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IMMUNOASSAYS FOR RESIDUE ANALYSIS OF AGROCHEMICALS: PROPOSED GUIDELINES FOR PRECISION, STANDARDIZATION AND QUALITY CONTROL

(Technical Report)

Prepared for publication by

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Pesticides report 33. Immunoassays for residue analysis of agrochemicals: Proposed guidelines for precision, standardization and quality control (Technical Report)

Synopsis Immunoassays (ELISA) for agrochemicals are analytical techniques which make use of antibodies as "biochemical detectors" for the quantification of active ingredients or their derivatives. In contrast to clinical chemistry the application of immunoanalytics to residue analyses of agrochemicals by industries and the acceptance of ELISA data by registration agencies is still in its infancy. Missing performance quality control criteria for ELISA in the past have raised questions about the reliability of this technique for the analysis of agrochemicals. This report gives basic technical information on immunoassays such as test formats and detection systems. Parameters critical for the reliability of this methodology such as matrix interference and cross reactivity are discussed. Guidelines are proposed for precision requirements, standardised evaluation procedures and the distribution of immunoanalytical methods for agrochemicals. Follow-up activities are summarised with the intention of generating a wide data base on the precision and applicability of immunoassays for agrochemical analysis.

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A. INTRODUCTION AND OBJECTIVES

Immunoassays for agrochemicals are analytical techniques which make use of antibodies as biochemical "detectors". When introduced into sample extracts they recognise and bind to the chemical compound they were produced against. The production of antibodies is induced in mammals which are immunised with conjugates of the agrochemical or a structurally related compound and a carrier (ref. 1). Due to their inherently different mode of detection immunoassays can be a valuable addition to the existing conventional analytical technology.

Immunoassays have been widely and successfully used in clinical chemistry and veterinary drug registration for many years. Extensive literature is available which indicates the potential of immunoassays as fast, reliable and cost efficient methods in residue analysis of agrochemicals (ref. 2, 3, 4). In contrast, the development of immunoassays by industries and their acceptance by registration agencies as analytical methods for the monitoring of agrochemical residues is still in its infancy.

Literature and the authors own experience show that immune analytical methods are as reliable and specific as chromatography based methods such as HPLC and GC, provided they are applied under equally high laboratory standards (ref. 5, 6, 7). In terms of speed and cost efficiency however, immunoassays can significantly outperform conventional methods. Immunoassays have been successfully applied to the analysis of agrochemicals in a wide range of sample matrices like water, soil, plant and animal samples as well as food items. In water ELISA assays are generally used as fast stand alone methods. In solid matrices they usually serve as detectors in sample extracts after clean-up (ref. 8, 9, 10, 11, 12, 13, 14). Recently the specificity and sensitivity of immunoassay (ELISA) detectors has been utilized also in hyphenated systems such as HPLC-ELISA and CE-ELISA couplings (ref. 21).

To date (with only a few exceptions), immunoassays have not been officially approved by national and international registration agencies as analytical or enforcement methods for the registration of agrochemicals. Immunoassay test kits for agrochemicals have been supplied to the market by various companies for a number of years already and a growing number of laboratories are using this technology. In contrast, only very recently efforts have been made to establish national and international guidelines for the standardisation and quality assurance of immune-analytical methods (ref. 15, 16). It is evident from the authors' own experience and the literature that immunoassays which do not fulfil certain quality criteria with respect to specificity, repeatability and reproducibility are very likely to yield incorrect results (ref. 17, 18, 7). The distribution and application of such immunoassays for the analysis of agrochemicals in the past have unneccessarily damaged the reputation of this methodology.

To support the introduction of immunoassays as a valid and reliable technique in the residue analytical laboratory, it is therefor necessary to define quality standards for immunoassays which meet the EC or EPA requirements for conventional residue analytical methods (ref. 19, 20). The future acceptance and application of immunoassays will depend on how well and how soon immunoassays comply with these quality standards while still maintaining high speed and cost efficiency as stand alone systems or in hyphenated systems.

With respect to quality criteria of immunoassays, the authors of this technical report do not differentiate between monoclonal and polyclonal antibodies used as "detectors" (immune probes) in these methods. It is obvious that the quality requirements proposed here must be fulfilled by all immune-analytical methods regardless of the type of antibody detector they use.

B. IMMUNOASSAYS: A TECHNICAL APPROACH

The "antibody detector"

Immunoassays for agrochemicals are analytical methods which make use of anti-bodies as specific "biological probes" for the detection and quantification of the parent compound and/or metabolites in sample extracts. The antibodies are produced by immunizing mammals with an analyte-carrier (usually a protein-conjugate)(ref. 1). Coupling of the analyte to a carrier is necessary to increase its molecular mass and thus generate immunogenic properties.

The antibodies can be used as a total population (polyclonal) of specific detectors after recovering in the blood serum of the host. Undesirable specificity may be eliminated from the serum by immune purification removal of certain antibody populations. As an alternative, the antibody-producing cells can be isolated from the host animal and subcloned in cell culture for the production of a single antibody clone (monoclonal antibodies).

Antibodies used in immunoassays usually belong to the IgG-subclass. These "Y-shape" proteins have a molecular mass of approximately 150 kDa. Antibodies usually have two binding sites for antigenes.

Principle of the assay

Despite the use of very different test formats, all immunoassays are based upon the same basic principle: The sample or sample extract containing the analyte is first incubated together with the antibodies resulting in the formation of the analyte-antibody complex (i.e. antigen bound to binding sites). In a second step, the number of the binding sites occupied by analyte is visualised. Visualisation is accomplished with a tracer which can be directly (or indirectly, after further chemical reactions) assayed.

The majority of immunoassays applied to the quantification of agrochemicals belong to the *competitive test type*. During the analyte-antibody reaction a small number of antibodies are competing for a surplus of analyte molecules. Among a wide range of different formats, competitive immunoassays used for residue and environmental analysis are characterised by a step in which the incubated analyte-antibody mixture is separated into a bound and a free fraction. These tests are called *heterogeneous competitive assays.* In most cases detection of the fraction bound to a solid phase is performed with the use of an enzyme tracer and subsequent substrate reaction.

Heterogeneous competitive enzyme immunoassay (EIA)

These tests use a lower concentration of antibodies as compared to the concentration of analytes in the sample. Antibodies are added to all samples and calibration standards in identical concentrations.

Two assay formats have gained wide acceptance:

Direct EIA

In this format the specific antibodies are immobilised on a solid phase prior to the assay (see Fig. 1). During the first incubation step in the procedure free analyte in the sample competes with an analyte-enzyme-conjugate (enzyme-hapten-tracer) for the antibody binding sites. Then the amount of the enzyme-tracer bound to the solid phase is quantified after addition of an appropriate enzyme substrate (Fig. 1a).

Indirect EIA (ELISA)

In this ELISA format the solid phase is coated with an analyte-carrier-conjugate prior to the assay. In most cases proteins serve as carriers as they allow the analyte to be tightly "glued" to the solid phase. In the first incubation step the free analyte molecules in the sample, compete with the bound analyte for binding sites of the specific antibodies which are present in the sample (Fig. 1b - d). These antibodies may be coupled to an enzyme (Fig. 1b), contain binding sites for an amplifier system (Fig. 1c) or may be used unmodified (Fig. 1d). In the first case the concentration of the antibodies bound to the solid phase can be quantified directly by an appropriate substrate reaction.

If nonmodified antibodies are used (Fig. 1d) they are labelled, in a second incubation step, with a second antibody-specific antibody-enzyme-tracer (anti IgG-antibody-tracer). The number of analyte specific antibodies bound to the solid phase can be visualised with an appropriate substrate reaction.

In indirect EIA-systems an additional incubation step is required compared to direct EIA methods and therefore tend to be longer procedures. However, since there is no direct contact of the sample matrix with the detection system, these tests tend to be more rugged, sensitive and reliable than direct EIA formats.

Solid phases

Heterogeneous immunoassays make use of a wide variety of different solid phases such as polystyrene tubes, plastic membranes, plastic microbeads and immunoaffinity columns. Most immunoassays for agrochemicals, however, utilize the 96-well microtiterplate (MTP) format. The MTP offers ease of handling, large sample capacity and simple test standardisation.

