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The Use of Immuno Based Techniques for Chemical Analysis

Robens Institute
University of Surrey

R&D Technical Report E8

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The Use of Immuno Based Techniques for Chemical Analysis

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Statement of Use
This document provides information on the current status of immuno-based techniques for chemical analysis of environmental samples and identifies areas where the Environment Agency could undertake further R&D.

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R&D Technical Report E8

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EXECUTIVE SUMMARY

This report presents the findings of a project to look at the feasibility of Environment Agency laboratories using immuno technology to monitor trace organics in environmental samples, particularly water. The study was mainly concerned with immunoassay test kits for laboratory use, particularly enzyme linked immunosorbent assays (ELISAs), and also considered immuno-extraction followed by chromatographic determination. The study involved a review of the scientific, technical and manufacturers literature, as well as meetings with kit manufacturers, users of immuno-technology and Environment Agency staff.

The report recommends that for trace organics, where suitable chromatographic methods exist, ELISA type tests offers no real advantage. For compounds where current methods are not satisfactory kit manufacturers should be encouraged to develop immuno-based methods that meet Environment Agency method performance criteria. It is recommended that a pilot study to evaluate immunoaffinity extraction be carried out once a suitable supplier of columns is identified.

KEYWORDS
Pesticide, Analysis, ELISA, Immunoassay, Immuno-extraction, Immuno-affinity, Water, Monitoring

1. INTRODUCTION

1.1 Background

This research contract was commissioned by the National Rivers Authority (which has now been subsumed into the Environment Agency). It involved an assessment of the suitability of immuno-based techniques for chemical analysis. The review mainly concerned commercially available kits but also considered immuno-extraction.

As part of its regulatory function the Environment Agency carries out a wide range of chemical and microbiological analysis mainly on water samples. The analysis of low concentrations of pesticides and related compounds such as polyaromatic hydrocarbons, dioxins and polychlorinated biphenyls require sensitive and accurate methods. These are available for most analytes, but require highly skilled analysts and sophisticated instrumentation and are thus relatively expensive.

In recent years increasing attention has been paid to the possibility of using immunoassays to monitor trace organics in the environment, particularly water, but also food, crops and contaminated land. The advantages claimed in product literature for immunoassays include:-

- Cost effective
- Many samples processed together on plate based assays
- Rapid, often allowing field testing
- Simple, requiring minimal sample preparation and available in kit form
- Sensitive, using very low sample volumes
- Selective, based on binding to specific antibodies
- Clean, producing little laboratory waste for disposal
- Accurate, showing good correlation with conventional methods

The Environment Agency thus wished to review the recently developed and emerging technology to assess whether such techniques were suitable and would improve efficiency in the analysis of trace organics undertaken in its laboratories. The study was principally concerned with water based samples but the formation of the Environment Agency meant that the analysis of air and solid samples has also been briefly covered. The review was concerned with laboratory based immuno-tests as field test kits were reviewed in a previous report by Bogue and Partners (1994).

1.2 Method

This programme of work has involved a review of the literature available on immuno-based techniques for chemical analysis including Enzyme linked immunosorbent assay (ELISA) type methods and immuno-extraction. The review has included scientific literature, immunoassay kit manufacturers literature, and other technical reports. Meetings and telephone conferences were arranged with staff within the Environment Agency, immunoassay kit manufacturers, laboratories using ELISA techniques for environmental analysis and other scientists working in this field. Future developments in immuno-based methods have also been considered for their likely impact on Environment Agency monitoring programmes.

2. CHROMATOGRAPHIC METHODS FOR TRACE ORGANIC ANALYSIS

The analysis of trace organics such as pesticides in water samples and other related environmental matrices has conventionally been carried out using chromatographic methods, particularly high performance liquid chromatography (HPLC) and gas chromatography (GC). A range of different detector options is used depending on the selectivity and sensitivity for particular analytes. Detectors in common use include UV, fluorescence and electrochemical detectors for HPLC and flame ionisation, electron capture, flame photometric nitrogen/phosphorous (NPD) and mass-spectrometry (MS) for GC. Recent developments have indicated that mass-spectrometry has now been more successfully interfaced with HPLC. With GC a wide choice of columns both packed and capillary are available and with HPLC different stationary phases are available, though many analyses are carried using C18 silica with various mobile phases. Both methods show good selectivity and sensitivity and many analytes can be individually determined simultaneously or with simple modifications to chromatographic conditions. Automation of injection and data handling is routine using modern instrumentation.

However most methods require treatment of samples before introduction into the instrument. Common methods of sample preparation include liquid-liquid extraction, solid-phase extraction, centrifugation and evaporation to dryness. Different matrices will usually require different sample pre-treatment for the same analytes. Despite the many advances in instrumentation sample preparation remains the rate limiting step for most assays and that most prone to errors.

Methods requiring sample preparation followed by chromatography require a high level of skill and need to be carried out in a laboratory rather than a field location. Chromatographic methods are well established in many environmental laboratories and have been shown capable of performing well in inter-laboratory comparisons and proficiency testing schemes. Chromatography coupled to mass-spectrometry is generally regarded as the definitive method if absolute certainty of the presence of a particular compound is needed. Even though chromatographic methods are considered mature techniques both scientific and manufacturer's literature continues to claim improvements in methodology.

