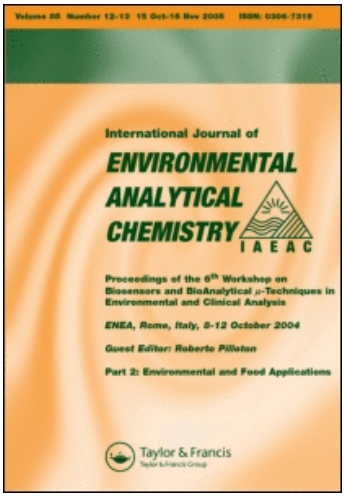

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Comparison of biosensoric and chromatographic methods for the detection of pesticides

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Comparison of biosensoric and chromatographic methods for the detection of pesticides

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Screen-printed biosensors with immobilized acetylcholine esterase (AChE) were used for measuring fruit and vegetable samples that had first been analysed using gas and high-performance liquid chromatography. The output signal for the biosensors is the current, which is used to calculate relative inhibition (RI), a measured quantity. RI is proportional to the inhibiting (toxic) effect of organophosphates and carbamates. Measurements with AChE biosensors are not easily reproducible. This problem is solved by the choice of an arbitrary toxicity standard of 1.25 μM Syntostigmin. Measurements were evaluated by the ratio of the relative inhibition of the sample against the relative inhibition of Syntostigmin. Results obtained from the biosensor match those of chromatography in 19 out of 38 total measurements made and for nine out of 19 positive samples. The confirmation rate was 50%. Future work must check the limit of 0.1 and the independent control of inhibiting pesticides contents after measurements using the biosensor.

Keywords: Acetylcholine esterase; Food analysis; Enzyme-based biosensor

1. Introduction

A number of methods are used for the detection of pesticides in food. They include immunoassays [1] chromatographic methods [2, 3], immunosensors, biosensors based on surface plasmon resonance (SPR), magnetic biosensors and biological methods [4]. This article compares standard gas and high-performance liquid chromatography analysis results, provided by the Czech Agriculture and Food Inspection Authority (CAFIA), against a method using screen-printed biosensors with immobilized

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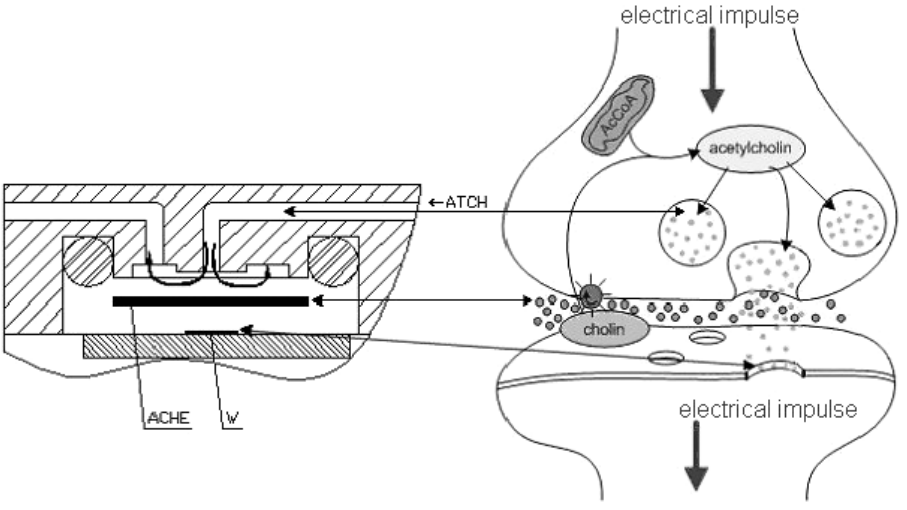

Figure 1. Comparison of artificial and biological synapses.

acetylcholine esterase (AChE). These sensors are underpinned by the sound physical principle of the well-reported interaction of AChE with organophosphorus (OP) and carbamate pesticides.

The basis of measurement using biosensors lies in the construction of an artificial model of a biological synapse (figure 1). It can be seen that the biological acetylcholine substrate is replaced with acetylthiocholine chloride (ATCh) in the sensor. This arrangement facilitates measurements because the decomposed substrate creates an electro-active substance, thiocholine, which releases electrons when combined with disulphide ions [5]. The result of this latter reaction is the current, which, at constant concentrations of acetylthiocholine, is proportional to the AChE activity.

