Determination of Organophosphorus and Carbamate Insecticides in Food Samples by High-Performance Thin-Layer Chromatography Multi-Enzyme Inhibition Assay

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My parents, my wife Manal,
and my children Mohammed Nizar and Noorullah

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Parts of this work have already been published in international peer-reviewed journals or were presented at international scientific conferences as oral or poster presentations.

FULL PAPERS

1. Rami Akkad and Wolfgang Schwack. Multi-Enzyme Inhibition Assay for Detection of Insecticidal Organophosphates and Carbamates by High-Performance Thin-Layer Chromatography. 1. Basics of Method Development. Journal of Planar Chromatography 21 (**2008**) 6, 411-415.
2. Rami Akkad and Wolfgang Schwack. Multi-Enzyme Inhibition Assay for the Detection of Insecticidal Organophosphates and Carbamates by High-Performance Thin-Layer Chromatography Applied to Determine Enzyme Inhibition Factors and Residues in Juice and Water Samples. Journal of Chromatography B 878 (**2010**) 1337-1345. This paper is part of the special issue “Bioanalysis of Organophosphorus Toxicants and Corresponding Antidotes”.
3. Rami Akkad and Wolfgang Schwack. Effects of Bromine Oxidation on the Multi-Enzyme Inhibition Assay for the Detection of Insecticidal Organophosphates and Carbamates by High-Performance Thin-Layer Chromatography. Journal of Chromatography A 1218 (**2011**) 2775-2784. This paper is part of the special issue “Planar Chromatography”.

ORAL PRESENTATION

1. Wolfgang Schwack, Rami Akkad. A New Multi-Enzyme Inhibition Test for the Detection of Insecticidal Organophosphates and Carbamates by HPTLC. International Symposium for Thin-Layer Chromatography, Helsinki, Finland, 11^{th} – 13^{th} June **2008**.

POSTER PRESENTATIONS

1. Rami Akkad and Wolfgang Schwack. HPTLC-EDA zur Bestimmung von Rückständen insektizider Organophosphate und Carbamate in pflanzlichen Lebensmitteln. 39. Deutscher Lebensmittelchemikertag, Stuttgart-Hohenheim, Germany, 20th – 22th September **2010**.

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LIST OF ABBREVIATIONS

λ_{max}	Maximum wavelength
‰	Per thousand
AChE	Acetylcholine esterase
Ac	Activity
ADC2	Automatic development chamber 2
AMD	Automated multiple development chamber
amu	Atomic mass unit
ATS4	Automatic TLC sampler 4
BS2	*Bacillus subtilis* esterase
BSA	Bovine serum albumin
ChE	Cholinesterase
CUT	Cutinase from *Fusarium solani pisi*
Da	Dalton (atomic mass unit), (kDa kilo Dalton)
DDT	Dichlorodiphenyltrichloroethane
Dev.%	Relative deviation
DFG	Deutsche Forschungsgemeinschaft
E. coli	Escherichia coli
EDA	Effect directed analysis
EI	Enzyme-inhibitor complex or Enzyme-inhibition
ES	Enzyme-substrate complex
ESI	Electrospray interface
EU	European Union
F_{254}	Fluorescence indicator by 254 nm
f_i	Enzyme inhibition factor
GC	Gas chromatography
GCB	Graphitized carbon black
HPLC	High-performance liquid chromatography
HPTLC	High-performance thin-layer chromatography
I	Inhibitor
k_i	Enzyme inhibition constant (bimolecular rate constant)
LC	Liquid chromatography
LOD	Limits of detection
LOQ	Limits of quantification
M (mol L^{-1})	Molarity
MRL	Maximum residue limit
MRM	Multi-residue method
MS	Mass spectrometry
MSD	Mass selective detector
m/z	Mass-to-charge ratio
OPP	Organophosphate pesticides

P	Substrate hydrolytic product
P_0	Inhibitor (pesticide) concentration
Pa	Pascal
ppm	Part per million
ppb	Part per billion
PSA	Primary secondary amine
psi	Pounds per square inch (a pressure unit)
QuEChERS	Quick, easy, cheap, effective, rugged, and safe
R^2	Determination coefficient
Rf	Retention factor
RLE	Rabbit liver esterase
RSD%	Relative standard deviation
S	Substrate
sdv	Standard deviation
SPE	Solid phase extraction
TLC	Thin-layer chromatography
TRIS	Tris-(hydroxymethyl)aminomethane
U	Unit; 1 U: Amount of enzyme required to hydrolyze 1 µmol of the substrate per minute under assay conditions
UV	Ultraviolet
v/v	Volume/volume
W/m^2	Watt per square metre
x g	Times gravity (Centrifugation speed unit)

CHAPTER I

GENERAL INTRODUCTION

1 Importance of pesticide trace analysis

Pesticides are modern-day miracles. They help food growing by elimination of pests, but unfortunately can also affect on both environment and human life. Their fate in the environment can be affected by many processes e.g. adsorption to soil particles, volatility, spray drift, runoff in water over a sloping surface, leaching in water through the soil, absorption by plants or microorganisms, and crop removal through harvest or grazing and as a result not all of the applied pesticides can reach the target (crops and soil) [1]. Humans can be exposed to pesticides either directly during or after they have been sprayed, or indirectly by eating or drinking contaminated foods or water leading to different health risks and diseases [2-12] (**Figure 1**). Since pesticides suffer from environmental reactions e.g. microbial breakdown, chemical reactions in soil, air, or water, and photo-degradation by sunlight [1], they can be found then in food in concentration down to trace levels (ppm or even ppb). Presently, the analysis of residues left on crops after pesticide application in food samples is considered the most promising application required to guaranty safe food consumption all over the world [2].

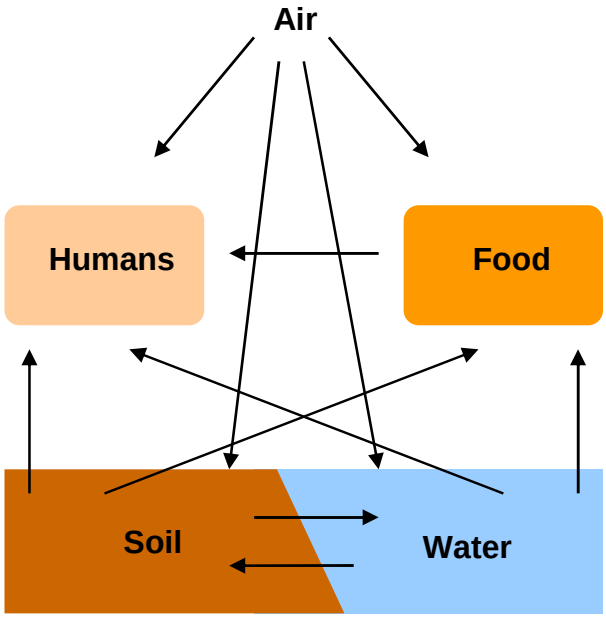


Figure 1 Routes of indirect exposure of humans to pesticides, modified from [2].

