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Development of an automated multienzymatic biosensor for risk assessment of pesticide contamination in water and food

Institute of Protein Biochemistry – National Research Council, Naples, Italy
Janis Rusko and Ferdinando Febbraio

Abstract

The goal of this research is to better address the problems related to the widespread presence of pesticides in the environment. Despite the unquestionable utility of the pesticides against various pests in the agricultural field, most pesticides and the corresponding pesticide residues are toxic to the environment and hazardous to human health. The recent literature on organophosphate compounds emphasises a clear correlation between their use and the occurrence of disorders in the nervous system, especially in children. The conventional systems for the detection and analysis of these compounds are expensive, time-consuming and require highly specialised operators; moreover, no online automated screening systems are yet available, that would allow the identification and quantification of the presence of these chemicals in samples from industrial sectors such as the food industry. Esterase-based biosensors represent a viable alternative to this problem. In this fellowship programme, we aim to develop a robust and sensitive methodology that enables the screening of toxic compounds using a streamlined process, using an automated robotic system to achieve a continuous monitoring for risk assessment of pesticides.

Keywords: organophosphate pesticides, biosensing device, thermophilic esterase, environmental monitoring

Correspondence: eu-fora@efsa.europa.eu

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Table of contents

1. Introduction

The presence of pesticide residues and the corresponding metabolites has been one of the major issues in food safety research. Although pesticides have improved agricultural productivity, they are associated with many health effects. Quantitative analysis of pesticides by chromatographic and spectroscopic technologies is limited by the time required to analyse a high number of samples, as the workloads and workforce involved in pesticide laboratories are immense. However, to perform food safety risk assessment much data are needed that is derived from large quantities of samples and a methodology that enables rapid quantitative analysis. To propose a solution for the issue, we are in the process of developing specific enzymes for the detection of select organophosphate pesticides (OPPs) and implementing the designed biosensing devices in a robotic system in combination with fluorescence and mass spectrometry (MS) detection.

Previous research has indicated several biosensors for OPPs detection, based on enzymatic or biological bioreceptors (Whangsuk et al., 2016; Hassani et al., 2017). Most widely used are biosensors based on the inhibition of acetylcholinesterase (AChE) (Guler et al., 2016). However, there are some limitations – the low stability of corresponding proteins and the susceptibility to react with inhibitors such as metals (Frasco et al., 2005; Amine et al., 2006), carbamates (Colovic et al., 2013) and even tetrahydrocannabinol (THC) (Eubanks et al., 2006), present in cannabis.

Esterase 2 from *Alicyclobacillus acidocaldarius* (EST2) is highly sensitive to the action of paraoxon, and appears to be endowed with a high affinity toward this OPP (Febbraio et al., 2008). EST2 is a carboxylesterase belonging to the hormone-sensitive lipase (HSL) family that includes several AChEs. Previous research has demonstrated the characteristics of EST2 – stability over time, prime resistance and activity at different pH values and temperatures (Mandrich et al., 2004, 2005; Foglia et al., 2007), as well as satisfactory stability in the presence of low concentrations of organic solvents and detergents (Del Vecchio et al., 2002; Mandrich et al., 2006), so supporting the potential further use in biosensing devices selective for a variety of inhibitors such as OPPs. In previous research, our group has already described the use of EST2 as a biological part of a colorimetric biosensor for paraoxon detection (Febbraio et al., 2011).

Furthermore, the EST2 3D structure has been solved at 2.6 Å resolution (De Simone et al., 1999, 2000), allowing modelling of the structure *in silico* and permitting molecular docking predictions. EST2's peculiarity for stability, in addition to the terms of irreversible inhibition, the sensitivity and selectivity toward phosphoryl OPPs, such as paraoxon and methyl paraoxon (Foglia et al., 2007; Febbraio et al., 2011), makes this enzyme a prime candidate for use as a bioreceptor in biosensors for qualitative and quantitative OPP detection.

Using our proposed bioassay methodology, analysis time for multiple samples by fluorescence measurement takes about 1 min, which is at least 10 times faster than common quantification methods. If any positives for OPPs are found, analysis is continued, provided by the extra selectivity of tandem MS (fragmentation patterns) or high-resolution MS (accurate mass and fragmentation). Generally, the system is optimised to minimise the means of sample preparation and analyse the native samples. Our methodology enables a rapid preliminary testing approach and works as a filter before chromatographic analysis, avoiding the testing of most negative samples.

