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USE OF IMMUNOASSAYS FOR THE ANALYSIS OF PESTICIDES AND SOME OTHER ORGANICS IN WATER SAMPLES

(Technical Report)

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Use of immunoassays for the analysis of pesticides and some other organics in water samples (Technical Report)

Synopsis. Immunoassay (IA) kits were first developed for use in the medical field where they now have extensive usage but over recent years many kits have been developed for determining pesticides and other organic chemicals in environmental samples. This paper provides a draft specification for EIA kits and an experimental design for validation of their performance. Enzyme immunoassay (EIA) kits available commercially for analysis of organic chemicals in water are reviewed in the light of the proposed requirements for analysis of pesticides in drinking water. Finally, some recommendations are given regarding the use of the kits under laboratory and field conditions.

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INTRODUCTION

IA analytical methods can offer an inexpensive, simple and rapid way of determining contaminants in environmental samples, including water (1-3). They are best applied to the determination of contaminants of concern that are relatively difficult to measure by conventional analytical methods and require frequent rapid monitoring. Many organic chemicals, including certain herbicides, fall into this category.

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The EC Directive relating to the quality of water intended for human consumption (1980) specifies a limit of 0.1 $\mu g\ l^{-1}$ for any individual pesticide in drinking water and monitoring pesticides at this level in water requires a very sensitive and specific method of analysis. Methods are available using high performance liquid chromatography (HPLC), gas chromatography (GC) and mass spectrometry but they are relatively costly, can be slow to produce results and do not always achieve the target performance in respect of sensitivity and selectivity.

IA techniques for low molecular weight compounds are usually of the competitive type. The determinand (chemical to be determined) and a known amount of a labelled form of the determinand compete for a limited number of binding sites on a specific antibody. The presence of unlabelled determinand results in less labelled determinand binding to the antibody and subsequently being detected in the assay. Hence, the determinand concentrations can be measured using a calibration curve produced for standard solutions.

Suitable antibodies are produced by injection of the determinand or the determinand conjugated to a larger protein molecule into an animal, usually a sheep or rabbit. As most pesticides do not have sufficiently large molecular weights (i.e. >10,000) they will not usually induce an immune response in an animal and consequently must be coupled (or conjugated) to a large protein molecule, such as thyroglobulin, before injection. Covalent coupling of the determinand to the protein molecule requires the presence of a reactive chemical group, such as an amino group ($-NH_2$) or a carboxylic acid group (-COOH). If the determinand does not have a reactive group, it is necessary to use an analogue of the determinand that contains such a group. Providing there is sufficient similarity between the analogue and the determinand, the antibody produced by injection of the analogue should respond successfully to the determinand.

PERFORMANCE AND TESTING SPECIFICATION FOR IMMUNOASSAY KITS

EIA method specification

In general, the methods currently used for specifying the performance requirements and performance testing of EIA kits are derived from their medical applications, not the needs of environmental analysis. In addition, there are a variety of different ways of specifying and testing the performance of EIA kits which makes comparison of the performance of EIA kits from different manufacturers and comparison of the performance of EIA kits with that of conventional analysis quite difficult. A suggested specification has therefore been drawn up based on the UK Government's Department of the Environment's Standing Committee of Analysts format for conventional analytical methods. This provides a suggested format for EIA analysis methods, including performance characteristics, by using a pesticide as the example determinand and drinking water as the example water matrix. However, the same standard format can be applied to other determinands in other water matrices by appropriate modification of the specific performance characteristics. Indeed, most of the points discussed regarding immunoassay methods apply whatever environmental water matrix is being analysed.

Performance characteristics

The values suggested in the performance characteristics (Table 1) are those recommended for analytical methods in the UK Department of the Environment booklet 'Guidance on Safeguarding the Quality of Public Water Supplies'. These recommendations are acknowledged to be demanding and some existing chromatographic methods for pesticides do not achieve the desired performance. However, they provide targets which the analytical methods should strive to achieve for the purpose of compliance monitoring. Immunoassay methods which fail these strict criteria may still be of use in pesticide analysis, particularly if the intended application is as a screening measurement rather than regulatory monitoring.

Principle of operation of immunoassay analysis

The main principle of immunoassay is the biochemical reaction between an antibody and an antigen, which for pesticide analysis is a pesticide. The assay is represented schematically in Fig. 1. The IA kit is