Detection systems

The test signal in most EIA and ELISA methods for agrochemicals is produced with an enzyme-tracer (in most cases horseradish peroxidase or alkaline phosphatase) together with an appropriate substrate. Depending on the type of substrate used both enzymes allow either UV/Vis-, fluorescence or luminescence detection. Most assays are based on UV/Vis detection, because MTP-compatible photometers are widely available and relatively inexpensive.

The most commonly used substrates for horseradish peroxidase are a solution containing H_2O_2 and tetramethyl-benzidine (TMB), and, for alkaline phosphatase, p-nitrophenylphosphate (PNP). Substrate reaction is assayed either at an endpoint (the reaction is stopped by adding acid or base) or in a kinetic mode. UV/Vis-absorption in all wells on the MTP can be assayed rapidly (in 3 to 5 s) with state-of-art MTP-compatible photometers.

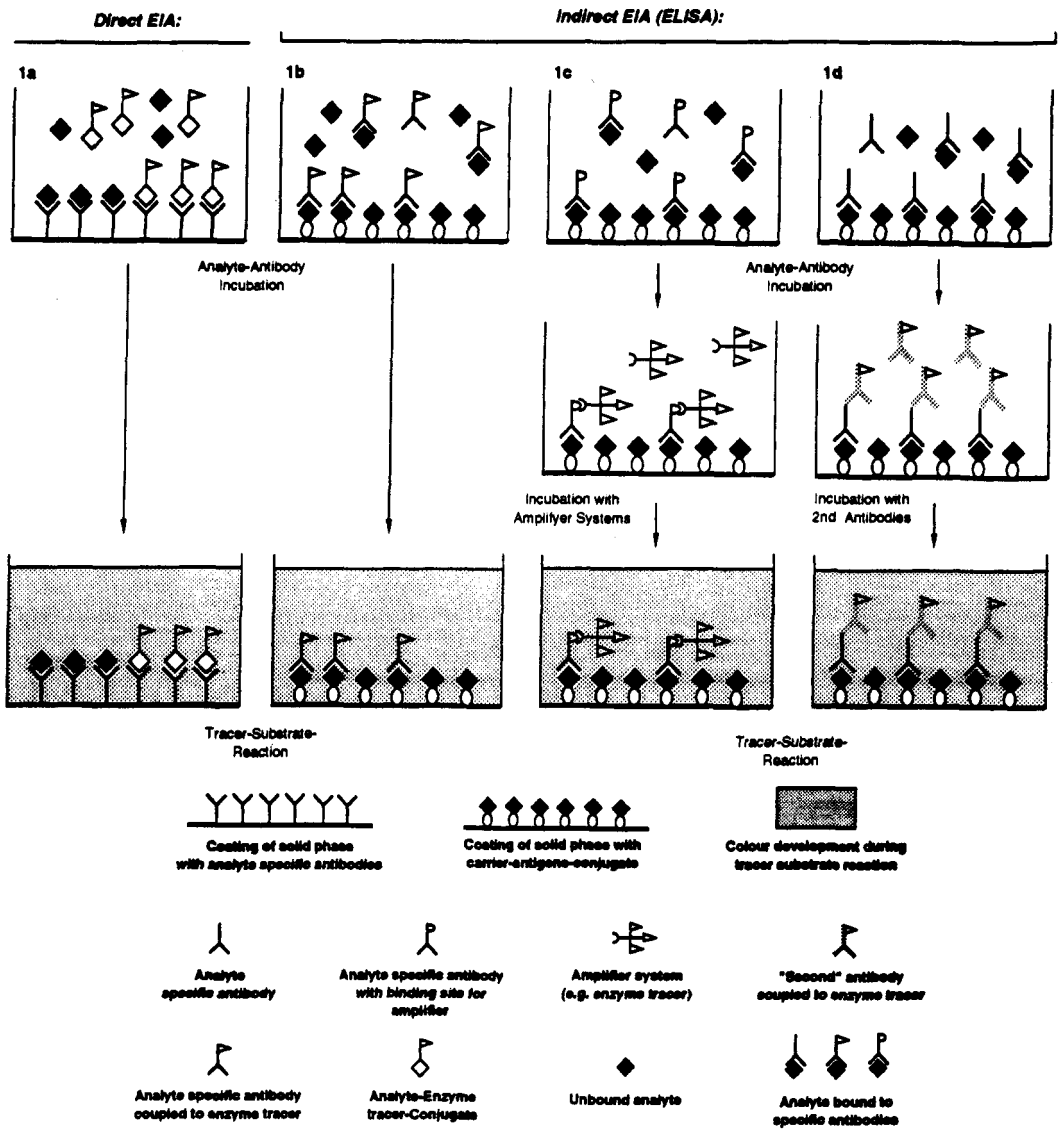

Fig. 1 Formats of direct and indirect, competitive immunoassay types

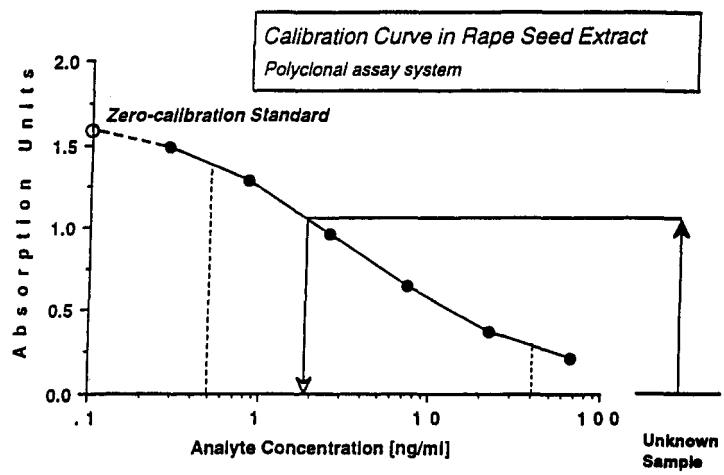

Fig. 2 Quantification of residues with ELISA-assays

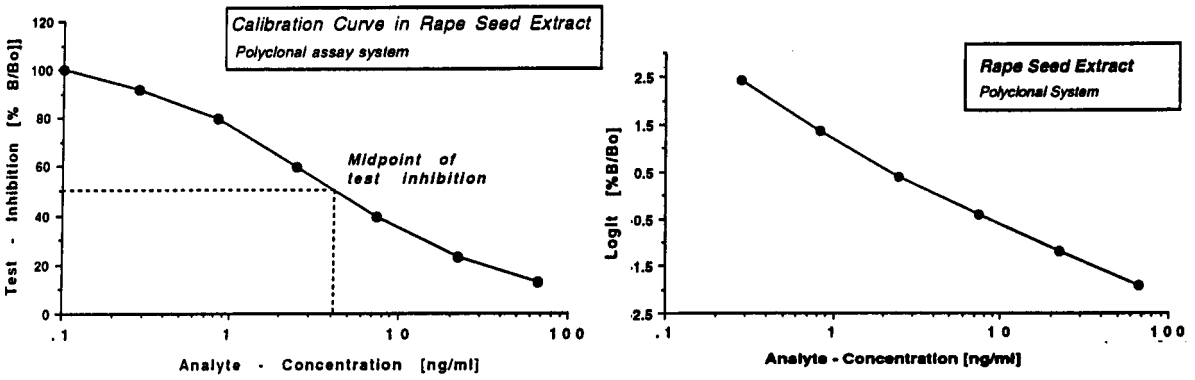

Fig. 3 ELISA standard calibration curves plotted in lin/log and logit/log transformed formats.

Quantification

In all heterogeneous, competitive immunoassays the intensity (coloration) of the test signal increases with the number of analyte-specific antibodies bound to the solid phase. The signal is inversely proportional to the analyte concentration in the sample (i.e. low coloration of the wells indicates high analyte concentrations in the sample).

