Anal Bioanal Chem (2007) 387:205–218
DOI 10.1007/s00216-006-0849-8

REVIEW

Optical immunosensors for environmental monitoring: How far have we come?

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Received: 26 June 2006 / Revised: 6 September 2006 / Accepted: 8 September 2006 / Published online: 27 October 2006

Abstract Immunosensing has proved to be a very interesting research area. This review discusses what has actually been achieved in the field of optical immunosensing for environmental screening, and what still needs to be done. The review is presented from a practical point of view. In terms of the basic design of the immunosensor, there is a trend towards decreasing assay time; indeed, this has been reduced from 15–20 minutes to less than 5 minutes. Another goal is to simplify the manifold, and label-free approaches combining indirect assay formats and the detection of antibody binding are popular. Rapid displacement assays have also been investigated thoroughly. In terms of some important features of immunosensing devices, the reusability of the sensing element has been studied in great depth, and working lifetimes of more than five hundred assays can now be found for all assay formats. Multianalyte assays are now being investigated, and current systems are able to monitor 2–3 target compounds, although this number is set to increase greatly (to >30) in the near future. In this sense, an increasing number of publications can be found on microarrays intended for multianalyte determinations. The application of immunosensing to real situations is the main challenge. Immunosensors are barely commercialized and are yet to be established as research or routine tools, due to a lack of validated protocols for a wide range of sample matrices. Regarding compounds considered as analytes, some significant pollutants such as dioxins or pharmaceuticals are rarely chosen as targets, although the current tendency is towards a broader spectrum of analytes. New immunoreagents should be raised for these compounds, for use in immunosensors that can be used as screening tools.

Keywords Immunosensor · Optical · Environment

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Introduction

Immunosensing is a very active research field. The inherent combination of the exquisite molecular recognition ability of antibodies and the philosophy of rapid, continuous, reversible and automatic analysis of chemical sensors utilized in immunosensors is very useful in many fields [1].

The popularity of immunosensing can be illustrated by a single fact: in the last five years, a large number of review and overview articles dealing directly or indirectly with immunosensing have been published. Some of the most relevant are focused specifically on environmental applications of immunosensors [2] and immunoanalytical techniques [3, 4], while others are devoted to the applications of various biosensors and related techniques to the environment [5–7], food [8] or to other fields [9].

Immunosensors have been around for over 15 years, but just how robust are current sensors? How far have we come in immunosensing? The main goal of this paper is to discuss the actual performances of current environmental immunosensors, in terms of the practical aspects of immunosensor design and their applications. Attention is paid to optical immunosensors, because transducers based on optical properties are very popular in environmental immunochemistry due to the choice available and the versatility of optical transduction enhanced by fiber optic technology.

As a starting point for this discussion, it is important that we define the goals to be reached in this field (in other

words, what an ideal immunosensor would do), bearing in mind that an immunosensor is developed to solve a concrete analytical problem or to open up new analytical pathways. Therefore immunosensors are developed in order to carry out analyses that are currently difficult to perform using other sensing methods. In this sense, immunosensors should be applied when:

- a high number of samples must be screened
- on-line control is necessary
- analysis is to be carried out in the field
- different analytes must be determined in a sample by different methods
- data should be presented within minutes or in real time
- samples need to be analyzed directly with no or hardly any pretreatment
- traditional methods do not work properly for the system under examination.

Thus, immunosensors have been targeted at fields such as process control, food safety and environmental monitoring.

With all of this in mind, the features of an "ideal" immunosensor can be described as follows:

- In general, the sensitivity of the immunosensor should be as high as possible, since this would allow the matrix to be diluted if necessary. When dealing with pollution monitoring, regulatory laws are becoming more and more strict regarding residue limits, so the analytical methods used to monitor for compliance to these laws need to be highly sensitive. In many cases, the working range should be below 1 μg/L.
- Selectivity should be sufficient that immunosensor can be applied to determine a single compound with minimal cross-reactivity, or that it can be applied to determine a whole family of related compounds with generic immunoreagents. Both of these approaches find real-world application.
- Rapidity is a parameter whose importance depends on the final application. For pollution monitoring, particularly in control programs or alarm stations, and especially when a large number of samples needs to be processed, the assay time should be as short as possible, yielding results almost in real time. However, the sensitivity and other analytical properties should not be compromised by the speed of the assay. An assay time of five minutes is a realistic value, taking into account the operations carried out in the assay protocols and the kinetics of analyte–antibody interactions and dissociation. In many applications, longer assay times might also be fine.
- The immunosensor should also be as reusable as possible, to ensure that the device can work continuously for very long periods while maintaining accuracy. In any case, the sensor should be reusable for a minimum of one hundred cycles in order to permit calibration, recalibration and the measurement of an acceptable number of samples to be achieved.
- For most multiparameter determinations, the device should manage 5–10 target compounds simultaneously. A higher number would be desirable in some applications, and this could be accomplished with an immunosurface array arrangement or by using different devices working in parallel. Also, the sensor should be versatile, in the sense of being able to process a new analyte immediately, provided that reagents are available.
- Finally, it should be robust and able to work properly under different conditions.

Another aspect that needs to be taken into account is that immunosensor development requires suitable immunoreagents for the analytes of interest, and the quality of reagents will have a great influence on the analytical properties of the final system. However, the availability of the immunoreagents does not ensure success in the creation of an immunosensor in terms of some performance requirements, because the principles of immunosensing are sometimes quite different from those of batch immunoassays [1].

If the two general immunochemical methodologies—batch immunoassays and immunosensing—are compared, it is found that the basic analytical properties of sensitivity and selectivity are nearly the same for both immunoassays and immunosensors, since they depend mainly on the immunoreagents employed. However, the sampling capacity of microplate assays is very high, because several hundreds of samples can be processed simultaneously. Immunosensors are designed to display the analytical signal after a short period of time, and they can work with complete automation and autonomy, while automation of batch immunoassays is possible but expensive. In this sense, batch immunoassays can solve analytical problems that require a high number of determinations, while immunosensing is the better choice when automation and rapidity results are needed.

Immunosensor design

Many of the parameters that define the quality of an immunosensor depend on the design of the system. Basically, when designing an immunosensor, four general aspects need to be considered: the mode of operation, the immunosupport, the assay format, and the signal transduction.