The goal of the research involved in the EU-FORA fellowship programme (Bronzwaer et al., 2016), granted by the European Food Safety Authority (EFSA), is to develop a method capable of providing rapid quality control and achieving continuous monitoring of the OPPs in the environmental (water and liquid food) samples.

2. Description of work programme

2.1. Aims

The work programme activities or aims were summarised in three principal parts. The first part was the participation of the selection and preparation of the suitable enzymes that would further be utilised in the second part of the work programme as bioreceptors for multienzymatic biosensors, developed using a robotic workstation. Third and final part of the work programme was dedicated to the collection and analysis of real world samples using the developed bioenzymatic method and to compare the achieved results with the use of conventional methods to further ensure confidence in the developed method. After data collection, the data were processed and analysed to provide the risk assessment of pesticide content in fruit juice and wine samples.

2.2. Methods

2.2.1. Part 1

The fellow accomplished the expression in mesophilic hosts, such as *Escherichia coli* strain BL21 (DE3), of several mutants of a thermostable esterase (Manco et al., 1998), sensitive to OPP inhibition (Febbraio et al., 2011), already available as transformed cells in the laboratory. Locally available equipment such as fermentation facilities, centrifuges, incubators and sonicators were used to accomplish this part. Proteins were further purified via thermoprecipitation steps, and size-exclusion chromatography (gel permeation chromatography (GPC)). Proteins were quantified using Bradford protein assays. Biochemical characterisation of purified proteins was performed by enzymatic activity assays using fluorescent substrates. The biochemical constants of inhibition in the presence of different concentration of OPPs for each enzyme were determined to catalogue them, depending on individual affinities for these compounds.

2.2.2. Part 2

In the second part of the work programme, the fellow was trained to use a robotic workstation Hamilton MICROLAB® STAR equipped with a robotic arm connected to a Multilabel Plate Reader PerkinElmer Victor X3, to develop methods that were used for application of real world sample analysis and further method development and investigation stages. In particular, residual activities of each selected enzyme for the respective OPPs were determined. Assay analysis times were optimised to obtain prime reproducibility of data and to better investigate the effects of unique residual activities. To support the bioenzymatic method and confirm the robustness, a conventional mass spectroscopy method was developed and verified using an AB Sciex QTrap 4,500 triple quadrupole MS system coupled to a Shimadzu Nextera x2 liquid chromatography system.

2.2.3. Part 3

In the last part of the work programme, various kinds of fruit juice, such as apple, apricot, peach and other commercial fruit juices were collected, to be used for method development and validation. The samples were collected at commercial sources in Naples, Italy. In total, there were more than 60 unique samples of both wine and juice. No information was available on possible previous treatment of these fruit juices or wines, except for products labelled with the 'BIO' label. The samples were shaken until homogeneous, two aliquots of 50 mL were transferred to screw-cap polypropylene sample tubes and stored at −20°C. Only one tube was thawed at one time to avoid unnecessary matrix product degradation.

Initially, the samples were analysed and the presence (or absence) of the OPPs was determined by use of the developed confirmatory MS method over a short period (48 h) of time after the initial acquisition of the samples. The method specificity and the occurrence of OPPs were studied. In particular, the bioenzymatic assays were optimised for the detection of OPPs in complex matrix solutions by measuring the efficiency of the assays in the presence of various matrix constituents. The obtained concentration data were used for the risk assessment of OPPs in liquid foods.

3. Conclusions

3.1. Method development

3.1.1. Part 1

Enzymes (EST2 mutants, displayed in Figure 1) were overexpressed in the mesophilic host *E. coli* strain BL21 (DE3) and purified. Protocols described by Manco et al. (1998) and Pezzullo et al. (2012) were used to obtain reference enzymes. As the purification method was excessive and not viable for large-scale preparation, final purification steps, including fast protein liquid chromatography (FPLC) and high-performance liquid chromatography (HPLC), were excluded. Two crude enzyme extracts, the first after thermoprecipitation and the second after thermoprecipitation followed by GPC were tested for the enzyme stability and activity against the reference purified enzymes. Results indicated that unpurified enzymes were more stable and active with respect to the purified ones. Even though the proteins obtained using the previous protocols were more pure, it was not viable to include the

redundant and time-consuming flash chromatography and FPLC steps, during which significant loss pf activity occurred. The experiment was continued using the final extracts obtained after a basic thermoprecipitation step, which allowed us to obtain all six of the mutant enzymes in a short period of time and in large quantities.

