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ENZYMATIC BIOSENSORS FOR THE ENVIRONMENTAL ANALYSIS OF PESTICIDES

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1. Introduction

Pesticides have been a common part of agricultural production for many years and thus pose a serious problem both in the processing of raw materials in the food industry and in direct consumption. They are used primarily to control pests during crop production, to treat crops in storage, or to prevent contamination during transport and sale. Examples of model pesticide substances are shown in Figure 1.

Because each crop is affected by a different pest mix, we may find multiple products containing different pesticides when determining the pesticide content in a sample. Regulations on pesticide use are harmonised within the European Union. The list of pesticide substances authorised for use and distribution in the Czech Republic is published by the Central Institute for Supervising and Testing in Agriculture in its regular bulletin[1].

A major problem is the persistent pesticides (currently, or even no longer used), which can circulate through the food chain by depositing in adipose tissue (e.g. DDT) or accumulating in soil or ground or surface water (glyphosate[2] , see Figure 1). Therefore, it is necessary to monitor the pesticide content of crops for consumption to avoid adverse health effects.

IUPAC defines a biosensor as a chemical detection device that uses an electrical signal, optical signal, or heat generated during a specific biochemical reaction mediated by isolated enzymes, immunosystems, tissues, organelles, or whole cells[3]. The ongoing enzymatic reaction, the presence of an antigen for the selected antibody, or the measurement of the electrochemical signal in relation to solutes in the sample play a crucial role. The basic element of a biosensor is the bioreceptor element (BE), according to which biosensors can be divided into catalytic and affinity biosensors depending on the interaction with the target molecule. The BEs of catalytic biosensors (which include enzymes, living cells, or tissue cultures) respond to changes in the environment and adjust their activity in relation to their environment. On the contrary, the BE of an affinity biosensor (antibody/antigen, nucleic acid, etc.) directly signals the presence of a particular molecular or cellular structure[4].

The most used enzymes for the determination of pesticides are discussed, together with an overview of the techniques used. The emphasis has been placed on the search for possible alternatives to enzymes widely used, as cholinesterase (ChE). In this paper, the reader will also find comments on some successful concepts suitable for field analysis developed mainly in the last 5 years.

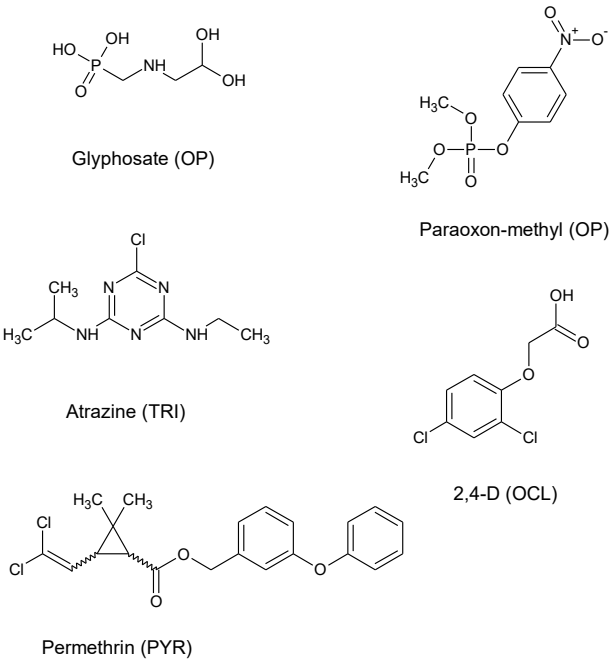

Fig. 1. **Structural formulae of different pesticide substances.** OP – organophosphate pesticides, TRI – triazines, OCL – organochlorine pesticides, PYR – pyrethroids

2. Enzyme as a biorecognition element

The principle of pesticide determination using enzyme biosensors is based on enzyme inhibition or enzyme-catalysed chemical conversion of the substrate, the pesticide. In biosensors, enzymes are bound to a sensing platform, e.g., a solid surface or a suspension of particles can be used as a substrate. For a more detailed overview of the techniques used for immobilisation of the enzyme on surfaces, the reader may refer to ref[5]. A detailed overview of the biosensors according to the used BE, with the detection principle and detection limit of the respective substance, is given in Table I. For interest, the determination time is also given with the preparation time, which together hardly exceeds 60 min. A graphical overview of the enzymes used in biosensors for pesticide determination is given in Fig. 2 together with the type of target substances.