Mode of operation

Nearly all optical immunosensors work under flow conditions. Hydrostatic systems are employed mainly in initial studies of new optical detection modes, for instance atomic force spectroscopy [10], imaging ellipsometry [11] or terahertz wave technology [12]. In the works mentioned here, only the generation of a signal related to a binding event involving antigen–antibody or other biological interactions is described. In the case of imaging ellipsometry, a recent application can be found in the immunosensing of *Salmonella typhimurium* [13], where a monoclonal antibody immobilized by means of protein G layer bound to a self-assembled monolayer (SAM) of 11-mercaptoundecanoic acid (11-MUA) on a gold surface is employed, with a lower detection limit of 103 CFU/mL.

When the main goal of a work is to develop and apply a whole immunoanalytical sensing device, and the work is at an advanced stage, it is performed in hydrodynamic mode. Flow systems offer versatility, ease of management and automation [14], so this option is preferred in all cases. Flow management is usually carried out by a computer-controlled continuous or sequential injection system, which is very versatile and easy to use.

Immunosurface

Traditional sorbents are currently employed as immobilization supports. For instance, polymer-derivatized silicon [15] is employed for the immobilization of capture proteins in a reversible immunosensor for atrazine that possesses very high sensitivity (LOD 6 ng L^{-1}), while agarose gel [16] is derivatized with trinitrobenzene sulfonic acid in a reversed-displacement immunosensor for TNT that is able to measure this compound when present at levels higher than 2.5 μg L^{-1}. The sensitivity can improved by employing the same format and reagents but using silanized glass as support [17], which decreases the LOD down to 0.25 μg L^{-1}.

However, new immobilization supports and methods that comply (or not) to the requirements of the new detection methodologies have been researched. As an example of sorbent development, a poly(glycidyl methacrylate-co-trimethylolpropane trimethacrylate) polymer disk [18] has been used for protein G immobilization, resulting in a capture assay for atrazine with improved sensitivity (the LOD drops from 0.2 to 0.03 μg L^{-1}) and selectivity (absence of matrix effect when analyzing surface water) when compared with a commercial protein G support.

Regarding immobilization methods, both classical and novel procedures can be found. In the former, direct physical adsorption has been employed, mainly on gold supports, for surface plasmon resonance (SPR) detection. The main advantages of this immobilization mode are its rapidity and simplicity. An example can be found in the determination of 2,4,6-trinitrophenol [19] with an indirect competitive immunosensor based on SPR detection, which is able to work in the range from 0.01 to 100 μg L^{-1}.

On the other hand, traditional methodologies for covalent binding have also been widely used, because covalent links are stable and there are well-established methodologies for anchoring antibodies and antigens onto a wide range of supports. One of the most popular classical immobilization methods, which can be used to attach a hapten to glass [17] or to immobilize an antibody or a hapten–BSA conjugate on a waveguide surface [20], is aminosilanization followed by glutaraldehyde linking. It should be noted that in this latter work [20] the combination of an indirect competitive format with the nonlabel detection of the antibody binding by means of optical waveguide lightmode spectroscopic detection produces an extremely sensitive immunosensor for the herbicide trifluralin, with an analyte concentration range from 2×10^{-7} to 3×10^{-5} μg L^{-1} in distilled water. The main disadvantages of this immobilization methodology are the need to block the remaining binding sites, and the inability to make use of oriented immobilization for antibodies.

New immobilization methodologies have also been investigated and applied beyond classical anchorage techniques. One approach that has proved popular recently is to use biological interactions. This approach makes use of the enhanced selectivity of biomolecular recognition. As an example, a combination of protein A antibody recognition and further covalent cross-linking with dimethylpimelimidate has been exploited to attach anti-paclitaxel antibodies to a glass column [21] in a displacement immunosensor for this analyte; this approach can yield a LOD of 1 μg L^{-1}.

Finally, immobilization based on the entrapment of antibodies in a sol-gel matrix has also been applied [22] in a highly reusable direct competitive immunosensor for isoproturon, which is able to measure this herbicide when present at 2–200 μg L^{-1}, based on a fluorescent tracer. This system has recently been improved by incorporating an on-line immunoaffinity preconcentration column containing the same antibody entrapped in sol-gel glass, and placing the immunosensing element inside the fluorometer flow cell [23]. The limit of detection reached with the aid of on-line preconcentration was 9.7 ng L^{-1}.

Assay format

Assay formats have evolved over the last few years, but only four different assay formats are employed in environmental immunosensing. The immobilized antibody format (Fig. 1a), also known as the competitive direct format, is not very popular these days, and very few works have been

