Rapid detection of neurotoxic insecticides in food using disposable acetylcholinesterase-biosensors and simple solvent extraction

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Abstract

The extensive use of pesticides to protect agricultural crops necessitates reliable tools for the detection of residues in food and water, thus ensuring environmental protection and consumer safety. Neuroinhibitors such as organophosphates and carbamates in particular, represent a potential hazard to human health. These compounds are frequently found in food but conventional methods of analysis are limited as they are either time consuming or not sufficiently sensitive. As a result, a rapid and sensitive biosensor test based on AChE-inhibition was developed. The disposable AChE-biosensor was directly applied in solvent extracts of food samples using isooctane as extraction solvent. A complete assay could be performed in less than 2 hours. Recovery rates of 84 % were obtained in tests with spiked orange juice samples. Tests in food samples with a lower water content resulted in reduced recovery rates (44 % for peach pap baby food). Phosphorothionate insecticides could be detected after direct oxidation in food with N-bromosuccinimide and solvent extraction. The assay displayed a detection limit of 2 µg/kg paraoxon which was sufficient for the monitoring of maximum residue limits in food according to EU regulations.

Introduction

Pesticides have been widely used in agriculture and industry throughout recent decades to ensure a supply of food for the world's growing population [1]. However, adverse effects of this use can be seen in the chemical residues found in drinking water or food. A monitoring program for pesticide residues in the European Union and Norway indicated that about 40 % of tested food samples contained detectable traces of these compounds [2]. Maximum residue limits (MRL) were exceeded in 3 % of all cases - frequently the samples contained organophosphate and carbamate insecticides. As some pesticides, especially organophosphates, display a high acute toxicity in humans and particularly infants, the European Union has set a very low limit for pesticides in baby food. According to this regulation infant formulae must not contain residues of individual pesticides at levels exceeding 10 µg/kg which is in practice the minimum detectable level using the officially admitted detection methods [3]. Consequently, there is a growing interest in faster and more sensitive detection systems. The standard methods for insecticide detection are based on gas chromatography (GC) or high performance liquid chromatography (HPLC) coupled with mass selective detectors (MSD) [4, 5]. Depending on the investigated food, e.g. citrus fruits, there are different multi-residue methods for compound specific detection [6]. The disadvantage of these methods is the long assay time and their inherent restriction to a limited number of pesticides, which can be identified by each multiresidue method. Hence there is a possibility of suspected false negatives in the results from unknown samples if not all pesticides are covered by the applied multiresidue method. As an alternative, acetylcholinesterase (AChE) inhibition tests, and the AChE-biosensors in particular, have been repeatedly described for use in insecticide detection [7-16]. As it is possible with these tests to determine the

presence of organophosphates or carbamates as sum parameter of AChE-inhibition with a high level of sensitivity in a relatively short time, it would be an advantageous supplement to precede the currently-used chromatographic methods with AChE-biosensors in order to increase the sample throughput and avoid suspected false negative results.

Most of the AChE-biosensors described until now were limited to water analysis due to their possible susceptibility towards matrix effects [7-16]. The few reports of cholinesterase-based biosensors for food testing either relied on laborious multi-step sample preparation or showed problems due to matrix effects reducing the accuracy of the results [17-21]. Furthermore, these tests were limited to carbamates and oxo-forms of organophosphates although most of the found organophosphorus insecticides are sulfur-containing compounds, especially those with a P=S moiety (e.g. phosphorothionates) [2]. These insecticide variants evolve their inhibiting ability only after oxidation by the target organism metabolism and display drastically reduced *in-vitro* inhibition potency compared to their corresponding oxo-forms. Phosphorothionates are therefore not detectable in commonly found concentrations by AChE inhibition. To circumvent this, phosphorothionates have to be oxidized prior to the inhibition tests to increase the sensitivity and to prevent suspected false negative results. Several authors have described methods of oxidation in buffer solution using N-bromosuccinimide (NBS) [22] or bromine [23] with a high yield. In a recent publication, the oxidation of phosphorothionates by a concentrated bromine solution in acetonitrile was described [24] but not applied for food analysis. However, the residual AChE-activity was measured in a buffer solution after evaporation of the solvent thus extending the time required for the overall test procedure.