3. IMMUNO METHODS FOR TRACE ORGANIC ANALYSIS

3.1 Immunoassays

Immunoassay methods such as radioimmunoassay (RIA) and ELISA have been widely used in clinical chemistry laboratories for many years. The basis of an immunoassay relies on the selective binding of analyte to antibodies to that compound. Unlabelled compound (i.e. the analyte in samples or standards) competes with labelled compound (added to all samples and standards) for the binding sites on the antibody. The presence of unlabelled analyte thus results in less labelled analyte binding to the antibody. Unbound compound is removed and the amount of labelled analyte measured (using the label). The label has previously been selected in the method development programme and thus can be determined at very low concentration. The earliest methods used radiolabels but on safety grounds these are now usually replaced by enzyme labels, (which catalyse a specific reaction with added reagents and are measured photometrically). A number of different formats are used along with a range of labels.

Immunoassays generally show very low limits of detection and high specificity. In the clinical laboratory they are frequently used with little or no sample preparation as the antibodies are compatible with biological matrices. A typical feature of ELISA assays is that the signal is inversely proportional to analyte concentration, blanks thus giving the highest reading. Another feature is that standard curves are on a semi log plot and are only linear over a central section of an S-shaped curve. A typical example of an ELISA calibration plot is shown in Figure 3.1.

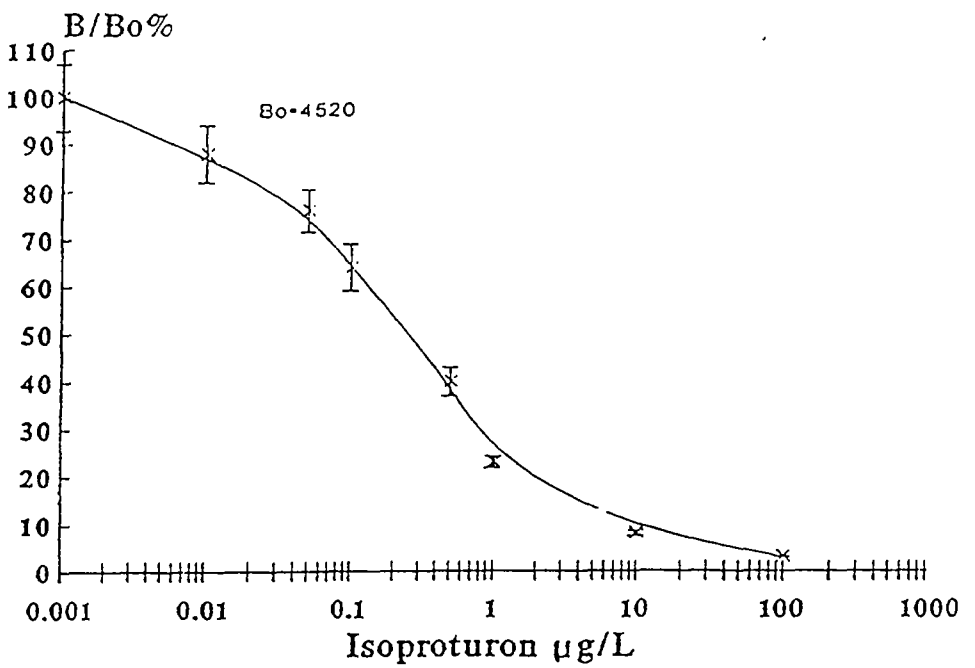

Figure 3.1 A typical example of an ELISA calibration plot

The largest drawback with immunoassays is the need to obtain antisera to the compound(s) of interest. Antibodies are raised in mammals such as sheep, rabbits, and mice. Small molecular mass compounds (less than 1000 daltons), such as pesticides, will not normally produce antibodies so they are conjugated to a carrier protein in order to elicit the immune response.

The chemical synthesis of the analyte-protein complex needs a functional group on the analyte capable of reacting with the carrier protein. If antibodies are produced it is hoped that they will recognise some part of the analyte molecule. If the analyte does not have suitable reactive functional groups a structural analogue of the analyte is synthesised for the conjugation. For a successful assay the antibodies produced must respond to the analyte as well as the structural analogue. The synthesis of structural analogues allows the possibility of bonding to the carrier protein at different functional groups on the analyte molecule. This approach has been tried as a means of obtaining group specificity by design, but has largely been unsuccessful. In some procedures a spacer arm such as an alkyl group is inserted between the analyte and carrier protein in order to produce better antibodies, Harrison et al, (1991).

Once analyte bound to carrier protein is injected into a suitable animal the production of antibodies is unpredictable; it may take between three months and two years and may not happen at all. Once harvested antibodies are stable with only simple precautions such as the addition of preservatives and low temperature storage. Larger mammals such as sheep give greater stocks of antibodies. The amount needed per assay varies but is generally very low. The unpredictability of antisera production means that for most laboratories it is only viable to consider development of a new method once antisera has been obtained from a commercial or University source.

One important feature of antibodies is that they are designed to bind selectively to the analyte only. In practice closely related compounds can bind to differing degrees (so called cross-reactivity). This leads to methods that can detect but not accurately determine the presence of closely related compounds, particularly other pesticides within a class, or metabolites of the analyte. Currently this cross-reactivity usually happens by chance rather than design, but it can be an advantage if a group of closely related compounds is to be measured.

Once developed immunoassays are regarded as economical, fast, easily automated and very sensitive. Very small sample volumes are required and most methods require little or no sample preparation compared with the same matrix analysed by HPLC or GC. Many tests can be carried out remote from the laboratory using a portable photometer. Antibodies used for environmental monitoring can be prone to "matrix effects" as they have been traditionally used with biological fluids, from which they originate. Some authors have overcome this by adding serum to the sample, Katmeh et al (1994).