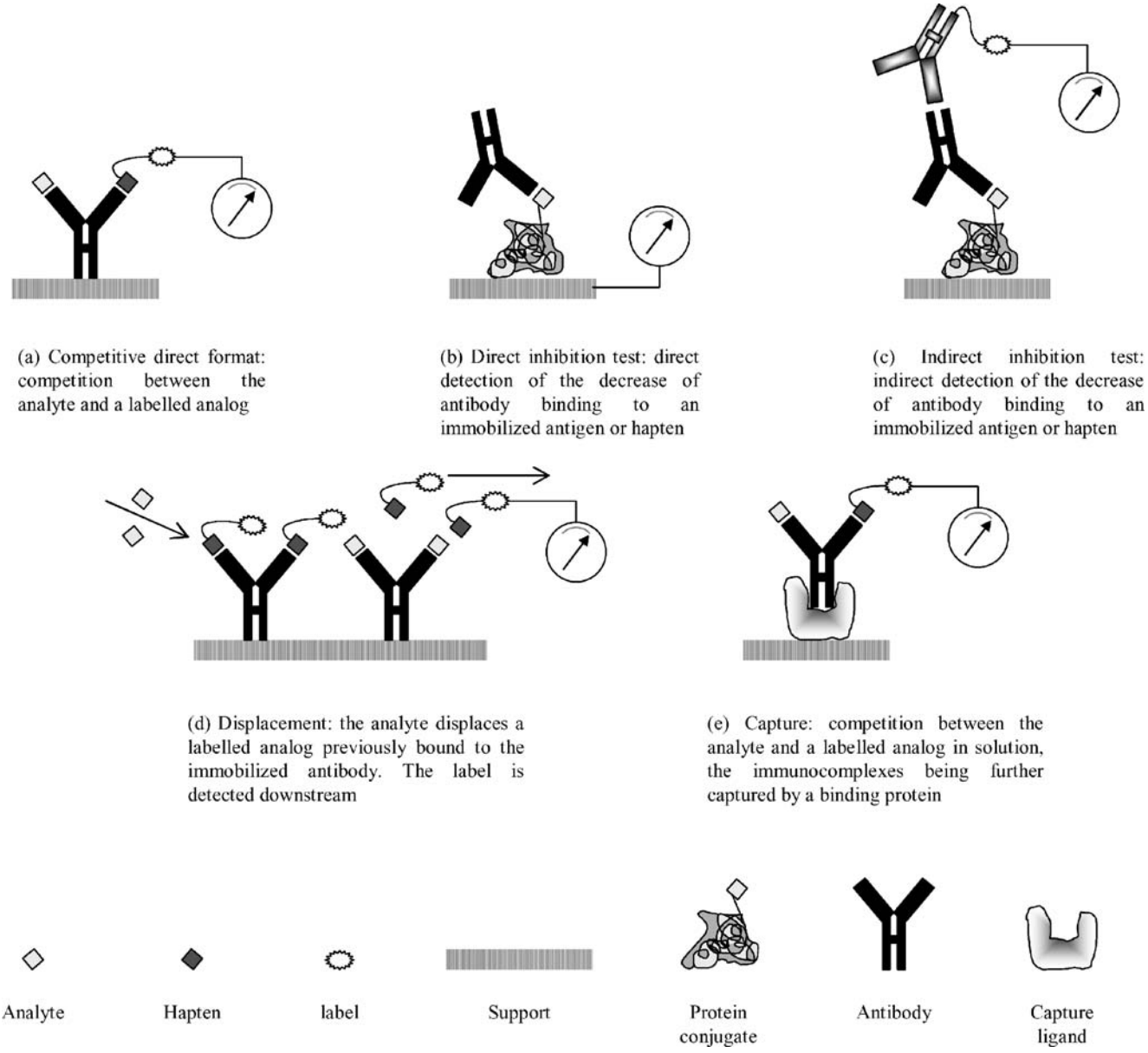

Fig. 1 Assay formats usually employed in optical immunosensors for pollutants

published on it lately. One example is the sol-gel immunosensor for isoproturon created by Pulido-Tofiño et al. [22, 23]. In a different work [24], coplanar PCBs are determined with a very high sensitivity (LOD 0.1 ng L^{-1}) disposable microimmunosensor working under the direct format with the antibody immobilized onto polystyrene by passive adsorption.

The immobilized conjugate format or inhibition test (Fig. 1b and c), previously known as the competitive indirect format, has been employed in more than 60% of the recent works due to its high versatility and the ability to work without labels (Fig. 1b). This option is very popular because all of the problems associated with labelling are avoided, and because the assay time is generally shorter.

An example of label-based indirect immunosensor is described for the determination of atrazine, estrone and isoproturon in river water [25, 26], based on total internal reflection fluorescence (TIRF) detection of Cy5.5 marker and the covalent immobilization of analyte–dextrane derivatives onto a transducer glass slide. LODs lower than 0.2 μg L^{-1} are achieved in all cases.

On the other hand, there are many examples of label-free indirect immunosensing systems that have been developed over the last few years, nearly all of them employing SPR as the detector. For instance, an immunosensor for the toxin deoxyvalenol has been developed [27] with an analyte–casein conjugate covalently immobilized onto a SPR chip sensor. The immunosensing device is applicable to an

analyte present at levels of 1 to 100 μg L^{-1}, while the working lifetime of the sensor exceeds 500 assay cycles.

The displacement immunosensor (Fig. 1d) is an interesting option that has been developed mainly by the US Naval Research laboratory [28] for the detection of explosives. Here, TNT and RDX are determined in water and soil samples [29] with three different systems: a bead-based microcolumn with a Sepharose or Emphaze AB1 support, a microporous nylon Immunodyne membrane, and a fused-silica glass microcapillary, employing antibodies that were covalently immobilized (details given) onto the supports. The sensitivities achieved for both analytes ranged from 5–50 μg L^{-1} with agarose microcolumns to 15–20 ng L^{-1} with the microcapillary. Applications of the reverse displacement format to the determination of TNT can be found in [16] and [17], where LODs of 2.5 and 0.25 μg L^{-1} were obtained, respectively.

Another alternative is to use the format based on the reversible capture of antibodies by immobilized protein A or G. However, only a few systems have been developed based on this approach. An example is found in [15] for the determination of atrazine, which has a sensitivity as high as LOD 6 ng/L and the ability to be regenerated for over eight months. In another instance, the antibody was selected from a pool of three in a capture immunosensor for atrazine [30]. LODs as low as 7 ng L^{-1} could be obtained, and reusability was higher than 600 assay cycles. In both cases, the analyte competes with a peroxidase-labeled analog, and peroxidase activity is measured by chemiluminometry and fluorometry, respectively.

Table 1 compares the features of the four assay formats. The table highlights the extreme sensitivity to estrone provided by the indirect format [31] when employing high-affinity antibodies immobilized on aminodextran-derivatized glass and optimizing the amount of antibody used. It is also worth noting the reusability obtained with the direct format (>1000 cycles) in a sensor for isoproturon employing antibodies entrapped in sol-gel [22]. The displacement format shows the shortest assay time in a sensor for TNT [17], due to the properties of this format. Finally, the capture format, when employed in sensors for atrazine, shows good features in terms of sensitivity and reusability [43]. The weakest property of this format is its long assay time.

Signal transduction

While classical detection techniques such as photometry and fluorometry are still used for immunosensors, other techniques such as total internal reflection fluorescence (TIRF) and SPR have also been developed and implemented. The advantages of the former are their robustness, availability, versatility, low cost, and their portability, which allows them to be used in the field, while their main drawback is the need for label. Important works using conventional fluorescence detection include those describing direct immunosensors for isoproturon [22, 23] or displacement immunosensors for explosives [28], while chemiluminescence has been employed in capture immunosensors for atrazine [15, 18].

Total internal reflection fluorescence detectors possess important features. For instance, they are compatible with glass and silica-based immunosorbents, so there is the potential for reagent immobilization, although most recent works employ immobilization via an aminodextran layer [25, 26, 31]. On the other hand, fluorometry is a very sensitive technique, and the detection of minute amounts of fluorescent analytes or labels is possible using it. Also, interferences caused by intrinsic fluorescent matrix components are minimized due to the fact that the excitation area is limited to the evanescent wave zone. Multiparametric array determination is also possible [25, 26], and this technique has been shown to be versatile and easy to apply in portable devices [32]. However, TIRF requires the use of fluorescent labels.