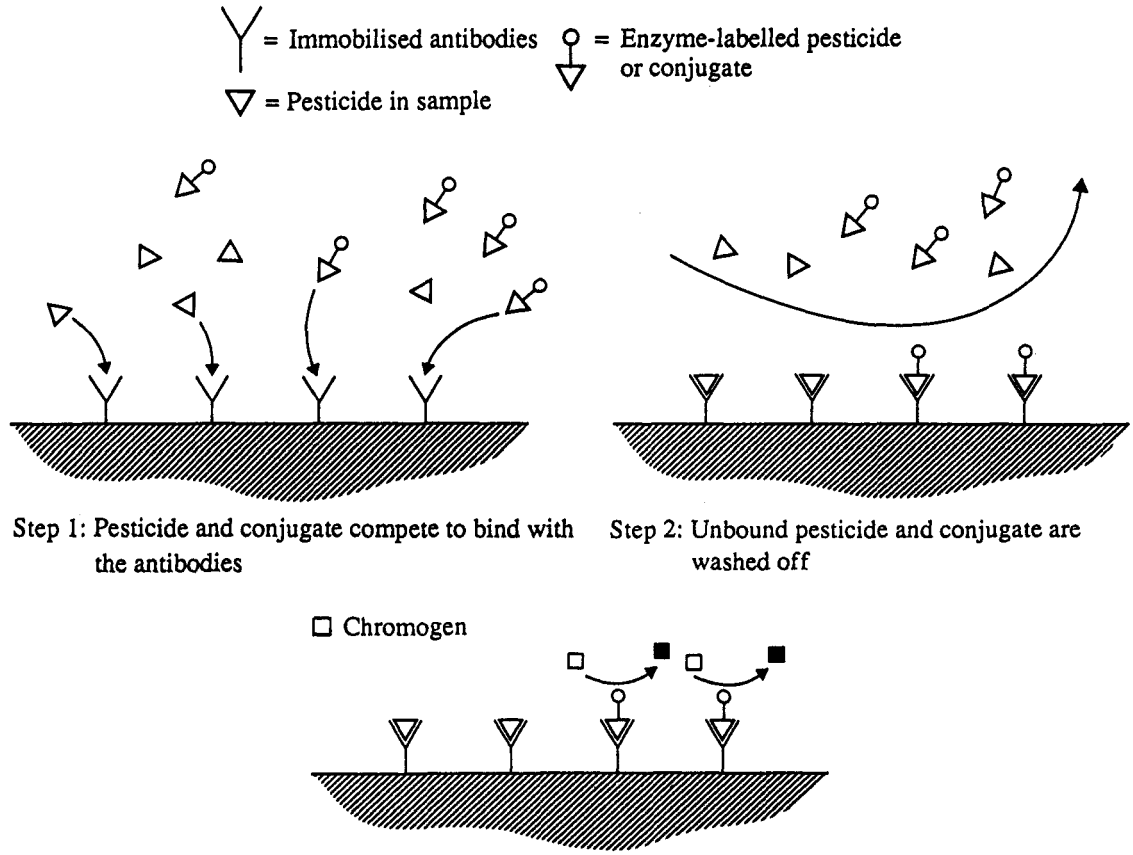

Fig. 1 Schematic of immunoassay

supplied with antibodies which have been raised to react selectively with compounds that resemble a specified pesticide(s). The antibodies are prepared by the IA kit manufacturers through stimulation of a mammalian immunosystem by a pesticide-hapten compound, which combines the functional group of the pesticide and the high molecular weight required to stimulate antibody formation. Once prepared in this manner, the antibodies may respond to the pesticide(s) specified and some antibody selection is needed.

The kit also includes a labelled form of the pesticide, often termed the 'conjugate'. The label is normally an enzyme (enzyme immunoassay, EIA, or enzyme-linked immunosorbent assay, ELISA), the presence of which can be determined through its enzymatic properties. In the assay (Fig. 1), pesticide in the water sample (typical volumes being 50-200µl) competes with the conjugate pesticide for a limited number of binding sites on the specific antibodies, which may be immobilised on the walls of a small cell or on suspended particles. The presence of unlabelled pesticide in the sample results in less label being bound to the antibody in the first stage of the assay. In the next stage, unbound pesticide and conjugate are removed through washing steps. In the final stage, the amount of bound labelled pesticide is determined by the action of the enzyme. The enzyme catalyses a reaction between added reagents (termed 'chromogen' and 'substrate') which produces a colour change in the solution. Pesticide concentrations in the sample are determined photometrically from comparison with standards. A consequence of this principle is that the response is inversely proportional to the amount of determinand in the water sample.

Interferences

These can be considered in two categories which reflect the way that the interference affects the IA kit. However, it should be noted that in general, IA kits are no more or less susceptible to interferences than conventional analytical methods.

TABLE 1. Performance characteristics

1	Substance determined	Depends on the choice of IA kit. Kits are available or being developed for atrazine, triazines, isoproturon, urons, phenoxy acids (2,4-D), acetamides (alachlor, metolachlor) and carbamates (carbofuran). This list is expected to grow.
2	Types of sample	Drinking water.
3	Basis of method	Pesticides in a sample compete with a labelled form of the pesticide(s) for a limited number of binding sites on a specific antibody. The presence of unlabelled pesticide results in less label being bound to the antibody and being determined in the assay. Pesticide concentrations are usually determined photometrically from comparison with standards.
4	Range of application	Drinking water concentrations down to 10 ng l^{-1}. The maximum concentration varies, up to more than 10 µg l^{-1}. In practice, pesticide concentra- tions above 5 µg l^{-1} in drinking water are rare.
5	Calibration curve	Linear on a semi-log plot over a central area of the application range.
6	Standard deviation*	2.5 ng l^{-1} or 5% of result, whichever is the greater.
7	Limit of detection (LOD)*	10 ng l^{-1}, 10% of the PCV.
8	Bias*	5.0 ng l^{-1} or 10% of result, whichever is the greater.
9	Total error*	10.0 ng l^{-1} or 20% of result, whichever is the greater.
10	Interferences	Potentially from other pesticides and from other impurities.
11	Time required for analysis	Depends on kit. Batches of about 60 samples may be analysed simultaneously, in about 3 h.

* Values in table are those recommended of analytical methods for regulatory monitoring of public drinking water supplies for pesticides.

Cross-reaction with other pesticides

The IA pesticide kit's antibodies may 'recognise' pesticides other than that which is being analysed, especially those pesticides within the same class as the pesticide of interest. For example, it may be better to regard some 'atrazine' IA kits as suitable for most triazines. This reaction to components other than the compound of interest is called 'cross-reaction'.

There are different ways of reporting cross-reactivities. The cross-reactivity of a substance in clinical immunoassay analysis is sometimes defined as the amount of substance which displaces 50 % of the labelled antigen (ID50) compared to the ID50 of the antigen. In pesticide analysis, this single parameter is only a guide to the relative sensitivity of the kit to various pesticides.

A pesticide IA kit may report the effect that other pesticides have on the assay. For example, the kit may state what concentration of simazine only would produce a 50% or 90% relative absorbance (which is a measure of pesticide concentration. Alternatively, an atrazine IA kit may state the apparent concentration of atrazine which the kit would yield if a given concentration of simazine alone were present. These apparent concentrations of atrazine are sometimes referred to as 'atrazine equivalents'. Neither of these measures of cross-reactivity is entirely satisfactory, since they do not take account of the effects of having, for example, both simazine and atrazine present in the sample. In this case, the antibody is potentially binding with three substances: labelled atrazine, the sample atrazine and the cross-reacting simazine. Complex factors, based on the kinetics and the strength of antigen-antibody binding, affect the outcome of these mixed assays. If competitive cross-reactions do occur in the IA, the resulting 'concentration' value for a real sample, containing a mixture of pesticides at differing concentrations, is not always the sum of the individual pesticide concentrations.

