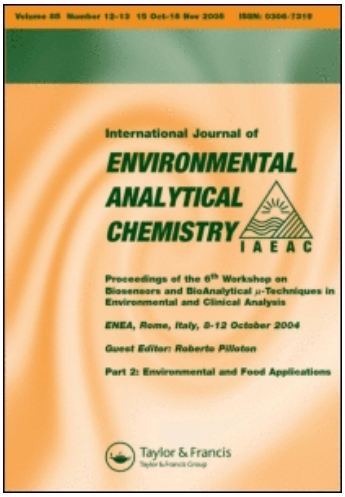

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Environmental Analysis Using Antibody and Receptor Based Techniques

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ENVIRONMENTAL ANALYSIS USING ANTIBODY AND RECEPTOR BASED TECHNIQUES

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Immunochemical analysis relies on the selective binding of antibodies to defined targets such as environmental compounds. Commercial applications in the environmental field are still restricted to a limited number of immunoassays and a few immunochromatographic applications. The main barrier to a broader exploitation is seen in the generation of a sufficient number of antibodies within an acceptable time period. Recombinant technologies are expected to eventually replace the circumstantial approach to obtain new Abs by new immunizations. Examples for recombinant singlechain fragments (scFv) and antigen binding fragments (Fab) directed against herbicidal s-triazines are given. If antibodies are replaced by receptors and other functional ligands, biological effects monitoring becomes available. As an example an enzyme-linked receptor assay (ELRA) for endocrine disruptors providing estrogen equivalents is presented. Finally, the concept of bioresponse-linked instrumental analysis is introduced for the tight coupling of effect monitoring to chemical analysis. In the first step, analyte binding by functional biomolecules is recorded to provide binding equivalents. The second step is targeted at chemical analysis of bioeffective analytes bound to the functional biomolecules.

Keywords: Antibodies; receptors; environmental analysis; effect monitoring

INTRODUCTION

Immunochemical techniques supplement traditional analytical methods in a useful way because they are extremely sensitive, selective, simple, and inexpensive. The assays are based on antibodies (Abs) as binding proteins. The main applications are found in the medical field, e.g., diagnostics and therapy followed by biochemical analysis. Table I lists the most relevant applications of immunochemical methods.

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TABLE I Main utilization of antibodies

Medical diagnostics	Viruses (e.g., HIV, hepatitis)
	Bacteria (e.g., *Heliobacter pylori, Borrelia burgdorferi)*
	Yeasts (e.g., *Pneumocystis carinii)*
	Pregnancy tests
	Autoimmune diseases (e.g., systemic Lupus Erythrematosus, Sclerodermia)
	Leucocyte markers
	Tumor markers (surface antigens)
	Serum and plasma components
	Hormones (e.g, insulin, steroids)
	Toxins, drugs
Therapy	Humanized antibodies (chimeric antibodies)
	Anti B1 antibodies directed against CD 20 antigen
	Anti B1 antibodies as ^{131}I conjugate
	Chimeric murine/human antibody 225 IgG1 (binds to the epidermal growth factor (EGE) receptor)
	Monoclonal antibody binding to the vascular endothelial growth factor (VEGF)
	Monoclonal antibody binding to specific surface antigens of activated fibroblasts (FAAP, fibroblast activation protein)
Molecular biology, biochemistry, cell biology	Reporter gene analysis
	Tags
	Cytoskeleton (actin, myosin, tubulin, intermediate filaments, enzymes, receptors)
	Cell cycle and cell nucleus proteins
	Chaperonins and heat shock proteins
	Signal transduction, neurology
	Growth factors, cytokines
	Cell surface markers
	Cell adhesion
	Extracellular matrix proteins
	Immunohistochemistry (antibody conjugates)
Food and environmental analysis	Mycotoxins, plant storage proteins, meat proteins, lectins, bacterial proteins
	Pesticides, environmental compounds

Environmental analysis is still limited by the availability of suitable Abs. Considering the large number of industrial chemicals found in the environment this is not surprising.