The analyte concentration in unknown samples may be quantified by comparing the assay signal (see Fig. 2) with a calibration curve which is simultaneously generated on the same microtiter plate. The typical sigmoidal calibration curves is generated by assaying a solution containing no analyte (zero -standard), a standard solution containing an analyte concentration which will saturate the antibodies (saturation standard) and other intermediate analyte standard concentrations as illustrated in Fig. 2.

Based on the absorption values the calibration curve is commonly established applying the 4- parameter logarithmic equation (ref. 22, see E.2.4.2) . As an alternative to the absorption values, test inhibition values (%B/Bo) for all standards may also be calculated and also used with this equation (see E.2.4 and Fig. 3). For special applications a logit/log transformation of the data may be useful for the linearisation of the calibration curves. However, linearised curves do not allow assessment of the reliability of the immunoassay at low analyte concentrations. Calculations may be performed manually or with a dedicated ELISA data processing software commercially available from suppliers of MTP-readers and other sources.

C. PRECISION OF IMMUNOASSAYS: CRITICAL TEST PARAMETERS

The use of antibodies as "detectors" of immunoassays allows high speed analysis because sample clean-up and concentration steps may be completely omitted. However, the possibility of sensitivities (*cross reactivities*) of the antibodies with chemical structures other than the analyte as well as the inhibition of antibody performance due to compounds present in the sample matrix (*matrix effects*) may curtail the reliability of immunoassays.

C.1. Cross reactivities

As outlined in chapter A. antibodies may also react, to varying degrees, with compounds other than the analyte they were designed for. If a sample or a sample extract contains several different chemical structures which react with the antibodies, the ELISA-assay acts as a total residue method (sometimes referred to as a "group specific ELISA"). However, if the cross reactivities of the antibodies with these "analytes" in the sample differ from each other, it becomes difficult to generate accurate residue data. In an ELISA-type immunoassay, unequal cross reactivities can lead to incorrect analysis-results.

Figure 4 shows an example of how an immunoassay with widely differing cross-reactivities towards two analytes may yield false results when being applied to unknown samples. In this example the ELISA - assay detects analyte 2 with only 30 % of the sensitivity compared to analyte 1. This leads to a calibration curve of analyte 2 which results in apparent higher (300 %) concentrations.

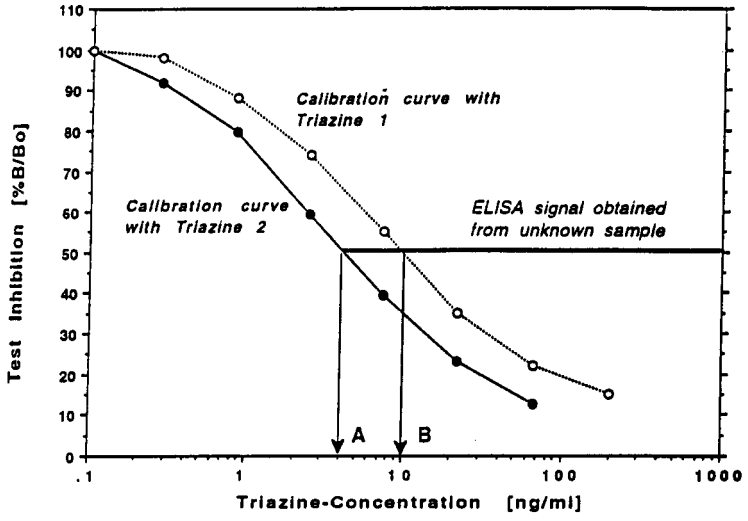

Fig. 4
Total residue concentration determined in an unknown sample using two different calibration curves.

In an unknown sample neither the analyte concentration nor the analyte composition (ratio of analyte 1 to analyte 2) are known. Therefore, depending on the calibration curve and the analyte composition in the sample, the model immunoassay illustrated in Fig. 4 deviates from - 67 % to up to + 300 % from the actual total residue in the sample.

The crucial effect of cross reactivities on the reliability of immunoassay results was highlighted in an interlaboratory trial of 14 immunoassays for s-triazines conducted by the Society of German Chemists (ref 23). The results and conclusions drawn from this study are summarised and discussed below.

All ELISA-assays tested had different sensitivities towards s-triazines such as atrazine, simazine, terbutylazine and other structurally related analytes. It is important to realize however that all of these compounds may be present (in unknown ratios) in a single sample .

All immunoassays tested in the inter-laboratory trial converted the signal intensity derived from unknown samples using atrazine calibration curves and expressed the residues as "atrazine-equivalents". Calibration standards other than atrazine were not supplied with the test kits.

The Triazine immunoassay illustrated in Fig. 5 detects atrazine with 100 %, simazine with 30 % and terbutylazine with 15 % cross reactivity. Such a range of sensitivities in one immunoassay was common and even exceeded by some of the immunoassays tested in the interlaboratory trials.

An unknown water sample containing total residues of 150 ng/l, composed of 50 ng/L each of atrazine, simazine and terbutylazine was analysed with this model immunoassay using an atrazine calibration curve. Due to the cross reactivities the theoretical total apparent residue should be 72.5 ng/L (48.3% of the "true" residue in this sample assuming 100 % recovery of the assay system). The actual apparent residue concentration obtained with this immunoassay was 51 ng (70.4 % of theoretical recovery).

It is obvious that the differences between actual analyte concentration in a sample and the corresponding ELISA - result strongly depends on the cross reactivity ratios and concentration ratios of the analytes in the samples as well as on the type of calibration curve. Increasing the percentage of s-triazines with low cross reactivity in the sample relative to the total residues of s-triazines results in a more pronounced deviation between the actual total residue concentration and the ELISA-result. This effect is illustrated in Fig. 6a and 6b.

Immunoassays are frequently proposed for the use as pre-screening methods prior to conventional re-analysis of samples identified as being "positive" by the ELISA assay. However, to identify "positive" samples, ELISA - assays with a broad range of cross reactivities can only be used in the "worst case" mode. For this the calibration curve has to be generated with the least detectable analyte (Fig. 6b).

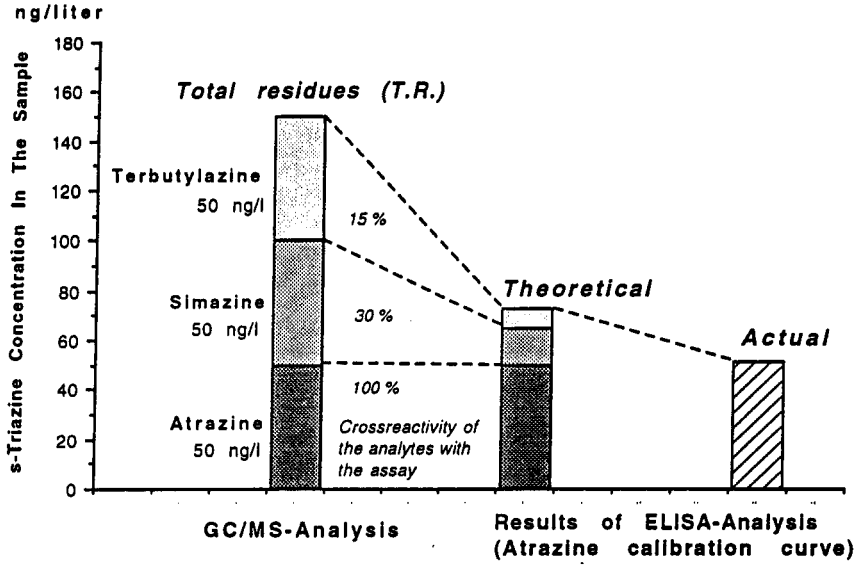

Fig. 5 The effect of antibody cross reactivity on the theoretical result of a model s-triazine ELISA-assay applied to a sample containing known amounts of atrazine, simazine and terbutylazine (calibration curve prepared with atrazine).