2 What is a pesticide?

A pesticide is any substance or mixture of substances intended for preventing, destroying, repelling, mitigating any pest. It applies to herbicides, fungicides, insecticides, and various other substances used to control pests. Pesticides can be classified according to their chemical family, and the most important families are organophosphate and carbamate pesticides [13]. Initially, inorganic compounds such as sulphur, mercury, and lead were used to control pests in agriculture. A great revolution was caused after discovering the insecticidal activity of dichlorodiphenyltrichloroethane (DDT) by Paul Müller in 1939 because of its great effect on pests causing different diseases like malaria and bubonic plague at that time. Peoples were shocked after publication of the book “silent spring” by Rachel Carson in 1962 which led them to know the toxic effects of DDT on birds. Due to the persistence and toxicity of organochlorine compounds, most of them have been banned and replaced with other pesticides e.g. organophosphates because of their low persistence and high effectiveness and therefore they are widely used as systemic insecticides for plants, animals, and soil treatments [2]. However, after all of these facts and progress achieved in the biological control and in the development of resistance of plants to pests, pesticides are still in use and their main role for protecting the world population from diseases could not be ignored [2].

According to the annual reports published by IVA (agricultural industry association, Germany) in the period between 2004 and 2009 [14-18], the European Union occupied 25% of the total world pesticides market in 2007 which is the same percentage as in 2004 even though the EU members were 25 in 2004 and increased to be 27 in 2007. Africa owns also same percentage of 4% without change over the years. Percentages of both Latin America and United States (inclusive Canada and Mexico) decreased 1% down to 19% and 22%, respectively, whereas it was 2% in Asia, and this may be due to the difference of currency exchange. On the other hand, there was an increase of 4% in East Europe (7% of total world market in 2007 over 3% in 2004). The net domestic sales were 1.377 billion Euros in 2008 with an increase of 28.9% over 2004 distributed between herbicides (decreasing), fungicides (increasing), insecticides, and other pesticides (**Figure 2**).

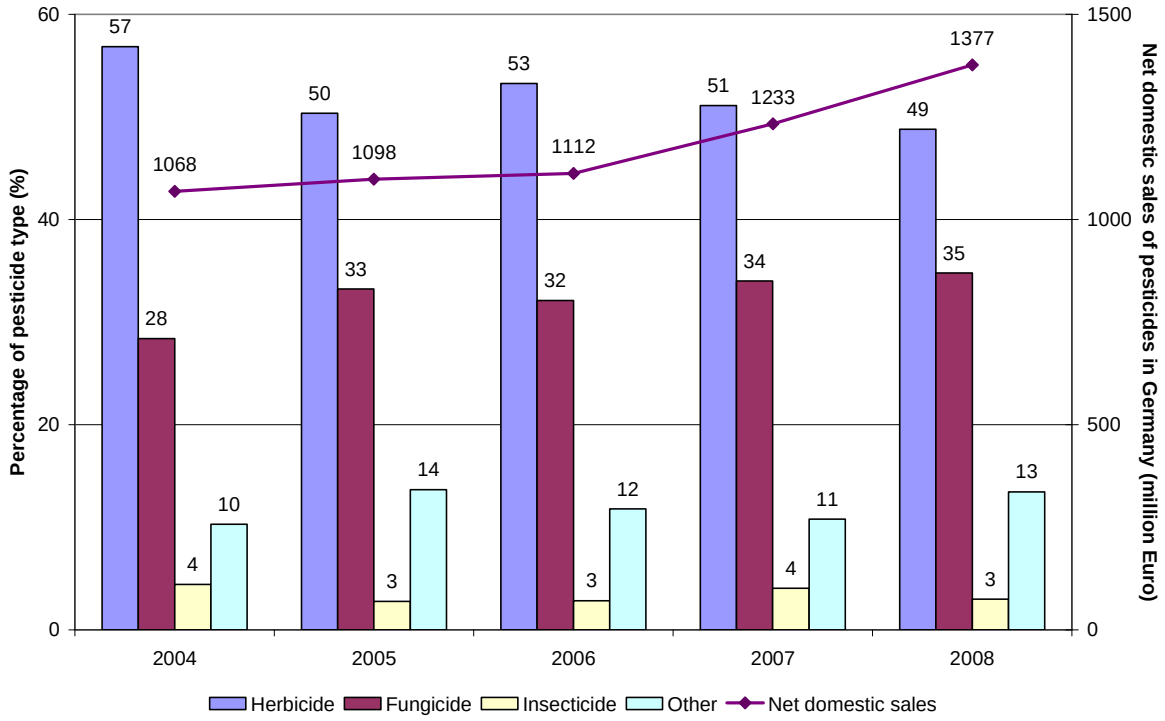


Figure 2 Percentage distributions of herbicides, fungicides, insecticides, and other pesticides sold in Germany in the period 2004-2008 and the net domestic sales of whole pesticides in Germany at same period (cf. [14-18]).

2.1 Maximum Residue Limit (MRL)?

Governments set limits on allowable levels of pesticide residues in food and animal feed as so called maximum residue limit (MRL) which is defined as the highest level of a pesticide residue that is legally tolerated in food or feed [19] and can be ingested daily during the whole life without showing an appreciable adverse effect [2]. The European Commission fixes MRLs and they are available online for all crops and all pesticides [20]. In the EU, pesticides can not be used unless it proves that they have no harmful effects on consumers, farmers, or environment in addition to having a sufficient effectiveness. Because of the excessive usage of pesticides all over the world, there was an increasing in percentage of samples with detected pesticides. Over the years, the percentage of samples with residues at or below the MRLs increased from 34.8% in 2000 to 43.8% in 2006 in the European Union (inclusive Norway, Iceland, and Liechtenstein) for fruit, vegetables and cereals [21-24] (**Figure 3**).

Since rotations of different pesticides on a crop are recommended to reduce the build-up of resistance by pests, mixtures of pesticides are often used for more effective control of pests and therefore multiple residues may be found on a commodity [25]. Some pesticides can be metabolized into other forms leading to increase the possibility of the multiple residues presence in food samples. Mixing of different crops of different sources which have been subject to different pesticide treatment regimes, can also lead to the detection of multiple residues in food samples [26]. The percentage of samples of fruit, vegetables, and cereals has increased from 14.7% in 2000 to 27.7% in 2006 with 29 pesticides as the highest recorded number in one sample in 2006 in comparison to only 10 pesticides in 2000 [21-24] (**Figure 3**).