To reduce the assay time, the direct application of the biosensor in the organic solvent would be advantageous. The stability of AChE in organic solvents has been described by Mionetto et al. [12]. According to Mionetto et al., the activity of immobilized AChE remained in hydrophobic solvents like hydrocarbons but decreased in polar solvents such as alcohols. Similar results were obtained by Campanella et al. [25]. They developed a bienzymatic amperometric oxygen-electrode with immobilized butyrylcholinesterase and choline oxidase for the detection of organophosphates and carbamates working in chloroform / *n*-hexane mixtures. Here we describe an amperometric biosensor test for food analysis based on disposable screen-printed AChE-biosensors, which were described in a former publication [15]. This test included direct oxidation of phosphorothionates in the food sample, an extraction step with organic solvents and direct incubation of the biosensor in the organic extract without re-dissolving or dilution with a buffer.

Experimental

Reagents

AChE (EC 3.1.1.7) from electric eel (Type V-S, 970 U/mg) was obtained from Sigma-Aldrich (Deisenhofen, Germany). Paraoxon (paraoxon-ethyl) was purchased from Riedel de Haën (Seelze, Germany). Insecticide stock solutions were prepared in ethanol. Isooctane, ascorbic acid and N-bromosuccinimide were received from Fluka (Buchs, Switzerland). All other reagents were of analytical grade as supplied by Sigma-Aldrich (Deisenhofen, Germany). Food samples were obtained from local

supermarkets: orange juice (Jope, Weinstadt, Germany), HIPP baby apple and HIPP peach with honey (HIPP, Pfaffenhofen, Germany).

Biosensor performance

Disposable biosensors with immobilized AChE were produced by screen-printing as described earlier [15]. All sensor experiments were carried out in a stirred buffer solution (0,01 M potassium phosphate buffer, 0,05 M NaCl, pH 7,5 (PBS)) at room temperature. Enzyme activity was determined by monitoring thiocholine formed by enzymatic hydrolysis of acetylthiocholine chloride (1 mM). Thiocholine was determined by oxidation at 100 mV versus Ag/AgCl. For inhibition experiments, the biosensor was incubated with a sample for 30 min at room temperature in a non-stirred solution and percentage of inhibition was calculated after residual activity measurement.

Reactivation of AChE-activity

To determine the AChE-activity reactivation rate, 1 mM pyridine-2-aldoxime methiochloride (2-PAM) in PBS was used as reactivating agent. The biosensor was incubated for 30 min in a stirred reactivation solution at room temperature. The final AChE-activity was then measured in a buffer solution.

Solvent extraction

A 50 g aliquot of the food sample was extracted with 100 mL isooctane for 30 min. After centrifugation at 17000 g and 4 °C for 15 min, a 10 mL aliquot of the upper organic phase was used as incubation solution of the AChE-biosensor. After 30 min

incubation in the organic phase, the remaining enzyme activity was measured in PBS. Recovery rates of spiked food samples were calculated using respective inhibition values obtained in PBS as reference.

Oxidation of phosphorothionates with N-bromosuccinimide in food

A volume of 500 µL N-bromosuccinimide (NBS) solution (0,4 g/L in water) was added to 50 g orange juice (final NBS-concentration: 4 mg/L) and mixed in an ultrasonic sound bath for five minutes. Then 500 µL ascorbic acid solution (4 g/L in water) was added to remove excessive NBS by mixing in an ultrasonic sound bath for five minutes.