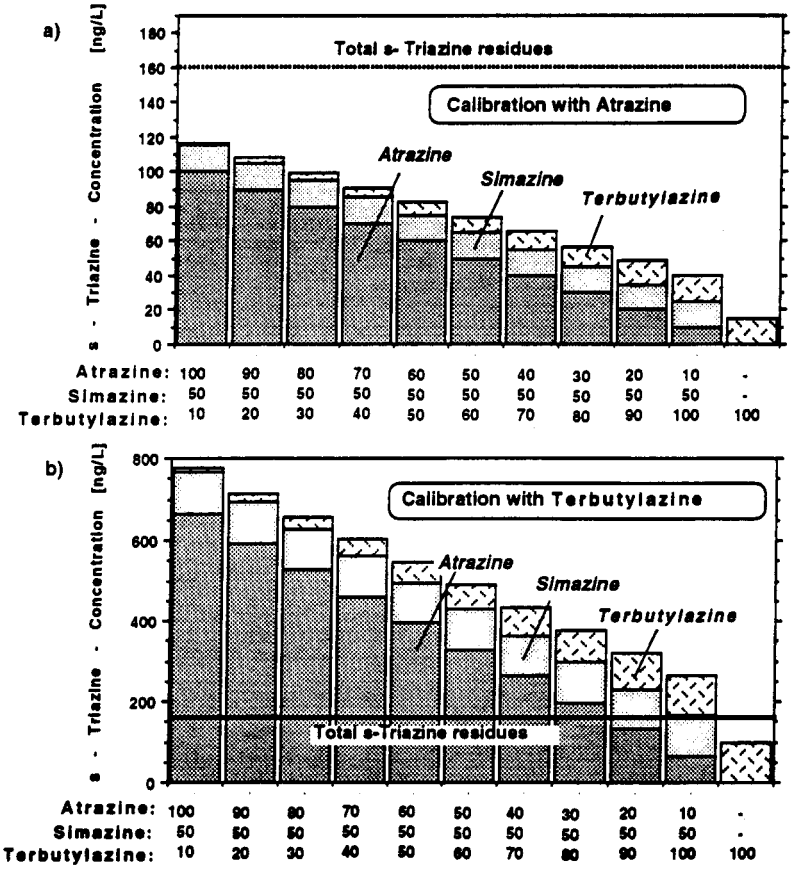

Fig. 6 a,b Results of a model immunoassay for s-triazines as affected by analyte concentration ratios in the sample. ELISA results were calculated, based on the cross reactivities of the test. All concentrations in ng/L.

Depending on the range of cross reactivities this can cause a large number of incorrect "positive" readings by the assay. In this case, the main advantage of an immunoassay used for pre-screening (i.e. saving conventional analysis time) would be lost.

These theoretical considerations highlight, how the accuracy of an immunoassay depends on the range of cross reactivities between the most and the least detectable analyte. The broader the range of reactivity the more inaccurate analysis will become. Thus, polyclonal antibodies used in immunoassays for total residue analysis, must have appropriate and similar sensitivities for all analytes present in the sample. Immunoassays with a wide range of cross reactivities for various analytes in a sample may still be successfully applied to residue analysis if they are coupled to conventional technology like HPLC (ref. 11).

Monoclonal antibodies usually are monospecific, i.e. they show high affinity only for a single chemical structure and only very little cross-reactivity to other compounds. This is the reason why immunoassays, based on monoclonal antibodies, with respect to the results obtained can be comparable to conventional residue methods for the analysis of individual compounds.

C.2 Matrix Effects

Immunoassay procedures are very rapid primarily because they usually do not require sample concentration and clean-up steps. However, this approach has some disadvantages as it may interfere with the reliability of immunoassays which are, due to the protein nature of the antibodies, prone to matrix interferences (ref. 26).

Chemical compounds present in samples or sample extracts (e.g. humic acids, solvents, heavy metal ions and others) can adversely and non-specifically effect the antibody affinity towards the analyte. Inhibition of the antibody-analyte recognition can also occur with inappropriately high or low ion concentrations and pH-values of the sample extract as well as insoluble material such as clay minerals suspended in the extracts. These so called "matrix effects" can lead to decreased antibody binding capacity and therefor can reduce the sensitivity and reliability of the immunoassay. Matrix effects are due to either non-specific alterations of the analyte (antigen) binding sites of the antibody, denaturation of the antibody or adsorption to insoluble material in the extract. In these cases the antibodies are removed from the reaction equilibrium (e.g. they do no longer bind to the solid phases provided). However, matrix compounds can also change the "appearance" of the analyte in the extract so that it can be no longer recognized by the antibody.

Matrix effects can be quantified by comparing a standard calibration curve produced in a defined buffer system with a calibration curve generated in a sample extract (Fig. 7).In immunoassays, matrix effects are characterised by a reduced rate of substrate turnover in a sample which contains matrix as compared to an equivalent buffer system. The slope of a standard calibration curve with matrix extracts is less steep than with the buffer system. Strong matrix effects can entirely inhibit the immunoassay.

Matrix effects are more pronounced in direct EIA systems (see chapter B.). In this format not only the specific antibodies are under matrix influence but also the tracer enzyme of the detection system.

In many ELISA assays the analysis results obtained from a sample with matrix effects are frequently calculated with a calibration standards made up in a buffer system. However, in these ELISAs even minor matrix effects may lead to a significant overestimation of the analyte residues in the sample (Fig.7) especially at low analyte concentrations. This is the main reason why such ELISA-assays are prone to false positive readings. In contrast, false negatice readings in ELISA are very rarely encountered.

Matrix effects may be reduced by an appropriate sample clean-up step (e.g. solid phase extraction). Also, they can be accounted for in the ELISA-assay by generating the standard calibration curves in untreated sample matrix, equivalent to the matrix of the actual residue samples.

The presence of analyte in a sample matrix considered to be untreated (contamination), or the presence of a compound non specifically interfering with the test significantly alters, even at very low concentration levels, two main assay parameters: The shape of the standard calibration curve produced in such a matrix

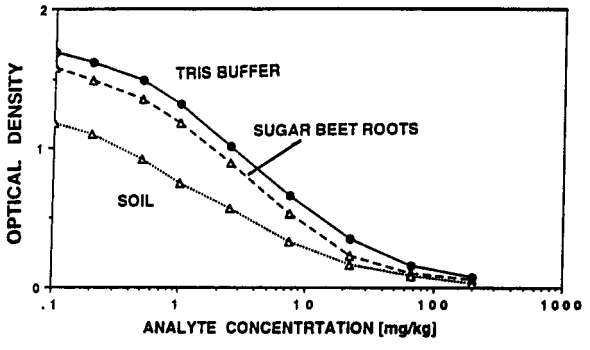

Fig. 7 Standard calibration curves of an immunoassay in a pure TRIS buffer system (pH 8.4), in TRIS buffered sugar beet extract (pH 8.4) and soil extracts.

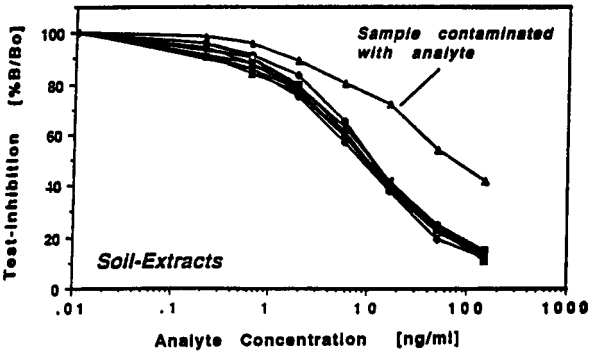

Fig. 8 Standard calibration curves of an immunoassay in TRIS buffered soil extracts (pH 8.4). Assay conditions were similar for all calibration curves.

will be characteristically changed (decreased slope, decreased test inhibition at defined concentrations; Fig. 8). Also, recovery experiments performed in the same sample material will yield results which are far above or below the acceptable levels (see E.2.2.2). The need for untreated sample material in ELISA analysis is not different from conventional analysis. For conventional analysis untreated samples must also be available because recovery experiments must be performed with samples free of analyte.