Since it is not possible to alter the binding properties of existing Abs, alternatives are required to circumvent the necessity of new immunizations, for instance to obtain monoclonal antibodies (mAbs) with altered cross-reactivities. This can be achieved at the DNA level by generating and expressing mutant Ab genes. This recombinant technology has considerable consequences. It will bridge the gap between artificial peptide receptors and Abs.

A totally different approach to environmental analysis has recently been undertaken to cope with the large number of chemicals that may contaminate the environment. It is the use of bioresponse-linked assays in order to detect the presence of toxic or pharmacologically relevant compounds. This is illustrated below for the detection of xenoestrogens by estrogen receptor assays, which are based on receptor binding of the bioeffective compounds and therefore indicate pharmacological equivalents.

However, this strategy cannot provide information on the chemical structure of the bioeffective substances. Therefore a novel approach to environmental analysis is introduced, which is based on the tight coupling of bioassays and chemical analysis. It is defined here as bioresponse-linked analysis. It combines biomolecular recognition, initiating a biological effect, and chemical analysis. The proper application of this strategy reduces chemical analysis to those samples or fractions of samples that contain bioeffective compounds.

IMMUNOANALYSIS

The most common immunological methods for environmental analysis are immunoassays, immunochromatography and immunosensors. Up to now, commercial applications are restricted to immunoassays and immunochromatography.

A typical example of immunoanalysis for herbicide screening is used here for illustration. Although the European Community has banned in 1991 atrazine in Germany, it is still found, mainly due to illegal use, but also continued and widespread application abroad, its mobility in the water cycle and its persistence. An enzyme-linked immunoassay (ELISA) was applied in our laboratory for screening atrazine residues in soil. A total of 2517 samples (including duplicates and repeats) were performed corresponding to 420 soil samples per year. The applied ELISA was initially developed to match the EC regulations for drinking water

analysis (Figure 1). The lower detection limit of the ELISA was determined at 0.02 µg/L atrazine. The upper test limit corresponded to 0.7 µg/L. Since the ELISA should provide a precise determination at the critical concentration of 100 µg/kg soil, which corresponds to atrazine applications in the same year, all samples were diluted 1:150 in bidistilled water after water extraction. Therefore, a sample with 100 µg/kg yielded 0.13 µg/L in the ELISA. Samples containing more than 300 µg/kg were additionally analyzed at a dilution of 1:500 in order to determine in the measuring range.