TABLE 2. Potential interferences in immunoassays

Potential source of interference	Concentration or range		Potential source of interference	Concentration or range	
Fulvic acid/Humic acid	10 mg l^{-1}		Water hardness:		
			Calcium	200 mg l^{-1}	
Metals:			Magnesium	50 mg l^{-1}	PCV
Iron	200 µg l^{-1}	PCV	Disinfectants and by-products:		
Copper	5 µg l^{-1}		Free chlorine	0.5 mg l^{-1}	
Lead	50 µg l^{-1}	PCV	Chloramine	0.5 mg l^{-1}	
Nickel	50 µg l^{-1}	PCV	Trihalomethanes (total)	100 µg l^{-1}	PCV
Zinc	5000 µg l^{-1}	PCV	Chloroform	50 µg l^{-1}	
Manganese	50 µg l^{-1}	PCV	Trichloracetic acid	50 µg l^{-1}	
			Surfactants:		
Chloride	400 mg l^{-1}	PCV	Linear alkylbenzene sulphonates	10 µg l^{-1}	
Nitrate	100 mg l^{-1}		Alkyl ethoxylates (nonionic)	10 µg l^{-1}	
Sulphate	250 mg l^{-1}	PCV	Di-hardened tallow cations	10 µg l^{-1}	
Bicarbonate	200 mg l^{-1}		pH	6-8.5	

* PCV = Prescribed Concentration or Value for Drinking Water.

Each kit should state the substances for which cross-reactivities were determined. The conditions of the cross-reactivity test should also be stated as the cross-reactivity depends on incubation timing, pH and temperature. Validation of the IA method in an analytical laboratory should encompass cross-reactivity testing.

Interference from other impurities

Other substances in the water sample may interfere with an immunoassay. The cause of the interference may be the inhibition of the pesticide-antibody or the enzyme-substrate reaction, the oxidation of the chromogen, or binding of the conjugate onto particulates. Interference can be a problem, particularly if river water is being analysed. Interferences from free chlorine, iron, calcium and humic acids have been observed for different kits.

While it is difficult to specify all potential interfering chemicals that may occur in a particular water or water type, some are commonly occurring and consequently it would be valuable if the robustness of the IA kit to the parameters in Table 2 be tested and stated by the manufacturer in the instructions. The suggested values for the parameters are guidelines, based on typical water analyses, and obviously the most relevant values to assess whether interferences are likely to occur are those which occur in the particular water being analysed.

Initial performance testing of the IA kit by a water laboratory should test for interferences. Ideally, a pesticide-free sample of the water matrix to be analysed, to which known amounts of the pesticide could be added and determined, would provide the most suitable test for interference effects. Practically, if pesticide-free matrix is not available, interferences may be most easily assessed by spiking the particular water to be analysed with a known amount of the determinand and analysing both the spiked sample and a sample of the water without added pesticide. Full recovery of the determinand from the spiked sample indicates that interference is not significant.

It is obviously preferable that an IA pesticide kit is not affected by impurities in the sample of drinking water. If the analysis is affected by a matrix constituent, it may be possible to successfully pretreat the sample to remove the cause of the interference by, for example, filtration or pH adjustment.

Hazards

All reagents must be assessed for adverse health and safety effects before use and the recommended control procedures implemented. Immunoassays do not require organic solvents and so are free of many of the associated flammability hazards. Pesticides may be toxic and their solutions, in the samples and in

the standards, should be treated as toxic. The antibodies may present a biological hazard and good hygiene is important. The stop solution is often a concentrated strong acid (toxic, corrosive). Safe methods of disposal should be used for all pesticides, solvents and other hazardous substances.

Reagents
The required reagents are supplied with the kits. Tap and pesticide-free water are normally the only extra reagents required.

An important point is that the shelf-life of these biological materials is usually shorter than that of conventional chemicals. Shelf-lives of six months for the antibodies and enzyme-labelled pesticide are common. The analytical laboratory may wish to inspect the kit manufacturer's test data to verify the claimed shelf-life. The kits should be stored in a refrigerator.

The diluted pesticide standards supplied with kits may also deteriorate on this timescale; the laboratory may prefer to make up fresh pesticide standards of its own.

Apparatus
As with all trace analysis, all apparatus should be clean and dry. Surfactant residues on glassware from detergents may interfere with the assay. A suitable procedure is to wash the glassware with a detergent, rinse with pesticide-free water and dry in an oven.

The equipment required varies for each manufacturer and supplier of IA kits. Some of the apparatus typically required for kits currently available are described in the following paragraphs.

The kits usually contain a plate of wells, typically 96 (8 x 12) in number, pre-coated with antibody. Some kits are supplied with the antibodies attached to small magnetic metal particles.

The following equipment may be required:

- A micro-titre plate or strip reader to measure the optical absorbance of the wells;
- Disposable-tip precision pipettes to dispense volumes in the range 10-100 µl (some kits are supplied with droppers for dispensing the reagents and samples);
- A timer to ensure correct duration of each of the assay steps;
- Some kits require an incubator to maintain the assay at constant temperature.
- An automatic plate-washer, a multi-channel pipette and an orbital mixer are optional but convenient. The methods are suitable for automation and an automatic pipetting station may be considered if sample throughput warrants it.
- A magnetic separation unit and vortex mixer are required if the kit is supplied with the antibodies on suspended magnetic particles.

Sample collection and storage
Sample bottles should be glass with PTFE-lined screw caps. Samples should be refrigerated at 2-6 °C. Most assays only require a few hundred microlitres of sample, but for convenience in sample handling, 50-100 ml should be collected if possible. If it is likely that confirmation of the result by conventional analysis will be required, then at least one litre should be collected.

It is known that some pesticides can adsorb onto glass and so may not be fully recovered in the analysis of the sample, whether the analysis is by immunoassay or chromatographic methods. If the pesticide is being extracted from aqueous solution into an organic solvent, a remedy for improving the pesticide recovery is to rinse the empty sample container with the organic solvent. This remedy is not generally applicable to IA methods which work on the aqueous sample. If pesticide adsorption is suspected (for example, if the recovery of pesticide in the analysis of spiked samples is low), the original sample container may be rinsed with a polar solvent (e.g. methanol) and the rinsings mixed with water and analysed by IA. Some IA kits tolerate the presence of polar solvents in the sample and rinsing of the original sample container with such a solvent may be successful provided that the solvent is first checked for interference with the assay (and of course provided that the pesticide is soluble in the solvent).

