Development of Acetylcholinesterase biosensors for neurotoxins detection in foods and the environment

Entwicklung von Acetylcholinesterase-Biosensoren zur Neurotoxinedetektion in Lebensmitteln und Umwelt

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For my family

„In all, three things remain:

The certainty that we are always starting…

The certainty that we need to continue…

The certainty that we will be stopped before the end…

Therefore, we must:

Turn the break into a new path…

The fall into a dance step…

The fear into a ladder…

The dream, a bridge…

The search, a finding…"

Fernando Sabino

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Table of contents

ABBREVIATIONS **7**

ENGLISH SUMMARY **9**

ZUSAMMENFASSUNG **11**

1. INTRODUCTION **18**

1.1. Biosensors 18
1.2. Acetylcholinesterase 20
1.2.1 Acetylcholinesterase from Nippostrongylus brasiliensis 24
1.3. Acetylcholinesterase biosensors 28
1.3.1 Amperometric acetylcholinesterase biosensors 32
1.4. Types of inhibition 36
1.5. Organophosphate and carbamate insecticides 38
1.5.1 Organophosphate and carbamate insecticides used in Brazil 42
1.5.2 Organophosphorothionate insecticides 47
1.6. Chloroperoxidase 49
1.7. Glycoalkaloids 54

2. OBJECTIVES **58**

2.1. General Objective 58
2.2. Specific Objectives 58

3. MATERIAL AND METHODS **59**

3.1. Material 59
3.1.1 Chemicals, biochemicals and equipments 59
3.1.2 Cultivation medium 62
3.1.3 Buffer, solutions and antibiotics 63
3.1.3.1 Buffers 63
3.1.3.2 Solutions 64
3.1.3.3 Antibiotics 65
3.2. Methods 65
3.2.1 Spectrophotometric enzymatic activity determination 65
3.2.1.1 Optical acetylcholinesterase activity assay 65
3.2.1.2 Optical chloroperoxidase activity assay 67
3.2.1.3 Bimolecular rate constant (k_i) 68
3.2.2 Determination of the protein concentration 70
3.2.3 Acetylcholinesterase biosensor test for food samples 70
3.2.3.1 Production of biosensors using screen printing method 70
3.2.3.1.1 Basic acetylcholinesterase biosensor production 70
3.2.3.1.2 Working electrode (TCNQ-graphite) 72
3.2.3.1.3 Acetylcholinesterase printing paste 72
3.2.3.2 Samples preparation 73
3.2.3.2.1 Chemical oxidation of organophosphorothionates in buffer solution 73
3.2.3.2.2 Chemical oxidation of organophosphorothionates in food samples 73
3.2.3.3 Acetylcholinesterase biosensor measurement 73
3.2.3.3.1 Acetylcholinesterase biosensors measurement tests for insecticide detection 73
3.2.3.3.2 Detection limit of the biosensors 76
3.2.3.3.3 Acetylcholinesterase biosensor measurement tests for glycoalkaloids detection 77
3.2.3.4 Food sample tests 78
3.2.3.5 Reactivation of the acetylcholinesterase activity 78
3.2.3.6 Multienzyme biosensors 79
3.2.3.7 Storage stability 80
3.2.4 Microbiological methods 80
3.2.4.1 Expression of acetylcholinesterase B from N. brasiliensis in P. pastoris 80
3.2.5 Molecular Biological methods 81
3.2.5.1 Sodium dodecacyl sulfate polyacrylamide electrophoresis (SDS – PAGE) 81

3.2.6 Analysis of phosphorothionate insecticides using a chloroperoxidase pretreatment and acetylcholinesterase biosensor detection ... 82
3.2.6.1 Chloroperoxidase enzymatic oxidation of phosphorothionates in food samples for GC/MS analysis 82
3.2.6.2 GC/MS analysis ... 83
3.2.6.3 Chloroperoxidase pretreatment of food samples for acetylcholinesterase biosensor measurement 83
3.2.7 Development of acetylcholinesterase biosensors tailor made for Brazil ... 84
3.2.7.1 Standard analytical methods ... 84
3.2.7.1.1 Supercritical fluid extraction (SFE) method ... 84
3.2.7.1.2 Liquid solvent based method ... 85
3.2.7.1.3 Measurement ... 86
3.2.8 Design of biosensors of acetylcholinesterase from N. brasiliensis for glycoalkaloids detection ... 87
3.2.8.1 Half maximal inhibition concentration (I_{50}) determination of glycoalkaloids ... 87
3.2.8.2 Preparation of the potatoes samples ... 87

4. RESULTS ... 88

4.1. Expression of the wild type and mutants of acetylcholinesterase B from *N. brasiliensis* ... 88
4.2. Analysis of phosphorothionate insecticides using a chloroperoxidase pretreatment and acetylcholinesterase biosensor detection ... 90
4.2.1 Acetylcholinesterase and chloroperoxidase stability test toward KCl and KBr ... 90
4.2.2 GC/MS analysis of the products of the reaction catalyzed by chloroperoxidase - Choice of pH and buffer of the reaction system ... 91
4.2.3 GC/MS analysis of the products of the reaction catalyzed by chloroperoxidase – Use of different halogen salts and insecticides ... 92
4.2.4 Determination of the bimolecular rate constant (k_i) of enzymatically activated phosphorothionates ... 97
4.2.5 Phosphorothionates conversion with chloroperoxidase pretreatment method and acetylcholinesterase biosensor assay in foods ... 100
4.3. Development of acetylcholinesterase biosensors tailor made for Brazil ... 103
4.3.1 k_i determination ... 103
4.3.1.1 Methamidophos ... 103
4.3.1.2 Acephate ... 104
4.3.1.3 Profenofos ... 104
4.3.1.4 Fenitrothion ... 105
4.3.1.5 Triazophos ... 106
4.3.1.6 Chlorpyrifos ... 107
4.3.2 Multienzyme biosensor ... 108
4.3.3 Sensitivity ... 110
4.3.3.1 Chlorpyrifos ... 110
4.3.3.2 Triazophos ... 111
4.3.3.3 Fenitrothion ... 112
4.3.3.4 Methamidophos ... 112
4.3.4 Validation ... 113
4.3.5 Storage stability ... 115
4.4. Development of acetylcholinesterase biosensors for the detection of glycoalkaloids ... 120
4.4.1 Half maximal inhibitory concentration (I_{50}) value determination ... 120
4.4.2 Acetylcholinesterase biosensor specificity ... 121
4.4.3 Kinetics of the inhibition activity ... 122
4.4.4 Food samples ... 127

5. DISCUSSION ... 132

5.1. Analysis of phosphorothionate insecticides using a chloroperoxidase pretreatment and acetylcholinesterase biosensor detection ... 133
5.1.1 pH ... 133
5.1.2 H_2O_2 ... 134
5.1.3 Tert-butyl hydroperoxide ... 135
5.1.4 Organophosphorothionate insecticides oxidation using chloroperoxidase ... 136
5.1.5 Halogen salts ... 136
5.1.6 Stability of the biosensor under oxidation conditions ... 138
5.1.7 Biosensor of acetylcholinesterase from N. brasiliensis in food samples ... 139
5.2. Development of acetylcholinesterase biosensors tailor made for Brazil ... 142

5.2.1 Acephate *142*
5.2.2 Sensitivity of acetylcholinesterase mutants from N. brasiliensis versus sensitivity of acetylcholinesterase mutants from Drosophila melanogaster *144*
5.2.3 Insecticides in foods and maximum residue limits accepted by the Brazilian legislation *145*
5.2.4 Stability of acetylcholinesterase mutants from N. brasiliensis at room temperature *147*
5.3. DEVELOPMENT OF ACETYLCHOLINESTERASE BIOSENSORS FOR THE DETECTION OF GLYCOALKALOIDS 149
5.3.1 Inhibition mechanism of α-solanine and α-chaconine over acetylcholinesterase from N. brasiliensis *149*
5.3.2 Ratio effect of α-chaconine: α-solanine *152*
5.3.3 Comparison of the toxicity between α-chaconine and α-solanine *153*
5.3.4 Comparison of sensitivity between butyrylcholinesterase biosensors and acetylcholinesterase biosensors from N. brasiliensis *154*
5.3.5 Acetylcholinesterase biosensor test in potato samples *156*
5.4. OUTLOOK 158

REFERENCES 160

6. PhD PRODUCTION 182

7. CURRICULUM VITAE 183

8. STATEMENT 184

Abbreviations

ε	Extinction coefficient
2-PAM	Pyridime-2-aldoxime methachloride
ACh	Acetylcholine
AChE	Acetylcholineterase
ANN	Artificial neural network
ANVISA	Agência Nacional de Vigilância Sanitária
ARfD	Acute Reference Dose
ATCh	Acetylthiocholine
BSA	Bovine serum albumine
BuCh	Butyrylcholine
BuChE	Butyrylcholinesterase
CA	Carbamate insecticides
ChE	Cholinesterase
ChO	Choline oxidase
CoPC	Cobalt phtalocyanine
CPO	Chloroperoxidase
CVUA	Chemisches und Veterinäruntersuchungsamt
DIN	Deutsches Institut für Normung
Dm	*Drosophila melanogaster*
DTNB	5,5´-Dithio-bis(2-nitrobenzoic acid)
E. coli	*Escherichia coli*
EFSA	European Food Safety Authority
EPA	Environmental Protection Agency
FAO	Food and Agriculture Organization of the United Nations
GA	Glycoalkaloid
GC	Gas chromatography
GOD	Glucose oxidase
HEC	Hydroxyethyl cellulose
HI-6	1-(2-hydroxyiminomethylpyridinium)-3-(4-carbamoylpyridinium)-2-oxa-propane dichloride
HPLC	High performance liquid chromatography
I_{50}	Half maximal inhibitory concentration
ISFET	Ion-selective field-effect transistors
k_i	Bimolecular rate constant
K_i	Dissociation constant for inhibitor binding
Km	Michaelis Menten constant
L	Liter
LOD	Limit of detection
MCD	Monochlorodimedon
MRL	Maximum residue level
MS	Mass spectroscopy
MSD	Mass selective detector
m. t.	Metric tons
Nb	*Nippostrongylus brasiliensis*

NBS	N- bromosuccinimide
OP	Organophosphate insecticides
PBS	Phosphate buffer solution
PDP	Pesticide Data Program
pH-FET	pH-sensitive field effect transistors
QuECHERS	Quick, Easy, Cheap, Effective, Rugged, and Safe
SDS-PAGE	Sodium dodecacyl sulfate polyacrylamide electrophoresis
SIM	Single ion monitoring
Tc	*Torpedo californica*
TCNQ	7,7,8,8-Tetracyanoquinodimethane
TNB	2-nitro-5-thioenzoic acid
U	Units
UN	United Nations
v/v	Volume per volume
WT	Wild type
w/v	Weight per volume
YPP	Yeast Extract Peptone Phosphate buffer

English Summary

Acetylcholinesterase (AChE) is responsible for the hydrolysis of acetylcholine in the nervous system. It is inhibited by several substances, like organophosphate and carbamate insecticides, glycoalkaloids, nerve gas and anatoxin-a(s). There is a growing need to develop new technologies to reduce time spent with sample preparation, discriminate between positive and negative samples, and to reduce analysis costs. Over the last decades, AChE biosensors have emerged as an ultra sensitive and rapid technique for toxicity analysis in environmental monitoring, food and quality control.

Acetylcholinesterase is only slightly inhibited by organophosphorothionate insecticides, the most applied organophosphate insecticides worldwide. This fact makes their detection analytically very difficult. A new enzymatic method for the activation and detection of phosphorothionates was developed with the capability to be used directly in food samples without the need of laborious solvent extraction steps. Chloroperoxidase (CPO) from *Caldariomyces fumago* was combined with *tert*-butyl hydroperoxide and two halides. Chlorpyrifos and triazophos were completely oxidized. Fenitrothion, methidathion and parathion methyl showed conversion rates between 54 – 61%. Furthermore, the oxidized solution was tested with an AChE biosensor assay. Chlorpyrifos spiked in organic orange juice was oxidized, and its oxon product was detected in concentrations down to 5 µg/L (final concentration food sample: 25 µg/L). The complete duration of the method took about 2 h.

An acetylcholinesterase B multisensor from *Nippostrongylus brasiliensis* (Nb) was developed to detect the most frequently used insecticides in Brazil. The objective was to establish a fast screening method, separating the negative samples from the positive ones. The four mutants, which together presented the widest sensitivity spectrum, were: F345A, M301A, W346V and W346A. The combination of these four mutants in a multienzyme biosensor array enabled the detection of 11 out of the 12 most important insecticides at concentrations below 20 µg/L. The biosensor test was compared with traditional analysis methods, and validated with food samples previously analyzed. The storage stability revealed that the biosensor remained stable for 40 weeks; however the sensitivity decreased with time.

Glycoalkaloids are secondary metabolites present in potatoes, which can be toxic to humans in high concentrations. α-Solanine and α-chaconine are the main examples of this group, and these substances show an anti-acetylcholinesterase activity. An Nb acetylcholinesterase B biosensor was designed to detect glycoalkaloids in buffer solutions and in foods. The two Nb AChE mutants that showed the highest sensitivity towards α-solanine and α-chaconine (lowest I_{50} value) were W303L and F345A. The Dixon and Cornish-Bowden plots demonstrated that the inhibition of these substances over Nb AChE was reversible and competitive. The achieved detection limits of α-chaconine and α-solanine were 0.1 and 0.5 µM, respectively. The designed biosensor was able to detect mixtures of α-solanine and α-chaconine in potatoes samples spiked with these glycoalkaloids in total concentrations higher than 300 µM.

Zusammenfassung

Pflanzenschutzmittel werden seit vielen Jahren in der Landwirtschaft in großem Umfang eingesetzt, um Ernteerträge zu steigern. Acetylcholinesterase (AChE), ein zentrales Enzym der Nervenreizleitung, wird durch Organophosphate und Carbamate gehemmt. Durch Hemmung der AChE kommt es im Gehirn an cholinergen Synapsen des autonomen Nervensystems und an motorischen Endplatten zur Anreicherung des Neurotransmitters Acetylcholin (ACh). Diese Pestizide agieren nicht nur auf Insekten-AChE, sondern wechselwirken auch mit AChE anderer Organismen, einschließlich der menschlichen (Fukuto 1990). Für die Routineanalytik von Organophosphaten wird Gaschromatografie mit Massenspektrometrie-Kopplung (GC/MS) eingesetzt. Die thermolabilen Carbamate können nur mittels Flüssigchromatografie mit Massenspektrometrie-Kopplung (LC/MS) nachgewiesen werden. Diese Methoden zeichnen sich durch einen hohen apparativen Aufwand und durch zeitaufwendige Analysen aus. Für den Nachweis von neurotoxischen Substanzen, z.B. Organophosphaten und Carbamaten, wurden, neben einem kommerziellen AChE-Test, eine Reihe von AChE-Biosensoren entwickelt. Die weitaus größte Zahl der AChE-Biosensoren basiert jedoch auf einer amperometrischen Detektion der AChE-Aktivität (Schulze et al. 2002).

Das erste Ziel war die Entwicklung einer Vorbehandlungsmethode mit dem Enzym Chloroperoxidase (CPO), um Organophosphorthionate Insektiziden zu aktivieren. Die Aktivierung ist nötig, weil Phosphorthionate in ihrer ursprünglichen, nicht metabolisierten Form, sehr schwache AChE-Inhibitoren sind. Diese Pestizide werden später durch einen AChE-Biosensor näher bestimmt (Abbildung 1).

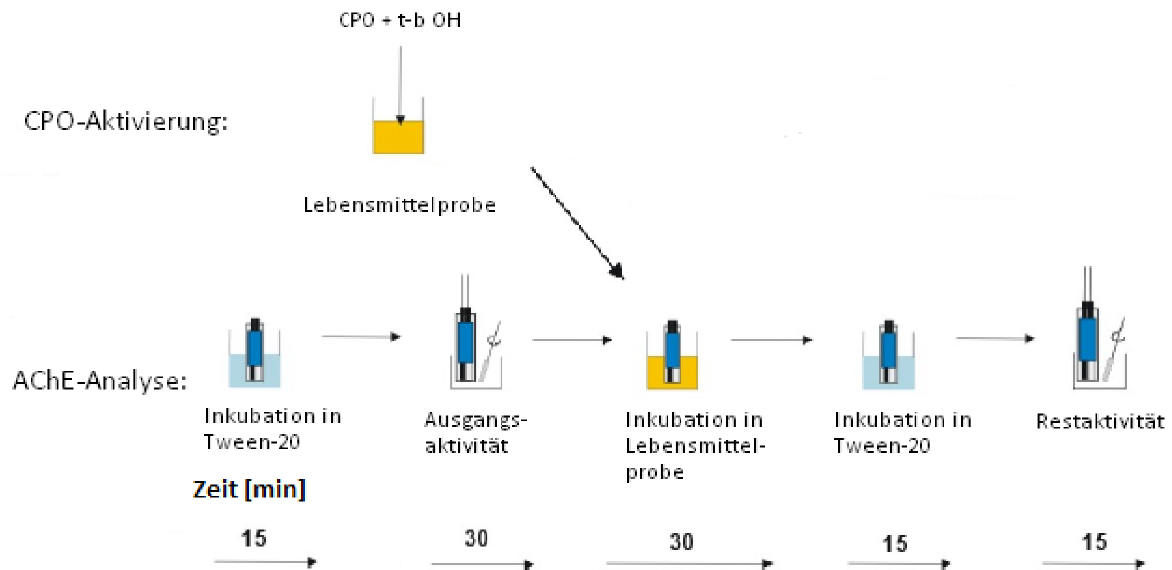


Abbildung 1 – Testprotokoll der CPO-Aktivierung- und AChE-Biosensortests für die Kontrolle von Organophosphorothionate Insektiziden

Hernandez et al. (1998) und Walz und Schwack (2007) zeigten, dass das CPO in der Lage ist, Phosphorthionate zu aktivieren. Mit GC/MS Analyse wurden dabei die nicht umgesetzten Edukte und die gebildeten Produkte im Reaktionsansatz nachgewiesen. Wie in Abbildung 2 zu sehen ist, bewirkte das CPO eine Transformation von Chlorpyrifos (Retentionszeit = 13.02 min) zu Chlorpyrifos oxon (Retentionszeit = 12.86 min). Bei der Reaktion ohne CPO entstand kein Chlorpyrifos oxon.

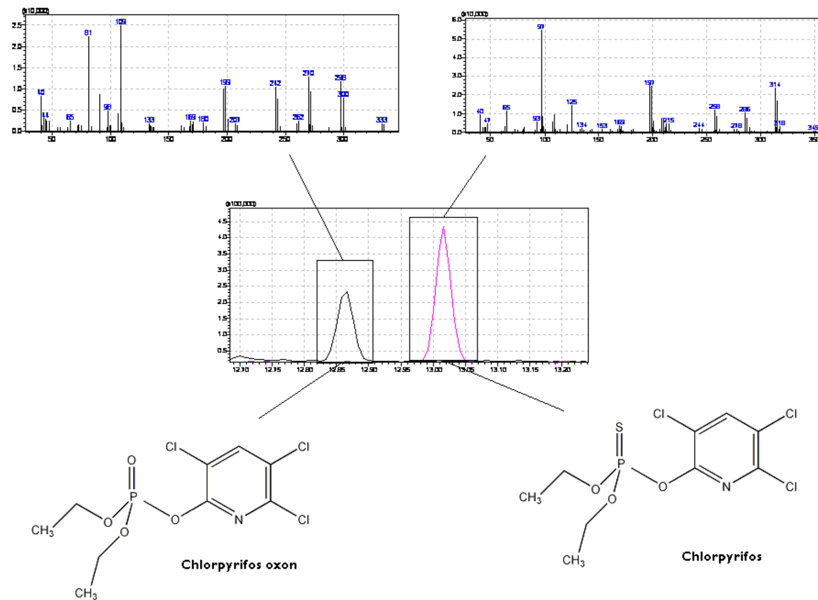


Abbildung 2 – GC/MS-Spektren der Reaktionsprodukte aus der Umsetzung von Chlorpyrifos mit (schwarz) und ohne CPO-Zugabe (rosa). Bei der Reaktion von Chlorpyrifos ohne CPO-Zugabe fand keine Umsetzung zu Chlorpyrifos oxon statt. Dagegen wird bei der Umsetzung von Chlorpyrifos mit der CPO-Zugabe Chlorpyrifos oxon gebildet.

Die Anwendbarkeit der neuen enzymatischen Aktivierungsmethode für komplexe Lebensmittelproben wurde untersucht. Die Versuche mit der KCl-Zugabe haben gezeigt, dass die Hemmung passiert ist, wenn das Pestizid abwesend war. Das bedeutet, dass diese optimierte Kondition nicht das Beste für die Methode war. Anderseits, wie in Abbildung 3 dargestellt ist, war die Kombination von der CPO-Aktivierungmethode mit der KBr-Zugabe zusammen mit dem AChE-Biosensortest erfolgreich, um Organophosphorthionaten zu aktivieren und zu bestimmen.

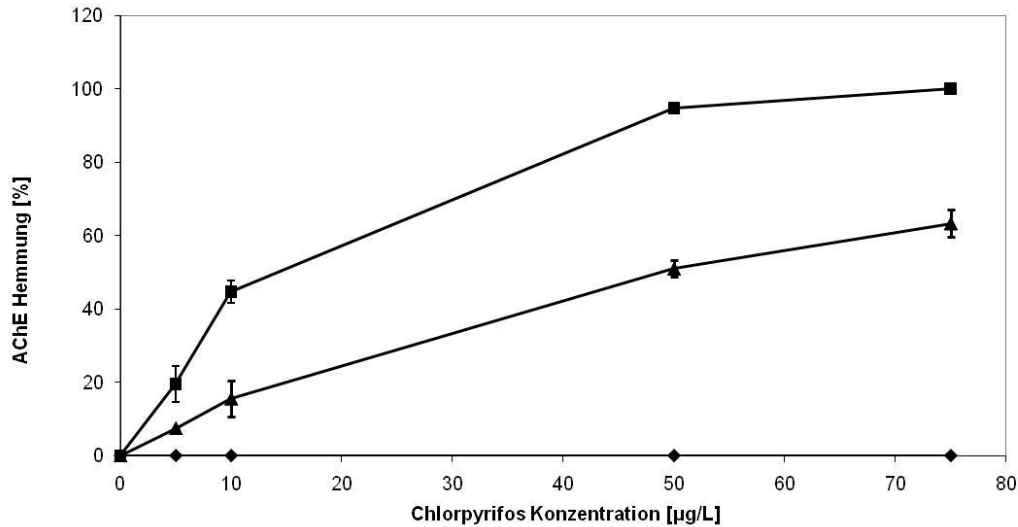


Abbildung 3 – WT Nb AChE-Hemmung in Abhängigkeit von der Konzentration von Chlorpyrifos in Orangensaft, bestimmt mit dem amperometrischen AChE-Biosensortest. Chlorpyrifos wurde vor der AChE-Inkubation mit CPO, t-b HP und KBr aktiviert: ♦ keine CPO Zugabe, Acetat Puffer 1 M pH 5.5; ■ CPO Zugabe, Acetat Puffer 1 M pH 5.5; ▲ CPO Zugabe, Bio Orangensaft (20%), Acetat Puffer 1 M pH 5.5. *n=3*

Es wurde bestimmt, dass CPO eine erfolgreiche Alternative für die Anwendung von AChE-Biosensor sein kann. Die Nachweisgrenze wurde in Lebensmittelproben auf 5 µg/L festgestellt (Endkonzentration in Lebensmitteln: 25 µg/L – 20% Bio Orangensaft-Zugabe).

Ein weiteres Forschungsziel war die Entwicklung eines AChE-Biosensors maßgeschneidert für Brasilien, der in dem brasilianischen Bundeskontrollprogramm von Pestiziden in Lebensmitteln benutzt werden kann. Das bedeutet, das Biosensor-Verfahren als *Screening* Test zu benutzen, um die Analysegeschwindigkeit zu beschleunigen. Die 12 am häufigsten gefundenen Pestizide in Gemüse- und Fruchtproben zwischen 2001-2007 wurden getestet, nämlich: Chlorpyrifos, Triazophos, Fenitrothion, Methidathion, Methamidophos, Profenofos, Ethion, Dimethoat, Parathion-methyl, Acephat, Carbaryl und Carbofuran.

Die Ergebnisse haben gezeigt, dass die Mutanten F345A, M310A, W346V und W346A die sensitivsten gegen die getesteten Insektizide waren. Es wurde später ein Multienzymsensor bestehend aus diesen vier verschiedenen Nb AChE Mutanten hergestellt.

Das Pestizid Acephat hat keine der getesteten AChE gehemmt. Suskayetrp und Plapp (1977) zeigten, dass Acephat einen indirekten AChE-Inhibitor war. Dieses Pestizid muss zuerst deacetyliert werden, damit es später als Methamidophos detektiert werden kann.

Das Verhalten der AChE-Mutanten in der immobilisierten Form auf dem Biosensor wurde mit den sensitivsten Mutanten (F345A, M310A, W346V und W346A) gegen die in Brasilien am häufigsten benutzten Pestizide getestet. Die Abbildung 4 zeigt die Nachweisgrenze dieses Multienzymsensors gegenüber Chlorpyrifos.

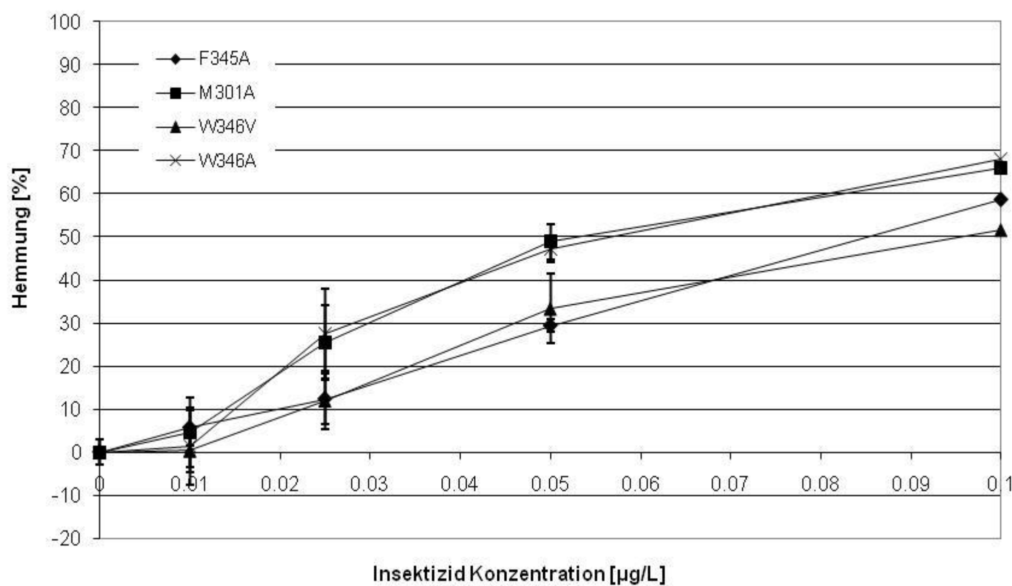


Abbildung 4 – AChE-Hemmung in Abhängigkeit von der Chlorpyrifos-Konzentration. Inkubation des Multienzymsensors in Lösungen mit unterschiedlichen Chlorpyrifos-Konzentrationen 30 min bei RT. Chlorpyrifos wurde vorher mit NBS chemisch oxidiert.

Gegenüber den Pestiziden Fenitrothion, Chlorpyrifos, Triazophos und Methamidophos stellte sich heraus, dass die entsprechenden Nachweisgrenzen des Multienzymsensors gegenüber den vier Pestiziden im Puffer 0.05, 0.025, 0.025 und 10 µg/L ist. Das Gesetz in Brasilien erlaubt in Lebensmitteln generell die Anwesenheit von bis zu 10 µg/L Chlorpyrifos, 50 µg/L Fenitrothion, 10 µg/L Triazophos und 10 µg/L Methamidophos. Das bedeutet, dass der Multienzymsensor geeignet ist, um als *screening tool* für das brasilianische Bundeskontrollprogramm eingesetzt zu werden.

Die Stabilität des Nb AChE Mutanten Biosensors durch Lagerung bei Raumtemperatur wurde untersucht. Einerseits hat die 40 wöchige Lagerung gezeigt, dass es zu keinem Verlust der Ausgangsaktivität kam. Anderseits stellte sich heraus, dass die Hemmung der Mutanten M310A, W346V und W346A durch 0.05 µg/L Chlorpyrifos über diesen Zeitraum hinweg leider nicht stabil bleibt.

Die dritte Aktivität konzentriertete sich auf die Entwicklung eines AChE Biosensors, um Glykosidalkaloide in Kartoffeln zu entdecken. Die giftigen Glykosidalkaloide α-Solanin und α-Chaconin kommen von Natur aus in kleinen Mengen in Kartoffeln vor - und sind für die Menschen toxisch. Zudem ist bei diesen zwei Substanzen AChE gehemmt. Ein Gesamtalkaloid-Gehalt (Summe von α-Solanin und α-Chaconin) von bis zu 200 mg/kg gilt bei Kartoffeln bislang als unbedenklich.

Die Ergebnisse haben gezeigt, dass die Mutanten W303L und F345A die sensitivsten gegen die getesteten Glykosidalkaloide waren. Es wurde später ein Multienzymsensor bestehend aus diesen zwei verschiedenen Nb AChE Mutanten hergestellt. Kinetik-Untersuchungen wurden durchgeführt, um herzufinden, was für ein Hemmungsmechanismus Nb AChE über α-Solanin und α-Chaconin hat. Die Dixon- und Cornish-Bowden-Plots zeigten, dass diese Glykosidalkaloide Nb AChE reversibel und konkurrenzfähig gehemmt haben. Die Nachweisgrenzen von α-Chaconine und α-Solanine wurden auf 0.1 and 0.5 µM festgelegt (Abbildung 5).

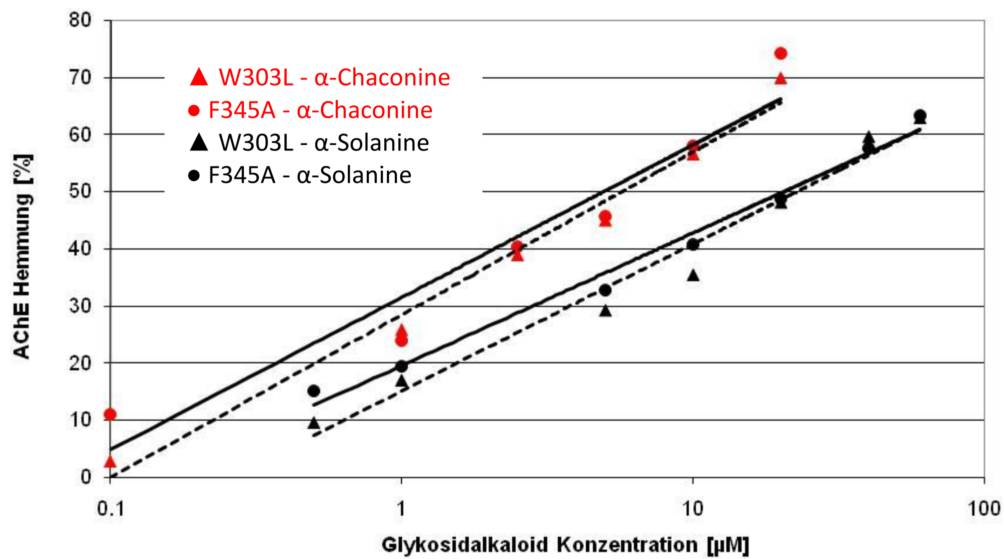


Abbildung 5 – Kalibriergerade von Nb AChE-Biosensor für Glykosidalkaloide-Detektion

Der Biosensor konnte in kontaminierten Kartoffelproben die Mischung von α-Solanine und α-Chaconine in Konzentrationen höher als 300 µM nachweisen.

1. Introduction

1.1. Biosensors

Biosensors are analytical devices that tightly combine biorecognition elements and physical transducers for the detection of target compounds. A sensitive and selective bioreceptor is attached onto the surface of a physical transducer able to respond to it. As a result of the biorecognition event between the bioreceptor and its target analyte, a biochemical and/or physicochemical property is transformed into a measurable signal (Turner et al. 1987, Thevenot et al. 2001, Patel 2002). Figure 1 illustrates the parts of a typical biosensor.

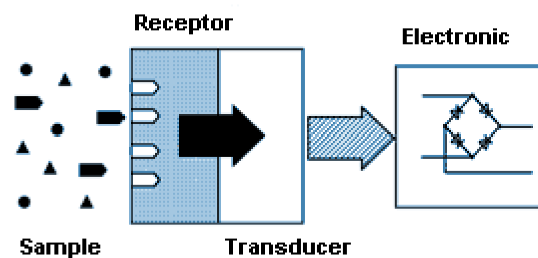


Figure 1 – Parts of a typical biosensor

The first biosensor was an enzyme sensor developed by Clark and Lions in 1962. This sensor contained the enzyme glucose oxidase (GOD) attached onto the surface of an amperometric oxygen electrode. This electrode was used to directly quantify the amount of glucose in blood, based on oxidation of glucose to gluconic acid (Clark and Lyons 1962). A biosensor used for the monitoring of glucose in blood is shown in Figure 2.

Figure 2 – Biosensor for glucose monitoring (3 Test 2010)

Biosensors have attracted extraordinary interest in recent years, becoming an important tool for the detection of chemical and biological components. They have exceptional performance capabilities, for instance, high specificity and sensitivity, fast response, low cost, relative compact size and user-friendly operation. In 2006, the biosensors market yielded revenues of U$ 5.11 billion, and it is expected that this sector will reach the sum of U$ 10.62 billion until 2013 (Frost and Sullivan 2007). According to Global Industry Analysts Incorporation, the market for medical biosensors is forecast to reach U$ 6.1 billion in 2012 (Global Industry Analysts 2008).

Analytical technology based on sensors is an extremely broad field, which impacts on many major industrial sectors such as pharmaceutical, healthcare, food and agriculture industries, as well as environmental monitoring. The application of biosensors for the detection of food contaminants and environmental pollutants has similar requirements of sensitivity, limit of detection, and stability as those used in medical applications. However,

the development and application of biosensors is more challenging, given the significantly more complex matrices, the need of large sample analysis, the required ruggedness, and the need of continuous monitoring in remote places (Baeumner 2003).

In enzyme-based biosensors, the biological element is the enzyme, which reacts selectively with its substrate (Guilbault et al. 2004). Two different approaches can be used for determining an analyte through an an enzymatic biosensor: (1) if the enzyme metabolizes the analyte, the analyte can be determined by measuring the enzymatic product, and (2) if the analyte inhibits the enzyme, the decrease of enzymatic product can be measured and correlated to the analyte concentration (Arduini et al. 2009).

Biosensors based on the principle of enzyme inhibition have been applied for a wide range of significant analytes such as organophosphate insecticides (OP), organochloride insecticides, heavy metals and glycoalkaloids (GA). The choice of enzyme/analyte system is based on the fact that these toxic analytes inhibit the normal enzyme function (Amine et al. 2006).

1.2. Acetylcholinesterase

Cholinesterases (ChE) are a family of serine hydrolases found in vertebrates and invertebrates that hydrolyze choline esters faster than other substrates (Fest and Schmidt 1973, Taylor 1991). In the human body, ChEs are responsible for the transmission of nerve impulses to the cholinergic synapses and are linked to human memory and Alzheimer's disease. More specifically, they are involved in the deactivation of acetylcholine at nerve endings, preventing continuous nerve firings, which is vital for the normal functioning of sensory and neuromuscular systems (Murphy 1986).

Two types of cholinesterases are known: acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE). Both enzymes show different kinetic properties and specificity toward various substrates and inhibitors. Acetylcholinesterase (EC 3.1.1.7) is an enzyme that preferentially hydrolyzes acetyl esters, for example, acetylcholine (ACh). Butyrylcholinesterase (EC 3.1.1.8) has a similar molecular structure to that of AChE, but it is characterized by different substrate specificity: it hydrolyzes butyrylcholine (Hosea et al. 1995). Another aspect that differentiates AChE from BuChE is the AChE inhibition by the excess of substrate. This property is related to substrate binding and the catalytic mechanism (Andreescu and Marty 2006). In human beings, AChE is localized in neurons and erythrocytes; BuChE is located in neurons, glia and blood serum (Giacobini 2004).

Acetylcholinesterase terminates transmission of neuronal impulses by rapid hydrolysis of ACh into acetate and choline (Barnard 1974). Acetylcholine is the major excitatory neurotransmitter that regulates motor functions (Rand and Nomet 1997) (Figure 3).

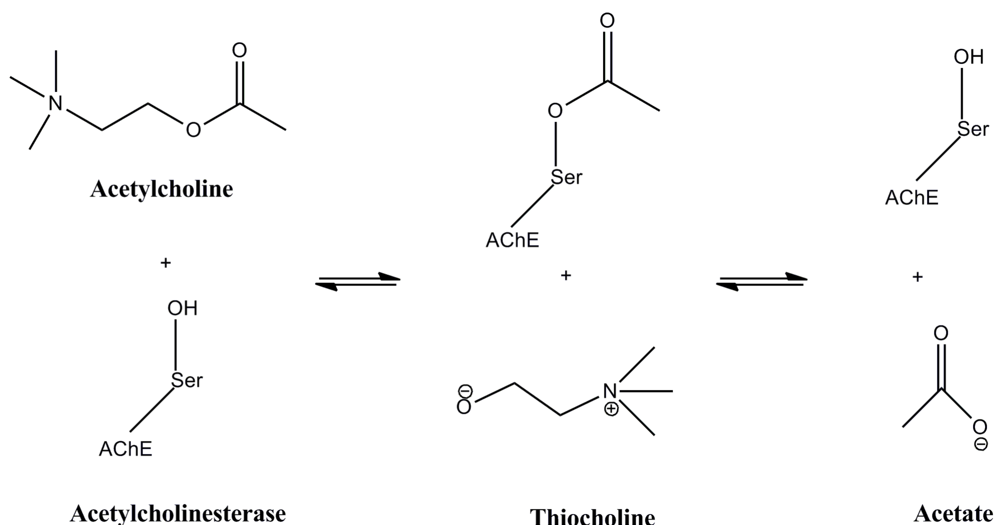


Figure 3 - Acetylcholine hydrolysis by acetylcholinesterase

In addition, AChEs are produced in other parts of the body, where they may have a variety of other roles, such as regulation of cell growth and adhesion, which appear to be unrelated to their catalytic properties (Soreq and Seidman 2001). On the other side, the function of BuChE remains unknown. Butyrylcholinesterase is involved in the degradation of succinylcholine, used as a myorelaxant in surgical operations. It also hydrolyzes drugs as heroin (Valentino et al. 1981) and physostigmine (Silver 1974).

The active site of AChE is classically considered as being composed of two subsites, the stearic subsite containing the active site serine, and the anionic subsite, which binds the quaternary ammonium residue of acetylcholine. Binding at this anionic subsite seems to involve hydrophobic as well as electrostatic interactions (Hasan et al. 1980). In addition, AChE possesses one or more additional binding sites for ACh and other quaternary ligands, called peripheral anionic site (Changeaux 1966). Figure 4 shows the region of the active site of *Nippostrongylus brasiliensis* (Nb) AChE.