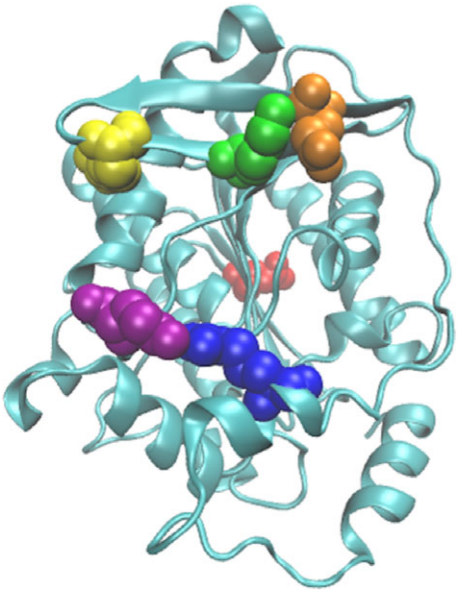

Figure 1: Representation of an EST2 3D structure in cyan, mutated groups are indicated using the van der Waals (VDW) structure, each colour referring to a different mutant

3.1.2. Parts 2 and 3

We proposed a modernised method that would allow a clean analysis process. The method development process started off with sample collection and preparation. During April 2018, we collected 60 combined commercial samples of fruit juice and wine. However, in the last months of the fellowship programme, we increased the variety and amount of the sample matrices.

The sample preparation was necessary to be robust and as simple as it could be. There were two opposite directions from which we had to decide on one. First direction was to improve the sensitivity and selectivity of the analysis using multiple sample preparation steps such as SPE (dSPE, μSPE), LLE or, the most common one, QuEChERS. Selective procedure helps to remove the excess matrix constituents such as sugars and antioxidants and enables the possibility of enrichment factor. Multiple sample preparation steps do come with a bigger price tag and generally take more time. The other direction was to be as easy as possible and enable high throughput analysis using simple sample preparation steps such as dilution, filtration and centrifugation. The issue with such basic sample preparation is that the matrix constituents are prone to diminish the detection efficiency and introduce bias in the analysis results. A problem was that we had to perform the confirmatory analysis using MS, which is very easily influenced by the matrix constituents providing signal suppression or enhancement effects, which can vary for individual samples.

We underwent preliminary method development steps using sample centrifugation, filtering with 0.22-μm polytetrafluoroethylene (PTFE) filters and dilution in water with final pH correction to 7.0 using 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer. Standard addition was performed to samples before the sample preparation procedure. Then 60 individual samples were tested using MS to determine that the matrix influence was minor and contributed to no more than 20% chromatographic signal peak area deviation from the standard addition (spiked) samples without the matrix (blank solvent). The results indicated that the best options were to choose convenience, use the previously mentioned rapid sample preparation methods and omit the extensive sample preparation practices. Then, we continued the streamlined method development, using the robotic workstation to prepare the samples automatically.

Investigation on the bioenzymatic assays in the presence of matrix influence was continued. To increase the threshold of OPP detection using the EST2 enzyme, we included the use of a fluorogenic substrate – 4-methylumbelliferyl butyrate (4-MUBu) to measure the residual enzymatic activity. Note

that all the matrix samples were diluted with HEPES buffer. The preliminary condition was to determine the effect of the matrix on fluorescence detection – whether there was signal suppression or enhancement. The hydrolysed substrate (4-MU) was used as the fluorescence agent. Matrix blank (containing no OPPs or inhibitors) samples were used for multiple concentration levels of 4-MU standard addition. Another condition was to determine whether 4-MUBu was hydrolysed within the matrix blank samples by standard addition of a single concentration level of the compound and by measuring the fluorescence emission change over time (kinetic measurements).

Furthermore, we continued to examine how different concentration levels of HEPES buffer and concentration of matrix in the final sample extracts affected the suppression or enhancement effects. Note that the amount of matrix used in the final sample affects the sensitivity of the method.