Care should be taken to ensure that sample bottles are pesticide-free if re-used. This can be checked by rinsing with water and analysing the rinsings by IA. Sample bottles may be cleaned by the cleaning procedure for glassware described above.

Analytical procedure

If interferences from a matrix component are found from the spiking procedures outlined above, the sample may need pretreatment, such as filtration or pH adjustment.

Manufacturer's instructions should be followed closely. These generally involve the following steps:

(a) Addition of pesticide-free water (as a negative control or blank), reference or calibrator solutions of pesticide and the test samples to separate wells, in duplicate or triplicate.

(b) Addition of enzyme-labelled pesticide (conjugate) to all wells.

(c) Mixing and incubation for a set time at a set temperature, which is usually between 4 and 37 °C.

(d) Washing off of unbound pesticide.

(e) Addition of substrate and chromogen solution(s) to all wells.

(f) Mixing and incubation for a set time (typically 30 min - 2 hrs) at a set temperature, which is usually between 4 and 37 °C.

(g) Addition of stop solution to all wells.

(h) Reading of "optical density" (OD), an absolute absorbance value, of the plates at a specified wavelength in a plate reader.

Calculation of results

Instructions should be followed on the conversion of OD values to relative absorbance (%B), usually relative to the negative control, and the construction of a semi-log calibration plot of %B versus $\log_{10}$ (pesticide concentration) using the reference pesticide solutions. The unknown pesticide concentrations are then read from the %B values for the samples. Some standards for the use of EIA methods (for example DIN) recommend the use of a log/log plot which enables both very low and very high concentrations to be incorporated.

A typical IA calibration curve is shown in Fig. 2. Two features of the curve are noteworthy:

(a) the slope is negative, i.e., the more pesticide in the sample, the lower the absorbance;

(b) linearity only extends over a central section of the S-shaped curve.

The latter point means that it is essential for quantitative assays that only the linear region of the curve between the reference points be used to convert %B readings to concentrations of unknowns. It is recommended that at least five standard solutions of pesticide are used to establish the range of linearity.

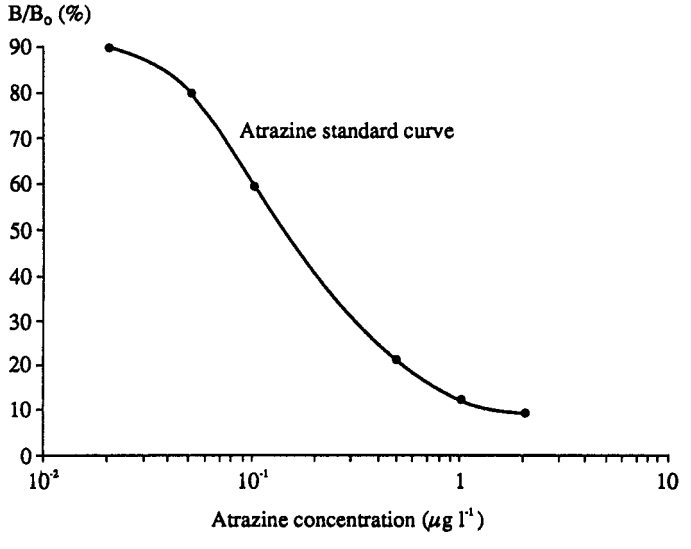

Fig. 2.
Example of an immunoassay calibration curve

Detectable responses may be obtained for samples containing low concentrations of the pesticide below the linear region.

Extrapolation outside of the standard pesticide concentrations is unreliable. If the absorbance of a sample is lower than that of the most concentrated reference solution, a fresh aliquot of the sample must be diluted at least tenfold in pesticide-free water and the assay repeated, including all calibration solutions. If the absorbance is higher than that of the most dilute standard, it is not possible to determine quantitatively the sample concentration and one may only conclude that the pesticide concentration is below that of the standard.

Analytical quality control

The quantitative use of IA kits to determine pesticide levels in potable waters should be subject to the same AQC measures as other analytical techniques. Initially the method must be demonstrated (either by the manufacturer of the kit or the water laboratory) to work for the desired pesticide, in the concentration range of interest and without interference from other water constituents including other pesticides. Initial performance testing should include analysis in random order of the following:

(a) blanks (pure, pesticide-free water);

(b) standard solutions of known amounts of pesticide in pure pesticide-free water;

(c) samples of unknown concentration of pesticide;

(d) if available, pesticide-free samples similar to the waters of interest, spiked with known amounts of pesticide;

(e) samples of the water to be analysed spiked with known amounts of pesticide, comparable to the amounts shown to be present before spiking, to check the recovery.

Since batch-to-batch variations in the antibodies are possible, reference samples must be included with every plate. Statistical monitoring of the results is recommended. Routine AQC measures should involve analysis of controls and use of QC charts.

Sources of error include those common to other methods of pesticide analysis (such as unclean apparatus and poor technique in operations such as pipetting) and those unique to immunoassay. The latter include within batch (or assay drift) batch-to-batch and day-to-day stability of the antibodies and timing errors on the incubation periods (which may not be run to completion). Operator training should be comprehensive, stressing the likely sources of error. Maintaining lot numbers of the reagents is recommended. It is possible to set test standards for initial testing of new batches of reagents.

A laboratory adopting IA methods to complement chromatographic methods may compare the two techniques by analysis of pesticide samples with both techniques and verify that they are in agreement. Interlaboratory testing of IA kits may also be done.

Summary

Immunoassay kits have the potential capability to analyse water samples for trace levels of pesticides. This capability could be very useful both to monitor compliance with the 0.1 $\mu g\ l^{-1}$ concentration limit set in the EC Drinking Water Directive (if the performance satisfies the criteria described earlier and to monitor levels of pesticides in river and groundwaters.

Pesticide analysis with IA kits offers several advantages to the analytical laboratory over conventional methods such as GC and HPLC:

- Many analyses can be done simultaneously;
- Capital equipment costs incurred are low;
- The aqueous samples may be analysed with little or no pretreatment;
- The volume of sample required is very small;
- The kits are specific for a particular pesticide or group of pesticides.