2.1. Biosensors based on enzyme inhibition

Even in recent years, enzyme biosensors for pesticide screening based on one of the two well-known and closely

Table I
Examples of enzymes used in biosensors for pesticide determination

Enzyme	Pesticide	Converter	LOD	Determination time (**D**etermination, **P**reparation)	Ref.
AChE	Carbofuran	optical (fluorimetry, quantum dots)	1,10 μg L^{-1}	D+P 30 min	54
	Malathion	electrochemical (amperometry)	99 ng L^{-1}	D+P 14 min	55
		Optical (ambient light sensor in smartphone)	0,45 mg L^{-1}	D 1 min, P 15 min	56
	Chlorpyrifos	Optical (ambient light sensor in smartphone)	3,3 mg L^{-1}	D 1 min, P 15 min	56
		electrochemical (amperometry)	0,2 μg L^{-1}	D 1 min, P 10 min	57
	Diazinon	optical (fluorimetry, nanoparticles)	0,05 μg L^{-1}	D 6 min, P 55 min	43
	Dichlorvos	electrochemical (amperometry)	0,28 μg L^{-1}	D immediately, P 15+15 min	58
	Permethrin	electrochemical (amperometry)	3,17 mg L^{-1}	–	59
	Parathion	optical (Hybond N+ colorimetric test strip)	1 μg L^{-1}	D 15 min, P 30 min	60
	Paraoxon-ethyl	optical (pH test strip)	13,8 μL^{-1}	D 2 min, P 10 min	37
ALP	2,4-D	electrochemical (amperometry, printed electrode)	50 μL^{-1}	D 2 min, P 5 min	14
	Acephate (specifically)	optical (fluorimetry (FL), spectrophotometry (SF))	0.4 μL^{-1} FL 0.9 μL^{-1} SF	D < 30 min, P 15 min	61
BChE	Paraoxon	electrochemical (amperometry, printed electrode)	2 μL^{-1}	D 2 min, P 5 min	14
	Dichlorvos	optical (spectrophotometry, optical fibre)	5.2 μL^{-1}	D 2 min, P 1 day	62
Lipasa	Paraoxon-ethyl	optical (colorimetry)	10,9 μg L^{-1}	D up to 20 min, P 20 min	12
	Parathion-methyl (specifically)	electrochemical (diff. pulsed voltammetry)	17,6 μg L^{-1}	_	63
OPH	Parathion-methyl	electrochemical (square-wave voltammetry)	2,6 μg L^{-1}	D 30 s, P overnight	64
Tyrosinasa	Atrazine	electrochemical (amperometry, printed electrode)	–	D 2 min, P 5 min	14
	Glyphosate	electrochemical (amperometry, printed electrode)	1,1 μg L^{-1}	D 1 min, P < 20 min	18
Ureasa	Dimethoate	optical (fluorimetry)	2 μL^{-1}	D 10 min, P 45 min	65
	Glyphosate	electrochemical (potentiometry)	0,5 mg L^{-1}	D 15 min, P 3 min	22

AChE – acetlycholinesterase, ALP – alkaline phosphatase, BChE – butyrylcholinesterase, OPH – organophosphate hydrolase, SPR – surface plasmon resonance

related ChEs, acetylcholinesterase (AChE, E.C. 3.1.1.7) and butyrylcholinesterase (BChE, E.C. 3.1.1.8), have retained their popularity. They are serine hydrolases that catalyse the hydrolysis of the neurotransmitter acetylcholine in the nervous system[6], BChE also acts as a detoxifying enzyme[7]. Unlike BChE, AChE is inhibitable by excess substrate, which is crucial to consider when prototyping a biosensor and known as a potential disadvantage[8]. Both ChEs are used in biosensors for the determination of organophosphate (OP) and carbamate (CM) pesticides[9], which covalently bind at the active site via the amino acid serine.