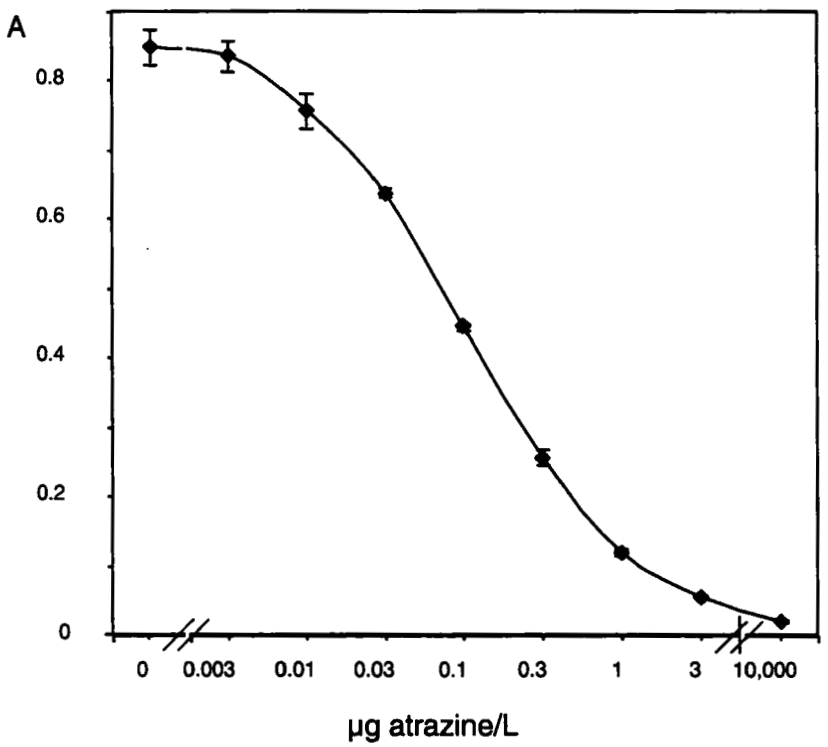

FIGURE 1 Calibration curve obtained by the heterogeneous, competitive enzyme immunoassay (based on polyclonal antibodies[1]) for the determination of atrazine in aqueous phase. Serum and POD tracer was diluted 1:10,000. The means and standard deviations are based on four repetitions

Soil samples annually collected in Southern Germany between 1993 and 1998 were removed from the top layer (0–5 cm) of cornfields each year on May/June subsequent to seasonal application and immediately processed. An average of 96.5 % of the samples contained less than 100 µg atrazine per kg soil and was

therefore regarded negative. This portion did not significantly alter during the monitoring period. Highly contaminated samples containing 500–1000 µg/kg atrazine were reduced from a mean portion of 3.0 % in 1993 and 1994 to 0.5 % in 1995–1998. Peak values exceeding 1000 µg/kg were only detected during the first year of monitoring. The portion of positive soil samples was therefore significantly reduced during the screening period in total as well as in the individual concentration categories.

TABLE II Comparison of the atrazine analyses, which were found within the critical concentration range between 50 and 150 µg/kg soil and performed by ELISA and HPLC

Monitoring year	*ELISA atrazine [µg/kg]*	*HPLC atrazine [µg/kg]*
1993	102 ± 8	109
1994	64 ± 11	96 ± 9
	109 ± 16	143 ± 13
	83 ± 13	111 ± 17
	79 ± 2	115 ± 20
	63 ± 6	141 ± 43
	124 ± 15	201 ± 4
	75 ± 8	91 ± 10
1995	103 ± 6	74
	120 ± 28	86
	72 ± 5	57
1996	169 ± 3	108 ± 17
	133 ± 5	111 ± 8
	123 ± 8	105 ± 10
	106 ± 5	113 ± 14
	170 ± 2	150 ± 7
	121 ± 4	91 ± 15
	85 ± 17	71 ± 23
	162 ± 4	108 ± 1
	145 ± 4	125 ± 2
	70 ± 5	65 ± 6
1997	120 ± 16	143 ± 11
	123 ± 5	145 ± 1
	114 ± 4	98 ± 0.5
	86 ± 1	109 ± 0.01
1998	[a]	[a]

a. Concentrations between 50 and 150 µg/kg atrazine were not found in 1998.

All positive samples and a selection of negative samples were validated by HPLC in the Bayerische Landesanstalt für Bodenkultur und Pflanzenbau at Freising (Germany). Comparison of ELISA and HPLC data yielded a correlation coefficient ranging between r = 0.958 and r = 0.981 (n = 18–47), except for the year 1995 where only a correlation of r = 0,864 (n = 18) was obtained. In toto, four samples were overestimated and four underestimated with respect to the atrazine threshold value of 100 µg/kg soil as revealed by HPLC validation (cf. Table II). Consequently, 99.7 % out of the 2517 analyzed samples were correctly evaluated. The precision and reproducibility of the ELISA turned out to be adequate for a prescreening tool. The low costs per sample and the high sample throughput is not yet achievable by conventional analytics.

Antibodies for immunoanalysis

Polyclonal sera often cause problems because of the limited amounts of Abs, which are produced by an individual animal, e.g. a rabbit, and because of the variability of Abs among different bleedings and different animals. To overcome these problems, it is advantageous to rely on mAbs. With the introduction of the hybridoma technology[2] immunochemical applications have entered a new dimension.

