Immunochemical techniques for environmental analysis II. Antibody production and immunoassay development

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Antibody production is the key step of any immunochemical technique. The antibody determines to a large extent the specificity and sensitivity of the resulting immunochemical technique. These features may be modulated by judicious design of the immunogen and by rational immunoassay development. Criteria for hapten design and the steps involved in the obtention of antibodies and the development of competitive assays are described.

1. Introduction

In our previous article [1] we described the most common immunochemical techniques employed for environmental analysis: immunoassays (IAs), immunoaffinity chromatography (IAC) systems, flow injection immunoanalysis (FIIA), and immunosensor technology. Immunochemical techniques offer many advantages compared to their limitations. However when small molecules are the target molecules of the analysis, the generation of the antibodies (Ab) and development of competitive assays become long and complex process which require the participation of highly qualified personnel who can combine experience in analytical chemistry, synthetic organic chemistry and immunology. As we will see for pesticide immunochemical analysis an important contributor to the cost of these techniques is the fact that most of the analytes are small molecules ($M_r < 1000$) which are unable to produce an immune response. In order to elicit Abs they must be presented to the immune system covalently linked to a carrier, usually a protein. Here we discuss the most important considerations for pollutant Ab production and the steps involved in the development of competitive assays. This process is shown schematically in Fig. 1.

2. Selection of the target molecule

A good knowledge of the analytical target, its chemical structure, its stability and its degradation routes are required before assay development

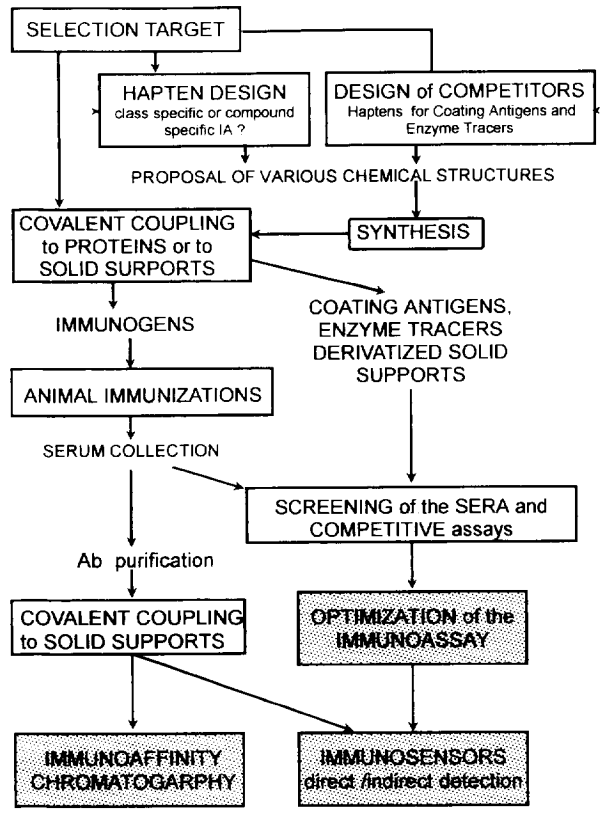

Fig. 1. Diagram showing the main steps involved in the obtention of antibodies for small molecules. The Ab obtention is an important early step in the development of competitive immunoassays, immunoaffinity columns or immunosensors.

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starts. As an example, we have developed antibodies to two analytes, carbaryl and 1-naphthol [2,3] which arise by rapid degradation of the carbamate in the environment, biological tissues, or during cleanup procedures. Similarly, the monitoring of parathion was intended by developing an enzyme-linked immunosorbent assay (ELISA) for 4-nitrophenol [4], its main degradation product. Another important requirement is to establish the purpose of the analysis—is it to be class-selective or analyte selective?. For use on an immunoaffinity column or some screening IAs a class-specific antiserum may be more convenient than a very selective Ab, particularly with regard to the fact that many environmental pollutants suffer degradation or biotransformation to other chemically related compounds. Finally, it is also important to know the kind of matrix to which the immunochemical analysis is going to be applied. Thus metabolites or protein adducts may be the targets for Ab obtention when the aim of the analysis is biological monitoring of exposure to toxicants and/or pollutants. Therefore the urinary metabolite, the N-acetylcysteine conjugate of naphthalene (NaphMA) (see Fig. 2), was selected as the target molecule for the development of an IA aimed towards biological monitoring of naphthalene exposure [5,6].

If the target compound contains functional groups such as NH_2, COOH, OH, SH, CO or CHO direct covalent coupling to the carrier molecule can be performed. However to avoid masking of essential groups for the Ab recognition, a *hapten* should be usually prepared. A *hapten* is a derivative of the target molecule that contains an appropriate group (*linker or spacer arm*) for attachment at a convenient place in the molecule.

3. Hapten design for immunogens

The design of a hapten is the most crucial step in the development of an immunochemical technique for pesticides or other low-molecular-mass environmental pollutants. The specificity and selectivity of an immunochemical technique are mainly determined by the Ab. Many literature examples prove that an appropriate hapten design determines the features of the resulting antibodies(e.g. [5,7–11], etc.). Although screening of several chemical structures is always recommended, we can give some important guidelines based on reported literature examples.