Results and discussion

Biosensor performance in buffer solution

As a reference the biosensor performance was tested with paraoxon as model analyte in a phosphate buffer solution. The calibration curve in Fig. 1 shows the percentage of AChE-inhibition caused by different paraoxon concentrations. Each experimental point was the mean of 5 measurements. The inhibition experiments showed a high level of intra-laboratory reproducibility with a coefficient of variation of 7 %. The working range of the system paraoxon electric eel AChE was between 1 and 60 µg/L paraoxon. The detection limit (signal to noise ratio of 3 : 1) was 1 µg/L paraoxon ($3{,}6 \times 10^{-9}$ mol/L) and thus sufficient for the control of maximum residue limits in food, which are in the range between 10 and 10 000 µg/kg depending on the kind of food and insecticide, according to EU regulations [26].

Direct measurement in food

In order to develop a simple assay system, direct measurement of untreated food with the AChE electrode was attempted. Aliquots of orange juice, peach and apple baby food were used as incubation samples. This treatment yielded a high AChE-inhibition for each sample (Table 1). One reason for this high inhibition was the low natural pH-value of fruits: adjustment to a neutral pH reduced the inhibition in the case of orange juice from 56 % to 41 %. It could be shown that the remaining AChE-inhibition was not caused by the presence of organophosphates or carbamates in the food samples, as the AChE-inhibition could not be reactivated with 2-PAM. Additional problems resulted from a much longer equilibration time of the thickfilm electrodes during activity measurements after incubation in the food sample.

Measurement in solvent extracts

To overcome the situation described above, an extraction step was introduced into the analytical method. As extraction of food samples should be performed with isooctane, the influence of this solvent on the activity of the immobilized enzyme was tested prior to inhibition studies. An incubation of 30 min of the AChE-biosensor in isooctane caused only a marginal reduction of enzyme activity of 3 %, which was within the degree of unspecific activity loss observed in PBS. Solvent extracts from unspiked orange juice, peach and apple baby food also caused virtually no unspecific AChE-inhibition (Table 1). In addition, the equilibration time remained within the normal range of 15 min. The accuracy of the biosensor test was checked by spiking the food samples with paraoxon. The AChE-inhibition observed after incubation in the solvent-extract was compared with the inhibition value caused by the same paraoxon-concentration in PBS. Using isooctane as the extraction solvent yielded an 84 % recovery of paraoxon in orange juice as can be seen in Fig. 2. As reactivation

of AChE-activity with nucleophiles such as 2-PAM is additional proof that the AChE-inhibition was caused by organophosphates or carbamates and not by other interfering compounds or matrix effects, the reactivation rate after incubation in the organic solvent was tested. The reactivation after incubation in isooctane was possible up to 97 %. In preliminary experiments also toluol was investigated as the extraction solvent, yielding 94 % recovery rate of paraoxon in orange juice. As the cholinesterase activity could not be reactivated after sensor exposure to toluol extracts of spiked solutions this possibility was not further investigated (data not shown).

In addition to liquid food such as orange juice, the biosensor test was also performed with apple and peach pap as food with a lower water content. A paraoxon concentration of 10 µg/kg in apple baby food caused an AChE-inhibition of 12 %, which corresponded to a recovery rate of 44 %. The negative control of unspiked apple pap caused no reduction in enzyme activity as shown in Fig. 2. The same amount of paraoxon in peach pap yielded a 14 % inhibition of AChE, - a 52 % recovery rate. Similar problems with low recovery rates in food samples were reported elsewhere [18, 19, 21]. Pogacnik et al. obtained recovery rates of 51 % in apple juice and 60 % in orange juice for 10 µg/kg paraoxon [21]. Skladal et al. reported recovery rates for carbofuran in potato juice [18, 19]: 28 % for 10 µg/kg carbofuran, 34 % for 50 µg/kg carbofuran and 99 % for 125 µg/kg carbofuran. At higher concentrations up to 125 µg/kg propoxur in carrot juice the recovery rate remained below 70 %. For 10 µg/kg propoxur the value was 14 %. Additionally Skladal et al. described problems due to a slower signal response presumably due to electrode fouling after incubation in food samples. Although direct incubation in the food sample would be advantageous, the low recovery rates and matrix effects would impede real sample application.