We continued to determine the specific kinetic constants for six of our expressed EST2 mutant enzymes under standard assay conditions in the presence of 60 unique matrices and a control blank solvent. 4-MUBu was used as a substrate in a range of concentrations. The kinetic constant values (K*m* and k_{cat}) were calculated by plotting the reciprocals of EST2 hydrolysis rates versus the substrate concentrations.

Next, we continued to perform the inhibition assays for each of the enzymes of the following 12 OPPs – ethyl-paraoxon, methyl-paraoxon, coumaphos, fensulfothion, methyl-parathion, ethyl-parathion, cyanophose, methyl-pirimiphos, diazinon, phosmet, chlorpyrifos and tolclofos-methyl. The inhibition assays were carried out under standard assay conditions in the presence of 60 unique matrices and blank solvent. The matrix added to final sample aliquots was constant and the amount of inhibitors added to the assays was in a continuous concentration parts per thousand (ppt) scale. Aliquots were tested for the residual activity of EST2 measured in the presence of 1 mM 4-MUBu.

Furthermore, we tested the inhibition assay performance in the presence of an oxidising agent *N*-bromosuccinimide (NBS), which transforms the sulfur-containing pesticides to the corresponding oxidated forms. Prior to the bioassay analysis, the NBS oxidation efficiency was tested using MS and deemed efficient. As for the bioassay, NBS was added to the samples (both blank and matrix) containing only the inhibitors and, after a brief period of incubation in room temperature, the inhibition assay protocol was continued and the residual activity of EST2 was determined.

3.2. Streamlined method

To propose a solution to planned issues such as lack of data, slow analysis methods and extensive resource use, our goal was to develop and apply a streamlined method for OPP analysis in various target matrices.

The developed method was designed to be equivalent to and possibly improve on the common pesticide analysis methods using MS methodologies, such as QuEChERS. A brief, illustrative comparison can be seen in Figure 2.

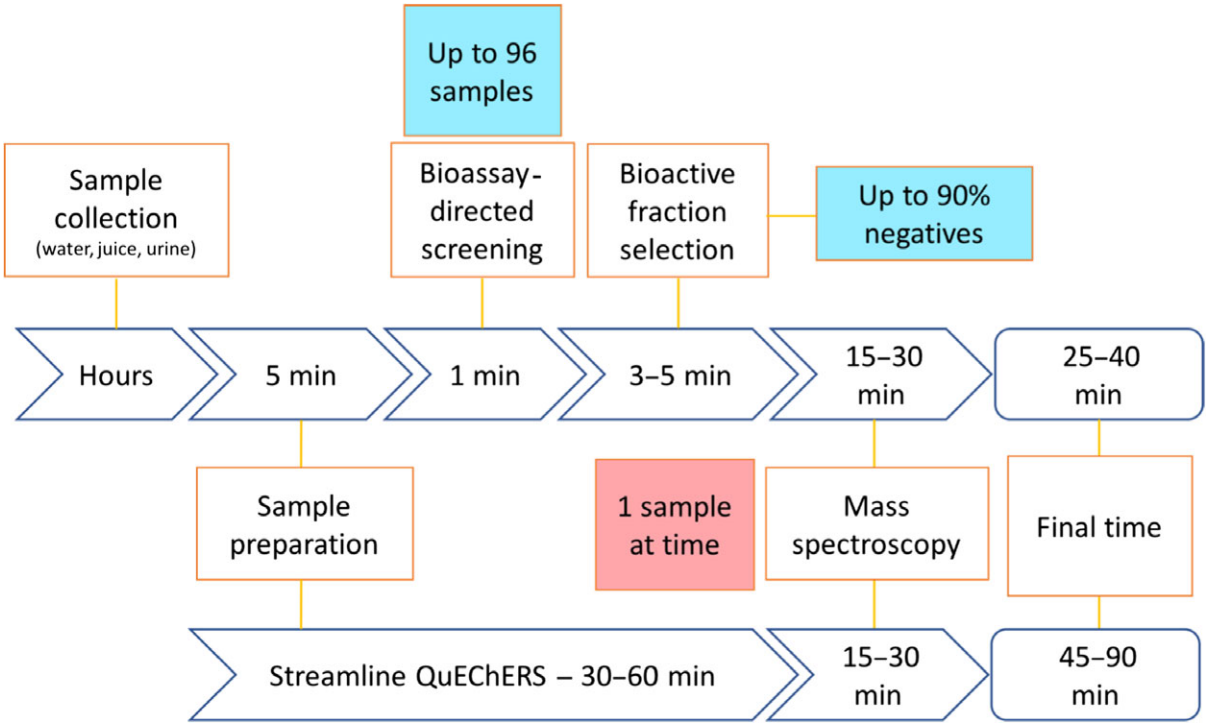

Figure 2: Analysis method process, in comparison with a conventional pesticide analysis method (QuEChERs + mass spectroscopy)