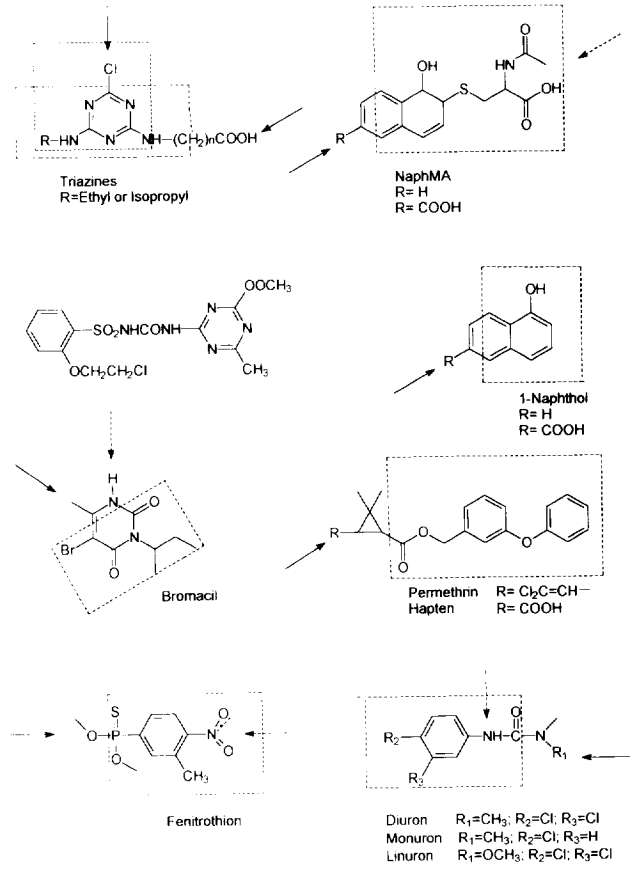


Fig. 2. Some examples of the strategies used in hapten design for several environmental contaminants. Solid arrows indicate the most favourable position for attaching the carrier molecule during the preparation of the immunogen. Dotted arrows signal less favourable coupling positions for preparing immunogens although sometimes they may be convenient for preparing competitors. Dotted boxes correspond to the target molecule moiety which is more exposed for Ab recognition when using the more favourable hapten.

3.1. Preservation of the chemical structure

A hapten should preserve as much as possible the chemical structure, electronic distribution and spatial conformation of the target compound. However, characteristic portions of the molecule are sometimes sufficient to generate valuable antibodies.

3.2. Linker location

Remote from the characteristic sites of the molecule. Exposure of the molecule to the immune system is greater for these sites placed further from the attachment point, thus determining the selectivity of the resulting antibodies. Diverse examples

support this fact. The selectivity of triazine assays [9–11] was determined by linking the hapten to a carrier protein through the alkylamino group (class selective) or by using the chloro group (compound selective) of the atrazine molecule (see Fig. 2). The antibodies generated by covalent coupling of the protein through the carboxyl group of the N-acetylcysteine residue [6] (dotted arrow) of the NaphMA, were more selective than the corresponding assay in which the immunogen was formed by attachment through ring A (solid arrow) because other urinary mercapturic acid (MA) conjugates were also recognized by the Ab [5]. Class-specific antibodies to pyrethroids were obtained by conjugation of a permethrin hapten through the distal part (solid arrow) of the 3-phenoxybenzyl group common to pyrethroid chemical structures [12].

Avoid using analyte functional groups (i.e., heteroatoms). Although it usually requires more elaborated chemistry, derivatization through the carbon atoms of the analyte is preferred since these atoms do not contribute as much to the steric and electronic properties of the target molecule. Using important functional groups of the target analyte leads to a reduction of the sites that could potentially help to stabilize the Ab–analyte immunocomplex. Antibodies with lower affinity may thus be obtained, leading to less sensitive assays. As an example, the antibodies to the herbicide bromacil generated by attaching the carrier protein through the methyl group gave a better IA than the antibodies obtained from the corresponding hapten having the linker on the amino group of the uracil ring [13] (see Fig. 2). Here, the conversion of a secondary to a tertiary amine reduced the possibilities of establishing electrostatic or Van der Waals interactions between the analyte and the Ab. The same explanation can be given for the difference in sensitivity encountered with the IAs developed for urea herbicides [14,15] with Abs raised using haptens whose carrier attachment point was the nitrogen or the methyl group (see Fig. 2). Antibodies obtained by coupling fenitrothion to the carrier protein through the nitro group led to assays with very poor sensitivity, while the best assay used antibodies raised to a hapten coupled through the thiophosphate group [16–19] which suggests participation of the nitro group in stabilizing the immunocomplex (see Fig. 2). Similarly, the highest affinity for NaphMA shown by the antibodies raised by coupling NaphMA through its ring A may be explained by the greater ability of the mercapturic acid (MA) moiety, rather than the aromatic ring to establish binding forces with the Ab [5,6].

3.3. Chemical structure of the linker

The length and structure should be chosen to reduce spacer recognition while maximizing target exposure to the immune system

Functional groups in the chemical structure of the linker should be avoided. The presence of heteroatoms or electron-withdrawing groups in the spacer arm could alter the electronic distribution of the target molecule while showing epitopes for its recognition. For example aminotriazole haptens prepared by using the hetero-bifunctional reagent maleidimidobenzoic acid N-hydroxysuccinimide (MBS) produced antibodies that recognize the hapten protein conjugate, but did not recognize the target analyte alone because of strong recognition of the bulky spacer [20]. Also, Hill et al. failed to obtain a good competitive assay when a bulky group was introduced as part of the linker in the immunogen for bioresmethrin [17] (see Fig. 2). In contrast, N-succinimidyl-4-(maleidimidomethyl)-cyclohexanecarboxylate (SMCC), another hetero-bifunctional bulky cross-linker, was used successfully to raise antibodies against a 33-amino-acid peptide, although in this case the molecular size of the target analyte was much greater than that of the linker [21]. Non-bulky alkyl spacers thus appear to be more convenient alternatives for the linker's chemical structure although this requirement becomes less strict for large analytes.

