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review

Fluorescence Polarisation Immunoassays for Determination of Pesticides and Biologically Active Compounds in Food Safety and Environmental Monitoring*

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Summary

Fluorescence Polarisation ImmunoAssay (FPIA) methods for rapid detection of pesticides and biologically active compounds are reviewed from 1989 onwards. The FPIA is a homogeneous (without separation) competitive immunoassay method based on the difference in fluorescence polarisation of free and antibody bound fraction of fluorescein labelled antigen. The advantages, limitations and recent innovations in FPIA are given.

Keywords: fluorescence polarisation immunoassay, pesticides, drugs, food safety monitoring, environmental monitoring

Introduction

The wide application of pesticides and other biologically active compounds, like veterinary drugs, in the food production is growing, but with this growth at the same time the contamination of food and of the environment is also increasing. It is obvious that monitoring of food and environmental samples for contamination with pesticides and related compounds is considered a matter of great importance. Millions of determinations of food and environmental contamination are performed daily in the world. Moreover, the samples must be assayed for multiresidue and/or selective single residue contents, and therefore, the analytical method for analysing food and environment samples must be simple, reliable, fast and cheap. Usual conventional analytical methods for pesticides detection are chromatographic methods, involving extraction and clean-up steps. These methods are time consuming, expensive and have some other disadvantages.

In the last ten years the importance and application of immunoassay (IA) methods, especially the enzyme linked immunosorbent assay (ELISA), have grown significantly. Several excellent reviews dealing with immunoassay methods used for pesticide analysis in food and environmental samples have been published *(1–4)*, and the recent advances in ELISA and new immunoassay techniques can be found in many chapters of two books of the American Chemical Society Symposium Series *(5,6)*. In general, ELISA has many advantages over the other techniques and allows to perform direct analysis of a large number of samples. However, this method requires a step of immunoreagent separation, and involves multiple washing steps, as well as an enzymatic detection.

Of course, the immunochemical methods are continuously improving and simplified. One of the new and very perspective direction in this area is the development of immunosensors. The major recognition elements in any biosensor are enzymes and antibodies, and analysis using these two types of bioactive compounds, can be either complementary or alternative to ELISA. The application of sensors is widely expanded for measuring different pesticide residues in environmental analysis. Excellent articles dedicated to the advances of (bio)chemical sensors have been recently published in two special issues of this journal *(7–8)* and some of their advantages and limitations of have also been reviewed *(9–11)*. Multianalyte immunoassays for simultaneous detection of several different pesticides, as a screening

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technique, have been also described *(12,13)*. The main limitation for using immunosensors is the regeneration of the receptor surface which should be at least washed or even entirely replaced. The additional problem for wider application of immunosensors, at this moment, is their poor stability, and, as a consequence, low precision of the assay.

The primary requirement for wider success of screening methods, for detection of pesticides and biological active compounds, is the simplification of the assay and from the author's point of view, the homogeneous methods of immunoassay, which are very simple and fast because they do not need any separation of immunoreagents or washing steps, are the most promising ones in this respect.

Fluorescence polarisation immunoassay (FPIA) is one of the techniques which fulfil appropriately the requirement for simple, reliable, fast and cost-effective analysis. FPIA is based on the increase in the polarisation of the fluorescence of a small fluorescein-labelled hapten (tracer) when bound by a specific antibody. If the sample contains unlabelled pesticides, these molecules will compete with the tracer for antibody binding and the polarisation signal will decrease. The theory and application of immunoassay methods based on fluorescence polarisation (FP) were described in different books on immunochemical techniques *(14–16)*. One of the most complete review on FPIA, which includes 195 references, was published in 1989 *(17)*. For this reason this review will focus only on the advances in the application of FPIA for detection of pesticides and relative compounds from 1989 onwards.

