DETERMINATION OF ORGANOPHOSPHORUS PESTICIDES IN LIVER SAMPLES USING AN OPTICAL BIOSENSOR

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ABSTRACT

Biosensors are a promising alternative of the existing chromatographic methods as GC, HPLC, etc. They are fast, easy to use and provide fully acceptable values of monitoring like sensitivity, LOD, LOQ, etc. In order to construct an optical biosensor, AChE is immobilized as a target enzyme for organophosphorus pesticides (OPPs) onto new membranes synthesized by a sol-gel technology. The designed biosensor is tested for determination of chlorpyrifos-methyl, parathion, pirimiphos-methyl and diazinon in liver samples. An appropriate method for sample preparation is also developed. The new method is validated in accordance with document SANTE/11945/2015 covering the required criteria for pesticides determination. The biosensor developed can detect levels lower than 30μg/kg. The latter is the maximum residue limit (MRL) for pesticides in food. Km of 2,2x10⁻³ M for acetylcholine and 4 months life-time of the biosensor are obtained.

Keywords: biosensor, acetylcholinesterase, organophosphorus pesticides, toxic compounds.

INTRODUCTION

The organophosphorus pesticides (OPPs) are generally esters, amides, or thiol derivatives of phosphorus acids. Some of these compounds are still widely used in agriculture as insecticides in crop protection and in veterinary practice (Fig. 1).

They are easily hydrolysed and thus do not persist in the environment for a very long time. However, the possibility of their accumulation in the food chain combined with their high or moderate toxicity can cause a risk for human health [1].

Therefore, the maximum residue levels (MRLs) of those pesticides in foods are set in Regulation (EC) No 396/2005 [2]. All Member States must analyze samples following the National Monitoring Plan in accordance with Council Directive 96/23/EC of 29 April 1996 [3]. The latter refers to measures of monitoring certain substances and residues thereof in live animals and animal products. In order to be able to carry out the Monitoring Programs, the laboratories must have methods for analysis that meet the requirements of the European legislation. All those

Fig. 1. General structures of thiophosphates (chlorpyrifos-methyl, parathion, pirimiphos-methyl and diazinon studied as OPPs).
methods need to be validated. The specific requirements in respect to the analytical quality control and validation procedures for pesticide residues analysis in food and feed are written in SANTE/11945/2015 [4].

Traditionally, the sample preparation of complex matrices, such as liver, is carried out in as a sequence of extraction and clean-up steps. Ref. [5] summarizes the most widely used pesticide extraction technique for foods of animal origin. They refer to solid–liquid extraction (the traditional Soxhlet extraction method), accelerated solvent extraction (ASE), microwave-assisted extraction and matrix solid-phase dispersion. The clean-up steps are mainly performed by freezing centrifugation, liquid–liquid partitioning, gel permeation chromatography (GPC), solid-phase extraction, etc. Nowadays, the QuEChERS method (quick, easy, cheap, effective, rugged and safe) combining extraction and clean up procedure is widely used for foods of plant origin [6]. Following the extraction and the purification procedures, the pesticides need to be separated and further identified. Gas chromatography coupled with mass spectrometric detectors, such as single quadrupole, ion trap and triple quadrupole is adapted to analyse pesticide residues in foods of animal origin [5]. However, this technic is quite expensive. A biosensor is a possible alternative as a fast, portable and easy to use method for preliminary test of samples. The majority of these biosensors are based on the inhibition reaction of acetylcholine degradation to choline and acetic acid catalyzed by the enzymes acetylcholinesterase (AChE) or butyrylcholinesterase (BuChE) [7-10] (Fig. 2).

EXPERIMENTAL
Analytical standards
Four certified standards solutions were used: chlorpyrifos-methyl (of 98 % purity), parathion (of 99 % purity), pirimiphos-methyl (of 99 % purity) and diazinon (of 99 % purity). All of them were purchased from Dr. Ehrenstorfer (Germany). The working standard solutions were prepared at 6 levels - 0,0, LOQ, 50 %, 100 % and 150 % from the maximum residue level (MRL) for each compound according to their MRL in accordance with Regulation 396/2005 (2005). All standards were diluted with acetonitrile (gradient grade) supplied by Supelco (USA) [23].