Length of the linker. With small molecules the use of a spacer arm helps to keep the target structure away from the shielding effect caused by the protein, favouring its recognition by the immune system. A medium size, with three to six atoms, has often been quoted as an optimal range, since longer spacer arms may lead to hapten folding, and then reducing the exposure to the immune system. Spacer arms of different lengths were evaluated during the development of an IA for bioresmethrin [17], failing to obtain good Ab titres with the zero spacer arm derivatives. The same results were obtained by Gee et al when raising antibodies against molinate [22]. Harrison et al. also reported a comparative study of the influence of the length of the spacer arm in the immunizing haptens for triazine [10]. Again, the length of the linker is not

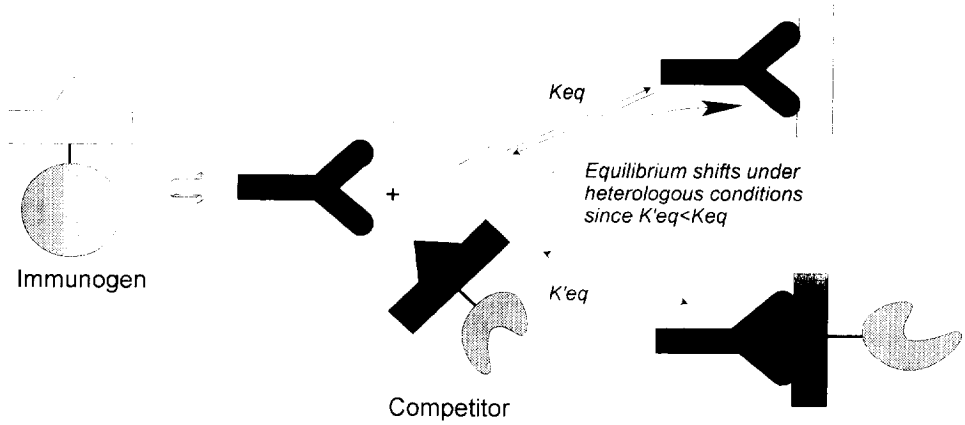


Fig. 3. Scheme representing the shift of the equilibrium when using heterologous systems in competitive immunoassays. The affinity of the Ab for the competitor is lower than for the target analyte because of its greater structural similarity to the immunogen.

so important when the size of the target molecule increases.

Contrary to the above guidelines, we succeeded in developing an acceptable assay for 1-naphthol [3] and the NaphMA [5] by using immunizing haptens which did not have either the desirable length or appropriate chemical structure (see Fig. 2). However, as we mentioned before, for stabilizing the immunocomplex with NaphMA, participation of the N-acetylcysteine residue seems to be greater than that of the aromatic ring, thus reducing the effect of the carboxyl group as an electron-withdrawing group.

4. Design of haptens for competitors

Most of the IAs and immunosensors developed for the analysis of environmental residues are working under competitive conditions (see Part I, our previous paper [1]). This method requires the preparation of competitors, usually as labelled-analyte conjugates (for direct assay) and protein–analyte conjugates or analyte derivatized surfaces, (for indirect assay). The requirements of the chemical structures of these analyte derivatives are not so strict as for the immunizing hapten. Although some *homologous* assays (using the same protein conjugate for immunogen and competitor) have been reported (e.g., [5,23]) competition from the target analyte is usually favoured by *heterologous* conditions (e.g., [15,24,26,27], etc.). With *heterologous* systems recognition of the competitor by the Ab is reduced. Consequently, small concentrations of analyte will be sufficient to shift the equilibrium conditions against formation of the Ab–analyte immune complex, thereby inhibiting association of the Ab to the competitor and leading to very sensitive assays (see Fig. 3). The structure of competitor may thus have a strong influence on the sensitivity of the resulting IA since it modulates the equilibrium conditions of the immunoassays. The desired IA heterology can mainly be accomplished at three different levels:

- *Hapten.* Different hapten chemical structures are used for both the immunogen and competitor. By providing hapten heterology the sensitivity of the chlorpyrifos IA increased from an I_{50} of 197 to 33 n*M* [28]. The competitor which performed better on a magnetic particle IA for triasulfuron had just the chloroethoxyphenylsufonylurea moiety instead of the whole molecule structure [24] (see Fig. 2). A fragment of the target analyte has also been used as competitor in an immunoassay for polychlorinated biphenyl compounds [29]. In this context, Schneider et al. have proposed a rational method to predict which hapten structures may be the best competitors, based on the cross-reactivity data of these haptens [14].
- *Site.* The attachment point is at a different location from that in the hapten used for immunization. This strategy has been used, for example, on one of the IAs developed for the NaphMA [5] using the less favourable immunizing hapten as a coating antigen (Ag) (see Fig. 2). Similarly, by using the nitrogen of the uracil ring of the bromacil molecule [12] or the secondary amine of the urea herbicides IA [14,15] as conjugation sites for the competitors in these assays

(see Fig. 2), a shift of the equilibrium vs. formation of the Ab–analyte complex was favoured by decreasing the Ab recognition of the competitor.

- *Linker.* The length, size or/and chemical structure of the spacer arm is different in the immunogen and competitor. In the bioresmethrin assay a bulky group on the spacer resulted in a lower recognition of the competitor, thereby increasing the analyte efficiency in the competition [17]. An interesting study of the influence of the competitor spacer arm's length on the sensitivity of the triazine assay has been reported [10], showing that the optimal length is also dependent on the kind of label chosen.