History of Fluorescence Polarisation Immunoassay

The principle of fluorescence polarisation, which is the base for detection in FPIA technique, was discovered in 1920s and described by Perrin (18). Next significant step was in 1960s, when Dandliker *et al.* reported the antigen-antibody interaction in which the changes in FP were utilised *(19)*. The term FPIA for measurement of concentration of various analytes in buffer was introduced by Dandliker *et al.* in 1973 *(20)*. At that time the FPIA had limited application due to the highly non-specific binding of tracer to serum proteins and also due to the non-availability of special instrumentation. Several years later, Landon and collaborators in St. Bartholomew's Hospital (London, UK) discovered that some tracers for drugs show a negligible non-specific binding to serum proteins. This group was the first (in 1976) to develop FPIA as an analytical method for detection of therapeutic drugs *(21,22)*, but they used the reverse order of the name for this method – polarisation fluoroimmunoassay (PFIA). Both names for this method are in use in the literature, although the name fluorescence polarisation immunoassay (FPIA) prevails. Therefore, when searching the literature one should always keep in mind that both names coexist and special care should be also taken with UK or US spelling of the word polaris(z)ation. The name FPIA is used in the *Current Contents, Analytical Abstracts, Chemical Abstracts, Medline* and some other data bases, and for this reason the name FPIA is used in this review.

Until 1980 the FPIA was not a widely used analytical technique due to the limited availability and high cost of polarisation fluorimeters. Fortunately in 1980s Abbott Laboratories developed a fully automated system, the Abbott TDx Analyser, for therapeutic drug monitoring *(23)*. This analyser was able to detect about one hundred of analytes *(24,25)*, and it should be mentioned that it is probably one of the most successful commercial innovation in the history of immunoassays.

Colbert and Coxon were the first who described FPIA for detection of pesticide *(26)*. They developed a FPIA for paraquat in serum samples and adapted this assay to be used in the Abbott TDx Analyser. In Table 1, successful applications of FPIA for different pesticides and drugs developed during the last ten years in the laboratory of Eremin's group are given, together with references of publications *(27–50)*.

Principle of Fluorescence Polarisation

The theory of fluorescence polarisation (FP) is well known and described *(51, 52)*, therefore, only the main principle of FP will be given here briefly. Some substances are able to absorb the light of some fixed wavelength, excite to the higher energy level and emit light of higher wavelength. This phenomenon is named fluorescence. Fluorescence is usually a relatively fast process, characterised by a first order decay with lifetime measured in nanoseconds. For example, fluorescein molecules have the maximum excitation and emission wavelengths of about 492 and 517 nm, respectively; and the time delay between excitation and emission of light is about 5 ns. If the fluorescein molecule is excited by polarised light, the molecules change their orientation due to Brownian movements and emitted light becomes additionally depolarised.

The fluorescence polarisation is determined by exciting the fluorescein compounds with vertically polarised light and measuring the intensity of both the vertically (Iv) and horizontally (Ih) polarised components of the emitted fluorescence. In other words, the emitted light parallel (Iv) and perpendicular (Ih) to excitation intensity is measured (Fig. 1). The polarisation (P) value is defined as the ratio of the difference and sum of these two components:

$$P = (Iv - Ih) / (Iv + Ih) \qquad /1/$$

Its value theoretically varies from 0 (full depolarisation) to 0.5 (for fixed molecules without movement). P could be measured very precisely, up to the 10^{-5} order of magnitude, and therefore in practice the »milliunits« of FP (*i.e.* mP values) are used.

It should be also mentioned that fluorescence anisotropy (A) could be also used in description of this phenomenon. A value is defined by Eq. 2, and the relations between P and A are expressed by Eqs. 3 and 4:

$$A = (Iv - Ih) / (Iv + 2Ih) \qquad /2/$$

$$A = 2P / (3–P) \qquad /3/$$

$$P = 3A / (2+A) \qquad /4/$$

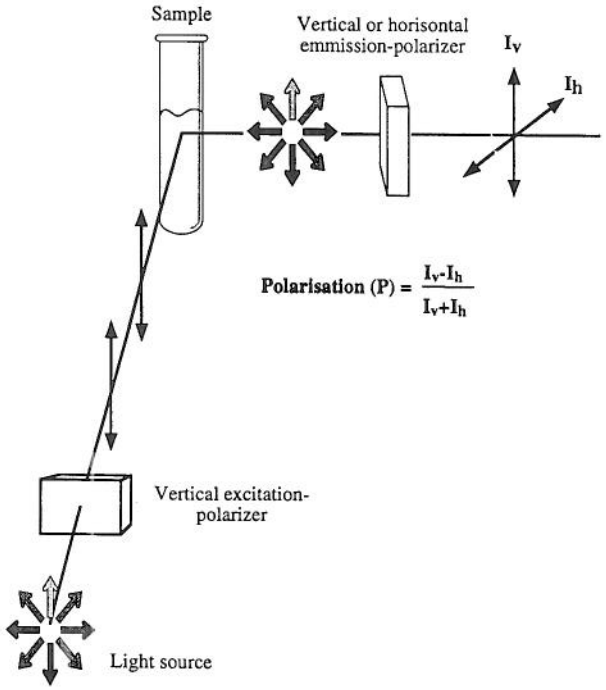

Fig. 1. Measurement of fluorescence polarisation

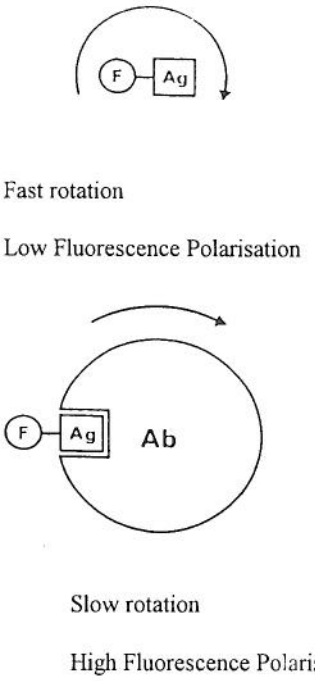

Fig. 2. Schematic illustration of the principle of fluorescence polarisation immunoassay

There exist several theoretical equations relating some other parameters to P and A, for simpler data manipulation. For example, the Perrin equations (5 and 6) describe the relationship between observed P or A, maximal Po or Ao for rigidly orientated molecules, the fluorescence lifetime (τ) of the fluorophore and its relaxation time (ϕ), could be presented as:

$$(1/P - 1/3) = (1/Po - 1/3)\,[1 + (\tau/\phi)] \qquad /5/$$

$$A = Ao\ /\ [1 + (\tau/\phi)] \qquad /6/$$

If the molecules are rotating rapidly (it means shorter rotational correlation time) relative to the fluorescence lifetime, the polarisation will approach zero value. If the molecules are rotating slowly (it means long rotational correlation time) relative to the fluorescence lifetime, the polarisation will approach its maximum value. It should be mentioned that the measurement of A does not provide additional information over the measurement of P, whose values are higher than those of A (Eqs. 2 and 3) although in arbitrary units. Therefore, the parameter P, expressed in mP, is more often measured and used for the immunoassay.

FP is a measure of the time-averaged rotational motion of fluorescent molecules. It is fortunate that molecular rotational relaxation time in solution is similar to typical fluorescence lifetime, because this fact enables the determination of the changes in molecular size. The relation between P and molecular size is expressed by Perrin equation, which could be written in the form:

$$1/P = 1/Po + (1/Po - 1/3)\,(RT/V)\,(\tau/\eta) \qquad /7/$$

where R = the gas constant, T = absolute temperature, V = molar volume of the rotating unit, and η = viscosity of the solution. From a practical point of view, it is important to keep in mind that decreasing the temperature, the value of P increases. As a consequence of this, the value of P increases with increasing the viscosity. At constant temperature and viscosity of the solution, P will be directly dependent on the molecular size of the fluorophore. For small molecules with high rotation, the values of P are lower, and opposite, for bigger molecules the measured values of P are higher (Fig. 2).

Principle of Fluorescence Polarisation Immunoassay

The FPIA, as any immunoassay for small molecules, is a competitive method based on detection of the difference of fluorescence polarisation between a small fluorescent-labelled antigen and its immunocomplex with a specific antibody. If the analyte is not present in the sample, the tracer will be completely bound to a specific antibody and the fluorescence polarisation value (P) will be higher (typically about 150–300 mP). If the analyte concentration in sample is significantly higher than the concentration of the tracer, the antibody binding centre will be occupied by the analyte, and the tracer will be free. This means that the P value of the reaction mixture will be lower (typically about 30–60 mP). The example of the FPIA standard curve obtained for 2,4-D pesticide is presented in Fig. 3, while dynamic ranges of FPIA for different pesticides and their acronyms are listed in Table 1.