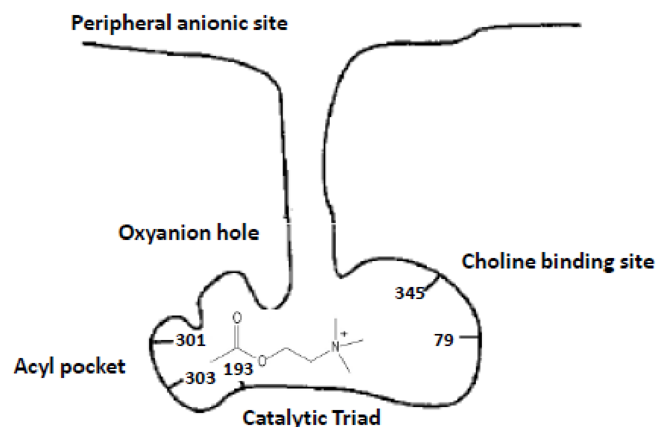


Figure 4 – Illustration of the regions of the active site of *N. brasiliensis* AChE with a molecule of acetylcholine in its interior

AChEs have a region in their active site called “catalytic triad”, which is formed by residues of the amino acids serine (Ser), histidine (His) and glutamate (Glu) (Rosenberry 1975, Shafferman et al. 1992). This active site is located near the bottom of a 20 Å deep, narrow gorge that is lined with 14 conserved aromatic amino acids. Sussman et al. (1991) described the structure of AChE from *Torpedo californica* (Tc). The enzyme monomer is an α/β protein that contains 537 amino acids. It consists of a 12-stranded mixed β sheet surrounded by 14 α helices. The active site is unusual, because it contains Glu, not aspartate (Asp), in the Ser-His-acid catalytic triad (Sussman et al. 1991).

Some compounds, such as natural and synthetic drugs, and pesticides, are known to be reversible and irreversible inhibitors of both cholinesterases. When AChE is inhibited, the neurotransmitter ACh is no longer hydrolyzed in the synapse, the postsynaptic membrane cannot be repolarized, and nerve influx is blocked (Devic et al. 2002). Some typical symptoms in humans of an acute intoxication with pesticides are hypotension, muscle weakness, respiratory depression, and coma, which could lead to death (Heath and Vale 1992). Effects of chronic exposure to insecticides may be for instance cancer, reproductive damage, and dysfunctions of the endocrine system. They may also damage the DNA or have a detrimental effect on the nervous systems of humans. Insecticides also affect wildlife and contribute to losses of biological diversity (Davignon et al. 1965, Worm and Vaupel 2008).

One AChE inhibitors known are the organophosphate and carbamate (CA) insecticides. They bind covalently to the stearic active site of the AChE, inhibiting the catalytic activity. Acetylcholinesterase is inhibited by both types of insecticides, but the mechanism of inhibition is different. In the case of CA the inhibition is slightly reversible while most OP insecticides induce an irreversible inhibition (Tran-Minh et al. 1990). They block the active site of the enzyme by phosphorylation or carbamylation of the Ser residue

of the catalytical triad, hence preventing the termination of a nerve impulse in the postsynaptic membrane (Schulze et al. 2005). The reaction between AChE and paraoxon, a typical organophosphate insecticide, is illustrated in figure 5.

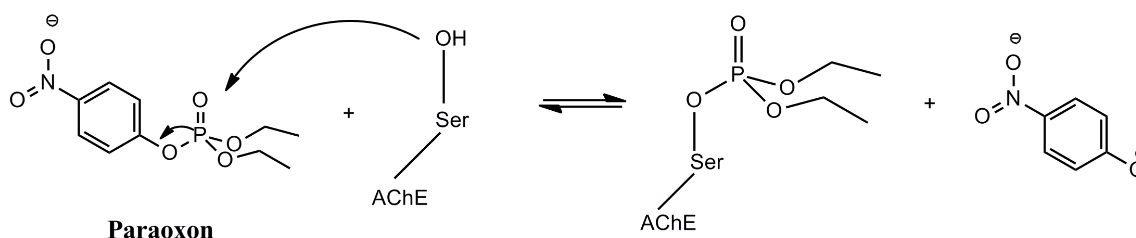


Figure 5 – Phosphorylation of AChE by paraoxon

Although the binding of insecticides to the active center is covalent, reactivation through nucleophilic compounds is possible. The irreversible inhibition over AChE can be reactivated with oxime type-recativation agents such as pyridine-2-aldoxime methachloride (2-PAM), 1,3-bis(4-hydroxyiminomethylpyridinium)-2-oxa-propane dichloride (obidoxime), 2-hydroxyimino-methyl-1-methylpyridium chloride (pralidoxime), and 1-(2-hydroxyimino methylpyridinium)-3-(4-carbamoylpyridinium)-2-oxa-propane dichloride (HI-6) (Tran-Minh et al. 1990, Kuca et al. 2007). This mechanism can be applied for the reactivation of AChE in a biosensor design, which makes repetitive use of the same biosensor after successive inhibition measurements possible (Andreescu and Marty 2006).

1.2.1 *Acetylcholinesterase from* Nippostrongylus brasiliensis

Many parasitic nematodes, in particular those which colonize the alimentary tract of their mammalian hosts, also release AChEs to the external environmental via specialized

secretory glands. Their possible functions have been interpreted in terms of hydrolysis of ACh in intestinal mucosal tissue (Ogilvie et al. 1973, Rhoads 1984). *Nippostrongylus brasiliensis* is a parasite that colonizes the rat intestine and secrets three AChEs. These enzymes are divided in forms A, B and C, which are distinguishable by their electrophoretic properties in non-denaturating gels (Ogilvie et al. 1973). All three forms are monomeric and hydrophilic, with molecular weights and acidic pIs estimated at 74 kDa and 4.0 for form A, 69 kDa and 3.8 for form B, and 71 kDa and 3.6 for form C (Grigg et al. 1997, Grigg 1994). They are expressed at subtly different time points in the life cycle of the parasite. Form A is produced immediately after entry of the fourth stage larvae into the duodenum, and forms B and C are secreted shortly after as the adult worms migrate to a more distal position in the jejunum (Edwards et al. 1971, Blackburn and Selkirk 1992). The reason why this happens is unclear.

The AChE forms from *N. brasiliensis* have truncated C-termini in comparison to AChE from other species. They are similar in this respect to AChEs from *Bungarus fasciatus* venom (Cousin et al. 1996) and from *Boophilus* (Baxter and Barker 1998), which possess short polar C-terminal peptides allowing the production of soluble secreted enzymes. Hussein et al. (2002) observed that three of the fourteen aromatic residues, which line the active site gorge in *T. californica* AChE, are substituted by non-aromatic residues (Y70T, W279D, and F288M) in *N. brasiliensis* AChE. All three variants of *N. brasiliensis* AChE have eight cysteine residues in conserved positions, including six that have been implicated in disulphide bonds in other AChEs (Hussein et al. 2002).

The form B of AChE from *N. Brasiliensis* was successfully expressed in *Pichia pastoris*, where the enzyme efficiently hydrolyzed acetylthiocholine (ATCh) and showed minimal activity against butyrylthiocholine (BuTCh) (Hussein et al. 1999). A recombinant form of

AChE C was also highly expressed in *P. pastoris*, and displayed a marked preference for ACh as a substrate (Hussein et al. 2000).

The structure of the active site of the *N. brasiliensis* AChE B (Figure 6) can be characterized in five regions (Ordentlich et al. 1996, Taylor and Radic 1994):

I. The catalytic triad, formed by Ser-193 (200), His-446 (440) and Glu-342 (327). The position of the amino acids is from the sequence of *N. brasiliensis* AChE B. The numeration between brackets corresponds to the same position in *T. californica*.
II. The choline binding site, formed by Trp-79 (84), Tyr-345 (330), Tyr-130 and Phe-346 (331). The ammonium region of choline binds at Trp-79 through cation-π interactions.
III. The acyl pocket, which defines the specificity of the substrate and is formed by two rests of phenylalanine (Phe-301 (288), Phe-303 (290)).
IV. The oxyanion site, which stabilizes the tetrahedrical transition state through the formation of hydrogen bonds (Gly-111 (118), Gly-112 (119), Ala-194 (201)).
V. The peripheral anionic site, formed by Trp-279, Tyr-65 (70), Tyr-121 and Asp-71.

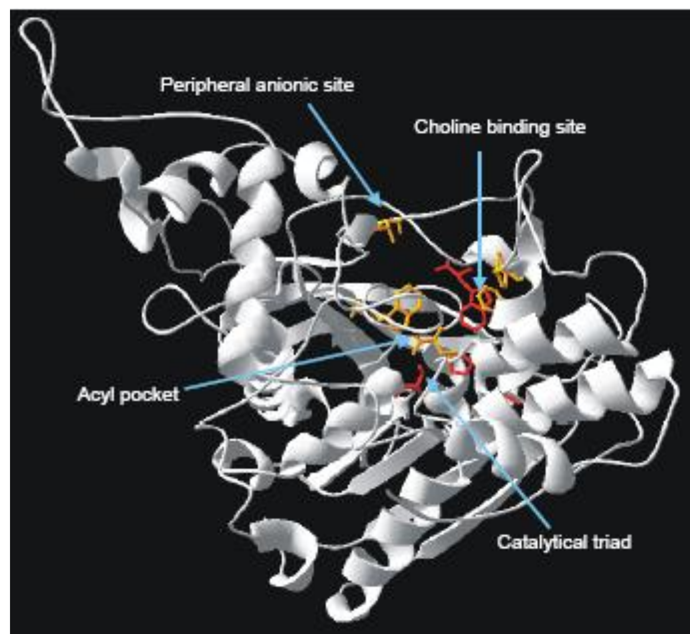


Figure 6 – Stereo representation of *N. brasiliensis* AChE B molecule. Red: residues of the catalytical triad (Ser-193 (200), His-446 (440), Glu-342 (327)) and Trp-79 (84), an essential part of the choline binding site. (Schulze et al. 2005)

A number of Nb AChE B mutants were produced at the Institute of Technical Biochemistry (ITB) of the University of Stuttgart using site-specific mutagenesis (Schulze 2003, Münch 2003, Gebhard 2005). These mutations had as an objective the enhancement of sensitivity of the enzyme toward insecticides, and the mutants were applied latter on biosensors. The wild type (WT) of Nb AChE B and all its recombinant forms were expressed in *P. pastoris*. Table 1 gives a summary of the mutations achieved.

Table 1 – Nb AChE B mutants developed at ITB

Single Nb AChE B mutants			
Nb AChE B position (Tc AChE position)	*Region of the active site*	*Original amino acid*	*Inserted amino acid*
65 (70)	Peripheral anionic site	Threonine	Tyrosine
301 (288)	Acyl pocket	Methionine	Alanine
			Tryptophan
303 (290)		Tryptophan	Glycine
			Alanine
			Leucine
346 (331)		Tryptophan	Alanine
			Valine
345 (330)	Choline binding site	Phenylalanine	Alanine Tyrosine
349 (334)	“Mid-gorge” binding site	Tyrosine	Glycine
			Leucine
Double Nb AChE B mutants			
Nb AChE B position (Tc AChE position)	*Region of the active site*	*Original amino acid*	*Inserted amino acid*
301 (288) 303 (290)	Acyl pocket	Methionine Tryptophan	Alanine Alanine
301 (288) 303 (290)	Acyl pocket	Methionine Tryptophan	Alanine Leucine
301 (288) 345 (330)	Acyl pocket Choline binding site	Methionine Phenylalanine	Alanine Alanine
301 (288) 345 (330)	Acyl pocket Choline binding site	Methionine Phenylalanine	Alanine Leucine
Insertion Nb AChE B mutants			
Between the amino acids threonine 309 and tyrosine 310: insertion of 26 amino acids			

1.3. Acetylcholinesterase biosensors

There is a growing need to develop new technologies to minimize sample preparation, to discriminate between positive and negative samples, to improve sample throughput, and to reduce analysis costs. Analytical technology based on sensors is an extremely broad field, which impacts on many major industrial sectors such as the

pharmaceutical, food and agriculture industries (Marco and Barceló 1996, Amine et al. 2006).

Over the last decades, AChE biosensors have emerged as an ultra sensitive and fast technique for toxicity analysis in environmental monitoring, food and quality control. Promising application areas for AChE biosensor techniques include public safety and military/antiterrorism. Its choice as a biorecognition element enables the simultaneous detection of a wide group of related toxic compounds.

Environmental monitoring and food control generally require the analysis of a larger number of samples and there is a need of low cost, fast and sensitive methods of analysis. So the main motivation for designing AChE biosensors for monitoring analysis is to provide a reliable alternative to classical methods currently used in analytical chemistry laboratories, like gas chromatography (GC) or high performance liquid chromatography (HPLC) coupled with mass selective detectors (MSD). These techniques are very powerful tools for monitoring toxic analytes, but unfortunately are very expensive, time-consuming, nor adapted for *in situ* and real time detection, and require highly trained staff (Andreescu and Marty 2006).

A successful AChE biosensor should offer comparable or even better analytical performances than the traditional chromatographic systems. They should be small, cheap, simple to handle and able to provide reliable information in real-time without or with a minimum sample preparation.

Analytical devices based on the inhibition of AChE have been described in the last years as an alternative for the detection of contaminants in food and environmental samples like OP and CA insecticides (Evtugyn et al. 1996, Abad et al. 1998, Bachmann and Schmid 1999, Schulze et al. 2002a, Schulze et al. 2005, Trojanowicz 2002, Solé et al. 2003), heavy

metals (Evtugyn et al. 2003), glycoalkaloids (Korpan et al. 2002, Arkhypova et al. 2003), aflatoxin B (Pohanka et al. 2008), and anatoxin-a(s) (Villatte et al. 2002, Devic et al. 2002).

Normally, the development of these biosensing systems relies on a quantitative measurement of the enzymatic activity before and after exposure to a target analyte. The percentage of inhibited enzyme (% I) resulted after the exposure to the inhibitor is quantitatively related to the inhibitor concentration and incubation time (Guerrieri et al. 2002, Ivanov et al. 2003). As a result, the residual enzyme activity is inversely related to the inhibitor concentration (Amine et al. 2006).

Substrates and inhibitors bind to three distinct binding sites on the AChE: (1) the size of the acyl pocket determines substrate specificity and mediates sensitivity towards the transition-state analogous inhibitors (OP and CA) of different size; (2) the choline binding site binds specifically to the substrate choline group and is blocked by tricyclic inhibitors (acridine, phenothiazine and their derivatives); (3) the peripheral anionic site is located near the entrance of the gorge and is blocked by charged mono and biquaternary inhibitors (propidium, decamethonium) (Radic et al. 1993).

Acetylcholinesterase biosensors have been shown to be functional, and various biosensors using cholinesterase as the bioanalytical element in combination with amperometric (Mionetto et al. 1994, Bernabei et al. 1992, Bachmann and Schmid 1999, Schulze et al. 2002a), potentiometric (Guindilis et al. 1996, Evtugyn et al. 1996, Lee et al. 2001), optical (Choi et al. 2001, Danet et al. 2000) and piezoelectric (Abad et al. 1998, Makower et al. 2003) transducers have been developed. The type of transducer and detection method is dictated by the choice of the substrate, enzyme system (mono- or multiple enzymes) and by the final application of the device (Andreescu and Marty 2006).

Nevertheless, the application of biosensors in food and environmental analysis is still limited. Acetylcholinesterase-based biosensors have a major drawback: they give a sum parameter of AChE-inhibition without any quantitative information about the individual analytes. That means that different AChE-inhibiting insecticides cannot be measured selectively (Schulze et al. 2003). Recent strategies developed to solve these problems rely on new methods of data analysis, using engineered enzymes, or combining different enzymes (Amine et al. 2006).

Genetically engineered AChE variants with specific and high inhibition constants for the desired analytes help in this way to improve the compound-specific multi-analyte detection. Boublik et al. (2002) conducted a study where the *Drosophila melanogaster* (Dm) AChE was engineered in order to increase its sensitivity and its rate of phosphorylation or carbamylation by organophosphates or carbamates. The greatest improvement was for the sensitivity to dichlorvos for which a mutant was 300-fold more sensitive than the *D. melanogaster* wild type and 288,000-fold more sensitive than the electric eel AChE. Engineered variants of *N. brasiliensis* AChE B were developed, focusing the increase of the sensitivity of insecticide detection. The combination of the WT enzyme with three mutants allowed the detection of 11 out of 14 important insecticides below 10 µg/kg (Schulze et al. 2005).

The application of multiarray sensors combined with chemometric data analysis using artificial neural networks (ANNs) could substantially improve the biosensor selectivity and allow the exact identification of the inhibitor or mixtures of inhibitors present in a sample. Bachmann and Schmid (1999) described for the first time a sensitive screen-printed amperometric AChE biosensor able to discriminate paraoxon and carbofuran in mixtures, applying the chemometric data analysis by ANN and the principle of AChE inhibition in four

types of native or recombinant AChEs. The association of engineered enzymes and ANN was also applied by Bachmann et al. (2000), who employed an array of multienzyme biosensors with four immobilized *D. melanogaster* AChEs (WT and three recombinant mutants).

However, the majority of AChE biosensors reported in the literature have been tested on standard solutions and not on real samples. This makes it difficult to fully evaluate the usefulness of these devices for real sample monitoring. Strategies used for testing in real samples involved spiking the sample (orange juice, water) with a known amount of insecticides and evaluating the inhibition degree in a real matrix (Albareda-Sirbent et al. 2001a, Albareda-Sirbent et al. 2001b). An amperometric AChE biosensor for the determination of carbamate insecticides directly in water, fruit and vegetable samples was proposed by Nunes et al. (1999), using a screen-printed biosensor strip modified by a layer of carbon paste mixed with cobalt (II) phthalocyanine and acetylcellulose. Schulze et al. (2002a) developed a highly sensitive and rapid food screening test based on disposable screen-printed AChE biosensors, which presented itself as suitable for monitoring infant food. The biosensor method could detect levels of insecticides lower than 5 µg/kg of food.

1.3.1 Amperometric acetylcholinesterase biosensors

In course of the years, several generations of ChE biosensors have been developed. These biosensors are categorized in three generations based on the mechanism of electron transfer.

The first generation of AChE biosensors, also known as bi-enzymatic AChE/ChO biosensors is based on the diffusion of the redox compound to/from the biocatalytic active layer. There are two enzymes involved: AChE and choline oxidase (ChO).

Acetylcholinesterase hydrolyzes ACh to choline and acetate; however choline is not electrochemically active. Consequently, a second enzyme, called choline oxidase, is used to produce hydrogen peroxide, which can be easily detected amperometrically at +650 mV versus Ag/AgCl (Figure 7).

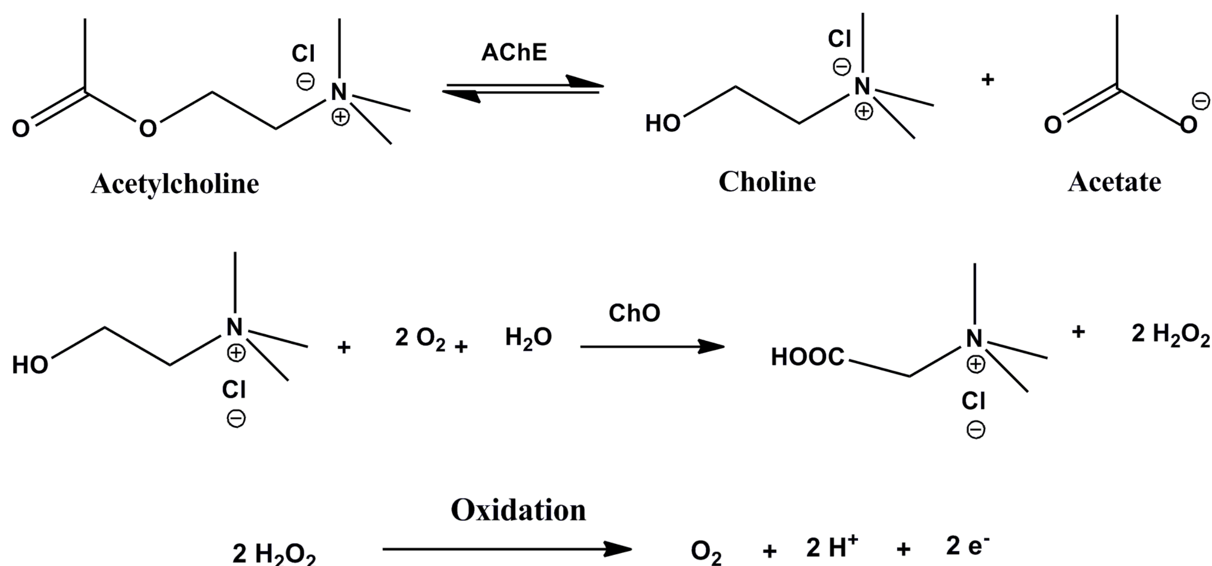


Figure 7 – First generation bi-enzymatic AChE/ChO amperometric biosensor

This indirect detection of AChE is difficult due to the high-applied potential, which leaves the sensor susceptible to interferences from other electro active species available in the reaction medium (Andreescu and Marty 2006).

On the other side, the second generation of AChE biosensors uses ATCh as substrate. The system is based on the direct amperometric detection of thiocholine, the product of the enzyme catalyzed reaction. Figure 8 illustrates this type of amperometric AChE biosensor.

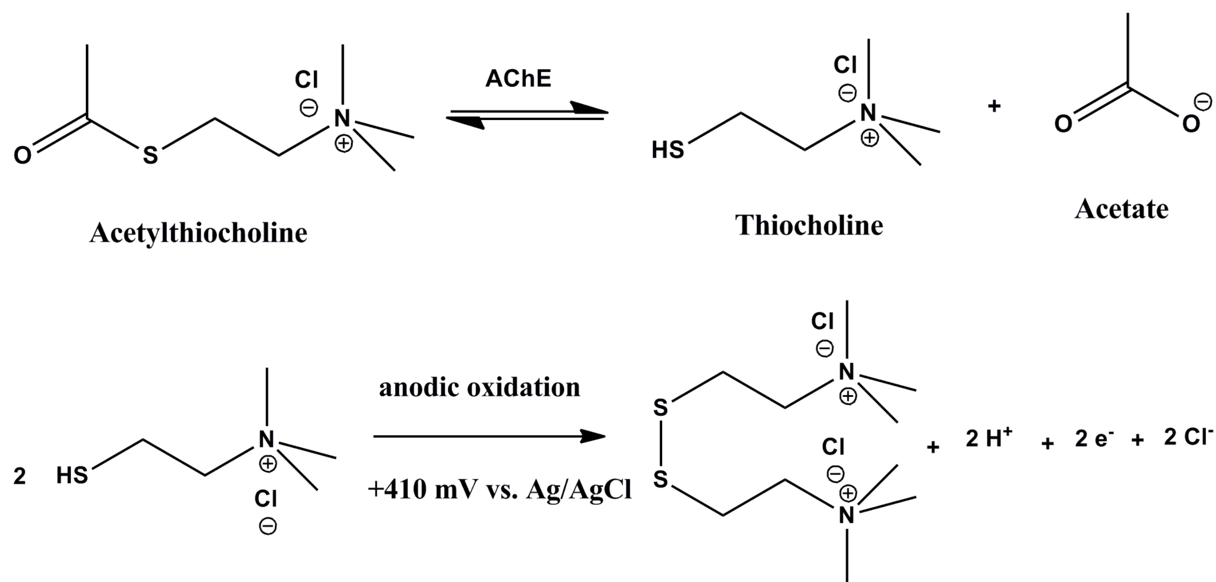


Figure 8 – Second generation AChE amperometric biosensor

This system has two major advantages over the bienzymatic AChE/ChO biosensors. First, the use of a single enzyme simplifies the design of the sensor and, secondly, the detection potential is lower than the one used for the oxidation of hydrogen peroxide (+410 mV compared to +650 mV versus Ag/AgCl). The only limitation of this system seems to be the slight spontaneous hydrolysis of the ATCh in the absence of enzyme, but this can be avoided by dilution in a NaCl (0.09%, w/v) solution and storage in ice (Andreescu and Marty 2006).

The use of electrochemical mediators such as tetracyanoquinodimethane (TCNQ) and cobalt phtalocyanine (CoPC) has improved the second generation AChE biosensors (Figure 9). They allow the working potential of the enzyme electrode to be determined by the oxidation potential of the mediator.

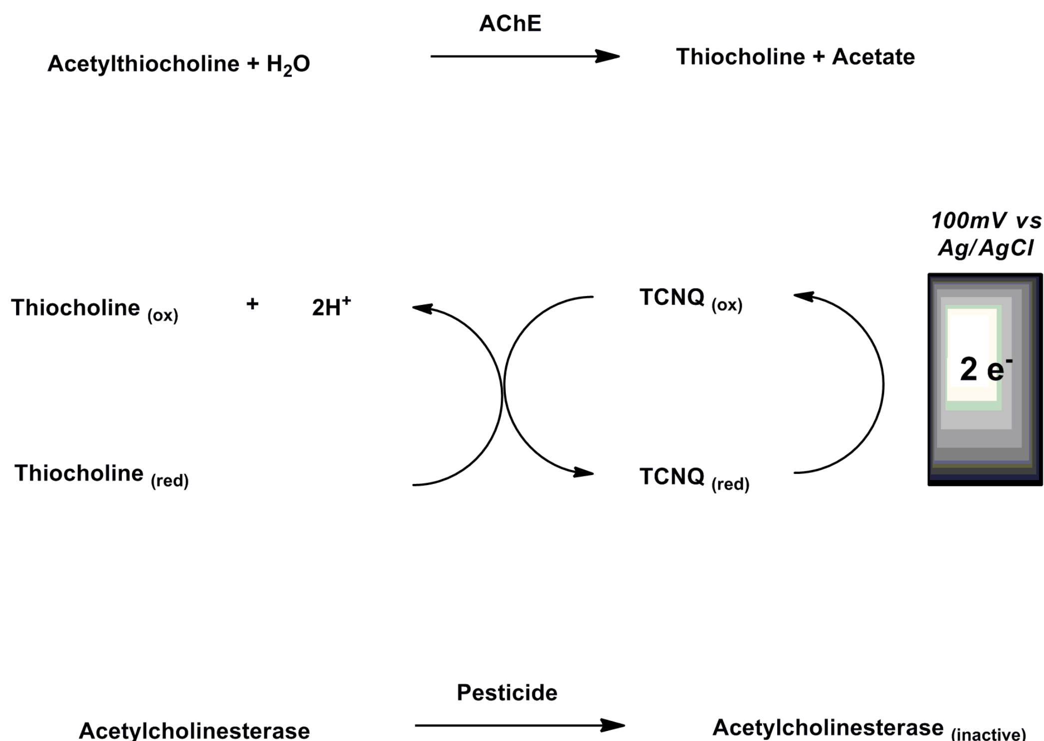


Figure 9 – Schematic representation of the amperometric detection of inhibitors of AChE with an electrode using TCNQ as an electrochemical mediator

Sensors with mediated detection are known to be less susceptible to interfering compounds due to the lower electrode potential. The mediators are normally immobilized onto the surface of the working electrode, in order to allow a fast electron transfer. This can be carried out in the same matrix as the enzyme or in a separate layer. For example, the mediator can be attached onto the surface of a graphite working electrode by simple mixing with the carbon paste. The composite layer can also contain the enzyme and bi-functional cross-linking reagents, such as glutaraldehyde (Hart et al. 1997).

The third generation of AChE biosensors is characterized by more complex electrode configurations involving three enzymes with or without mediator. A mediatorless tri-enzyme system was reported by Ghindilis et al. (1996). The sensor is very similar with the bi-enzyme amperometric configuration; however it uses a third enzyme (a peroxidase) to detect the hydrogen peroxide generated in the ChO catalyzed reaction.

A recent trend in AChE biosensor manufacturing is to use nanostructured materials and nanocomposites to obtain a high surface area and an increased sensitivity. Because of their high electronic conductivity combined with a high surface area, these materials are especially attractive for electrochemical biosensors (Andreescu et al. 2005). Examples of materials used with this aim are gold nanoparticles (Du et al. 2008a, Du et al. 2009), quantum dots (Du et al. 2008b), silicon nanowire (Su et al. 2008), and carbon nanotubes (Chen et al. 2008, Joshi et al. 2005, Lin et al. 2004).

1.4. Types of inhibition

Enzymatic inhibition can be characterized in two types: irreversible and reversible. Irreversible inhibition is known by the covalent bonding between enzyme and inhibitor and thus requires either a new biosensor after the inhibitor measurement or a reactivation of the biosensor in use. Reversible inhibition, on the other hand, is characterized by noncovalent interaction between inhibitor and enzyme with the consequent restoration of the initial activity after the inhibition measurement. This is what renders measurements based on reversible inhibition especially cost-effective, fast, easy, and reproducible (Korpan et al. 2002). The same biosensor can be used for several analyses provided there is a simple washing step with buffer solution after the inhibition and reaction time (Arkhypova et al. 2003, Dzyadevych et al. 2004a).

In order to optimize the analytical performance of biosensors based on reversible inhibition, it is essential to know the mechanism of inhibition. The different types of reversible inhibition depend on the different mechanisms involved in interactions between

enzyme and inhibitor. They are: competitive, non-competitive, uncompetitive and mixed (Figure 10).

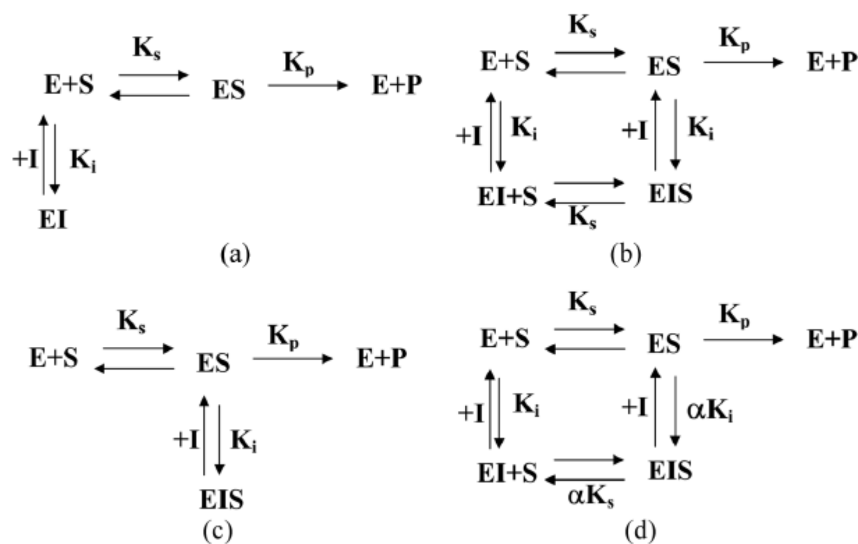


Figure 10 – Scheme of mechanism for competitive, noncompetitive, uncompetitive, and mixed inhibition type. (a) competitive, (b) non-competitive, (c) uncompetitive, (d) mixed (Arduini et al. 2009)

In competitive inhibition, the inhibitor may bind to the active site center and competes with the substrate for the active site. This equilibrium is regulated by the constant of inhibition that describes the affinity of the inhibitor for the enzyme. A high concentration of substrate competes with the inhibitor and prevents the detection of a low concentration of inhibitor. For this reason, to reach a low detection limit, the substrate concentration should be chosen as a compromise between having good analytical signal and an inhibition effect still detectable for the expected level (Arduini et al. 2009).

In the case of non-competitive inhibition, the inhibitor binds to the enzyme and the enzyme-substrate complex, most likely at a site other than the active site. Consequently, the inhibitor does not compete with the substrate, and the degree of inhibition does not depend on the substrate´s concentration. Therefore, it is much easier to reach a low detection limit

of the analyte/inhibitor, because there is no limitation in using an amount of substrate giving the optimal analytical signal (Arduini et al. 2009).

In the uncompetitive inhibition, the inhibitor binds only to the enzyme-substrate complex, so that the degree of inhibition does not depend on the substrate when the concentration of substrate is higher than the Michaelis-Menten constant (K_m) value. On the other hand, in the mixed inhibition, the inhibitor binds to the enzyme and the enzyme-substrate complex with different affinities as regulated by the binding affinity of the inhibitor (K_i) in the case of of inhibitor-enzyme complex and αK_i for the inhibitor-enzyme substrate complex. The inhibition is designated mixed, predominantly competitive when $\alpha<1$, mixed predominantly uncompetitive when $\alpha>1$, and noncompetitive when $\alpha=1$. Hence noncompetitive inhibition is only a case of mixed inhibition (Arduini et al. 2009).

For the evaluation of the type inhibition and determination of K_i, an exact evaluation of type of the mechanism can be made by use of the Lineawever-Burk plot, Dixon plot, and Cornish-Bowden plot (Lineawever and Burk 1934, Dixon 1953, Cornish-Bowden 1974). It should be noted that K_i is equal to the concentration, giving 50% inhibition when the inhibition is noncompetitive or uncompetitive and the concentration of substrate is superior to K_m.

1.5. Organophosphate and carbamate insecticides

One of the solutions found to grant sufficient nutrition supplies for a rapidly increasing global population was the production of pesticides. According to the United Nations (UN), the human population worldwide in 2050 will be of approximately 9 billion of people (UN 2009) (Figure 11).

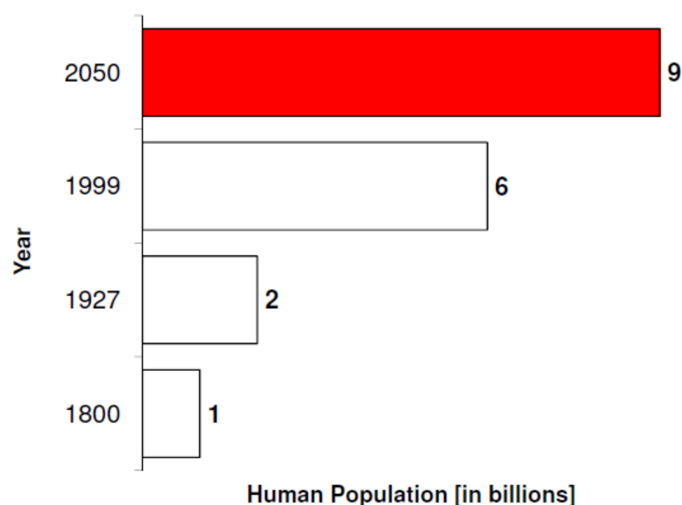
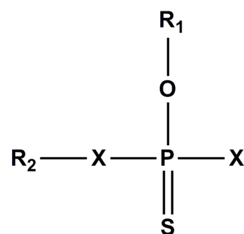
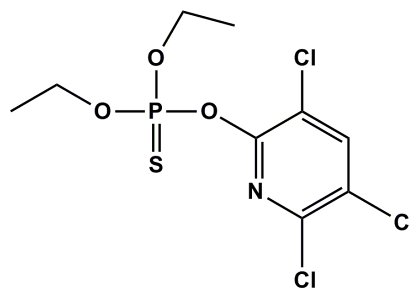


Figure 11 – Estimation of the world population (UN 2009)

Pesticides are used in agriculture in order to increase yield and control fungi, insects and weeds. Since the banning of organochlorides, organophosphates and carbamates are the most used insecticides, due to their high activity and relatively low persistence (Amine et al. 2006, Mora et al. 1996). According to the statistical data published by the Food and Agriculture Organization (FAO) of the UN, the consumption of organophosphate insecticides worldwide in 2001 was of about 22,000 metric tons (m.t.), and of 13,000 m.t. of carbamates (FAO 2004). The disadvantage of the use of these insecticides is the contamination of drinking water and food. Besides, it has been proved that they show chronic and acute toxicity also against humans (Jeyaratnam 1990).

Organophosphate insecticides contain a central phosphorus atom with a double bond to sulfur or oxygen, R_1 and R_2 groups that are either ethyl or methyl in structure, and a leaving group which is specific to the individual organophosphate. The general structure of organophosphates and a specific example (chlorpyrifos) are shown below (Figure 12):

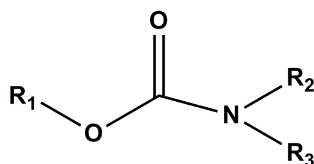


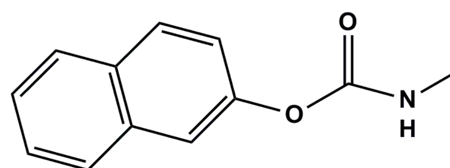


General chemical structure **Chlorpyrifos**

Figure 12 – General chemical structure of organophosphate insecticides and an example

On the other hand, carbamate insecticides are organic compounds derived from carbamic acid, featuring the carbamate ester functional group (Figure 13).





General structure **Carbaryl**

Figure 13 – General chemical structure of carbamate insecticides and an example

Acetylcholinesterase is inhibited by organophosphates and carbamates (Fukuto 1990). Since these insecticides not only inhibit insect AChE, but also interfere with neural transmission in other organisms, including humans, they represent a potential hazard for human health and environmental food chains, and thus require continuous assessment (EC Council Directive 1980). Monitoring of insecticides has increased in recent years since most countries have established maximum residue level (MRL) for insecticides in food products, in order to protect consumers (European Commission 2008a). Therefore, there is an urgent demand, especially in developing countries like Brazil, for rapid sensitive and cost-effective insecticide detection technologies.

The classical and standard assays for insecticide detection are based on gas chromatography or high performance liquid chromatography coupled with mass selective detectors (Martinez et al. 1992, Pylypiw 1993). The disadvantage of these methods is the relatively long assay time, applicability only in central laboratories and their inherent restriction to a limited number of insecticides, which can be identified by multiresidue methods (Schulze et al. 2002a).

As an alternative, AChE inhibition tests, and AChE biosensors in particular, have been repeatedly described as tools for insecticide detection. Thus they are regarded as highly promising candidates for on-site insecticide detection (Schulze et al. 2005, Nagatani et al. 2007, Hildebrandt et al. 2008). Acetylcholinesterase-based test strips have been extensively used in insecticide residual detection in fruits and vegetables. Compact and portable devices specifically designed for in-field analysis are also described in the literature. No et al. (2007) developed an AChE coated membrane strip for organophosphate and carbamate insecticide assays. In another study, Kim et al. (2007) presented an AChE-based kit for monitoring of insecticides present in agricultural samples. However, most of these tests strips are based on colorimetric methods that are only qualitative and easily interfered with by colors present in real samples (Chen et al. 2008).

In this regard, electrochemistry offers an ideal tool for reliable AChE-based inhibition assays that provides quantitative signals and is in principle not susceptible to even heavily colored samples. The more common AChE biosensor design is based on the immobilization of only AChE and acetylthiocholine as substrate in combination with an amperometric transducer. The reaction product thiocholine is oxidized at the working electrode (Gulla et al. 2002, Del Carlo et al. 2006).