SPR has become popular in the last few years, not only for immunosensors but also for other biosensing applications, as reviewed in [33]. The main benefit of this technique is the ability to develop label-free sensors. In the past, the drawbacks to SPR were the large size and cost of the apparatus, although these drawbacks have recently become less important because compact and cheaper devices have been made available. A recent application of miniaturized portable SPR is described by Kim et al. [34] in the determination of 2-hydroxybiphenyl via an indirect immunosensor employing a BSA–hapten conjugate immobilized onto the sensor surface using physical adsorption and a monoclonal antibody. This system yields an LOD of 0.1 μg L^{-1} and a reusability of 30 assay cycles.

Table 1 Best performances achieved with each assay format used in immunosensing

Assay format	Analyte	Label	Detection system	LOD (ng L^{-1})	Sensor life (cycles)	Assay time (min)	Ref.
Direct	Isoproturon	Fluorescein	Fluorometry	9.7	1000	5	[23]
Indirect	Estrone	Cy5.5	TIRF	0.2	400	12	[31]
Displacement	TNT	Cy5	Fluorometry	250	“High”	3	[16]
Capture	Atrazine	Peroxidase	Fluorometry	7	600	20	[43]

Classical optical detection, TIRF and SPR are all established detection techniques nowadays. For a comparison between the different detectors, refer to Table 2. This shows the properties of different immunosensors for atrazine, an analyte that has been widely studied and so a great deal of data are available on it. The SPR sensor has a limit of detection of 50 ng L^{-1} [35], while the LOD for TIRF is 155 ng L^{-1} [25] and those for classic detectors are lower than these LODs [21, 43]. However, these figures are not conclusive, because both the immunoreagents and the assay formats used are not the same. On the other hand, no conclusions can be drawn about the response time either, because this parameter depends on the assay format and other features, rather than the detection system. Finally, photometry and fluorometry equipment, which have been routine analytical instruments for decades, are less expensive than novel transductor equipment.

It should be noted that in label-based immunosensing, the labels employed are the classical ones: enzymes and fluorescent dyes. In fact, enzymes are popular for classic optical detection. For instance, in the works by Yakovleva et al. [15] and Jain et al. [18], peroxidase is employed along with 4-iodophenol-enhanced chemiluminescence detection of luminol. Also, in basic studies performed by our group into multiparametric capture immunosensing [36] and comparison of labels for immunosensing [37], peroxidase and alkaline phosphatase were used. For peroxidase, a classic fluorogenic substrate, 3-(*p*-hydroxyphenyl) propanoic acid, and fluorometric detection allows the determination of three pesticides (carbaryl, atrazine, Irgarol 1051) to below 0.1 μg L^{-1}. For alkaline phosphatase, an 1,2-dioxetane derivative was shown to be an excellent luminogenic substrate, allowing the determination of carbaryl in pure organic solvent (methanol) at the μg L^{-1} level.

Fluorophores are now used more often than enzymes, because they are more stable in solution and the signal is displayed immediately, shortening the assay. There are many examples of fluorescent labeling. For instance, rhodamine [21] and fluorescein [22] conjugated to the analyte are employed for paclitaxel and isoproturon, respectively, in immunosensors based on conventional fluorescence detection. In a different approach, 2,4,6-trichlorophenol (TCP) is determined in urine with a LOD of 1.6 μg L^{-1} through the use of a homogeneous quenching laser fluorescence immunoassay performed in microdroplets [38]. The tracer employed is fluorescein–TCP conjugate, and the quenching takes place when antibody binds to the tracer.

However, the most popular dyes are Cy5 and its analogs, which are employed along with classic fluorometry in many displacement immunosensors [16, 17], and also along with novel TIRF detection. Nearly all of the recent applications of TIRF in immunosensing are used to measure Cy5 and Cy5.5. A good example, that describes the work performed by a European consortium [39, 40], is the development of a real multianalyte immunosensor applicable to the monitoring of pesticides and other pollutants such as pharmaceuticals and estrogens in water. One possibility that has hardly been exploited thus far is to use time-resolved fluorescence with rare-earth chelates used as labels. In recent work by our group [37], a terbium chelate was attached to a generic antibody in the development of an indirect immunosensor for carbaryl, 1-naphthol and Irgarol 1051, which gave a LOD for carbaryl of 0.13 μg L^{-1}.

A new generation of labels for use in biosensing is on the horizon, and research efforts are being focused on the development and applications of nanomaterials [41]. Quantum dots are perhaps the most commonly studied nanoprobes, and their application to sensing and biosensing has been reviewed recently [42]. As examples of the application of nanomaterials, Eu(III) chelate-dyed nanoparticles have been employed as antibody label in an indirect competitive fluoroimmunosensor for atrazine that uses an indium tin oxide waveguide as immobilization support, achieving a sensitivity, expressed as IC_{50}, of around 1 μg L^{-1} [43]. Also, polymer-encapsulated europium-doped gadolinium oxide nanoparticles have been employed to labeling antibodies in a microarray-arranged indirect immunosensor for phenoxybenzoic acid that employs confocal fluorescence microscopy [44]. The LOD shown is 1.4 μg L^{-1}.

It should be pointed out that the use of fluorescent markers implies the conjugation of them to an antibody or a hapten. In this sense, labeling reactions involve organic synthesis, which illustrates the difficulties associated with them, and the results obtained can be uncertain. Indeed, in a study of Tb chelate intended for use as label [37], direct coupling of the marker to a hapten was not successful, and

Table 2 Performances of the atrazine immunosensors with different detection systems

Detector	Assay format	Support	LOD (ng/L)	Assay time (min)	Reference
Fluorometry	Capture	Polymer–protein A/G	7	20	[43]
Photometry	Capture	Polymer–protein G	33	6	[18]
TIRF	Indirect	Glass	155	15	[25]
SPR	Direct	Gold	50	15	[35]

only a format based on labeled antibodies could be developed. On the other hand, fluorescent labels are displayed when bound onto the surface, and this could lead to light scattering phenomena, at was discussed in [37].

An interesting research line would be the possibility of a universal labeling methodology. This might be achieved via a molecular bridge between the label and the antibody/hapten. The bridge would be a doubly functionalized molecule that would bind the label (enzyme, dye) on one side and the binder on the other side. In this sense, commercial derivatives of fluorescent dyes that are easy to bind to haptens are being researched.