It should be pointed out that the FP can be also useful for antibody assessment. In this case the FP measurement is performed after mixing of the antibody solution in several dilutions with a constant aliquot of the tracer

Table 1. Minimal detectable quantity and working ranges for FPIA of some pesticides and biologically active compounds

	Min. detectable quantity / ng	Range of detection c / μg mL^{-1}	Reference
Pesticide name			
Atrazine	0.1	0.001–1	*27,28*
2,4-Dichlorophenoxyacetic acid (2,4-D)	5	0.1–100	*28–33*
[(±)-2-(2,4-Dichlorophenoxy)propanoic acid] (Dichlorprop, 2,4-DP)	30	0.01–100	*34*
Isoproturone	4	0.5–10	*35*
2-Methyl-4-chlorphenoxyacetic acid (MCPA)	500	2–200	*36*
2-Methyl-4-chlorbuthyric acid (MCPB)	50	1–100	*37*
Methabenzthiazurone	0.4	0.02–5	*38,39*
Paraquat	1.5	0.025–2	*26*
Propazine	0.5*	10–1000**	*40*
Simazine	0.15	0.001–1	*28, 41*
Triazine	0.25	0.001–1	*42,43*
2,4,5-Trichlorophenoxyacetic acid (2,4,5-T)	4	0.2–10	*28, 44, 45*
Drugs			
Aminoglucoside	2	1–50	*46*
2-Aminobenzimidazole (degradation product of Benomyl)	0.3	0.001–0.1	*47*
Benzylpenicillin	2	0.1–10	*30*
Chloramphenicol	5	0.1–10	*30*
Gentamicine	2.5	0.05–0.8	*25, 48*
Potato Glycoalkaloids	10*	20–200**	*49*
Sulphamethazine	0.2	0.05–1	*30, 50*