1.5.1 Organophosphate and carbamate insecticides used in Brazil

In most countries, before it can be marketed and used in order to manage a pest problem, insecticides must be registered in a government agency responsible for regulating the sale, the distribution and the use of insecticide products. Though insecticide regulations differ from country to country, insecticides and products on which they were used are traded across international borders. To deal with inconsistencies in regulations among countries, delegates of FAO adopted a code in 1985, named the “International Code of Conduct on the Distribution and Use of Pesticides”. The objective was to create voluntary standards of insecticide regulation for different countries. This code was updated in 1998 and 2002 (FAO 2010).

In the United States, the Environmental Protection Agency (EPA) establishes the MRLs, while the U.S. Department of Agriculture organizes the Pesticide Data Program (PDP). Data obtained between the years 2004 and 2007 shows lower occurrence of detection of insecticides in food samples than in 2003, going from 53.9 % of total samples in 2003 to 23.1% in 2007. The largest increase in the detection of insecticides in the period 2003-2008 occurred in those samples where four or more residues where present. This increase was of almost 11% (EPA 2010).

In the European Union, the European Commission reviewed and simplified the rules related to pesticide residues, creating the Regulation EC No. 396/2005. This new regulation covers approximately 1,100 pesticides that are currently or were formerly used in agriculture in or outside the EU, and it lists the MRLs for 315 agricultural products. When a pesticide is not specifically mentioned, a general default MRL of 0.01 mg/kg applies (European Commission 2008b). The annual report on pesticide residues, published by the European

Food Safety Authority (EFSA), showed that 96.5% of the samples analyzed were in accordance with the legal MRLs and 3.5% exceeded them (EFSA 2010).

In 2008 Brazil became the world´s biggest pesticide consumer market, record previously held by the USA (ANVISA 2009). According to data provided by the Brazilian Association of the Chemical Industries (ABIQUIM) about pesticides in Brazil, it is known that their consumption enhanced significantly in the last years, and that in 2009 the total amount was of US$ 6.3 billion (ABIQUIM 2010). In a study done with Brazilian export fruits, 23.2% were positive to insecticide residues, and 14.3% of the samples even exceeded the European Union MRLs (Ciscato et al. 2009). Furthermore, due to differences in the legislation that regulates the insecticide market, Brazil also imports high amounts of highly toxic, severely restricted or even banned insecticides from developed countries (FASE 1998, Smith 2001). Gebara et al. (2005) observed in a monitoring study, done between 1994 and 2001 for insecticides residues in vegetables and fruits sold in the city São Paulo, that 32.6% of the analyzed samples contained one or more insecticide residues. Violations of the MRL were observed in 3.0% of the vegetables and 0.9% of the fruit samples.

The Health Ministry is responsible for the legislation and the control of pesticides in Brazil. The Regulation of Pesticides No. 7802/1989 determines that only pesticides approved by the National Sanitary Agency (ANVISA), a department of the Health Ministry, can be commercialized in Brazil (ANVISA 2008). In order to protect the population against the risks associated with insecticides, ANVISA has been promoting since 2001 a nationwide monitoring program for insecticide residues in fruits and vegetables, called the PARA Program (Oliva et al. 2003). In 2009, this program analyzed 20 types of fruits and vegetables, and it showed that the use of non authorized insecticides is still very high in the country. Moreover, these substances were also used in concentrations higher than accepted by the

legislation. 29% of the samples analyzed were irregular (ANVISA 2010a). In a study published by Caldas et al. (2006), the cumulative exposure of twenty five AChE inhibiting insecticides through the consumption of nine fruits and vegetables by the Brazilian population was assessed, using the residue data provided by the PARA program between 2001 and 2004. The exposure to AChE inhibiting insecticides for the general population at percentiles P99.9 represented 33.6% of the acute reference dose (ARfD) as methamidophos and 70.2% ARfD as acephate. One worrying conclusion was that the exposure of children up to 6 years was 2.4 times higher than the exposure for the general population. Tomato represented 67% of the total intake of AChE inhibiting insecticides.

The results of this program between the years 2005 - 2007 can be observed in figure 14 (ANVISA 2008). The percentages shown are from fruits and vegetables samples with insecticides residues against the total amount of samples analyzed.

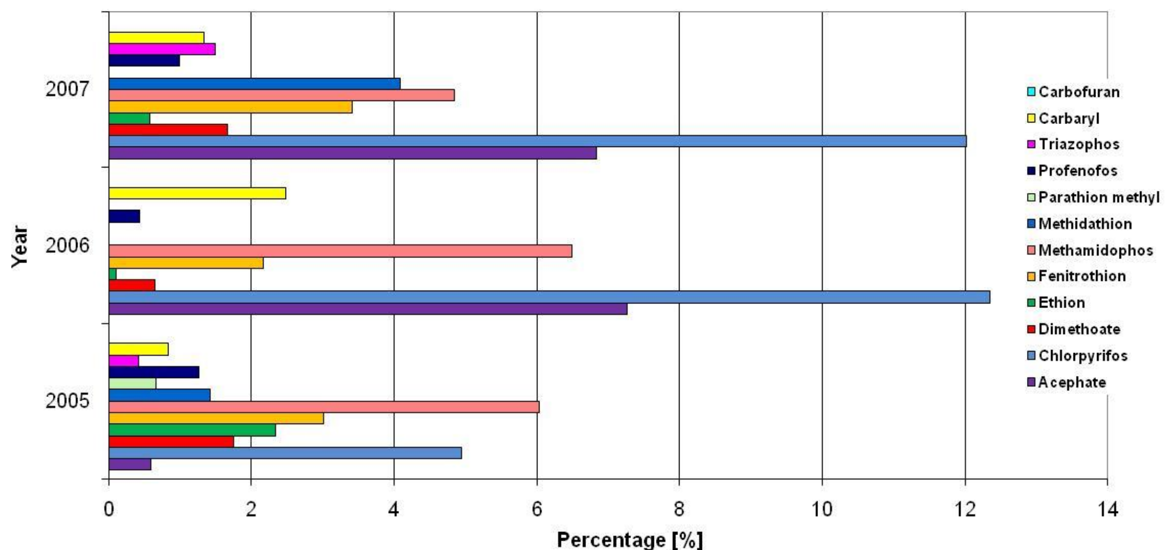
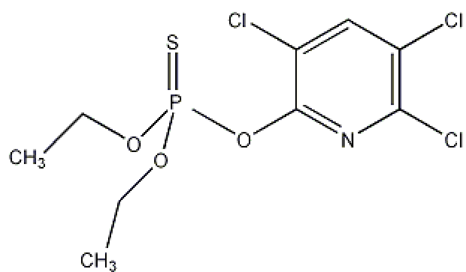
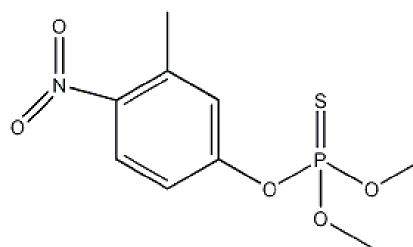


Figure 14 – Detection of carbamate and organophosphate insecticides in food samples by the PARA Program between the years 2005-2007

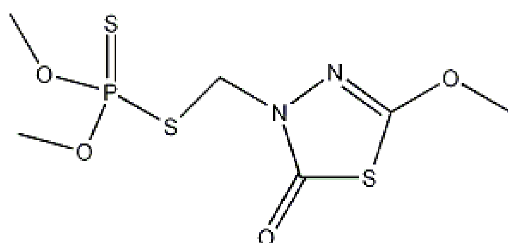
The analysis of the results showed that the organophosphate insecticides with the highest occurrence in vegetables and fruits commercialized in Brazil were: acephate, chlorpyrifos, dimethoate, ethion, fenitrothion, methamidophos, methidathion, parathion-methyl, profenofos, and triazophos (Figure 15). The most present carbamates insecticides were: carbofuran and carbaryl (Figure 16).



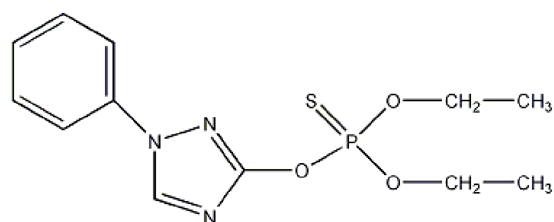
Chlorpyrifos



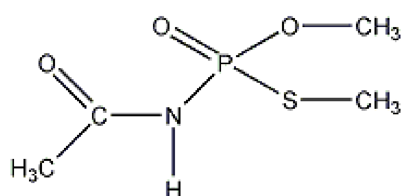
Fenitrothion



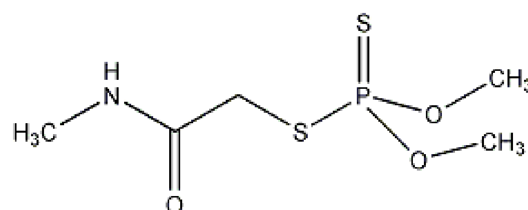
Methidathion



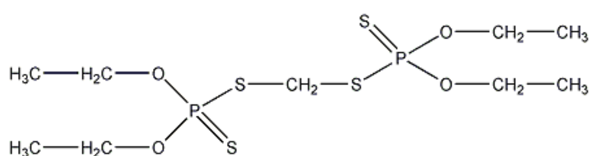
Triazophos



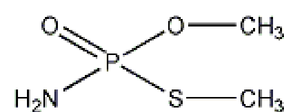
Acephate



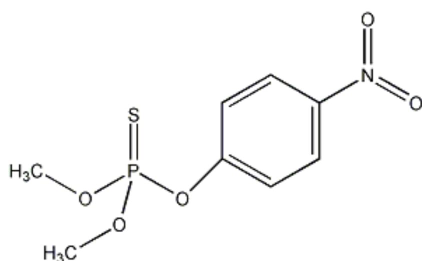
Dimethoate



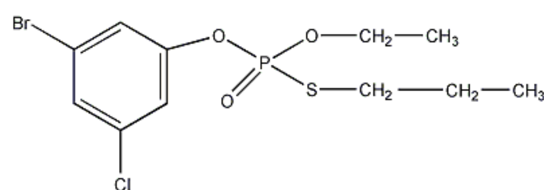
Ethion



Methamidophos



Parathion-methyl



Profenofos

Figure 15 – Organophosphate insecticides used in Brazil detected by the PARA Program

Figure 16 – Carbamate insecticides used in Brazil detected by the PARA Program

1.5.2 Organophosphorothionate insecticides

The majority of the organophosphate insecticides applied worldwide belongs to the group of phosphorothionates. They are lipophilic and characterized by one thione moiety (P=S) and three other radical groups attached to a phosphorus atom, whereas their respective oxidized analogues are more polar, characterized by a double phosphorus oxygen bound (P=O) (Chambers 1992) (Figure 17).

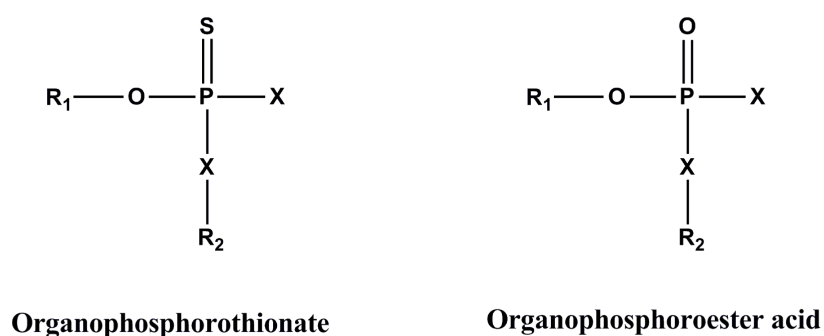


Figure 17 – Structure of an organophosphorothionate insecticide and its oxon form

This group of insecticides usually exhibit a considerably reduced inhibitory effect towards AChE due to the lower reactivity of the P=S group. This is caused by the smaller electronegativity of sulfur compared to oxygen, thus complicating the attack of nucleophilic agents like the serine hydroxyl of esterase (Fukuto 1990). Despite of this, the AChE inhibition ability of these insecticides is greatly increased *in vivo*, as a result of their transformation into their oxon analogues by cytochrome P-450 monooxygenase, found in living organisms (Jokanovic 2001).

Phosphorothionates are not detectable in commonly found concentrations by AChE inhibition, and therefore are an analytical challenge. To solve this problem, they have to be oxidized into their oxon analogues before the execution of the inhibition tests, in order to enhance the sensitivity and prevent false negative results.

According to DIN 38415-1 (Deutsches Institut für Normung), oxidations can be performed using *N*-bromosuccinimide (NBS), and the excess of reagent is destroyed by ascorbic acid (DIN 2005). However, this method revealed itself unsuitable for the analysis of food samples, due to a less oxidizable food matrix than water (Schulze et al. 2004). Because NBS exhibits restricted water solubility, increasing the concentration of this oxidant is rather limited (Herzsprung et al. 1992). Besides, ascorbic acid, a widespread food additive and a natural ingredient, can block the chemical oxidation with NBS in food samples, what does not occur in water (Schulze et al. 2004). In a study using NBS and ascorbic acid in food samples, the addition of a 50-fold concentration of NBS and a 500-fold concentration of ascorbic acid higher than the organophosphorothionates was successful for the oxidation of this group of insecticides (Kralj et al. 2006). However, this assay was only applied in apple juice samples, which naturally have a low concentration of the antioxidant ascorbic acid. On the other hand, in a study using an apple puree baby food spiked with 20 µg/kg parathion,

where the sample was submitted to chemical oxidation and analyzed with the AChE biosensor assay, 20 mg/L of NBS were unable to generate an inhibition over the AChE biosensor after 30 min incubation (Schulze et al. 2004). Fauser (2003) observed that the oxidation of parathion on the surface of apples by means of NBS according to DIN 38415-1 (DIN 2005) did not result in any paraoxon formation (Fauser 2003).

The application of a prokaryotic cytochrome P450 mutant for the oxidation of organophosphorothionates was shown to successfully increase the sensitivity of the AChE biosensor assay (Schulze et al. 2004, Waibel et al. 2006).

1.6. Chloroperoxidase

Chloroperoxidase (CPO) (EC 1.11.1.10) is produced by the marine fungus *Caldariomyces fumago*. This enzyme is a glycosylated hemoprotein containing iron (III) protoporphyrin as the prosthetic group, which has a molecular weight of 42 kDa (312 amino acid residues, predominantly acidic). Chloroperoxidase shows a p*I* value in the range 3.2 – 4.0 (Hager et al. 1966, Pickard et al. 1991, Sundaramoorthy et al. 1995). Figure 18 illustrates the stereo representation of the CPO molecule from *C. fumago*.

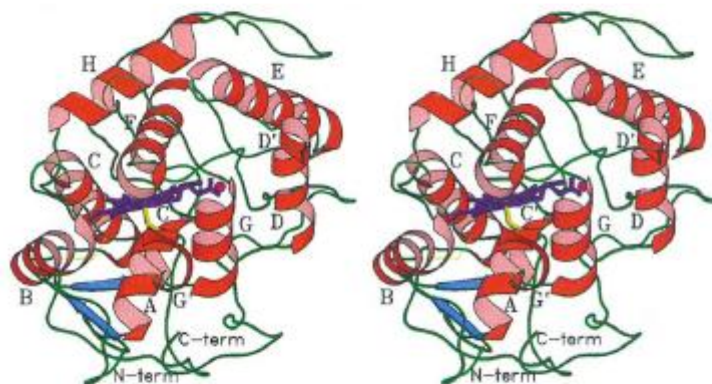


Figure 18 – Stereo representation of *Caldariomyces fumago* CPO molecule. α-helices are shown in red and are labeled with upper-case letters from A-H and 3_{10} helices are labeled with primed letters. The β-pair is shown as blue arrows. The cation is indicated as a pink sphere near a heme propionate. The N and C termini are only 5.6 Å apart and are bridged by a solvent molecule (not shown) (Sundaramoorthy et al. 1995)

Chloroperoxidase uses hydrogen peroxide (H_2O_2) as oxidant and does not require any cofactor. This enzyme also has a broad substrate range, is easily isolated in large quantities and is relatively stable under nonoxidizing conditions. These properties turn the CPO as one of the most attractive and promising peroxidase enzymes for synthetic applications (Van Deurzen et al. 1997). Chloroperoxidase shows cytochrome P450-like monooxygenase activity, in addition to peroxidase, halogenase and catalase activity (Hernandez et al. 1998, Andersson et al. 2000).

Chloroperoxidase and cytochrome P-450 present a number of similar physical properties as shown by electronic absorption, electron paramagnetic resonance, and Mossbauer spectroscopy. Chloroperoxidase, like P-450, forms a reduced +CO complex absorbing at an abnormally long wavelength (443 nm). This happens due to thiolate anion as the proximal ligand of the heme iron (Hollenbe and Hager 1973, Dawson et al. 1976). The fifth axial ligand of the catalytically active iron of CPO is, analogously to the P450 enzymes, a cysteine instead of the usual histidine. Furthermore, the iron atom is more exposed than in

any peroxidase known. It has been named “a heme peroxidase-cytochrome P450 functional hybrid” for these reasons (Sundaramoorthy et al. 1995, Van Deurzen et al. 1997).

Chloroperoxidase employs hydrogen peroxide as the natural source of oxygen in direct oxygen transfers to its substrates via an iron (IV) oxo porphyrin radical cation (compound I) (Kiljunen and Kanerva 2000). The main difference between CPO and monooxygenases is that the monooxygenases require reduction with a cofactor (like NAD(P)H) and oxidation with the more expensive molecular oxygen in the formation compound I (Van Deurzen et al. 1997, Colonna et al. 1999, Van de Velde et al. 1999). The proposed mechanism of action of CPO is illustrated in figure 19.

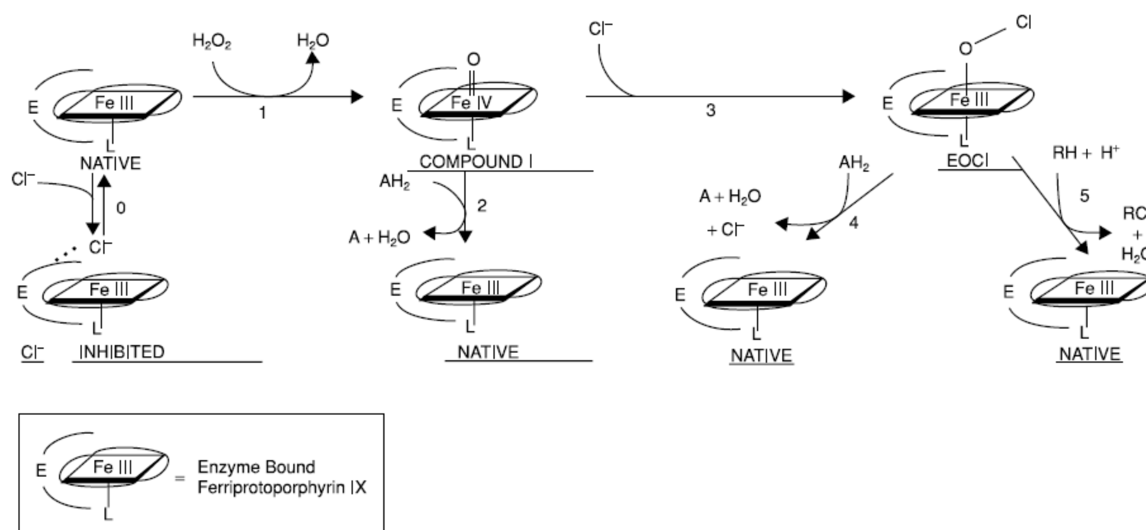


Figure 19 – Proposed mechanism of action of CPO (Amitai et al. 2003)

The main limitation of all heme-containing peroxidases is their low operational stability, mostly due to their rapid deactivation by hydrogen peroxide or organic hydroperoxides. The inactivation of CPO occurs very rapidly in the presence of hydrogen peroxide; thus its concentration is critical for enzymatic oxygen transfers. Deactivation of heme-containing peroxidases by hydrogen peroxide is thought to involve the oxidation of

the porphyrin ring and the formation of the inactive intermediate compound III (Van Deurzen et al. 1997, Vazquez-Duhalt et al. 1993, Mylrajan et al. 1990).

In order to increase the operational stability of CPO toward peroxide (oxidative inactivation), the concentration of H_2O_2 has to be maintained at a low level. This can be achieved by continuous addition of H_2O_2 (Van Deurzen et al. 1994), or, preferably, by feed-on-demand (Van Deurzen et al. 1997). To avoid high local concentrations, H_2O_2 can be generated *in situ*, for example by the oxidation of glucose mediated by glucose oxidase (Neidleman et al. 1981).

In many synthetic applications of the enzyme, *tert*-butyl hydroperoxide is now replacing hydrogen peroxide as an oxygen donor. An advantage of *tert*-butyl hydroperoxide is that the catalase side reaction can be avoided. In this reaction, H_2O_2 dismutates into water and molecular oxygen, which causes problems with pressure build up and the evaporation of volatile substrates (Van Rantwijk and Sheldon, 2000). The use of *tert*-butyl hydroperoxide is also reasonable because *tert*-butyl alcohol, its product, showed a stabilizing effect on CPO (Van Deurzen et al. 1997). However, a major disadvantage of *tert*-butyl hydroperoxide is that its rate is much lower than that of H_2O_2 (Van Rantwijk and Sheldon, 2000). It has also been shown that in concentrations up to 30%, *tert*-butyl hydroperoxide can be harmful to CPO (Van Deurzen et al. 1997).

Another strategy that has been used is to increase the stability of peroxidases toward hydrogen peroxide; this means the improvement of these enzymes through protein engineering, using site-directed and random mutagenesis (Van de Velde et al. 2001). Random mutagenesis was used to obtain mutants that were resistant to the suicide deactivation, via N-alkylation of the heme that occurs with the native CPO during the epoxidation of 1-alkenes (Rai et al. 2000). However, a major drawback of the expression of

the *cpo* gene in its original organism is the possibility that both wild-type and mutant CPO are being produced in the *C. fumago* constructs. Heterologous expression of CPO in another host could solve this problem. Indeed, the *cpo* gene was expressed in the filamentous fungus *Aspergillus niger*. The *A. niger* strain produced an active extracellular enzyme (Conesa et al. 2001).

The application of CPO to perform oxidations of organophosphorothionate insecticides, that means the transformation of P=S into P=O, was described by Hernandez et al. (1998). Ten organophosphorus insecticides containing the phosphorothionate group were oxidized by CPO in the presence of H_2O_2 and chloride ions. The products were identified as oxon derivatives (phosphates), where the sulfur atom from the thiolate group is substituted by an oxygen atom. Figure 20 demonstrates the mechanism for phosphorothionates oxidation executed by CPO from *C. fumago*.

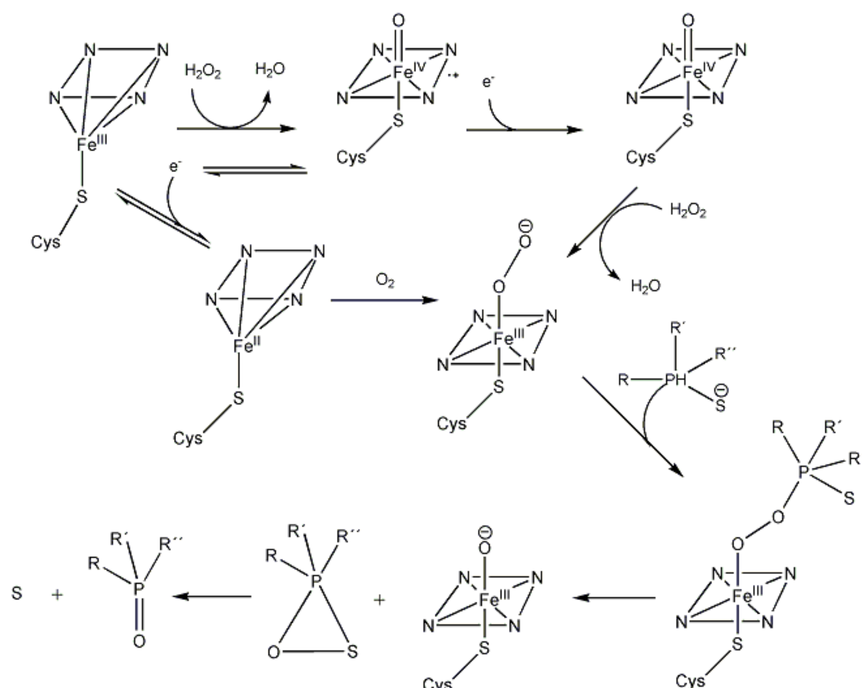


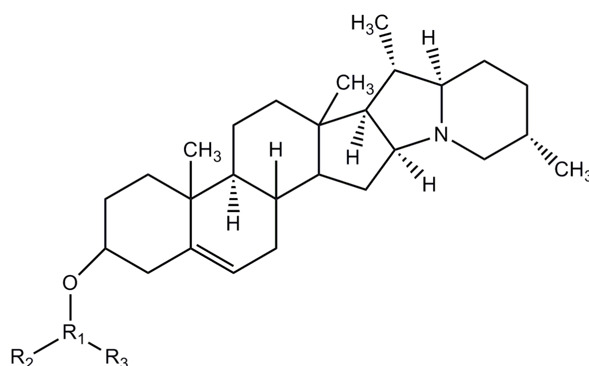
Figure 20 – Proposed mechanism for phosphorothionates oxidation performed by means of chloroperoxidase from *Caldariomyces fumago* (Walz and Schwack 2007)

Walz and Schwack (2007) combined a CPO pre-oxidation step, followed by a solvent extraction of the food samples and a spectrophotometric enzymatic assay. The idea was to transform organophosphorothionate insecticides into their more potent serine esterase inhibiting oxon analogues, and then measure their inhibition over the enzyme cutinase.

1.7. Glycoalkaloids

Potato (*Solanum tuberosum* L.) is one of the most important staple crops for direct and processed consumption. It is widely grown in over 80% of countries worldwide, which makes it the world´s number one non-grain food commodity. However, it produces potentially toxic glycoalkaloids (GA) during growth and after harvest.

Glycoalkaloids are nitrogen-containing derivatives of higher plants steroid glycoside-saponins of *Solanaceae* plant family, which includes potatoes and tomatoes (Gee et al. 1996). α-Solanine and α-chaconine are among the most common solanaceous glycoalkaloids (Morris and Lee 1984). The structures of these two glycoalkaloids are illustrated in figure 21.



alfa-solanine	**alfa-chaconine**
R_1 = D-galactose	R_1 = D-glucose
R_2 = D-glucose	R_2 = L-rhamnose
R_3 = L-rhamnose	R_3 = L-rhamnose

Figure 21 – Structure of α-solanine and α-chaconine

Because glycoalkaloids are present in mainly almost all commercial potatoes, they are a widely consumed dietary secondary metabolite. A person consuming 500 g of potatoes may ingest up to 100 mg of glycoalkaloids (Hopkins 1995). Initial levels of the total potato glycoalkaloids are genetically determined, and significant differences occur according to varieties and production locations. They are supposed to be involved in some disease and insect resistance mechanisms. Various factors, such as annual or regional variations, exposure to light (green potatoes), insect damage, disease, chemicals or mechanical damage (harvesting) may result in a substantial increase in the initial glycoalkaloids concentration (Ahmed and Müller 1978, Cronk et al. 1974, Hlywka et al. 1994, Percival et al. 1996). Neither α-solanine nor α-chaconine levels are affected by food processing and preparation (Bushway and Ponnampalam 1981, Zhao et al. 1994).

A safety level of steroidal GAs in potatoes has been established at 200 mg/kg (250 μM) for acute toxicity, but this level does not account for possible subacute or chronic effects. A more rigorous limit of 60 – 70 mg/kg (75 – 87.5 μM) has been proposed for cultivars to be selected for human consumption (Valkonen et al. 1996). Commercially available potatoes used for consumption in Europe and USA contain about 20-150 mg of total glycoalkaloids per kg of unpeeled tuber. The lethal oral dose for humans of α-chaconine was estimated to range from 3 to 6 mg/kg (Morris and Lee 1984).

The consumption of potatoes containing higher than normal levels of steroidal glycoalkaloids is associated with human deaths, poisonings and a lot of livestock deaths. High concentration of alkaloids may cause acute poisoning, including gastrointestinal and neurological disturbances in humans, with death caused by central nervous system depression (Ripakh and Kim 1968, McMillan and Thompson 1979). Moreover, these substances have been shown to be teratogenic, genotoxic and embryotoxic. There is an

increased risk for brain, breast, endometrium, lung and thyroid cancers (Baker et al. 1987, Keeler et al. 1990, Morris and Lee 1984). The mechanism of toxicity described in the literature consists of two processes: disruption of phospholipids in membranes (Roddick 1989) and inhibition of acetylcholinesterase (Orgell et al. 1958).

The anti-cholinesterase activity of solanaceous glycoalkaloids was first demonstrated by Pokrovskii (1956) and has been confirmed by a number of works both *in vitro* and *in vivo* (Orgell et al. 1958, Bushway et al. 1987, Roddick 1989). Kinetics studies have shown that α-solanine and α-chaconine are reversible inhibitors of AChE and BuChE (Nigg et al. 1996, Benilova et al. 2006). Roddick (1989) described that the inhibition caused by α-chaconine and α-solanine over the bovine and human AChE occurred at a concentration of 100 μM. It was also demonstrated that BuChE is more sensitive to potato glycoalkaloids than AChE (Arkhypova et al. 2003).

Therefore, there is a strong need of higher control of glycoalkaloids in the agriculture, food analysis and health care. The methods that are routinely used to detect these alkaloids are colorimetry (Bushway 1980), gas chromatography (Herb et al. 1975), high performance liquid chromatography (Jonker et al. 1992, Crabbe and Fryer 1980), and, more recently, immunoassays (ELISA) (Morgan et al. 1983). All these methods are complex, and need an expensive and bulky instrumentation with high power consumption and well-trained operators.

The ChE biosensors could be a promising tool to replace the traditional methods. Several pH-sensitive field effect transistors (pH-SFET) have been developed for this purpose (Korpan et al. 2002, Arkhypova et al. 2003, Dzyadevych et al. 2004a). Korpan et al. (2002) designed a biosensor sensitive to glycoalkaloids using BuChE as a biorecognition element and with a detection limit of 0.5 μM for α-chaconine and of 2.0 μM for α-solanine. A

biosensor based on potentiometric pH-SFET was developed utilizing BuChE from horse serum (Arkhypova et al. 2003). Moreover, a potentiometric biosensor based on ion-selective field-effect transistors (ISFET) and immobilized AChE and BuChE was studied by Dzyadevych et al. (2006).

2. Objectives

2.1. General Objective

1. Develop biosensors based on acetylcholinesterase from *Nippostrongylus brasiliensis* for the detection of neurotoxic substances in foods and the environment.

2.2. Specific Objectives

1. Design a multisensor of acetylcholinesterase from *N. brasiliensis* specific for the detection of the most applied organophosphates and carbamates insecticides in Brazil;

2. Propose a method to activate and to detect phosphorothionate insecticides, composed by a pretreatment using the enzyme chloroperoxidase and a detection analysis using Nb acetylcholinesterase biosensors;

3. Design a biosensor of acetylcholinesterase from *N. brasiliensis* for the screening of the glycoalkaloids α-solanine and α-chaconine in buffer and food samples.

3. Material and Methods

3.1. Material

3.1.1 Chemicals, biochemicals and equipments

All reagents were supplied by Sigma-Aldrich (Deisenhofen, Germany) or Fluka (Buchs, Switzerland) in analytical grade, unless stated otherwise.

Glycoalkaloids stock solutions were prepared as 2 mM solutions in 5 mM acetic acid and stored at - 20° C. Dilutions were prepared daily in 5 mM acetic acid and stored at - 4° C.

The food samples for the validation of the biosensor tailor made for Brazil were supplied by the *Chemisches und Veterinäruntersuchungsamt* (CVUA) Stuttgart. The potato samples were acquired in supermarkets in Stuttgart, Germany, as well as the organic orange juice applied in the method for activation and detection of organophosphorothionate insecticides.

The chemicals, biochemicals and equipments used in the experiments are listed in table 2.

Table 2 – Utilized chemicals

Chemicals	**Producer**
α-chaconine	Carl Roth (Karlsruhe, Germany)
α-solanine	Carl Roth (Karlsruhe, Germany)
Electrotag 423 SS (graphite paste)	Acheson (Scheemda, Niederland)
Electrotag 6037 SS (Ag/AgCl paste)	Acheson (Scheemda, Niederland)
Electrotag PF-410 (silver paste)	Acheson (Scheemda, Niederland)
Insecticides (Pestanal)	Riedel de Haën (Seelze, Germany)
Marabu dilutor UKV2	Marabuwerke (Tamm, Germany)
Marastar SR 057	Marabuwerke (Tamm, Germany)
Polyvinylchloride sheets	SKK (Denzlingen, Germany)
T15 graphite	Lonza (Sins, Switzerland)
Tween 20	Riedel de Haën (Seelze, Germany)
Zeocin	Invitrogen (Karlsruhe, Germany)
Polyester screens	Steinmann GmbH (Stuttgart, Germany)
Discovery DSC-18 Solid Phase Extraction Cartridge	Sigma-Aldrich (Deisenhofen, Germany)
Bradford reagent	BIO-RAD Laboratories (Munich, Germany)

Insecticide stock solutions were prepared as 1 g/L solutions in ethanol and stored at -20° C. Dilutions for sensor measurements were made daily in 50 mM phosphate buffer solution (PBS) pH 7.5 and stored at - 4° C. The insecticides and biochemicals applied in this work are listed in tables 3 and 4, respectively.

Table 3 – Insecticides used as inhibitors for the acetylcholinesterase biosensor measurements

Insecticide
Acephate
Carbaryl
Carbofuran
Chlorpyrifos
Dimethoate
Ethion
Fenitrothion
Methamidophos
Methidathion
Omethoate
Parathion methyl
Profenofos
Triazophos

Table 4 – Utilized biochemicals

Biochemical	**Producer**
SDS-PAGE-Standard Page Ruler Unstained Protein Ladder (SM0661)	Fermentas (Burlington, Canada)
Chloroperoxidase (EC 1.11.1.10) (CPO) from *Caldariomyces fumago* (suspension in 0.1 M sodium phosphate pH 4.0; concentration higher than 10,000 U/mL)	Sigma-Aldrich (Taufkirchen, Germany)

The library of acetylcholinesterase (AChE) from *N. brasiliensis* developed by ITB (wild type and 19 mutants) was applied in this work (for more information about the mutants, see table 1). They were previously expressed in a *Pichia pastoris* X33 strain (Invitrogen, Karlsruhe, Germany) transformed with a pPICZαB vector (Invitrogen, Karlsruhe, Germany) bearing the *N. brasiliensis* AChE gene (Hussein et al. 1999). Mutagenesis was performed by primer extension using the Quickchange kit (Stratagene, La Jolla, USA). The sequence was checked by DNA sequencing.

Table 5 describes the equipments used in the experiments.

Table 5 – Equipments used in the experiments

Equipment	Description	Producer
Autoclave	PACS 2000	Getinge AB (Getinge, Sweden)
pH-Meter	Digital pH-mV-Thermo Meter GPRT 1400 AN	Greisinger Electronic (Regenstauf, Germany)
Four channel potentiostat	MCP 94	Bank-Wenking (Clausthal-Zellerfeld, Germany)
GC/MS	QP 2010 HP 5890 Series II	Shimadzu (Kyoto, Japan) Hewlett-Packard (Palo Alto, USA)
LC/MS	HP 1100 series	Hewlett-Packard (Palo Alto, USA)
HPLC	1200 Series	Agilent Technologies (Santa Clara, USA)
Centrifuge	5417 R 5810 R Sorvall RC 5C Plus	Eppendorf (Hamburg, Germany) Eppendorf (Hamburg, Germany) Thermo Scientific (Waltham, USA)
UV visible Spectrophotometer	Nicolet Evolution 100	Thermo Scientific (Waltham, USA)
Incubator	HT	Infors (Bottmingen, Germany)
Rotamix	RM1	Elmi (Riga, Latvia)
Screen printer	DEK 249	DEK Ltd. (Weymouth, England)
Evaporator	EZ 2 Plus	Genevac (Valley Cottage, USA)
Rotatory evaporator	Laborota 4000 Efficient	Heidolph (Schwabach, Germany)
Mixer	KPG mixer RW-20	Janke & Kunkel IKA Labortechnik (Staufen i. Br., Germany)
Ultra-Turrax	T 25	Janke & Kunkel IKA Labortechnik (Staufen i. Br., Germany)
Multichannel recorder	BD111 BD 101 Linseis L 200 E	Kipp & Zonen (Solingen, Germany) Kipp & Zonen (Solingen, Germany) Linseis (Selb, Germany)
Vacuum manifold / Vacuum regulator	QIAvac 6S	Qiagen (Hilden, Germany)
Ultrasonic bath	Sonorex Super RK 514H	Bandelin Electronics (Berlin, Germany)

3.1.2 Cultivation medium

Double deionized water was utilized for the production of mediums and buffers. The sterilization was done under 121° C for 20 min in an autoclave. A digital pHmeter was used for the pH determination.

YPP Medium (Yeast Extract Peptone Phosphate buffer)

Bactopeptone	20 g/L
Yeast extract	10 g/L
Phosphate buffer (1 M, pH 7.5)	q.s.

Glycerin stock cultures

Glycerin (86%)	500 µL
Culture (OD_{600} = 1)	500 µL

3.1.3 Buffer, solutions and antibiotics

3.1.3.1 Buffers

50 mM Phosphate buffer (pH 7.2)

0.5 M KH_2PO_4 solution	71.7 mL
0.5 M K_2HPO_4 solution	28.3 mL

50 mM Phosphate buffer (pH 7.5)

0.5 M KH_2PO_4 solution	83.4 mL
0.5 M K_2HPO_4 solution	16.6 mL

100 mM Phosphate buffer (pH 6.0)

1.0 M KH_2PO_4 solution	13.2 mL
1.0 M K_2HPO_4 solution	86.8 mL

1 M Phosphate buffer (pH 7.5)

10 M KH_2PO_4 solution	83.4 mL
10 M K_2HPO_4 solution	16.6 mL

50 mM Acetate buffer (pH 5.5)

6.804 g $C_2H_3NaO_2$.3 H_2O in 1000 mL of water. The pH was adjusted with 50 mM acetic acid.

1 M Acetate buffer (pH 5.5)

136.08 g $C_2H_3NaO_2.3\ H_2O$ in 1000 mL of water. The pH was adjusted with 1 M acetic acid.

10 mM Acetate buffer (pH 7.5), 50 mM NaCl

$C_2H_3NaO_2.3\ H_2O$	1.361 g/L
NaCl	2.922 g/L
Water	q.s.

The pH was adjusted with 10 mM acetic acid.

10 mM Phosphate buffer (pH 7.5), 50 mM NaCl

0.1 M KH_2PO_4 solution	83.4 mL
0.1 M K_2HPO_4 solution	16.6 mL
NaCl	0.2922 g

3.1.3.2 Solutions

Methanol mineral stock solution

$CuSO_4.5\ H_2O$	3.00 g
NaI	0.04 g
$MnSO_4.H_2O$	1.50 g
$CoCl_2$	0.25 g
$ZnCl_2$	10.00 g
H_3BO_4	0.01 g
$Na_2MoO_4.2\ H_2O$	0.10 g
$FeSO_4.7\ H_2O$	32.50 g
Biotin	0.10 g
6N H_2SO_4	15 mL
100% methanol	q.s. 500 mL

Methanol mineral solution

1.2 mL in 100 mL of 100% methanol.