Preparation of membranes and AChE immobilization
Acetylcholinesterase used in this study was isolated from Electrophorus electricus (electric eel) Type VI-S, lyophilized powder, 200 units/mg protein -1,000 units/mg protein. In addition the following reagents were used - polyamidoamine (PAMAM) dendrimer, ethylenediamine core, a generation 4.0 solution of the formula of [NH2(CH2)2NH2](G=4), dendri PAMAM(NH2)64 of a molecular mass of 14214.17, methyltriethoxysilane (MTES) and cellulose acetate propionate (CAP), all purchased from Sigma-Aldrich. 3g of CAP in 40ml chloroform were homogenized for 2 h (solution A).

1ml MTES was mixed with 3mL of ethanol and 1 drop of conc. HCl (solution B).
The hydrolysis reaction was conducted at a room temperature with vigorous stirring at 25°C for 1 h in a Becher glass. Solution A was combined with solution B and 100µl PAMAM dendrimer were added. The obtained mixture was stirred for 3 h, removed to a petri and dried at a room temperature.

The synthesized membranes were activated by treatment with 12.5 mL solution of formaldehyde (HCHO) in a phosphate buffer (0.1M pH = 7.5) at a temperature of 45°C and stirring for 4 h in a closed container. At the end of reaction time, the activated membranes were washed with distilled water. Finally, a covalent immobilization of enzyme AChE on the membranes was carried out by adding 1% of the enzyme solution in 0.1 M phosphate buffer (pH 5.5). It was spread over the surface of the hybrid membranes and put at 4°C in dark for 8 h.

The residual activity of the immobilized enzyme was measured analyzing the samples. It was converted to a relative activity (%) and compared with the relative activity of a blank sample. The inhibition percent was calculated as:

\[
100 - \text{relative activity of the sample (\%)} = \text{inhibition \%}.
\]

The results were obtained on the ground of a calibration curve obtained using samples spiked with an inhibitor (parathion) at 5 levels: LOQ; 0.5 MRL; 1.0 MRL, 1.5 MRL and 2.0 MRL. This was done with each series of samples.

**Enzyme activity determination**

The enzyme activity of AChE was determined on spectrophotometer VWR634-6001, UV-1600PC in accordance with the Worthington methodology [16]. The specific activity of free AChE was defined as:

\[
\frac{[\text{A blank} - \text{A test}]}{\text{mg}} \times \frac{270}{\text{A blank}}
\]

**Enzymatic assay**

The method used was based on ACh disappearance determined by using ferric-acethydroxamic acid complex. One unit of AChE activity was equivalent to the disappearance of one micromole of ACh per minute at 25°C.

**Sample preparation**

5g of the homogenized sample (swine liver) were placed in a 50 mL centrifuge tube. 5mL of the buffer-substrate solution were added as described in the Worthington Enzyme Manual [16]. The obtained mixture was put on vortex for 2 min followed by 10 min brake. The sample was then transferred using filter paper to a glass tube containing 100 mg of membranes with an immobilized enzyme. The obtained mixture was again subjected to vortex for 2 min. The solvent was separated from the membranes and then AChE activity was determined. The reactivation of the enzyme was performed with 1 mM 2-pyridinealdoxime methyl chloride (2-PAM) water solution for 15 min immediately after the enzyme inhibition.

Each sample sequence contained a blank sample for enzyme activity control and tree samples spiked of concentrations corresponding to LOQ, 0.5MRL and MRL levels from each of the targeted OPPs aiming to obtain a calibration curve.

**Method validation**

The validation was performed in accordance with the recommendations of SANTE/11945 [4]. Blank liver samples spiked with OPPs at levels corresponding to 0.5, 1 and 1.5 MRLs were used for evaluation of the recovery and the precision (repeatability and within-laboratory reproducibility). The spiked samples at each concentration level were analyzed in three series, each on the different day, and each in six replicates. The accuracy determined by the average recovery was calculated by comparing the determined concentrations of the spiked samples to their target level. The precision was determined by calculating the coefficient of variation (CV). The limit of quantification (LOQ) of OPPs was tested as the minimum concentration of the analyte that could be quantified with acceptable accuracy and precision. The uncertainty was estimated in accordance with the recommendations of Eurachem/CITAC Guide, 2011, [22].