* amount in pmol, ** concentration in nM

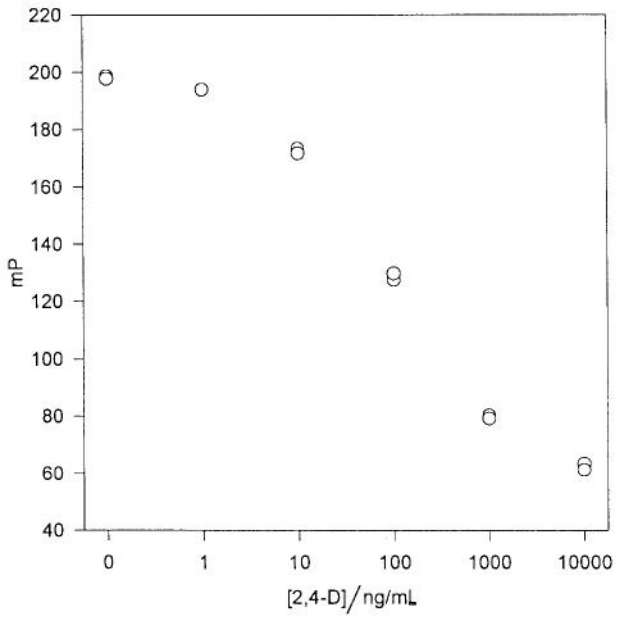

Fig. 3. FPIA standard curve for 2,4-D pesticide

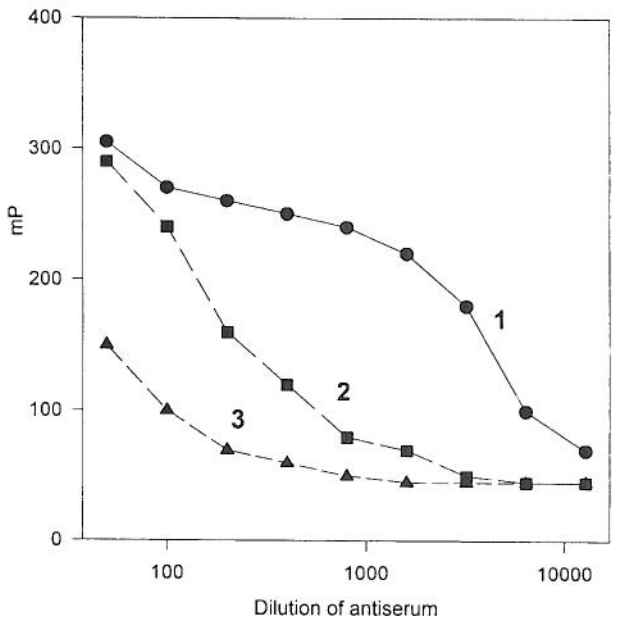

Fig. 4. Dilution curve of antiserum to methabezthiazurone by fluorescence polarisation technique (1 and 2,antiserum for two different immunogens, 3,non-immune serum)

solution. A typical antibody dilution curve is presented in Fig. 4. The titer of the antibody, defined as the dilution of antibody which gives 50% of tracer binding, is a quality characteristic of an antibody and depends on the concentration and affinity of the antibody. The non-specific binding of non-immune serum after dilution more than 100 times is negligible (Fig. 4). Therefore, the fast determination of the titre of an antibody by FP technique is a very useful tool for antibody assessment, if different antibodies have to be compared.

Again, the FPIA method is a homogeneous method, which is carried out in solution. From a practical point

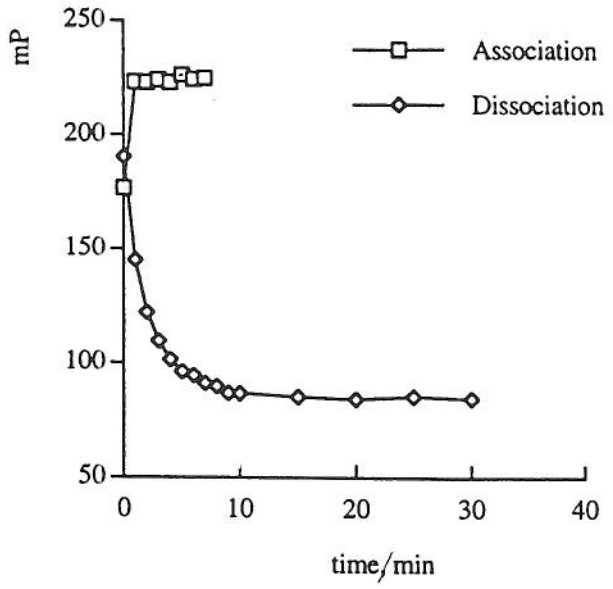

Fig. 5. Kinetics of association of fluorescein labelled 2,4-D and specific antibody in solution and dissociation of tracer from immunocomplex with excess of antigen measured using fluorescence polarisation technique

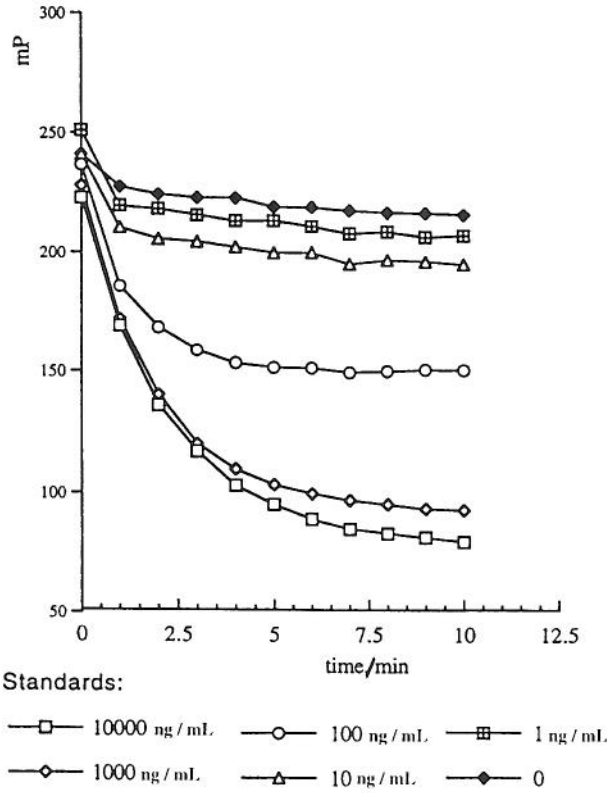

Fig. 6. Kinetic curves for single-reagent FPIA of 2,4-D measured at different times after addition of standards to pre-mixed tracer/antiserum reagent