The resulting increased throughput and speed of pesticide analysis could permit the use of IA pesticide

kits as a method of screening many water samples for undesirably high levels of pesticides, prior to confirmatory analysis using conventional analytical methods.

The main disadvantage of IA is that the specificity of the analysis for the given pesticide or group of pesticides may be affected by cross-reactivity to other pesticides or other substances in the sample. Positive results (showing levels at or above the PCV) should be confirmed with established techniques, such as GC-MS, which identifies the pesticide more definitively than IA.

Requirements for performance testing and test design

The EC Drinking Water Directive sets maximum admissible concentrations (MACs) for many parameters, including pesticides, for which an MAC of 100 ng l^{-1} for each individual compound is set. In order to monitor validly these MACs for pesticides, analytical methods must meet specific performance characteristics. These characteristics and the design of tests to check them are set out in a document produced by the UK Department of the Environment's Drinking Water Inspectorate (DWI) entitled 'Guidance on Safeguarding the Quality of Public Water Supplies' (HMSO 1989). These guidelines can be broken down into (1) Accuracy Targets and (2) Method Performance Test Design, as follows:

1. Accuracy Targets: For pesticides in drinking water the accuracy targets are:
   (a) limit of detection of 10 ng l^{-1} or better
   (b) total standard deviation of individual results not exceeding 2.5 ng l^{-1} or 5% of the determinand concentration, whichever is the higher, and
   (c) bias not exceeding 5 ng l^{-1} or 10% of the determinand concentration, whichever is the higher.

2. Method Performance Test Design: Determinations should be made in duplicate, in random order, in a sufficient number of analytical batches to provide at least ten degrees of freedom for estimates of total standard deviation (this is guaranteed by 11 batches of analysis). Tests should be carried out on the following solutions as a minimum. (These performance test solutions should be treated as samples, i.e. the calibration procedure should be that used routinely and would be implemented for each batch of analysis.)
   (a) A blank of water used to prepare the calibration standards (if no response is obtained for zero concentration samples then this should be spiked to a level where responses can be measured, e.g. 5 ng l^{-1}).
   (b) A standard solution at or near to 100 ng l^{-1}.
   (c) A standard solution at levels typically found in routinely analysed samples, say 1000 ng l^{-1}. Standard test samples a) and b) should be prepared using pesticide from a different source to that of the calibration standards.
   (d) A drinking water sample at 20 ng l^{-1} (spiked if necessary).
   (e) The drinking water sample used for d) spiked to 800 ng l^{-1}.

If samples of substantially different matrix are to be analysed routinely by the method then each matrix should be tested as in d) and e) to provide precision and recovery data.

In addition, tests are required to determine the effects (if any) of interference from substances which may cross-react with the antibodies used in an immunoassay system (e.g. other pesticides and by-products) and from other substances occurring in drinking water which may interfere with the test (e.g. humic materials, metals, anions). The design of such interference tests requires comparison of the results obtained from analysis of the determinand at two concentrations with and without the addition of the interferent. The number of batches required for interference tests is fewer than for the precision and accuracy tests since the most powerful experimental design estimates interference effects from tests within one batch of analyses. It is accepted that it is impractical (and probably prohibitively expensive) to test for all possible interferences and combinations thereof, but a representative range of the most likely interferences is required. A list of suggested interferences and concentrations is given in Table 2 above.

If an analytical method produces acceptable performance in these tests then it is considered by DWI to be suitable for drinking water analysis. The performance tests required by DWI also meet the recommendations of the Standing Committee of Analysts for methods produced in the 'Blue Book' series (Methods for the Examination of Waters and Associated Materials). Hence an immunoassay showing adequate performance when validated in this way should be suitable for production as a 'Blue Book' method.

A similar approach should be used to test the performance of EIA kits for water samples other than drinking water. In this case the 'maximum admissible concentration' might be the environmental quality standard set for the chemical or may be the 'level of interest' for the particular study. The accuracy targets are then derived as follows, where C is the MAC or level of interest: total standard deviation 0.05C, systematic error 0.1C, total error 0.2C. Further information on the derivation of these method performance targets is provided in Gardner *et al.* (4).

REVIEW OF IMMUNOASSAY KITS

Commercially available

Until recently, the impetus for production of the EIA kits for organic pollutants in environmental samples had been provided by the North American market which has different requirements to the European market as a result of different Regulations. Thus, the performance required from the kits is substantially lower than that required for example to monitor pesticides in drinking water in the EC and this is reflected in the performance that can be achieved by the kits. Increased interest is now being displayed by manufacturers in the European market, but until very recently this had not been reflected in significant improvements in the performance of EIA kits.

The two major producers of EIA kits for analysis of aqueous environmental samples are J T Baker and Millipore. J T Baker are a Dutch owned company who sell EIA kits produced by an American company, Ohmicron Corporation. Millipore are an American company whose EIA kits are produced by Immunosystems Incorporated. Both of these companies were approached and kindly supplied up-to-date information on the determinands for which they had EIA kits available or under development. These are shown in Tables 3 and 4.

Assessment of the analytical performance of EIA kits is carried out using a biological/biochemical approach rather than a chemical analytical approach. For example, the limit of detection is expressed as the 'lowest detectable dose' (LDD) which is the amount of determinand that produces an absorbance (B) equivalent to 90% of the absorbance produced by a blank (Bo) i.e. LDD ≡ 90% $^{B}/_{Bo}$. Some manufacturers provide different versions of the kits, for example Millipore offer both tube and plate versions, but the tables quote data for the most sensitive version of the kit for each determinand.

Few, if any, of the kits exhibit performance which is close to being satisfactory for analysis of pesticides in drinking water. For pesticides where the limit of detection does approach that required there is often a problem of cross reactivity to other pesticides of similar structure. This is the case for the atrazine and isoproturon kits, for example. Although some precision data (mostly within-batch, but in some cases also between batch) has been obtained for most of the EIA kits, none has been validated using an experimental design of the type recommended earlier.