All the considerations mentioned above are very important, especially when dealing with small molecules. However, in this case the preparation of multiple haptens is also crucial, for the future screening of several heterologous or homologous assays

5. Synthesis of the haptens

After considering the guidelines mentioned above, whenever possible it is wise to use commercially available starting materials or intermediates to prepare the haptens. Sometimes, known metabolites or degradation products can be used as starting materials on the synthetic route. Most of the assays described involved at least two or three synthetic steps for hapten preparation (e.g., triazines [9], urea herbicides [14], *p*-nitrophenol [4]). Nevertheless, one must balance the benefits derived from an initial synthetic effort on the steps of the assay development since, as mentioned above, appropriate hapten design can direct the Ab specificity and selectivity. The synthesis of the most favourable hapten for the NaphMA involved about eight steps, but the resulting IA increased the sensitivity by a factor of 100 compared to the previously developed assay [5,6] (see Fig. 4, scheme A). The synthesis of the immunizing hapten for bioresmethrin also involved several steps [17] (see Fig. 4, scheme B).

6. Covalent attachment to carrier molecules

Methods used for protein conjugation have been extensively reviewed [30,31] and Table 1 shows

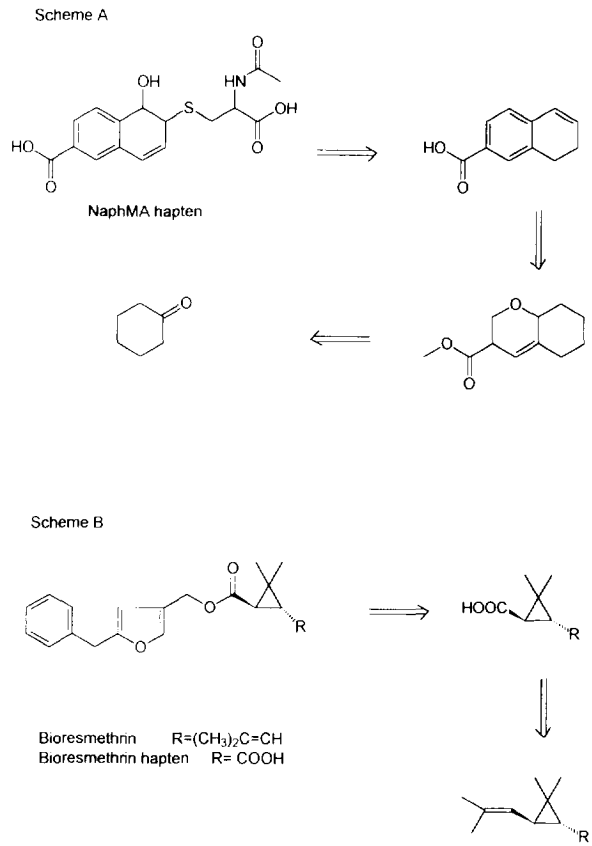


Fig. 4. Retrosynthetic schemes used in the preparation of haptens for immunogens to produce antibodies against NaphMA (A) and the pyrethroid insecticide bioresmethrin (B)

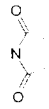
a summary of some common coupling strategies. The functional group of the hapten governs the selection of the conjugation method to be used. However, the stability of the hapten is an important factor. Hill et al. [17] reported the difficulties of keeping unaltered the furan moiety of the insecticide bioresmethrin while preparing the hapten protein conjugate (see Fig. 4). Thus, functional protection strategies may be required prior to protein conjugation. For example, the reactive primary amine of aminotriazole was protected by using a chromophore which provided simultaneous monitoring of the protein conjugation reaction [20].

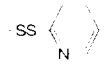
Keyhole limpet hemocyanin (KLH), tyroglobulin (TG), conalbumin (CONA), bovine serum albumin (BSA) or ovalbumin (OVA) are the carrier proteins most frequently used as either immunogens or coating Ags. However, in recent years the use has also been described of other materials such as synthetic polymers or immunoglobulins. As competitors, enzymes are the most commonly used labels in IA, but a variety of chemiluminescent or fluorescent labels is also available and increas-

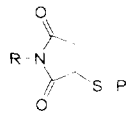
Table 1
Principal strategies for preparing protein conjugates

Protein **P** (reactive groups)	Hapten **R** (reactive groups)	Bond type
P–NH_2 (lysine and **N**-amino terminal)	**R**–COOH (**N**HS esters or mixed anhydrides)	**R**–CO–**N**H–**P**
	R–N=C=S	**R**–**N**HCS–**N**H–**P**
	R–CHO	**R**–CH_2–**N**H–**P**[a]
	R–SO_2Cl	**R**–SO_2–**N**H–**P**
	R–CH_2–halogen	**R**–CH_2–**N**H–**P**
P–SH (cystine, *cysteine* and *methionine)*	**R**–$COCH_2$–halogen[b]	**R**–$COCH_2$–S–**P**
	R–maleidimide[c]	**R**–succinimide–S–**P**[d]
	R–pyridyl–disulfide[e]	**R**–S–S–**P**
	R–halogen	**R**–CH_2–S–**P**
P–COOH (glutamic and *aspartic*)	**R**–NH_2	**R**–**N**H–CO–**P**
	R–**N**H–NH_2	**R**–**N**H–CO–**P**
P–CHO (periodate oxidation of carbohydrate residues)	**R**–NH_2	**R**–**N**H–CH_2–**P**[a]
	R–**N**H–NH_2	**R**–**N**H–CH_2–**P**[a]
P–OH (tyrosine)	**R**–COOH	**R**–CO–O–**P**[f]
P–Ar (tyrosine)	**R**–N_2	**R**–NH–Ar–**P**

[a]After $NaBH_3CN$ imine reduction.
[b]Halogen is usually iodine.
[c]Maleidimide:

[d] 

[e]Pyridyl disulfide: 

[f]Unstable bond. 

ingly being used [32]. This is specially the case when optical transducer elements are used such as evanescent wave (EW) or surface plasmon resonance (SPR) systems.