Currently, as well as the novel labels, there are new detection techniques under development: for instance microcantilevers with optical read-out [10, 45] interferometry [46, 47], and the newest, emerging nanophotonic technology for biomolecular detection [48] and other nanotechniques such as localized surface plasmon resonance spectroscopy and surface-enhanced Raman scattering [49]. Although they are still in their infancy and are far from being practical immunosensors, all of these techniques are really promising, because they can monitor many kinds of interactions without labels in an integrated, small, low-cost, disposable chip with great potential for parallel determinations. However, they still have issues related to fluidics and, in some cases, the response time. Other drawbacks are associated with sample conditioning, portability, and the cost of the devices, considering the scope of environmental applications.

Other aspects of basic immunosensor design

Another trend in environmental immunosensing involves the use of analyte-ligands that are different from conventional monoclonal or polyclonal antibodies. Immunoglobulin fragments have been employed in the past. An example is a system for the determination of the anticancer drug alpha-(difluoromethyl)ornitine [50], employing enzyme-labeled Fab fragments and a column with immobilized analyte. In the absence of analyte, fragments were captured in the column, while in the presence of analyte, the analyte binds to the fragment and the complex passes through. Currently, basic research is still being performed into applications of antibody fragments, native or recombinant, since the possibility of obtaining monovalent antibodies that also have suitable groups that enable them to be linked to a surface via a specific site is still to be exploited. A recent example is given in [51], which describes the engineering of a single-chain fragment variable antibody to contain two histidines within the linker peptide used to join the light and heavy chains. This makes it possible to attach the fragment covalently to a gold surface with the correct orientation while maintaining its activity.

A different approach to the development of bioaffinity-based analytical systems is the use of nucleic acid sequences (RNA and DNA) as molecular recognition elements. An interesting variant is the use of aptamers; that is, artificial nucleic acid ligands that can be generated against a target analyte, molecular or not. Aptamers have already been employed as recognition tools in analytical procedures, and initial applications of them have recently been reviewed [52, 53]. These ligands appear to have a promising future in environmental and other applications.

Finally, an important and rapidly maturing approach involves the use of molecularly imprinted polymers (MIPs) as biorecognition-like synthetic molecular receptors, instead of antibodies. The advantages of MIPs over real biological ligands are their cost-effective and easy preparation, the variety of formats (bead, block, film), and their higher chemical and thermal stabilities, while their main drawbacks are their lower catalytic capabilities and the heterogeneity of the binding site [54]. The development of analytical and sensing schemes based on MIPs is a popular trend at the moment, although overview articles on the application of MIPs to biosensing can be found [55]. A recent example can be found in the work by Benito-Peña et al. [56] for the determination of penicillin G with a direct competitive format employing fluorescently labeled β-lactams as competitors, where a dynamic range of 3–890 μM was achieved in 99:1 acetonitrile–water solution.

Important features of immunosensing

After discussing the basic design of the immunosensor, we now switch our attention to some important properties of immunosensors, because the final quality and actual applicability of the developed system depends directly on these.

Reusability and sensor life

The ability to regenerate the sensing element and its working lifetime are key points [57]. Some authors prefer the concept of a disposable “one-shot” immunosensor, which reduces the assay time and avoids difficulties associated with the analyte–antibody dissociation. However, this has two limitations: the immunosurface must be reproducible and calibration can be difficult. In this sense, a throwaway sensor could be of most use when a binary response (yes/no) is needed.

An example of a disposable immunosurface has been described by Soh et al. [58] for the monitoring of 2,4-dichlorophenol using a gold chip with antibody immobi-

lized on it via a gold-binding polypeptide. SPR detection of BSA, used as hapten label, was employed, and an LOD of 20 μg L^{-1} was obtained. Another is described for the sensitive determination of coplanar PCBs (LOD 0.1 ng L^{-1}) in 10.5 min via a microflow immunosensor chip [24], where the immunosorbent consists of antibody adsorbed on polystyrene beads. Finally, it is worth mentioning work by Mastichiadis et al. [59] in which a four-band disposable optical capillary immunosensor permits the simultaneous determination of mesotrione, hexaconazole, paraquat and diquat in around 35 min with LODs of 0.04, 0.06, 0.09 and 0.10 μg L^{-1}, respectively. Here protein–hapten conjugates adsorbed on poly(methylpentene) and a competitive indirect assay format are used.

On the other hand, the advantages of the disposable sensor are the drawbacks of the regenerable one. However, the regeneration of the sensing element is usually considered to be a positive aspect of an immunosensor, because it implies that immunoreagents can be saved, it ensures that the immunosurface is reproducible, and it makes automation and autonomy easier. This option is therefore preferred in general. However, the results reported in the literature on sensor reusability are somewhat varied [57]. Our experience has shown that sensor life depends on the assay format, type of support, immobilization procedure, immunoreagent properties and the dissociation procedure. Indeed, when working with carbaryl (Table 1), it was stated that the immobilized conjugate has a longer life than the immobilized antibody, and the reusability of the latter was different when different antibodies, supports, linking methods and dissociation solutions were used [60, 61]. It was concluded that the immunosensor reusability should be studied in any immunosensor development. However, the reusability of the immunosurface is not made clear in some reports. For instance, the regeneration procedure is described (injection of 50 mM H_3PO_4) in [18], as it is in [20], where regeneration is carried out by injecting 0.01 M HCl, as well as in [25], in which SDS solution at pH 1.9 is employed. However, reusability data are not given in any of these three papers.

Anyway, there are many cases in which the working lifetime of the immunosensor has been described in terms of number of adsorption–desorption cycles, as well as the storage time. Some examples of successfully resolved regenerable systems can be found in Table 2. In the direct format, entrapment on sol-gel and mild regeneration at pH 3 gives good results (>1000 cycles) [22]. In the indirect format, a treatment combining acids and organic solvents ensures the dissociation of the antibody for 400 cycles without damaging the immunosorbent [31]. The displacement format does not require regeneration after each assay, and the life of the sorbent depends on the amount of reagent immobilized [16]. In our research work [43], immunosorbent regeneration proved to be a huge problem during the first stages of basic studies into immunosensing, when assaying the direct and indirect formats. The immunocomplex capture format was developed in order to solve this problem, and it was really successful in this sense, because the sorbent could be used for more than 600 cycles.

One can even find recent works dealing mainly with the regeneration of immobilized antibodies, as in the work by Kandimalla et al. [62], which describes the effect of different dissociation solutions (such as glycine/HCl at different acidic pH values with or without organic modifiers and other regeneration agents) on the dissociation and reusability of anti-ethyl parathion antibodies immobilized on silanized glass. This work found that the optimal dissociation reagent was glycine–HCl at pH 2.3 containing 1% DMSO, which yielded a column reusability of 13–14 assay cycles.