3.1.3.3 Antibiotics

The characteristics of the antibiotic zeocin applied in the tests are listed in table 6.

Table 6 – Applied antibiotic

Antibiotic	*Stock solution concentration*	*Used concentration*
Zeocin (Bleomicine derivate)	100 mg/mL in H_2O	1 µL/mL

3.2. Methods

3.2.1 Spectrophotometric enzymatic activity determination

3.2.1.1 Optical acetylcholinesterase activity assay

Acetylcholinesterase activity was determined spectrometrically at room temperature (Ellmann et al. 1961). The method is based on the formation of the product 2-nitro-5-thiobenzoic acid (TNB), a substance with a yellow coloration. The ATCh present in the solution will be hydrolyzed by AChE, producing thiocholine and acetate. The thiocholine will react with 5, 5´- dithiobis-2-nitrobenzoic acid (DTNB), generating the desired product (Figure 22).

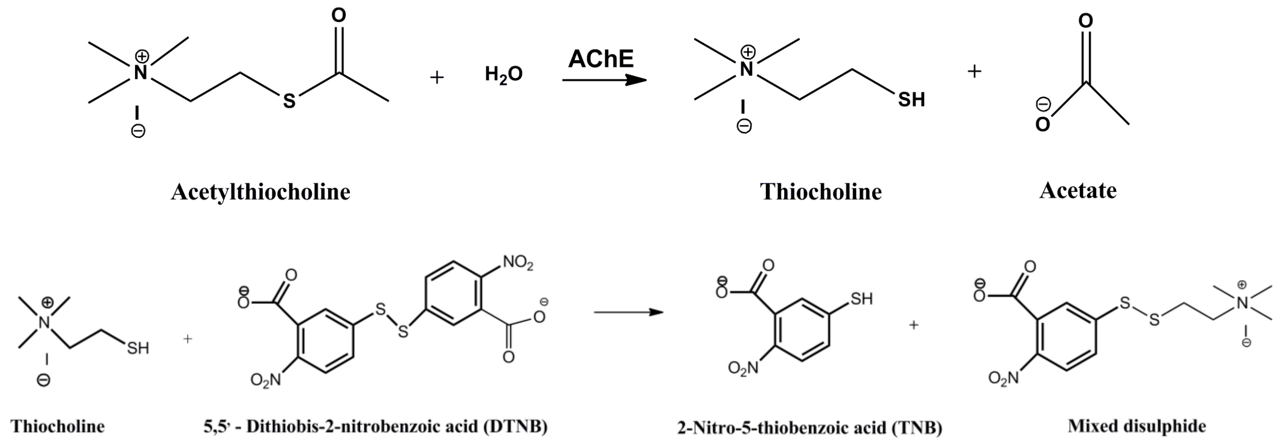


Figure 22 – Principle of the Ellman Test

In brief, the assay containing 790 µL of 50 mM PBS pH 7.2, 100 µL of 7.8 mM DTNB (in 50 mM PBS pH 7.2) and 10 µL of 10 mM acetylthiocholine iodide solution (in water) was started by adding 10 µL of enzyme solution. The increase in absorption was followed at 412 nm.

Using a spectrophotometer, the modification of the absorbance was observed for a determined time (ΔA/Δt). The enzyme activity could be calculated using the Lambert-Beersches law:

$$(1)\quad A = \varepsilon . c . d$$

A = absorbance

d = cuvette length (1 cm)

ε = extinction coefficient (13,600 L $mol^{-1}.cm^{-1}$)

c = molar concentration of the absorbing substance ($mol.L^{-1}$)

(2) $Activity\ (Act) = \frac{n}{t}$

(3) $A = \varepsilon . Act . t . d$

(4) $Act = \frac{\Delta A}{\Delta t} . \frac{V}{\varepsilon . d}$

The activity was calculated in Units per milliliter: $[Act] = \frac{\mu mol}{\min . mL} = \frac{U}{mL}$

3.2.1.2 Optical chloroperoxidase activity assay

The method of CPO activity determination assay measures the oxidation of pyrogallol to purpurogallin by chloroperoxidase when catalyzed peroxide at 420 nm (Figure 23) (McCarthy and White 1983).

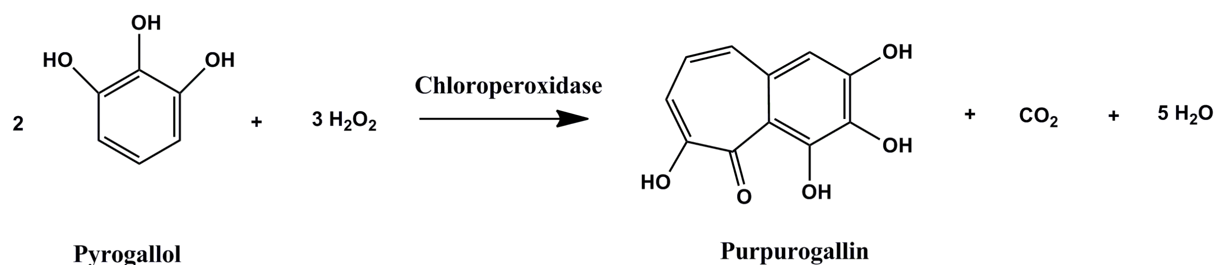


Figure 23 – Principle of the optical CPO activity assay using pyrogallol

The determination of CPO activity was recorded spectrometrically at room temperature. The assay containing 800 µL of 0.1 M PBS pH 6.0, 100 µL of 5.33% pyrogallol solution (w/v) and 67 µL of 0.4% H_2O_2 solution was initialized by adding 33 µL of enzyme

solution. The increase in absorption was followed at 420 nm. The volumetric CPO activity was calculated in U/mL.

The activity of CPO was obtained using the equation (4). The extinction coefficient (ε) of purpurogallin is 12,000 L $mol^{-1}.cm^{-1}$.

The activity was calculated in Units per milliliter: $[Act] = \frac{\mu mol}{\min .mL} = \frac{U}{mL}$

3.2.1.3 *Bimolecular rate constant (k_i)*

The rate of phosphorylation or carbamylation of CPO is expressed in terms of bimolecular rate constant (k_i), characterizing the inhibitory capacity of insecticides. The determination of the k_i values was performed according to Aldridge (1950) (Figure 24).

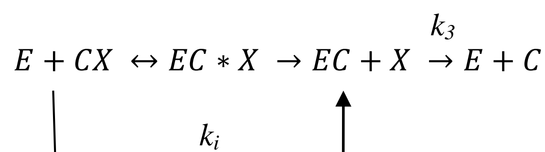


Figure 24 – Inhibition mechanism of AChE (Aldridge, 1950); E = enzyme, CX = carbamate or organophosphate compound, X = leaving group, C = remaining group, k_3 = decarbamylation rate constant and k_i = bimolecular rate constant

To estimate them, the enzymes (E) were incubated for various periods of time with different AChE inhibitors concentrations ([I]) (with [I] being at least 10-fold higher than [E] in 50 mM PBS pH 7.5 at 25° C). The change of the concentration of the free enzyme [E] over

time was estimated by recording the remaining activity measured as the turnover of the acetylthiocholine substrate (1 mM) and follows a pseudo-first-order kinetics:

$$(5)\ \ln \frac{[E]}{[E_o]} = -ki.[I].t$$

$[E_o]$ = initial enzyme concentration

[E] = free enzyme concentration after incubation with inhibitor

k_i = bimolecular constant

[I] = inhibitor concentration

t = time of incubation

The determination of the k_i value is based in the following correlation:

$$(6)\ ki = -\frac{m}{[I]} \quad [M^{-1}.min^{-1}]$$

m = obtained slope from the relation between the logarithm from the AChE remaining activity and the incubation time

[I] = inhibitor concentration (mol/L)

3.2.2 Determination of the protein concentration

In order to determine the concentration of protein present in the samples, 800 µL of sample and 200 µL of Bradford reagent were mixed in a cuvette, and exposed to the light. After 5 min, the absorbance was determined at 595 nm. The protein concentration was calculated using a calibration curve (Bradford 1976).

3.2.3 Acetylcholinesterase biosensor test for food samples

3.2.3.1 Production of biosensors using screen printing method

3.2.3.1.1 Basic acetylcholinesterase biosensor production

Disposable biosensors with immobilized AChE were produced by screen-printing as illustrated in figure 25 using a DEK 249 screen printer and polyester screens (Bachmann and Schmid 1999).

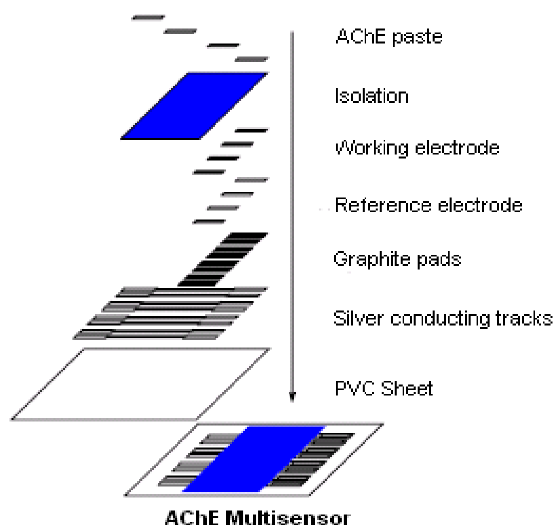


Figure 25 - Screen printing method for the production of AChE multisensors

As a support for the transducer, flexible polyvinylchloride sheets (10 X 10 X 0.05 cm) were used. Different pastes were printed onto these sheets, one after the other. After printing the fifth layer (isolation layer), the biosensors were dried for 1 h under 90° C in a drying chamber. Finally, the transducers were stored under 4° C.

The pastes were printed over a sieve with the help of a squeegee. The different parameters applied are listed in table 7.

Table 7 - Screen printing parameters for the production of basic acetylcholinesterase biosensors

Printing step	**Paste**	**Squeegee Pressure**	**Gap (mm)**	**Squeegee speed (mm/s)**
Silver conducting tracks	Electrodag PF-410	4	1.8	50/50
Graphite pads	Electrodag 423 SS	5	1.3	70/70
Reference electrode	Electrodag 6037 SS	5	1.4	70/70
Working electrode	15% (w/w) TCNQ-graphite in 3% (w/w) HEC solution	7	1.4	50/50
Isolation	Marastar SR 057 90% in Matt Vinyl R14	5	2.0	70/70
AChE paste	2 U/mL AChE in 5% (w/w) BSA in 1% (w/w) HEC	6	1.5	70/70

3.2.3.1.2 Working electrode (TCNQ-graphite)

For the production of the TCNQ-graphite working electrode, 100 mg 7,7,8,8-Tetracyanoquinodimethane (TCNQ) was dissolved in 300 mL acetone and mixed with 4 g T15 graphite. The acetone was evaporated using a rotatory evaporator. For the printing paste, the obtained TCNQ-graphite was applied in a 15% (w/w) solution with 3% (w/v) hydroxycellulose (HEC) solution. This paste was mixed for 1 h.

3.2.3.1.3 Acetylcholinesterase printing paste

For each AChE listed on the item 3.2.3.6 a separated printing paste was prepared according to the produced biosensor. 5% bovine serum albumine (BSA) was dissolved in a 1% (w/v) hydrohyethyl cellulose (HEC) solution. In order to produce a 2 U/mL AChE printing paste, the unpurified *P. pastoris* supernatant obtained in the expression experiments was diluted using the HEC/BSA solution. Screen printing parameters are shown in Table 7. Wild type and mutants of *N. brasiliensis* AChE were immobilized on top of the working electrodes by cross-linking with glutaraldehyde vapor (15 min, room temperature) after printing. The storage of the biosensor was made in Petri plates at 4°C.

3.2.3.2 Samples preparation

3.2.3.2.1 Chemical oxidation of organophosphorothionates in buffer solution

A 40 µL NBS solution (0.4 g/L) was added to 3.92 mL of the organophosphorothionate sample solution (final NBS concentration, 10 mg/L) and incubated in an ultrasonic bath for 5 min. After that, 40 µL of ascorbic acid solution (4.0 g/L) was added to remove excessive NBS by mixing in an ultrasonic bath for 5 min.

3.2.3.2.2 Chemical oxidation of organophosphorothionates in food samples

The samples (10 g) were mixed with 10 mL 1 M PBS pH 7.5 to ensure a sufficient amount of water at a neutral pH value. A 200 µL NBS solution (0.4 g/L) was added to 19.60 mL of the sample and buffer mixture and ultrasonicated for 5 min. After that, 200 µL of ascorbic acid solution (4.0 g/L) was pipetted into the solution; this final solution was exposed to the ultrasonic bath for more 5 min.

3.2.3.3 Acetylcholinesterase biosensor measurement

3.2.3.3.1 Acetylcholinesterase biosensors measurement tests for insecticide detection

All biosensor experiments to determine AChE activity were carried out in a stirred buffer solution at room temperature. The enzyme activity was determined by monitoring thiocholine formed by enzymatic hydrolysis of acetylthiocholine chloride (final

concentration, 1 mM). Thiocholine was detected by oxidation at +100 mV versus Ag/AgCl. The reaction scheme of thiocholine oxidation is shown in figure 26.

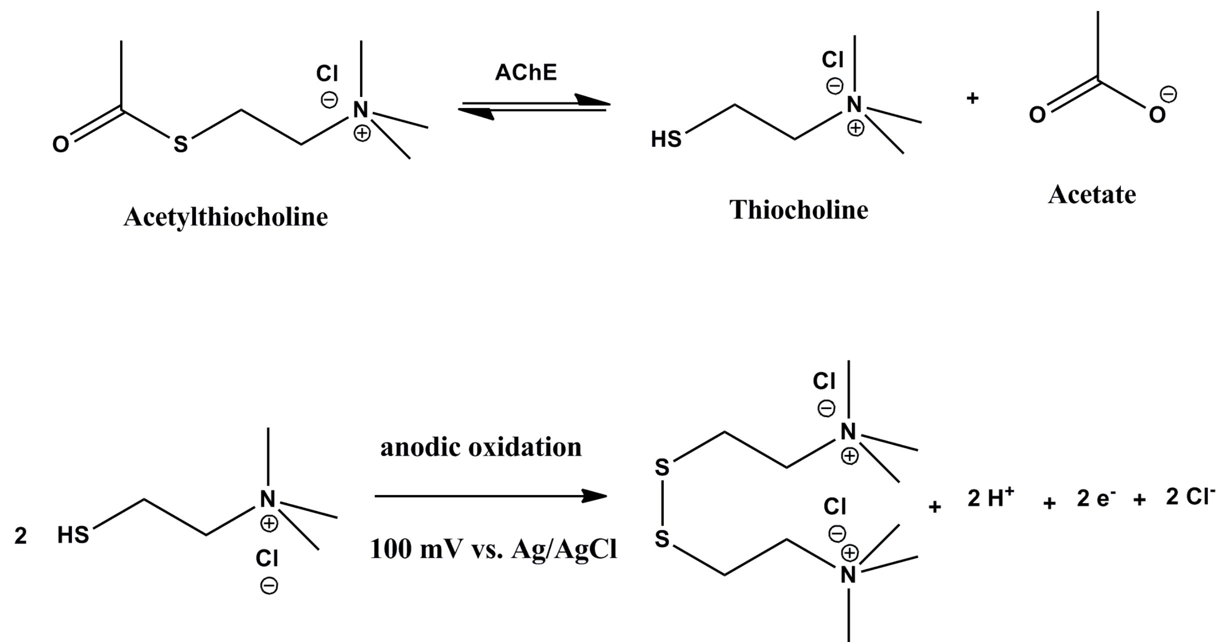


Figure 26 – Reaction scheme of acetylthiocholine hydrolysis and subsequent amperometric thiocholine oxidation

The AChE biosensors were connected to a 4-channel potentiostat, and the outsignal was monitored with a multichannel recorder (Figure 27).

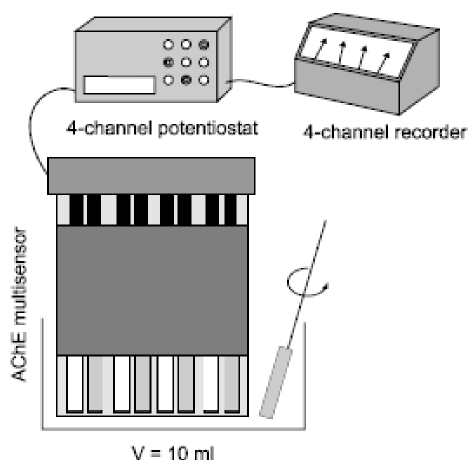


Figure 27 – Acetylcholinesterase multisensor measurement using a 4-channel potentiostat

The measurement tests for the detection of insecticides using the Nb AChE biosensor tailor made for Brazil were executed according to the protocol described in table 8. In the tests applying chloroperoxidase and AChE biosensors, 10 mM acetate buffer pH 7.5 was used instead of 10 mM PBS pH 7.5.

Table 8 – Protocol for the AChE biosensors measurement tests for insecticide detection

Measurement Test Step	***Description***
1st Step Measurement of enzyme activity without inhibition (a_0)	AChE biosensors were incubated in a stirred 10 mL 10 mM PBS (with 50 mM NaCl, pH 7.5) and the 100 mV voltage was turned on. After 10 min, 100 µL of 100 mM ATChCl was added to the solution (end concentration: 1 mM). The voltage was turned off after 5 min, and the biosensors washed with 10 mM PBS pH 7.5.
2nd Step Incubation in the insecticide solution	AChE electrodes were incubated for 30 min in a non-stirred sample solution
3rd Step Measurement of enzyme activity after incubation with the sample (a_i) – remaining activity 1	See 1st Step
7th Step Reactivation with 2-PAM	The electrodes were incubated for 30 min in 10 mL stirred 2-PAM solution (1 mM in PBS pH 7.5)
8th Step Measurement of the remaining activity 2	See 1st Step

Acetylcholinesterase activity was calculated as %H from steady state currents monitored in an acetylthiocholine concentration of 1 mM, according to the formula:

$$(7) \% H = \frac{I_O - I_H}{I_O} . 100$$

%H = percentage of the AChE activity inhibition

I_O = Current signal before the biosensor incubation (Initial activity)

I_H = Current signal after the incubation (Remaining activity)

To determine the AChE activity reactivation rate, the following formula was used:

$$(8)\% \ RA = \frac{I_{RA} - I_H}{I_O - I_H} .100$$

%RA = percentage of the reactivation rate

I_O = current signal before incubation in the sample (initial activity 2)

I_H = current signal after incubation (remaining activity 1)

I_{RA} = current signal after incubation with 2-PAM (remaining activity 2)

3.2.3.3.2 Detection limit of the biosensors

The limit of detection, expressed as the concentration, cL, is derived from the smallest measure, xL, which can be detected with reasonable certainty for a given analytical procedure. The value of xL is given by the equation:

$$(9)\ xL = \bar{x}bi + k\ sbi$$

where $\bar{x}bi$ is the mean of the blank measures, sbi is the standard deviation of the blank measures, and k is a numerical factor chosen according to the confidence level desired (IUPAC 1997). The numerical factor chosen for the analysis was 3 (99% confidence level).

3.2.3.3.3 Acetylcholinesterase biosensor measurement tests for glycoalkaloids detection

The measurement tests for the detection of glycoalkaloids using the Nb AChE biosensors were carried out using a two-step addition method (Figure 28). In this method, two different measurements are made, the first one in the absence of the inhibitor, after which the working solution is replaced with a new working solution to which the inhibitor is then added. Subsequently, the substrate is added, and the signal obtained at the steady state corresponds to the residual activity. The protocol is described in table 9.

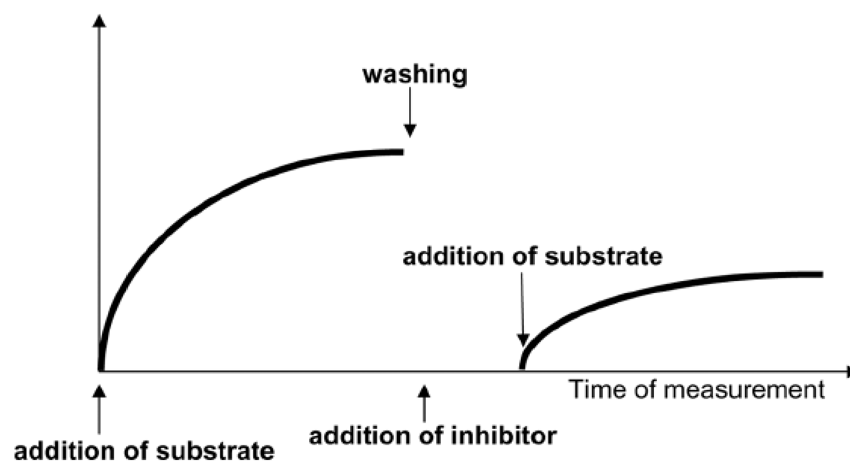


Figure 28 – Two-step addition method for glycoalkaloids determination

Table 9 – Protocol for the AChE biosensor measurement tests for glycoalkaloids detection

Measurement Test Step	***Description***
1st Step Measurement of enzyme activity without inhibition (a_0)	AChE biosensors were incubated in a stirred 4 mL 10 mM PBS (with 50 mM NaCl, pH 7.5) and the 100 mV voltage was turned on. After 10 min, different volumes of 100 mM ATChCl were added to the solution (end concentration: 0.25; 0.50 and 0.75 mM). The voltage was turned off after 5 min, and the biosensors washed with 10 mM PBS pH 7.5.
2nd Step, Measurement of enzyme activity with inhibition (a)	AChE biosensors were incubated in a stirred 4 mL 10 mM PBS (with 50 mM NaCl, pH 7.5) and the 100 mV voltage was turned on. After 10 min, different volumes of 2 mM α-solanine or α-chaconine solution in 5 mM acetic acid were added. The incubation with the inhibitor lasted 3 min, then different volumes of 100 mM ATChCl were added to the solution (end concentration: 0.25; 0.50 and 0.75 mM). The voltage was turned off after 5 min, and the biosensors washed with 10 mM PBS pH 7.5.

Acetylcholinesterase activity was determined using the formula (7).

3.2.3.4 Food sample tests

Before measuring the activity of the enzyme in the buffer solution, the electrodes were placed in 10 mM PBS pH 7.5 containing 1 vol-% Tween-20 for 15 min and then washed with a 10 mM PBS pH 7.5 before measuring the initial AChE activity. The AChE biosensor was incubated in the sample for 30 min. After incubation in the food sample, the biosensor was once again partially immersed in 10 mM PBS pH 7.5 containing 1 vol-% Tween-20 for 15 min and then washed with 10 mM PBS pH 7.5, before measuring the residual activity.

3.2.3.5 Reactivation of the acetylcholinesterase activity

In order to test the AChE-activity reactivation rate, 1 mM pyridine-2-aldoxime methochloride (2-PAM) in 10 mM PBS pH 7.5 was applied as a 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 10 mM PBS pH 7.5 containing 50 mM NaCl.

3.2.3.6 Multienzyme biosensors

With exception of the AChE biosensor used for the activation and detection of organophosphorothionates, all the biosensors used in the tests were multi-enzymatic. An illustration of the position of each electrode in the biosensor can be seen in figure 29.

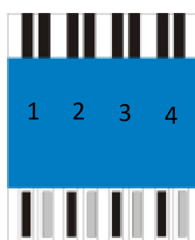


Figure 29 – Multi-enzymatic biosensor

Table 10 shows the distribution of AChEs on the different biosensors developed.

Table 10 - Distribution of AChE variants on the electrode positions of each biosensor

Biosensor	**Position 1**	**Position 2**	**Position 3**	**Position 4**
AChE Biosensor tailor made for Brazil	F345A	M301A	W346V	W346A
AChE Biosensor for glycoalkaloids detection	F345A	W303L	F345A	W303L

Only the Nb AChE WT was immobilized on the biosensor used in the AChE biosensor detection test after pretreatment with CPO.

3.2.3.7 Storage stability

A shelf life study was conducted with the AChE biosensor tailor made for Brazil. A set of 33 multi enzymatic biosensors was printed using the mutants mentioned in table 10, according to the method previously described. They were stored at room temperature in Petri dishes, and no drying agents were added. The AChE activity was determined comparing the current output before and after the incubation of the biosensor in a 0.05 µg/L chlorpyrifos solution, previously oxidized using chemical oxidation. The biosensors were evaluated for 40 weeks.

3.2.4 Microbiological methods

3.2.4.1 Expression of acetylcholinesterase B from N. brasiliensis *in* P. pastoris

The strains were cultivated in a complex medium, containing 2% (w/v) peptone, 1% (w/v) yeast extract, 1% glycerol (w/v), and 100 mM PBS pH 7.5. After 24 h incubation at 30° C and shaking at 200 rpm, this medium was exchanged by 200 mL of medium without glycerol. Induction of Nb AChE B expression was started by the addition of 0.5% (v/v) methanol. During shaking flask expression experiments, 0.5% (v/v) methanol was added every 24 h. After 5 days, protein expression cultures were centrifuged at 5,000 g for 20 min, and the supernatant was used as the source of the enzyme.

3.2.5 Molecular Biological methods

3.2.5.1 Sodium dodecacyl sulfate polyacrylamide electrophoresis (SDS – PAGE)

In order to confirm the presence of the recombinant protein produced and its molecular weight, the samples were submitted to the SDS-PAGE analysis. A 12.5% resolving gel was prepared, which was covered after the polymerization with a 4% stacking gel. Before the electrophoresis the samples were 5:6 diluted with a SDS loading buffer and denaturized (10 min, 95° C). The protein samples were loaded, and the electrophoresis was first run for 15 min at 20 mA (stacking gel), and finally for 90 min at 40 mA (resolving gel). The detection of the protein bands after the run was possible using Coomasie Brilliant Blue R250 as stain. The excess dye incorporated in the gel was removed by destaining with the same solution but without the dye. The proteins were detected as blue bands on a clear background.

<u>Resolving gel (12.5%)</u>

Acrylamide solution 30% (w/v)	3.33 mL
4x Resolving gel buffer (Tris/HCl, 1.5 M; pH 8.8; 0.4% (w/v) SDS)	2.00 mL
H_2O	2.67 mL
TEMED	4.00 µL
APS (10% (w/v))	40.00 µL

<u>Stacking gel (4%)</u>

Acrylamide solution 30% (w/v)	0.52 mL
4x Stacking gel buffer (Tris/HCl, 0.5 M; pH 6.8; 0.4% (w/v) SDS)	1.00 mL
H_2O	2.47 mL
TEMED	4.00 µL
APS (10% (w/v))	40.00 µL

2x-SDS sample buffer

Tris	100 mM
DTT	200 mM
SDS	4.0% (w/v)
BPB	0.2% (w/v)
Glycine	20.0% (v/v)

Electrophoresis buffer

Tris/HCl, pH 8.3	3.0 g/L
Glycine	14.4 g/L
SDS	2.0 g/L

Coomassie Briliant Blue stain

Coomassie Briliant Blue R-250	1.0 g/L
Methanol	30% (v/v)
Acetic acid	10% (v/v)

Coomassie Briliant Blue destain

Methanol	30% (v/v)
Acetic acid	10% (v/v)

3.2.6 Analysis of phosphorothionate insecticides using a chloroperoxidase pretreatment and acetylcholinesterase biosensor detection

3.2.6.1 Chloroperoxidase enzymatic oxidation of phosphorothionates in food samples for GC/MS analysis

The organophosphorothionate insecticides were dissolved in 0.4 g of juice (final concentration, 2,500 µg/L) and the mixture was preincubated with 34 U/mL CPO, acetate buffer (1 M, pH 5.5) and the halogen ion solution. The applied concentration of the halogens

ions was of 0.67 M for KCl and 0.1 M for KBr (in 1 M acetate buffer, pH 5.5). The suspension was stirred for 5 min; the reaction was started by adding 800 mM *tert*-butyl hydroperoxide (t-b HP) (70% aqueous solution) in the case of the KCl tests, and 100 mM in the case of the KBr tests (final amount, 4 g). The reaction was kept for 45 min with a Rotamix RM1 at room temperature, at 10 rpm.

3.2.6.2 GC/MS analysis

The reaction solutions were extracted with 1 mL ethyl acetate, and once more with 1 mL ethyl acetate after 1 mL of saturated NaCl solution was added to the aqueous phase. The combined ethyl acetate phase was dried over sodium sulfate, centrifuged and concentrated with N_2.

The analysis was performed with a Shimadzu QP 2010 gas chromatograph, equipped with a FS Supreme 5 column, coupled with mass spectrometry detector (MSD). The following oven program was used: 100° C for 1 min, then 20° C/min to 200° C, followed by 7° C/min to 300° C, and finally 300° C for 10 min. The temperature of injection was of 250° C.

3.2.6.3 Chloroperoxidase pretreatment of food samples for acetylcholinesterase biosensor measurement

1.6 g as well as 3.2 g of the food sample spiked with chlorpyrifos was mixed with acetate buffer (1 M, pH 5.5), 34 U/mL CPO solution and 0.1 M KBr. After 5 min of preincubation, 100 mM t-b HP (70% aqueous solution) was added to a final sample amount

of 16 g. The solution was further mixed for 45 min using a Rotamix RM1 at room temperature, at 10 rpm.

3.2.7 Development of acetylcholinesterase biosensors tailor made for Brazil

The following standard analytical procedures were used by CVUA in order to analyze insecticides in food samples.

3.2.7.1 Standard analytical methods

3.2.7.1.1 Supercritical fluid extraction (SFE) method

A representative sample portion of 7.5 g was weighed into a 100 ml beaker. Hydromatrix (6 g) (Varian, no. 0019/8004) was added and the mixture stirred intensively to achieve good homogeneity. An aliquot of 4.5 g (2.5 g sample) was transferred into the extraction thimble. To prevent particles from being extruded during extraction or depressurization, which could result in tube plugging, a fine grade glass wool paper was placed at both ends of the extraction thimble. The loaded thimbles were placed into a freezer immediately after filling and kept there until further processing because previous storage experiments had shown that some analytes (such as ethiofencarb, methiocarb, benfuracarb, furathiocarb, carbosulfan, dichlorvos) degraded significantly in the extraction cells when kept at room temperature (Anastassiades 2001). Freezing of the loaded thimbles (at -18° C) efficiently prevented degradation. A second 4.5 g aliquot of the sample/Hydromatrix mixture was stored in a glass vial for further use in case the extraction

had to be repeated. Extraction of the samples by SC/CO_2 and reconstitution of the extracts in acetonitrile was performed automatically. The extraction conditions used are shown in Table 11.

Table 11 - SFE method parameters

Parameters	
Extraction pressure	329 bar
Extraction temperature	55° C
CO_2-density	0.89 g/mL
Static extraction time	2 min
Dynamic extraction time	25 min
CO_2-flow	1.8 mL/min
Trap material	ODS, 10° C during extraction, 40° C during elution
Elution solvent	Acetonitrile (2 X 1.5 mL, flow 0.5 mL/min)
Solvent for trap clean-up	Cyclohexane/ethyl acetate 1:1 (4 mL, flow 0.8 mL/min)
Solvent for trap reconditioning	Acetonitrile (3 mL, flow 0.8 mL/min)

3.2.7.1.2 Liquid solvent based method

A modified version of a multiresidue method was used to extract organophosphates and carbamates, which was developed at CVUA Stuttgart for the analysis of pesticide residues in citrus fruits (Anastassiades and Scherbaum 1997). After sample comminution, a 20 g sample was extracted with 50 mL acetone with an Ultra Turrax and filtrated. Water and 10 mL saturated NaCl solution was added to the extract. Two liquid/liquid partitioning steps were performed with 20 mL cyclohexane/ethyl acetate (1:1). The combined extracts were dried with Na_2SO_4 and concentrated, followed by reconstitution with acetonitrile to a defined volume of 10 mL. Determination was performed with GC in SIM mode or LC-MS in ESI-positive mode.

3.2.7.1.3 Measurement

The instrumental conditions used during LC-MS in ESI (pos.) mode for the identification and quantification of the carbamates are listed in Table 12. An internal standard calibration was performed for quantification. Determination limits for the carbamates were 0.01 mg/kg or lower. Organophosphates were analyzed using GC-MS in single ion monitoring (SIM) mode in order to achieve good determination limits (equipped with uncoated precolumn 3 m/0.25 mm, press fit connection to the analytical column HP5MS, 30 m/0.25 mm, 0.25 mm, carrier gas helium constant flow). The determination limit was 0.01 mg/kg or lower for most compounds.

Table 12 - Instrumental conditions used at LC-MS

ESI (pos.) mode	
LC parameters	Column: 2.1 x 150 mm, 3.5 m, ZORBAX XDB C-18 Injection volume: 5 mL Mobile phase A: 5 mm aqueous - H_3COONH_4:acetonitrile (90:10) Mobile phase B: 5 mm aqueous CH_3COONH_4:acetonitrile (10:90) Flow: 0.3 mL/min Gradient: 30% B → 90% B in 8 min, temperature: 50° C
MSD parameters	Drying gas: nitrogen 10 L/min, 300° C, Vcap: 4000 V

3.2.8 Design of biosensors of acetylcholinesterase from *N. brasiliensis* for glycoalkaloids detection

3.2.8.1 Half maximal inhibition concentration (I_{50}) determination of glycoalkaloids

In order to select the most sensitive Nb AChE toward the glycoalkaloids α-solanine and α-chaconine, the I_{50} was determined. The method was adapted using a method previously mentioned by Roddick (1989).

The following solutions were mixed in a cuvette – 885 µL 50 mM PBS pH 7.2, 65 µL of the 2 mM glycoalkaloid sample in 5 mM acetic acid, 30 µL of 50 mM PBS pH 7.2 containing 10 mM 5,5-dithiobis-2-nitrobenzoic acid (DTNB) and 1.5 mg/ml $NaHCO_3$, 5 µL of 75 mM acetylthiocholine chloride, 15 µL (5 U/mL) aquous Nb AChE WT and mutants diluted in 50 mM PBS pH 7.2. After 3 min at room temperature, absorbance was measured at 412 nm and enzyme activity expressed as ΔA_{412}/min.

3.2.8.2 Preparation of the potatoes samples

The potato samples were bought in local supermarkets. First, they were washed and cut into small pieces. The peel was not removed. After that, a mixer was used to chop and homogenize the samples. The mixture was transferred to Falcon tubes and centrifuged for 10 min at 10° C, at 4,100 g. The supernatant was spiked with the standard solutions of α-chaconine and/or α-solanine, resulting 100, 200 and 400 µM α-chaconine, and/or 100 and 200 µM α-solanine. These solutions were then added to the buffer solution of the Nb AChE biosensor test in different volumes, resulting different final concentrations.

4. Results

4.1. Expression of the wild type and mutants of acetylcholinesterase B from *N. brasiliensis*

The WT and 19 mutants of Nb AChE B, previously developed at ITB, were expressed in a *P. pastoris* X33 strain for 5 days, using YPP medium. Samples were taken daily, and the fermentation process achieved its maximal enzymatic production after 5 days, when the AChE activity stabilized. Figure 30 exemplifies the activity of the WT strain during the fermentation process.

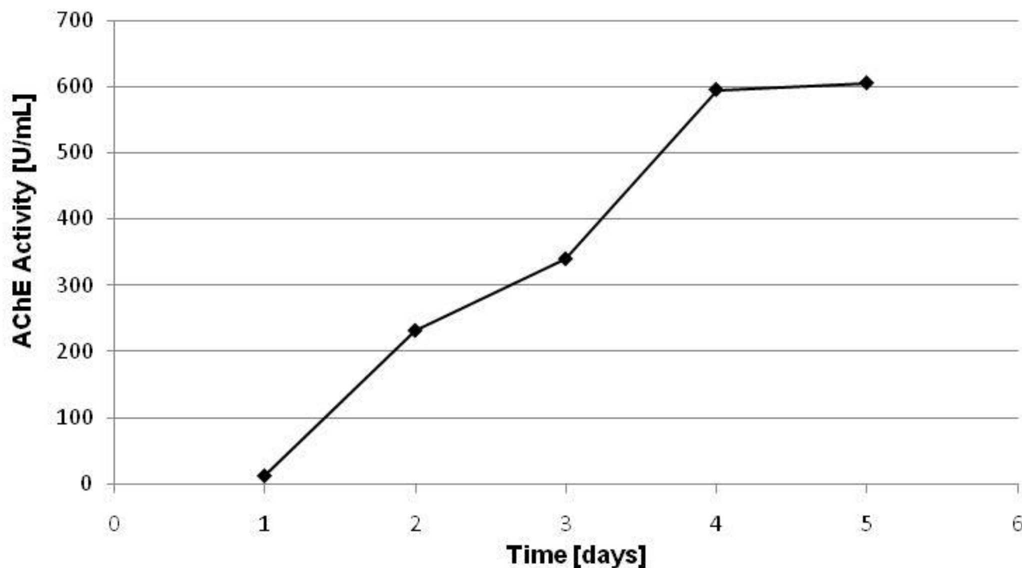


Figure 30 – Activity of Nb AChE WT during the fermentation process for 5 days

In order to confirm the molecular weight of the expressed proteins and to characterize them, a SDS-PAGE analysis was conducted. Nb AChE B has a molecular weight of 69 kDa (Hussein et al. 1999). All the recombinant enzymes produced in the fermentation process showed a molecular weight similar to the one described in the literature (Figure 31).

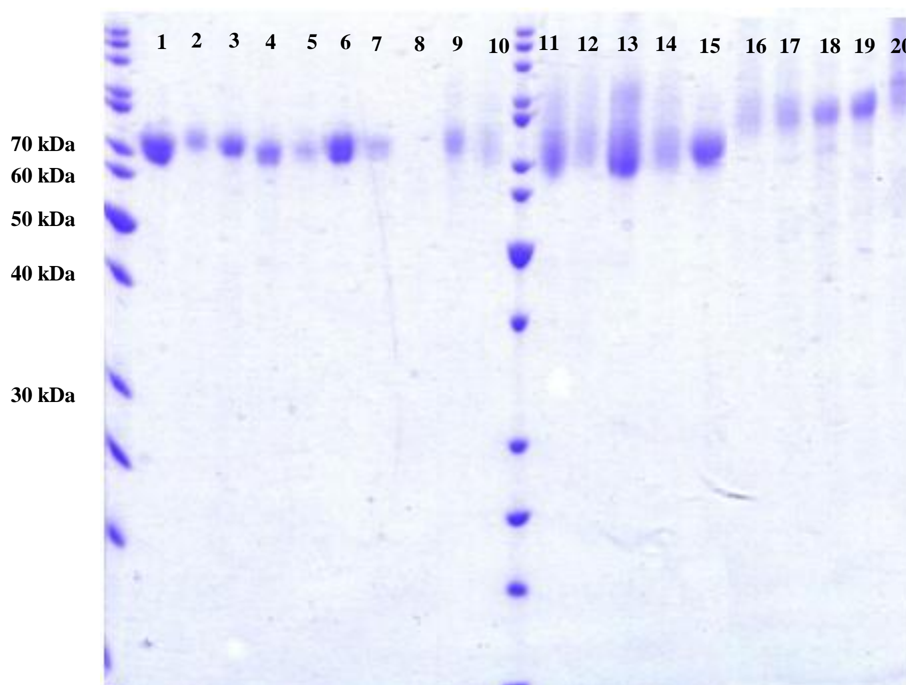


Figure 31 – SDS – PAGE of the recombinant Nb AChE WT and mutants. Legend: 1 – WT, 2 - M301W/W303A, 3- M301W/W303L, 4- M301W/F345A, 5- M301W/F345L, 6- W303A, 7- W303G, 8- W303Ains, 9- W303L, 10- Y349L, 11- T65Y, 12- M301W, 13- M301A, 14- F345Y, 15- F345A, 16- Y349G, 17- M301L, 18- M301V, 19- W346V, 20- W346A

AChE B from *N. brasiliensis* demonstrated a high stability at 4° C. Over a period of time of more than two years, the recombinant enzymes either maintained their original activity, or showed low activity loss. Figure 32 illustrates the stability of Nb AChE WT and of 3 mutants (M301W/W303A, M301W/W303L, M301W/F345A and M301W/F345L) over a two-year period of time.