D. USE OF IMMUNOASSAYS

Immunoassays may be used as:

1. *analytical methods* for the monitoring programs
2. *analytical residue methods (research methods)* performed under GLP-compliance for the generation of residue data in the registration process
3. *enforcement methods*
4. *pre-screening methods* for the identification of 'positive' samples prior to conventional re-analysis

This technical report proposes quality standards for immunoassays used as quantitative residue analytical methods. The standards listed below are intended to provide a maximum level of accuracy of data derived from immunoassays which are used for registration and/or enforcement purpose. The quality standards for immunoassays proposed here are similar to those applied to conventional analytical methods.

E. STANDARDISATION AND QUALITY-CONTROL

The proposals for standardisation and quality assurance of immunoassays as discussed below are based on the EPA Evaluation Procedures for Tolerance Method Validations (7), on the EU guidelines for residue analytical methods which are in preparation (23), as well as on EPA views on the use of immunoassays for agro-chemicals. The personal experience of the authors who have successfully applied immunoanalytics to generate residue data for registration purpose are also being used.

E.1. Guidelines for Analytical Methods

The main criteria utilized by the EPA for the assessment of the validity of residue analytical methods by the EPA (7) are listed in table 1.

Of the EPA assesment criteria listed in table 1, items 4 and 11 are the most crucial demands with respect to ELISA technology. Proposals on how immunoassay technology can comply are discussed in detail in chapters E.2.2 and E.4 of this report.

TABLE 1. EPA criteria for the assessment of analytical methods for enforcement purpose

1.	The chemist must be able to run the method without communicating with the registrant or any laboratory that has run the method.
2.	The method must not take more than 24 hours.
3.	The method should not use carcinogenic or explosive reagents. There should be no refluxing or reaction step when laboratory personnel are not present.
4.	All equipment must be commercially available in the U.S. The method should use laboratory equipment commonly found in Federal and state regulatory laboratories performing pesticide residue analysis.
5.	Recoveries of the analyte must be in the range of 70 - 120 %.
6.	A standard of the analyte must be available from EPA Research Triangle Park Repository.
7.	The methods should be rugged enough so that equivalent columns and equipment may be substituted unless the method specifically states otherwise.
8.	The method must be tested on all commodities at analyte levels requested by OPP.
9.	If the final analytical step requires GLC or HPLC chromatography, the peaks of interest will be sufficiently resolved from other peaks in the chromatogram to enable unambiguous identification.
10.	The control samples must not contain analyte levels which will interfere with the quantitative determination of the pesticide or metabolite used as a spike.
11.	The method must not require the use of an untreated commodity or a blank.
12.	The method should describe how to obtain a homogeneous and representative sub sample from a larger sample.

In addition, there are several internal EPA papers on the use of ELISA techniques and what these must contain to make EPA more "comfortable" with this methodology. They stress the need to confirm both positive and negative findings with conventional techniques, depending on the expectations, of ELISA-methods .

The proposals discussed below are also based on the EEC Council Directive which established Annex II, III and VI of the "Uniform Principles " (Directive 91/414/EEC) with respect to residue analytical methods (14).

E.2 Proposed Quality Standards

E.2.1 Materials and Supplies

E.2.1.1 Chemicals

All chemicals supplied and used with immunoassays must be of "analytical grade" standard.

E.2.1.2 Antibodies

- Type and source of the antibodies must be specified in the method rotocol. The cross reactivities of the specific monoclonal or polyclonalantibodies (or sera) supplied with the immunoassay must also be listed in the method protocol.

E.2.1.3 Solid phase materials

- If precoated solid phase material is supplied with the immunoassay, the type of coating (antigen-conjugate or antibodies) must be specified in the test manual. If the solid phase material has to be coated by the user, a detailed protocol must be supplied with the immunoassay.

It is also important to specify in the method protocol the requirement for a blocking agent (protein) in buffer-systems if they should be necessary for the immunoassay. For storage stability requirements of solid phase materials see E.2.1.3.

E.2.2 Specifications of the Assay

E.2.2.1 Test specificity

The cross reactivity of antibodies with chemical structures other than the analyte can significantly reduce the reliability of residue results (see C.1). Therefore it is necessary to define limits of cross reactivities in an immunoassay above or below which this test can not be used as a residue analytical method without further modifications. To comply with these standards it may be required to mix antisera or antibody populations with differing cross-reactivities. This should pose no problem as long as the antisera-mix or the antibody population is clearly defined in the immunoassay they are applied to.

With respect to cross reactivities a DIN (German Industry Norm) standardisation proposal currently in preparation for an ELISA assay for water analysis defines the following standards:

- Immunoassays used for group-specific analysis (total residue method) must allow, curve applied, the correct quantification of total residues within a range of +/- 20 % of the actual total residues obtained in a sample.

- In immunoassays designed for the quantification of individual analyte concentrations (individual residue methods) the cross reactivities of antibodies with compounds other than the analyte should not exceed 2 %. This leads to a maximum deviation of + 50 % from the actual residue concentration in the presence of a 50-fold concentration of likely cross reactants. In samples with unexpectedly high levels of analyte confirmatory analysis with chromatography based methods should be performed.

If an ELISA-assay does not meet the specifications proposed above, compounds interfering with the antibodies must be removed from the sample extracts prior to analysis. The clean-up steps to accomplish this must be specified in the method protocol too.

Follow up activities include:

» Request producers of commercially available test kits to supply data about the cross-reactivities of the antibodies with chemicals other than the analyte.

» After mathematical evaluation of the cross reactivity data compile a list of those immunoassays which meet the specifications listed above.

» Define maximum allowable cross reactivity of monoclonal antibodies in view of the accuracy of the residue result in the presence of the highest probable concentration of cross reactants.

E.2.2.2 Fitting of standard calibration curves

The precision of data derived from immunoassays depends on the number of reference standards which are used to shape the sigmoidal calibration curve and on the method of curve fitting applied. Due to the

sigmoidal shape of the curve it is paramount to use enough levels of reference standards to define the upper and lower limit of quantitation (linear range of the calibration curve), the maximum response and the limit of detection of the test. Since the consitency of the solid phases (MTP) may vary it is essential to calibrate each analytical series on a MTP. Due to the lin/log conversion of the dose/response curve it is advisable not to consider the untreated calibration standard for the curve fitting.

- The shape of the standard calibration curve of immunoassays should be defined with reference standards (calibration standards) at not less than five levels of concentration plus an analyte free standard for the definition of the maximum response of the test. Two calibration standards should be chosen which are close to the lower and higher limits of quantitation of the assay. One calibration standard should be close to the 50% inhibition level of the assay.

- Each routine analytical series on a MTP must be calibrated with a calibration curve.

- Curve fitting of standard calibration curves of immunoassays should be performed with the 4-parameter-logistic equation of Rodbard (13) as specified in equation 1:

$$Y = d + \frac{a - d}{1 + (x/b)^b} \qquad (1)$$

x	:	Analyte concentration
y	:	Test signal (absorption value)
a, b, c, d	:	Constants

The constants a and b represent the upper and lower asymptotes of the curve, c represents the analyte concentration at the midpoint of the test (= 50 % B/Bo) and b represents the slope of the curve at c. Dedicated software based on this equation is commercially available.

- Curve fitting must be done without of the untreated calibration standard.

- The variation of replicate calibration standards should not exceed the values given below:

Level of calibration	Variation (+/- %)
Lim. o. Quantitation (low)	25
Midpoint (50 % B/Bo)	15
Lim. o. Quantitation (high)	25

- Levels of reference standards for the calibration curve should be defined which allow results from fortification experiments at the lower limit of quantitation to fall between two actual calibration standards (not between the untreated control and the lowest calibration standard).

Follow-up activity

» Supply literature data or use available raw data to define the most appropriate procedure for the fitting of the standard calibration curve for immunoassays.

E.2.2.3 Limit of determination, method-validation

Frequently only limits of detection of immunoassays are published in the literature. These are determined from standard calibration curves generated in buffer-systems or equally 'clean' matrices. However, the limit of detection [e.g. the smallest concentration in an analytical sample for which the method yields significant signals (level of confidence = 95 %)] in an analytical method is at best only of very minor interest (ref. 14). Much more meaningful for the performance and sensitivity of a residue method is its limit

of determination. It is defined as the lowest concentration at which an acceptable recovery of analytes is obtained (ref. 14).