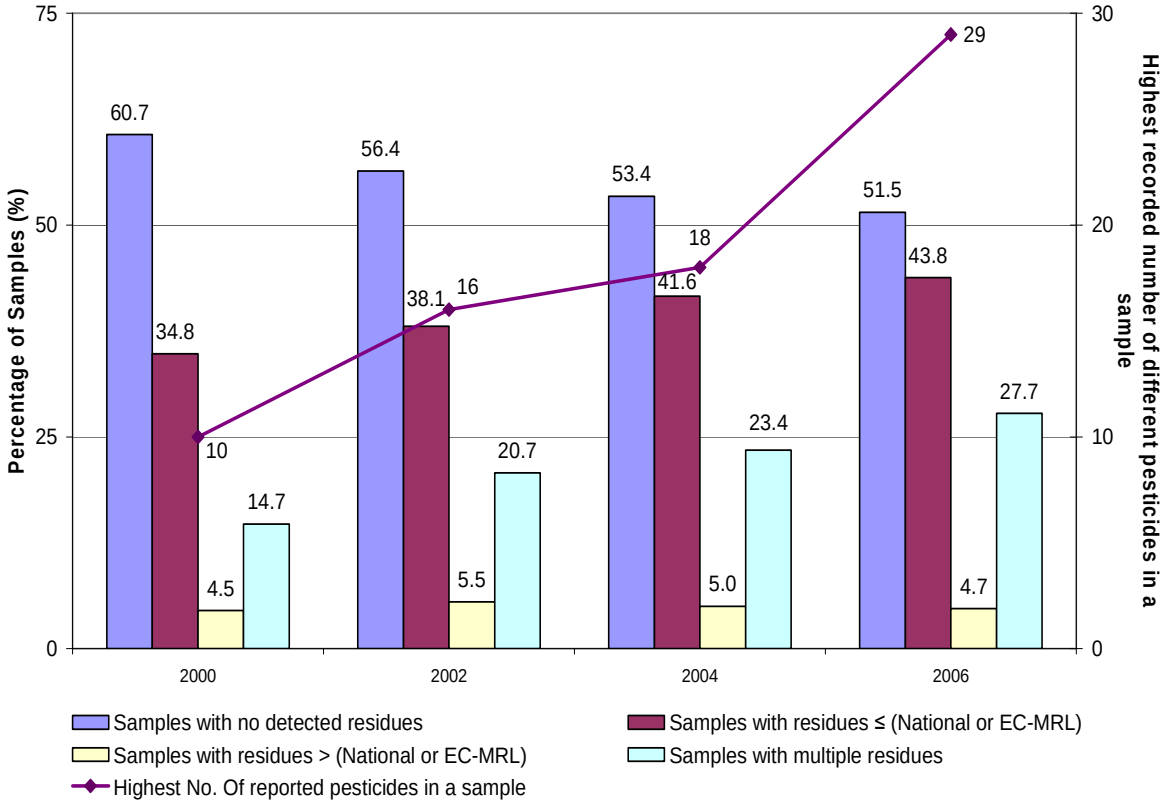


Figure 3 National monitoring results 2000-2006 for fruit, vegetables and cereals: percentage of samples with no residues detected, with residues below and above (national or EC-MRL), and with multiple residues and the highest number of reported pesticides in a sample in the period of 2000-2006 period (cf. [21-24]).

Analytical methods with improved sensitivity, which allow the detection of lower residue concentrations leading also to increase the number of pesticides detected in

single samples, may be one of the reasons for the increased detection of multiple residues. However, it is not possible to clarify whether the multiple residues are from application of different pesticides on the crop or from other sources e.g. mixing of crops from different sources [26].

Since a greater variety of pesticides are used in growing fruits and vegetables than for any other food items, it is logical to find that the percentage of samples with detected pesticides either below or above national or EU-MRL is greater in fruit and vegetables than in cereals and other food stuffs (**Figure 4**).

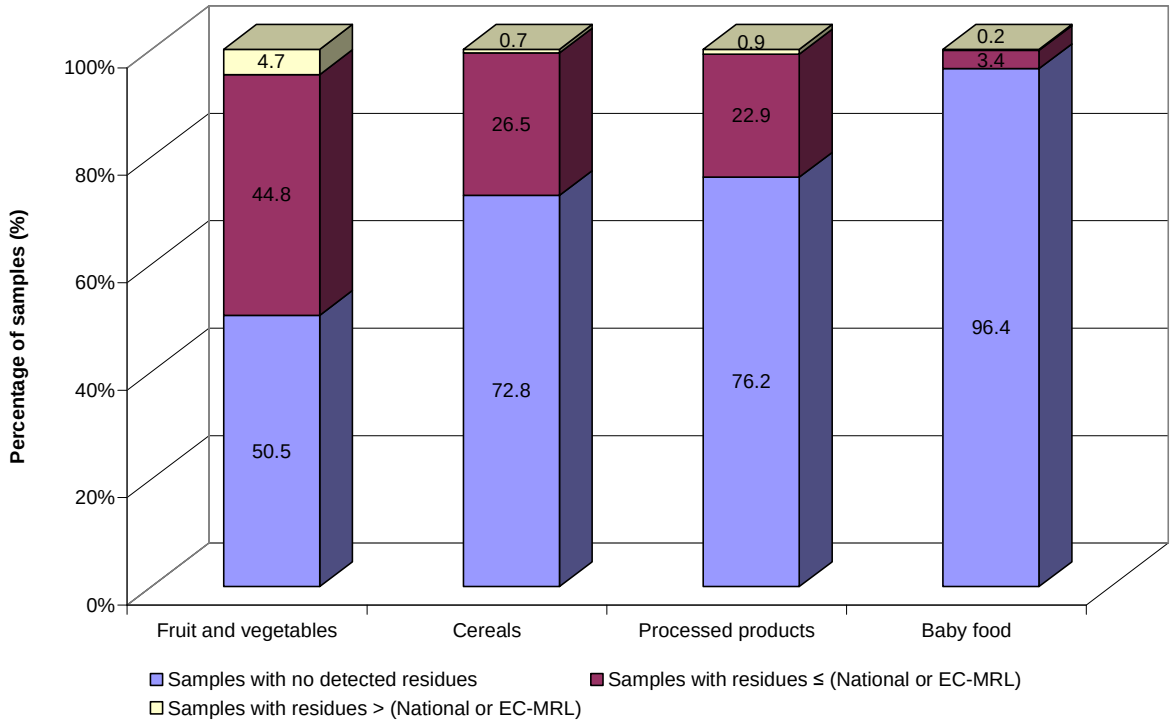


Figure 4 Monitoring results for fruit/vegetables, cereals, processed products (excl. baby food) and baby food, surveillance samples only recorded in the EU in 2006 (cf. [24]).