of view, the performance of FPIA is a sequential addition of the sample (or standard), tracer and antibody solutions. After a brief incubation (several minutes or even seconds) the FP are measured and the concentration of analyte in the sample is calculated. The FP depends on viscosity and temperature, and this must be kept constant within few degrees. The kinetics of the immunoreaction in solution is so fast (Fig. 5), that equilibrium in the reaction mixture is reached in minutes or even in seconds. Therefore, it is not necessary to make an incubation step in FPIA, as it is for other immunoassay methods. As a consequence of this, total time for detection of an analyte using FPIA is only several minutes, depending mainly on the time of pipetting.

Advantages and Limitations of Fluorescence Polarisation Immunoassay

The FPIA have many advantages common for immunoassays. Moreover, the FPIA has several unique characteristics specifically related to the use of fluorescein as the label and for measure of FP as the analytical signal.

At first FPIA is a homogeneous method which does not need any separation or washing steps. Secondly, FPIA is a simple and fast method because all what this method requires is the addition of sample, tracer and antibody solutions and measurement of FP, after few minutes or even seconds of incubation. Indeed, the only technically difficult step is the need for accurate pipetting of the reagents. Therefore, FPIA method is very precise, the coefficient of variation (CV) is usually < 3–5% (see Fig. 3) and the method could be easily automated. Today several fluorimeters with a polarisation unit are on the market (Perkin-Elmer LS-50, Merck VITALAB, SLM-8000 etc.) and they are fully automated like Abbott TDx, Abbot IMx and Roche Cobas Fara II analysers.

The synthesis of fluorescein labelled hapten (tracer) could be achieved under gentle and simple conditions using commercially available fluorescein derivatives (Molecular Probe or other companies). More frequently used derivatives are: fluorescein isothiocyanate (FITC), (aminoacetoamido)fluorescein, 4'-(aminomethyl)fluorescein hydrochloride, 5-(4,6-dichloro-triazinyl)aminofluorescein (DTAF), carboxyfluorescein and many others. The tracers are stable in storage and retain the ability of fluorescein to fluorescence with high quantum yield and immunoreactivity of the hapten.

As a consequence of the tracer's high stability and simplicity, the FPIA standard curve is very constant, minimising the need for frequent measurement of multiple standards routinely performed in an assay. This also is a very important advantage of FPIA when only one or few samples have to be measured.

As any analytical method, FPIA has some disadvantages, or more correctly limitations. The sensitivity of FPIA is not so good as for ELISA. The minimum detectable quantity of FPIA is between 0.1 and 10 ng of analyte, and the working range for FPIA is in the ng/mL to μg/mL concentration interval (Table 1). The FPIA method is matrix dependent, needs special instrumentation for FP measurement and could be applied first of all for detection of small molecules. It is essential to have a clear understanding of the limitations of any

currently used analytical technique, because today's instrumental development is so fast that many disadvatanges might be improved in the very near future.

Recent innovations and an outlook on FPIA

Single-reagent FPIA

The FPIA method is very simple in performance and its rate determining step is the pipetting. Anyway, this method could be even more simplified by using the single-reagent format, which was developed for the first time for abused drugs *(53)*. The single-reagent FPIA is a pre-equilibrated solution of antibody with tracer, which could be used as a direct immunoreagent for the measurement of displacement of tracer from immunocomplex after sample addition. The couple of antibody and tracer for a single-reagent format must be chosen such that it has a fast dissociation kinetics (Fig. 5). The change of FP depends on the concentration of an analyte in the sample and the time of displacement. For semi-quantitative assays, the results could be obtained after 2–3 minutes of incubation. A precise FPIA standard curve using single-reagent could be obtained after 15–30 min. Recently, the single-reagent FPIA was developed for detection of methabenzthiazurone *(39)* and some other pesticides *(54)*. This methodology gives quite unique advantages of FPIA in comparison with any other immunoassay procedure.