Response time and rapidity

Another important aspect is response time. Immunosensors are supposed to be devices that generate results in real time, but there is always some delay between sample collection and data display. The shorter this delay, the better the sensor in terms of assay speed. However, the value of rapidity depends on the final application. In some cases, for instance alarm situations, the response time must be as short as possible, although in this case other analytical properties (such as sensitivity) are diminished. In other scenarios, such as continuous routine monitoring, sensitivity is more important than assay time, and a response time of 15 min or longer might well be fine.

Again some of the works in this field contain no data on assay response time—for instance the one by Székács et al. [20]. However, most reports on immunosensors do mention the total assay time and even the times at which the events happened in each assay run. In this case, the data are not as varied as for regeneration, and some general tendencies can be inferred.

Many immunosensors show a total assay time of around 15–20 minutes, and these correspond mainly to those using an immobilized antigen format assay: for instance in the sensitive (LOD<0.2 μg L^{-1}) determination of atrazine, isoproturon and estrone with TIRF detection [25], or in the SPR indirect immunosensor for benzo[*a*]pyrene and 2-hydroxybiphenyl [63] using analyte–BSA conjugates adsorbed on the SPR chip, with dynamic ranges of 0.01–300 and 0.01–1000 μg L^{-1}, respectively. It should be noted that in many instances, the time between sample introduction and signal display is lower than the total assay time, because the sensor is regenerated after the data has been presented. In other cases, the response time is higher than the published value [15], because a previous step of

analyte–antibody incubation (which could take from 5 to 30 minutes to perform) should be included, and this period is not taken into account when establishing the total assay time. However, in such approaches, the sampling rate can be improved simply by performing the different steps of the assay simultaneously, so that the data presentation step for one sample occurs while the next sample is being incubated.

In examples of sensors working with immobilized antibodies, the total assay time is shorter, five minutes in some cases [22], and this period includes the enzyme label reaction which generates the signal, as well as sensor regeneration. The most rapid immunosensors are those based on the displacement assay format. An analysis time as short as two minutes has been described for the capillary immunosensor for RDX [64], which is sensitive down to low $\mu g\ L^{-1}$ and has a useful lifetime of greater than 10 h. In these systems, no incubation time is required, and immunosurface regeneration is not performed after each assay. The optimal assay format, in terms of speed and simplicity, is the displacement one. However, this is difficult to set up.

In terms of the final application, the rapidity is a property that authors generally try to optimize in order to develop a really competitive device that can work under almost real-time conditions, with data displayed within few minutes. In general, the total assay time has been reduced over the last decade, without changing assay formats or other basic features. In some cases this reduction has led to systems with an acceptable response time of fifteen minutes, although an additional reduction would make them really competitive. In other cases, the developed immunosensors can almost be considered to work in real time, with response times lower than five minutes, even when assay formats (immobilized antibody) are used that yielded slow immunosensors in the past. Hence, low response time is a very important feature in immunosensing, and research aimed at decreasing it further should continue, even when developing new assay formats or even to the point of reducing assay sensitivity in order to maximize rapidity.

Multianalyte capacity

From a practical point of view, one very important aspect that needs to be studied is the multianalyte capacity of the immunosensor, because a multiresidue immunosensor could be successful applied to scenarios that are difficult to address by traditional methods; for instance, the simultaneous determination of three pesticides with different chemical properties and reference determination method such as carbaryl, atrazine and glyphosate; or the monitoring of several targets present at different concentration levels. In this sense, a multianalyte immunoassay method is highly advantageous over other analytical methodologies.

The main issue to solve in this regard is to design a system that is able to monitor several immunoreactions, and the solution to this is nearly always the multichannel spatial separation of the immobilized reagents and the use of a multiple detector and/or labels, which is similar to having a battery of immunsensors working in parallel, whatever the assay format or detector. In most cases, the number of target compounds is low. An example is given in [63] of a nonlabel SPR-based indirect immunosensor for two polycyclic aromatic hydrocarbons. The determination of three targets is the most common multianalyte scenario, and is described in [25, 26] for an indirect immunosensor for estrone, isoproturon and atrazine employing fluorescent labeling and TIRF detection. Also, in a recent work [65], hapten–BSA conjugates carbodiimide-linked to alkanethiol self-assembled monolayers on gold are employed in a nonlabel SPR-based immunosensor for the determination of DDT, chlorpyrifos and carbaryl insecticides in natural water samples. This sensor gives LODs of 20, 50 and 900 $ng\ L^{-1}$, respectively. A device that is theoretically able to monitor thirty-two compounds (the RIver ANAlyzer, which has become the Automated Water Analyser Computer Supported System, AWACSS) is currently being constructed [39, 40]. This system has 32 separate sensing patches on a chip surface and a fiber-coupled detection array that is used to monitor 32 separate fluorescence signals; this is a typical arrangement for a multianalyte device based on the spatial separation of immunoreactions. The system has been developed to determine several target compounds (estrone, propanil, progesterone, sulfonamides) with high sensitivities (LODs lower than 1 $ng\ L^{-1}$ in most cases), and will be employed to monitor for the presence of 19 compounds (pesticides, PAHs, endocrine disruptors, etc.) in European rivers at μg/L levels or lower. An actual multianalyte system has been successfully used to determine six compounds simultaneously.

A slightly different approach is based on the concept of antibody array, which can be used for multianalyte and/or multisample determinations. Arrayed biorecognition reagents is an important research field these days, and although most array-based devices employ nucleic acid sensing, there is an increasing shift towards the use of other bioactive reagents, such as antibodies, as stated recently in a review by Sheehan et al. [66]. Examples of array-based multianalyte immunochemical devices have been described, mainly for hazardous biomaterials. A representative example is the 3×3 antibody array [67] used for the detection of nine different toxins and bacterial antigen targets, which is based on a sandwich immunoassay. The device was further miniaturized and the final prototype, which weighs less than six kg, is able to monitor six analytes being tested for

staphylococcal enterotoxin B, ovalbumin and chicken IgY [68]. More recently, the same research group has developed a multisample displacement array-based immunosensor for TNT [69], by employing a biotinylated antibody immobilized onto NeutrAvidin-coated Luminex beads and Alexa Fluor as label. The analyte can be detected at 0.1 μg L^{-1}.