**RESULTS AND DISCUSSION**

To confirm that the developed method is suitable for its intended use, and to fulfil the aim of this study, a validation process is carried out. Since the result from the biosensor is given as a sum of the pesticides, the validated method is a screening one. All validation results obtained for OPPs determinations in spiked liver
samples are listed in Table 1. The quantification of the sum of OPPs is obtained using a calibration curve obtained on the ground of samples spiked with parathion. The estimated validation parameters of the method are satisfactory. The accuracy of the method is expressed as mean recoveries. They are found higher than 70 % for all spiked levels and all tested OP pesticides. The repeatability of the measurements and the within-laboratory reproducibility, expressed as coefficients of variation (CV), are lower than 7.5 % and 15.7 %, respectively. The regression curve of all pesticides shows a good linearity with a regression coefficients (R²) value higher than 0.95. The parameters illustrating the accuracy and the precision obtained for liver samples at LOQ level of 0.006 μg/g are listed in Table 1. The LOQ values are considerably lower than those of MRLs as required by SANTE 11945/2015 [4].

The inhibition rate of AChE is the lowest (48.8 %) with parathion (Fig. 3).

Therefore, the results referring to a sum of pesticides

Table 1. Validation parameters for a liver sample.

<table>
<thead>
<tr>
<th>Pesticide</th>
<th>Level [μg/g]</th>
<th>Linearity</th>
<th>Recovery [%], (n=18)</th>
<th>Repeatability CV [%], (n=6)</th>
<th>Within laboratory Reproducibility, CV [%], (n=18)</th>
<th>Measurement uncertainty [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorpyrifos-methyl</td>
<td>0.025</td>
<td>y = 725.11x + 5.6839</td>
<td>76,6</td>
<td>7,47</td>
<td>14,17</td>
<td>17,73</td>
</tr>
<tr>
<td></td>
<td>0.050</td>
<td>R² = 0.9655</td>
<td></td>
<td>78,6</td>
<td>5,06</td>
<td>12,93</td>
</tr>
<tr>
<td></td>
<td>0.075</td>
<td></td>
<td>75,9</td>
<td>4,92</td>
<td>9,06</td>
<td>15,47</td>
</tr>
<tr>
<td>Parathion</td>
<td>0.025</td>
<td>y = 739.97x + 5.5265</td>
<td>76,3</td>
<td>7,34</td>
<td>15,63</td>
<td>18,00</td>
</tr>
<tr>
<td></td>
<td>0.050</td>
<td>R² = 0.965</td>
<td></td>
<td>76,5</td>
<td>7,09</td>
<td>13,76</td>
</tr>
<tr>
<td></td>
<td>0.075</td>
<td></td>
<td>75,7</td>
<td>4,92</td>
<td>11,12</td>
<td>15,03</td>
</tr>
<tr>
<td>Pirimiphos-methyl</td>
<td>0.025</td>
<td>y = 956.72x + 6.5002</td>
<td>73,3</td>
<td>4,62</td>
<td>12,42</td>
<td>15,36</td>
</tr>
<tr>
<td></td>
<td>0.050</td>
<td>R² = 0.9564</td>
<td></td>
<td>75,7</td>
<td>2,94</td>
<td>11,94</td>
</tr>
<tr>
<td></td>
<td>0.075</td>
<td></td>
<td>74,6</td>
<td>5,35</td>
<td>9,12</td>
<td>14,54</td>
</tr>
<tr>
<td>Diazinon</td>
<td>0.015</td>
<td>y = 1473.5x + 6.833</td>
<td>72,1</td>
<td>3,86</td>
<td>14,91</td>
<td>15,72</td>
</tr>
<tr>
<td></td>
<td>0.030</td>
<td>R² = 0.9579</td>
<td></td>
<td>73,7</td>
<td>2,39</td>
<td>12,57</td>
</tr>
<tr>
<td></td>
<td>0.045</td>
<td></td>
<td>74,7</td>
<td>2,22</td>
<td>12,11</td>
<td>14,19</td>
</tr>
</tbody>
</table>

Table 2. LOQ of the optical biosensor developed.