The figure illustrates the processes involved in the streamlining of both our developed method and the common MS analysis method for pesticides. For a single sample, the preparation takes significantly less time than using QuEChERS. The latter is then directly followed by MS analysis. In our developed methodology, sample preparation is followed by rapid bioassay analysis and the preliminary result evaluation. Positive samples are confirmed by MS.

The major difference is that our methodology provides a preliminary bioassay mass-screening of up to 96 (or even 384) samples at a time, compared with the conventional pesticide analysis method, which allows the processing of a single sample at time using MS detection. This approach is slightly more convenient as the results provided by the bioassays allowed us to discard up to 90% of the samples that had negative results. Therefore, the large-scale sample analysis workload and time of use of the MS systems is decreased.

3.3. Future goals

Direct influence on further method development by allocating more time would allow the incorporation of technology also in solid samples, which typically require extra treatment (SPE, QuEChERS, etc.) before pesticide data procurement. Continuous research would allow the application of genetically modified sensors to bind new, more complex target molecules and enable the application of analysis to new environmental and food matrices. Also, there is a possible line of research in binding the designed enzymes to a stationary phase, developing a SPE approach, so opening the possibilities of more sensitive and selective methods.

In future, it is possible to develop systems for people to use even in household applications, by performing the analysis using a simple set up of mobile phone and a sample cell.

The ultimate goal is to develop an accessible methodology in which only the biosensor would be used. However, we are limited by the extensive resources needed to develop such a method, as we would further require professionals with a background in bioinformatics and software development that would help in the development of software packages for data analysis and profiling. Such a system would allow accurate fingerprinting of the OPPs. Data processing and elaboration for fluorescence measurements and fingerprint determination would be continued by employing artificial neural networks (ANNs). ANN databases are collected through the determination of patterns and relationships in data and so are trained via experiences from sample dataset.

The possibility of further research would enable not only the ability for fast routine screening in environmental or quality control (QC) laboratories, but also for on-site operation in places significantly remote for the immediate identification of hazard, risk assessment and action. This approach would allow immediate operation, so preventing the placement of positive products on the market locally.

3.4. Extracurricular activities

The fellow participated in scientific seminars of interest and also presented the current research idea pitch in a mutual and poster presentation at EFSA organised Risk Assessment Research Assembly (RARA).

3.5. Disclaimer

The results obtained from the method development, sample analysis and risk assessment are not included in this report to avoid certain copyright claims, as the research is still ongoing over the last months of the fellowship programme and these results will be subsequently published in other scientific journals.

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Glossary and Abbreviations

AChE	acetylcholinesterase
ANN	artificial neural network
dSPE	dispersive solid-phase extraction
EST2	esterase 2 from *Alicyclobacillus acidocaldarius*
EU-FORA	The European Food Risk Assessment Fellowship Programme
FPLC	fast protein liquid chromatography, technique used to analyse purified mixtures of proteins
GPC	gel permeation chromatography, technique of size-exclusion chromatography used to separate analytes on the basis of their molecular size
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	high-pressure liquid chromatography, technique used to separate, identify and quantify each component in a mixture
HSL	hormone-sensitive lipase
LLE	liquid–liquid extraction
4-MUBu	4-methylumbelliferyl butyrate
4-MUBu	4-methylumbelliferyl butyrate
MS	mass spectrometry
NBS	*N*-bromosuccinimide
OPPs	organophosphorus pesticides
PTFE	polytetrafluoroethylene
QC	quality control
QuEChERS	'Quick Easy Cheap Effective Rugged Safe', dispersive solid phase extraction method for detection of pesticide residues in food
RARA	Risk Assessment Research Assembly
μSPE	micro solid-phase extraction
THC	tetrahydrocannabinol
Thermoprecipitation	Purification of sample using multiple high and low temperature steps to separate the impurities from target compound