The search for additional enzymes and approaches for pesticide analysis is ongoing. Figure 2 shows that ChE-based biosensors can already be partially replaced by other enzymes. In the following lines, we will briefly introduce them.

An alternative to cholinesterase biosensors for organophosphate determination can be, for example, esterase 2 (EST2, EC 3.1.1.1) isolated from the thermophilic bacterium *Alicyclobacillus acidocaldarius*[10]. This is a more stable enzyme with similar catalytic properties. Its active site is covalently modifiable by a mechanism similar to that of ChE. EST2 inhibitors esterify the amino acid serine at the active site of enzyme[11].

Furthermore, worth mentioning are lipases – another potential substitute for ChE. Lipases also belong to the group of serine hydrolases. A representative is triacylglycerol-acylhydrolase (E.C. 3.1.1.3), which appears to be a more affordable replacement for ChE. It can be isolated from bacteria, such as the psychrophilic bacterial strain *Psychrobacter sp.* originally found in Antarctica, whose lipase has relatively strong activity[12,13].

Other enzymes used in biosensors for the determination of pesticides are alkaline phosphatase (ALP), tyrosinase (E.C. 1.14.18.1) or urease.

ALP (E.C. 3.1.3.1) catalyses the hydrolysis of phosphoric acid monoesters. There are several types of ALP depending on the pH of the environment in which they are commonly found. Acid and alkaline phosphatase can be found in the human body. They are known as biomarkers in clinical biochemistry. For the determination of pesticides by alkaline phosphatase, 1-naphthyl phosphate can be used, whose hydrolysis by ALP produces electroactive 1-naphthol, the activity of ALP is influenced, for example, by 2,4-dichlorophenoxyacetic acid, an organochlorine pesticide (OCL) known by the acronym 2,4D (ref.[14–16]).

Tyrosinase is a metalloenzyme with two copper atoms in the active centre. As monooxygenase, it catalyses the hydroxylation of monophenols to *o*-chinones with *o*-diphenols as intermediates[17]. A fungus-isolated tyrosinase is used for biosensing, where the change in electrical current is detected by electrochemical reduction of *o*-chinones to *o*-diphenols[14]. Tyrosinase inhibitors include the herbicides atrazine, glyphosate, and 2,4-D. Except for glyphosate, these are its competitive inhibitors[14,18,19].

Urease (E.C. 3.5.1.5) is a naturally occurring enzyme produced by plants, bacteria, and fungi[20]. It has two active sites in its molecule, and at the centre of each active site is a nickel atom. Urease catalyses the conversion of urea to ammonia and carbon dioxide in two steps. Its inhibitors include the herbicides glyphosate[21,22] and atrazine, which is a non-competitive inhibitor[23]. An interesting finding is that urease activity in soil will also be affected by the presence of microplastics[24]. This interference will have to be considered in the design of biosensors for longer-term monitoring of pesticides (for hours to days), e.g., in surface water.

2.2. Biosensors based on enzyme catalysis

As mentioned above, the second group of enzyme biosensors is based on the catalytic reaction of the target substance and the selected enzyme.

A representative of such an enzyme in biosensors for pesticide determination is organophosphate hydrolase, also known as phosphotriesterase (OPH or PTE, E.C. 3.1.8.1).