Recently, the indirect competitive immunoassay format has also been applied in a multisample array arrangement to the determination of aflatoxin B1 [70], by employing the avidin–biotin interaction to anchor the analyte to a glass support, Cy5-labeled monoclonal antibody, and a special 6- or 12-channel assay flow cell to build the array. The limit of detection reached was 0.3 μg L^{-1}.

Our contribution to the study of multianalyte immunosensing has been based on the universal features of the capture format [36]. The ability of protein A/G to capture immunoglobulins has been exploited in three different multianalyte approaches for the pesticides carbaryl, atrazine and Irgarol 1051 as a model system, employing only immunosorbent, enzyme label and detector, without spatial separation of immunointeractions. Employing the sequential analysis mode, the analytes can be quantitatively determined just as if three different sensor systems were used, but all of them were on the same device, with limits of detection of 9, 8 and 8 ng L^{-1} for the insecticide, the herbicide and the antifouling agent, respectively. The additive analysis mode led to results that can only be employed for qualitative purposes. Finally, using the simultaneous analysis mode, along with a cocktail of antibodies and tracers, the capture immunosensor can be used as a screening device, with a single measurement provided for the three targets. As can be seen in Fig. 2, the signal generated by the sample permits the presence of one or more analytes to be deduced, with a limit of detection of 11 ng L^{-1} when the three targets are simultaneously present, or 103, 40 and 80 ng L^{-1} if either carbaryl or atrazine or Irgarol 1051, respectively, are present alone in the sample. This could provide an interesting approach to screening pollutants that have alarm levels. However, the application of these formats to more than three targets is not practical.

Applications

The most relevant features of immunosensing development have now been discussed, but this analysis and the conclusions that were drawn are meaningless if we do not take into account the applications they are intended for. With this in mind, two different issues need to be addressed: sample treatment, which will enable the application of immunosensors to real problems, and target compounds of interest.

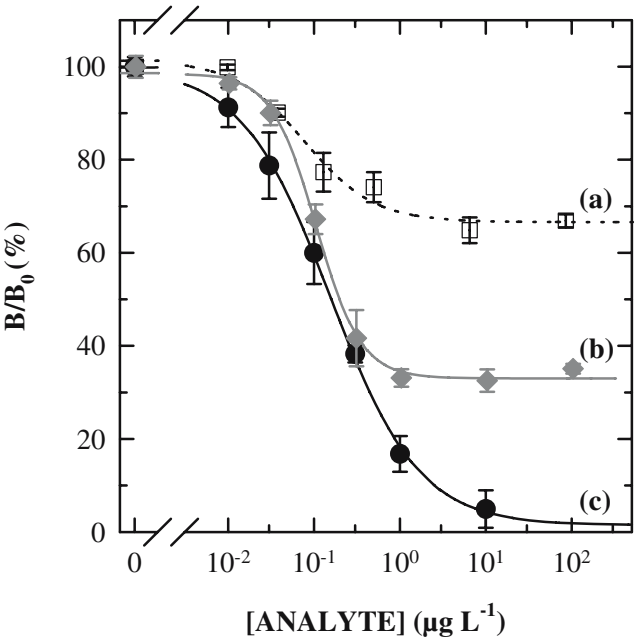

Fig. 2 Calibration curves obtained with an immunosensor working in the simultaneous protocol for (*a*) carbaryl, (*b*) carbaryl and atrazine, and (*c*) carbaryl, atrazine and Irgarol 1051. Each point represents the mean±SD of three replicates

Sample treatment in immunosensing

The sample preparation step is generally the bottleneck in an assay, and it can determine the feasibility of the whole method. The development of new immunosensors should be accompanied by corresponding sample processing, and if sample treatment is not well-developed and adjusted, the immunosensor is of little practical value. In our opinion, the sample treatment should be simple, even on-line with the assay, although other analytical properties such as sensitivity are partially lost, so operations such as dilution, solid-phase extraction and interference masking are recommended.

This is also the general feeling of most authors, and in works on immunosensing that also deal with sample preparation the proposed sample treatments are as simple as possible. For instance, in the work by Rodriguez-Mozaz et al. [25], the only study performed is a calibration in a matrix and a comparison with a calibration in a buffer. In this work, it is concluded that ground and river water samples can be analyzed without treatment. The same approach is employed for the determination of isoproturon in seawater [22]. In this case, the standards are prepared in 3% NaCl aqueous solution.

A different example is the work by Gauger et al. devoted to the determination of the explosives TNT and RDX by means of a displacement immunosensor with a LOD of 10 μg L^{-1}, where preparation steps involving extraction with acetone, evaporation and reconstitution in buffer are

used [71]. Another approach, employed for fruit juice, is simple dilution with buffer, and this has been studied in the work by Yakovleva et al. [15] on a capture immunosensor for atrazine, for example. In this case, the dilution factor applied must be higher than 400 in order to eliminate matrix effects.

Another example where organic solvents are employed is the extraction of isoproturon from potatoes by means of methanol, and dilution with buffer up to a maximum solvent percentage of 25% [22]. When a sol-gel immunosorbent is used, recovery values are excellent, which is not the case when the same antibodies are immobilized onto a different support, presumably due to the inability of macromolecules to enter the sol-gel matrix. The same authors have recently developed a novel improved immunosensor based on the same immunosorbent, but with on-line extraction of the analyte by means of a preconcentration immunocolumn containing the antibody entrapped in the sol-gel matrix [23]. The enrichment factor achieved in the on-line preconcentration step is 66.6.

Finally, there is a very interesting work devoted to the application of commercial membranes for the SPE extraction of explosives (TNT and RDX) from groundwater and seawater samples, followed by elution with solvents (methanol, acetonitrile, methanol/acetone 50/50 v/v), and then further determination by means of a displacement immunosensor [72]. The extraction efficiencies obtained were between 80 and 100%.