In the list of the top ten pesticides which are found most often in fruits, vegetables and cereals according to the national monitoring programmes recorded in the European Union, Norway, Iceland, and Liechtenstein from 2002 to 2006, the most frequently found pesticides in fruits and vegetables were mainly fungicides, whereas just chlorpyrifos and chlorpyrifos methyl were recorded in this list. On cereals, the pesticides found were mainly insecticides. However, this is in line with the finding of previous years 2002 and 2004 [22-24] (**Table 1**).

Table 1 Organophosphorus pesticides found most often according to the national monitoring programmes in the EU, Norway, Iceland, and Liechtenstein for fruits, vegetables and cereals. (cf. [22-24]).

Organophosphorus pesticide	2002	2004	2006
Fruits and vegetables	Chlorpyrifos	Chlorpyrifos	Chlorpyrifos
			Chlorpyrifos methyl
Cereals	Fenitrothion	Chlorpyrifos	Chlorpyrifos
	Chlorpyrifos methyl	Chlorpyrifos methyl	Chlorpyrifos methyl
	Dichlorvos	Dichlorvos	Dichlorvos
	Malathion	Malathion	Malathion
	Pirimifos methyl	Pirimifos methyl	Pirimifos methyl

2.2 Organophosphorus pesticides

Since organophosphorus pesticides (OPP) are the most commonly used insecticides and still form the largest group of the world wide sales, it is worthwhile to highlight their success although other newer and more specific insecticides were developed, and this may be due to their mode of action, physical properties and metabolism [27-28]. This group of pesticides tend to degrade rapidly on exposure to sunlight, air and soil, and some of them have high volatility which limits their persistence after foliar application. They are accordingly used at relatively high application rates (0.25-2 kg/ha) in most crop protection outlets [13, 28].

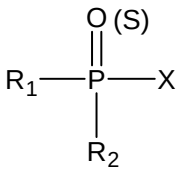


Figure 5 Schematic diagram of the general structure of organophosphate pesticides.

Organophosphate insecticides have a general structure (**Figure 5**), where R_1 = R_2 = methoxy or ethoxy and X is the leaving group after hydrolyses or reaction with cholinesterase (ChE). Forty seven compounds of them are thiono type (P=S) which is more stable than the analogue oxon (P=O), has higher volatilities, better penetrability of insect cuticle, but the most important have lower mammalian toxicities and are very poorer inhibitors of acetylcholinesterase [28]. This is because of the lower

electronegativity of sulphur compared to oxygen. Polarization of the P=O linkage results in a higher positive partial charge of the phosphorus atom (**Figure 6**), which facilitates attack on phosphorus by nucleophilic agents e.g. the serine hydroxyl of ChE.

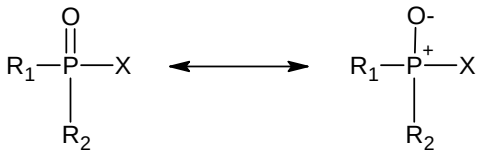


Figure 6 Polarization of the P=O linkage in organophosphate insecticides, modified from [16].

These compounds can be activated into more toxic oxon analogues either by oxidation in the environment or by thiono-thiolo rearrangement reactions [27-29] (**Figure 7**).

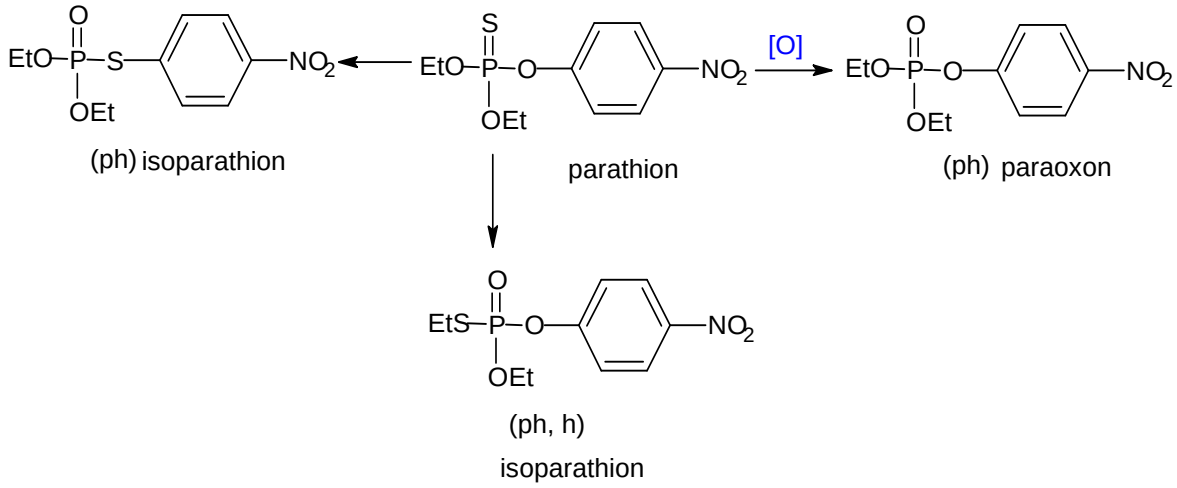


Figure 7 Rearrangement and oxidative desulfuration of parathion [28].

Since insects have highly developed nervous systems and many of their sensory receptors are exposed to the atmosphere outside the insect body, the great majority of insecticides used today are classified under nerve poisons [30]. To understand the mode of organophosphorus pesticides action, it is important to explain the catalytic mechanism of acetylcholinesterase (AChE). This enzyme is able to hydrolyse acetylcholine (the neurotransmitter) into choline and acetic acid, but it can be phosphorylated by OPP. The acetylated enzyme is very rapidly hydrolysed releasing the enzyme again whereas the phosphorylated enzyme is hydrolysed at an extremely slow rate resulting in enzyme inhibition and blocking its functional role [28].

Insects with cholinergic nervous systems are very sensitive to this pesticide group. They show, after exposure to organophosphorus and carbamate insecticides or their active metabolites, overt signs of excitation, exhaustion and death at sufficient doses [30].

2.3 Carbamate pesticides

Carbamates are generally represented as methyl esters of carbamic acid (**Figure 8**), where X is an oxime or phenyl rest, and R is either a hydrogen or methyl group [27].

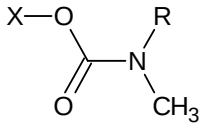


Figure 8 Schematic diagram of the general structure of carbamate pesticides.

They inhibit acetylcholinesterase forming a reversible complex which is reactivated again through decarbamoylation. The reactivation rate of the carbamoylated enzyme is very slow and measured in hours but still much faster than those of organophosphorus-inhibited enzymes which are measured in days, weeks or even longer [28] (**Figure 9**).