In contrast to polyclonal Ab production, hybridoma technique requires considerable investments in time, personnel, and capital. Nevertheless, the effort is worthwhile; it is possible to produce by *in vitro* cultures of hybridoma cells unlimited amounts of mAbs with constant properties such as defined isotype and affinities. mAbs from hybridomas established in our laboratory were successfully applied in immunoassays and immunohistochemistry. The selection of high-affinity mAbs now guarantees assay sensitivities, which are comparable to those of antiserum-based assays. For instance an ELISA with the mAb K4E7 developed in our group allows the determination of atrazine in a concentration range from 0.05 µg/l to 1 µg/l with a middle of the test (IC_{50}) at 0.1 µg/l[3].

In spite of the advantages of the hybridoma technology, the necessity to carry out new immunizations followed by screening and cell cloning can not be circumvented if new Abs with different properties are required. This restriction can be overcome by the use of genetic engineering for Ab production. Efforts are targeted at the generation of the natural Ab repertoire *in vitro*, followed by the selection of variants and further modifications at the gene level. An intensively applied strategy for the generation of functional Ab fragments is the phage display method, which was already developed in 1988[4]. It requires the gene fusion of Ab fragments (scFv or Fab, respectively, cf. Figure 2) with truncated phage surface proteins. The Ab fragments are then displayed at the surface of phages as

phage Abs. Today several commercial kits are available for all steps of phage display technology. A detailed description of a representative approach for the generation of functional *s*-triazine recombinant antibodies (rAb) has been given by Kramer[5]. Improved technologies now allow the production of larger amounts of functional soluble Ab fragments.

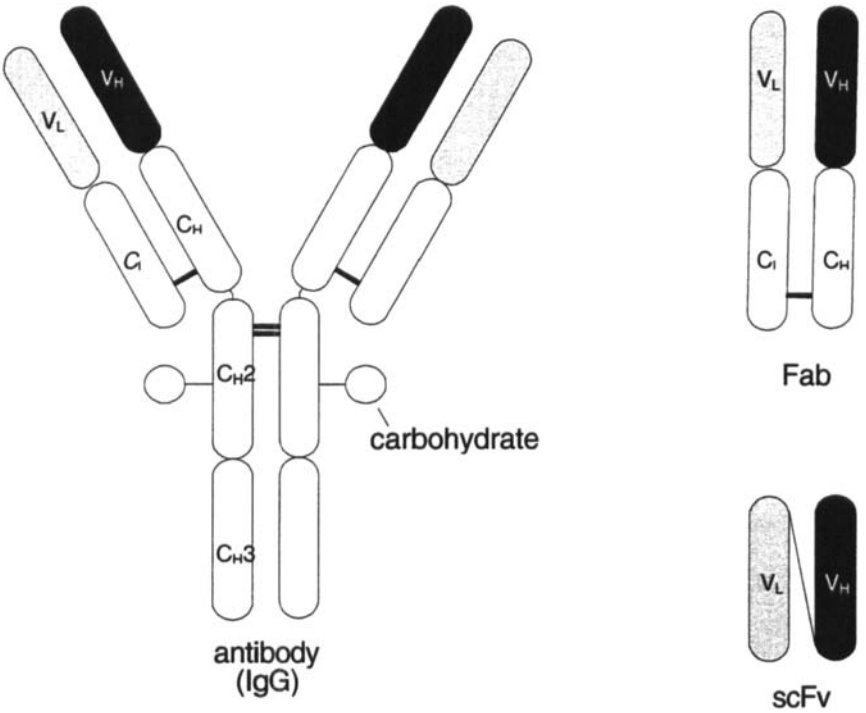

FIGURE 2 Domain structure of antibodies (IgG, Fab and scFv)

An important aspect in the exploitation of rAbs is the long-term stability. It has been noted before that mAbs for atrazine suffered only moderate activity losses after a 10-day incubation at 37 °C, which could by demonstrated by shifts of the calibration curves[6]. Now rAb stability was tested for long-term storage at 4 °C. Recombinant Fab and scFv fragments were synthesized by cloning the variable heavy and light chain encoding genes of the triazine specific hybridoma line K4E7[3] into the vectors pASK 85[7] and pCANTAB 5 E[8], respectively. Figure 3 shows the results for the respective ELISAs (zero inhibition) carried out with scFv and Fab. It is obvious that stability is greatly improved in the case of

Fabs. This is attributed to the kind of the linker, which connects the variable domains of scFv, the presence of the constant domains C_1 and a superior purification strategy applied for the Fab fragments.