In a biological synapse, the neuronal transmitter, acetylcholine, is released from the vesiculae as illustrated in figure 1. This natural biological process is simulated in the artificial synapsis, also shown in figure 1, where the equivalent process is represented by ATCh solution flow around the working electrode of the sensor. If an inhibitor is added to the input solution of ATCh, it competes with ATCh for active sites of AChE on the sensor surface. The rate of ATCh substrate decomposition due to the AChE catalysis is thus decreased, and this decrease is signalled, during the sensor measurement, by a decrease in output current value. This observed decrease in current value is exactly proportional to the lowering of the enzyme activity value.

Pesticide toxicity is determined by the exposure of laboratory animals to different concentrations of pesticides. The median lethal dose (LD_{50}) is then set and tabulated. It is a dose expressed in milligrams of pesticide per kilogram of tested animal, which kills 50% of tested organisms. The pesticide concentration in samples is measured and compared with tabulated values. For each pesticide, a toxicological study of its action must be undertaken.

The approach to toxicity is very different when using the principle of AChE inhibition. An AChE enzyme is isolated from an appropriate organism and immobilized on the working electrode of a sensor. Such an arrangement simulates what happens in the region of a biological synapse where the action of AChE-inhibiting

pesticides takes place. The current output values contain not only information about OP pesticide concentration values but also information about the OP reaction with AChE, possible important synergy effects, and stimulation or suppression of toxic action by other chemicals present in the sample. This wide-ranging information is directly measured as a current value, and in comparison with the measurement of concentration via GC and HPLC methods, such equivalent information can only be obtained by independent toxicological studies made on animals.

The output signal of biosensors is the current. The measured current was used for calculation of relative inhibition (RI). Relative inhibition is defined by equation (1):

$$\mathrm{RI} = \frac{\mathrm{d}I/\mathrm{d}t}{I_{\mathrm{ss}}}, \tag{1}$$

where I_{ss} is the steady-state current value after the substrate addition, and $\mathrm{d}I/\mathrm{d}t$ is the rate of current decrease observed after the addition of a sample containing pesticide [6]. It can be seen that the value of RI is proportional to the inhibiting effect of the organophosphates and carbamates. The value of RI is also proportional to the concentration value, but the constant of proportionality differs for each pesticide.

To quantify the above observations, it would be necessary to tabulate the values of constants of proportionality and determine the particular OP type by subsequent analysis. Immobilized AChE is a very sensitive biochemical system with many effects influencing its stability – ageing, humidity, temperature, etc. Consequently, it is difficult to reproduce measurement values with AChE biosensors. One approach is to adopt an arbitrary toxicity standard based on the behaviour of Syntostigmin measured under the same conditions as the sample. Measurements are evaluated as the ratio of the relative inhibition of the sample to the relative inhibition of Syntostigmin. Both sample and Syntostigmin are added within one measurement cycle.

Again, it is stressed that the artificial synapse simulates the toxic action of OP in a biological system, and its response also includes eventual stimulations, cross-effects, and matrix influences. It is suggested that the innovative step demonstrated by these measurements is that knowledge of RI values gives more information from a single measurement than concentration data obtained from GC/HPLC methods.

The approach of using screen-printed electrodes based on AChE for detection of AChE inhibiting pesticides is well known [7–10]. The soundness of the approach in this article is that samples of fruit and vegetables contaminated by pesticides, previously analysed by gas and high-performance liquid chromatography, have been used as the test samples.

2. Experimental

2.1 *Czech Agriculture and Food Inspection Authority (CAFIA) procedures*

CAFIA analyse their food samples using GC and HPLC. Preparation for GC analysis starts with ethyl acetate extraction and 2 min of homogenization using an Ultraturax Polytron. Subsequent steps include PL gel filtration and evaporation at 35°C on a rotary vacuum evaporator. The sample is then transferred to a 1 : 1 ethyl acetate : cyclohexane mobile phase and cleaned-up by Gel Permeation Chromatography.

Table 1. Samples not containing AChE-inhibiting pesticides.

Sample no.	Matrix	CAFIA quantitative analysis	CAFIA concentration ($mg\,kg^{-1}$)
225	Lettuce	–	–
315	Apple	–	–
316	Kohlrabi	–	–
347	Cauliflower	–	–
349	Potatoes	–	–
385	Kohlrabi	–	–
130	Banana	TBZ	0.111
		Imazalil	0.153
178	Banana	TBZ	0.083
149	Cabbage	–	–

Finally, there is evaporation on a rotary vacuum evaporator and dilution with toluene. Detectors: NPD/ECD/MSD (neutral particles det., electron capture det., mass selective det.).