- The limits of determination of an immunoassay must be statistically validated with fortification experiments in each sample material the test is applied to. All validation must be performed according to EPA or EEC standards or guidelines of the German Research Organisation DFG (15, 23, Tab. 1 and D.2).

For the validation of an immunoassay as an analytical method for agrochemicals a minimum of 4 samples must be fortified with the concentration of analyte(s) representing the proposed limit of determination of the assay, as well as its ten- and hundred fold concentration. These samples must be analysed in parallel with the immunoassay. The average recovery of the fortification experiments at each level of fortification must be in the range of 70 - 120 % with a variation coefficient of +/- 20 % to fulfil EPA standards (see Tab. 1). The proposals for the new EEC guidelines define mean recoveries of 70 - 110 % (+/- 20 %) (ref. 14). The immunoassay must be adapted (validated) for all matrices it is supposed to be used in. Matrices the assay was validated for must be specified in the method protocol.

- The correct function of the immunoassay must be revalidated with each sample series analysed. Each sample series must contain a minimum of one untreated sample spiked with an analyte concentration which represents either the limit of determination of the assay or which falls within the concentration range of the unknown samples. The recovery from this fortification experiment must fall within the ranges specified above.

E.2.2.4 Precision

Precision of immunoassays include two parameters which must be evaluated for each anlytical method in accordance with German (BBA) and EEC guidelines (ref. 14, 20):

Repeatability:

Repeatability is defined as the value below which the absolute difference between two single results, obtained with the same method on an identical test sample under the same conditions (including the same analyst) may be expected to lie with a probability of 90 %. As with conventional methods the difference between the maximum and minimum of four test results of an immunoassay at about the limit of determination or higher analyte concentrations must be less than the following values:

Residue level (mg/kg)	Difference (mg/kg)	Difference
0.01	0.010	100.0 %
0.10	0.025	25.0%
1.00	0.125	12.5 %
>1.00		12.5%

Reproducibility:

Reproducibility is defined as the value below which the absolute difference between two single results, obtained with the same method on an identical test sample but under different conditions (different analyst, different time) may be expected to lie with a probability of 90 %. As with conventional methods the difference between the maximum and minimum of three test results of an immunoassay at about the limit of determination or any other analyte concentration must meet the specifications listed below:

Residue level (mg/kg)	Difference (mg/kg)	Difference
0.01	0.02	200.0 %
0.10	0.05	50.0%
1.00	0.25	25.0 %
>1.00		25.0%

As immunoassays in general involve less, if any, sample clean-up steps the precision requirements with respect to reproducibility and repeatability should be quite easily fulfilled.

- The repeatability and the reproducibility of an immunoassay should be specified in the method protocol for each sample material the immunoassay is validated for.

E.2.2.5 Practical working range

Due to the typically sigmoidal shape of the standard calibration curves of immunoassays only the linear part of the calibration curve, centred around the midpoint of test inhibition, should be specified as the practical working range of the assay.

- The working range of an immunoassay must be specified in the method protocol for each sample material the immunoassay is validated for.

E.2.2.6 Handling of the matrix effects

As outlined in C.2 the accuracy of immunoassays may be significantly decreased by the effects of matrix compounds on the antibodies. Several strategies which can be adopted to eliminate or compensate for these matrix influences are as follows:

- Sample extracts may be purified by means of clean-up steps or ELISA coupling techniques to allow the use of standard calibration curves generated in buffer systems, when analysing unknown samples.

In some cases such clean-up procedures may be as time consuming as conventional methods.

- Samples or sample extracts may be analysed without prior clean-up using standard calibration curves which are prepared in untreated sample material.

This technique requires untreated control matrices which is in conflict with EPA-requirements (see Tab. 1).

- Analysis may be performed without prior extract clean-up using "matrix modifiers". Sample signals are evaluated with standard calibration curves prepared in buffer systems which are supplemented with appropriate "matrix-modifiers" (see Fig. 9) These "matrix-modifiers" which are produced from untreated sample extracts, must be standardised and are supplied by the producer of the immunoassay.

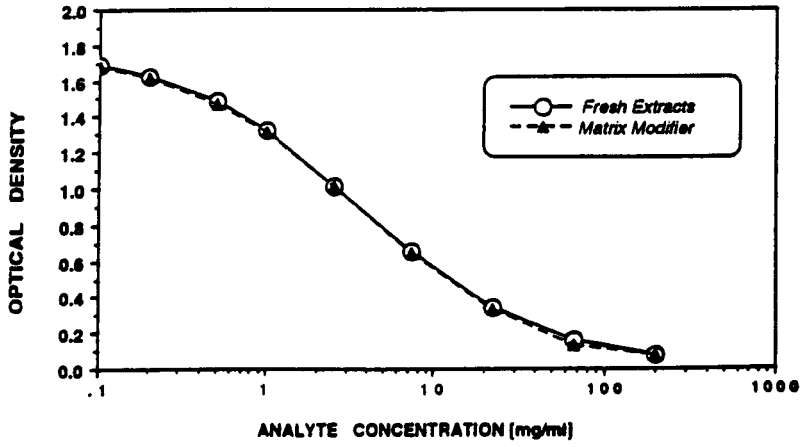

Fig. 9 Comparison of standard calibration curves of an immunoassay in sugar beet extract and in a buffer system supplemented with a "matrix modifier" which had been stored for 6 months prior to analysis.

Follow-up activities

» Collect data on the use of matrix modifiers in conventional residue methods. Compare these methods with the matrix modification of ELISA standard calibration curves.

» Collect data from users of immunoassays or establish standardised procedures which make it possible to identify the analyte or other interfering coumpound(s) which contaminate the control matrices used for calibration curves .

» Standardise procedures for the production, quality control and storage stability of matrix-modifiers. Specify procedures in the method protocol.

» Evaluate methods for ELISA compatible, rapid sample clean-up procedures.

E.2.3 *Experimental Procedure*

E.2.3.1 Sample size

Due to high sensitivity of the antibodies, immunoassays are generally performed in very small sample volumes However, representative aliquots from a laboratory sample may not be obtained if the subsample for the ELISA-assay becomes too small. This can result in a loss of accuracy and reliability of the test results due to nonhomogenous distribution of the residue in the sample.

- The size of the extracted samples in immunoassays must allow for representative sub sampling. Specify in the method protocol a minimum sample size for extraction. Continue the procedure with an appropriate aliquot of the extract.

Follow-up activity

» Supply literature data (or provide experimental data) on the correlation of the test precision and the sample size for extraction in different matrices.

E.2.3.2 Preparation of extracts, clean-up procedures

- The preparation of sample extracts, all clean-up or analyte concentration steps, and the pH-value to which the final extract must be adjusted prior to analysis must all be specified in the method protocol.

E.2.3.3 Number of replicate analyses

In order to use to a full extent the sample capacity of immunoassays and other considerations the number of replicate analyses is often reduced to two to the disadvantage of the reliability of the results.

- In accordance with a proposal for a DIN-standardisation (German Industry Norm) of an ELISA-assay (see E.2.1.4) each unknown and calibration sample should be analysed at least in three, better four replicates with immunoassays. In MTP-formats the border wells should not be used for analyses except if specialised equipment such as incubator shakers are available. For the maximum allowable variation between the individual results of unknow or fortified samples refer to chapter 2.2.4 (Precision) of this report.

Follow-up activity

» Supply literature data or use available raw data for the correlation of the number of replicate analyses and reliability of results of conventional and/or immunochemical residue analysis

E.2.4 *Evaluation of Data*

E.2.4.1 Standardised data formats, % B/Bo values

The results of immunoassays may be calculated with the use of the absorption values obtained with the photometer.

- To allow the direct comparison of calibration curves for the assessment of the validity of individual assay series (see chapter C.2), absorption values may be converted into their corresponding test-inhibition values (% B/Bo-values) using equation 2:

$$\% B/B_0 = \frac{(A - A_{sat})}{(A_0 - A_{sat})} \times 100 \qquad (2)$$

A : Absorption values of sample or standard calibration samples
A_0 : Absorption value of the zero-control sample
A_{sat}: Absorption value of the analyte saturated sample (< 5 mg/litre)

The % B/Bo value represents the ratio (in %) of the tracer bound to the solid phase in the presence and absence of the analyte. The inhibition values range from 100 % (absence of the hapten) to n % (hapten saturated antibodies).