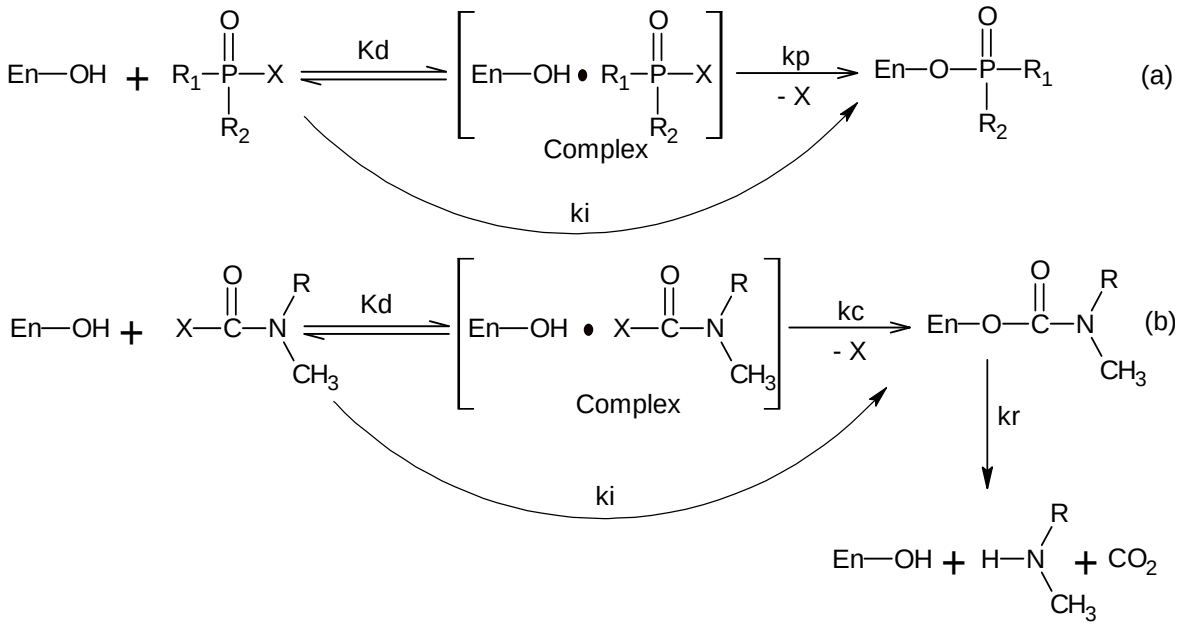


Figure 9 The proposed reaction of AChE with (a) organophosphorus and (b) carbamate pesticides, where X the leaving group, K_d dissociation constant, k_p phosphorylation constant, k_i bimolecular rate constant, k_c carbamylation constant, and k_r regeneration constant (modified from [29]).

3 Thin-Layer Chromatography Enzyme Inhibition Assay (TLC-EI)

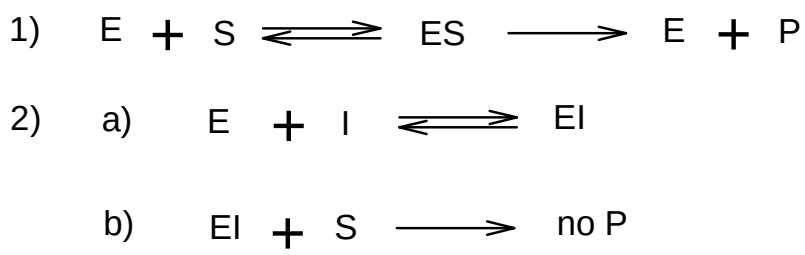
The traditional method for determining pesticide residues is by gas chromatography (GC) [31-33] or high-performance liquid chromatography (HPLC) [34]. High-performance thin-layer chromatography (HPTLC) is used routinely for metabolism, degradation, and other studies of pesticides in plants, animals, environment, and migrations through soils. It complements GC and HPLC but gained more popularity with time as an important analytical tool for analysis of pesticides because of its many advantages over column chromatography e. g. simplicity of development; high sample throughput with low operating cost because multiple samples can be run simultaneously with standards on a single plate using a very low volume of solvent [35-36]; high resolution through automated multiple development (AMD) [37] or two- or multi-dimensional development on a plate [38]; selective and sensitive postchromatographic detection and identification with a very wide variety of chromogenic and fluorogenic reagents coupled with spectrometric techniques [35, 36, 39-41]; and high resolution with accurate quantification especially with automated sample application, development, and densitometric scanning methods.

Since organophosphorus and carbamate insecticides are inhibitors for cholinesterases [27], acetylcholinesterase (AChE) was used in a most specific and effective detection method for determination of organophosphorus and carbamate insecticides [42-43]. Many studies were published concerning the effect of the structural hindrance and steric effects of pesticides on the activity of the cholinesterase from different sources [44-47].

Because of the high sensitivity of biological assay for organophosphate and carbamate pesticides using cholinesterase enzymes from different sources and the numerous advantages and benefits of thin-layer chromatography in separation pesticides, combining both methods represented a very sensitive, effective, and quick bio-analysis of the cholinesterase-inhibiting pesticides. Mendoza et al. described extensively the new analysis method as so called thin-layer

chromatography enzyme inhibition assay (TLC-EI). TLC-EI has been developed for studies of metabolic pathways of pesticides and residue analyses in the extracts of different fruit and vegetable samples with or without clean-up [48-55]. Depending on the survey described by Mendoza and under conditions differing in substrate, pH, temperature, method of oxidation, and even the enzyme source, numerous studies were published in the last few decades [56-60].

Mendoza [55] proposed the simplified mechanism of the enzyme-inhibitor reaction on thin-layer chromatography plates as follows



where E = Enzyme, S = Substrate, I = Inhibitor, and P = Substrate hydrolytic product.

When the enzyme is reacted with an inhibitor, its active sites are blocked and cannot catalyze the substrate hydrolysis. Therefore, no hydrolysis product will be obtained from reaction 2 and the area on the plate where the inhibitor is located will appear as a spot against a uniform background.

In all thin-layer chromatography enzyme inhibition methods, whatever the used substrates are, the quantification depends on the photometric scanning of the non-coloured spots in reverse scan mode, either by absorption in the reflectance mode or by detecting the differences of colour intensities by a videodensitometric scanner [59]. Because the spectrum of the enzymatic detected spot looks inverted after normal scan, the reverse scan mode gives the negative image of the of the previous spectrum (**Figure 10**).