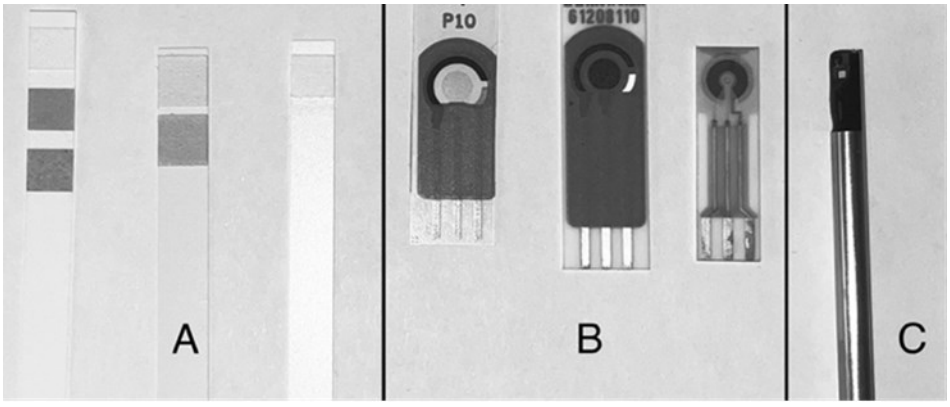

Fig. 2. **Examples of test resources.** A – Test strip for colorimetric pH determination with one, two and three test surfaces, B – Printed electrodes for electrochemical determination, C – ISFET electrochemical module

It is an enzyme of soil bacteria that catalyses the hydrolytic cleavage of molecules with P-F, P-O, PCN, and PS bonds (ref.[25,26]). These bonds are found in organophosphate pesticides such as paraoxon, chlorpyrifos, etc. Thus, OPH can be considered to some extent as an interesting alternative to ChE-based biosensors. The advantage of using OPH in biosensors is the possibility of its reusability compared to ChE biosensors when blocked by the substance to be determined[27].

2.3. Multi-enzyme biosensors

More than one enzyme can be found in biosensors for pesticide determination. We also encounter systems that works as a catalytic cascade. In addition to the main enzyme, the target of substance, other added enzymes act as intermediaries to transmit the signal, and thus refine the resulting response. These are usually electron producers for fluorescent probes or mediators of the redox reaction with accompanying colour changes. Examples of such systems together with a description of the principle of determination, detection limit, and detection time are shown in Table II.

Coupled enzymes in two- and multienzyme systems tend to be choline oxidoreductase or horseradish peroxidase. Choline oxidase (ChO, E.C. 1.1.3.17) is an oxidoreductase catalysing the two-step oxidation of choline to betaine and hydrogen peroxide. It carries flavin coenzymes as cofactors in its molecule. Horseradish peroxidase (HRP, E.C. 1.11.1.7), an enzyme with a molecule of hem as a cofactor, is one of the most widely used enzymes for biosensors. It is used as a fluorescent probe in biological assays (e.g. ELISA, immuno-histochemistry). It exhibits robust activity over a wide pH and temperature range[2,28]. In conjunction with quantum dots, HRP is used for fluorescence detection of organophosphates in combination with ChE (ref.[29]).

3. Signal detection of enzyme biosensors

In the case of an enzyme biosensor, the physicochemical transducer responds to the enzyme reaction that occurs. It converts the signal into a form that can be measured by the sensoric part of the biosensor. The most common transducers applied in biosensors are electrochemical and optical[30]. Patterns of possible transducers for immobilising enzymes such as BE can be seen in Figure 3.

3.1. Electrochemical transducers

Electrochemical transducers generate a signal depending on partial changes in electrochemical parameters such as voltage, current magnitude, potential difference, etc. Electrochemical biosensors come in two forms. Either it is a solid electrode with immobilised BE (ref.[31]), or BE is immobilised, e.g. on an ISFET module[32],

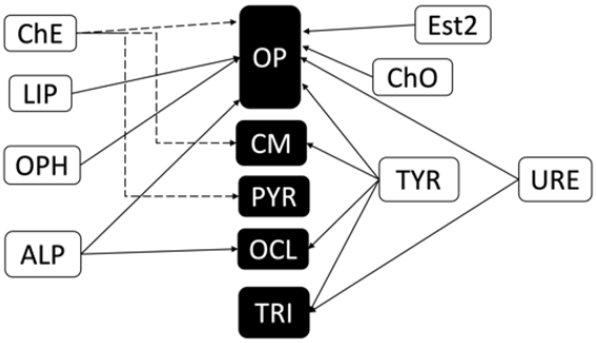

Fig. 3. **Enzymes and their target substances – possibilities for the determination of pesticides (black) with different enzymes (white),** ChE – cholinesterase, Cho – choline oxidase, Lip – lipase, est2 – esterase 2, OPH – organophosphate hydrolase, Tyr – tyrosinase, ALP – alkaline phosphatase, URE – urease, OP – organophosphate pesticides, CM – carbamate pesticides, PYR – pyrazines, OCL – organochlorine p., TRI – triazines