E.2.4.2 Fitting of standard calibration curves

- For definition, fitting and precision of the calibration curves see chapter 2.2.2

Follow-up activity

» Supply literature data or use available raw from routine analyses about the variability of replicate calibration standards of the standard calibration curve for immunoassays.

E.2.5 *Reporting of Data*

Residue analysis data should be reported as µg analyte/kg (i.e. in water) or mg analyte/kg sample material.

E.3. Standardised Test Format

As outlined in chapter B. a wide range of test formats are used for immunoassays. A survey of the market for ELISA instrumentation indicates that immunoassays formatted for microtiter plates are still favoured (ref. 24). The MTP-format offers high sample capacity, low cost, ease of handling and the potential for complete automation.

Depending on their intended use and in order to speed up the standardisation process it seems advisable to promote only few test formats for those immunoassays which are intended to be used as enforcement or research residue methods .

This also keeps the need for different types of instrumentation at a reasonably low level. If the microtiterplate format is favored, strip-plates should be offered since this format offers more flexibility.

- Immunoassays used as enforcement or residue analytical methods should be formatted as heterogeneous direct or indirect assays. MTPs should be favoured as solid phase. In the first stage detection systems should be limited to enzyme tracers for the use with UV/Vis- or fluorescence-detection.

Follow-up activity

» Supply literature for information or use own data on the performances of different test formats with respect to reliability, precision, ease of handling and cost efficiency.

E.4. Supply and Storage of Immunoassays

In contrast to conventional technology analysts who want to use immunoassays require an entire assay kit in addition to a detailed method protocol. Alternatively they need to be able to acquire at least those components which were specifically produced for the immunoassay [specific antibodies, coating conjugates and hapten enzyme tracers (if applicable)].

Acceptance of immunoassay technology will require easy and rapid access of registration agencies and prospective users to the immunoassays and/or their components. Storage and distribution of immunoassays and materials can be contracted out to specialised subcontractors as well. For analytical immunoassay methods which are solely used internally, supply and storage of entire tests or components thereof may be organized differently from the suggestions proposed below.

- Entire immunoassays or the specific assay components which are listed below have to be kept readily available for the prospective user. Customised test components are:

  1. Antigen-specific antibodies or antisera **or** precoated MTPs or tubes
  2. Coating antigen **or** precoated MTPs or tubes
  3. Antigen-enzyme tracers

Follow-up activity

» Decide which immunoassay format should be promoted with respect to potential commercial users and registration agencies.

Upon request EPA representatives seemed to favor transfer of larger quantities of test components (antibodies, tracers etc.) to their facilities in order to have the ELISA methods readily available.

- Immunoassays must specify the storage time or a date of expiration after which the assay, or parts of it, must not be used. Immunoassays must be tested, at regular intervals, for their compliance with the specifications defined in the method protocols. These quality control checks must be performed by the producer, or suppliers. The results of the quality control checks must be cited in the method protocol or otherwise supplied with the method..

This is of particular importance not only with respect to the quality of antibodies used in the assay but also for the stability of the reference substances supplied.

E.5. Contents of Method Protocols

Commercially available immunoassays frequently are supplied with only very brief instructions and information about the most basic handling steps.

- Immunoassays must be supplied together with comprehensive instruction manuals which allow the correct use of the assays (see Tab.1.1). These method protocols must contain detailed information about materials and instrumentation as well as the handling of the test including all sample preparation and extract clean-up steps.

The method protocols must specify all relevant data on the quality standards of the assay as listed in D. Data on the storage stability of reagents must be included as well as instructions for trouble shooting procedures. The method protocol must specify all sample matrices the assay is approved for.

Follow-up activity

» Define standards for the minimum requirements for format and contents of method protocols of immunoassays for agrochemicals.

F. GENERATION OF BRIDGING DATA

During an introductory phase residue data produced with immunoassays likely will have to be supported by data produced with conventional methods which were applied to the same samples ('Bridging data').This requires, at least for a limited time, the use of conventional residue methods in parallel to immuneanalytical procedures. Bridging data may be produced with any of the following conventional techniques: HPLC, GC, HPTLC and 'accountability studys' (i.e. application of a new analytical method to radioactive samples in which metabolism of an active ingredient was studied). Accountabilities prove the correct performance of analytical methods with respect to extraction and detection of relevant analytes in "natural" samples. Analytical results in an accountability must reflect the findings of metabolism with respect to concentration and type of analyte present in the sample.

Follow-up activity

» Inquire with registration agencies and prospective customers regarding the number of bridging data required to support immunoassay data.

G. INTERLABORATORY EXPERIMENTS

Proof of the reliability and ruggednes of an immunoassay as a new analytical technique may be obtained by conducting independent laboratory validations (validation by selected and qualified laboratories). These studies have become common practice with conventional analytical methods and are excellent means to generate confidence (for both developer and user) about the reliability of a new technique or method.

- Interlaboratory studies should involve the analyses of samples with known analyte composition and concentration (fortified samples) as well as 'unknown' residue samples. These samples may be provided or may be generated by external contract laboratories. The fortified samples should be analysed in several laboratories with identical immunoassays to show interlaboratory variation of the results (handling of the test kits). Preferably selected samples should be re-analysed with conventional methods to check for biases.

Follow-up activities

» Provide literature or experimental data about independent laboratory validations and/or interlaboratory experiments with ELISA assays.

» If necessary organise (among the members of the panel) an interlaboratory trial with a selected ELISA assay.

As discussed above, two interlaboratory trials of immunoassays for s-Triazines were conducted by the Society of German Chemists (ref. 23, 25).

H. NATIONAL APPROACHES FOR STANDARDISATION

In recent years several working groups in Europe and the United States have been established which, by different approaches, ultimately aim at defining standards for the use and quality of immunoassays when applied to agrochemicals. In Germany, a working group of the Society of German Chemists (GdCH) with members from universities, water works, agrochemical industries and research laboratories have met on a regular base since 1990. This group has performed two interlaboratory trials with commercially available immunoassays for s-Triazines (ref. 23, 25). Very recently a guideline protocol for an ELISA assay for water analysis was forwarded to the DIN-Guideline Commission of the German Industry for approval. A similar working group, the American Environmetal Immunochemical Consortium (AEIC) has been established in 1993, with the aim to propose giudelines and quality criteria for ELISA residue analysis (ref. 15, 16). It comprises members from agrochemical industries, commercial ELISA-kit suppliers, universities and representatives from the EPA.

In the United Kingdom a discussion panel, comprising representatives from industries universities and independent laboratories was established, with the aim of generating a wider data base on the reliability and applicability of immunoassays for agrochemicals. This working group, in its fact finding stage, has mainly relied on data and experience from its own members. It is also attended by representatives of the Ministry of Agriculture, Food and Fisheries (MAFF).

It is obvious that all three panels, even though using different approaches, are ultimately aiming at a similar goal: to define nationally accepted guidelines and standards for the use of ELISA-methods for agrochemicals. Once achieved, this would allow industries as well as independent laboratories to use ELISA-methods for registration and monitoring purposes. It would be highly desirable to promote an exchange of information between these groups. With representatives from agrochemical industries, registration agencies, commercial kit suppliers and research laboratories involved this exchange should result in internationally accepted, "uniform principles" for the application of ELISA technology.

Follow-up activities

- » Collect comprehensive information about the participants, current activities and goals of working groups in Europe, the United States and other countries involved in the application, standardisation and quality control of ELISA assays for agrochemicals.

- » Provide appropriate means for a regular exchange of information approaches and ideas between these panels with the aim to formulate internationally accepted guidelines for the use of ELISA-methods

I. SUMMARY OF RECOMMENDATIONS

Test specificity

Producers of commercially available test kits should routinely supply data on the cross-reactivity of antibodies with chemicals other than the analyte. This will enable mathematical evaluations of cross-reactivity data to be made.