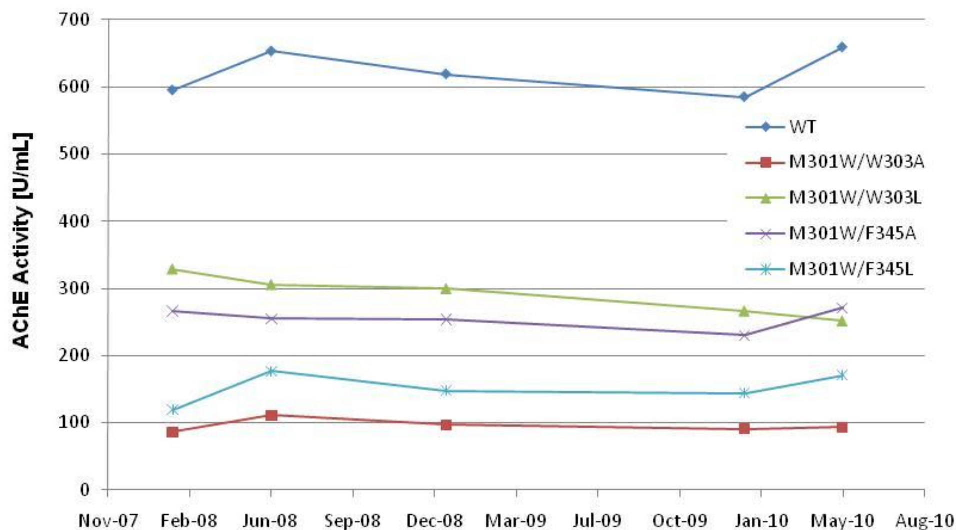


Figure 32 – Stability of Nb AChE WT, M301W/W303A, M301W/W303L, M301W/F345A and M301W/F345L over time at 4° C

Of all five enzymes illustrated in Figure 32, only M301W/W303L showed a decline in the enzymatic activity over the storage time. The other enzymes remained stable for the whole period.

4.2. Analysis of phosphorothionate insecticides using a chloroperoxidase pretreatment and acetylcholinesterase biosensor detection

4.2.1 Acetylcholinesterase and chloroperoxidase stability test toward KCl and KBr

In order to stabilize the reaction system for both enzymes, AChE and CPO, different pHs and buffers were tested. Studies in our laboratory revealed that acetate buffer was the ideal buffer to be applied in a system shared by these two enzymes. Additionally, AChE was inhibited by pHs lower than 5.5. CPO remained stable under a broad pH range (data not shown).

The stability of AChE toward different concentrations of KCl and KBr in acetate buffer (1 M pH 5.5), which are halogens required as cofactors in the oxidation reaction catalyzed by CPO, can be observed in figure 33. Nb WT AChE showed itself unstable in the presence of concentrations of KBr higher than 0.1 M (8.8% inhibition in the presence of 0.2 M KBr). KCl did not influence the stability of this enzyme until concentrations of 1.0 M.

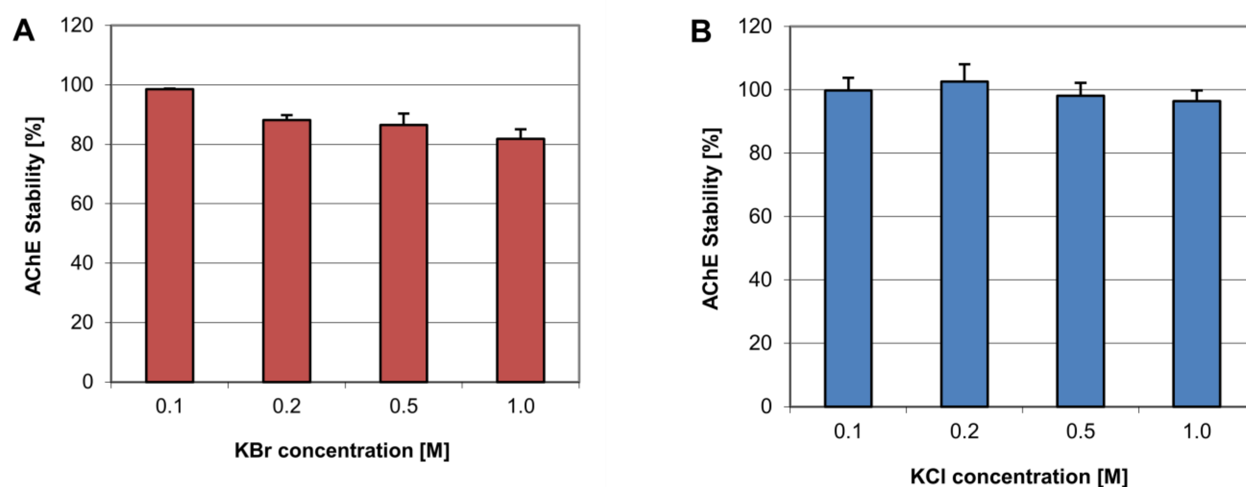


Figure 33 - Stability of *N. brasiliensis* AChE WT toward different concentrations of halogens: (A) KBr and (B) KCl

Both halogen salts did not show any inhibitory activity toward CPO. Concentrations until 1.0 M were tested, as previously done in tests using AChE.

4.2.2 GC/MS analysis of the products of the reaction catalyzed by chloroperoxidase - Choice of pH and buffer of the reaction system

Tests for biocatalytic oxidation of chlorpyrifos under different pHs were conducted using CPO. Figure 34 shows that the higher the pH of the system, the lower the oxon product conversion rate.

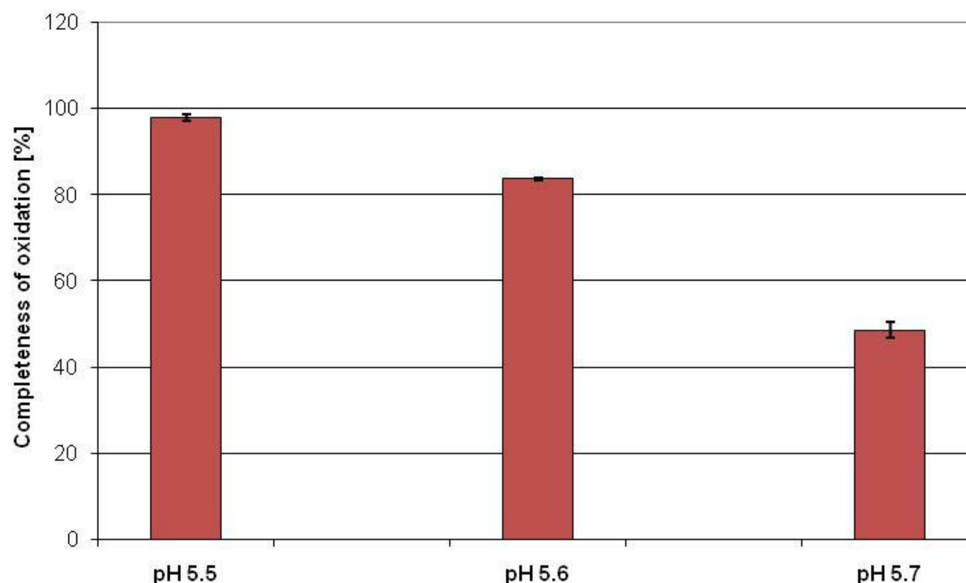
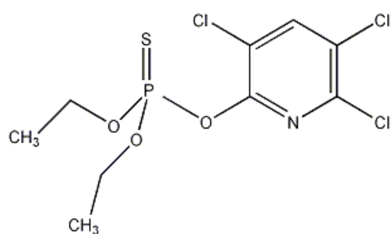


Figure 34 - Completeness of oxidation of chlorpyrifos using CPO under different pHs. The reaction system was formed by CPO, t-b HP, KCl and acetate buffer 1 M. *n=3*

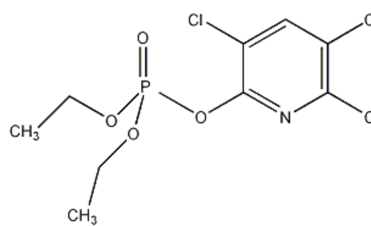
The oxidation reaction using pH 5.5 indicated a conversion of about 100% of chlorpyrifos into chlorpyrifos oxon, while CPO could only convert about 44% of this insecticide in a system with pH 5.7. The control tests (no addition of CPO) showed the absence of oxidation of chlorpyrifos after exposed to the reaction system. Tests using pHs lower than 5.5 were not executed, due to the inhibition activity of pH values lower than 5.5 over AChE.

4.2.3 GC/MS analysis of the products of the reaction catalyzed by chloroperoxidase – Use of different halogen salts and insecticides

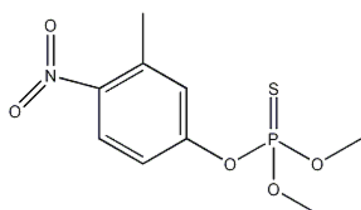
To investigate whether CPO could transform organophosphorothionate insecticides, we incubated this enzyme with orange juice samples spiked with these insecticides, in the presence of t-b HP and two halogen salts: KCl and KBr. The tested insecticides and their oxon forms are shown in figure 35.



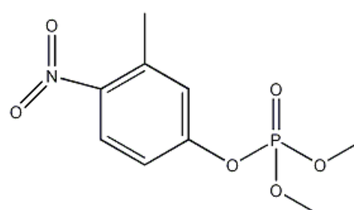
Chlorpyrifos



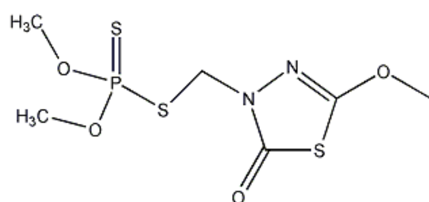
Chlorpyrifos oxon



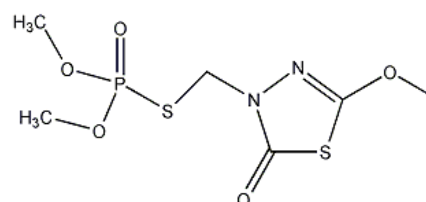
Fenitrothion



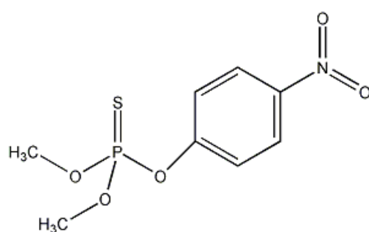
Fenitrothion oxon



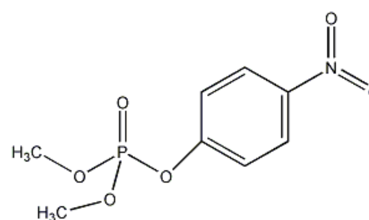
Methidathion



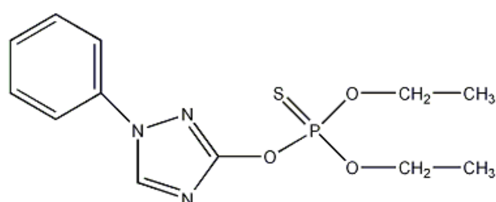
Methidathion oxon



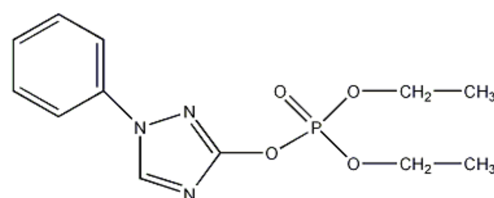
Parathion methyl



Paraoxon methyl



Triazophos



Triazophos oxon

Figure 35 - Organophosphorothionate insecticides used in this study and their correspondent oxon forms

The products obtained after the oxidation reaction in the presence of orange juice were analyzed using GC/MS. The results showed that CPO was able to activate all the five organophosphorothionate insecticides into their oxon derivative forms. Figure 36 illustrates the GM/MS spectra of two reactions conducted with and without the presence of CPO.

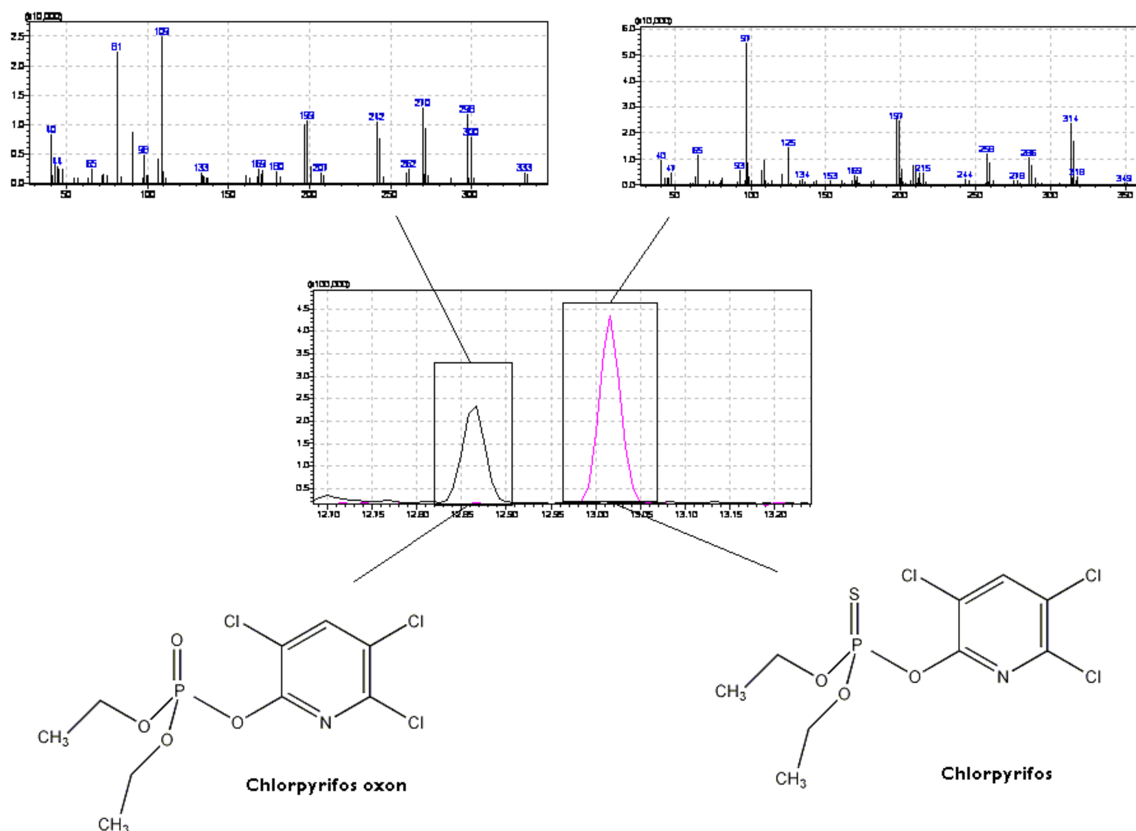


Figure 36 – GC/MS spectra of the reaction products obtained with (black) and without (pink) addition of the enzyme CPO. In the reaction without the presence of CPO there was no formation of the oxon form. The addition of CPO in the reaction system generates the oxidation of chlorpyrifos into chlorpyrifos oxon

In the reaction done without CPO, only the peak from chlorpyrifos could be observed, consequently there was no oxidation of the organophosphorothionate insecticide. On the other side, when this enzyme was added to the reaction system, the product chlorpyrifos oxon was formed, confirming that the substitution of a sulfur atom present in the molecule for an oxygen one was successful.

The data described in table 13 were obtained by GC/MS analysis of the products of five phosphorothionates submitted to the enzymatic oxidation.

Table 13 - Retention times (t_R) and MS characteristics of the obtained product of the oxidation of organophosphorothionate insecticides using CPO and KCl/KBr detected by GC/MS

Original Insecticide		**Obtained product**		
Name of the insecticide	*M.W.*	*Mass fragments of main products (m/z)*		
		M+	*Base*	*Other*
Chlorpyrifos	349	333	270	298, 272, 242, 109
Fenitrothion	277	261	109	244, 79, 127
Methidathion	302	286	85	145, 142, 109, 125, 229
Parathion methyl	263	247	96	230, 200, 109
Triazophos	313	297	161	269, 241, 188

All insecticides produced one major degradation product, and the decrease of the molecular ions (16 *m/z* smaller than the original substrate) of the products suggests the oxidation of the thiophosphoryl bond (P=S) into phosphoryl bond (P=O) by mass spectroscopy (MS). No oxidation reaction could be detected when CPO or t-b HP was alone added in the presence of the five tested phosphorothionate insecticides.

The addition of the halogen salts in the medium under a pH of 5.5 generated an important increase in the conversion rates. The results of the conversion of the five phosphorothionates using KBr and KCl are shown in figure 37.

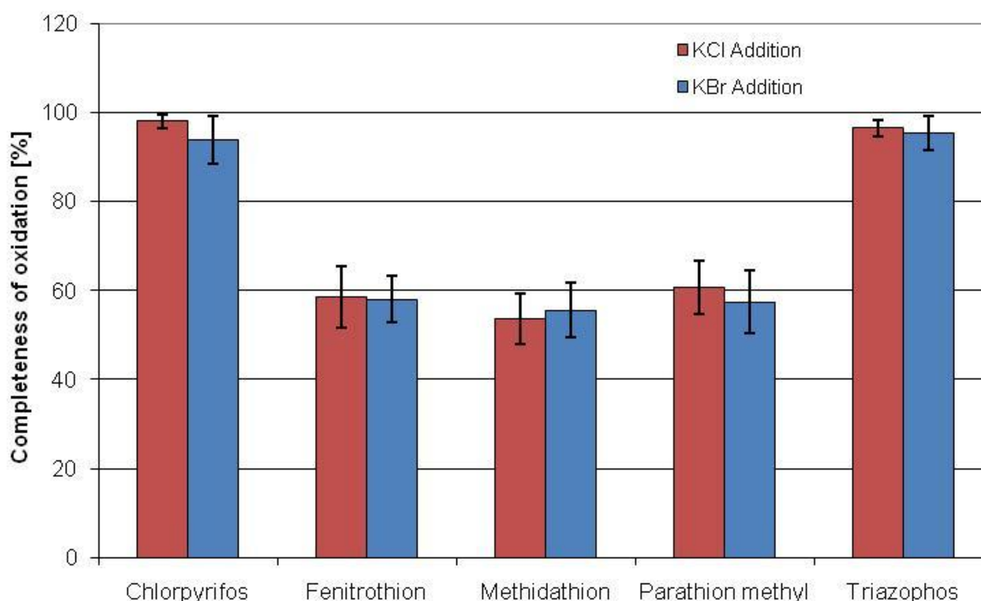


Figure 37 - Completeness of oxidation of different organophosphorothionate insecticides using CPO. The reaction system was formed by CPO, t-b HP and acetate buffer 1 M. *n=3*

The conversion tests adding CPO with absence of chloride or bromide ions generated a low biocatalytic activity (data not shown). In contrast, the conversion rates with addition of KCl and KBr were very similar, when each insecticide was observed individually. Chlorpyrifos and triazophos were completely oxidized by CPO into chlorpyrifos oxon and triazophos oxon, respectively. Fenitrothion, methidathion and parathion-methyl were partially oxidized adding both types of halogen salts.

It is important to point out that the concentration of KBr added to the reaction was six-times lower than the KCl one. Even though the KBr concentration added to the reaction could only reach 0.1 M due to stability problems of AChE, a better conversion rate of the organophosphorothionates was obtained, when compared with the same concentration of KCl. The use of this same KCl concentration (0.1 M) did not cause the oxidation of the studied insecticides. Additionally, the quantity of t-b HP necessary for the oxidation reaction was 10-times lower in the KBr tests (100 mM) than in the KCl ones (1,000 mM).

The formation rates of the oxon forms in the presence of CPO varied from each other. Chlorpyrifos and triazophos were completely oxidized, however a complete conversion rate of the insecticides fenitrothion, methidathion and parathion-methyl was not achieved. 58% of the fenitrothion was converted into its oxon form adding both halogen salts. Meanwhile, 54 and 56% of the methidathion was enzymatically oxidized in the presence of KCl and KBr, respectively. Parathion methyl was transformed into paraoxon methyl with a conversion rate of 61% using chloride ions, and of 57% applying bromide ions.

CPO has the ability to utilize several hydroperoxides and peroxy acids as electron acceptors (Spreti et al. 2004, Colonna et al. 1990) in order to execute its reactions. However, preliminary tests showed that the addition of H_2O_2 to food samples generated absent or low conversion rates (data not shown). *Tert*-butyl hydroperoxide replaced successfully H_2O_2 as an oxygen donor in the tests.

4.2.4 Determination of the bimolecular rate constant (k_i) of enzymatically activated phosphorothionates

We wanted to ensure that the product obtained after the enzymatic oxidation could inhibit the enzyme Nb AChE. Therefore, the bimolecular rate constant k_i of the oxon forms from the enzymatic oxidation was determined. The k_i value illustrates the sensitivity of AChE towards insecticides. Additionally, it measures the rate of phosphorylation of this enzyme, characterizing the inhibitory capacity of the phosphorothionates.

In order to determine the bimolecular rate constant, Nb AChE WT was incubated for a certain period of time with different concentrations of the insecticides oxidized by chloroperoxidase with addition of KCl or KBr. Additionally, the k_i value of the same

insecticides using chemical oxidation was established. The remaining activity of Nb AChE was established using the Ellman test, which has the acetylthiocholine iodide as substrate.

Figure 38 exemplifies the principle of the k_i value determination. The k_i values of the products obtained in the chemical oxidation of chlorpyrifos, and in the enzymatic oxidation with CPO and KBr were calculated.

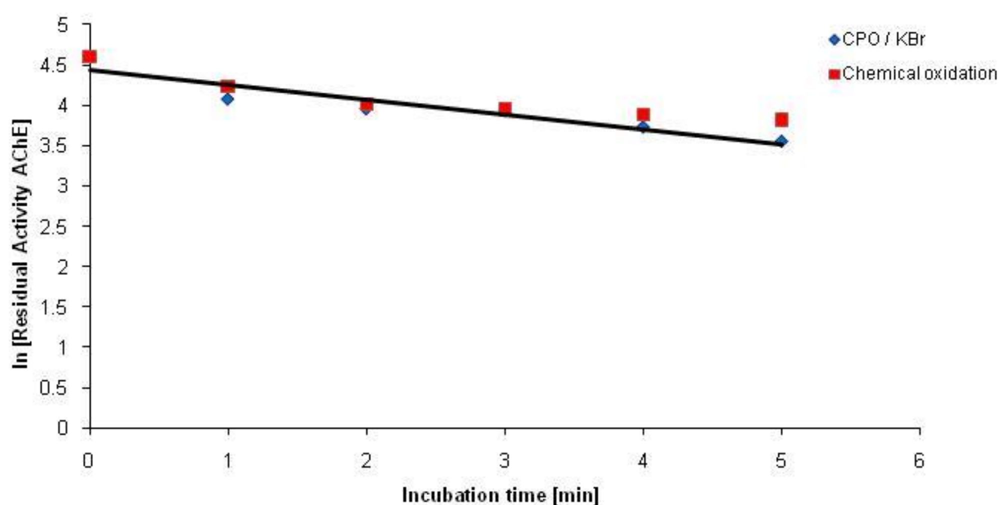


Figure 38 – Principle of the k_i value determination. Enzyme: Nb AChE WT. Insecticide: 10 µg/L chlorpyrifos

The inhibition effect of the investigated insecticides toward Nb AChE WT is shown in table 14. The k_i values of the products obtained in the enzymatic oxidation were compared with the k_i values of the standard oxon form. This last one resulted from the analysis of the chemically oxidized form.

Table 14 - Effect of the enzymatic oxidation with CPO and chemical oxidation over the bimolecular constants (k_i) of different phosphorothionate insecticides. *n=3*

	k_i **[L/mol.min]**		
	Oxon standard [(a)]	*KCl Addition*	*KBr Addition*
		After exposure to oxidation reagents	After exposure to oxidation reagents
Chlorpyrifos	4.92×10^6 +/- 0.18×10^6	5.47×10^6 +/- 0.10×10^6	4.87×10^6 +/- 0.12×10^6
Fenitrothion	2.42×10^5 +/- 0.20×10^5	1.34×10^5 +/- 0.11×10^5	9.71×10^4+/- 0.85×10^4
Methidathion	5.30×10^4+/- 0.35×10^4	3.15×10^4 +/- 0.19×10^4	2.58×10^4 +/- 0.03×10^4
Parathion methyl	1.70×10^5 +/- 0.12×10^5	1.33×10^5 +/- 0.13×10^5	1.35×10^5 +/- 0.06×10^5
Triazophos	1.02×10^7+/- 0.10×10^6	1.19×10^7 +/- 0.06×10^7	9.36×10^6 +/- 0.05×10^6

[(a)] Obtained using chemical oxidation with NBS and vitamin C.

For most oxidized thionates, the deviations of k_i values from those presented by the oxon standards were low. Nevertheless, higher deviations happened in the tests with addition of KBr than in the ones using KCl. With the exception of chlorpyrifos, all other thionates presented k_i values after the enzymatic oxidation with KBr lower than the standard oxon chemically oxidized. The most remarkable difference was obtained during the tests with fenitrothion, where a negative deviation of about 60% was obtained. Moreover, deviations also occurred in the case of methidathion and fenitrothion, when chloride ions were added during the tests. Comparing the k_i values, a negative deviation of about 45% was obtained for fenitrothion, and of about 41% for methidathion.

A comparison between the results obtained in the determination of the bimolecular constant and the GC/MS analysis of the products showed that a correlation between both results could be found. The two insecticides with which the enzymatic oxidation reaction was most successful (chlorpyrifos and triazophos) were also the same ones with the lowest deviation rates of the k_i values.

4.2.5 Phosphorothionates conversion with chloroperoxidase pretreatment method and acetylcholinesterase biosensor assay in foods

The applicability of the CPO activation method combined with the detection assay using AChE biosensors for food analysis was tested. The oxidation of phosphorothionates using CPO was executed in a solution with neutralized food sample. The 1 M acetate buffer was applied in order to neutralize the sample, and consequently to maintain the stability of both enzymes, CPO and AChE.

First, the stability of AChE in the reaction system without the presence of chlorpyrifos (blank) was tested. No inhibition was observed after the incubation for 30 min with the solution obtained from the reaction between 34 U/mL CPO, 100 mM t-b HP and 100 mM KBr (data not shown). However, the blank sample of the reaction with 34 U/mL CPO, 1,000 mM t-b HP and 670 mM KCl inhibited 100% of the Nb AChE WT immobilized on the biosensor (Figure 39). Even the addition of lower concentrations of t-b HP inhibited AChE. Consequently, the additional tests were done using bromide as cofactor.

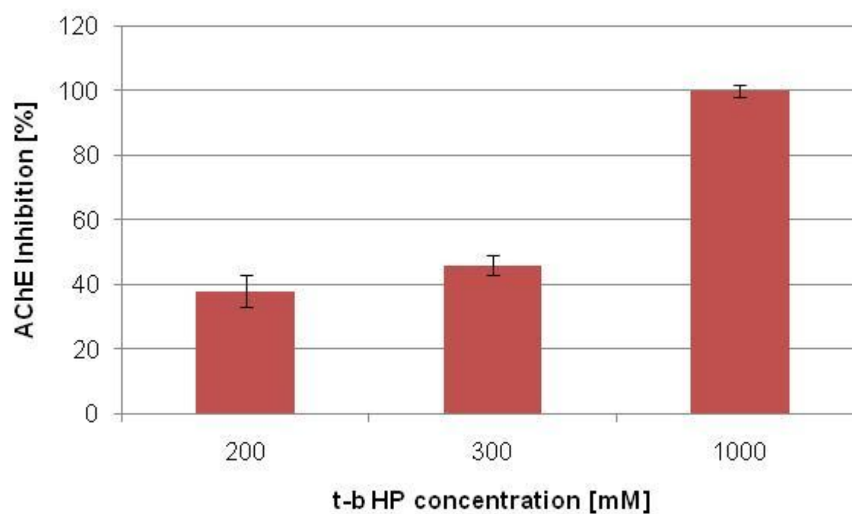


Figure 39 – Inhibition of Nb AChE WT immobilized on the screen printed biosensor after 30 min of incubation in the reaction solution with CPO, t-b HP and KCl

To determine whether the enzymatically oxidized product could inhibit the AChE biosensor, different concentrations of chlorpyrifos were exposed to the enzymatic reaction. Two conditions of the system were tested: the addition of only acetate buffer (1 M, pH 5.5), and the extra addition of 20% organic orange juice (Figure 40).

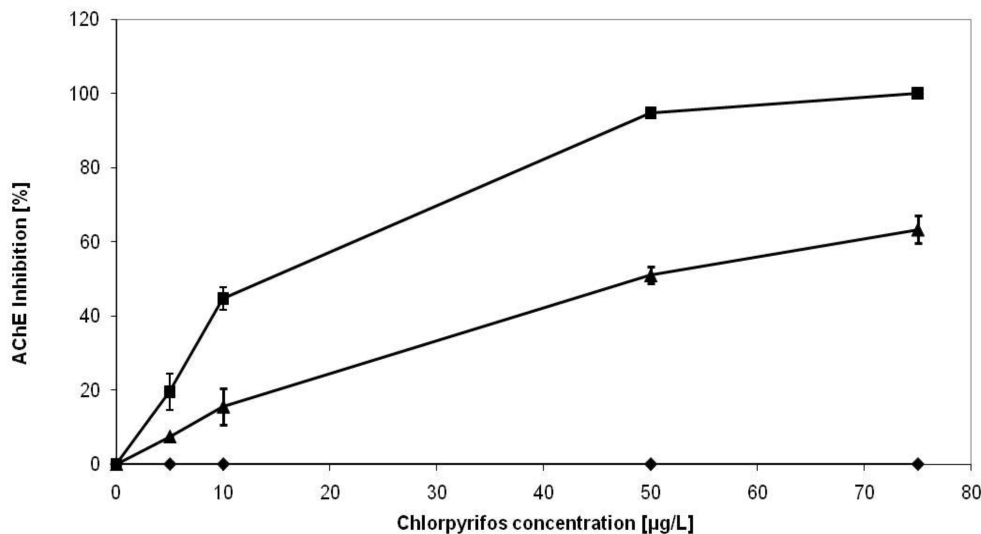


Figure 40 - AChE inhibition caused by different chlorpyrifos concentrations in acetate buffer and food samples after activation with CPO, t-b HP and KBr using amperometric measurement with immobilized AChE: ♦ no CPO addition, acetate buffer 1 M pH 5.5; ■ CPO addition, acetate buffer 1 M pH 5.5; ▲ CPO addition, organic orange juice (20%), acetate buffer 1 M pH 5.5. *n=3*

The conversion rate of chlorpyrifos into chlorpyrifos oxon in food samples was slightly lower than the one obtained in the solution with only acetate buffer. The application of the enzymatic method to orange juice in combination with a disposable AChE biosensor enabled detection of chlorpyrifos at concentrations down to 5 µg/L, with a final concentration of this insecticide in food samples of 25 µg/L. The inhibition of chlorpyrifos was proportional to its concentration from 5 - 75 µg/L in 20% organic orange juice, with a correlation coefficient of 0.9845.

The MRL established by the European Union for chlorpyrifos is 50 µg/kg food (Official Journal of European Union 2008). Consequently, the chloroperoxidase pretreatment and the acetylcholinesterase biosensor detection were able to detect the recommended limit by the legislation. More diluted and concentrated samples (10 and 30% organic orange juice) were also tested; however this did not further enhance the assay performance.

4.3. Development of acetylcholinesterase biosensors tailor made for Brazil

4.3.1 k_i determination

The studied organophosphate (acephate, chlorpyrifos, dimethoate, ethion, fenitrothion, methamidophos, methidathion, parathion-methyl, profenofos, triazophos) and carbamate (carbofuran, carbaryl) insecticides were selected due to their high occurrence in vegetables and fruits commercialized in Brazil (ANVISA 2008). The AChE B WT from *N. brasiliensis* and 19 mutants were tested toward six organophosphates. The k_i values of the remaining six insecticides were obtained in previous studies in our laboratory (Schulze et al. 2005).

The rate of phosphorylation or carbamylation of acetylcholinesterase is expressed in terms of bimolecular rate constants (k_i), characterizing the inhibitory capacity of insecticides. Alternatively, the k_i values were determined from the slope of the logarithmically plotted residual activity against the insecticide concentration (mol/L, in the inhibition batch).

4.3.1.1 Methamidophos

In the case of methamidophos, a mutation in the acyl pocket (Nb W346(331)V) increased the k_i value 1,130 times, the largest enhancement between all the evaluated insecticides in this study (Figure 41).

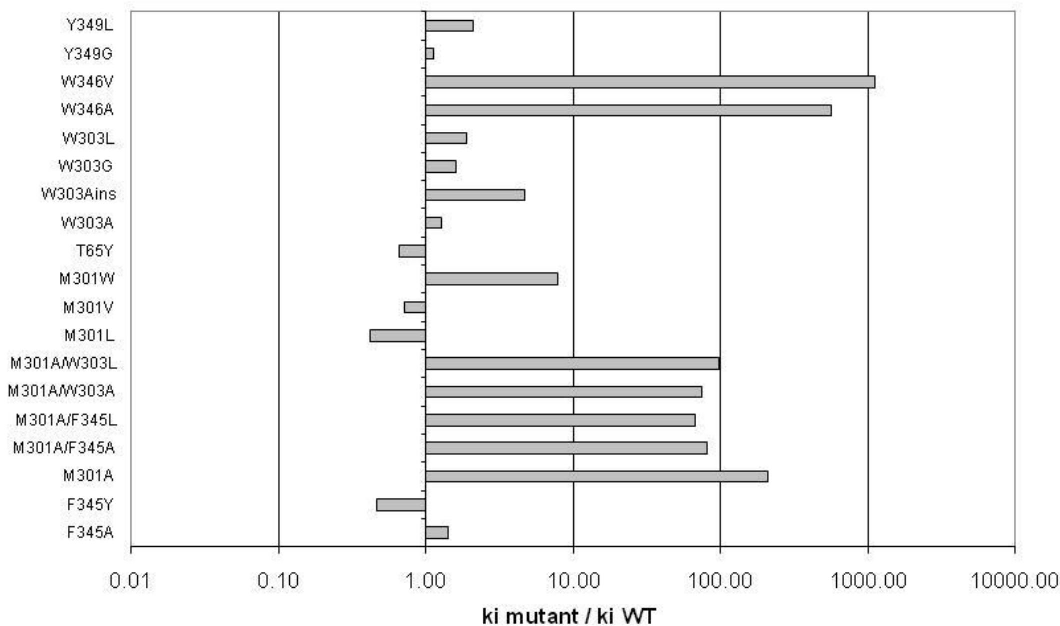


Figure 41 – Ratio of the k_i values of the Nb AChE mutants and WT toward methamidophos. The incubation was done at room temperature. $n \geq 3$

4.3.1.2 Acephate

None of the mutants was inhibited by acephate. This insecticide was already described in the literature as an indirect AChE inhibitor (Rojakovick and March 1972, Suksayretrup and Plapp 1977).

4.3.1.3 Profenofos

The substitution in position 346(331) of tryptophan for alanine generated a 10-fold increase of sensitivity toward profenofos. A change in the same position for valine enhanced the k_i value from 4.38 x 10^4 $M^{-1}.min^{-1}$ (obtained for the WT Nb AChE) to 6.24 x 10^5 $M^{-1}.min^{-1}$, therefore a k_i value 16 times higher (Figure 42).

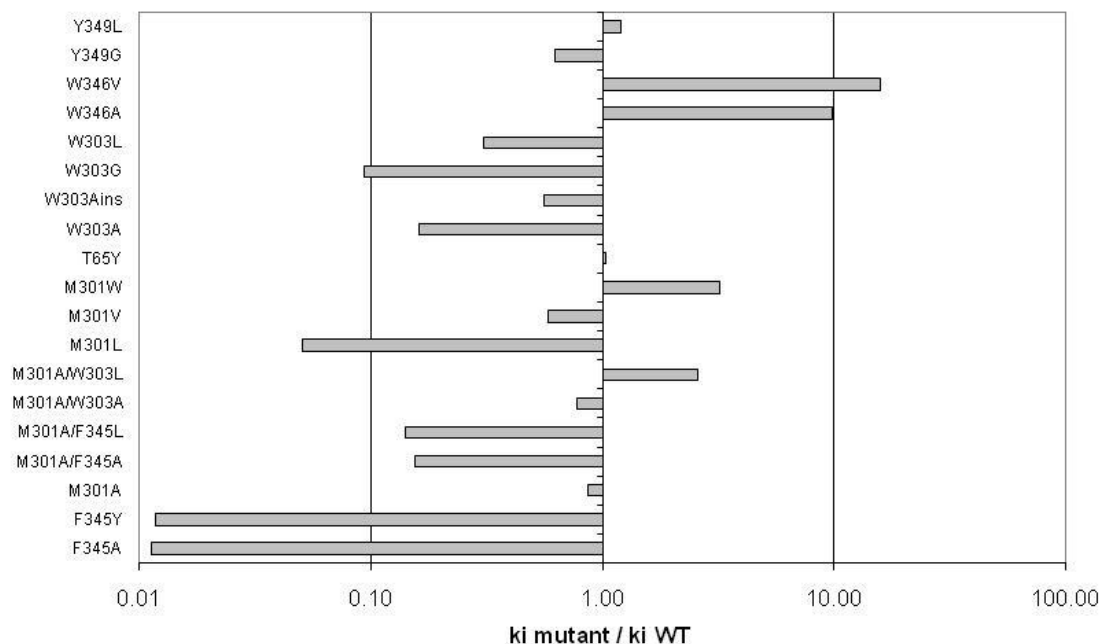


Figure 42 – Ratio of the k_i values of the Nb AChE mutants and WT toward profenofos. Profenofos was chemically oxidized using a pretreatment with NBS. The incubation was done at room temperature. $n \geq 3$

4.3.1.4 Fenitrothion

The mutation in the position 301(288) of a methionine into an alanine, and also the combination of this mutation with the change in the position 303(290) of a tryptophan for a leucine, was successful to enhance the sensitivity toward fenitrothion. The mutant M301(288)A was 10 times more sensitive, and the double mutant M301(288)A/W303(290)L showed a sensitivity 7.5 times higher than the WT NbAChE (Figure 43).