Two types of antibody have been produced. Almost all work described has been carried out on polyclonal as opposed to monoclonal antibodies. When an animal is exposed to the antigen a mixture of antibodies is produced. These can be produced to the analyte, the spacer arm or the protein. Only antibodies to the target analyte are useful. The antibodies are harvested from the serum of the animal so once it dies the supply will dry up and further animals must be

immunised. Monoclonal antibodies are obtained by removing tissue from an animal producing polyclonal antibodies. Cells from this tissue are then fused with tumour cells and the resulting hybridoma cells can be isolated and grown using tissue culture techniques. An unlimited source of antibodies is thus available. Monoclonal antibodies are more specific but their production is much more time consuming and expensive. Other than when large amounts of antibody are required monoclonals have shown few advantages over polyclonals, hence for the majority of analytical methods polyclonal antibodies have been used, Vanderlaan et al (1991).

3.2 Commercially available immunoassay kits for environmental analysis

There are several commercial companies selling immunoassay test kits in the UK. The range of compounds that can be measured and the kit suppliers are shown in Table 3.1 (page 11). The use of immunoassays for monitoring pesticides in water has recently been reviewed, Watts and Hegarty (1995). Manufacturer's literature does not provide much validation and performance data. In this report, Watts and Hegarty (1995), Guildhay, Millipore and J T Baker provided a list of performance data for their kits, including lowest detectable dose, within batch precision and analytical performance range. Few of the kits approached the performance required for satisfactory determination of pesticides in water. The authors concluded that the method performance required for water analysis was being approached but not currently met.

Typical laboratory equipment required would include an ELISA plate reader and plate-washer as well as multichannel pipettes. These vary in cost depending on the level of automation and sophistication required but typically range from a few thousand pounds to £25k. The most commonly quoted assays are for atrazine or triazines and isoproturon plus other phenylureas. Most of the assays quoted are capable of monitoring down to the 100 ng/l EEC limit for pesticides in water, with little or no sample preparation. Kits usually consist of a 96 well microtitre plate or tubes onto which antibody has been coated. Reagents and standards are also supplied. If only antisera and labelled analyte are available it is relatively easy for trained analysts to adapt assays for their own particular purpose.

Immunoassay test kits for a wide range of analytes are available in the North American market. This has different regulatory requirements to the European market and assay performance requirements are not as stringent. The companies offering the widest range of kits are J T Baker who sell the Ohmicron range known as RaPID Assays and Millipore who sell the Immunosystems range of kits known as EnviroGard. Ensys, another American company, have set up a UK base in Mortlake which they have recently relocated to Alton. Until recently they have concentrated on soil samples. Guildhay a UK company based in Guildford offer a wide range of kits and antisera to clinical, food and veterinary products. They now also offer test kits for atrazine and isoproturon and antisera to paraquat, chlortoluron and simazine. Another new company Environmental Sensors Ltd based in Cambridge offers assays for atrazine and isoproturon with other kits under development.

The Millipore Enviroguard range includes both test tube and ELISA plate based assays. The tube range costs from £180 to £215 per kit for 12 unknown determinations. A filter photometer for laboratory or field use is available for £900. Plate kits cost from £330 - £396

for up to 44 determinations done in duplicate, and require the use of an ELISA plate reader. Limits of detection quoted are typically down to 0.01-1 ug/l for individual pesticides.

The Ohmicron range is offered by J T Baker and now Tepnel Life Sciences based in Knutsford. The Ohmicron system uses one micron magnetic particles as a solid support for the antibodies. Separation of bound and free analyte is thus accomplished by the application of a magnetic field. Assays are thus carried out in tubes that do not need coating with antibody. Tubes cost from about £190 for a 30 tube kit. A magnetic rack costing about £500 is also needed. A photometer is also supplied (£4000) to allow determination of concentration. Limits of detection are typically 0.01-1 ug/l.

Ensys Europe are expanding the range of kits offered. As this report is being compiled they have purchased the Enviroguard range from Millipore. The exact product line to be retained is likely to be reviewed. They have concentrated on soil and amongst their products are kits for dioxins, polyaromatic hydrocarbons, polychlorinated biphenyls and hydrocarbons. They do point out that sample preparation is needed prior to the analysis. They also sell a portable photometer and sample preparation system.

Environmental Sensors Ltd is a new company currently offering atrazine and isoproturon methods with several other pesticide methods shortly to be available. Their kits are designed to be used with the instrument package they have developed. The assays are almost completely automated and the atrazine assay is claimed to meet Drinking Water Inspectorate (DWI) guidelines and is being used by Water Companies.

Guildhay sell two pesticide kits and a range of other antisera. They have several new pesticide products under development. They have a lot of experience of antisera development and custom assay development.

Several manufacturers point out that they can customise their kits to achieve detection limits different to those quoted, and for some analytes different kits are required for different analyte concentration ranges.