In this context one should note the effect of the resulting hapten–protein ratio in the conjugates. Usually it is considered that a high ratio of immunogens increases the strength and specificity of the immune response, but for competitors (an enzyme, protein or derivatized solid surface) a moderate value is more desirable, favouring the sensitivity of the resulting competitive IA. Theoretically, when the amount of hapten in the competitor is limited there is a requirement for less analyte in solution to compete for the specific Ab. There is also a risk that a high degree of substitution could affect the activity of enzymes or antibodies when these are the labelled immunoreagents. A correlation study on the influence of the hapten density of the competitor on the ELISA sensitivity for sulfamerazine has been reported recently [33]. Verification of the coupling reaction and determination of the hapten density can be accomplished mainly by using spectrophotometry [30,31,34], electrophoresis [35], radiolabelled haptens [36], or trinitrobenzenesulfonic acid (TNBS) [34] to evaluate free lysine residues, matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) [37,38], electrospray ionization mass spectrometry (ESI-MS) [36] or by studying the amino acid composition [39] of the protein conjugates. All these methods have advantages and disadvantages, but their applicability is often limited when KLH is the carrier molecule, because of its high molecular size.

7. Immunization protocols

For obtaining antibodies, essentially any vertebrate can be used. Sheep, goats and cows offer the

possibility of obtaining larger amounts of Ab, but rabbits are widely used because they are easy to care for and produce moderate amounts of serum. The obtention of polyclonal antibodies is relatively simple but monoclonal antibodies offer the advantage of containing a defined Ab type and, being produced by established cell lines, yielding unlimited quantities of antibodies as long as the hybridoma line is stable. However, for both research and commercial purposes, polyclonal antibodies are often sufficient, and reduce the cost of the research. Nevertheless, the quality of the antibodies (either polyclonal or monoclonal) depends mainly on the animal's immune system, the immunogen, and the immunization schedule or protocol used. A number of immunization procedures has been described but, because of animal variability and the diversity of immunogens used, no conclusion can be drawn on which are the more efficient methods. A normal protocol we use in our laboratories involves injecting intradermally into the back of the animal 10 to 20 spots of the immunogen (approximately 100 μg) mixed with the adjuvant (commonly complete Freund) to enhance the immune response. Booster injections (incomplete Freund) are then administered to the animal every four or five weeks. After each boost serum samples are collected to monitor the Ab titre. The booster injections continue (usually for three to six months, although longer times are also possible) until no increase in the Ab titre is observed. Additional information on immunizations and different adjuvants has been published [39]. Antibodies can be used as serum or ascites fluid, or well purified. The purification methods most commonly used include ammonium sulfate purification and affinity purification. Once the affinity of the resulting sera or ascites fluid for the target analyte has been proved by titration experiments, the purified antisera can be used directly for the preparation of immunoaffinity columns or direct immunosensors (see Fig. 1). However, for the competitive configurations needed in IA and for some immunosensors, additional parameters have to be optimized.

8. Screening of the sera and competitive assays

Because of animal variability at least three animals should be employed for every immunizing hapten prepared. The difficulties mentioned above of predicting which will be the best hapten to raise antibodies mean that often the research will end with many different sera (6 to 12), each of which need to be screened for its Ab titre in the presence of a collection of different possible competitors (i.e, three haptens each coupled to two proteins and/or two enzymes, a total 12 competitors). Usually two-dimensional titration experiments are performed with each of the Ab–competitor combinations. Eight concentrations of the Ab are screened simultaneously in front of several concentrations of the competitor (e.g., 12) to complete the usual 8×12 configuration of the microtitre plates. These experiments allow one to determine roughly the affinity of the Ab versus the competitors and to estimate the concentration range of the immunoreactives for future competitive experiments. A key factor in the experimental design is that the investigator should be able to select the most promising combinations and eliminate the least promising with minimum work.

In the next stage, the combinations which give the better titres are subject to evaluation of their performance under competitive configurations. The aim of these experiments is to estimate the capability of the analyte to displace the competitor on binding the Ab. It is not unusual for the analyte to show low efficiency in inhibiting the binding of the competitor to a serum having high titres for the corresponding protein conjugates. In this stage of the IA development, the scientist will evaluate the advantages of designing other possible immunizing haptens or better competitors if the existing results are not completely satisfactory (see Fig. 1).

9. Optimization of the immunoassay

One of the most promising combinations of Ab–competitor is usually chosen for further optimization. The performance of the immunoassay can be improved by varying several factors such as the following:

- *The concentrations of the sera and competitor can be adjusted.* Usually an increase in the dilution factor leads to an improvement of the sensitivity, although the signal may be reduced.
- *The format* chosen has an strong influence on the sensitivity and specificity of the assay. Indirect immunoassay configurations have been shown to be more sensitive in detecting metolachlor in soil samples [25] and superior in terms of precision and accuracy for detecting picloram [40]

whilst also reducing the effects of the matrix. In contrast, Skerritt et al. found the direct assay to be more sensitive in detecting permethrin [18]. One advantage of direct assays is that the analyses are carried out in a shorter time by reducing the number of steps necessary for the assay. Immunoassays based on magnetic particles have been reported to have faster reaction kinetics while working under homogeneous conditions —e.g., for triasulfuron [24] and triazines [41], etc.). Schneider and Hammock [15] evaluated three different microplate ELISA formats using monoclonal antibodies to *s*-triazine herbicides and reported the benefits of using Ab orientating reagents such as protein A or anti-IgG.