Samples for HPLC analysis are extracted and homogenized for 2 min with acetonitrile using an Ultraturax Polytron. This is followed by filtration and shaking with 4 : 1 dichloromethane : acetone before evaporation on a rotary vacuum evaporator at 35°C. The final step before injection to LC/MS is transfer into a methanol–water mixture.

2.2 *Brno University of Technology (BUT) procedures*

The Czech Agriculture and Food Inspection Authority (CAFIA) supplied BUT with frozen food samples together with quantitative and qualitative analysis of the pesticides in each sample. Samples were separated into two groups:

(1) Negative samples that did not contain pesticides inhibiting AChE; see table 1.
(2) Positive samples that contained AChE inhibiting pesticides; see table 2.

Anti-AChE sample activity was tested on prototype instruments being developed for this purpose within the ANTOPE project (Analyser of Toxicity of Pesticides, FD-K2/53, Ministry of Industry and Trade of the Czech Republic). The measuring system was very simple, consisting of a conventional electrochemical vessel covered by a lid, which carries the body of the micro-flow insert. This contained the cell for an acetylcholine esterase biosensor. One to 5% of the analysed liquid flows around the sensor (the cell was marked AS – artificial synapse), while the remaining 95–99% of the sample is pumped through the open channel ensuring intensive stirring of the solution. Additions of substrate and samples were injected into the reaction vessel. The liquid flow is illustrated in figure 2.

These measurements were amperometric following time–current dependence. The proposed evaluating biosensors and measuring device were as follows:

(1) equipment used: a Microflow System (MFS) incorporating an AChE biosensor AC1.W2.RS (BVT Technologies Ltd), a Bioanalyser, and a PC with the OFBio software;
(2) MFS content: 10 mL of phosphate buffer (3.3 mM KH_2PO_4, 63 mM Na_2HPO_4, pH 8.3).

Table 2. Samples containing AChE-inhibiting pesticides.[a]

Sample no.	Matrix	CAFIA quantitative analysis	CAFIA concentration ($mg\,kg^{-1}$)
133	Grapefruit	Captan	0.002
		Chlorothalonil	0.005
		Chlorpyrifos	0.19
		Dichlofluanid	0.02
		Methidathion	0.65
		Imazalil	0.59
		TBZ	1.06
		O-Fenylfenol	12.2
175	Mandarin	**Methidathion**	0.17
		Carbendazim	0.006
		TBZ	0.006
		Imazalil	1.53
		O-Fenylfenol	14.7
177	Grapefruit	**Chlorpyrifos**	0.09
		Metalaxyl	10.2
		Methidathion	0.19
		Phosalon	0.71
		TBZ	0.044
		Imazalil	1.05
		O-Fenylfenol	12.7
299	Apple	**Carbendazim**	0.21
		Captan	0.4
335	Kohlrabi	**Malathion**	0.07
345	Radish	**Carbendazim**	0.003
381	Apple	**Chlorpyrifos**	0.19
31	Orange	**Carbendazim**	0.002
		Imazalil	0.032
32	Grapefruit	**Carbendazim**	0.002
		TBZ	1.953
		Imazalil	0.032
60	Grapefruit	**Malathion**	0.05
		TBZ	0.023
		Imazalil	0.033
131	Tomato	Chlorothalonil	0.026
		Carbendazim	0.003

[a] Pesticides shown **in bold** inhibit acetylcholine esterase.

Additions were as follows:

(1) 20 µL of acetylthiocholine chloride (ATCh) substrate;
(2) 1 mL of filtered negative sample juice;
(3) 1 mL of filtered positive sample juice;
(4) 10 µL of Syntostigmin (AChE-inhibiting drug, active substance – neostigmin methyl sulphate).

The buffer was poured into the MFS vessel. A biosensor, with immobilized AChE, was inserted into the MFS cell and the system started. ATCh substrate was injected after current stabilization and decomposed to induce a current response. This was followed by a negative sample addition, which caused a rapid current decrease caused by the buffer and the substrate solution dilution in a 1 : 10 ratio. The positive sample and Syntostigmin were injected last, with Syntostigmin being used as an inhibition standard.

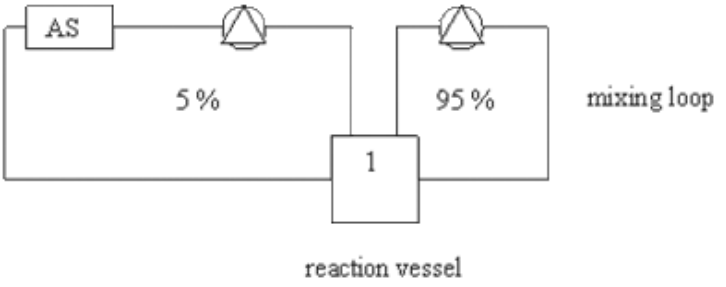

Figure 2. Liquid distribution in MFS.