Table II
Examples of biosensor systems composed of multiple enzymes

Sensory system	Detection principle	Analyte (LOD detection)	Determination time (**D**etermination, **P**reparation)	Ref.
AChE/ChO/HRP	chemiluminescence	Chlorpyrifos-methyl (83.5 ng mm^{-2})	D 6 min, P < 15 min	51
AChE/ChO/TMB	AM and CM	Paraoxon (6 pg L^{-1} AM, 10 ng L^{-1} KM)	D < 15 min, P 30 min	66
ChO/HRP/ABTS	colorimetry	Paraoxon-methyl (14.33 mg L)$^{-1}$	D+P 12–60 min	67
AChE/HRP	differential pulse voltammetry	Monocrotophos (1 ng L^{-1})	D < 15 min, P 10 min	29
AChE/ChO/MnO_2	colorimetry	Paraoxon (0.5 mg L^{-1})	15 minutes	68

ABTS – 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), AChE – acetylcholinesterase, AM – amperometry, HRP –

Fig. 2C). Furthermore, we can use screen-printing techniques and print the electrodes on a substrate of different materials[33], these are then so-called screen-printed electrodes (SPE), see Figure 4B. Inspired by commercial Origami SPE electrodes, Arduini et al. used filter paper to create a folded disposable 3-enzyme biosensor for pesticide determination[14]. The electrodes were printed using graphite and AgCl inks. They fabricated the reaction space using a wax printing and drying oven. This biosensor allows measuring the activity of ALP (OCL screening of herbicide 2,4-D), BChE (OP screening) and TYR (TRI screening) amperometrically after a five-minute incubation. The reaction is triggered by the enzyme substrate, and the biosensor is connected by a clamp. The chronoamperometric analysis takes 2 minutes and the decrease in generated current is monitored. This measurement can give more information about the composition of the sample than a single enzyme analysis. Mishra et al. tested the possibility of creating an electrochemical system with a disposable examination glove[34]. They printed the electrodes on the index finger of the glove, which were connected via a strip of contacts, and the signal was transferred to a portable potentiostat using cables. A spot was created on the thumb to wipe the sample. From a technical point of view, this concept is already very close to the field analysis of surfaces of commercially grown crops. In addition to work using commercial BE signal conversion devices, we can also see projects involving "open-source" electronics (see Chapter 4)[35].

We can also encounter a somewhat unconventional approach to the use of standard devices. Tang et al. have been able to adapt a personal glucometer to determine OP (ref.[36]). A flaw in this approach is the need to adapt commercially available electrodes ready for capillary blood collection.

3.2. Optical transducers

Optical transducers are based on display the color changes as a signal in depends of BE response.

The optical transducers of the BE signal monitors the colour change associated with the BE response. For example, if there is a change in pH in a solution, an acid-base indicator, either dissolved or immobilised[37,38] (e.g. on a test strip), monitors the pH by the change in its colour. We could also determine the products showing that a chemical or enzymatic reaction has taken place colourimetrically (cleavage of Ellman's reagent to give a yellow orange product, hydrolysis of indoxylacetate to give the blue pigment indigo[13,39]). The signal is then detected by the naked eye, while a more accurate determination is made using instrumental techniques.