Table 3.1 Commercially available immunoassay kits

Compound	Supplier(s)	Compound	Supplier(s)
2,4- D	O, M	Giardia	EN
2,4,5-T	M	Heptachlor	M
6-Hydroxyatrazine	M	Isoproturon	M, G, ES,
Alachlor	O, M	Lindane	M
Aldicarb	O, M	Linuron	M
Aldrin	M	Metalxyl	M
Ametryn	M	Methoprene	M
Atrazine	O, M, G, ES	Metolachlor	O
Benomyl/Carbendizim	O, M	Metsulfuron	M
Benzene	EN	PAH	O,M
Captan	O	Paraquat	O,M,G
Carbaryl	O,	Parathion/methyl parathion	M
Carbofuran	O, M	PCB	O,M,EN
Chlorothalonyl	O	Pentachlorophenol	O,M,EN
Chlorsulfuron	M	Petroleum Hydrocarbons	M,EN
Chlorotoluron	M, G	Pirimithos-methyl	M
Chlorpyriphos	O	pp-DDT	M
Chlorpyriphos-Methyl	M	pp-DDE	M
Chlorpyriphos-Ethyl	M	Procymidone	O,M
Cyanazine	O, M	Prometon	M
Cyclodienes (Chlordane)	M	Prometryn	O,M
Deethylatrazine	M	Propazine	O,M
Diazinon	M	Simazine	O,M,G
Didealkylatrazine	M	Simetryn	M
Dieldrin	M	Toxaphene	M
Dioxin	EN	TNT	M
Diuron	M, ES,	Terbuthylazine	M
Endosulfan	M	Trietazine	M
Endrin	M	Triasulfuron	M
Fenitrothion	M	Urea Herbicides	M

Key to manufacturers
O = Ohmicron, sold in the UK by J T Baker, Tepnel Life Science and Supelco
M = Millipore
G = Guildhay Antisera
ES = Environmental Sensors Ltd
EN = Ensys Europe, sold directly and through Thames Chromatography

3.3 Scientific and other literature

3.3.1 Immunoassays

Methods are continually being developed (see a selection in the bibliography) and published in the scientific literature. As with much analytical scientific literature these do not usually provide enough validation and performance data to allow a judgement of the possibility for routine assay. This is not necessarily a criticism of the authors as some editors will not want too great a level of detail. A draft specification for evaluating immunoassay kits for water monitoring has been suggested by Watts and Hegarty (1995).

The availability of antisera from authors publishing in the scientific literature (mainly research laboratories) is also questionable. Commercial kit manufacturers are quite likely to buy and evaluate antisera from such laboratories if new opportunities arise. The performance data quoted by manufacturers is often based on the publication of the laboratory developing the method.

Meulenberg and Stoks (1995) have reported the use of Baker and Millipore kits for screening Rhine water for 2,4-D. They carried out a comparison with GC-MS and found no false negatives with either kit but they did report matrix interference. Marco et al (1995) have reported good agreement between immunoassay kits and HPLC-MS for carbaryl and 1-naphthol determination in water. Mouvet et al (1995) carried out a comparison of several test kits for triazine analysis with chromatographic methods. Samples were surface, ground and lysimetric plate water. They found good agreement between the chromatographic and ELISA technique for the sum of the triazines. They did conclude that good results required dedicated equipment and operator training. Goolsby et al (1991) carried out a comparison of atrazine test kits with GC-MS and found a good correlation for water samples. They estimated the cost per assay as $15 for the immunoassay and $200 for GC-MS. This did not allow for simultaneous determination of other analytes by GC-MS.

Ohmicron Diagnostics have recently published a paper, Hayes et al, (1996) detailing an evaluation of their magnetic particle immunoassay for atrazine. This was an inter-laboratory collaborative study involving 14 laboratories in North America. Laboratories analysed 42 samples of municipal, well and surface water both spiked and with incurred levels. Concentrations ranged from 150- 4500 ng/l. Average recoveries were 104%, ranging from 80-120%. Samples at concentrations below 240 ng/l had a relative standard deviation of ±23% and samples at a concentration of 470 ng/l or above of ±10 %. The inter-laboratory agreement was comparable with chromatographic methods. The assay used was optimised to maximise performance at 3 ug/ml, the regulatory limit for the USA.

3.3.2 Immunoaffinity

Solid phase extraction is now commonly used for the isolation and pre-concentration of trace organics in environmental matrices. Recently several groups have proposed the use of immobilised antibodies as a highly selective solid phase extraction system. Immunoaffinity attempts to use the highly specific and selective interaction between antibodies and antigen. Antibodies are immobilised onto an activated matrix such as silica or glass. When a sample is passed through the immobilised antibody column the antibody should retain the antigen. The

columns can be washed to remove potentially interfering compounds and then if a suitable eluent can be found the target analyte can be released from the antibody. The analyte can then be determined by other methods such as chromatography or immunoassay. Binding of the antigen to the antibody takes place by formation of multiple non-covalent bonds such as hydrogen bonds, electrostatic bonds, Van der Vaals and hydrophobic interactions.

Many different matrices are available for use in immunoaffinity chromatography such as agarose, cellulose, acrylamides, silica and controlled pore glass. For analytical purposes silica and controlled pore glass are preferred due to their mechanical strength. The matrix requires chemical activation in order to react with the antibodies. The most common methods are cyanogen bromide or glutaraldehyde activation. In general only small amounts of antibody are bound so the columns are of low capacity.

The antibody columns are most stable when used with aqueous eluents at neutral pH. Extremes of pH, or high concentrations of organic solvent will denature the columns. Hence when developing an analytical protocol the range of eluents to desorb analyte is limited. Analyte elution is achieved by lowering pH, adding a proportion of a water miscible organic solvent, specific displacer molecules, changing ionic strength, chaotropic reagents or denaturing the antibody. As supply of antibody is usually limited columns and protocols are often designed to allow multiple use of the columns. Most immunoaffinity protocols have used non-selective desorption with a mixture of low pH and addition of ethanol or methanol to the elution buffer. Although the immunoaffinity columns are designed to give selective extraction of analyte non-specific interactions are common. This is probably due to interactions with the matrix onto which the antibodies are bound.