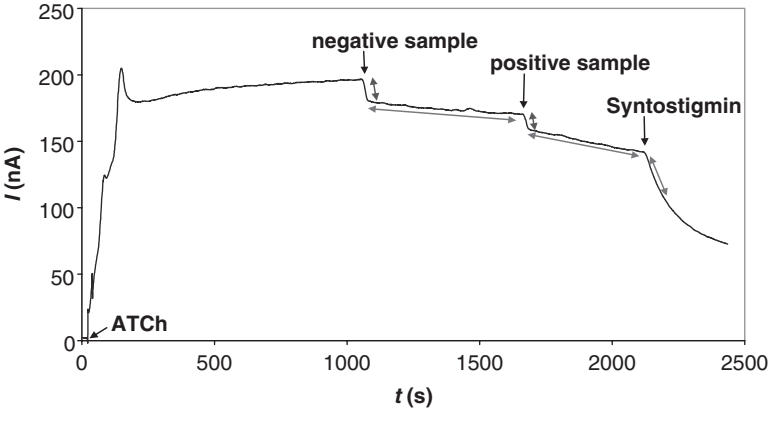

Figure 3. Graph from a run of the experiment.

A typical run of the experiment is shown in figure 3. Arrows, with legends, show the time of each addition. Vertical double arrows correspond to the dilution effect. Horizontal double arrows indicate the graph parts used to evaluate the inhibition effect.

3. Results and discussion

Two series of measurements were carried out, and the following findings were observed. CAFIA data identified only a small number of samples which contained OP or carbamate pesticides. This fact is consistent with the known decrease in OP usage in agriculture during recent years. Table 3 shows the analytical results of the biosensor measurements.

The first approach was to arbitrarily choose a limit of $0.1 = \mathrm{RI}_{\mathrm{sample}}/\mathrm{RI}_{\mathrm{toxicity\ standard}}$ to signify positive toxic effects (the toxic standard is 1.25 μM Syntostigmin). For this choice of standard, the BUT results matched those of the CAFIA data in 19 cases out of 38 measurements and also for nine out of 19 measurements for the positive samples. Figure 4 illustrates this in a descriptive way. Clearly, a detection rate of 50% is not satisfactory, and future work must check the validity of the 0.1 limit, and an independent control of inhibiting pesticide content after biosensor measurements must be established. In current experiments, it has been impossible to prove whether

Table 3. Results of biosensor analysis.

Sensor no.	Sample no.	Matrix	AchE inhibition[a]	ATCh response	RI_s[b] for sample (per second)	RI_0[c] for Synt (per second)	RI_s/RI_0
A-80	130	Banana	No	124	2.40E−04	3.40E−03	7.06E−02
	131	Tomato	Yes		4.90E−04		1.44E−01
A-81	130	Banana	No	101.5	8.00E−04	3.00E−03	2.67E−01
	131	Tomato	Yes		1.40E−03		4.67E−01
A-82	178	Banana	No	171	1.50E−04	1.20E−03	1.25E−01
	32	Grapefruit	Yes		1.10E−03		9.17E−01
A-86	149	Cabbage	No	174.5	6.30E−05	3.20E−03	1.97E−02
	31	Orange	Yes		1.30E−04		4.06E−02
A-88	149	Cabbage	No	122	4.40E−05	1.80E−03	2.44E−02
	60	Grapefruit	Yes		2.70E−04		1.50E−01
G-19	347	Cauliflower	No	86.2	4.34E−04	1.61E−04	2.70E+00
	177	Grapefruit	Yes		8.71E−04		5.41E+00
G-20	347	Cauliflower	No	85.6	1.53E−04	3.20E−04	4.78E−01
	177	Grapefruit	Yes		1.11E−03		3.45E+00
G-39	225	Lettuce	No	99.2	2.66E−04	0.001203	2.21E−01
	175	Mandarin	Yes		1.86E−03		1.55E+00
G-40	225	Lettuce	No	93.6	1.08E−04	1.28E−03	8.44E−02
	175	Mandarin	Yes		1.77E−03		1.38E+00
G-86	385	Kohlrabi	No	41.4	3.05E−05	1.33E−03	2.29E−02
	381	Apple	Yes		7.37E−05		5.52E−02
G-87	385	Kohlrabi	No	41.2	9.28E−06	1.50E−03	6.18E−03
	381	Apple	Yes		7.54E−05		5.02E−02
G-91	315	Apple	No	45.9	2.15E−04	1.59E−04	1.36E+00
	133	Grapefruit	Yes		1.82E−03		1.15E+01
G-98	315	Apple	No	122.7	3.15E−04	2.66E−04	1.18E+00
	133	Grapefruit	Yes		8.85E−04		3.33E+00
G-92	385	Kohlrabi	No	61.2	3.15E−04	1.16E−03	2.72E−01
	299	Apple	Yes		7.80E−05		6.72E−02
G-93	385	Kohlrabi	No	60.3	4.36E−05	1.01E−03	4.32E−02
	299	Apple	Yes		4.53E−05		4.49E−02
G-96	316	Kohlrabi	No	70.6	3.23E−04	3.11E−03	1.04E−01
	345	Radish	Yes		1.34E−04		4.31E−02
G-97	316	Kohlrabi	No	88.8	5.77E−05	2.21E−03	2.61E−02
	345	Radish	Yes		9.71E−05		4.39E−02
G-102	349	Potatoes	No	65	7.56E−05	1.24E−03	6.10E−02
	335	Kohlrabi	Yes		2.17E−05		1.75E−02
G-103	349	Potato	No	59.8	4.70E−04	1.84E−03	2.55E−01
	335	Kohlrabi	Yes		3.00E−05		1.63E−02