Stopped-flow FPIA

As in any homogeneous immunoassay, the main limitation of FPIA, when it is applied to the analysis of real samples, is its relatively low sensitivity. The high detection level for this method is a result of the relatively higher level of the background signal, which is caused partly by scattered light and partly by the sample matrix. However, an alternative approach to avoid or minimise this effect is to use the initial rate of the immunochemical reaction as an analytical parameter instead of the signal obtained when the reaction reaches or it is close to the equilibrium. Since the competitive antigen-antibody reactions are usually very fast (Fig. 5), kinetic data can be obtained using stopped-flow (SF) mixing technique. The SF allows to perform the measurements shortly after mixing the reagents, and, in addition, enables to automatize this step of the analytical process. Both single-reagent and SF FPIA have shown their usefulness in clinical analysis for determination of therapeutic drugs and monitoring of their abuse *(55–56)*, yielding lower detection limits than conventional FPIA. The first application of SF FPIA technique in environmental and food analysis was made recently by developing a method for the determination of 2,4-D *(57)* and atrazine *(58)* in river water, orange juice and white wine samples. The SF FPIA method reduces the time of reactant manipulations and shows a high sample throughput, as the measurement step takes only one second. Moreover, compared with the conventional FPIA method, the kinetic methodology allows direct analysis and decreases 10-times the detection limit because the dynamic measurement is obtained at the beginning of the reaction between the tracer and antibody, thus avoiding or minimising the background signal and the potential interferences from the sample matrix.

FPIA in organic solvent

Reverse micellar systems of surfactants in non-polar organic solvents are known as homogeneous organic media which are able to solubilise biologically active substances like antibody, giving optically clear solutions. Recently a FPIA method has been developed to analyse pesticides using the specific antibodies solubilised in reverse micelles of sodium bis(2-ethylhexyl)sulfosuccinate surfactant in octane (Aerosol OT). The specific activity of the antibodies is retained after their solubilisation in reverse micellar systems. The advantage of these systems is that the analyte can be added when dissolved in a non-polar organic solvent. The performance of FPIA for 2,4-D (59), atrazine *(60)* and propazine *(40)* in reverse micellar systems was described. The detection limit of FPIA in organic solvent is comparable with that in aqueous medium.

Another perspective way for an improvement of FPIA performance is to extract the sample with a water-miscible organic solvent as methanol, acetonitryl or others. As it was found for a FPIA of 2,4,5-T pesticide *(45)*, the specific antigen-antibody interaction tolerated up to 10% of the water-miscible solvent in the mixture reaction. Therefore, the analyte from complex food or environmental samples could be directly measured in the organic extract by FPIA.

Instruments for FPIA

Probably the main factor which impedes wider application of FPIA is the scarce availability of sensitive polarisation fluorimeters, as compared with the ubiquitous presence of UV/VIS spectrophotometers used for ELISA. Fortunately, relatively less expensive polarisation fluorimeters are now available on the market. The new instruments such as FPM-1 (Dynatech) and Beacon 2000 (PanVera) are relatively cheap (about 16000–20000 US $). General information and currently updated bibliography are included in the Application Guide Manuals of these instruments while additional informations can be found in WWW Home Page: http://www.jolley.com and http://www.panvera.com. These new generations of instruments are more sensitive for FP detection and, therefore, the sensitivity of FPIA will be significantly improved *(61)*. Moreover, the same instrument can be used for kinetic measurement and calculations of affinity constants for antibodies.

Structure of immunoreagents for FPIA

The antibody is a key component for the specificity and sensitivity of immunoassays. The structure of the labelled antigen (tracer) also affects the sensitivity. Using the same antibody, the analytical parameters of FPIA could be improved by careful choice of the tracer's structure *(28)*. In some cases the sensitivity is greater using the shortest chemical "bridge" between the antigen and the fluorescent label *(27,30,34)*. Labelled antigens, that were structurally homologous or heterologous to the primary target analytes, were investigated and results indicated that FPIA is more sensitive when structu-

rally heterologous tracers were used *(28)*. The affinity of antibodies to atrazine are strongly dependent on the immunogen used for antibody production which can be detected by FP method *(61)*.