Our contribution to the study of sample treatment has been based on the applicability of immunosensors to organic extracts. In a first work [73], a basic study on the behavior of reagents—haptens, conjugates, antibodies, enzyme labels—in solvent–buffer mixtures was carried out, and the effect of using these mixtures on the final performances of the immunosensors, for different assay formats, was investigated. It was concluded that the assay sensitivity dropped when shifting from aqueous to organic mixture samples, but this effect could be compensated for by the preconcentration inherent to the extraction process. It was also observed that selectivity—expressed as cross-reactivity—was different in solvent-based sensors; in general it was better, because the cross-reactivity values diminished. Further work also addressed the applicability of immunosensors to organic extracts. It was possible to determine carbaryl [74] in vegetables after solvent extraction (two different methods were proposed) using a capture-format immunosensor working with a 50% v/v methanol–buffer mixture, which had a sensor life of >400 cycles and an analyte dynamic range of 2–50 μg L^{-1}. Atrazine was also determined in water and vegetables [75], after solid-phase extraction and elution with methanol, in a sensor used for the same organic mixture, which had a LOD of 0.15 μg L^{-1}. Also, the atrazine in olive oil was determined after methanol extraction at low temperature [76], giving a LOD for this herbicide of 50 μg L^{-1} in the native oil sample.

Another contribution to the study of sample treatment dealt with the application of an on-line analyte derivatization step to a very sensitive capture immunosensor for glyphosate that employed a previous analyte acetylation reaction in order to enhance antibody affinity [77]. The final immunosensor was applied to water and aqueous KOH soil extracts, and gave a LOD of 21 ng L^{-1} in a completely automated system with a total analysis time (including derivatization) of 25 min.

All of the examples mentioned above describe treatments that were found to be either suitable or unsuitable when combined with immunosensing, but in general, the study of sample preparation has been done systematically in only a few cases, which is an aspect of immunosensor research that needs to be addressed. Therefore, we can make the following conclusion: immunosensors are consolidated analytical systems, but they are barely commercialized and are yet to be established as routine tools, due to a lack of validated protocols for a wide range of sample matrices. In this sense, sample treatment is now the main challenge, because this stage determines the viability of the whole assay.

Target compounds

Another important aspect that needs to be studied deals with the target compounds in immunosensing. If we consider the data in Table 3, which provides only a general overview of this issue, it is clear that a lot of analytes are being considered: pesticides (especially herbicides), explosives, PAHs and PCBs, although other compounds are less studied, such as pharmaceuticals [21, 78], toxins [27, 79], or dioxin precursors [58]. However, the current trend is to broaden the spectrum of compounds that are considered pollutants, for instance pharmaceuticals and surfactants [80]. The efforts should focus on those compounds that could be dangerous from an environmental point of view, for instance those appearing in Annex X of the European Union Directive on priority substances [81], or persistent organic pollutants as defined by the United Nations [82] and USEPA [83]. Some of these pollutants, such as atrazine, isoproturon and benzo-α-pyrene, have been used as targets in immunosensing for years, but other compounds (chlorobenzenes, pentabrominated diphenylethers, di(2-ethylhexyl)phthalates, and others) have not been studied at all. It is therefore interesting to develop immunosensors for these compounds, and the hardest task is currently to obtain antibodies and other specific immunoreagents. If all the tools needed were assembled and running, and only new reagents had to be raised and

Table 3 An overview of target compounds for environmental optical immunosensing[a]

Analyte family	No. of references	Percentage of literature
Pesticides	26 (>80% herbicides)	36
Explosives and nitrocompounds	15	21
PAHs	11	15
PCBs	7	10
Endocrine disruptors[b]	6	8
Pharmaceuticals	4	6
Toxins	2	3
Dioxin precursors	1	1

[a] Literature searched from 2000 to 2005, both years included
[b] Compounds not belonging to another family from the table

applied, the total cost has been estimated at sixty thousand euros, considering staff wages and other expenses for one year. This is a very competitive cost if a real analytical problem needs to be solved.

Real applications

The final challenge in immunosensing is possibly the most important stage in the development of a new analytical technique: its validation, commercialization and actual application to routine analysis. All the time, effort and money invested in immunosensing development is worthless if no immunosensor devices are applied to real analysis. This stage is now being considered by researchers, but almost everything is still to be done. The commercial optical instruments applied in general biosensing—detectors, mainly SPR—have been on the market for years, and they have been increasing used in recent years, as is apparent from extensive annual reviews performed by Rich and Myszka [84–87]. The merchandising of some kinds of biosensors, especially those applied in the clinical area, has been accomplished in some cases (such as for the blood glucose biosensor), or is now being developed, as can be inferred from the recent Ninth World Congress on Biosensors, held in May 2006 [88], which had sessions devoted to commercial developments, manufacturing and markets. However, when immunosensing is applied to the environment, spiked, blind or even real unknown samples are analyzed with the immunosensor but the results are then compared with data from other established or reference methods. An example is the determination of glyphosate in water and native and spiked soil samples using immunosensing and liquid chromatography [77], where real contamination from this herbicide was found in some samples using the two methodologies. However, real validation interlaboratory assays do not appear in the literature. Prototype immunosensors that can be applied to real samples have also been described in some works [28, 39, 40], but information on commercial optical immunosensors nor routine applications of immunosensing devices could not be found.

Conclusions

The main conclusions that can be drawn from this discussion are as follows:

- Immunosensors are well-developed analytical devices, because many of the requirements for a good immunosensor have been achieved. In fact, prototype immunosensors, even for multianalyte applications, such as the European RIver ANAlyzer and AWACSS, have been developed. Practical aspects such as sensitivity, sensor working lifetime and applicability are good, but improvements in them should continue. Research into immunosensors with a multianalyte capacity (the ability to determine multiple targets simultaneously) as well as new detection modes and measurement methodologies for biosensing should also continue.
- The main challenge at present is to apply the immunosensors to real samples. Sample treatment protocols must be developed, and they must be rapid and simple if they are to fit with continuous and automatic immunosensors. Sample treatment should be designed to take into account the actual application of the final immunosensor (i.e., to solve an analytical problem).
- Many emerging pollutants are the targets of the future. If we want to study new environmental problems, new sensors must be developed for these compounds, new immunoreagents must be synthesized, and this development process should be inexpensive.
- Finally, the development of immunosensors is at a very advanced stage, because multianalyte systems with rapid response times systems that are able to detect minute amounts of pollutants can be found in the literature. However, from a practical point of view, these devices must be validated if they are to reach the market, and routine analysis has to be performed. Batch bioassays and immunoassays for monitoring water are already commercialized and validation tests are currently being performed in order to assess their potential as robust analytical tools, but immunosensors are not yet at this stage. Commercial equipment that can be used in immunosensing (e.g., SPR) has been on the market for years, but the final goal—the construction of truly practical immunosensors—is yet

to be achieved. This is the real challenge for researchers in this field at present.

Acknowledgements Authors gratefully thank the financial support of Spanish CICYT projects BQU 2003–02729 and BQU 2003–02677.

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