The potential for cross-reactivity from chemicals with similar structures to those of the determinand has been mentioned above and the extent of this is determined by the specificity of the antibodies used. However, because the kits use colorimetric reactions there is potential for other chemicals to interfere by affecting the chemistry used in the test. Among those that can affect the kits are chlorine (at levels in excess of 1-2 ppm) and pH (at <6.5 or >7.5). Other chemicals e.g. sodium chloride or calcium carbonate, do not interfere even at quite high (≥100 mg l^{-1}) concentrations.

Recently ENSYS, who have rapidly established a market-leading position in the USA for IA kits for contaminated land analysis, have indicated that they are about to enter the European markets. Their kits are designed to give a yes/no answer for a group of contaminants rather than a specific qualitative or

TABLE 3. EIA kits produced for water analysis by J T Baker (Ohmicron)

Substance	Analytical Performance		
	LDD ($\mu g\ l^{-1}$)	Range ($\mu g\ l^{-1}$)	Precision - within batch*
2,4-D	0.7	0.7-50	8% (36 $\mu g\ l^{-1}$)
Alachlor	0.05	0.05-5.0	6% (0.5 $\mu g\ l^{-1}$)
Aldicarb	0.25	0.25-100	17% (12 $\mu g\ l^{-1}$)
Atrazine (high sensitivity kit)	0.015	0.015-1.0	7% (0.16 $\mu g\ l^{-1}$)
Atrazine (and other triazines)	0.046	0.05-5.0	5% (2 $\mu g\ l^{-1}$)
Benomyl/Carbendazim	0.1	0.1-5.0	6% (3 $\mu g\ l^{-1}$)
Captan	10	0.01-3.0	10% (0.6 $\mu g\ l^{-1}$)
Carbaryl	0.25	0.25-5.0	8% (2 $\mu g\ l^{-1}$)
Carbofuran	0.056	0.06-5.0	9% (2 $\mu g\ l^{-1}$)
Chlorothalonil	0.07	0.07-5.0	5% (1.5 $\mu g\ l^{-1}$)
Chlorpyriphos	0.1	0.1-3.0	5% (1 $\mu g\ l^{-1}$)
Cyanazine	0.035	0.04-3.0	10% (0.5 $\mu g\ l^{-1}$)
Metolachlor	0.05	0.05-5.0	6% (0.7 $\mu g\ l^{-1}$)
Paraquat	0.02	0.02-0.5	7.5% (0.225 $\mu g\ l^{-1}$)
PCB	0.1	0.1-5	10% (0.1 $\mu g\ l^{-1}$)
Pentachlorophenol	0.06	0.06-10	8% (3 $\mu g\ l^{-1}$)
Procymidone	0.8	0.8-100	5% (20 $\mu g\ l^{-1}$)
+Prometryn (and other triazines)			
+Propazine (and other triazines)			
+Simazine (and other triazines)			

* The figure in brackets is the concentration of the pesticide in the water used for replicate analyses
\+ See Atrazine (and other triazines)

TABLE 4. EIA kits produced or under development by Millipore (Immunosystems Incorporated) for analysis of water

Substance	Analytical Performance		Substance	Analytical Performance	
	LDD ($\mu g\ l^{-1}$)	Range ($\mu g\ l^{-1}$)		LDD ($\mu g\ l^{-1}$)	Range ($\mu g\ l^{-1}$)
2,4-D	0.5	0.5-100	Isoproturon (and other 'urons')	0.01	0.01-0.4
2,4,5-T	3	3-500	Lindane	20	20-1000
Alachlor	0.1	0.1-2.5	Linuron (and other 'urons')	3	3-120
Aldicarb	1.0	1.0-20.0	Metalaxyl	0.1	0.1-2.5
Aldrin (and other 'drins')	4.5	4.5-850	Methoprene	1000	1000-10 000
Atrazine (and other triazines)	0.1	0.1-2.0	*PAH	100	100-1000
Benomyl/Carbendazim	0.4	0.4-10.0	Paraquat	0.03	0.03-0.15
Benzene/Toluene/Xylenes	2000 (soil)	2000-60 000 (soil)	PCB	1000 (soil)	1000-50 000 (soil)
Carbofuran	0.1	0.1-10.0	Pentachlorophenol	5 (soil)	5-50
Chlorotoluron	6	6-250	p,p'-DDT	100	100-1000
Chlorpyrifos	0.08	0.08-1.0	p,p'-DDE	180	180-2000
Dieldrin (and other 'drins')	2	250	Procymidone	6	0.05-1.0
Diuron (and other 'urons')	7	7-250	Propazine	0.014	0.014-0.3
Endosulfan	0.6	1.0-150	Simazine (and other triazines)	3	3.0-30.0
Endrin (and other 'drins')	1.0	1.0-120	TNT	0.5	0.5-50
Fenitrothion	100	100-2000	Triasulfuron	0.05	0.05-1.0
Heptachlor	4	4-100			

* = under development

Target CV is 10%
all ≤ 10%

quantitative value. Although their main products are for soil analysis, they also produce IA kits for analysis of PCB, petroleum and pentachlorophenol in water. The current performance of these kits is not suitable for drinking water analysis, but they are actively investigating the market for IA kits for pesticide analysis in drinking waters. In the USA their kits have been approved by the EPA and have draft method status, hence they are well aware of the importance of satisfying regulatory requirements for IA kit performance and validation.

Information on the EIA kits produced by the only UK manufacturer, Guildhay Antisera, is provided in Table 5. These manufacturer's data suggest that these kits may be capable of achieving the required performance for analysing pesticides in drinking water.

TABLE 5. EIA kits produced by Guildhay Antisera

Substance	LDD ($\mu g\ l^{-1}$)	Precision	(Within batch) ($\mu g\ l^{-1}$)
Atrazine	0.0055	24%	(0.043)
		7%	(0.24)
		9%	(0.85)
Isoproturon	0.009	18%	(0.01)
		8%	(0.3)
		7%	(0.7)

Comparison of the cost, complexity and performance of IA kits with that of conventional instrumental analytical methods is difficult, but some comparative information is useful in assessing the potential rôle of IA kits. Instrumental analysis methods for pesticides typically will determine suites of similar compounds rather than a single compound. Assuming a cost of about £50 for analysis of a suite of 10 pesticides in a sample then the unit cost per determinand per sample will be £5.00. IA kits typically will analyse about 35 samples for a single determinand at a cost of about £350, equivalent to £12.00 per determinand per sample. Hence the relative cost of conventional analysis is apparently less. However, IA will work out cheaper if the requirement for monitoring is for only a few pesticides, if there is a need to analyse small sample sizes (e.g. porewaters) or if analysis is required in the field.