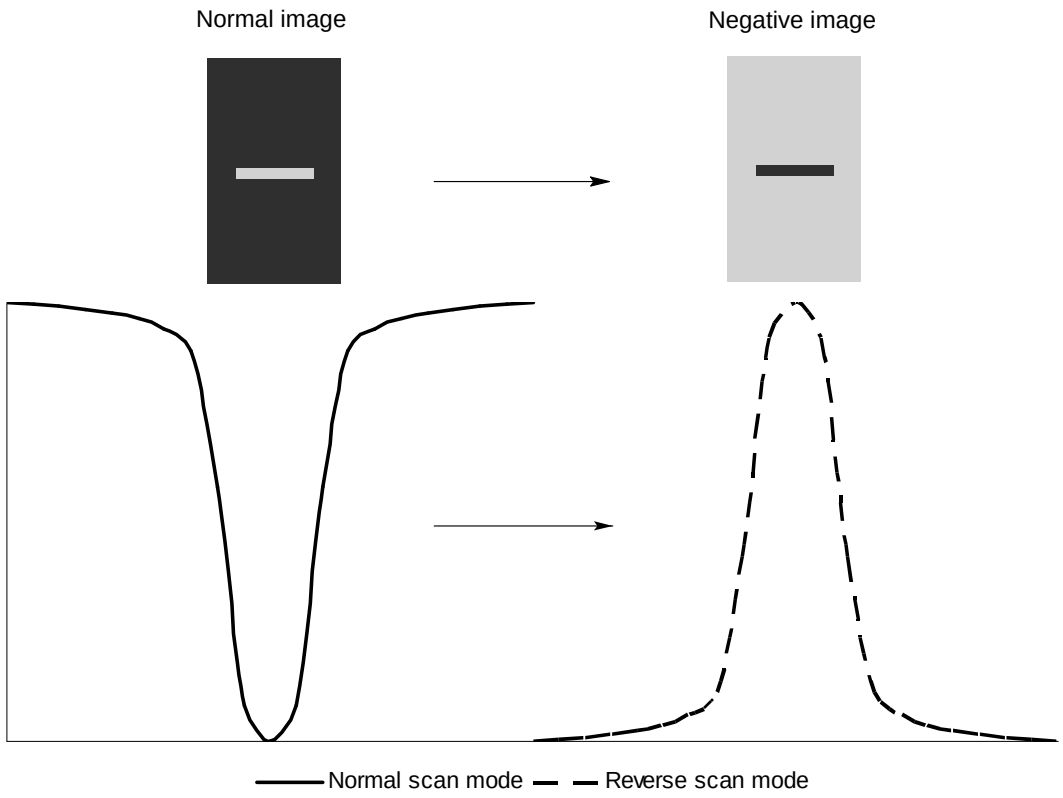


Figure 10 Simplified schematic diagram of the reverse scan mode.

By the enzymatic detection of inhibitors on thin-layer chromatography plates, the amount of the signal depends on some factors like the amount of the active enzyme in the dip solution which is affected by its storage time and conditions and on the sorbent (therefore in most cases it was added small quantities of BSA (bovine serum albumin)) and the reaction time on the plate [61].

4 Esterases

Esterases which metabolize organophosphorus insecticides can be divided into three groups: A-esterases are not inhibited by organophosphorus insecticides but hydrolyze them, e.g., phosphatises, which detoxify many organophosphorus insecticides especially phosphates in insects. B-esterases are susceptible to organophosphorus inhibition e.g. carboxyl esterases, which play significant roles in degrading organophosphates and carbamates in insects, and C-esterases, which are uninhibited by these insecticides but do not degrade them [62]. Carboxyl esterases have, as well as lipases, α/β-hydrolase fold with a definite order of α-helices and β-sheets forming the catalytic triad Ser-Asp-His (serine, aspartic acid, and histidine, respectively) [63] (**Figure 11**).

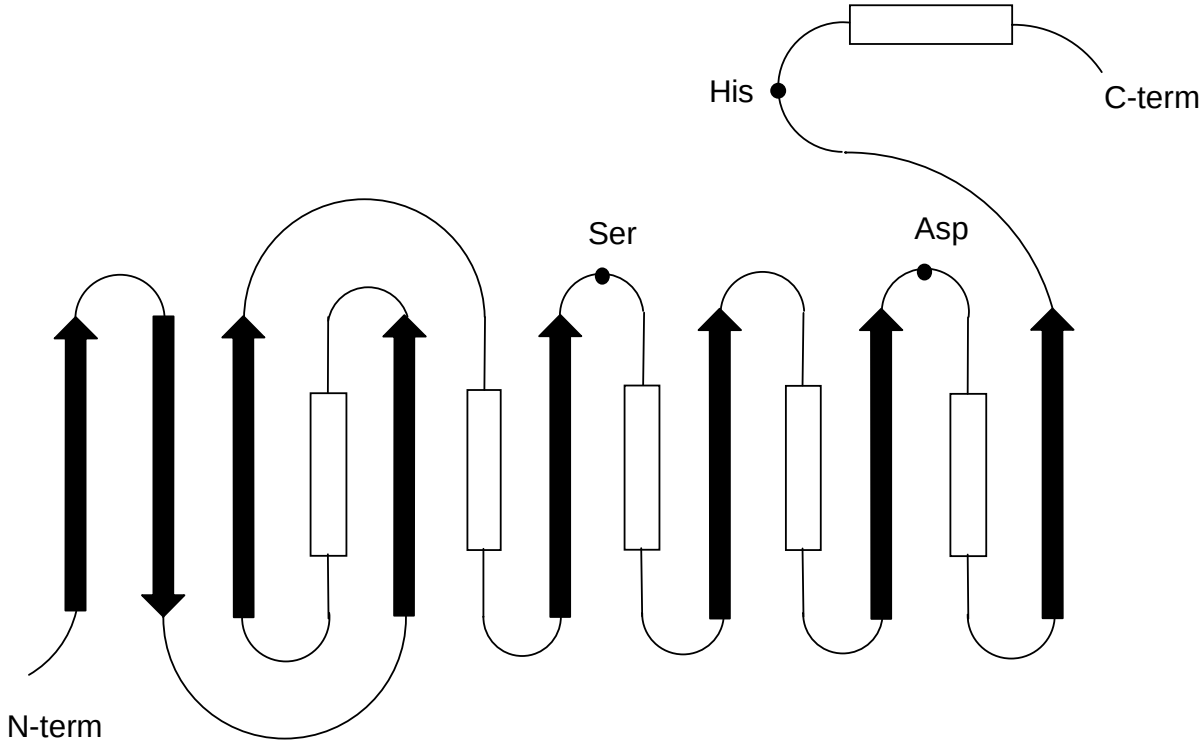


Figure 11 The α/β-hydrolase fold of esterase where the active site triad (Ser, Asp, and His) is indicated together with eight β-strands (black arrows) and six α-helices (modified from [63]).

Nowadays, there is a tendency to isolate esterases from other sources with better sensitivities toward organophosphorus and carbamate insecticides to be applied in their bioassay methods. Three enzymes (rabbit liver esterase (RLE), esterase from *Bacillus Subtilis* (BS2), and cutinase from *Fusarium solani pisi* (CUT)) proved their ability to be inhibited by these pesticides forming a suitable alternative of acetylcholinesterase [64-68].