Immunoextraction requires the binding of antibodies onto a suitable rigid support such as silica or controlled pore glass without the loss of antibody activity. These columns use aqueous eluents and thus create little toxic laboratory waste. Potentially this technology combines the selectivity of antibodies with the confirmation possible with spectroscopic detectors in chromatography. The amount of antibody used per column is higher than in an ELISA so columns have to be reusable to prove economical.

Immunoaffinity columns have been used in automated systems via column switching in HPLC or as typical solid phase extraction methods. This type of work has been used for monitoring veterinary drugs for some years but has recently been proposed for pesticides in water by Pichon et al (1995a and b) for phenylureas, by Thomas et al (1994) for atrazine, and by Shahtaheri et al (1995) for chlortoluron. These papers are research based showing the feasibility of using immunoaffinity but did not contain extensive validation data.

Immunoaffinity columns can be used for pre-concentration followed by classical ELISA as well as chromatography. The columns allow pre-concentration of isoproturon and chlortoluron from up to 1 litre of drinking water and then elution in just 1 ml of phosphate buffer /ethanol, 50/50. No further sample preparation was necessary before introduction into HPLC with UV detection. Such a large pre-concentration is probably not necessary if the methods were adapted for automated routine use with specific detection. These authors have also demonstrated that retention was almost entirely due to antibody-antigen interactions.

The mass capacity of immunoaffinity phases is limited. Although it will vary widely, as will the quality of antibodies, a very rough estimate is that 1 ul of antibody will retain 1 ng of analyte. Much of the development work has used columns capable of retaining 10-100 ng of analyte. This is very compatible with current chromatography detection systems. As the protocols used extraction cartridges of the same dimensions as are currently used in automated systems such as the Gilson ASPEC, then automation of sample preparation ought to be possible.

Pesticides within the same class often cross-react with antibodies so more than one may bind to an antibody raised to a particular analyte. Generally speaking this is a disadvantage in an ELISA type assay as it makes calculation and reporting of individual values difficult. If such group specificity were by design then the immunoaffinity cartridge could be used to extract a class of compounds. The chromatographic methods are efficient at separating closely structurally related compounds such as the pesticides within a group. The combination of immunoaffinity with chromatography thus offers the potential to extract groups of compounds and then separate, confirm and accurately determine them. Current reports, for example Pichon et al (1995a), show some phenylureas cross-reacting though not the whole group. As cross-reactivity is different for different antisera there is the possibility of using mixed bed antibodies where more than one antiserum is used in the same column.

The field of immunoaffinity extraction followed by chromatography is in its infancy for pesticide residue analysis. The current disadvantages are the lack of availability of antisera, and no commercial companies are offering immunoaffinity phases though several offer matrices suitable for the more common use in the clinical field. Clifmar, a company of the University of Surrey, has developed techniques for the immobilisation of antibodies to pesticides on silica and controlled pore glass reported by Shahtaheri et al (1995). These have proven suitable for selective extraction and pre-concentration of pesticides from drinking water. A typical scheme for immunoextraction is shown in Figure 3.2.