Conventional analysis using, for example GCMS with a one litre sample extracted and concentrated to 100 µl with 1 µl analysed needs to detect the equivalent of 100-200 pg of material to achieve a method limit of detection of 10-20 ng. A typical IA kit using a 200 µl water sample has to detect 2-4 pg of material to achieve a method limit of detection of 10-20 ng.

Carrying out IA based analysis is relatively straightforward and requires only simple equipment. Analyses are run on 96-well plates with duplicates used for each sample determination. The results of analysis of a batch of 20-35 samples can be available in 2-3 hours from the start of analysis. With conventional instrumental analysis using chromatographic methods the results from a similar batch of samples will probably take more than 48 hrs to produce once extraction and concentration are taken into account. IA kits offer advantages where results are required quickly, where there is a desire or need to avoid the use of sophisticated analytical instrumentation, where analysis is required in the field or where only small sample sizes are available.

New developments

The open scientific literature up to 1992 on immunoassay methods for pesticides has been the subject of several recent reviews (see Wilson and Gale (5) and references therein). Subsequent developments in immunoassay of water samples are reviewed here.

A tube-based EIA for atrazine (Millipore) was used to measure atrazine in lake and river water and results were compared with those from conventional analysis using extraction and GC-NPD (6). Limits of detection for atrazine were 62 and 180 ng l^{-1} in lake and river water respectively. Comparison of analytical results from EIA and GC-NPD for 124 water samples showed a linear relationship with a correlation coefficient of 0.919. Over the course of 65 assays the variability of the controls was generally within two standard deviations of the mean response of the assay. The EIA kit was recommended for use as a screening technique and as an inexpensive way of monitoring triazine levels in waters known to be contaminated. The tube-based atrazine assay has also been applied to analysis of 67 groundwaters from 30 boreholes and results compared with GCMS (7). A reasonable, linear relationship was obtained between the EIA and GCMS method results and statistical evaluation showed the EIA to be suitable as a monitoring method in the routine control of groundwater.

An EIA plate kit for alachlor analysis (Millipore) was compared with conventional analysis by GC-NPD and GCMS for borehole samples in the USA (8). Poor correlation was obtained between EIA and conventional results with many false positives being indicated by EIA. This was found to be due to the cross-reactivity of the EIA kit with an ethanesulphonate metabolite of alachlor which was often present in the samples although alachlor itself was not. A tube-based EIA for alachlor analysis (J T Baker) using magnetic particles was applied to 15 groundwater samples and the results compared to a GCMS method (9). Good linear correlation was found over a range of 0.2 - 6 μg l^{-1} with a regression coefficient of 0.984. A precision test (5 replicates over 5 days) at four concentration levels in spiked groundwater showed within assay coefficients of variation (CV) of 3.4 - 11.1% and between assay CVs of 1.9-5.5%.

Pentachlorophenol was analysed in four samples each of surface, ground and drinking waters using a plate type EIA (Westinghouse Bio Analytic Systems) and the results compared to analysis using a GCMS based method (10). EIA results showed higher variability than GC results, but a good linear correlation with a regression coefficient of 0.92, over a range of 0 - 3.2 μg l^{-1}. No false negatives were found for the EIA and it was concluded that EIA was an attractive, low-cost alternative to conventional GC based methods of analysis.

A tube-based EIA developed for PCB in soil samples (ENSYS) could also be applied to waters (11). The limit of detection was 5 μg g^{-1} based on the soil concentration and there was little interference from different soil matrices. Cross-reactivity to other chlorinated aromatics and some chlorinated pesticides was significant in a few cases.

A major study was carried out by the US Geological Survey using EIA and GCMS based analysis of atrazine, desethylatrazine, metolachlor, arachlor and simazine to determine the levels, sources and fate of herbicides in surface waters of the mid-western USA (12, 13). The study used both plate and tube EIA kits (Millipore) and found limits of detection of 0.05 and 0.2 μg l^{-1} respectively for 100 ml samples. Neither false positives or negatives were found and comparison with GCMS results for 127 surface waters showed good correlation with a simple non-linear regression coefficient of 0.86. The authors concluded that EIA is a viable tool for triazine analysis of surface waters, groundwaters and rainwaters.

The influence of humic acids, heavy metal ions, anions and solvents on EIA of triazines in natural water was examined in detail (14) and conventional instrumental analysis used to validate the results. Heavy metals inhibited peroxidase (used as the tracer enzyme) at levels of several mg l^{-1}. Solvents influenced the sensitivity and cross-reactivity of the test, probably by modification of the antibody binding sites. Measurements of atrazine in groundwaters, used for drinking water, over one year showed that EIA was viable for routine monitoring of water with a known matrix. This enabled the intervals between analysis by conventional techniques to be increased with a resultant saving in time and costs. In general, no false negatives were produced by EIA, so it could be used for screening a large number of samples.

Two reviews of pesticide testing by EIA using commercial kits have been published recently. The first considers the use of EIA for environmental and agricultural samples (15) and concludes that the kits are simple to use (even in the field), sensitive (with detection limits of 10 - 20 μg l^{-1}), specific (to classes or individual compounds), rapid (results available in as little as 6 - 7 min.) and inexpensive. However, they

are not always the most suitable method and care must be taken to avoid 'false-positives'. The second reviews the application of immuno-enzymatic methods to the detection of pesticides (16).

A potentially significant new development concerns immobilisation of antibodies onto a graphite electrode to produce direct potentiometric immuno-electrodes (17). Such an electrode using atrazine antibodies has been shown to have a useful concentration range of 20 - 250 ng l^{-1} for water samples. However, since the electrode is sensitive to ions in natural waters, these must be removed by ion-exchange prior to determination of atrazine. With further development this may prove to be a valuable tool for on-line monitoring of pesticides and hence real-time control of treatment processes.