[a] Whether chromatographic analysis identified pesticides inhibiting AChE in the sample.
[b] RI_s: relative inhibition of sample.
[c] RI_0: relative inhibition of Syntostigmin – standard of toxicity ($c = 1.25\,\mu mol\,L^{-1} = 0.418\,mg\,L^{-1}$).

the observed discrepancy is caused by pesticide dissociation in the sample or by the inherent inaccuracy of the method using AChE biosensors.

Chromatographic measurements provide both quantitative and qualitative information, but their performance is very slow (one sample takes 2–3 days), and the technical operations involved require a very skilled workforce.

In contrast, measurements with biosensors can be carried out rapidly and without operator expertise, and provide information about the bio-toxic action of the sample on organisms, which is in addition to the knowledge of the concentration value. On the other hand, the biosensor data are limited, because they can only show that a sample contains AChE inhibitors and in this respect fall short of the information provided by qualitative chromatographic analysis. The task now is to find

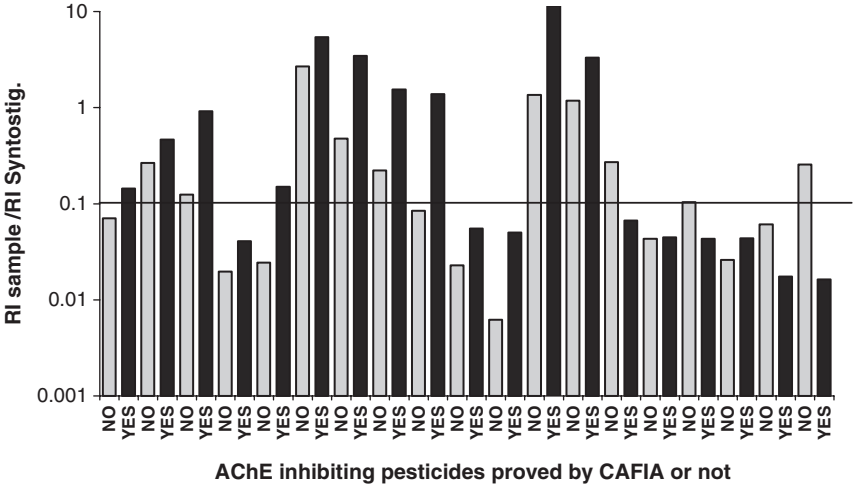

Figure 4. Comparison of chromatographic and biosensor results.

methodologies for obtaining better detection limits, and especially for the detection of compounds that are not soluble in water solutions. Again, it is stressed that the main advantages of measurements with biosensors are their fast performance (10–60 min) and handling simplicity.

4. Conclusion

In this initial study, it was demonstrated that an AChE biosensor is suitable for OP and carbamate pesticide detection in food samples. Future research will focus on evaluating the optimization of the biosensor method and include a wide range of different types of sample testing. Where CAFIA and BUT results are found to differ in value, another standard chromatographic analysis would be carried out. In this way, the content of inhibiting pesticides would be analysed again in order to demonstrate that the pesticides found in the first analysis were still intact in the sample and had not decomposed over the time of storage.

Acknowledgements

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