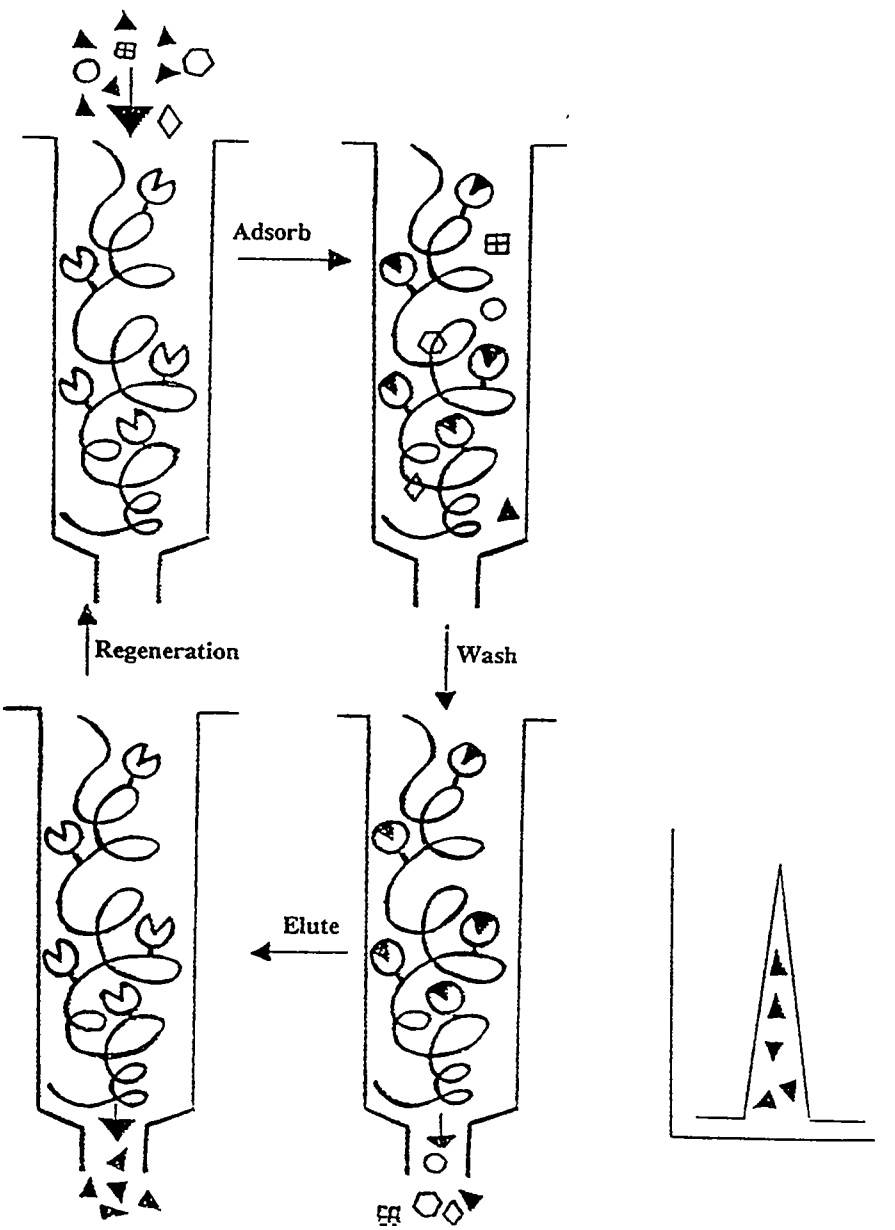

Figure 3.2 Immunoextraction - Basic Principles

3.4 Technical literature

The Foundation for Water Research has published a report by Gale et al (1994) on the "Development of Immunoassay kits for the Analysis of Pesticides and other Organics in Water". A draft "Blue Book" style specification for a hypothetical immunoassay kit for pesticide analysis was prepared.

The report has several important conclusions. A survey of water industry analysts has revealed that immunoassay kit performance was improving and that the best kits came close to meeting DWI guidelines for water analysis but none as yet achieved them. Where kits did approach the limit of detection required there was cross- reactivity with other pesticides.

The unit cost of immunoassay kits was considered similar to instrumental analysis if a suite of ten analytes was measured, but turnaround was quicker for immunoassays. Analysts also thought that immunoassay kits would be useful for the more difficult determinants that were poorly extracted, needed derivatisation or for which a sensitive specific detector was not available. These included analytes such as paraquat, glyphosate, trichloroacetic acid and asulam. Several water industry analysts are now using kits to evaluate water treatment. There was also mention of an interest in selective extraction or clean-up using immobilised antibodies, preferably with group specificity.

The report estimated assay of a single determinand at about £6 assuming 70 samples were assayed together. Assuming a cost of £50 for chromatographic analysis of a suite of ten pesticides these costs are similar. Immunoassay will work out cheaper if many samples of only a few determinands were needed, GC or HPLC was cheaper for multi analyte assays. The report also concluded that immunoassays produced results in 2-3 hours whereas sample preparation followed by GC or HPLC may take 48 hours. Chromatographic methods required up to 1 litre of sample whereas a typical ELISA used 50-200 ul of sample.

One of the perceived advantages of ELISA type techniques was the possibility of screening more than one compound due to antibody cross-reactivity. However the authors pointed out that this caused difficulty in reporting results as a sample containing a mixture of pesticides at differing concentrations was not always the sum of the individual pesticides. Kits therefore needed to state cross-reactivities and the conditions under which they were determined as they depended on incubation time, temperature, pH etc. There were many potential interfering compounds in water other than those that are structurally related. These may inhibit analyte-antibody binding, interfere with the enzyme-substrate reaction, the oxidation of the chromagen or binding of conjugate onto particulates. Potential sources of interferences included metals (Fe, Cu, Pb, Ni, Zn, Mn, Ca, Mg), fulvic and humic acid, chloride, nitrate, sulphate, bicarbonate, chlorine, disinfectants and surfactants as well as pH. The report noted that if samples of substantially different matrices were assayed routinely then precision and recovery data from at least eleven batches were required.

A detailed evaluation of the Guildhay atrazine kit was described in Gale et al (1994). This gave a limit of detection of 5 ng/l. Interassay relative standard deviation was ± 24% at

43 ng/l, ± 7% at 244 ng/l and ± 9% at 846 ng/l. Cross reactivity was high with propazine (71%) and simazine (18%) and some with prometryne and desethyl atrazine. Interference tests with metals, detergents, humic acid, chloride, chlorine, bicarbonate, methanol and pH showed little interference at likely concentrations other than with levels of humic acid at 2 mg/l and above. In a comparison with GC-NPD 172 samples of raw water abstracted for drinking water treatment, water at different stages of treatment and potable water there was good correlation between the two methods (r = 0.828). These were over the concentration range 10-990 ng/l. Better correlation (r = 0.922) was obtained on the treated water from a single source.

The report also described the development of an isoproturon ELISA and it's validation. The kit had a limit of detection of 9 ng/l, an interassay relative standard deviation of ±18% at 65 ng/ml, ±15% at 248 ng/l and ±9% at 736 ng/l. There was no cross-reactivity with other uron herbicides.

There was encouragement in the report to kit manufacturers to continue their dialogue with the water industry and to improve the performance of their immunoassay kits. They were also encouraged to use "Blue Book" specifications to validate their methods and to submit some methods to the Standing Committee of Analysts.