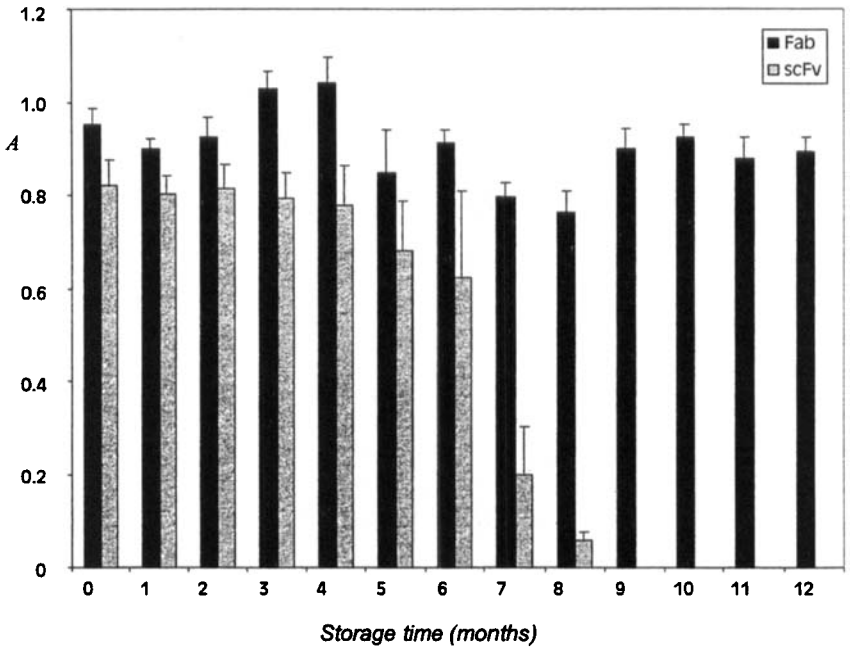

FIGURE 3 Stability studies with scFv and Fab directed against atrazine. The concentration of rAb fragments was adjusted to 0.1 mg/ml by dilution in PBS buffer (40 mM, pH 7.2). The antibody solutions were permanently stored at 4 °C. Aliquots were removed with sterilized pipette tips at the indicated intervals to investigate the immunochemical functionality of Fab and scFv by direct ELISA. Applying zero dose of atrazine in the ELISA, the maximum absorbance was determined at 450 nm in a Multiscan ELISA reader after 20 min of peroxidase substrate incubation. The indicated values constitute the mean absorbance derived from three (for the Fab) and six (for the scFv), respectively, separate antibody preparations

Further examples for immunochemical analysis with rAbs have been provided for the herbizide atrazine[9,10], diuron[11], parathion[12], as well as for several toxins such as Fumonisin B1[13], Botulinum NT[14], Restrictocin[15], Tetanus toxoid[16], *Clostridium difficile* toxin A[17], and anthrax toxin[18].

In the near future, the appearance of mutant Abs generated by genetic engineering is expected. Phage display can be used to present Ab diversity *in vitro*. The immune system covers the diversity of possible foreign antigens by presenting a huge and diverse repertoire of Abs and carries out an affinity maturation with the aid of somatic hypermutations[19,20]. These steps can be simulated *in*

vitro with Ab libraries in combination with phage display technique. Phage display is considered an excellent tool for the selection of defined variants because of its tight coupling of DNA and protein functions. Subsequent cycles of (1) production of new variants, (2) selection and (3) reproduction correspond to the principle of natural selection (survival of the fittest), as it is being used in evolutionary biotechnology[21], but also in combinatorial chemistry[22,23]. The advantage of evolutionary biotechnology is based on the direct connection between protein function and DNA sequence.