- *The establishment of incubation times, pre-incubation steps, sequential or simultaneous addition of reactants, etc.* In one of our assays the sensitivity was improved by a factor of four by pre-incubating the analyte on the Ab-coated plate for about 30 min prior to addition of the enzyme tracer and additional incubation of the whole mixture for 10 min more [42]. This strategy is often used to give an advantage to the analyte versus the enzyme tracer while reacting with the Ab.
- *The use of blocking agents*, if necessary, to reduce background noise
- *The effect of the ionic strength and pH of the buffers.* Ions such as calcium have been shown on some occasions to reduce variability among the samples.
- *Selection of the label.* As well as enzymes, many different labels have been reported and reviewed recently for their application to immunoassay (i.e., [43]). To enhance detection in most fluoro-immunoassays to pesticides, a fluorophore is generated enzymatically rather than being used them as a label. The use of fluorescent lanthanide labels [i.e., Eu(III), Tb(III), or Sm(III) chelates] has allowed the development of multi-analyte immunoassay devices by detecting the individual labels in time-resolved assays at different emission wavelengths. However, few examples have been reported of this method's application to environmental analysis, apart from recent detection of triazines [44]. Other perspectives include the use of organometallic markers such as $Cr(CO)_3$, $Mn(CO)_3$, $Co_2(CO)_6$ and their detection by Fourier transform infrared spectrometry (FT-IR). As a consequence of the differences in their spectral profiles, this kind of labels could also allow the development of multi-analyte procedures [45,46].

10. Characterization of the assay and validation

Immunochemical technologies offer many practical advantages. However, their acceptance is dependent on the demonstration of their quality and validity in comparison with more traditional methods. Although some agencies are now working on establishing adequate guidelines for the consistent validation of immunochemical environmental methods [47,48], no final regulation has yet been proposed. In this section we will focus on some aspects we consider to be fundamental at this point in immunoassay development.

The optimized competitive IA may be characterized first by the parameters which define the standard curve, using measurements in the usual buffer system. In this context, the usual four-parameter equation frequently employed to fit sigmoid curves gives us important information: $y=(A-D)/[1+(x/C)^B]+D$, where A is the maximal absorbance; B is the slope; C is the I_{50} concentration inhibiting 50% binding of the Ab to the competitor, and D is the minimal absorbance.

Sensitivity. This is expressed as the *limit of detection* or the smallest concentration of analyte that produces a signal statistically different from zero. Often the value given from most of the reported immunoassays refers to the dose that inhibits 20% or 10% of the binding of the Ab to the competitor.

Precision. The precision of the assay gives information regarding the agreement between replicate analyses. Since the response of IAs is non-linear and the variance is non-uniform it would be desirable to establish the precision profiles by calculating the standard deviation, or the percent of the coefficient of variation (%CV) versus concentration. This would show the interval of concentrations where precision is maximum (usually given as 20% to 80% inhibition of the maximum absorbance). Finally, it is interesting to know the standard deviations within runs in different bands of the measurement range (i.e., doses inhibiting 20, 50 and 80% of the maximum absorbance).

Reproducibility. The reproducibility is the ability of the technique to give the same result between and within analyses and laboratories.

Accuracy. The accuracy of the assay reflects its capability to measure true values for an analyte. An easy test consists in preparing several dilutions of the standard sample and measuring them. A plot of the observed concentration of analyte(s) versus the expected concentration should be linear and have a gradient of unity. Another test is to calculate recoveries by spiking real samples with known amounts of the target analyte. Evaluation will be completed by comparing results obtained using other analytical techniques and establishing correlations between the methods over a broad range of concentrations.

Specificity. This may be established by assaying a number of structurally related analytes and determining their cross-reactivity in the assay, usually as a percentage of the response given by the target analyte by the I_{50}. These studies will help to determine which other analytes may interfere in the assay or give false positives, or also whether degradation products are still being detected.

Matrix effects. Studies of matrix effects are usually carried out by preparing standard curves on the matrix and comparing their parallelism with the curve prepared using a usual IA buffer (e.g., PBS). A shift to the right (lost of sensitivity) or to the left (increase of the sensitivity) whilst keeping parallelism may indicate the need of performing measurements by preparing the standard curve on the matrix. An alternative is to add modifiers to the assay buffer (i.e., changing ionic strength, adding proteins etc.) in order to mimic the effect of the matrix. These kinds of studies are unique for every Ab system. Whenever a matrix effect is affecting the reaction analyte–Ab giving non parallel curves, the sample preparation methods must be developed. In this context, analytical chemists must consider the small amount of sample needed and the preference for using highly-volatile or water-miscible solvents, since immunoassays are predominantly run in aqueous media. Organic solvents usually used in IA are ethanol, dimethyl sulfoxide, acetonitrile, tetrahydrofuran, dioxane, methanol or propylene glycol, although the resistance of the assay should be assayed. Tolerances up to around 10% are often reported (e.g., [3,15,22], etc.), although occasionally this may be increased without the assay slope and I_{50} been affected [14]. Recently a hexane-compatible ELISA method has been developed to analyze directly for parathion in extracts by using the specific antibodies encapsulated in reverse micelles, although the sensitivity did not reach the same values as when the assay was performed in aqueous media [49].