Dialkyl phthalates in water have been determined by a time-resolved fluoro immunoassay (FIA) at levels down to 125 ng l^{-1} with a range to 500 µg l^{-1} (18). The FIA responds almost equally to dimethyl, diethyl, dibutyl, butylbenzyl and dioctyl phthalate, with little cross-reactivity to iso- or tere-phthalates.

EIA methods rely on simple chemistry and colorimetric reactions and consequently in principle they can be adapted for flow-injection analysis to provide an automated analytical method. A flow-injection system based on immunochemical reaction has been described (19) which allows automated, quasi-continuous measurement of triazines in water samples. Flow injection immunoanalysis (FIIA) of atrazine and propazine, which both cross-react with the antibodies used, in water samples showed detection limits of about 30 ng l^{-1} with a range to 500 ng l^{-1}.

A recent review critically assessed the use of immuno-detection techniques for the enumeration of micro-organisms and toxins in water (20). While the techniques offered the advantages of simpler, quicker, and cheaper methods than conventional procedures, they can show false positives due to interferences in the water samples or cross-reactivity with a wide range of organisms. In addition, the methods cannot indicate the viability of organisms detected in the water samples.

Guidelines for the precision requirements, standardised evaluation procedures and quality control of immunoassays for residue analysis of agrochemicals have recently been produced (21). A series of recommendations were made to improve the comparability of different IA kits and to improve the performance testing and reliability of the kits.

CONCLUSIONS AND RECOMMENDATIONS

Some of the immunoassay kits commercially available are now approaching the performance required for routine compliance monitoring of pesticides and other organics in drinking and other waters. Some further development is required to increase sensitivity and specificity and decrease variability of analytical results. Confirmatory analysis using conventional analysis is recommended if MACs are exceeded.

A draft specification has been produced for IA methods, together with a recommended experimental design for performance testing. It is recommended that these should form the basis of the information on immunoassay kits provided to users by manufacturers. In addition, the performance testing procedure should be used by laboratories to validate the performance of the kits prior to their routine use.

Immunoassay kits are ideally suited for use in locations remote from conventional laboratory facilities. However, considerable operator skill and training is required to obtain reliable results. IA kits are particularly valuable where results are required for only a few determinands, for a large number of samples, over a short period of time or for small sample volumes.

An immunoassay based technique offers the potential for on-line continuous monitoring of trace organics in water using determinand specific electrodes. Development of an instrument of this type would be invaluable, e.g. for real time control of treatment processes in drinking water plants or for monitoring levels of organic chemicals in river water *in situ*.

Some immunoassay kits can cross-react with chemicals of similar structure to the chemical of interest, thereby reducing their specificity. In addition, some substances in water may interfere with the test if they are present at high enough levels. Consequently the use of IA kits in dirty waters, e.g. sewage or industrial effluents is not recommended.

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REFERENCES

1. Bushway, R.J., Perkins, B., Savage, S.A., Lekouski, S.J. and Ferguson, B.S. *Bull. Environ. Contam. Toxicol.*, **40,** 647-654, (1988).
2. Harrison, R.O., Gee, S.J., Hammock, B.D. In: *Biotechnology for crop protection. American Chemical Society. Symposium Series,* **379,** 316-330 (1988).
3. Sauer, M.J., Foulkes, J.A., Morris, B.A. In: *Immunoassays in Food Analysis,* edited by B.A. Norris and M.N. Clifford, Elsevier, London (1985).
4. Gardner, M.J., Cheeseman, R.V., Wilson, A.L. WRc Report NS-30, ISBN 0 902156 85 3 (1989).
5. Wilson, K. and Gale, P. Foundation for Water Research Report FR 0349 (1993).
6. Sherry, J.P. and Borgmann, A. *Chemosphere,* **26,** 12, 2173-2184 (1993).
7. Weins, C., Becker, B., Kirn, M.-R. *Vom Wasser,* **78,** 377-385 (1992).
8. Baker, D.B. Bushway, R.J., Adams, S.A. and Macomber, C. *Environ. Sci. Technol.* **27,** 562-564 (1993).
9. Lawruk, T.S., Hottenstein, C.S., Herzog, D.P. and Rabio, F.M. *Bull. Environ. Contam. Toxicol.* **48,** 643-650 (1992).
10. Van Emon, J.M., Seiber, J.N. and Hammock, B.D. In: *Analytical methods for pesticides and plant growth,* XVII, 217-261 (1989).
11. Mapes, J.P., McKenzie, K.D., Stewart, T.N., McClelland, L.R., Studabaker, W.B., Manning, W.B., Friedman, S.B. *Bull. Environ. Contam. Toxicol.* **50,** 20, 219-225 (1993).
12. Thurman, E.M., Goolsby, D.A., Meyer, M.T., Mills, M.S., Pomes, M.L., Kolpin, D.W. *Environ. Sci. Technol.* **26,** 12, 2440-2447 (1992).
13. Thurman, E.M., Meyer, M., Pomes, M., Perry, C.A. Schwab, A.P. *Anal. Chem.* **62,** 2043-2048 (1990).
14. Ruppert, T.W., Weil, L., Niessner, R. *Vom Wasser,* **78,** 387-401 (1992).
15. Ferguson, B.S., Kelsey, D.E., Fan, T.S., Bushway, R.J. *Sci. Tot. Environ.* **132,** 415-428 (1993).
16. Pillette, J.R. *Eau, Industrie, Nuisances,* **163,** 46-50 (1993).
17. Engel, L. and Baumann, W. *Fresenius Z. Anal. Chem.,* **346,** 745-751 (1993).
18. Ius, A., Bacigalupo, M.A., Meroni, G., Pistillo, A., Roda, A. *Fresenius Z. Anal. Chem.* **345,** 589-591 (1993).
19. Kraemer, P.M. and Schmid, R.D. *Pest. Sci.* **32,** 451-462 (1991).
20 Kfir, R. and Genthe, B. *Wat. Sci. Technol.* **27,** 3-4, 243-252 (1993).
21. Krotzky, A., Zeeh, B. *Pure Appl. Chem.* In press (1994) .