4.1 Rabbit liver esterase (RLE)

Rabbit liver esterase ES-1A is a glycoprotein and classified as a carboxyl esterase (EC 3.1.1.1). The molecular mass of the isolated native protein from rabbit liver microsomes/ lysosomes was found of about 183 kDa with a subunit mass of 63 kDa [69]. Rabbit liver carboxyl esterase as compared to AChE is able to hydrolyze choline esters [70] and was found to be inhibited by paraoxon [69, 71] as well as other variety of organophosphate and carbamate insecticides [67]. ES-A1 works well at an optimum pH-value of 9.2 [72].

4.2 Esterase from *Bacillus Subtilis* (BS2)

Bacillus Subtilis esterase (BS2) is also a carboxyl esterase (EC 3.1.1.1) and shares a significant amino acid sequence homology with eukaryotic acetylcholine esterases and liver carboxyl esterases [73]. It is a recombinant enzyme and was cloned and functionally expressed in Escherichia Coli (E. coli). This bacterial esterase, with a theoretical molecular mass of 54 kDa, has an ability to hydrolyse esters of tertiary alcohols showing highest activity and stability under mild basic conditions of about pH 8-9 and temperature 37 ℃ [7 4]. BS2 was identified as one of the most active enzyme in the selective hydrolysis on *tert*-butyl ester moiety [75] and this high activity comes from the short-chained amino acid sequence GGG(A)X (where G denotes to glycine, X is any amino acid, A is alanine, and G may be replaced by A in few enzymes) [76] which is located in the active site contributes to the formation of the so-called oxyanion hole [77]. BS2 is inhibited quite effectively by a wide set of organophosphate and carbamate insecticides and showed also a relatively high sensitivity toward these inhibitors [67].

4.3 Cutinase, esterase from *Fusarium solani pisi* (CUT)

Cutinase (CUT) is an extracellular lypolytic enzyme catalyzes hydrolysis of cutin (an insoluble polymer and the structural component of plant cuticle) and excreted by some fungal sources [78-80] and more recently from several bacteria [81]. It is the smallest lipase/esterase enzyme with a molecular mass of about 22 kDa [78, 79, 82, and 83] and was isolated and purified for the first time from *Fusarium solani* f. sp. *pisi* [78]. Cutinase belongs to the class of serine esterases (EC 3.1.1.74) [79, 84-88] and to the family of the α/β hydrolases [79-82]. Cutinase consists of five β-strands and four α-helices constitute the folded protein containing the active site triad Ser-Asp-His [83, 85-87, 91] (**Figure 12**). The binding site of all serine esterases and lipases investigated is located inside a pocket on top of the central β sheet and cutinase has much less hydrophobic fatty acid binding site inside this pocket and thus resembling the esterases, but the shape of cutinase pocket is lipase-like [88]. Thus, cutinase can be considered as a bridge between true esterases and true lipases, since it has the capabilities of both families [91-93]. The catalytic serine is located on the surface and not buried under the active-site covering lid like in most lipases [86, 94]. The fungal infection of plants is one of the major agriculture problems because of the enzymatic

digestion of the cuticular polymer cutin and might be possibly prevented by chemicals targeted against this enzyme [84, 95]. Cutinase has high sensitivity to inhibition by organophosphates e. g. paraoxon and diisopropylfluorophosphate because of its esterase properties [78, 79, 83, 84, 92, 96, and 97]. And more recently, a variety of organophosphate and carbamate insecticides were found as potent inhibitors for cutinase from *Fusarium solani pisi* [64-68]. Cutinase exhibits highest activity at pH maximum of 8.5 [98] and is unstable above 45 ℃ and loses more than 80% of its activity in 1h at 60 ℃ [97].

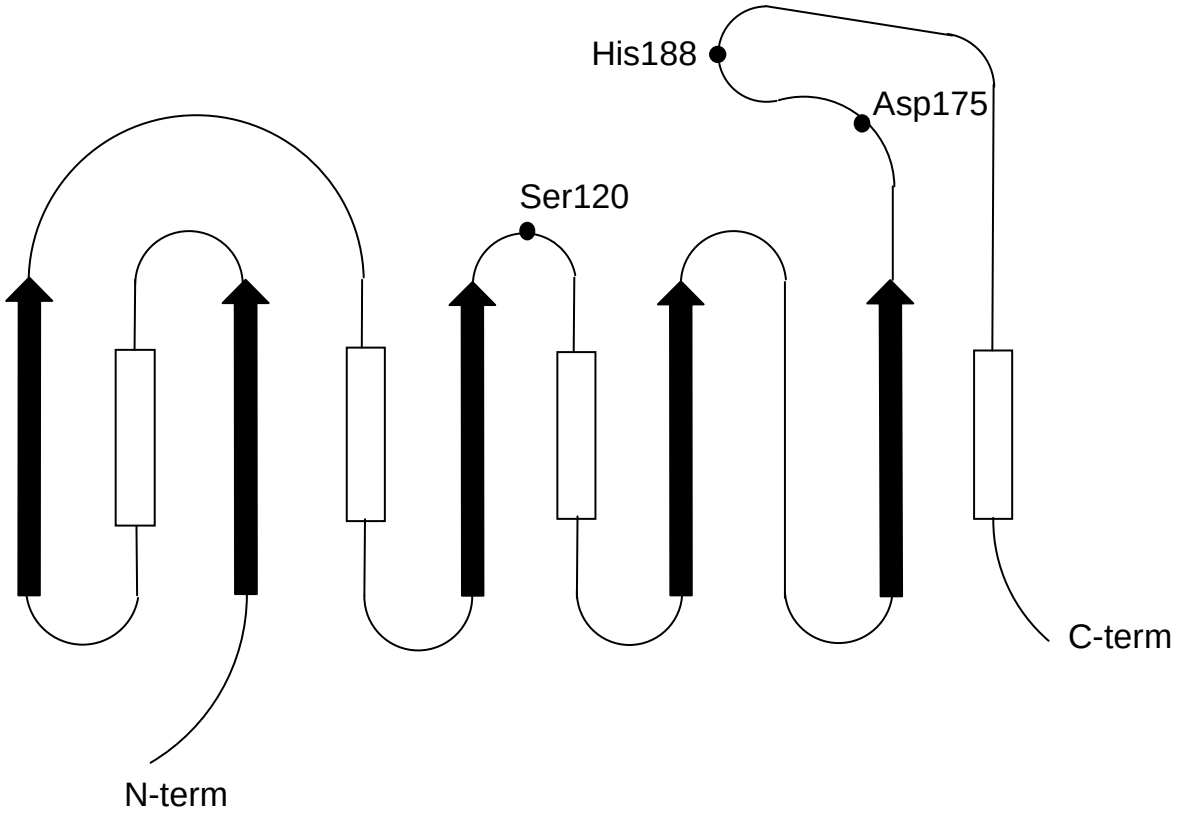


Figure 12 The α/β-hydrolase fold of cutinase where the active site triad (Ser120, Asp175, and His188) is indicated together with five β-strands (black arrows) and four α-helices (modified from [91].