The biosensor food screening test was fast compared to chromatographic methods. Information about cholinesterase inhibiting residues was obtained after 90 minutes in contrast to one day which is needed for standard chromatographic procedures. In contrast with biosensor tests described earlier or other validated GC-MS methods, neither time-consuming evaporation nor clean-up steps were necessary. The AChE biosensor test is suitable for food with a high water content, but will have to be adapted for food with a low water content. A possible strategy would be the variation of the extraction conditions. The coefficient of variation was on average 18 % (corresponding to a value of 1 µg/kg paraoxon in the case of orange juice) in the three tested food matrices. The recovery rate of paraoxon in orange juice of 84 % was in the range of the legislative regulations that require a recovery rate of between 70 and 110 % [27].

Oxidation of phosphorothionates in food

Parathion, the thio analogue of paraoxon, was used to investigate the possibility of oxidizing thio-variants of organophosphates directly in food. Without oxidation parathion did not cause noteworthy inhibition in a concentration range between 20 and 2000 µg/L (max. 7 % AChE-inhibition at 2000 µg/L). These results clearly indicated the need for an oxidation step to increase the analyte spectrum of the biosensor test towards phosphorothionates. Preliminary results indicated an optimal NBS concentration of 4 mg/L which ensured a quantitative oxidation of the phosphorothionates (data not shown). Treatment of unspiked orange juice with NBS/ascorbic acid followed by isooctane extraction yielded a 3 % decrease of enzyme activity. The analysis of a sample spiked with 10 µg/kg parathion caused an

AChE-inhibition of 17 % (Fig. 3). Taking the paraoxon values in buffer as a reference this meant a recovery rate of 63 %. This biosensor method is, to the best of our knowledge, the first one for the detection of phosphorothionates in food. The recovery rate of the test is close to the required value of 70 % even at very low parathion-concentrations. In the European study on pesticide residues in the European Union and Norway, four organophosphates (out of seven pesticides), namely acephate, chlorpyrifos, chlorpyrifos-methyl and methamidophos, were included in the study. The values found in the study ranged up to 0,79 mg/kg for chlorpyrifos in apples and 0,59 mg/kg for chlorpyrifos-methyl in strawberries. It would clearly have been possible to detect such concentrations using the described biosensor test.

Conclusion

The development of a biosensor test for the detection of organophosphates and carbamates in food was described. The biosensor met the requirements set by EU regulations with respect to detection limits for all tested food matrices and according to recovery rates for orange juice. The test was also capable of detecting phosphorothionates by adding an oxidation step into the procedure. While the biosensor performed well in food with a high water content, additional optimization would be needed for more solid samples. As the test could be performed within less than two hours and detected anti-acetylcholinesterase activity as a sum parameter, it would be a valuable supplement to multi-residue methods as a pre-screening process to reduce the number of false negatives and increase the sample throughput. Currently, work is under way to further integrate food analysis with the AChE-biosensor to cover a larger number of food products with an increase in both accuracy and speed.