RECEPTOR ANALYSIS

In spite of the elegance of the immunochemical approach, there is no relation between the affinity of the analyte(s) to a given Ab and potential toxic or pharmacological effects. The advantage of Ab production by the immune system is due to its ability to discriminate between self, i.e. components of the body, and non-self such as pathogens or xenobiotics, which are detected by Abs. However, other approaches are available, which can be performed as binding assays similar to ELISAs, but provide information on potential biologic effects. They use biomolecular recognition processes as they occur in signal transduction processes. Table III lists presently available options.

As an illustrative example estrogen receptor (ER) binding assays are presented. The development of an enzyme-linked receptor assays (ELRAs) in microwell-plate format has recently been reported[24]. The assay employs the same principles as competitive immunoassays, which are based on ligand-protein interactions. Plates are coated with a 17β-estradiol-BSA conjugate. After washing and blocking the plates, samples are added together with the recombinant human ER produced in yeast. After removing the non-bound receptor by a further washing step, the bound receptors form an immune complex with a biotinylated anti-ER Ab. Followed by another washing step, a streptavidin-POD-biotin complex is added. The plates are washed again and the peroxidase-substrates (TMB; H_2O_2) are incubated. Substrate turnover is stopped, and the plate is measured with a plate reader. Similar to the ELISA, the ELRA shows an inverse relation between the free estrogen concentration and the color intensity (cf. Figure 4). A detection limit of approximately 0.1 μg/l for 17β-estradiol can be reached with the present ELRA. Therefore the sensitivity is comparable to radioreceptor assays[25]. Table IV shows cross-reactivities of the ER to other estrogenic compounds, including natural estrogens as well as xenoestrogens.

TABLE III Biomolecular recognition principles which can be exploited for bioeffects-related analytics

Targets	*Example*	*Function in*	*Interference by*	*Effects at the level of*
Proteins Enzymes	acetylcholine esterase	synapses	organophosphorous and carbamic compounds	neurotoxicity
	protein phosphatase 1 and 2A	liver cells	microcystins	hepatotoxicity
Ion channels	Na^+ channel (voltage gated channel)	synapses	saxitoxin, tetrodotoxin, procaine	neurotoxicity
Transport proteins	SHBG, CBG, TBG	transport of hormones in blood	endocrine disruptors	growth, reproduction
Receptors	estrogen receptor	cell nucleus	endocrine disruptors (e.g. o,p-DDT, nonylphenol)	growth, reproduction
	nicotinic acetylcholine (ACh) receptor (ligand-gated channel)	vertebrate muscle endplate (neuromuscular transmission)	anatoxins	neurotoxicity
Electron caniers	Q_B protein	chloroplasts	photosynthesis II herbicides (e.g. s-triazines, phenylureas), phytotoxins	photo-synthesis (plants)
Nucleic acids DNA	DNA double strands	chromosomes	intercalating polycyclic aromates (ethidium, acridine, coffein); DNA adducts (metabolites of chloracetamide herbicides)	genotoxicity
Cytoskeleton	tubulin	cell division	colchicin, taxol;	cytotoxicity (general)
			anti-tubulin herbicides (e.g. trifluralin, oryzalin)	(plants)
Ribosomes	rRNA (ricin)	protein synthesis	ribotoxins (ricin, abrin, Shiga toxin)	cytotoxicity

TABLE IV Cross-reactivities of the uman estrogen receptor, determined with the ELRA. The IC_{50} for 17β-estradiol was arbitrarily set to 100%