The second option for optical signal conversion from BE is to capture the emission of radiation associated with the ongoing reaction. Quantum dots are popular: semiconducting CdSe nanocrystals with a ZnS shell, or other hybrid surfaces that can emit a quantum of energy during an ongoing oxidation reduction process in their surroundings (e.g. oxidation of hydrolysed choline by ChO to betaine and hydrogen peroxide)[40,41]. More information on the preparation and properties of quantum dots can be found in ref.[42]. Another example is the reaction of the hydrolysis product acetylthiocholine, which displaces copper ions from the nanoparticles, triggering a fluorescent signal[43]. Fluorophores can also alternate in response depending on whether the enzyme is inhibited by the pesticide or not[44]. Thus, by comparing the two signals, the analyst can have an even clearer indication of the sample content compared to the situation where he is observing the rate of change in fluorescence when there is a weak contamination by a single fluorophore.

4. Use of commonly available electronics

However, in the analysis with biosensors, we do not have to rely only on expensive equipment and analytical instruments, which research on newly designed devices is often carried out, and the analysis in the field will not be possible by its design.

For determinations outside the lab, we can use a mobile phone[45], or build our own device fitted with simpler electronics[46,47]. To improve the optical biosensors, we can use 3D printing and prepare aids to record the signal: chambers for photography, and finally the bodies of the analysers themselves[11,47–52]. Printing of reaction arrays for optical determination of enzyme activity is another possibility for preparing the determination of contaminants outside the laboratory setting. (An example of 2D and 3D printed arrays for colorimetric measurements can be found in papers ref. [46,53]). We can also turn a smartphone into a data collector and determine contaminants using other simple devices built on single-chip computers. There are already several promising portable devices usable as photometers or electrochemical analysers. Although they have some disadvantages so far, such as the fixed wavelength range of the light source or the need for certification for commercial operation, their advantages include that they are easy and cheap to manufacture, and that they can be tuned quite imaginatively to suit the needs.

A successful example of in-house design is the instrument developed by Chao et al.[51]. It allows the determination of organophosphates using the AChE/ChO/HRP enzyme system immobilised on the hydrogel disc. The testing was carried out on surface of vegetable samples and the concentration of chlorpyrifos-methyl was measured by chemiluminescence. A similar method of testing will be particularly advantageous for the screening of banned pesticides due to its high sensitivity.

5. Conclusions

Enzyme biosensors are still a valuable tool in pesticide analysis. The active search for efficient ways to detect pesticides can be boldly based on biosensors in environmental control, agriculture, food, or medicine.

They can be used to perform field analyses in units of minutes to tens of minutes. At the same time, they do not place such demands on sample preparation compared to chromatographic or MS systems[9]. A large proportion of new biosensors still rely on cholinesterases, but in recent years, they have been supplemented by urease, tyrosinase, lipase, choline oxidase, and organophosphate hydrolase. At the same time, there has been an important shift in the design of biosensor systems. Increasingly, "open-source" electronics are being used in the design of portable devices.

List of abbreviations used

2,4-D	(2,4-dichlorophenoxy)acetic acid
ABTS	2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid
AChE	acetylcholinesterase
ALP	alkaline phosphatase
BChE	butyrylcholinesterase
BE	biorecognition element
CM	carbamate pesticides
DDT	1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane
HR	Horseradish peroxidase
ChE	cholinesterase
ChO	choline oxidase
ISFET	ion-sensitive field effect transistor
OCL	organochlorine pesticides
OP	organophosphate pesticides
OPH	organophosphate hydrolase
PDMS	polydimethylsiloxane
PET	polyethylene terephthalate
PYR	pyrethroids
TMB	3,3′,5,5′-tetramethylbenzidine
TRI	triazines
TYR	tyrosinase
URE	urease

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Abstract

This review article describes the background of enzyme-based biosensors and discusses selected examples of pesticide detection using these platforms. Although cholinesterases are still the most common enzymes for the analysis of commonly used pesticides, alternative enzymes for commonly used pesticides are also important and are highlighted. This article shows the current status of enzyme-based biosensors for the analysis of pesticides in the environment and discusses the prospects for future developments, in particular the use of open source electronics as a promising interface for wireless biosensing in the environment.

Keywords: biosensor, enzyme, pesticide, point of care testing, environmental analysis, portable biosensors

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