Acknowledgement

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Figures

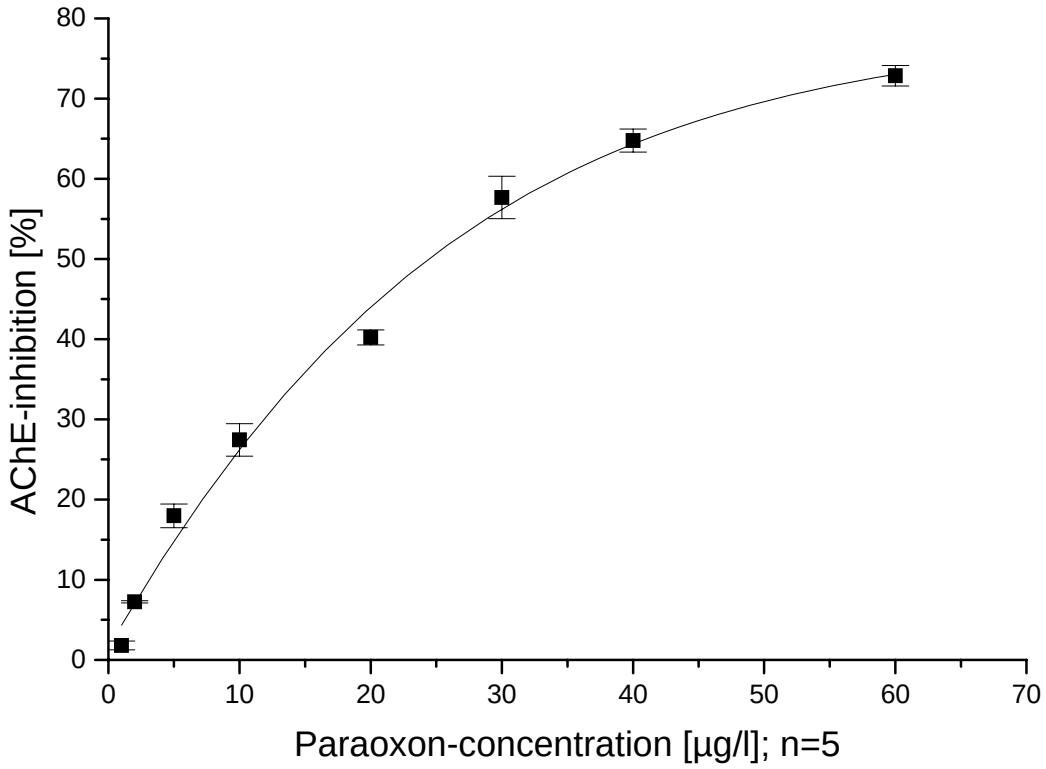

Figure 1

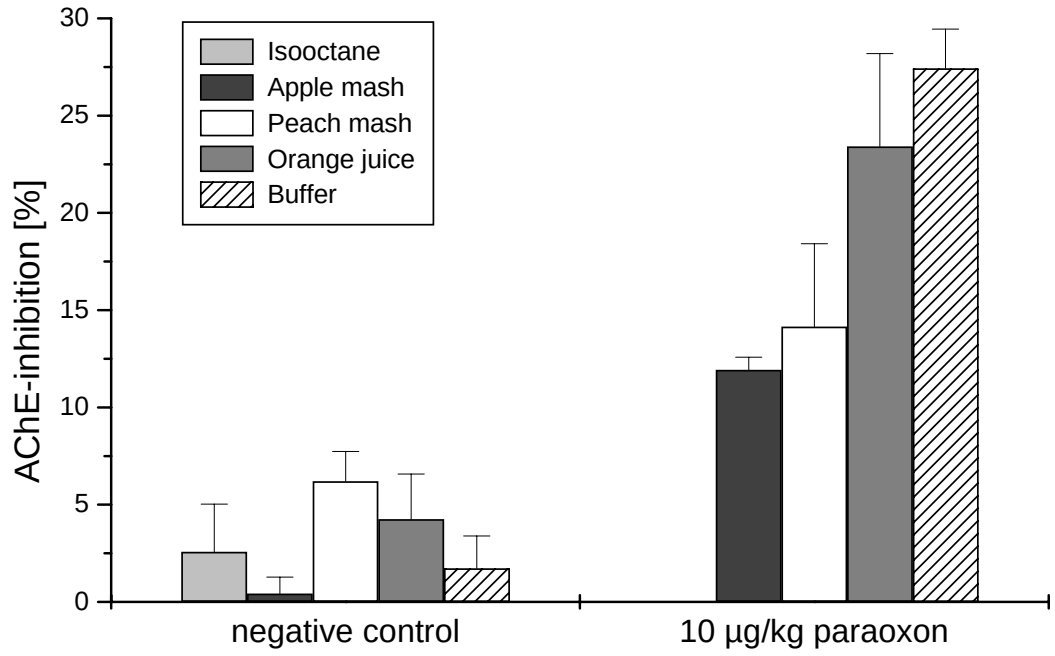
30
25
20
15
10
5
0
AChE-inhibition [%]
Isooctane
Apple mash
Peach mash
Orange juice
Buffer
negative control
10 µg/kg paraoxon