<table>
<thead>
<tr>
<th></th>
<th>MRL [μg/g]</th>
<th>LOQ [μg/g]</th>
<th>Recovery [%], (n=20)</th>
<th>Within laboratory Reproducibility, CV [%], (n=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorpyrifos-methyl</td>
<td>0.05</td>
<td>0.006</td>
<td>75.8</td>
<td>18.1</td>
</tr>
<tr>
<td>Parathion</td>
<td>0.05</td>
<td>0.006</td>
<td>75.6</td>
<td>19.8</td>
</tr>
<tr>
<td>Pirimiphos-methyl</td>
<td>0.05</td>
<td>0.006</td>
<td>72.8</td>
<td>15.4</td>
</tr>
<tr>
<td>Diazinon</td>
<td>0.03</td>
<td>0.006</td>
<td>72.0</td>
<td>16.7</td>
</tr>
</tbody>
</table>
in the sample are estimated on the ground of a calibration curve obtained using samples spiked with parathion (Fig. 4). In case of an inhibition percentage result higher than 40 %, the sample has to be run with the application of a confirmatory method. The number of papers reporting biosensor analyzes conducted with real samples and complying with the requirements in the food control area is limited. Experiments with food of plant origin and water are conducted among the available ones because of the low fat content which facilitates the sampling. Biosensor results of the analysis of carbamates pesticides in milk are presented in ref.[19]. A sample preparation is not applied in that case because the matrix is in a liquid state. The resulting LOQ for paraoxone refers to 0.001 μg/g, while for carbaryl – to 0.020 μg/g. This is one order of magnitude higher than those obtained in the present work. In addition, the applied biosensor [19] is designed for a single use of the enzyme.

The validation process in the present study is performed in accordance with SANCO. This guidance document describes the method of validation and the analytical quality control requirements supporting the validity of the data used to check compliance with the maximum residue limits, the enforcement actions, or the consumer exposure assessment to pesticides in EU. This document is complementary and integral to the requirements of ISO/IEC 17025, 2005 [20]. The validation parameters obtained for the liver matrix demonstrate that the developed analytical method corresponds to the method performance acceptability criteria (mean recoveries in the range of 70 % - 120 %, precision with CV < 20 %, LOQ < MRL).

In contrast to other biosensor tests described in the literature that reach far lower recoveries (mostly below 70 %) [6], the recovery data values obtained in the present investigation exceed the required minimum of 70%. Schulze et al. report 101 % yield of paraoxon-ethyl in peach purée and 106 % yield in apple purée [17]. The biosensor constructed by those authors is incubated directly in the food, which is not applicable in the present case due to the type of sample being tested, namely liver. It is well known that samples of an animal origin cause far more interfering effects than those of a vegetable origin because of the fat content.

The higher the pesticides concentration in the sample, the better is its repeatability. At the second validation level, all repeatability results are below 7 %, which is less than half of the requirement. This means that the biosensor works very precisely and the results are with minimal deviations in recurring conditions. The lowest value of CV in respect to repeatability is obtained with diazinon at a validation level of 3 % - 2.22 %, i.e. almost 10 times below the criteria.

Using the repeatability and reproducibility data, it can be stated that the biosensor is working more precisely when the pesticide concentration in the sample is increased. This is a prerequisite to consider that the possibility of obtaining false negative results is minimal. Moreover, because of the high specificity of the reaction, practically all AChE inhibitors can be detected even at the low levels demanded by food control. This is extremely important for screening methods, as it is far more dangerous to have a false negative result (FN) than a false positive one (FP). There are also stringent measures - no
more than 5 % of samples (2002/657/EC, 2002) [21].

CONCLUSIONS

The newly developed biosensor offers many advantages over the chromatographic methods. The most important of them refer to its short response time, much lower cost, and easier maintenance. The main disadvantage of the biosensor analysis is that the result is obtained for a sum of inhibitors in the sample. Nevertheless, it is very suitable for a preliminary control aiming a positive or negative response prior to further proceeding to more expensive and time consuming analytical methods. The low cost, the speed of the analysis and the high sensitivity also make it a good alternative as a screening method in food analysis. Demonstrating the applicability of the method through validation in accordance with the requirements of SANTE /11945/2015 [4], it can be concluded that the newly constructed optical biosensor meets the requirements of the European Union related to the parameters of the food testing methods as sensitivity, detection limit, linearity, within-laboratory reproducibility. The reproducibility values in analyzing OPPs in liver by the developed optical biosensor are within the range of 4%-15 %. The precision increases with the increase of the pesticide concentration in the sample. The results obtained in the current work demonstrate without any doubt that biosensors can be introduced to food control as an alternative screening method for pesticide analysis.

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