3.5 New developments

Group specific antibodies have arisen by serendipity rather than design but laboratories have reported on the possibility of achieving this using recombinant antibody fragment techniques. A different approach is to use molecular imprinting to produce antibody mimics. These are synthetic polymers produced around a template analyte.

3.5.1 Group Specific Antisera

Optimisation of hapten design may allow antibodies to exhibit group selectivity e.g. to the triazine or uron group. So far attempts to do this have not proven successful. Using recombinant antibody techniques it is now possible to modify antibody binding techniques at the DNA level in organisms such as E. Coli. though these are not yet ready to use for pesticide analysis, Hock et al (1995). These engineered antibodies might allow individual pesticide or group specificity by design rather than accident. Clones of interest can be modified and eventually produced on a larger scale. Once available these antibodies could be used in typical ELISA formats, in immunoaffinity columns for sample extraction or in immuno-based biosensors.

3.5.2 Molecular Imprinting

The difficulty and unpredictability in obtaining antibodies from animals have led to efforts to synthesise antibody mimics using synthetic polymers. Molecular imprinting involves polymerisation around a target analyte using a monomer selected because of its ability to form around the target compound. Subsequent removal of the target analyte leaves a cavity in the polymer. These recognition sites allow the polymer to selectively bind to analyte. Thus imprinted polymers may mimic antibodies in their binding but with the obvious advantage that they arise via laboratory synthesis rather than unreliable biological origin.

Siemann et al (1996) have recently described the synthesis of imprinted polymers to selectively bind atrazine. These were used in ligand binding studies and as an HPLC column. The author also pointed out the possibility of using the polymers for selective solid phase extraction, or to carry out immunoassay type methods in non-aqueous solvents.

3.6 Discussions with Environment Agency laboratories

In discussions with one laboratory it described how it carried out three types of tests:- routine, incidents and surveys.
Routine work was carried out in the field wherever possible and where analyte stability was a problem. Samples for analytes such as pesticides were sent to a central laboratory for analysis. This meant a 24 hour delay which was quite acceptable. Field tests were calibrated weekly at each office by a mobile laboratory.

Incident teams used field kits wherever possible to trace incidents and movement upstream. Surveys of a five to ten mile stretch were planned well in advance, utilising field test kits as well as laboratory samples.

The responsibility for selecting an appropriate test method was the obligation of the testing laboratory. The ELISA test kits might be useful for tracing pollution incidents providing they have been fully validated.

In another meeting it was pointed out that over five million laboratory tests were carried out per year of which over one million were pesticides. Most of these are on dirty samples. Some 30-40 pesticides are screened routinely as a statutory requirement. Dioxins, polyaromatic hydrocarbons and polychlorinated biphenyls were also screened and were difficult assays. Dioxins were contracted out. The "drins", "urons", organochlorines and organophosphates were also a priority. A broad spectrum screen was thus necessary. The formation of the Environment Agency will increase the number of tests and the diversity of matrices analysed.

The current range of ELISA tests was not viewed as adequate to meet the need to screen for so many individual compounds. Tests for whole classes of compound would be an advantage as a screen but were not yet available. The lack of validation data for these was not seen as a problem as any new methods would have to show acceptable performance in validation tests This would add to start-up costs.

The position with field kits was similar in that the complete range for organics was required and the range of immunoassays currently available was limited. Samples would still have to be sent to a laboratory for the analytes not covered by the field test. As chromatographic methods were multi-analyte there was often minimal extra cost in reporting several analytes. Immuno-based tests for viruses and dioxins could be an advantage.

In a further meeting there was some interest in the use of immunoassays for monitoring trace organics particularly pesticides. It was indicated that when placing contracts with the laboratory it was basically the decision of the analytical laboratory as to which method was used. However in setting up the service agreement with the analytical laboratory the client would expect to discuss which methods were to be used if it was proposed to change to immunoassay methods. They would wish to see the full validation data and performance

criteria for the assay before becoming convinced that ELISA type techniques would satisfy their requirements. Semi-quantitative results would be adequate especially if screening assays were available. It was felt however that the current performance of immunoassay kits was not adequate.

The situation would improve if better validation and comparison with chromatographic methods were carried out. It would need approval of a standard method by the standing committee of analysts to their criteria to convince clients of the suitability of ELISA type methods.

There were however several compounds for which current methodology was not adequate, or was very expensive. These included glyphosate, polyaromatic hydrocarbons, dioxins, fungicides and algal toxins. Immunoassay kits might prove useful for tracing sources of pollution, particularly if they could monitor at levels of biosignificance. An immuno-extraction project to measure microcistene (from toxic algae) was underway. Method validation was indicating that this had not been as successful as originally hoped.

3.7 Immunoassay kit suppliers

Guildhay offer a range of antisera in the human , veterinary and food diagnostics area and also the environmental area. They also offer a custom antisera development service and have several products under development. In addition to the atrazine and isoproturon kits they are also selling antisera to paraquat, chlortoluron, simazine and solanine. Antisera under development include MCPA, mecoprop and diuron.

They are involved in several research programmes, Support for products under Research (SPUR), and Small firms Merit Award for Research and Technology (SMART) part funded by the Department of Trade and Industry (DTI) and are increasing the portfolio of compounds to be monitored in water, particularly drinking water. They are developing both plate-based and field kits. As an approximation they have estimated a cost of about £60k to custom design a new assay. Some of their projects have been carried out in collaboration with the water industry.