Figure 2

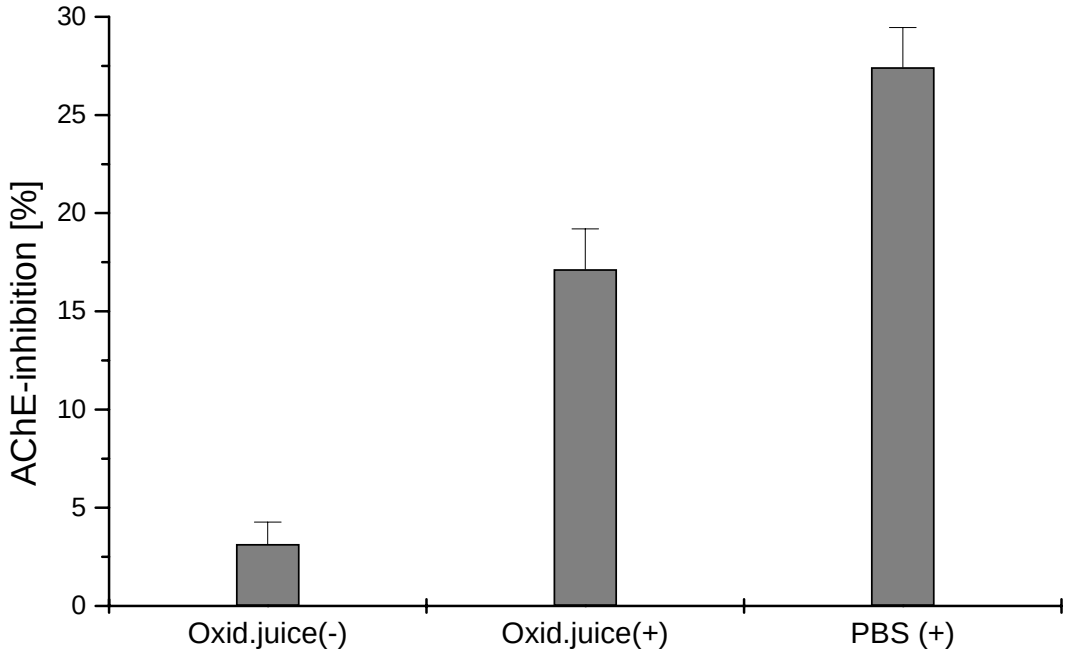

Figure 3

Table 1: Effect of untreated food samples on the activity of AChE in comparison to isooctane extracts of corresponding foodstuff (unspecific inhibition):

	AChE-inhibition [%]		
	pH 4	pH 7	Isooctane extraction
Orange juice	56	41	4
Peach baby food	21	16	6
Apple baby food	19	12	0

Figure legends:

Figure 1: Calibration curve; electric eel AChE-inhibition caused by different paraoxon-concentrations; n=5

Figure 2: AChE-inhibition caused by incubation in isooctane-extracts of different food samples and in buffer spiked with 10 µg/kg paraoxon, compared with incubation in isooctane-extracts of unspiked food samples and in pure isooctane (negative control); n_{min}=4

Figure 3: AChE-inhibition caused by incubation in isooctane-extracts of orange juice spiked with 10 µg/kg parathion (+) and without parathion (-) after oxidation of the food sample with NBS; n=4; PBS (+): AChE-inhibition caused by 10 µg/kg paraoxon in buffer solution.