Conclusions

The FPIA for pesticides is a simple, fast, accurate and cost-efficient method for environmental and food safety control. Of course, the FPIA is not restricted only to detection of pesticides. A lot of articles have been recently published for validation of FPIA for drugs and hormones *(62,63)*. The papers dealing with development of methodology of FPIA for barbiturate *(64)*, amitriptyline and nortriptyline *(65)*, imipramine and desipramine *(66)*, thyroxine *(67)*, progesterone *(68)*, methamphetamine and benzphetamine *(69)*, methadone *(70)*, N-desmethylzopiclone *(71)*, tobramycin *(72)*, homocysteine *(73)* and epitope of *Neisseria meningitidis (74)*, are recommended to the readers. More detail information about recent FPIA publications could be available from Medline or other data bases. Any question concerning FP could be disscussed with a fluorescence polarisation group (E-mail: fluorescence-polarization@itis.com).

The same principle of FPIA, which uses antibodies as the recognition element was applied for a protein binding assay for detection of the SH2 domain of tyrosine kinase pp60 *(75)*, and for biotin in a biotin-conjugated protein *(76)*.

The FPIA method could be developed not only for small haptens, but also for high-molecular-weight antigens based on a long-lifetime Ru-ligand complex *(77)* or using synthetic peptides as tracer antigen *(78)*.

One interesting application of FPIA is a fast detection of specific serum antibodies to *Mycobacterium bovis* extracellular protein MPB70 *(79)* and to *Brucella abortus (80)*. This type of immunoassay should be applied to the diagnosis of other infectious diseases, especially those in which the causative agents induce an antibody response in the host. The demonstration of such response by fast FPIA method is a new and very important indicator of the infection *(79)*.

The principle of FP is very powerful not only for FPIA method, it could be also used for measurement of specific protease activity *(81)* and detection of proteases and their inhibitors *(82)*. In the last years, FP is more and more applied for any type of interaction in which the fluorescein-labelled ligand changes its molecular size. As for example, rapid detection of complementary and mismatched DNA sequences *(83)* or analysis of protein-DNA and other bio-molecular interactions using fluorescence polarisation were recently reviewed *(52,84)*.

The FP technique is also used for strand displacement amplification (SDA) and transient-state fluorescence polarisation detection of *Mycobacterium tuberculosis* DNA *(85)* and of *Chlamydia trachomatis* DNA *(86)*. Two reviews dealing with applications of FP method for cells and molecular biology *(87)* and protein-protein interactions *(84)* have been recently published.

The FP technique can be used not only as analytical methods, but also as a tool to determine microviscosity and structural order in food systems (88). The use of FP to probe the structure and aluminium complexation of three molecular weight fractions of a soil fulvic acid was also reported (89).

At the end of this review, the author of this article would like to point out that the hormone and drug detection for medical diagnostic in the 1960s and 1970s was performed mainly using chromatographic methods, while today they are generally measured by immunoassay methods. In the author's opinion the same can happen in food control, and in the nearest future the routine detection of certain pesticides can be done mainly by immunoassays methods. Moreover, if therapeutic drug monitoring and abused drug screening tests are today performed more by FPIA than by ELISA, the same trend can be expected for pesticide determination, because FPIA is today, by the author's opinion, one of the most promising technique in environmental and food analytical chemistry.

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Flurescencijsko-polarizacijska imunoodređivanja pesticida i biološki aktivnih spojeva pri praćenju kakvoće hrane i motrenju onečišćenja okoliša

Sažetak

Dan je pregled (od 1989. godine do danas) fluorescencijsko-polarizacijskih imunoodređivanja (FPIO) pesticida i biološki aktivnih spojeva. Navedena FPIO-metoda je brza, jednostavna, ne zahtijeva prethodna odjeljivanja; analit se određuje u homogenom mediju, a temelji se na kompetitivnom imunoodređivanju pri kojemu se mjeri fluorescencijskom polarizacijom razlika udjela slobodnog i na antitijelo vezanog antigena označenog fluoresceinom. U radu su navedene prednosti, ograničenja i najnovija unapređenja te metode.