0.42 | 2.77 | 0.00 | 0.00 | 0.27 | 3.29 | 0.00 | | |
| 3 | 0 | 0.5 | 2.69 | 0.49 | 0.28 | 0.41 | 2.41 | 0.66 | 0.41 | | |
| 3 | 0.5 | 0 | 2.59 | 1.13 | 0.00 | 0.00 | 2.54 | 1.09 | 0.00 | | |
| 3 | 3 | 3 | 2.38 | 3.38 | 2.57 | 2.59 | 2.32 | 3.38 | 2.57 | | |

Table 8
Linear regression analyses of amount found on amount added for each analyte in a 3-analyte mixture (from 9 samples common to both standard and additional plates), showing the coefficient of determination ($R^2$), intercept ($a$) and slope ($b$) of the least squares line, with standard errors in brackets

| | Standard | | | Additional (adj.) | | | Additional (unadj.) | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Prometryne | OH–atrazine | Atrazine | Prometryne | OH–atrazine | Atrazine | Prometryne | OH–atrazine |
| $R^2$ (%) | 99.3 | 95.4 | 98.7 | 99.8 | 97.5 | 96.8 | 99.6 | 94.8 | 96.1 |
| $a$ | 0.02 (0.05) | 0.19 (0.15) | −0.04 (0.06) | 0.08 (0.03) | 0.01 (0.11) | −0.01 (0.09) | −0.03 (0.03) | 0.20 (0.18) | −0.00 (0.09) |
| $b$ | 0.85 (0.03) | 1.00 (0.08) | 0.82 (0.04) | 0.95 (0.02) | 1.00 (0.06) | 0.73 (0.05) | 0.82 (0.02) | 1.14 (0.10) | 0.70 (0.05) |

on an additional plate, and that the estimation is improved by re-evaluating the $A$ and $D$ parameters as suggested.

### 3.4. Confirmatory experiment — multi-analyte

Comparison of estimates is shown in Table 7 for the nine samples assayed on both standard and additional plates. Again estimation on the additional plate is performed with and without adjustment of the $A$ and $D$ parameters.

Assessment of performance for multi-analyte determination is not obvious since estimates of the components of a mixture tend to be correlated [15]. Independent regression analyses for the nine common mixtures (Table 8) does not indicate inferiority of the estimates from the additional plates: the slope is a little worse for atrazine, but a little better for OH–atrazine. If the $A$ and $D$ parameters are not re-estimated, the slope is a little worse for all analytes.

A similar regression analysis for all 27 mixtures on the additional plate (Table 9) shows that a large number of mixtures can be assayed accurately by using an additional plate. In this case, not adjusting the parameters as we suggest led to a better slope for atrazine but a much worse slope for prometryne and a slightly worse slope for OH–atrazine.

## 4. Discussion

Our main concern here was to discover if we could use additional plates of samples for multi-analyte ELISA, in order to overcome the limitation of the small amount of space available on the plate containing the standard curves. Our second confirmatory experiment suggests that this can be done without a significant loss in accuracy.

The importance of re-estimating the $A$ and $D$ parameters is clearer in the single-analyte results. Our analysis of the components of variation suggest that the variation in these parameters is greater between plates than between different locations on the same plate. Clearly there will be occasions when the $A$ estimated from one plate will be closer to the true $A$ at one location on a second plate than the estimated $A$ from a different location on the second plate; in the long run, however, re-estimating $A$ on each plate should lead to greater accuracy.

Since $B$ and $C$ vary little from plate to plate, it might even be possible to prepare calibration curves

Table 9
Linear regression analyses of amount found on amount added for each analyte in a 3-analyte mixture (from all 27 samples on the additional plate), showing the coefficient of determination ($R^2$), intercept ($a$) and slope ($b$) of the least squares line, with standard errors in brackets

| | Additional (adj.) | | Additional (unadj.) | | |
|---|---|---|---|---|---|
| | Prometryne | OH–atrazine | Atrazine | Prometryne | OH–atrazine |
| $R^2$ (%) | | | | | |
| $b$ | | | | | |

just once per day per lab and to use these for subsequent assays. Alternatively Bayesian methods, in which plausible values for the parameters are up-dated using observed data, could be used to get accurate estimates using fewer calibration points [28].

An important point of general concern in the use of microtiter plate-based immunoassay is the extent to which the relationship between the response and the analyte concentration varies from one location to another on the same plate (see also [17]). Earlier generations of 96-well plates often showed high variability of protein attachment when used in ELISA; this was especially true of the outer wells usually on two of the four sides. The optical and adsorptive properties of plates have improved dramatically, although it is still advisable to check a shipment of plates for within-plate consistency. In addition to the plates themselves there are experimental contributions to within-plate variation. There may be ways of reducing or accommodating this variation: some procedural, such as preventing a temperature gradient from developing across the plates; some statistical, such as the judicious design of the plate template, or estimating and adjusting for spatial correlation. Such methods could lead to further improvement in what is already a sensitive and accurate technique.

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