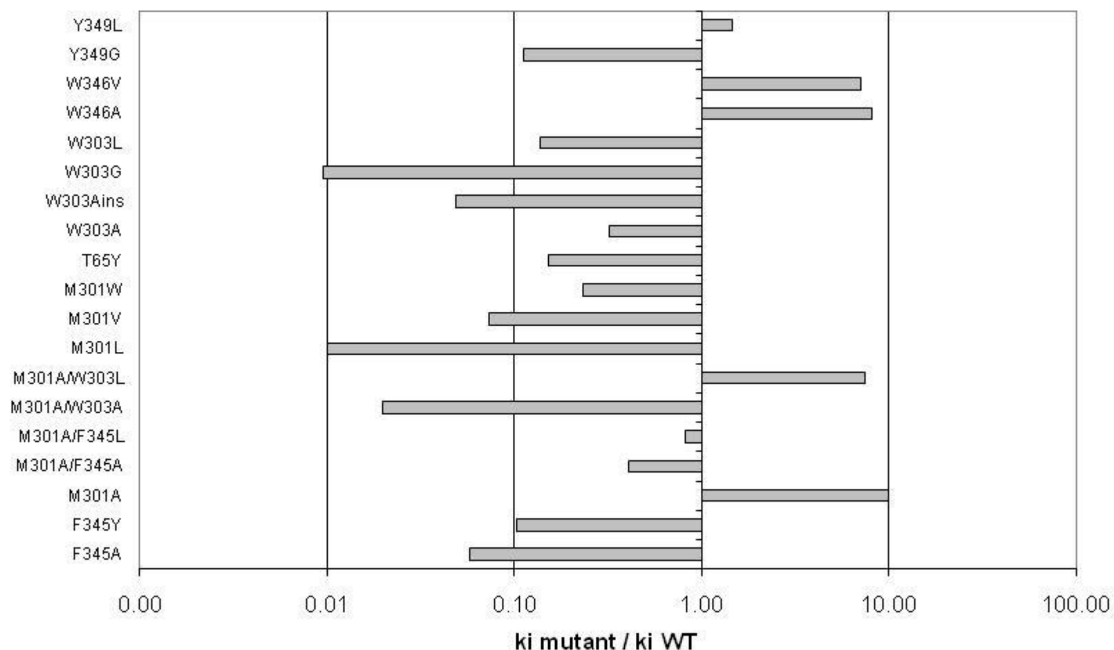


Figure 43 – Ratio of the k_i values of the Nb AChE mutants and WT toward fenitrothion. Fenitrothion was chemically oxidized using a pretreatment with NBS. The incubation was done at room temperature. *n ≥ 3*

4.3.1.5 Triazophos

Normally the k_i determination should be done at room temperature, but in the case of triazophos, it was observed that for some mutants, there was no linear relation between the logarithm of AChE residual activity and the incubation time. Therefore, a second-order reaction could be visualized. Following the same methodology, but using the incubation temperature of 4° C allowed a reduction of the reaction velocity, and consequently, a resulting pseudo-first-order kinetics. The mutant W346(331)V showed the best k_i value toward triazophos, which was of 3.46 x 10^7 $M^{-1}.min^{-1}$. This value was 3.4 times higher than the one found for the WT Nb AChE (Figure 44).

The mutants that needed an incubation temperature of 4° C were: W303A, W303G, W303L, M301A, F345A, M301W/W303A, M301W/W303L, W346V and W346A.

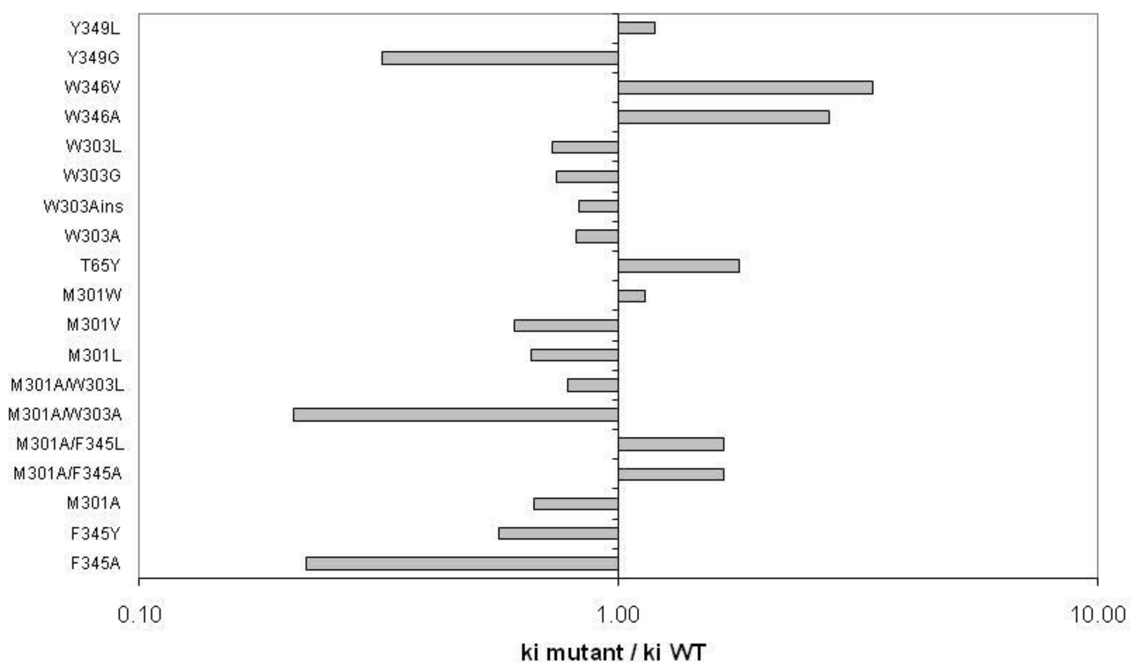


Figure 44 – Ratio of the k_i values of the Nb AChE mutants and WT toward triazophos. Triazophos was chemically oxidized using a pretreatment with NBS. The incubation was done at 4° C for the mutants listed above, and the room temperature was applied for the tests with the WT Nb AChE and other mutants. *n ≥ 3*

4.3.1.6 Chlorpyrifos

Like triazophos, the k_i value of chlorpyrifos toward some mutants had to be measured under incubation temperature of 4° C, allowing the pseudo-first-order kinetics. Chlorpyrifos showed the lowest increase of sensitivity among the designed mutants. The only mutant that obtained a better k_i value, when compared with Nb AChE WT, was Y349(334)L, which presented a mutation on the substrate entrance region. The result was 1.22 times higher than the one from the wild type (Figure 45).

The mutants that needed an incubation temperature of 4° C were: W303A, W303G, W303L, W303Ains, F345A, M301W/W303A, M301W/W303L, M301W/F345A, W346V and W346A.

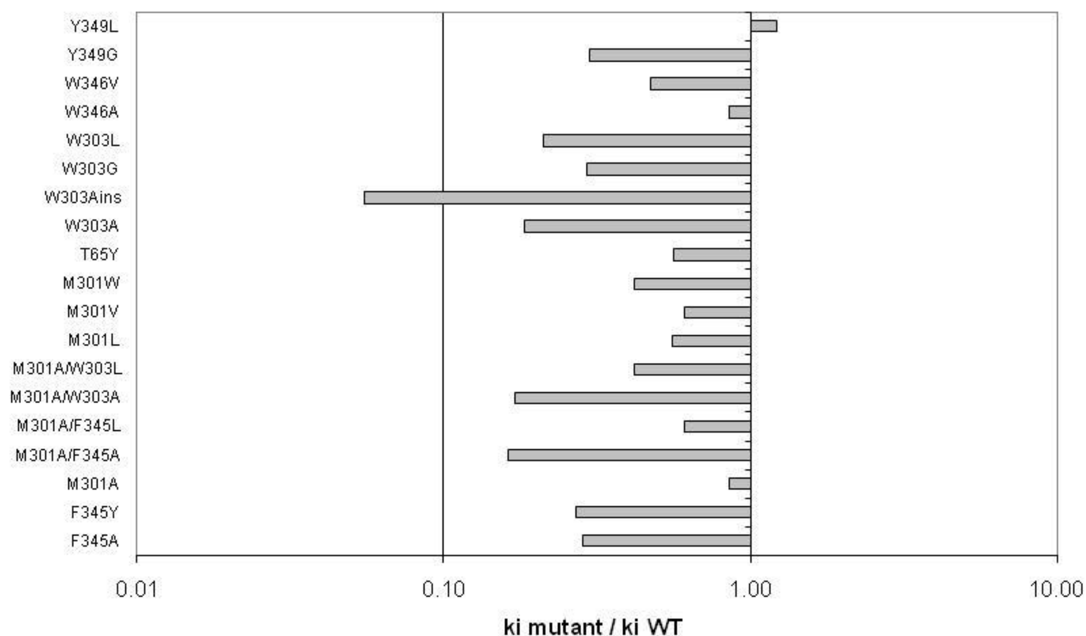


Figure 45 – Ratio of the k_i values of the Nb AChE mutants and WT toward chlorpyrifos. Chlorpyrifos was chemically oxidized using a pretreatment with NBS. The incubation was done at 4° C for the mutants listed above, and the room temperature was applied for the tests with the WT Nb AChE and other mutants. $n \geq 3$

The tests showed that the AChE B WT from *N. brasiliensis* is already very sensitive toward chlorpyrifos; nevertheless the mutants conserved this characteristic.

4.3.2 Multienzyme biosensor

Table 15 compares the k_i values of Nb AChE WT, of the most sensitive mutant, and of the four mutants chosen for the multienzyme biosensor towards all insecticides studied.

There was an increase of the k_i value of every analyzed insecticide, except for acephate, when compared the values obtained for WT and mutants.

Table 15 – Bimolecular rate constants ($M^{-1}.min^{-1}$) of the Nb AChE WT, of the most sensitive mutants, and the four mutants chosen for the multienzyme sensor towards organophosphate and carbamate insecticides

	k_i [L/mol.min]					
Insecticide	**Nb AChE WT**	**Nb AChE mutant**	**F345A**	**M301A**	**W346V**	**W346A**
Acephate	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition	No inhibition
Carbaryl [a]	3.1×10^4	1.2×10^5 (F345A)	1.2×10^5	5.3×10^4	3.3×10^4	3.7×10^4
Carbofuran [a]	4.4×10^5	7.9×10^5 (M301L)	1.5×10^5	3.7×10^5	1.6×10^5	3.4×10^5
Chlorpyrifos [b]	2.0×10^7	2.4×10^7 (Y349L)	5.7×10^6	1.7×10^7	9.5×10^6	1.7×10^7
Dimethoate [a, b]	5.2×10^2	5.6×10^4 (M301A)	3.6×10^2	5.6×10^4	1.5×10^4	1.1×10^4
Ethion [a, b]	3.0×10^4	6.5×10^6 (W346A)	3.1×10^4	1.3×10^5	5.8×10^5	6.5×10^6
Fenitrothion [b]	2.4×10^5	2.4×10^6 (M301A)	1.4×10^4	2.4×10^6	1.7×10^6	2.0×10^6
Methamidophos	5.4×10^1	6.1×10^4 (W346V)	7.8×10^1	1.2×10^4	6.1×10^4	3.1×10^4
Methidathion [a, b]	1.5×10^4	3.1×10^6 (W346V)	2.9×10^3	1.7×10^5	2.4×10^5	3.1×10^6
Parathion-methyl [a, b]	1.7×10^5	5.3×10^6 (W346A)	1.6×10^5	1.5×10^6	3.1×10^6	5.3×10^6
Profenofos	4.4×10^4	7.0×10^5 (W346V)	4.9×10^2	3.8×10^4	7.0×10^5	4.3×10^5
Triazophos [b]	1.0×10^7	3.5×10^7 (W346V)	2.3×10^6	6.8×10^6	3.5×10^7	2.8×10^7

[a] Insecticides previously studied (Schulze et al. 2005).
[b] Insecticides oxidized chemically with NBS.

Two insecticides presented k_i values below 10^5 $M^{-1}.min^{-1}$: dimethoate and methamidophos. A value higher than this one is necessary to obtain detection limits below 10 µg/L (Schulze et al. 2005).

The four identified enzymes, which together presented the widest sensitivity spectrum toward the most used insecticides in Brazil, were: F345A, M301A, W346V and W346A. Mutant F345A, with an alanine in position 345, showed a high sensitivity toward carbaryl. A mutation in the acyl pocket in mutant M301A caused k_i values that were 108 times higher for dimethoate and 10 times higher for fenitrothion, when compared with the wild strain. The largest increase of sensitivity was obtained with mutant W346V, where the mutation caused a 1,127 times higher sensitivity toward methamidophos. Mutant W346A was highly inhibited in the presence of almost all insecticides tested, especially by organophosphorothionates such as chlorpyrifos and triazophos.

4.3.3 Sensitivity

The calibration plots of Nb AChE B multisensor toward chlorpyrifos, fenitrothion, triazophos and methamidophos revealed a high sensitivity of the developed biosensor toward these insecticides. This sensitivity was established by the detection limit of the biosensor toward the evaluated substances.

4.3.3.1 Chlorpyrifos

For chlorpyrifos, the detection limit was 0.025 µg/L. The most inhibited mutants were M301A and W346A, which in the presence of this insecticide concentration were inhibited up to 25 and 27%, respectively (Figure 46).

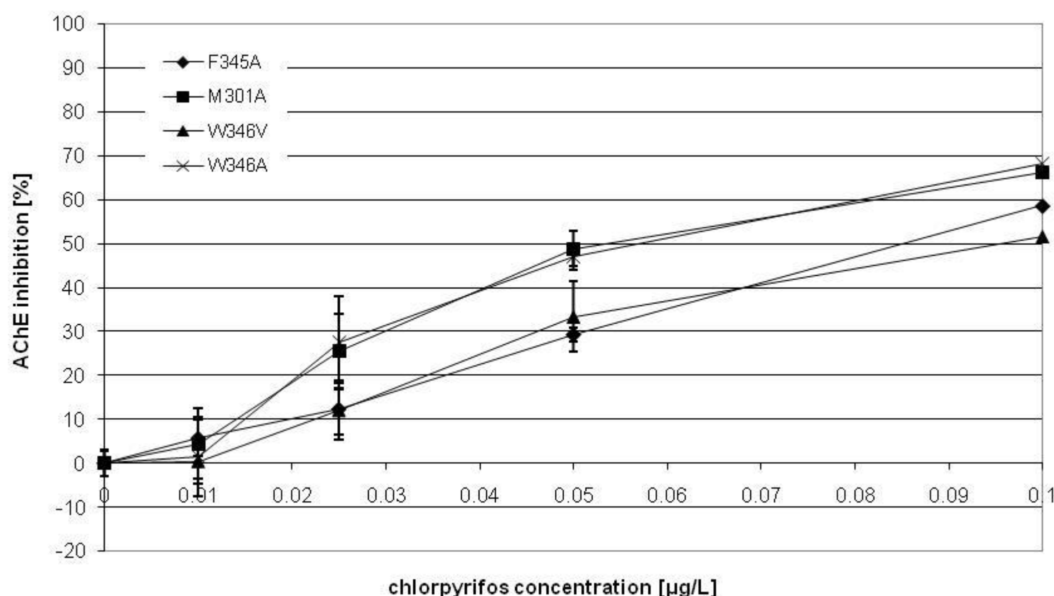


Figure 46 – Calibration curve of the multienzyme biosensor toward chlorpyrifos. Chlorpyrifos was chemically oxidized. Incubation time of 30 min. *n=3*

This detection limit is in accordance with the Brazilian legislation, where this insecticide is allowed only in concentrations lower than 10 µg/L.

4.3.3.2 Triazophos

The AChE biosensor showed the same sensitivity toward triazophos. The detection limit of 0.025 µg/L of this insecticide generated a high inhibition activity over all mutants, with exception of F345A (Figure 47).

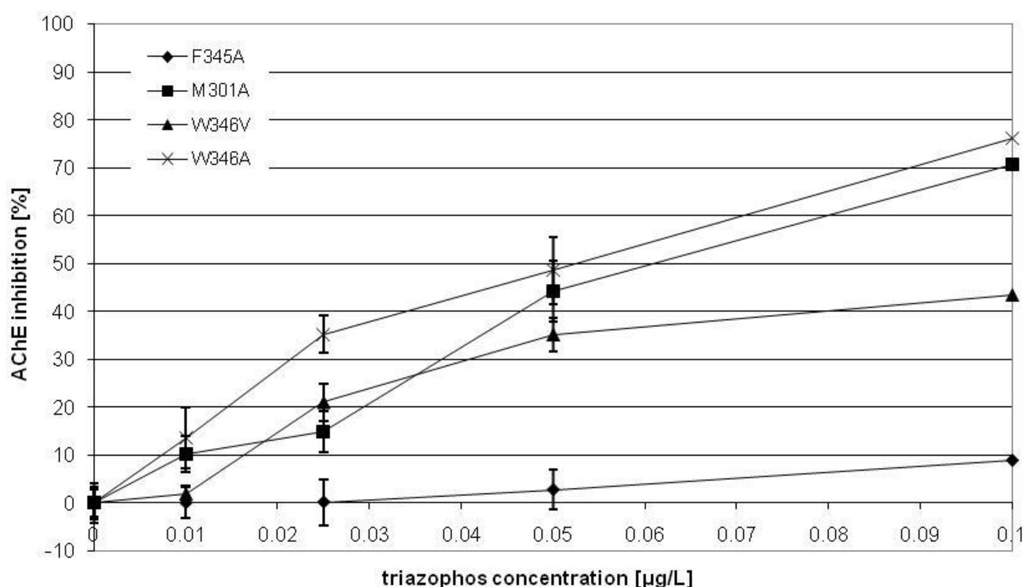


Figure 47 – Calibration curve of the multienzyme biosensor toward triazophos. Triazophos was chemically oxidized. Incubation time of 30 min. *n=3*

This insecticide is, like chlorpyrifos, only allowed in foods in concentrations lower than 10 µg/L. Consequently, the developed biosensor also fits Brazilian requirements regarding this insecticide.

4.3.3.3 Fenitrothion

Fenitrothion was first detected in concentrations higher than 0.05 µg/L, inhibiting the mutants M301A and W346A in rates of 26% both (Figure 48).

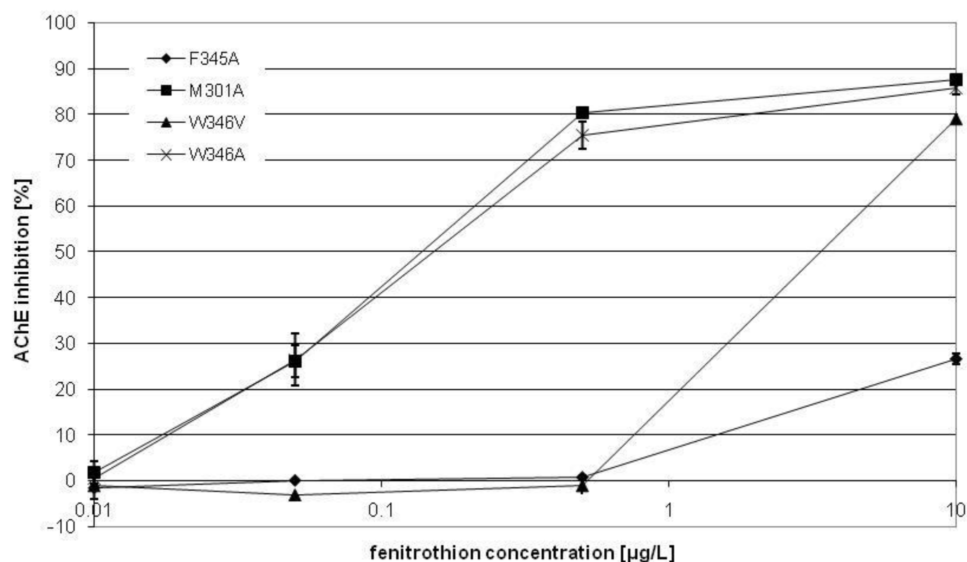


Figure 48 – Calibration curve of the multienzyme biosensor toward fenitrothion. Fenitrothion was chemically oxidized. Incubation time of 30 min. *n=3*

Additionally, there is a good correlation of this detection limit with the MRL accepted by the Brazilian legislation, which is 50 µg/L.

4.3.3.4 Methamidophos

For methamidophos, the detection limit found was higher when compared with the other analyzed insecticides, 10 µg/L, shown in Figure 49. The mutant W346V, which showed the highest k_i value improvement among the mutants toward this insecticide (6.13 x 10^4 $M^{-1}.min^{-1}$), was 15% inhibited in the presence of this concentration in PBS.

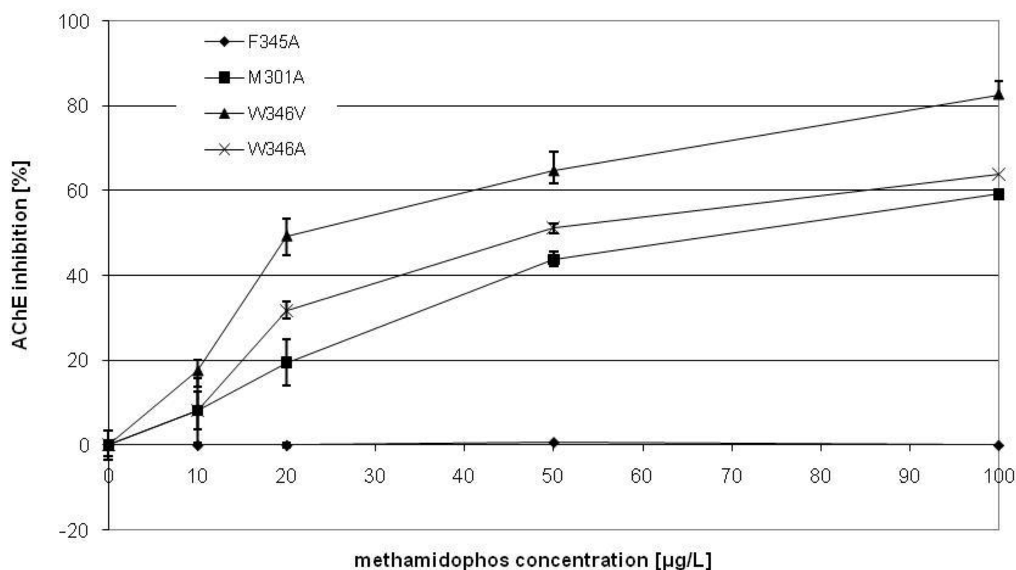


Figure 49 – Calibration curve of the multienzyme biosensor toward methamidophos. Incubation time of 30 min. *n=3*

This result is different from previous results, which showed that a k_i value of 10^5 $M^{-1}.min^{-1}$ was necessary in order to obtain a detection limit below 10 µg/kg (Schulze et al., 2005).

The legislation in Brazil establishes that no more than 10 µg/L methamidophos is allowed in food samples. Despite of the fact that the detection limit of this insecticide in buffer solution was achieved, in analysis with food samples there is a necessity of a dilution step 1:1 with 1 M PBS. Consequently, the detection limit for foods would be 20 µg/L, higher than the limit set by legislation.

4.3.4 Validation

The developed multienzyme biosensor analysis was validated through the comparison with standard analytical methods. The food samples were previously analyzed

by the CVUA Stuttgart, and then investigated using the AChE biosensor analysis. The results are shown in table 16.

Table 16 - Validation tests: comparison between the results obtained using standard analytical methods (GC/LC-MS) and the AChE biosensor assay

Food sample	**AChE inhibition (%)**				**AChE reactivation (%)**				**GC/LC-MS**
	F345A	M301A	W346V	W346A	F345A	M301A	W346V	W346A	
1-Pear	39	30	26	28	100	97	93	95	Chlorpyrifos: 0.029 mg/kg Chlorpyrifos- methyl: 0.005 mg/kg
2-Tamarillo	32	47	33	49	94	93	86	95	Omethoate: 0.010 mg/kg
3-Cranberry	45	41	45	46	98	87	91	89	Carbaryl: 0.009 mg/kg
4-Orange	25	41	33	38	91	96	79	93	Chlorpyrifos: 0.094 mg/kg
5-Pepper	37	35	37	29	95	68	91	68	Chlorpyrifos: 0.023 mg/kg
6-Tomato	9	36	20	32	97	75	96	97	nd
7-Strawberry	0	38	44	40	0	31	20	49	nd
8-Grape	16	25	45	28	35	52	40	39	nd
9-Pepper	14	20	17	15	100	97	93	95	nd
10-Tomato	13	23	11	21	57	64	69	57	nd
11-Strawberry	3	26	8	15	96	75	99	94	nd
12-Grape	0	16	0	0	0	59	0	0	nd
13- Pepper	0	5	0	3	0	21	0	0	nd

In the case of the first five samples described in table 16, there was the detection of one or more insecticides using the GC/LC-MS, followed by the inhibition of Nb AChE mutants present in the AChE multisensor. Among the identified insecticides are two organophosphorothionates (chlorpyrifos, chlorpyrifos-methyl). This class of insecticides needs to be oxidized first, in order to increase the AChE inhibiting strength (Fukuto 1990). The samples were previous the biosensor analysis chemically oxidized. However, the inhibition rates in the biosensor analysis were lower than the ones expected.

No insecticides were detected in samples 6 to 11 using standard analytical methods. In contrast, some of these samples had a significant inhibitory activity over Nb AChE mutants in the biosensor. In some samples, like samples 7 and 8, the AChE reactivation rate after the treatment with 2-PAM was low.

4.3.5 Storage stability

The stability of *N. brasiliensis* AChE B WT at room temperature was already tested (Schulze et al. 2005). However, we wanted to verify if Nb AChE B mutants could also remain stable under this condition after a certain period of time. In order to do that, the AChE activity, represented by the output current of the biosensor, and the sensitivity toward chlorpyrifos in buffer solution, chemically oxidized before the analysis, were measured for several weeks after the production of the AChE biosensor. The shelf life analysis is an important point to be evaluated in order to market a biosensor.

Figure 50 shows the output signal current of Nb AChE M301A for 40 weeks. Its activity remained stable after 40 weeks at room temperature. Similar behavior was shown by the mutants F345A, W346V and W346A printed in the biosensor (Figures 51, 52 and 53).

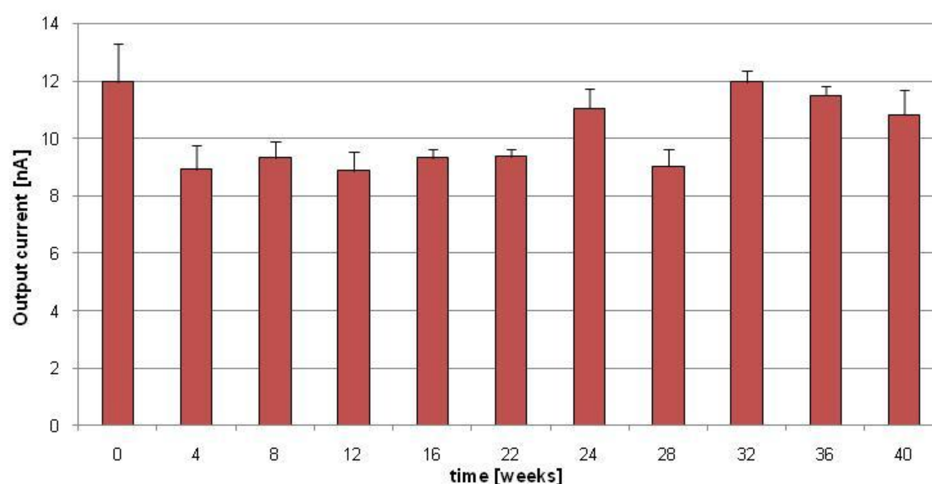


Figure 50 – Room temperature stability of *N. brasiliensis* AChE B M301 immobilized on thick-film electrodes. AChE activity, represented as current output, of the biosensor after having been stored for 40 weeks at room temperature. *n=3*

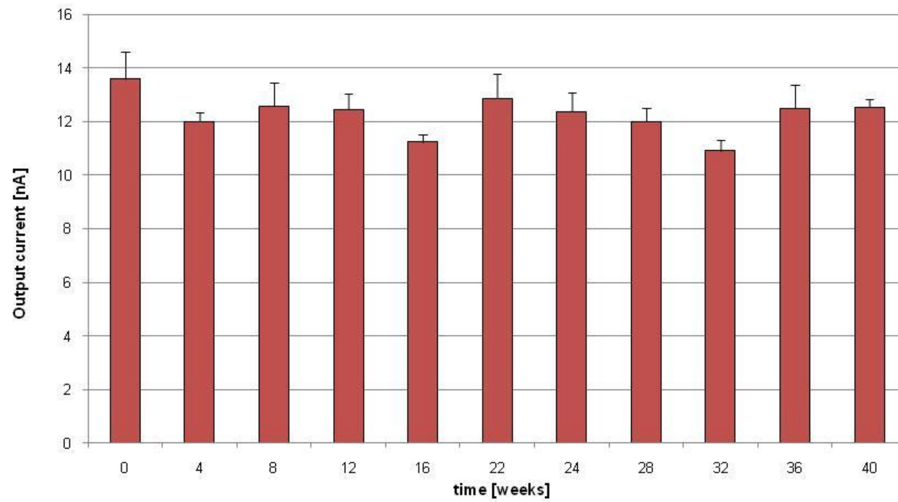


Figure 51 – Room temperature stability of *N. brasiliensis* AChE B F345A immobilized on thick-film electrodes. AChE activity, represented as current output, of the biosensor after having been stored for 40 weeks at room temperature. *n=3*

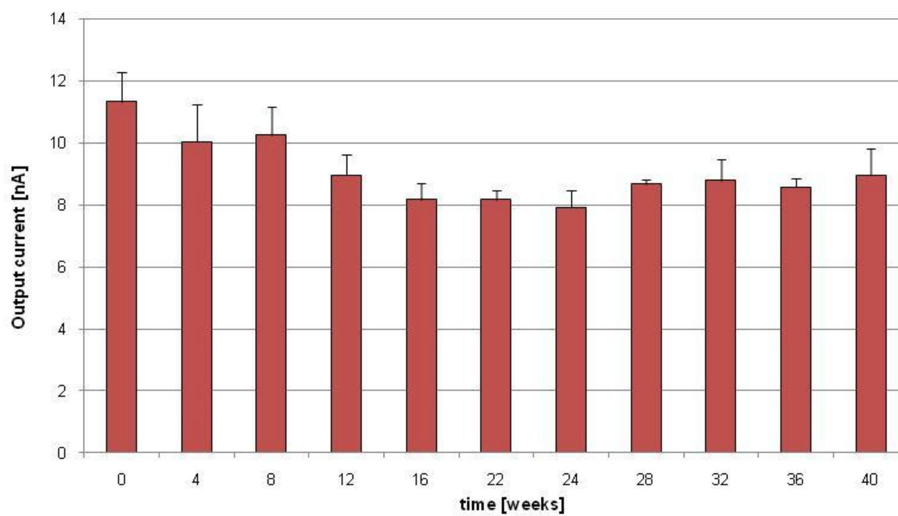


Figure 52 – Room temperature stability of *N. brasiliensis* AChE B W346V immobilized on thick-film electrodes. AChE activity, represented as current output, of the biosensor after having been stored for 40 weeks at room temperature. *n=3*

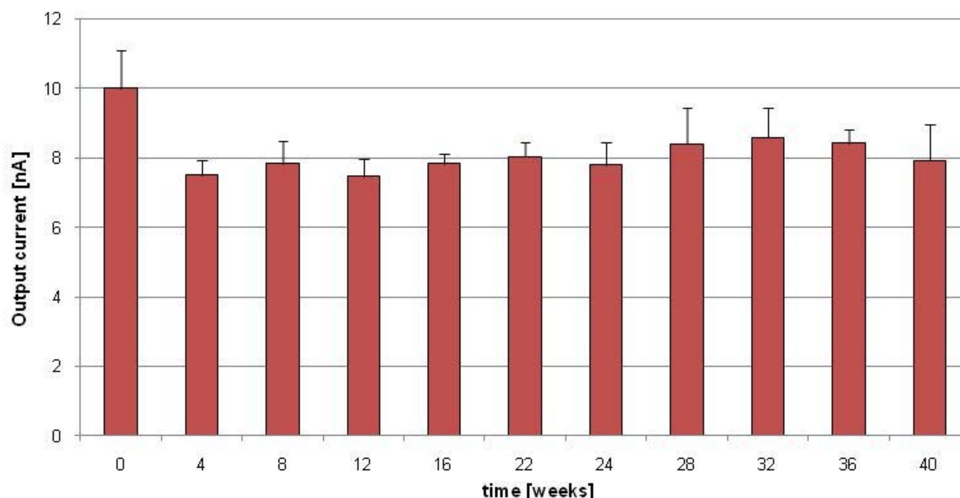


Figure 53 – Room temperature stability of *N. brasiliensis* AChE B W346A immobilized on thick-film electrodes. AChE activity, represented as current output, of the biosensor after having been stored for 40 weeks at room temperature. *n=3*

However, it could be observed that the sensitivity of the mutants M301A, W346V and W346A toward 0.05 µg/L chlorpyrifos decreased slowly with the time, when stored at room temperature (Figures 54, 56 and 57). F345A was the only mutant in the biosensor that conserved this characteristic for the whole analysis period (Figure 55). However, this mutant presented the lowest sensitivity toward chlorpyrifos among the four enzymes immobilized on the biosensor.

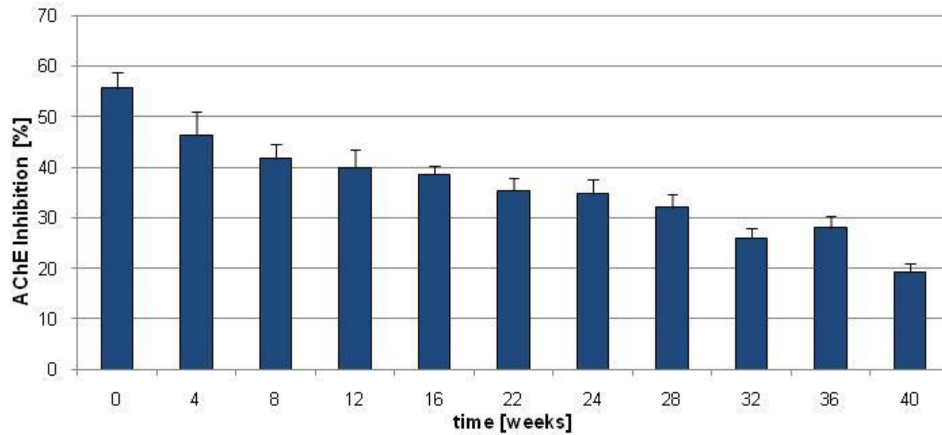


Figure 54 - Stability of *N. brasiliensis* AChE B M301 immobilized on thick-film electrodes at room temperature. Biosensor sensitivity over 40 weeks, referred to as AChE inhibition caused by 20 min incubation with 0.05 µg/L chlorpyrifos. *n = 3*

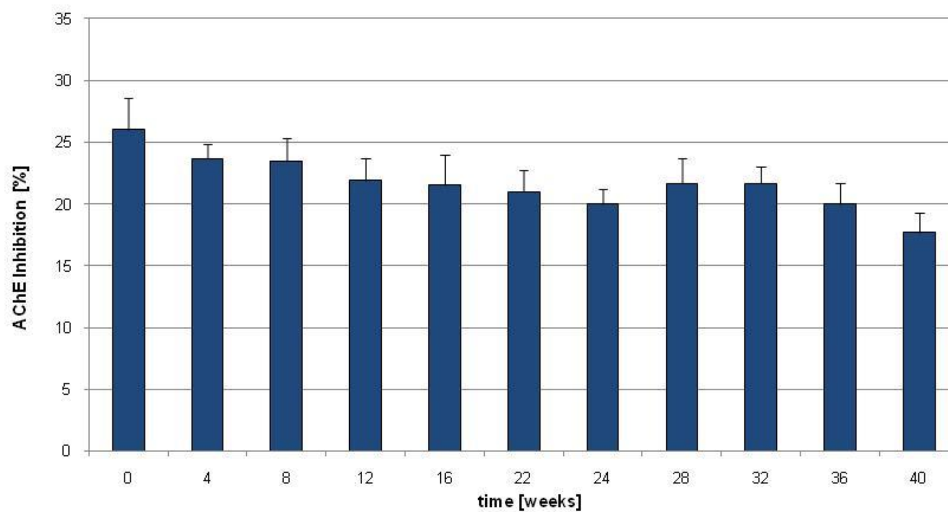


Figure 55 - Stability of *N. brasiliensis* AChE B F345A immobilized on thick-film electrodes at room temperature. Biosensor sensitivity over 40 weeks, referred to as AChE inhibition caused by 20 min incubation with 0.05 µg/L chlorpyrifos. *n = 3*

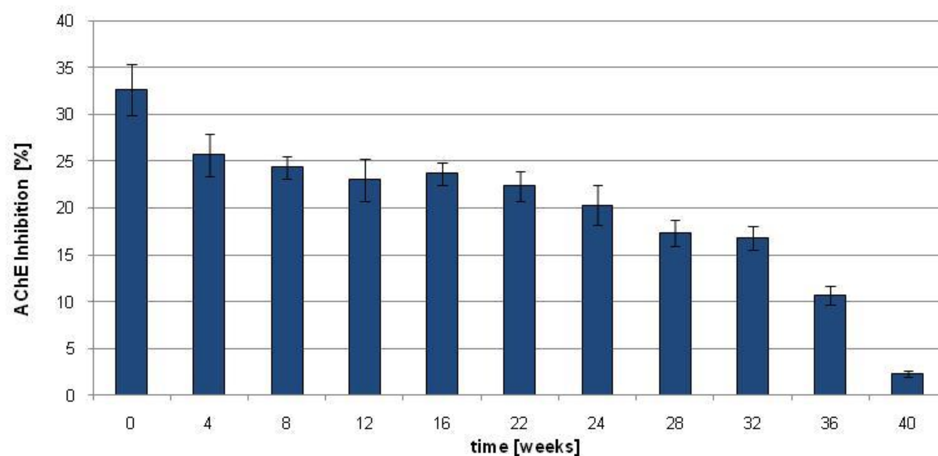


Figure 56 - Stability of *N. brasiliensis* AChE B W346V immobilized on thick-film electrodes at room temperature. Biosensor sensitivity over 40 weeks, referred to as AChE inhibition caused by 20 min incubation with 0.05 µg/L chlorpyrifos. *n = 3*

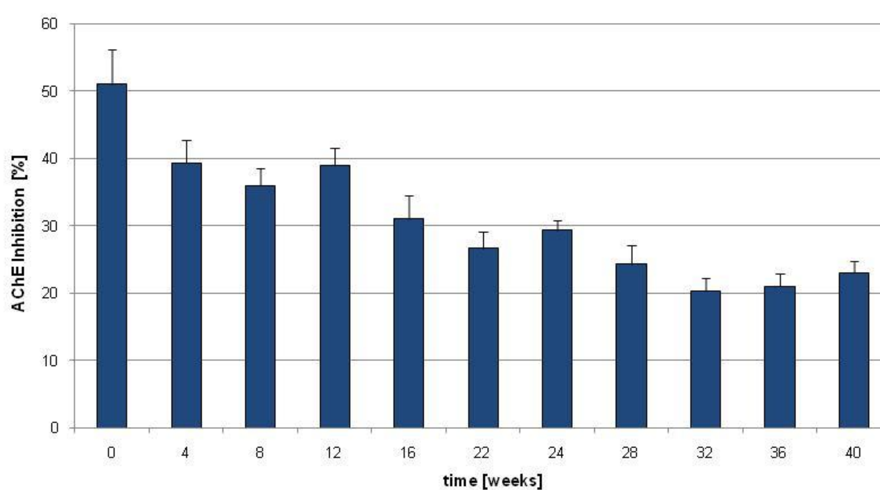


Figure 57 - Stability of *N. brasiliensis* AChE B W346A immobilized on thick-film electrodes at room temperature. Biosensor sensitivity over 40 weeks, referred to as AChE inhibition caused by 20 min incubation with 0.05 µg/L chlorpyrifos. *n = 3*

4.4. Development of acetylcholinesterase biosensors for the detection of glycoalkaloids

4.4.1 Half maximal inhibitory concentration (I_{50}) value determination

In order to determine which Nb AChE had the highest sensitivity towards α-chaconine and α-solanine, the I_{50} value, which is the concentration of inhibitor able to generate 50% of inhibition over the enzyme to be studied, was calculated. Table 17 demonstrates the obtained results.