11. Conclusions

Despite their high cost and the long process involved in their development, immunochemical techniques may soon have a broad applicability in the environmental field. We have described in this paper the factors involved in Ab obtention and competitive immunoassay development. It is evident that Ab production is the key step of any immunochemical technique. We have emphasized how hapten design largely determines the success of the final assay. Once appropriate chemical structures have been proposed, the synthesis of the haptens constitutes one of the more arduous steps in immunoassay development for environmental applications. In this context, chemical companies and/or pesticide producers are valuable sources of chemical compounds structurally related to the target analytes. These compounds may be easily derivatized and used as potential immunizing haptens or competitors, thus reducing the cost of long synthetic pathways in the early steps of the assay development. Chemical companies could thus be valuable collaborators in developing immunochemical techniques. However, for this interaction to be fruitful immunoassay development should be initiated early in the evaluation of a chemical product.

It is likely that recombinant DNA technology will affect the development of immunoassays in many ways. At present, the development of recombinant antibodies is very expensive, time consuming and risky. However, as the techniques become better established it should become much less expensive than the development of monoclonal antibodies and may rival polyclonal antibodies for low cost. Recombinant DNA technology will probably supplement but not replace polyclonal antibodies in immunoassays. One field that is developing rapidly, but clearly cannot compete with classical Ab production, includes the development of antibodies in microbes by screening recombinatorial systems analogous to panning for gold. The strategy is to use a hapten to select a binding molecule somehow associated with its gene. This then allows the production of a binding molecule without the use of vertebrate animals. This technology is being developed using antibodies for therapeutic purposes. However, other pro-

teins can be used for generation of binding proteins by screening. There are also other biotechnologies which are likely to affect immunoassay. For example, when one clones and expresses genes for poly- or monoclonal antibodies many advantages can be realized. An expression system allowing the production of high levels of antibodies could facilitate work in affinity chromatography or even in environmental clean-up. Once cloned the non-binding portions of antibodies could be modified to improve biosensors or other detection systems by designing attachment sites or fusing the Ab to another biological molecule. As our ability increases of using computer modelling to predict Ab structures, we may be able to improve the affinity or vary the selectivity of the Ab itself. Antibodies have proved to be exceptionally difficult molecules to handle using recombinant technologies. However, techniques for cloning and engineering antibodies and other binding molecules are evolving rapidly and will have a major beneficial input for the immunoassay field.

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References

[1] M.-P. Marco, S.J. Gee and B.D. Hammock, Trends in Anal. Chem., 14 (1995) in press.
[2] M.-P. Marco, S.J. Gee, H.M. Cheng, Z.Y. Liang and B.D. Hammock, J. Agric. Food Chem., 41 (1993) 423.
[3] P.M. Krämer, M.-P. Marco and B.D. Hammock, J. Agric. Food Chem., 42 (1994) 934.
[4] Q. Li, M. Zhao, S.J. Gee, M. Kurth, J.N. Seiber and B.D. Hammock, J. Agric. Food Chem., 39 (1991) 1685.
[5] M.-P. Marco, B.D. Hammock and M.J. Kurth, J. Org. Chem., 58 (1993) 7548.
[6] M.-P. Marco, M. Nasiri, M.J. Kurth and B.D. Hammock, Chem. Res. Toxicol., 6 (1993) 284.
[7] R.P. Vallejo, E.R. Bogus and R.O. Mumma, J. Agric. Food Chem., 30 (1982) 572.
[8] R.O. Harrison, M.H. Goodrow, S.J. Gee and B.D. Hammocks, in M. Vanderlaan, L. H. Stanker, B.E. Watkins and D.W. Roberts (Editors), Immunoassays for Trace Chemical Analysis: Monitoring Toxic Chemicals in Humans, Food, and the Environment, American Chemical Society, Washington, DC, 1991, p. 14.
[9] M.H. Goodrow, R.O. Harrison and B.D. Hammock, J. Agric. Food Chem., 38 (1990) 990.
[10] R.O. Harrison, M.H. Goodrow and B.D. Hammock, J. Agric. Food Chem., 39 (1991) 122.
[11] M.H. Goodrow, J.R. Sanborn, D.W. Stoutamire, S. J. Gee, and B.D. Hammock, in J.O. Nelson, A.E. Karu and R.B. Wong (Editors), Immunoanalysis of Agrochemicals. Emerging Technologies, American Chemical Society, Washington, DC, 1995, p. 119.
[12] L.H. Stanker, C. Bigbee, J.V. Emon, B. Watkins, R.H. Jense, C. Morris and M. Vanderlaan, J. Agric. Food Chem., 37 (1989) 834.
[13] F. Szurdoki, H.K.M. Bekheit, M.-P. Marco, M.H. Goodrow and B. D. Hammock, J. Agric. Food Chem., 40 (1992) 1459.
[14] P. Schneider, M.H. Goodrow, S.J. Gee and B.D. Hammock, J. Agric. Food Chem., 42 (1994) 413.
[15] P. Schneider and B.D. Hammock, J. Agric. Food Chem., 40 (1992) 525.
[16] D.P. McAdam, A.S. Hill, H.L. Beasley and J.H. Skerrit, J. Agric. Food Chem., 40 (1992) 1466.
[17] A.S. Hill, D.P. McAdam, S.L. Edward and J.H. Skerritt, J. Agric. Food Chem., 41 (1993) 2011.
[18] J.H. Skerritt, A.S. Hill, D.P. McAdam and L.H. Stanker, J. Agric. Food Chem., 40 (1992) 1287.
[19] A.S. Hill, H.L. Beasley, D.P. McAdam and J.H. Skerritt, J. Agric. Food Chem., 40 (1992) 1471.
[20] F. Jung, A. Szekacs, Q. Li and B.D. Hammock, J. Agric. Food Chem., 39 (1991) 129.
[21] M.-P. Marco, G. Fabriàs and F. Camps, Arch. Insect Biochem. Physiol., 1995, in press.
[22] S.J. Gee, T. Miyamoto, M.H. Goodrow, D. Buster and B.D. Hammock, J. Agric. Food Chem., 36 (1988) 863.
[23] J. Schlaeppi, W. Meyer and K.A. Ramsteiner, J. Agric. Food Chem., 40 (1992) 1093.
[24] J.-M.A. Schlaeppi, A. Kessler and W. Fory, J. Agric. Food Chem., 42 (1994) 1914.
[25] J. Schlaeppi, H. Moser and K. Ramsteiner, J. Agric. Food Chem., 39 (1991) 1533.
[26] S.I. Wie and B.D. Hammock, J. Agric. Food Chem., 32(6) (1984) 1294.
[27] E.H. Gendloff, W.L. Casale, B.P. Ram, J.H. Tai, J.J. Pestka and L.P. Hart, J. Immunol. Methods, 92 (1986) 15.
[28] J.J. Manclus, J. Primo and A. Montoya, J. Agric. Food Chem., 42 (1994) 1257.
[29] R.E. Carlson, in J.O. Nelson, A.E. Karu and R. B. Wong (Editors), Immunoanalysis of