17β-estradiol	100.0
ethinylestradiol	81.2
diethylstilbestrol	80.3
4-OH-tamoxifen	79.5
dienestrol	61.1
α-zearalanol	57.2
α-zearalenol	54.2
4-OH-estradiol	34.4
hexestrol	31.1
ICI 182,780	30.1
zearalenone	28.1
estriol	26.2
β-zearalanol	19.4
2-OH-estradiol	17.1
estron	14.2
17α-estradiol	13.1
zearalanone	12.7
mestranol	5.1
genistein	3.0
nafoxidin	2.6
clomiphen	2.5
tamoxifen	2.1
androsten	2.0
β-zearalenol	1.2
o,p′-DDD	0.43
4-nonylphenol	0.36
norethynodrel	0.33
bisphenol-A	0.25
o,p′-DDT	0.16
o,p′-DDE	0.08
estrone-3-sulfat	0.07
resveratrol	0.04
5α – dihydrotestosteron	0.03
methoxychlor	0.01
2,4-D	< 0.01
atrazin	< 0.01
benomyl	< 0.01
cholesterol	< 0.01
cortisone	< 0.01
p,p′-DDE	< 0.01
p,p′-DDT	< 0.01
pregnenolone	< 0.01
progesteron	< 0.01
β-sitosterol	< 0.01
testosteron	< 0.01

It was demonstrated that even in mixtures of substances with very different receptor affinities, additivity is obtained. Therefore the ELRA reflects the sum of receptor relevant substances in a sample which can be expressed as estradiol equivalents. Since the ELRA detects all substances, which are able to bind to the ER, it is an effects-related test. However, it is not possible to distinguish agonists from antagonists with this approach. This can even be an advantage if environmental samples containing complex mixtures of chemicals are to be measured. Regardless of the simultaneous presence of agonists and antagonists the ELRA gives correct results for receptor binding equivalents. Assays that do distinguish agonists and antagonists, like cell proliferation assays, may cause problems in this particular instance as the signals could compensate each other.

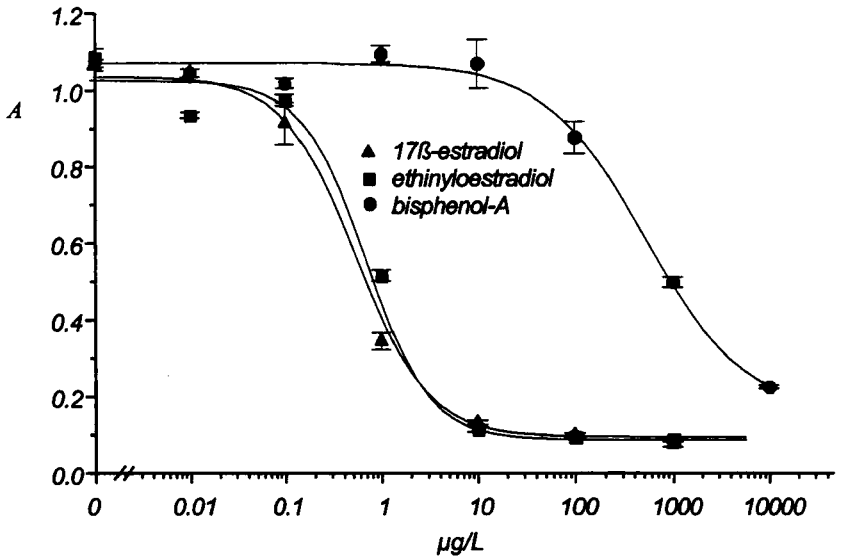

FIGURE 4 Calibration curve for the ELRA performed with the human estrogen receptor α

To prove the applicability of the ELRA to environmental monitoring, real water samples were measured. No relevant matrix effects were observed with the optimized ELRA in water samples from lakes, and even a sewage plant outflow could be measured correctly without sample preparation or sterilization. Figure 5 shows the results of comparative measurements performed with the ELRA and HPLC for lake water samples spiked with ethinylestradiol, a contraceptive, frequently found in the outlet of sewage plants. The close correspondence illustrates the applicability of the ELRA for environmental monitoring, but also for screening of chemicals for estrogenic effects.