Table 17 – I_{50} values of Nb AChE WT and 19 mutants in the presence of α-chaconine and α-solanine

Strain	**I_{50} value - α-chaconine (μM)**	**I_{50} value - α-solanine (μM)**
WT	22.97	26.90
W303G	13.80	26.78
W303Ains	6.99	28.47
W303A	13.68	31.34
W303L	10.01	18.76
Y349G	613.59	11,662.00
Y349L	58.89	121.22
T65Y	28.01	23.19
M301W	8.14	12.31
M301A	127.12	1,928.00
F345Y	20.59	29.17
F345A	4.57	41.48
M301A/W303A	26.63	88.26
M301A/W303L	15.41	16.30
M301A/F345A	14.87	68.26
M301A/F345L	179.47	574.48
M301V	27.03	40.39
M301L	29.87	50.14
W346V	3.91	9.99
W346A	7.24	16.95

One position where the mutations caused a positive influence over the inhibitory power of the glycoalkaloids was the position 303, where there was previously a tryptophan. All four mutants with a mutation in this position showed a good sensitivity towards α-

chaconine and α-solanine. The mutant W303L was finally selected to be used in the biosensor, due to its lowest sensitivity towards organophosphate and carbamate insecticides among all four mutants.

Mutations in the position 346, where originally a tryptophan was present, also showed good results of I_{50} value. However, mutants W346V and W346A demonstrated a high sensitivity towards organophosphates in previous tests. These mutants were used in the biosensor tailor made for Brazil, in order to detect insecticides in food samples.

The mutant F345A showed a good inhibition rate when in the presence of α-chaconine. Consequently, it was chosen to be applied in the AChE biosensor for the glycoalkaloids detection. A negative characteristic shown by this enzyme is that it has a high sensitivity toward the insecticide carbaryl.

The Nb AChE biosensor for the detection of α-chaconine and α-solanine was formed by the enzymes Nb AChE W303L and F345A.

4.4.2 Acetylcholinesterase biosensor specificity

The inhibition of glycoalkaloids over cholinesterases is classified as reversible (Nigg et al. 1996, Benilova et al. 2006). Consequently, one of the first points to be observed was the behavior of Nb AChEs immobilized on the biosensor in the presence of glycoalkaloid inhibitors and of different concentrations of the substrate, acetylthiocholine chloride.

As it can be seen in Figure 58, the inhibition rate of both substances over Nb AChE F345A declined in the presence of a final concentration of ATCh higher than 0.25 mM.

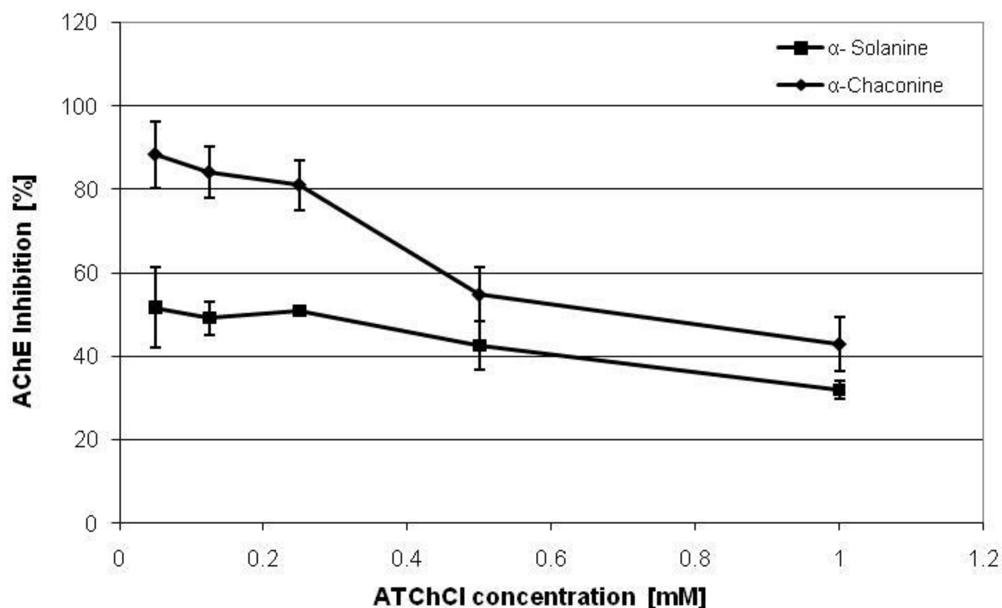


Figure 58 – Specificity of Nb AChE F345A in the presence of different concentrations of acetylthiocholine chloride and 20 µM α-chaconine / 20 µM α-solanine

The best sensitivity of the biosensor to glycoalkaloids was achieved at low substrate concentrations (0.05 - 0.25 mM), and this phenomenon could be considered as one of the attributes of competitive inhibition. For substrate concentrations higher than 0.25 mM, the acetylthiocholine started to compete with α-solanine and α-chaconine for the active site of Nb AChE, and the sensitivity decreased. The concentration of ATChCl of 0.25 mM was further applied in the biosensor assays using food samples.

4.4.3 Kinetics of the inhibition activity

The type of inhibition shown by α-chaconine and α-solanine over Nb AChE was studied using different concentrations of the inhibitors and of the substrate, acetylthiocholine. Previous studies revealed the independence of the variable "time of incubation", meaning that the inhibition could be a reversible one (data not shown).

A reversible inhibition can be classified in competitive, non-competitive, uncompetitive and mixed. The best way to study this type of inhibition is plotting the results obtained in the kinetics studies as Dixon (1/output current X inhibitor concentration) and Cornish-Bowden plots (substrate concentration/output current X inhibitor concentration).

A reversible and competitive inhibition can be characterized when 3 lines intercept at a single point in the second quadrant above x-axis in the Dixon´s coordinates; and when 3 parallel lines are observed in the Cornish-Bowden plot. This description matches the results shown by the inhibition behavior of α-chaconine over Nb AChE F345A (Figures 59 and 60). The mutant W303L was also competitively inhibited by α-chaconine (data not shown).

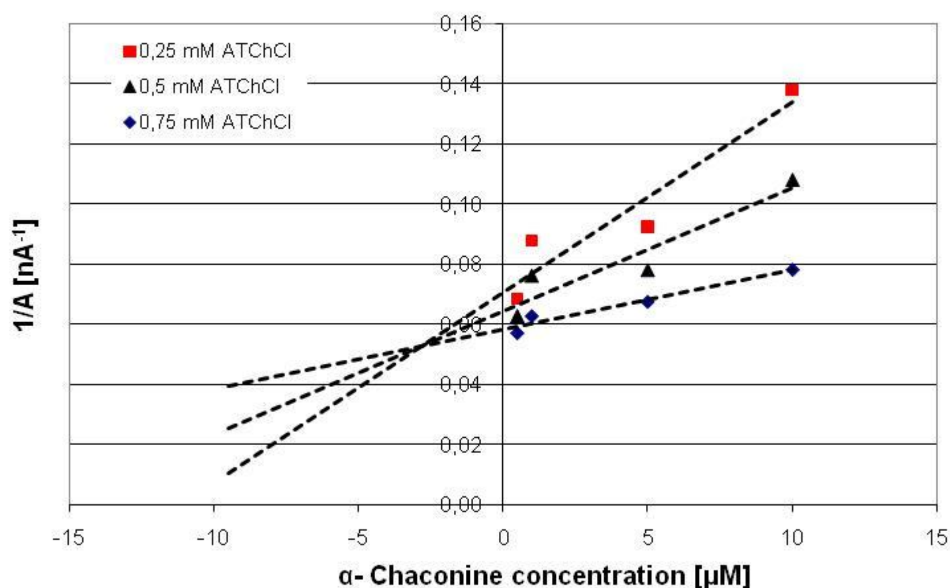


Figure 59 – Dixon plot of mutant F345A in the presence of different concentrations of α-chaconine

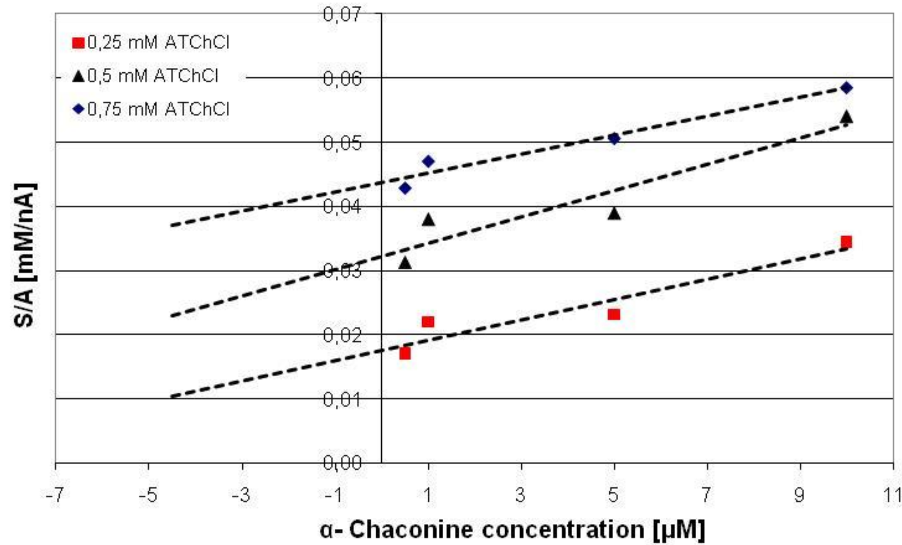


Figure 60 – Cornish-Bowden plot of mutant F345A in the presence of different concentrations of α-chaconine

The inhibition of α-solanine over F345A was reversible and competitive (Figures 61 and 62), like already observed for α-chaconine. The Dixon and Cornish-Bowden plots of mutant W303L also revealed a similar inhibition behavior of α-solanine over this enzyme (data not shown).

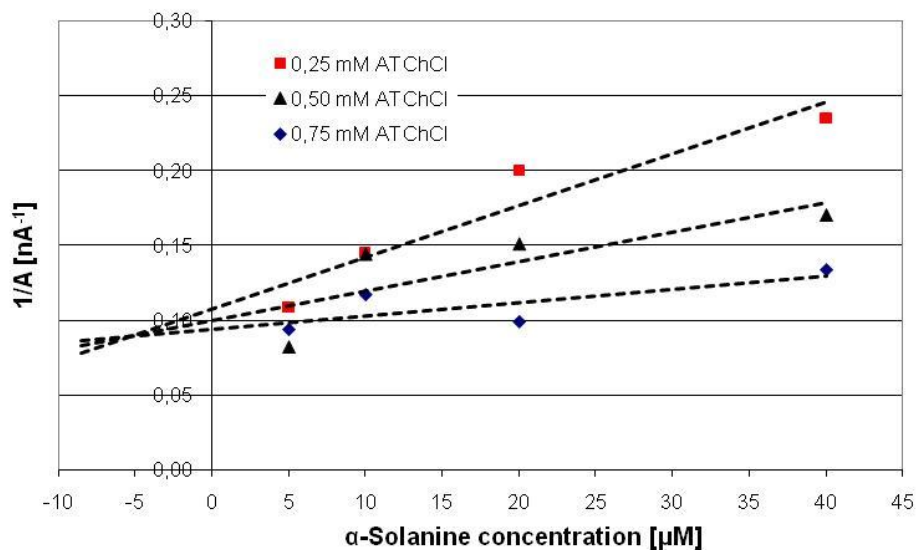


Figure 61 – Dixon plot of mutant F345A in the presence of different concentrations of α-solanine

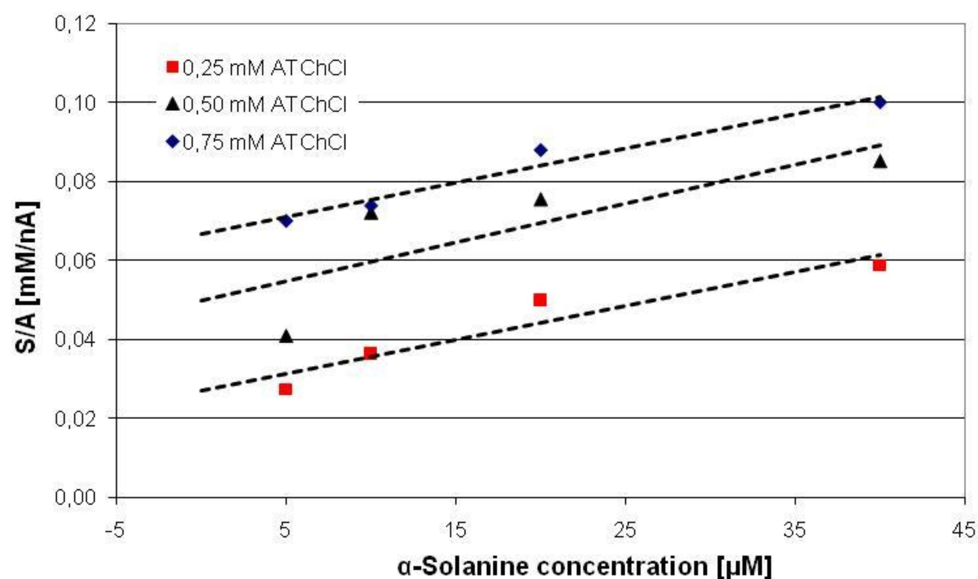


Figure 62 – Cornish-Bowden plot of mutant F345A in the presence of different concentrations of α-solanine

The values of the apparent inhibition constant (K_{iapp}) and half maximal inhibitory concentration (I_{50}) can be evaluated in table 18.

Table 18 – Apparent kinetic parameters of Nb AChE F345A and W303L immobilized on a biosensor measured in 10 mM PBS pH 7.5 in the presence of α-chaconine and α-solanine

Inhibitor	AChE F345A K_{iapp} [μM]	AChE W303L K_{iapp} [μM]	AChE F345A (0.25 mM ATChCl), I_{50} [μM]	AChE W303L (0.25 mM ATChCl), I_{50} [μM]
α-chaconine	3.0	4.3	7.6	8.4
α-solanine	4.0	12.0	23.2	25.4

It was observed that the K_{iapp} values were lower than the respective I_{50} values. For example, the mutant F345A in the presence of α-solanine showed a K_{iapp} value of 4.0 μM, in comparison the I_{50} value for the same system was 23.2 μM.

Figure 63 illustrates the calibration line of the developed biosensor in the presence of the two studied glycoalkaloids.

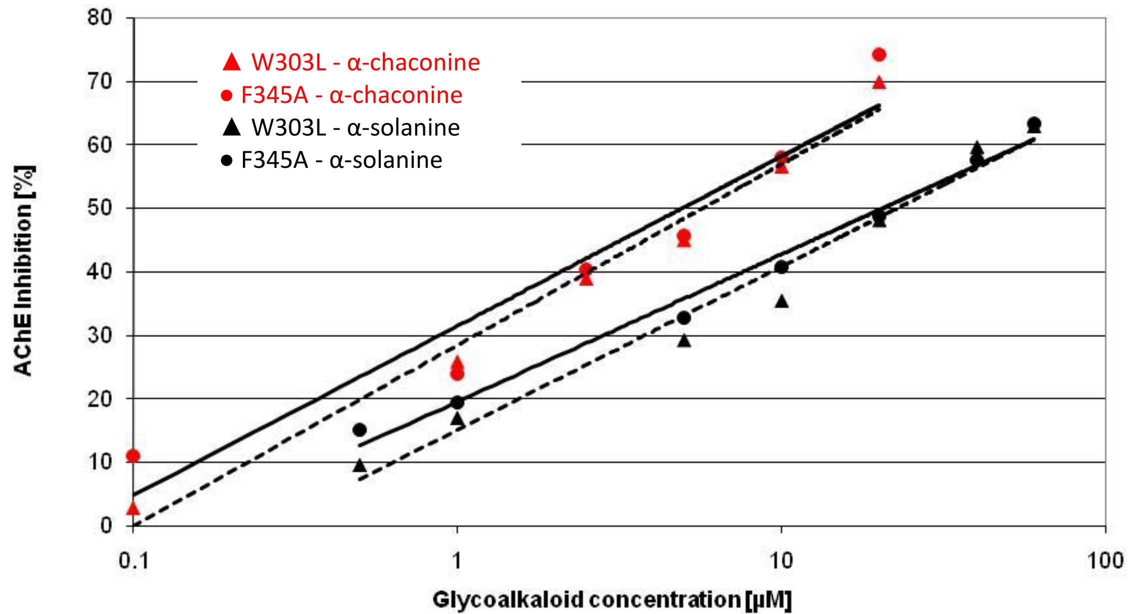


Figure 63 – Calibration lines of the Nb AChE biosensor for α-chaconine and α-solanine

The line equations obtained were the following:

y = 9.0698 . ln(x) + 20.122 (in the calibration line α-solanine X F345A)

y = 8.4045 . ln(x) + 16.082 (in the calibration line α-solanine X W303L)

y = 14.23 . ln(x) + 24.756 (in the calibration line α-chaconine X F345A)

y = 11.529 . ln(x) + 27.964 (in the calibration α-chaconine X W303L)

The detection limit of the developed biosensor for α-chaconine was 0.1 µM, and for α-solanine, 0.5 µM. Both enzymes W303L and F345A showed similar inhibition rates. The total glycoalkaloid concentrations in potato tubers destined for human consumption is limited to 200 mg/kg potatoes, which correspond to 250 µM (Valkonen et al. 1996). This means that the sensitivity of the biosensor (0.1 µM for α-chaconine, and 0.5 µM for α-solanine) is in accordance with the levels requested by legislation.

Additionally, it is mentioned in the literature that α-chaconine has a higher inhibitory activity over cholinesterases than α-solanine (Fewell and Roddick 1997). The obtained

results confirmed this information, based on the detection limits of the biosensor (0.1 µM for α-chaconine, and 0.5 µM for α-solanine).

4.4.4 Food samples

The concentrations of α-chaconine and α-solanine in the food samples were determined using the linear region of the calibration curve obtained in the kinetic studies (section 4.4.3). The biosensor analysis was validated using potato samples spiked with standard solutions of α-chaconine and α-solanine. The safety level allowed by legislation for glycoalkaloids, in order to avoid acute intoxications in humans, is 250 µM (Valkonen et al. 1996).

First the sensitivity of the Nb AChE biosensor towards α-chaconine was tested. This glycoalkaloid is described as three times more toxic to humans as α-solanine (Fewell and Roddick 1997). Figure 64 shows the results obtained with samples contaminated with 100 µM of α-chaconine. The final concentration of α-chaconine was its concentration in the solution in the container where the biosensor was immersed. To achieve the different glycoalkaloid concentrations in this solution, different volumes of the sample were added to the container. The recovery rate of the inhibition was calculated using the inhibition rate after the sample was added, and the inhibition rate of the standard curve. The detection limit of α-chaconine was determined in 0.1 µM (Figure 63).

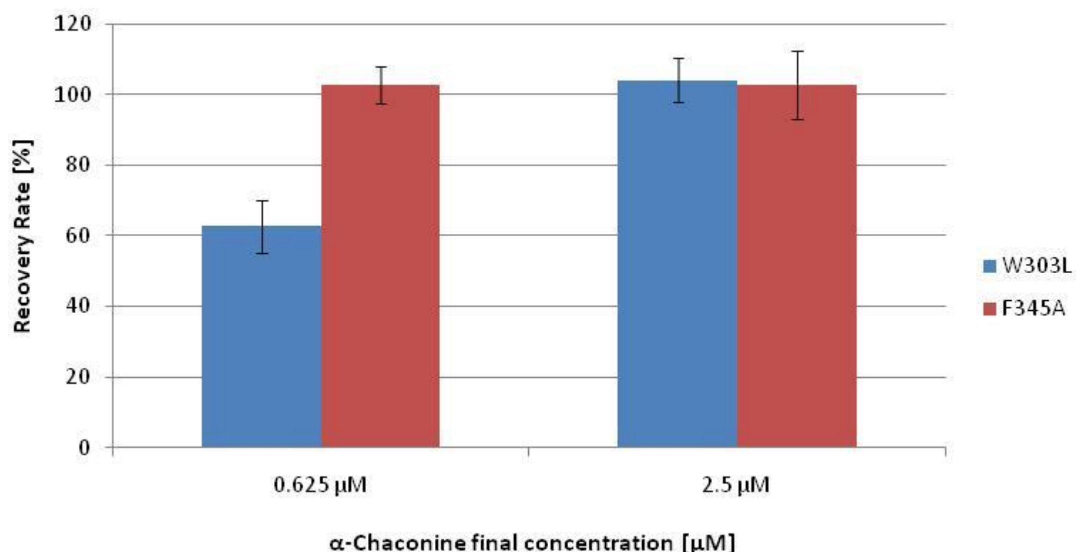


Figure 64 – Recovery rate of the inhibition over mutants W303L and F345A after incubation in the solution with different potato sample volumes, contaminated with 100 µM α-chaconine. *n=3*

The results showed that mutant F345A was more sensitive to detect α-chaconine than mutant W303L. The recovery rates of the inhibition over F345A in the presence of 0.625 and 2.5 µM α-chaconine were approximately 100%, while mutant W303L showed a recovery rate of around 62% from the inhibition signal expected in the presence of 0.625 µM α-chaconine. Table 19 illustrates the results obtained with samples spiked with 100, 200 and 400 µM α-chaconine with the same mutants.

Table 19 – Recovery rates of the inhibition over mutants W303L and F345A after incubation with 100, 200 and 400 µM α-chaconine

α-chaconine concentration in the potato sample (µM)	*α-chaconine final concentration in solution (µM)*	*Inhibition rate of W303L (%)*	*Recovery rate of the inhibition over W303L (%)*	*Inhibition rate of F345A (%)*	*Recovery rate of the inhibition over F345A (%)*
100	0.625	14.12	62.64	18.55	102.71
	2.5	40.13	104.15	38.89	102.91
200	1.25	13.79	45.15	29.58	105.91
	5.0	45.60	98.02	50.06	105.06
400	2.5	39.62	102.83	39.53	104.60
	10.0	46.86	85.97	56.87	104.31

As already observed with 100 µM α-chaconine, mutant F345A was more sensitive to this glycoalkaloid than mutant W303L in the presence of 200/400 µM α-chaconine. This mutant had previously shown a higher sensitivity towards α-chaconine in the I_{50} value determination tests (Table 17) than mutant F345A.

Depending on the variety, potatoes may contain α-chaconine and α-solanine at concentration ratios of α-chaconine to α-solanine raging from around 1.2:1 to 2.4:1 (Friedman et al. 2003). We chose to spike the potato samples at a concentration ratio of α-chaconine:α-solanine of 2:1. The total inhibition rate was calculated as the sum of the inhibition rates of the correspondent final concentrations of α-chaconine and α-solanine. The detection limit of α-chaconine was determined in 0.1 µM, and of α-solanine in 0.5 µM (Figure 63).

The concentration of total glycoalkaloids tested were 300 (200/100 µM α-chaconine/α-solanine) and 600 µM (400/200 µM α-chaconine/ α-solanine). It is important to point out that the detection limits of the biosensor for both substances (0.1 µM for α-chaconine and 0.5 µM for α-solanine) enable the detection of lower quantities of these GAs in samples.

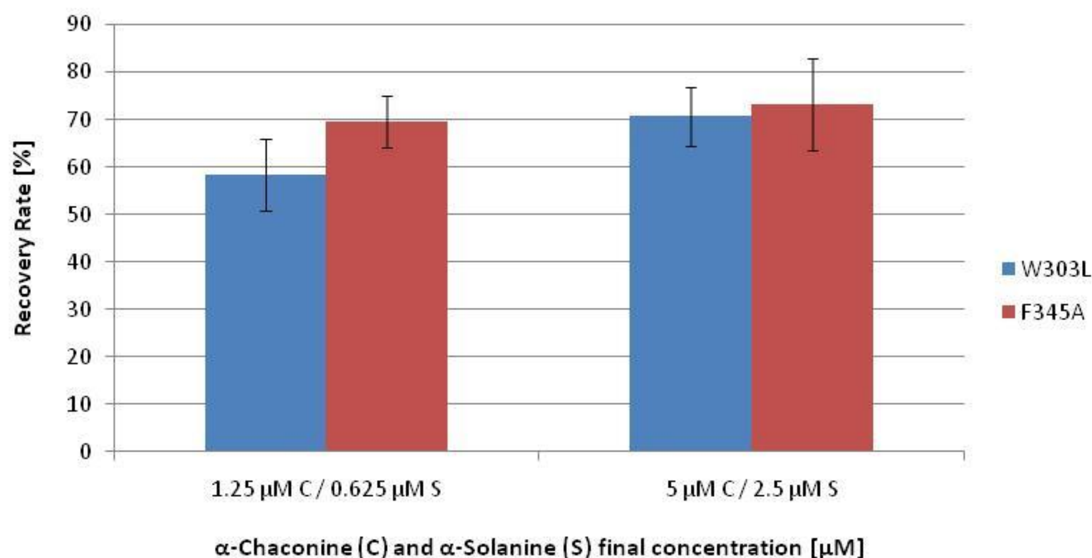


Figure 65 - Recovery rate of the total inhibition over mutants W303L and F345A after incubation in solution with different potato sample volumes, contaminated with 200 µM α-chaconine (C) and 100 µM α-solanine (S). *n=3*

The recovery rate of the inhibition over the biosensor using samples spiked with 200/100 µM α-chaconine/α-solanine was around 70%, lower than expected. This means that the ratio 2:1 of the mixture of α-chaconine/α-solanine generates an antagonistic effect over the total inhibition, with both glycoalkaloids competing for the active site of Nb AChE. The recovery rates of the inhibition over both mutants in the presence of 200/100 µM α-chaconine/α-solanine and 400/200 µM α-chaconine/ α-solanine are shown in table 20.

Table 20 – Recovery rates of the inhibition over mutants W303L and F345A after incubation with 200/100 µM and 200/400 µM α-chaconine/ α-solanine

α-chaconine / α-solanine concentration in the potato sample (µM)	***α-chaconine/ α-solanine final concentration in solution (µM)***	***Inhibition rate of W303L (%)***	***Recovery rate of inhibition over W303L (%)***	***Inhibition rate of F345A (%)***	***Recovery rate of inhibition over F345A (%)***
200/100	1.25/0.625	24.92	58.40	30.47	69.58
	5.0/2.5	49.69	70.68	55.67	73.17
400/200	5.0/2.5	33.33	59.01	42.84	71.48
	20.0/10.0	60.79	72.27	63.37	68.70

The ratio 2:1 of 400/200 μM α-chaconine/α-solanine also obtained results similar to the ones with 200/100 μM. The inhibition rate obtained after the sample addition was lower than the inhibition rate of the standard curve.

5. Discussion

The aim of this work was to develop Nb AChE biosensors able to detect neurotoxic substances in foods and in the environment. They should be easy to operate, be highly sensitive, and obtain results in a short amount of time. The specific objectives established in the beginning of this work were successfully fulfilled:

1. A new methodology using a pretreatment with chloroperoxidase and a detection system of Nb AChE biosensors was able to activate and to detect phosphorothionate insecticides in foods;

2. A multisensor using four specific mutants of Nb AChE detected the most applied organophosphate and carbamate insecticides in Brazil in fruit and vegetable samples. The detection limits of the biosensor respected the residual limits requested by the Brazilian legislation;

3. The detection of the glycoalkaloids α-solanine and α-chaconine in potato samples was possible using Nb AChE biosensors up to a concentration of 300 µM total glycoalkaloids.

5.1. Analysis of phosphorothionate insecticides using a chloroperoxidase pretreatment and acetylcholinesterase biosensor detection

The oxidation of phosphorothionate insecticides increases the AChE inhibiting strength (Fukuto 1990, Jokanovic 2001). In order to improve the detection capability of AChE biosensors toward these substances, a new method was successfully developed, combining an oxidation pretreatment with the enzyme CPO and a detection step using AChE biosensors. This methodology could be applied in food samples directly, without needing an extraction step. The enzyme CPO was chosen due to its ability to oxidize thionates into their oxon forms (Hernandez et al. 1998, Walz and Schwack 2007).

5.1.1 pH

One important point to be evaluated is the pH of the reaction system. The study of the pH gives information about the stability of the enzymes involved, and it also clarifies in which pH range the reaction occurs. The results showed that AChE is inactivated in pHs lower than 5.5, while the activity of CPO remained stable under the same conditions. Acetylcholinesterase is described in the literature as having a p*I* of 5.3. Below this pH, an aggregation of the enzyme occurs, due to changes in its conformation (Dziri et al. 1997). In contrast, CPO retains its greatest oxidation activity at pH 5-6, which may produce a more favorable ionization state of the key amino acid (Glu-183), thus reducing radical formation (Park and Clark 2006). However, this enzyme is irreversibly deactivated at pHs higher than 6 (Van Deurzen et al. 1997). The pH chosen to be used in the system was 5.5.

5.1.2 H_2O_2

Chloroperoxidase has the ability to utilize several hydroperoxides and peroxy acids as electron acceptors in order to execute its reactions (Colonna et al. 1990, Spreti et al. 2004). The conversion of thionates by CPO-catalyzed oxygen release from H_2O_2 in pHs lower than 4.8 was previously demonstrated (Hernandez et al. 1998).

H_2O_2 was chosen as the first choice of natural source of oxygen for the oxidation experiments using CPO and organic orange juice. However, preliminary tests showed that the addition of H_2O_2 to the sample generated absent or low conversion rates. One explanation for these results could be that this peroxide reacts with antioxidants found in foods, like the ascorbic acid, remaining nothing left to react with CPO.

Furthermore, CPO is deactivated by H_2O_2 at very low concentrations, and its quantity in the reaction system is critical for enzymatic oxygen transfers. Deactivation of heme-containing peroxidases by hydrogen peroxide is thought to involve the oxidation of the porphyrin ring and formation of the inactive intermediate compound III (Van Deurzen et al. 1997, Vazquez-Dulaht et al. 1993, Mylrajan et al. 1990). Oxidation of methionine, cysteine, tryptophan and/or tyrosine is usually one of the major chemical reactions that render a loss in enzymatic activity. Chloroperoxidase contains three cysteines, where two of them form a S-S bridge (Cys 87 and 79) and one acts as a ligand to the heme iron in the form of a thiolate (Cys 29) (Andersson et al. 2000).

5.1.3 *Tert*-butyl hydroperoxide

In contrast to the results obtained using H_2O_2, *tert*-butyl hydroperoxide revealed itself a successful oxygen donor to CPO. 800 mM t-b HP (70% aqueous solution) could be added in the case of the KCl tests, and 100 mM in the case of the KBr tests, without causing any problem to the stability of CPO, or to the conversion rates. Chlorpyrifos and triazophos were completely oxidized by CPO into chlorpyrifos oxon and triazophos oxon, respectively. Fenitrothion, methidathion and parathion-methyl were partially oxidized adding both types of halogen salts.

An advantage of the use of *tert*-butyl hydroperoxide is that the catalase side reaction can be avoided. In this reaction, H_2O_2 dismutates into water and molecular oxygen, which causes problems with pressure build up and the evaporation of volatile substrates (Van Rantwijk and Sheldon 2000). Enhanced stability of CPO toward t-b HP allows its addition in high concentrations. As a result, this peroxide can be used as oxidant for the PO-catalyzed oxidation reactions. Walz and Schwack (2007) had already demonstrated the hability of CPO to use this peroxide to oxidize organophosphorothionate insecticides. But the sample had to be extracted before the oxidation step, with the aim of extracting the insecticides in the food matrix. This added an extra step to the method, and consequently, a longer analysis time.

Additionally, the use of t-b HP is reasonable, since the *tert*-butyl alcohol, which is the product from t-b HP, had previously shown to exert a stabilizing effect on CPO (Van Deurzen et al. 1997, Park and Clark 2006). However, the addition of *tert*-butyl alcohol in concentrations higher than 30% has a negative influence on CPO stability towards oxidizing conditions (Van Deurzen et al. 1997).

In this work, *tert*-butyl hydroperoxide was successfully applied in the oxidation of phosphorothionates using CPO, without interfering with the stability of the AChE immobilized on the biosensor.

5.1.4 Organophosphorothionate insecticides oxidation using chloroperoxidase

The results of GC/MS tests revealed that CPO could successfully activate phosphorothionates when applied directly to food samples. This can be affirmed, because all insecticides exposed to CPO produced one major degradation product, its oxon form. Besides, the decrease of the molecular ions (16 *m/z* smaller than the original substrate) of the products reveals the oxidation of the thiophosphoryl bond (P=S) into phosphoryl bond (P=O) by MS.

The activation of organophosphorothionates using CPO is similar to those performed by cytochromes P450 in *in vivo* and *in vitro* systems. Nevertheless, the major difference between both biocatalysts is that a further cleavage of oxons, which is typical of the P450-catalyzed reaction (Hernandez et al. 1998), was not observed with CPO.

5.1.5 Halogen salts

Furthermore, the need of adding halogen salts in the oxidation system was evaluated, and if so, which halogen salt, KCl or KBr, would be more effective in the oxidation system.

Chloroperoxidase exists in two active forms: the acidic form was described to catalyze halide-dependent, and the neutral form, halide-independent reactions. The

transition between the two forms occurs between pH 3 and 5. Furthermore, the neutral form of CPO shows a broad pH optimum at around pH 5-6, where the enzyme is responsible for various halide-independent oxidation reactions (Kiljunen and Kanerva 2000).

The first results revealed that the conversion reaction with absence of chloride or bromide ions had a low biocatalytic activity. This data correlates to a previous report, where chloride had a great influence over a successful oxidation of phosphorothionates using CPO at pH 4.8 (Walz and Schwack 2007). On the other hand, the conversion rates obtained with addition of KCl and KBr were very high and similar, when the insecticides were analyzed individually. Chlorpyrifos and triazophos were completely oxidized by CPO into chlorpyrifos oxon and triazophos oxon, respectively. Fenitrothion, methidathion and parathion-methyl were partially oxidized adding both types of halogen salts.

In acid pHs, Amitai et al. (2003) showed that the degradation of 10 µM VX, a toxic warfare chemical agent, by CPO together with urea hydrogenperoxide (UPER, 0.5 mM) and 0.5 M NaCl was completed within 1 min in a pH 2.75, indicating low activation energy for the enzymatic formation of hypochlorous ions/free chlorine. Halides ions (X^-) are also substrates for peroxidases; however they behave differently from conventional reduced substrates (Torres et al. 2003).

It was also observed that lower concentrations of bromide salt were required in the oxidation experiments than of chloride salt. One possible explanation is that bromide has a lower redox potential than chloride, hence the level of activation of the active site is reduced. Among the halides other than fluoride, which is apparently not oxidized by CPO, chloride is always the least active and iodide the most active substrate. Acceleration of oxidation in the presence of chloride/bromide may be explained by the formation of a certain content of chlorine/bromine, catalyzing the oxygen release. In the case of the

oxidation of organophosphorothionates, a reactive Fe^{III}-O-O^- intermediate may be formed by reaction of the active site with O_2, alternatively to a reaction with two molecules of peroxide, which as a two-step reaction is expected to occur with slower speed than the former one-step reaction (Walz and Schwack 2007). It has already been shown that CPO has a two- to threefold higher halogenation activity for monochlorodimedon (MCD) in the presence of bromide ions than chloride ions (Hager et al. 1966). In another study, the initial activities of halohydrin formation using CPO increased 3-times applying bromide ions, from 4.22 U/mg with chloride to 12.22 U/mg with bromide, when compared with the addition of chloride ions (Kaup et al. 2007).

Another important fact verified during the k_i determination tests was that higher deviation values occurred in the tests with addition of KBr than in the ones with KCl. The use of KBr as a catalyst of the oxidation of phosphorothionates by CPO was described for the first time in this work.

5.1.6 Stability of the biosensor under oxidation conditions

Experiments were conducted to test the stability of Nb AChE immobilized on an amperometric biosensor in the reaction system. No inhibition was observed after the incubation of the biosensor for 30 min in the solution obtained from the reaction between 34 U/mL CPO, 100 mM t-b HP and 100 mM KBr. On the contrary, the blank sample from the reaction with 34 U/mL CPO, 1,000 mM t-b HP and 670 mM KCl inhibited 100% of Nb AChE WT.

In preliminary tests, Nb AChE WT remained stable in a solution under the same concentrations of KCl and t-b HP used in the oxidation tests for 45 min. However, the two

reagents were tested separately, what brings to the conclusion that a secondary product was formed during the conversion reaction in a concentration able to inhibit AChE. This product could be *tert*-butyl alcohol, which is a secondary product of reactions using t-b HP. The addition of *tert*-butyl alcohol in concentrations higher than 30% has a negative influence on the stability of CPO towards oxidizing conditions (Van Deurzen et al. 1997). On the other hand, it was found that the activity of AChE from erythrocytes in alcohol-addicted subjects is much lower than the one measured in healthy subjects (Mionetto et al. 1994). Aliphatic alcohols are able to inhibit membrane-bound forms of AChE. The extent of enzyme inhibition by short aliphatic alcohols usually increases with the length of the alcohol hydrocarbon chain (Lasner et al. 1995). Arsov et al. (2005) found that AChE from erythrocytes was inhibited at high concentrations of *n*-butanol, and that two alcohol molecules usually interact with AChE under this condition.