Environmental Sensors is a relatively new company in the environmental monitoring field. They were established with part funding from Anglian Water. They have developed an instrumentation package (called LumIA) for an automated laboratory based monitoring system. This is based on an enhanced chemiluminescent ELISA assay. It comprises a sample processor, an ELISA plate washer and a luminometer along with a PC based controller. They sell pre-coated ELISA plates and all the reagents and standards to perform the assay. The operator only has to put the plate in place along with reagents. Currently two assays are being sold, atrazine and isoproturon.

The complete instrument costs approximately £40k or £23k without the sample processor, though they strongly recommend the complete package. They have invested a lot of effort in meeting the performance criteria laid down in the DWI guidelines for analytical method performance. They believed the level of automation was needed to achieve these criteria.

Kits soon to be launched included diuron, mecoprop, chlortoluron and trichlopyr. Kits under development included glyphosate, benazolin, imazapyr and bentazone. New developments included a uron specific kit and a triazine specific kit. They would also consider custom method development and would estimate very approximately £75k-£90k for a method and kit for their instrument.

The atrazine kit has been tested in trials by Anglian and other (UK &/or European) water companies. The protocol was in accordance with DWI guidelines. The limit of detection was about 10 ng/l on spiked water (surface raw, surface treated, bore hole raw and bore hole treated). Precision was less than ±5% within batch and less than ±10% between batches. They have also shown good correlation between their method and GC-MS performed in four water company laboratories over the range 15-400 ng/ml. In that trial they did allow for known cross-reactivity (simazine). The company also have under development single use printed circuit disposable dipstick assays and portable heavy metal sensors based on anodic stripping voltammetry.

EnSys Europe Ltd have recently acquired the Enviroguard range of products from Millipore and would be reviewing the product range though they expected to maintain much of it. They have concentrated on residues in soil and foods where only one pesticide is needed. They have an antibody to dioxin though this was selective to the 2,3,7,8- congener as it was deliberately designed that way. They have not focused on water analysis in the UK as they believed that the requirement to report an individual result with error limits was not well suited to immunoassays. This may change as the expanded range of antisera is reviewed.

3.8 Current users of immuno technology

Anglian Water have helped develop the Environmental Sensors LumIA instrumentation and kit for atrazine and are shareholders in the company. They have evaluated the system and have recently introduced it into their Milton Keynes Laboratory. They identified the need for faster, cheaper methods to monitor a small number of pesticides, particularly atrazine, isoproturon, diuron and mecoprop. Under DWI guidelines they do not have to analyse every pesticide. The initial interest in atrazine arises from the need to screen for atrazine at water treatment works. This was not a regulatory compliance analysis but was needed to decide whether expensive ozone and carbon treatment was needed. In these circumstances method precision or cross-reactivity with simazine was not important.

They have also started to use the atrazine assay for drinking water as it now satisfies the DWI guidelines. They believed that ELISA techniques have a role to play in this area. They also considered glyphosate and polyaromatic hydrocarbons to be a problem from the analytical point of view.

4. CONCLUSIONS

- Commercial immunoassay kits are available for a wide range of pesticides and other trace organics and these can be used with minimal sample preparation on water samples

- Very few of these kits meet the requirement to report individual pesticide values below 100 ng/l with satisfactory precision, though with dedicated instrumentation this is possible for atrazine and isoproturon, though even then cross-reactivity occurs

- Immunoassays are quicker than procedures needing sample preparation and chromatography and can be used for rapid screening and on-site analysis

- Satisfactory performance criteria is now available for atrazine ELISA kits and is becoming available for other analytes such as isoproturon in water

- Antisera to a wider range of analytes is becoming available including some compounds that cannot be satisfactorily analysed by conventional methods

- Currently available ELISA tests are for single analyte measurements and do not offer group specificity

- More complex matrices such as effluent waters and solid samples require sample preparation before analysis

- Air samples which are collected onto filters or adsorbents are desorbed using solvents that are incompatible with the direct use of antibodies

- Immunoassays are less expensive than chromatography for analysis of large numbers of samples if only a small number of analytes are required in a single sample

- GC and HPLC is more cost effective if a suite of analytes is required

- Immuno-extraction has been demonstrated as feasible though not rigorously validated

- Immunoaffinity sorbents to pesticides or other organics of interest are not commercially available

- Custom development of an ELISA type assay would cost over £50k for a single client with no guarantee of success and antisera to problem compounds are likely to become available via other sources if development is possible

5. RECOMMENDATIONS

1. For compounds where suitable chromatographic methods exist immunoassay currently offers no real advantage for laboratory testing. The use of ELISA tests for the majority of trace organics is therefore not recommended.

2. For problem compounds particularly where these are a single analyte ELISA type tests merit consideration.

3. If antisera is available for these, or is under development, discussions with kit manufacturers and other interested user groups should be initiated to clearly define Environment Agency method performance requirements.

4. A pilot study to evaluate immuno-extraction in Environment Agency laboratories is recommended if a reliable source of columns is identified. Clifmar Associates, based at the University of Surrey, have been producing and using immuno-affinity columns for sample clean-up and analysis for specific analytes. They should be contacted if target analytes are selected for this approach. Immuno-affinity columns combined with immunoassays should be considered as well as sample preparation for chromatography.

5. Environment Agency laboratories requiring on-site testing might find the availability of test kits useful. The performance of these test kits should be evaluated by laboratories carrying out conventional methods of analysis. A standard protocol for the conduct of such studies should be agreed to include method comparison on the same samples.

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