Agrochemicals. Emerging Technologies, American Chemical Society, Washington, DC, 1995, p. 140.
[30] S.S. Wong, Chemistry of Protein Conjugation and Cross-linking, CRC Press, Boca Raton, FL, 1991.
[31] M. Brinkley, Bioconjugate Chem., 3 (1992) 2.
[32] A. Mayer and S. Neuenhofer, Angew. Chem. Int. Ed. Engl., 33 (1994) 1044.
[33] S.W. Garden and P. Sporns, J. Agric. Food Chem., 42 (1994) 1379.
[34] R.B. Sashidhar, A.K. Capoor and D.J. Raman, J. Immunol. Methods, 167 (1994) 121.
[35] C. Kamps-Holtzapple, R.J. Carlin, C. Sheffield, L. Kubena, L. Stanker and J.R. Deloach, J. Immunol.Methods, 164 (1993) 245.
[36] K.M. Straub and M.J. Levy, Bioconj. Chem., 5 (1994) 194.
[37] M.M. Seigel, H.-R. Tsuo, B. Lin, I.J. Hollander, A. Wissner, M. Karas, A. Ingendoh and F. Hillenkamp, Biol. Mass Spectrometry, 22 (1993) 369.
[38] J.P. Briand, S. Muller and M.H.V. Van Regenmorte, J. Immunol. Methods, 93 (1985) 9.
[39] M.H.V. Van Regnmortel, J.P. Briand, S. Muller and S. Plaue, Synthetic Polypeptides as Antigens, Elsevier, Amsterdam, 1988.
[40] R.J.A. Deschamps and J.C. Hall, Food Agric. Immunol., 3 (1991) 135.
[41] F.M. Rubio, J.A. Itak, A.M. Scutellaro, M.Y. Selisker and D.P. Herzog, Food Agric. Immunol., 3 (1991) 113.
[42] F. Iglesias, G. Fabriàs and M.-P. Marco, unpublished results.
[43] A. Mayer and S. Neuenhofer, Angew. Chem. Int. Ed. Engl., 33 (1994) 1044.
[44] M. Wortberg, K. Cammann, K. Strupat and F. Hillenkamp, Fresenius J. Anal. Chem., 348 (1994) 240.
[45] G. Jaquen, A. Vessieres and I.S. Butler, Acc. Chem. Res., 26 (1993) 361.
[46] M. Salmain, A. Vessieres, P. Brossier and G. Jaouen, Anal. Biochem., 208 (1993) 117.
[47] C. A. Mihaliak and S.A. Berberich, in J.O. Nelson, A.E. Karu and R.B. Wong (Editors), Immunoanalysis of Agrochemicals. Emerging Technologies, American Chemical Society, Washington, DC, 1995, p. 288.
[48] J. Rittenburg and J. Dautlick in in J.O. Nelson, A.E. Karu and R.B. Wong (Editors), Immunoanalysis of Agrochemicals. Emerging Technologies, American Chemical Society, Washington, DC, 1995, p. 301.
[49] J.M. Francis and D.H. Craston, Analyst, 119 (1994) 1801.

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book reviews

Charge transfer devices in spectroscopy

Charge Transfer Devices in Spectroscopy, edited by Jonathan V. Sweedler, Kenneth L. Ratzlaff and M. Bonner Denton, VCH, New York, 406 pp., 1994, US$ 95, ISBN 1-56081-060-2

This is the first comprehensive book with detailed information on the operation of charge coupled devices (CCD) and charge injection devices (CID) which addresses their utilization in spectroscopic applications. The book brings contributions from twelve different experts in the field of charge transfer detectors (CTD). The editors were pioneers in the application of charge transfer devices to the field of spectroscopy. The book reflects the years of experience that each contributor has in the selection, utilization and application of the appropriate CTD to the various fields of analytical spectroscopy. While the book, at first, may be difficult for those unfamiliar with solid-state silicon detectors, many chapters repeat the key concepts and present them in different ways. This repetition ultimately enables even a scientist unfamiliar with the topic to understand and use these devices.

The book serves as a resource for a thorough understanding of the fabrication and fundamental operational principles of CCD and CID detectors. The different architectures of CCDs are clearly explained with guidance to selection of the appropriate architecture for spectroscopic applications. Charge storage principles; noise sources; specialized read-out modes; inherent silicon device properties; and the effects of polysilicon gates on UV responsivity are presented with a minimum of the often confusing device physics and associated mathematics. The authors have distilled and succinctly translated the minimal and often confusing information available