Define maximum allowable levels of deviation between actual total residues in a sample and the result of an ELISA-assay which is used for group specific detection. Considering the variability of residue results with conventional methods, a maximum value of +/- 20 % for the deviation between ELISA-result and actual residues would be acceptable. This must be decided (on a case-to-case base) in accordance with relevant guidelines.

Handling of matrix effects

Promote the acceptance of matrix-modified standard calibration curves for analytical methods in general and for immunoassays in particular with the registration agencies.

If the use of matrix modifiers is acceptable to authorities, recommend standardised procedures for their production, quality control and storage stability.

Sample Size

Minimum sample sizes for sampling and extraction of representative aliquots should be defined.

Number of replicate analyses

More information is needed on the correlation of replicate analysis and reliability of results of conventional and/or immunoassay methods.

Fitting of standard calibration curves

Evaluate the most appropriate procedure for the fitting of standard calibration curve for immunoassays.

Supply and storage of immunoassays

Because the application of immunoassays to residue analysis for agrochemicals is relatively new, the minimum requirements for format and contents of protocols should be defined.

Generation of bridging data

A fully validated analytical method should be acceptable in its own right irrespective of the basis of the method. However, at this stage of the development of immunoassays for agrochemicals it is recommended that bridging data with existing chromatographic methods be obtained. The extent of data needed should be assessed on a case-by-case basis.

Interlaboratory experiments

It is recommended that further interlaboratory studies similar to those conducted by the ELISA working group of the Society of German Chemists should be conducted. Results of such studies can be of considerable assistance in speeding up the acceptance of the immunoassay approach both by industries and regulatory authorities.

ELISA working groups

The activities and approaches of working groups or panels (whose aim is to propose national guidelines and set standards for the use of ELISA-methods) should be made public and coordinated. An exchange of information and the cooperation between these groups should be promoted by appropriate means (workshops, technical exchange meetings, circulation of papers) with the aim to set internationally accepted guidelines for the use of ELISA-methods.

K. REFERENCES

1. R.O. Harrison, M.H. Goodrow, S.J. Gee, and B.D. Hammock. In *Immunoassays for Trace Chemical Analysis: Monitoring Toxic Chemicals in Humans, Food and the Environment.* (Vanderlaan, M., L.H. Stanker, B.E. Watkins and D.W. Roberts eds.), pp.14-27, ACS Symposium Series 451(1989).

2. P.Y.K. Cheung, S.J. Gee and B.D. Hammock.In: *The Impact of Chemistry on Biotechnology: Multidisciplinary Discussions.* (Philips, M.P., S.P. Shoemaker, R.D. Middlekauff and R.M. Ottenbrite, eds.), pp. 217-229, ACS Symposium Series No. 362, American Chemical Society, Washington D.C. (1988).

3. B.D. Hammock. In *Biotechnology for Crop Protection* (P.A. Hedin, J.J. Menn and R.M. Hollingworth eds., pp. 298 - 305, ACS Symposium Series No. 379, American Chemical Society, Washington D.C. (1988).

4. F. Jung, S.J. Gee, R.O. Harrison, M.H. Goodrow, A.E. Karu, A.L. Braun, Q.X. Li and B.D. Hammock. *Pest. Sci.* **26**, 303-317 (1989)

5. J.F. Brady, G.F. LeMasters, R.K. Williams, J.H. Pittman, J.P. Daubert and M.W. Cheung. *Abstr. Pap. Am. Chem. Soc.* (204 Meet., Pt. 1, AGRO 43), (1992)

6. R.J. Bushway , L.B. Perkins, L. Fukal, R.O. Harrison, and B.S. Ferguson. *Arch. Environ. Contam. Toxicol.*, **21**, No. 3, 365-70 (1991)

7. B.D. Hammock, S.J. Gee, R.O. Harrison, F.Jung, M.H. Goodrow, Q.X. Li, A.D. Lucas, A. Szekacs and K.M.S. Sundaram. In *Immunochemical Methods for Environmental Analysis.*, (J.M. Van Emon and R.O. Mumma, eds), pp. 112-139, ACS Symposium Series 442 (1990)

8. Newsome W.H. and P.G. Collins. *Bull. Environ. Contam. Toxicol.* **47**, No.2, 211-16 (1991).

9. Wigfield Y.Y. and R. Grant. *Bull. Environ. Contam. Toxicol.* **49**, No.3 , 342-47 (1992).

10. J.H. Skerrit , A.S. Hill, S.L. Edwards, H.L. Beasley, and L.P. McAdam. *Abstr. Pap. Am. Chem. Soc.* (203 Meet., Pt. 1, AGRO 151) (1992)

11. J.H. Skerrit , A.S. Hill, H.L. Beasley, S.L. Edward and D.P. McAdam. *Journal of AOAC International* **75**, No.3, 519-527 (1992)

12. Brandon D.L. and R.G. Binder. *J. Agric. Food Chem* **40**, No. 9, 1722-26 (1992).

13. K.S. Goh, S.J. Richman, J. Trojano, C.L. Garretson, J. Hernandez and J Hsu. *Bull. Environ. Contam. Toxicol* **48**, No. 4, 554-560 (1992)

14. Proposals for a Councel Directive establishing Annex II, Annex III, Annex VI of Directive 91/414/EEC; Uniform Principles (1993). Brussels

15. C.A. Mihaliak and S.A. Berberich. In *Immunoanalysis of Agrochemicals* (J.O. Nelson, A.E. Karu, R.B. Wong eds.), pp 288-300, Americam Chemical Society, Washington (1995)

16. J. Rittenburg and J. Dautlick. In *Immunoanalysis of Agrochemicals* (J.O. Nelson, A.E. Karu, R.B. Wong eds.), pp 288-300, American Chemical Society, Washington (1995)

17. Lee S.M. and S. Richman. *J. Assoc. Off. Anal. Chem.* **74**, No. 6, 893 (1991).

18. D.B. Baker, R.J. Bushway, S.A. Adams and C Macomber. *Environ. Sci. Technol.* **27**, No.3, 562-564 (1993)

19. Rückstandsanalytik von Pflanzenschutzmitteln, Mitteilung VI der Senatskommission für Pflanzenbehandlungs-, Pflanzenschutz- und Vorratsschutzmittel; Methodensammlung der Arbeitsgruppe "Analytik",Deutsche Forschungsgemeinschaft, VCH Verlagsgesellschaft mbH, Weinheim (1991)

20. **Bewertung von Pflanzenschutzmitteln im Zulassungsverfahren: Biologische Bundesanstalt für Land- und Forstwirtschaft, Braunschweig, Paul Parey, Berlin, 1992**

21. **A.D. Lucas, S.J. Gee and B.D. Hammock.** *Journal of AOAC International* **78, No. 3, 585-591 (1995)**

22. **D. Rodbard.** *Clin. Chem.* **20**, 1255 (1974).

23. P.D. Hansen, B. Hock, R. Kanne, A. Krotzky, U. Obst, U. Oehmichen, C. Schlett, R. Schmid and L. Weil. Z. *Wasser-Abwasser-Forsch.* **24**, 20 -25 (1991).

24. O. Janssens. Diploma Thesis, Agricultural University Press Gembloux, Belgium, ISBN 2-4532-6675-8 (1993)

25. P.D. Hansen, B. Hock, R. Kanne, A. Krotzky, U. Obst, U. Oehmichen, C. Schlett, R. Schmid and L. Weil. Z. *Wasser-Abwasser-Forsch.* **24**, 65-70 (1991).

26. A.J. Krotzky. Z.*Wasser-Abwasser-Forsch.* **24**, 83-89 (1991)

27. D.P. Wright. *Publication of the United States Environmental Protection Agency, Office of the Pesticide Programs*, Washington, DC 20460 (1989)

28. C. Wittmann and B. Hock. *Nachr. Chem. Techn. Lab.* **39**, Nr. 11, 2-40 (1991).

29. C. Wittmann and B. Hock. *J. Agric. Food Chem* **41**, Nr. 9, 1421-1425 (1993)