5.1.7 Biosensor of acetylcholinesterase from *N. brasiliensis* in food samples

The main objective of this project was to propose an alternative method able to oxidize organophosphorothionate insecticides in foods, followed by a fast and cheap detection tool. The application of the enzymatic method to organic orange juice in combination with a disposable AChE biosensor successfully detected chlorpyrifos at concentrations down to 5 µg/kg, with a final concentration of this insecticide in foods of 25 µg/kg. The MRL established by the European Union for chlorpyrifos is 50 µg/kg of food (Official Journal of European Union 2008).

Table 21 compares the different methods listed in the literature for the oxidation and detection of organophosphorothionate insecticides. The method developed in this work

showed a low LOD, which was similar to the one obtained by Schulze et al. (2004), and better than the one using the same enzyme (CPO) in the oxidation step (Walz and Schwack, 2007).

Table 21 – Comparison between different methods combining oxidation pretreatment step and insecticides detection

Reference	***Method***	***Observation***	***LOD /Determined concentration (µg/kg)***
This work	Enzymatic oxidation with CPO / AChE biosensor analysis	Detection of insecticides was made using organic orange juice	LOD: 25 (chlorpyrifos)
Walz and Schwack (2007)	Enzymatic oxidation with CPO / cutinase spectrophotometric analysis	Necessity of an extra extraction step between the oxidation and detection step	30% recovery (300 µg/kg chlorpyrifos) – cherry 84% recovery (500 µg/L parathion-methyl) – apple juice
Schulze et al. (2004)	Enzymatic oxidation with P450 BM3 / AChE biosensor analysis	Detection of insecticides was made using apple puree for babies	LOD: 20 (chlorpyrifos and parathion)
Waibel et al. (2006)	Enzymatic oxidation with P450 BM3 (CYP102-A1)/ AChE biosensor analysis	P450 BM3 was immobilized on the biosensor. Oxidation step executed during detection analysis. Measurements done only in buffer solution.	LOD: 10 (parathion)
Schulze et al. (2002b)	Chemical oxidation with NBS and vitamin C / AChE biosensor analysis	Necessity of an extra extraction step between the oxidation and detection step	LOD: 10 (parathion)

One way to transform phosphorothionates into their oxon form is using chemical oxidation. At the standard oxidation method for water, the DIN 38415-1 method, oxidations are performed using NBS, followed by a step where the excess of reagent is destroyed by ascorbic acid. However, this method revealed itself unsuitable for the analysis of food samples, due to the fact that food matrices are more difficult to oxidize than water. In a previous study using an apple puree baby food spiked with 20 µg/kg parathion, the sample was submitted to chemical oxidation, combined with the AChE biosensor assay. In this case, 20 mg/L of NBS were unable to generate an inhibition over the AChE biosensor after the 30 min incubation (Schulze et al. 2004). Due to a restricted water solubility of NBS, increasing this oxidant's concentration is rather limited (Walz and Schwack 2007). Besides, the ascorbic

acid or other antioxidants present in foods can also prevent the chemical oxidation of existing organophosphorothionate insecticides with NBS (Schulze et al. 2004).

Walz and Schwack (2007) described a spectrophotometric enzyme inhibition assay using the enzyme cutinase from *Fusarium solani* pisi (EC 3.1.1.74), combining a preoxidation step using CPO from *C. fumago*, H_2O_2 and chloride. This method fulfilled successfully the demands to be used as a screening method. However, the food sample needed to be extracted using the QuECHERS method before the oxidation step, in order to extract the insecticides in the food matrix. This process takes a certain amount of time, and the use of several solvents and reagents is necessary.

Cytochrome P450 monooxygenases catalyze *in vivo* the insertion of an oxygen atom, derived from molecular oxygen, into a wide variety of organic substrates. The application of a prokaryotic cytochrome P450 mutant for the oxidation of organophosphorothionates was shown to successfully increase the sensitivity of the AChE biosensor assay (Schulze et al. 2004, Waibel et al. 2006). However, the cytochrome P450 mutant needs the addition of the cofactor NAD(P)H, which demands a complex regeneration system. On the other side, the biocatalysis reaction using CPO does not require the use of a cofactor. Chloroperoxidase exhibits higher storage stability than cytochrome P450 BM-3 (CYP102A1) from *Bacillus megaterium*, and it is stable for weeks at room temperature and under pH control (Pickard et al. 1991). In contrast, the half-life of P450 BM-3 was characterized as being of 26 days at 4° C in solution (Maurer et al. 2003), and of 4 weeks in a sol-gel entrapped biosensor (Waibel et al. 2006). Additionally, to our knowledge, this is the first time that a method for the activation and determination of phosphorothionate insecticides using a pretreatment with CPO and an AChE biosensor assay is described.

5.2. Development of acetylcholinesterase biosensors tailor made for Brazil

5.2.1 Acephate

None of the mutants was inhibited by the insecticide acephate. This insecticide was already described in the literature as an indirect AChE inhibitor (Rojakovick and March 1972, Suksayretrup and Plapp 1977). Acephate is an organophosphorus insecticide produced by the N-acetylation of methamidophos (Figure 66).

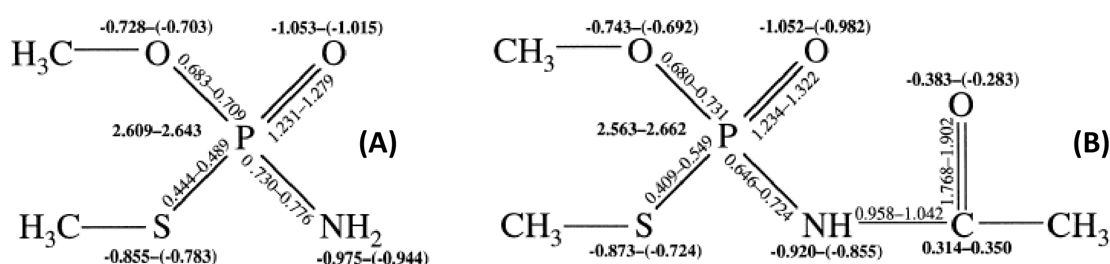


Figure 66 – Bond order and electronic charge in methamidophos (A) and acephate (B)

But, unlike methamidophos, which has two ionizable groups, acephate has three ionizable groups: P=O, C=O and –NH groups. Therefore, it shows a more complex ionization scheme and charge delocalization occurring between O, P, N and carbonyl (Singh et al. 1998). The N-acetylation of methamidophos does not alter its insecticidal properties; however its mammalian toxicity is reduced by 50% (Singh 1984). Figures 67 and 68 illustrate the interaction mechanisms of methamidophos and acephate with bovine erythrocyte AChE (mAChE), respectively.

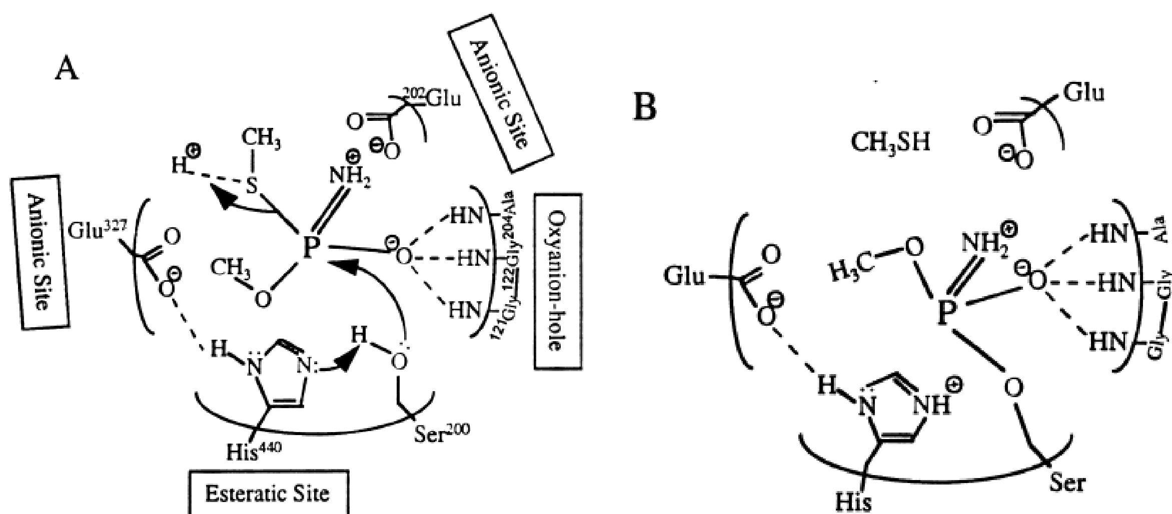


Figure 67 – Proposed mechanism of interaction of methamidophos with mAChE. A: The P-O$^-$ (the electronic form of P=O at physiological pH) of methamidophos forms hydrogen bonds within the oxyanion-hole and the NH_2 group interacts with Glu. The leaving group is facing the opening of the "gorge" toward Asp[74]. B: Hydrolysis of the P-SCH_3 bond results in the phosphorylation of Ser[200] (Singh et al. 1998)

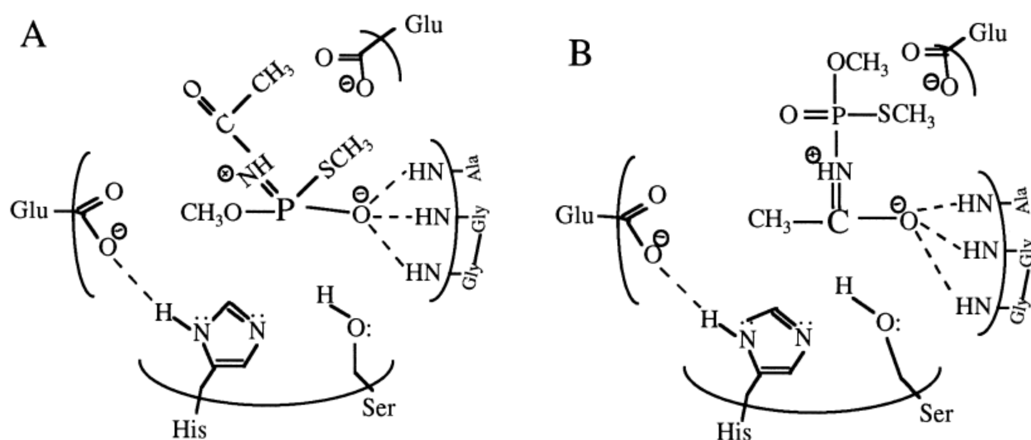


Figure 68 – Proposed mechanism of interaction of acephate with mAChE. A: The P-O$^-$ (the electronic form of P=O at physiological pH) of acephate forms hydrogen bonds within the oxyanion-hole. This may result in possible repulsion between the C=O$^{\delta-}$ group of acephate and the Glu of AChE. B: The C-O$^-$ (the electronic form of C=O at physiological pH) of acephate forms hydrogen bonds within the oxyanion-hole, resulting in a non-functional interaction between acephate and AChE. In both cases the Ser[200] residue is prevented from attacking the P atom of acephate. Thus, phosphorylation is prevented (Singh et al. 1998).

5.2.2 Sensitivity of acetylcholinesterase mutants from *N. brasiliensis* versus sensitivity of acetylcholinesterase mutants from *Drosophila melanogaster*

We identified in a library containing the wild type and 19 mutants of Nb AChE which ones had the widest sensitivity spectrum toward the most used insecticides in Brazil. The mutants F345A, M301A, W346V and W346A were the four enzymes that together presented this characteristic.

The exact positions and introduced amino acids were selected according to results of similar studies on humans (Ordentlich et al. 1996), rat brain (Pleiss et al. 1999) and *D. melanogaster* AChEs (Villatte et al. 2000, Boublik et al. 2002). A mutation in the choline binding site in the position 345 revealed itself efficient to enhance the sensitivity toward carbaryl. A phenylalanine was replaced by an alanine (mutant F345A).

Former studies showed that the replacement of the acyl pocket residue 301(288) by smaller aliphatic ones caused an increase in sensitivity toward several inhibitors (Ordentlich et al. 1996, Pleiss et al. 1999, Villatte et al. 2000, Boublik et al. 2002). In the case of *N. brasiliensis* AChE, there is a methionine at position 301(288). The replacement of this amino acid by an alanine (mutant M301A) caused a 108 times increase of the k_i value of dimethoate and a 10 times increase of the same value of fenitrothion, when compared with the wild strain. Previous studies in our institute had already shown that high k_i values were obtained when organophosphate insecticides were put in contact with this mutant (Schulze et al. 2005).

Two mutants involving the position 346, related to the region of the acyl pocket, obtained excellent results. Zischka (2005) had previously demonstrated a high sensitivity of these two mutants towards organophosphates. The amino acid tryptophan in this position seems to limit the docking of butyrylcholine (Hussein et al. 2002). In the mutants, this

position was replaced by alanine and valine. Mutant W346V presented the largest increase of sensitivity between all insecticides evaluated, where the mutation caused the sensitivity toward methamidophos to increase 1,127 times. Besides, mutant W346A was highly inhibited in the presence of almost all insecticides tested, especially by organophosphorothionates such as chlorpyrifos and triazophos.

The comparison of *N. brasiliensis* AChE mutants with *D. melanogaster* AChE mutants also demonstrated that exact forecasts about the effect of mutations on the properties of AChEs are not possible. Nevertheless, certain similarities were observed with mutations in the choline binding site and the substrate access gorge. The acyl pocket was the region where the largest differences were found (Schulze et al. 2005).

5.2.3 Insecticides in foods and maximum residue limits accepted by the Brazilian legislation

The proposal of a rapid screening method for residue analysis of insecticides applied in Brazil is of a huge importance, selecting negative from positive samples, and accelerating the samples analysis process. The Brazilian legislation establishes different MRLs for each carbamate and organophosphate insecticides in foods (ANVISA 2010b). Table 22 illustrates the MRLs values of the insecticides studied in this work.

Table 22 – MRLs of the most applied insecticides in Brazil, determined by the Brazilian legislation (ANVISA 2010b)

Insecticide	***MRL (μg/kg)***
Acephate	200
Carbaryl	20
Carbofuran	50
Chlorpyrifos	10
Dimethoate	1,000
Ethione	1,000
Fenitrothion	50
Methamidophos	10
Methidathion	20
Parathion methyl	50
Profenofos	50
Triazophos	10

All insecticides selected were successfully detected by the Nb AChE multisensor, with two exceptions. The biosensor was unable to identify acephate in solution, due to an indirect inhibition mechanism of this insecticide over AChE (Rojakovick and March 1972, Suksayretrup and Plapp 1977). Furthermore, the biosensor could only detect twice as much methamidophos in foods (20 μg/kg) as of the concentration proposed by legislation (10 μg/kg).

The tests in foods showed good correlation between the standard analytical tests and the AChE biosensor tests. In the samples where the GC/LC-MS analysis detected one or more insecticides (like samples number 1 to 5), their presence was confirmed by the Nb AChE multisensor.

Among the identified insecticides are two organophosphorothionates (chlorpyrifos, chlorpyrifos-methyl). These substances need to be oxidized first, in order to increase the AChE inhibiting strength (Fukuto 1990). One way to transform phosphorothionates into their oxon form is using chemical oxidation. In the standard oxidation method for water, the DIN 38415-1 method, oxidations are performed by NBS, followed by a step where the excess of

reagent is destroyed by ascorbic acid. However, this method had problems when applied to food samples, due to an excess of oxidizable compounds like ascorbic acid or other antioxidants in certain foods (Schulze et al. 2004). This would justify the absence of strong inhibition rates, as originally expected. The pretreatment method using CPO was not applied in this work, because the two projects were developed simultaneously.

In other samples (samples 7 and 8) there was low AChE reactivation rate after the treatment with 2-PAM. For irreversible inhibitions, like the one generated by insecticides, the AChE can be reactivated with oxime type-reactivation agents such as 2-PAM (Tran-Minh et al. 1990). This suggests that the substance causing the inhibition is not an organophosphate or a carbamate insecticide.

It is important to point out that the AChE biosensor assay allows the identification of certain organophosphate and carbamate insecticides in detection levels much lower than the ones allowed by the standard analytical methods, like GC/MS or HPLC/MS. The detection limit of these equipments varies between 1-20 µg/kg (Schulze et al. 2002a).

5.2.4 Stability of acetylcholinesterase mutants from *N. brasiliensis* at room temperature

The shelf life analysis at room temperature revealed a strong stability of Nb AChE mutants applied on the biosensor. Their activity remained unchanged after 40 weeks. Schulze et al. (2005) observed this same behavior with Nb AChE WT after a period of 60 weeks, revealing a high stability. Nb AChE seems to have a higher stability than other types of AChE. One reason for this behaviour could be the presence of an additional cysteine in the

N. brasiliensis AChE B sequence. This protein could then form four intramolecular disulfide bonds instead of three like other AChEs (Hussein et al. 1999).

An electric eel AChE immobilized on chemically modified poly-(acrylonitrile-methyl-methacrylate-sodium vinylsulfonate) membranes showed 75% of its initial current response after storage for 20 days at 4° C, and 25% after 30 days (Marinov et al. 2010). No sensitivity studies were done with this biosensor. Ivanov et al. (2010) described a biosensor with the same enzyme and immobilization method that retained 61% of the initial current response after 120 days of storage. On the other hand, amperometric biosensors printed with the WT and genetically modified (B394) AChE from *D. melanogaster*, where the enzymes were immobilized on cobalt(II) phthalocyanine-modified electrodes by entrapment in a cross linkable polymer (PVA-AWP), exhibited a storage stability for 7 months. The storage temperature during the shelf life analysis was not mentioned in the article (Valdes-Ramirez et al. 2008).

The immobilization of enzymes on biosensors has as an objective a larger storage stability. In this work, it was possible to compare the evolution of the shelf life analysis of the AChE biosensor developed only with Nb AChE mutants with the results obtained by Schulze et al. (2005), using the Nb WT under the same conditions. Three (M301A, W346A and W346V) of four mutants showed a decrease of sensitivity over time. This result reveals a lower stability of Nb AChE mutants than WT, where the sensitivity of Nb WT AChE remained unchanged even after having been stored for 17 months at room temperature.

These findings may be explained by the fact that the changes made in enzyme structure induce a decrease in sensitivity. Bucur et al. (2006) reported that the storage stability of WT AChE was higher than the one shown by the AChE mutants; however they did not report sensitivity studies during the shelf life analysis. They observed that, under a

storage temperature of – 18° C, WT AChE based biosensors did not significantly lose the initial enzymatic activity during the first month and a decrease of only 17% was observed after the second month. Under the same conditions, the signal of the mutant-based electrodes decreased by 27% after 1 month and by 50% after 2 months.

5.3. Development of acetylcholinesterase biosensors for the detection of glycoalkaloids

5.3.1 Inhibition mechanism of α-solanine and α-chaconine over acetylcholinesterase from *N. brasiliensis*

We wanted to evaluate the type of inhibition mechanism of α-solanine and α-chaconine over Nb AChE. Glycoalkaloids behave differently toward cholinesterases, when compared with organophosphorus and carbamate insecticides. This may be due to differences in binding affinities to the stearic and/or serine active sites of the enzyme. Although the anticholinesterase activity of GAs was revealed more than 50 years ago (Pokrovskii 1956), the mechanisms of inhibition by these natural toxins are still not clear. Only few investigations were done on the kinetics of BuChE inhibition by α-solanine, α-chaconine and α-tomatine, but the inhibition mechanism of these substances over AChE is still unknown.

Our results demonstrated that Nb AChE was inhibited reversibly and competitively by α-solanine and α-chaconine. This was confirmed by studying the kinetic of the inhibition mechanism. Two plots were created with the results: the Dixon plot and the Cornish-Bowden plot. The reversible and competitive inhibition mechanism shown by Nb AChE

toward glycoalkaloids is in accordance with the literature, where the inhibition of glycoalkaloids over cholinesterases was classified as reversible (Nigg et al. 1996, Benilova et al. 2006). The kinetics of butyrylcholinesterase inhibition by α-solanine, α-chaconine and α-tomatine was described in the literature. Benilova et al. (2006) found out that α-solanine, α-chaconine and α-tomatine inhibited horse and human immobilized BuChE reversibly. They also acted in competitive and mixed modes towards horse and human BuChE, respectively.

The mechanism of inhibition by glycoalkaloids probably involves non-covalent competitive binding to the active site of the enzyme. Additional structure-inhibitory activity relationships showed that (a) the unshared electron pair on the ring nitrogen on the aglycone may be required for formation of bioactive iminium ions (Kim et al. 1996) and (b) the nitrogen-containing E/F ring of the aglycone is a more important determinant of anticholinesterase activity than is the carbohydrate side chain (Roddick et al. 2001).

The naturally occurring steroidal alkaloid solanidine is assumed to bind to the charged anionic site component within the normal human BuChE (Quinn 1987). It interacts selectively with mammalian BuChE (Harris and Whittaker 1962), and inhibits its catalytic activity, but not with AChE (Roddick 1989). On the other hand, the glycoalkaloids α-solanine and α-chaconine are inhibitors of both AChE and BuChE (Schwarz et al. 1995). Neville et al. (1992) observed that variants of Gly70 of human BuChE failed to interact with solanidine, although they bound to some extent with α-solanine and effectively to the rhamnose enriched α-chaconine. Since no major change in charge is involved, these graded inhibition patterns suggest that Asp 70 asserts a primary binding capability, although the hydrophilic and structural elements of the sugar moieties stabilize this binding. Moreover, it is likely that the presence of sugar residues repositions the electrostatic components that are required for the active site binding of these alkaloids. These observations indicate that structural

interactions exist between N´ and C´ terminal domains in ChEs, which contribute to substrate and inhibitor binding, and suggest a crucial involvement of both electrostatic and hydrophobic domains in the build-up of the ChE active center.

The inhibition kinetic studies showed a difference between the I_{50} values and the K_{iapp} values. The K_{iapp} values were lower than the I_{50} ones, for both glycoalkaloids. For the characterization of the inhibitor efficiency, it is normal to determine the inhibition coefficient, or I_{50}. This parameter is borrowed from routine usage in pharmacology and revels the effect concentration at which the enzymatic reaction is inhibited (Kosterin et al. 2005). However, I_{50} is insufficient for adequate evaluation of both inhibition type and affinity of reversible inhibitor toward the target enzyme, because I_{50} depends on the inhibition mechanism. Consequently, I_{50} cannot be accepted as a complete characteristic of affinity in the case of competitive inhibition, because of its strong dependence on the initial substrate concentration (S_o) and the equilibrium constant of the dissociation of the enzyme-substrate complex (K_s).

The inhibition of both glycoalkaloids over Nb AChE is reversible and competitive. In competitive inhibitions the substrate concentration is a critical point to be observed; consequently the presence of substrate in higher concentrations would mask the inhibitory action of the glycoalkaloids. Therefore, K_i serves as a measure of inhibitor effectiveness, meaning the affinity toward the enzyme, and K_i=I_{50} only in the case of full non-competitive inhibition.

5.3.2 Ratio effect of α-chaconine: α-solanine

The two glycoalkaloid compounds α-chaconine and α-solanine account for 95% of the GA present in *Solanum tuberosum*, the potato (Smith et al. 1996). Depending on the variety, potatoes may contain α-chaconine and α-solanine at concentration ratios of α-chaconine:α-solanine ranging from 1.2:1 to 2.4:1 (Friedman et al. 2003).

Experiments were conducted in order to verify if the combination of α-chaconine and α-solanine showed a synergic, additive or antagonistic effect over the inhibition kinetics. The ratio of α-chaconine:α-solanine used was of 2:1. The results revealed that the recovery rate of the inhibition over the mutants was around 70%; consequently this ratio of the mixture of both glycoalkaloids had an antagonistic effect.

Roddick (1989) described that both glycoalkaloids "pair" appear equally inhibitory against AChE and do not synergize. On the contrary, there is evidence too that such "paired" glycoalkaloids may interact synergistically (Roddick et al. 1988, Fewell and Roddick 1993). Friedman (2006) affirmed that mixtures of α-chaconine and α-solanine were found to be slightly antagonistic. Another study revealed that in assays which combined α-chaconine, α-solanine and solanidine, inhibition of BuChE was less than additive (Nigg et al. 1996).

Interestingly, it is demonstrated that this magnitude varies with the α-chaconine: α-solanine ratio (Fewell and Roddick 1993, Roddick et al. 1988). Whether the synergy between these GAs is of relevance to toxicity in animals, including humans, is not yet known. However, this is highly likely, given that this synergy has been observed in the case of glycoalkaloids-mediated damage of blood cells (Roddick et al. 1988).

Moreover, it may not always be possible to predict effect of mixtures of glycoalkaloids, possibly because α-chaconine and α-solanine may compete for receptor sites

on enzyme surfaces. Such competition could result in either antagonistic, additive, or synergistic effects (Friedman et al. 2005).

Because these two glycoalkaloids, which share the common aglycone solanidine but not the same trisaccharide side chain, appear to be synthesized via distinctly different biosynthetic channels, it is possible that the rates of biosynthesis of the two glycoalkaloids in the different channels are cultivar dependent (Choi et al. 1994). These considerations imply the mutual dependence exists when alterations in the genes responsible for encoding enzymes involved in the biosynthesis of α-chaconine and/or α-solanine are made (Friedman 2006).

5.3.3 Comparison of the toxicity between α-chaconine and α-solanine

The K_{iapp} values, considered as a characteristic of inhibition, of Nb AChE mutants immobilized on the amperometric biosensor confirmed that α-chaconine had a higher inhibition activity over Nb AChE mutants than α-solanine. Consequently, α-chaconine exerts a higher toxic effect over this enzyme than α-solanine. This same conclusion was proposed by Fewell and Roddick (1997), and by Rayburn et al. (1994). On the other hand, Bushway et al. (1987) affirmed that α-chaconine and α-solanine were equally potent inhibitors of AChE. Roddick (1989) found similar results, where α-chaconine and α-solanine were about equal in potency with regard to *in vitro* inhibition of bovine and human AChE.

During the evolutionary process, it is likely that nature initially created only one glycoalkaloid, probably α-solanine. As phytopathogens became adapted over time to resist to the effects of α-solanine, the plant created a second, biologically more potent glycoalkaloid, by modifying the trisaccharide side chain. This GA was probably α-chaconine.

Another possibility is that both glycoalkaloids were created concurrently to exert the observed synergistic effects. Other possibilities are that one compound might be more effective against one set of pests and the other for a different set or that the availability of the different sugars required for the synthesis of the side chains dictates the formation of two glycoalkaloids (Friedman 2006).

It is important to point out that the structure of the steroid appears to be more important than that of the sugar side chain in determining AChE inhibition. However, the presence of a sugar side chain is required for the AChE inhibition to occur (Friedman 2006).

5.3.4 Comparison of sensitivity between butyrylcholinesterase biosensors and acetylcholinesterase biosensors from *N. brasiliensis*

The biosensor using Nb AChE mutants could successfully detect glycoalkaloids in low concentrations, revealing a high sensitivity. The detection limit of α-chaconine was of 0.1 µM, and the detection limit of α-solanine of 0.5 µM. Table 23 compares these results with studies made with biosensors using AChE and BuChE.

Table 23 – Comparison of limits of detection of AChE and BuChE biosensors for glycoalkaloids detection

Reference	Enzyme	Transducer	LOD
This work	Nb AChE	Amperometric	0.1 μM α-chaconine 0.5 μM α-solanine
Arkhypova et al. (2003)	Electric eel AChE	Potentiometric	> 150 μM α-chaconine
Arkhypova et al. (2008)	Electric eel AChE	Potentiometric	80 μM α-chaconine
Arkhypova et al. (2008)	Bovine erythrocytes AChE	Potentiometric	2 μM α-chaconine
Arkhypova et al. (2008)	Horse serum BuChE	Potentiometric	1.0 μM α-chaconine
Korpan et al. (2002)	Horse serum BuChE	Potentiometric	0.5 μM α-chaconine 2.0 μM α-solanine
Arkhypova et al. (2004)	Horse serum BuChE	Potentiometric	1.0 μM α-chaconine 1.0 μM α-solanine
Arkhypova et al. (2003)	Horse serum BuChE	Potentiometric	0.2 μM α-chaconine 0.5 μM α-solanine
Arkhypova et al. (2008)	Human serum BuChE	Potentiometric	< 0.1 μM α-chaconine

Obs: LOD - limit of detection

The Nb AChE amperometric biosensor developed for the detection of the total concentration of glycoalkaloids was the first described in the literature with this purpose. The biosensors mentioned in the literature until now are potentiometric, using pH-sensitive field-effect transistors. AChE was described as having a lower sensitivity to glycoalkaloids than BuChE (Arkhypova et al. 2003, Arkhypova et al. 2008). However, the LOD of Nb AChE mutants were similar (Arkhypova et al. 2008) or even lower (Korpan et al. 2002, Arkhypova et al. 2003) than the ones registered for BuChE. Only the human serum BuChE showed a higher sensitivity (Arkhypova et al. 2008). Consequently, Nb AChE confirmed promising use in the future in biosensors for the detection of glycoalkaloids. It is important to point out that all results listed in Table 23 were obtained from immobilized cholinesterases in biosensors inhibited by glycoalkaloids in buffer solution.

5.3.5 Acetylcholinesterase biosensor test in potato samples

The total glycoalkaloids content in potato juice was determined using a calibration curve. The linear part of the calibration curve previously obtained was used for determination of glycoalkaloids in juice. The safety level of glycoalkaloids established by the legislation is 250 µM. This level is determined in order to avoid acute intoxications in humans (Valkonen et al. 1996). The developed Nb AChE biosensor successfully detected α-chaconine in concentrations below 100 µM in contaminated samples. Besides, the mixture of α-chaconine/α-solanine 200/100 µM caused a significant inhibition over the Nb AChE mutants. In this case, the total concentration of glycoalkaloids was 300 µM, slightly higher than the limit established by legislation. However, the detection limits of both substances (0.1 µM for α-chaconine and 0.5 µM for α-solanine) would enable the detection of lower quantities of these GAs in food samples.

Glycoalkaloid-contaminated potatoes and potato products are widely consumed. The concentration of GAs in commercial potato chips is known to vary between 0.1 and 0.7 g/kg of chips (Sizer et al. 1980). For instance, the daily per capita intake of glycoalkaloids from potatoes in the United Kingdom is estimated to be around 14 mg (Hopkins 1995). Although the levels of GA in commercial tubers are normally less than the widely accepted "safety limit" of 200 mg/kg fresh weight, the concentration can increase substantially on exposure of potatoes to light and as a result of mechanical injury. Surveys showed values in excess of this limit in 2 - 9% of samples (Van Gelder 1990, Hellenäs 1986). Friedman (2006) reported that a commercial potato protein concentrate contains significant amounts of glycoalkaloids (around 200 mg/100 g). If potato isolates are to assume greater role in animal and human nutrition, a need exists to reduce their glycoalkaloids content.

Despite of that, peeling usually removes most of the GA in the tuber, as shown in Table 24. The majority of glycoalkaloids in the potato tuber is located within the first 1 mm from the outside surface and decrease toward the center of the tuber (Friedman et al. 2003). The most reliable methods for protecting consumers from GAs poisoning are to improve handling practices and to reduce the rate of alkaloid synthesis.

Table 24 – Levels of glycoalkaloids in various commercial potato products and preparations

Product or preparation	***GA concentration (mg/kg)***	***Reference***
Boiled peeled potato	27-42	Mondy and Gosselin (1988)
Baked jacket potato	99-113	Bushway and Ponnampalam (1981)
Fried skins	567-1450	Bushway and Ponnampalam (1981)
Crisps (with skin)	95-720	Sizer et al. (1980)
Crisps (UK: potato chips)	32-184	Davies and Blincow (1984)

As a result, the development of tools that are cheap, fast and highly sensitive has a remarkable importance for the detection of GAs in food samples. Arkhypova et al. (2008) studied the practical application of biosensors based on pH-sensitive field-effect transistors and BuChE to glycoalkaloids analysis in potato tubers. The biosensor was applied to quantitative analysis of glycoalkaloids in tubers of different potato varieties. The results showed that the total glycoalkaloids content in peelings (234 mg/kg wet weight) and in the tuber parenchyma (105 mg/kg) of Agata potatoes differed considerably. Glycoalkaloid concentration varied also depending on growth locality. Soldatkin et al. (2005) found similar results using the same type of biosensors and enzyme. Dzyadevych et al. (2004b) used the biosensor based on pH-sensitive field-effect transistors and BuChE to detect α-tomatine, another glycoalkaloid, in tomato samples. The inhibition results obtained in the biosensor tests were compared to HPLC analysis, and a good correlation with the known real content was revealed.

5.4. Outlook

This work showed that the detection analysis using multi-enzymatic biosensors based on Nb AChE is viable and successful. The developed biosensors were able to operate in natural samples with little or no treatment, as well as to function for on-line performance, in order to facilitate the quality control of products during processing, which is of great importance in the food industry.

A method associating a pretreatment with the enzyme chloroperoxidase and a detection analysis using AChE biosensors allowed the oxidation of organophosphorothionate insecticides in food samples. Further work could be done to immobilize chloroperoxidase and Nb AChE together on the biosensor, in order to execute the conversion of organophosphorothionates into their oxon form during the inhibition test.

The Nb AChE biosensors tailored for the detection of the most used insecticides in Brazil were able to qualitatively identify these substances in food samples. The next step would be to analyze real samples from the PARA Program using this technology, and compare the results with the conventional methods. Further studies of the shelf life of the developed biosensor are required at a storage temperature of 4° C. One strategy to enhance the stability of the Nb AChE mutants on the biosensor would be the use of nanoporous materials, like activated carbon, fullers, and carbon nanotubes, avoiding the decrease of sensitivity over the time.

Acetylcholinesterase from *N. brasiliensis* was successfully applied on biosensors to detect glycoalkaloids in foods. Future studies could compare the results obtained from samples analyzed using conventional methods and the results using the biosensor designed

especially for glycoalkaloids in potatoes. The same biosensor could also be tested to detect the glycoalkaloid α-tomatine in tomato samples.

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6. PhD production

List of publications

- Roepcke, C. B. S., Muench, S. B., Schulze, H., Bachmann, T. T., Schmid, R. D., Hauer, B. Development of an acetylcholinesterase-based biosensor: detection of glycoalkaloids. Sensors and Actuators B: Chemical. *Article submitted.*
- Roepcke, C. B. S., Muench, S. B., Schulze, H., Bachmann, T. T., Schmid, R. D., Hauer, B., 2011. Tailoring biosensors for Brazil: detection of insecticides in foods. *Food Control* 22, 1061 – 1071.
- Roepcke, C. B. S., Muench, S. B., Schulze, H., Bachmann, T. T., Schmid, R. D., Hauer, B., 2010. Analysis of Phosphorothionate Pesticides Using a Chloroperoxidase Pretreatment and Acetylcholinesterase Biosensor Detection. *J. Agric. Food Chem.* 58(15), 8748-8756.

Oral presentation

- Tailoring biosensors for Brazil: detection of pesticides in foods. In: *4th International Congress on Bioprocess in Food Industries*, 08.10.2010, Curitiba, Brazil.

Poster presentations

- Tailoring biosensors for Brazil: detection of pesticides in foods. In: *4th International Congress on Bioprocess in Food Industries*, 2010, Curitiba, Brazil.
- Development of an acetylcholinesterase biosensor for the detection of glycoalkaloids. In: *4th International Congress on Bioprocess in Food Industries*, 2010, Curitiba, Brazil.
- Detection of phosphorothionate pesticides in food: chloroperoxidase and acetylcholinesterase biosensors. In: *20th World Congress on Biosensors*, 2010, Glasgow, Scotland.
- Development of an amperometric acetylcholinesterase B biosensor tailor made for Brazil: detection of relevant insecticides. In: *Effost Conference - New challenges in food preservation*, 2009, Budapest, Hungarn.
- Screening of Acetylcholineterases B of *N. brasiliensis*: inhibition toward relevant insecticides applied in Brazil. In: *Kongress Lebensmitteltechnologie (GDL)*, 2009, Lemgo, Germany.

7. Curriculum Vitae

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Name: Clarisse Brüning Schmitt Roepcke
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City of birth: Blumenau
Nationality: Brazilian

EDUCATION

Nov 07 – May 11 **University of Stuttgart** Stuttgart, Germany

PhD in Chemistry (Focus Area: Analytical Biotechnology)

Mar 05 – Jul 07 **Federal University of Paraná** Curitiba, Brazil

Master of Science in **Biotechnology** (Focus Area: Fermetation)

Aug 00 – Dec 01 **Federal University of Santa Catarina** Florianópolis, Brazil

Bachelor of Science with Honors in **Food Technology**. GPA: 9.20 /10.0

Mar 97 – Jul 00 **Federal University of Santa Catarina** Florianópolis, Brazil

Bachelor of Science with Honors in **Pharmacy**. GPA: 9.04 /10.0

Mar 86 – Dec 96 **Sagrada Família School** Blumenau, Brazil

Primary and secondary school.

WORK EXPERIENCE

Oct 11 – Today **Prodiet Nutrição Clínica** Curitiba, Brazil

R&D Coordinator – R&D Department

Nov 07 – Jun 11 **University of Stuttgart** Stuttgart, Germany

DAAD PhD Scholarship Holder – Institute of Technical Biochemistry

“Development of Acetylcholinesterase Biosensors: neurotoxic substances detection in foods and the environment”.

Jul 06 – Jun 07 **SENAI´s Technology Prospection and Dissemination Observatory** Curitiba, Brazil

Senior Researcher – Technology Prospection Department

Mar 05 – Jun 06 **Federal University of Paraná** Curitiba, Brazil

CNPq Master Scholarship Holder – Biotechnology Process Department

“Development of a bioprocess for the production of yeast biomass enriched with organic zinc”.

ROEPCKE, C. B. S.; VANDENBERGHE, L. P.; SOCCOL, C. R. Optimized Production of *Pichia guilliermondii* Biomass with Zinc Accumulation by Submerged Fermentation. *Anim. Feed Sci. Tech*. 163(1), 33-42.

Aug 01 – Mar 05 **Bunge** Gaspar, Brazil

Junior Researcher – Food R&D Department

Jul 99 – Jul 01 **Federal University of Santa Catarina** Florianópolis, Brazil

Intern – Natural Products Chemistry Laboratory

MALHEIROS, A.; CECHINEL FILHO, V.; SCHMITT, C. B.; YUNES, R. A.; ESCALANTE, A.; SVETAZ, L.; ZACCHINO, S.; MONACHE, F. D., 2005. Antifungal activity of drimane sesquiterpenes from *Drimys brasiliensis* using bioassay-guided fractionation. *J. Pharm. Pharm. Sci.* 8(2): 335-339.

MALHEIROS, A.; CECHINEL FILHO, V.; SCHMITT, C. B.; SANTOS, A. R. S.; CALIXTO, J. B.; DELLE MONACHE, F.; YUNES, R. A., 2001. A new sesquiterpene drimane with antinociceptive activity from *Drimys winteri* barks. *Phytochemistry* 57: 103-107.

8. Statement

I herewith declare that I have completed this work by my own means and with the aid of the listed resources and literature.

Stuttgart, May 20th 2011.