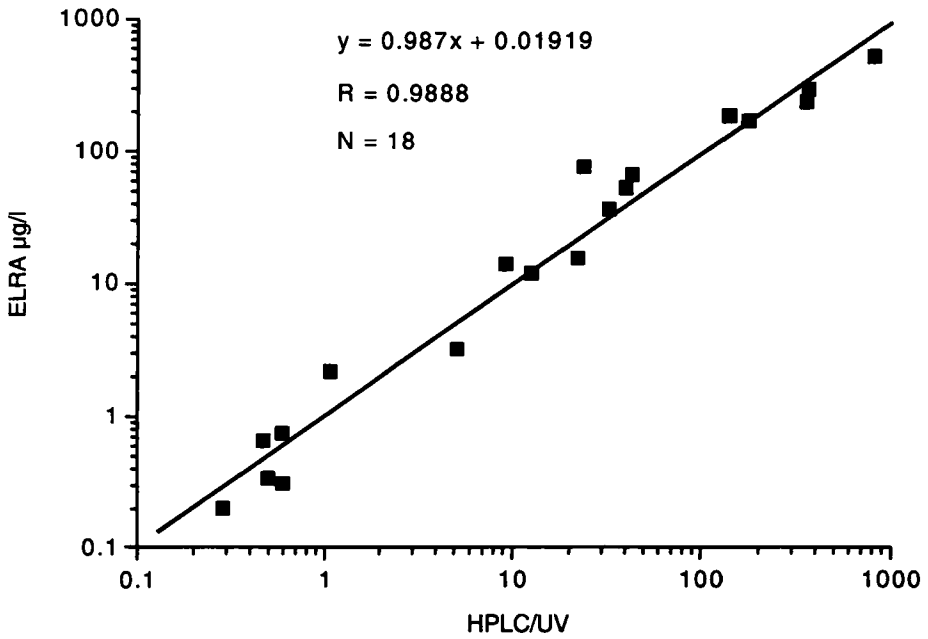

FIGURE 5 Correlation between ELRA and HPLC/UV. Water samples from a lake were spiked with different anounts of ethinyl estradiol

BIOEFFECTS-RELATED ANALYSIS

The principle of biomolecular recognition can be exploited for binding assays that provide data on binding equivalents. However, this approach does not provide information on the chemical structure of the bioeffective substances. Therefore bioeffective binding should be linked to chemical analysis. This can be achieved in different ways. The most elegant approach is seen in the application of hyphenated technologies, which enable online coupling of binding assays with chemical analysis. Although this technology is only at its beginning, the power of the strategy is obvious. It simultaneously provides information on biological effects with respect to the applied target structures and on the chemical identity of the active substances present in a sample. A further significant advantage is the possibility to perform quasi-continuous analyses. Several variants of coupling between the biomolecular and chemical part appear to be feasible: (1) Determination of the ratio of free and bound target structures such as

enzymes, receptors and other binding proteins followed by chemical analysis of bound analytes. The pharmacological or toxicological potential of the sample can be derived from the ratio of bound versus free fraction of the target molecules, whereas the identity of the active substances is revealed by chemical analysis. A feasible approach for the chemical analysis appears to be LC-MS. (2) Direct analysis of bound and free target structures by ESI-Q-TOF, ESI-TOF [26].

PERSPECTIVES

The power of bioeffects-related analytics is based on the combination of biomolecular recognition principles with chemical analysis of bioeffective substances. The proper application of this strategy is expected to reduce chemical analysis to those samples or fractions of samples that contain bioeffective compounds.

However, the scope of this strategy is restricted by the availability of representative target structures. For instance, DNA molecules only apply to DNA-relevant ligands, estrogen receptors only to certain classes of endocrine disruptors and so on. Therefore, the effectivity of this type of analytics depends on the proper choice of biological target structures. It can be foreseen that this problem will be solved by a proper combination of different and representative target structures. It may become necessary to combine specific targets (such as receptors) with more integral ones (such as some kinases or phosphatases), which may be realized by modular test batteries. The success of this concept depends on the optimal combination of the individual modules that can be exchanged according to the specific requirements. Presently, modules providing data on cell toxicity, genotoxicity, neurotoxicity and immunotoxicity appear to be most important. It is also obvious that the applicability of bioeffects-related analytics depends to a great deal on the availability of biochemical target structures. Recombinant approaches for their biosynthesis appear to be indispensable.

Acknowledgements

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