5 Objectives of the work

Despite of restricted rules of pesticides usage in food crops protection, food samples with detected pesticides below or above MRLs listed by governments recorded significant proportions especially in fruit and vegetables more than in other food stuffs. Therefore, there is an increased interest for more sensitive detection methods of pesticides down to MRLs or even below. Since organophosphorus and carbamate insecticides share a common effect of the inhibition of choline esterases,

an effect directed analysis (EDA) using esterases from different sources after separation on high-performance thin-layer chromatographic plates presents a very sensitive detection method and can be classified under so called multi-residue methods because of many samples can be run in parallel (on chromatographic plates). Therefore, development of a sensitive, convenient, and fast high-performance thin-layer chromatography effect directed analysis (HPTLC-EDA) of pesticides in food samples was the aim of this study and to achieve this point, the following issues were addressed:

- Transfer the highly effective multi-enzyme assay [67] to HPTLC (**Chapter II**).
- Apply the HPTLC-enzyme inhibition assay (HPTLC-EI) to 21 organophosphorus and carbamate insecticides with a trial to determine HPTLC-EI related enzyme inhibition constants for the insecticides under study (**Chapter III**).
- Improve the detection of organophosphate thiono insecticides by application an additional oxidation step with a trial to study the oxidation effect on the rest of insecticides under study (**Chapter IV**).
- Transfer the developed method to be applied for detection some insecticides under study in fruit and vegetable samples (**Chapter V**).

To achieve these goals, a group of twenty one insecticides, which includes the organophosphorus compounds acephate, chlorfenvinfos, chlorpyrifos, chlorpyrifos-methyl, chlorpyrifos oxon, chlorpyrifos-methyl oxon, demeton-S-methyl, dichlorvos, malathion, malaoxon, monocrotofos, parathion, parathion-methyl, paraoxon, and paraoxon-methyl, and the carbamates carbaryl, carbofuran, ethiofencarb, methomyl, pirimicarb, and propoxur, was used for HPTLC-EDA using rabbit liver esterase (RLE), esterase from *Bacillus Subtilis* (BS2), and cutinase from *Fusarium solani pisi* (CUT). For application on food samples, samples of drinking water, apple juice, apple, cucumber, grapes, nectarines, plums, and tomatoes were analysed after spiking with some pesticides under study at their MRLs determined by the European Commission.

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CHAPTER II

MULTI-ENZYME INHIBITION ASSAY FOR DETECTION OF INSECTICIDAL ORGANOPHOSPHATES AND CARBAMATES BY HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY. 1. BASICS OF METHOD DEVELOPMENT

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Key words:

Enzyme inhibition, cutinase, rabbit liver esterase, *Bacillus subtilis* (BS2) esterase, organophosphorus insecticides, carbamate insecticides.

1 Summary

A recently introduced microtiter–plate multienzyme inhibition assay using rabbit liver esterase (RLE), *Bacillus subtilis* (BS2) esterase, and cutinase from *Fusarium solani pisi* has been successfully transferred to high–performance thin–layer chromatography. Paraoxon, malaoxon and carbofuran as esterase inhibitors with high, medium and low inhibitory activity, respectively, were used to optimize the method performance with regard to enzyme concentration, incubation time, and time of immersion in α-naphthyl acetate-fast blue salt B substrate. For paraoxon as strongest inhibitor, limits of detection (LOD) of 1.3, 1.2 and 540 pg/band were determined using RLE, BS2 and cutinase, respectively. Respective LODs were 7.9, 7.4 and 760 ng/band for malaoxon, and 33, 54 and 1420 ng per band for carbofuran. With regard to the LODs of strong, medium and weak inhibitors, the detectability range is favorably reduced for the low-sensitive cutinase (0.54–1420 ng per band) whereas it was approximately 3×10^4 and 5×10^4 for RLE and BS2, respectively.

2 Introduction

Since the banning of organochlorine insecticides, organophosphates and carbamates are the most widely used insecticides throughout the world for plant and store protection, against animal and household pests, and for control of disease vectors. As compounds of high acute toxicity, organophosphates are occasionally used in food extortion threats and as neurotoxins in chemical warfare. According to statistical data, in the European Union consumption of organophosphates has reached more than 3,600 tonnes compared with approximately 400 tonnes of carbamate insecticides [1]. Consequently, there is great interest in rapid and sensitive methods for detection of these compounds as residues and contaminants.

Despite the development of rapid sample-preparation and extraction methods [2-5], identification and quantification of pesticide residues and contaminants is rather time–consuming and, especially, cost–intensive when mass spectrometry coupled to gas or liquid chromatography (GC–MS(-MS) or LC–MS(-MS)) are used. Thus, a rapid screening test to identify positive samples seems to be a promising approach to save time and cost, and to enhance sample throughput.

We recently, therefore, introduced a multi–enzyme inhibition assay in a microtiter–plate format by using three different esterases - cutinase from *Fusarium solani pisi*, rabbit liver esterase, and *Bacillus subtilis* (BS2) esterase [6-9]. This assay enabled convenient, rapid, and highly sensitive detection of organophosphorus and carbamate insecticides at maximum residue limits for fruits and vegetables. With regard to different enzyme–dependent inhibition constants for each insecticide, the assay can provide both qualitative and quantitative results. In contrast, the known cholinesterase tests give only the sum of inhibitors expressed, for example as paraoxon equivalents [10].

In terms of an effect-related detection method, cholinesterase inhibition has also been used in planar chromatography, referred to as thin–layer chromatography-enzyme inhibition (TLC–EI), bioautography, or bioactivity staining, offering a second dimension of chromatographic separation. Most previously published methods for organophosphorus or carbamate insecticides based on cholinesterase inhibition have been reviewed by *Mendoza* [11-13]; a few more recent publications concern modifications and applications [14-20]. Besides the detection of pesticides, TLC–cholinesterase assays have also been reported for identification of inhibitors in plant extracts to be used for treatment of *Alzheimer's* disease [21, 22].

Published methods usually follow the steps sample application, (HP)TLC development, dipping the plate into enzyme solution, incubation, spraying with or dipping into substrate solution, stopping the enzyme reaction by heat, and evaluation. Depending upon substrate systems, in the region of pesticide bands substrate transformation does not occur, because of enzyme inhibition, thus revealing bands different in color from the background. As a fluorogenic reagent, maleimide CPM was used by *Hamada* and *Wintersteiger* [19]; this reacts with thiocholine enzymatically released from acetylthiocholine to form a strong blue fluorescent background. In this way, the sites of enzyme inhibition by inhibitory pesticides can be identified as dark spots. Three different assays using chromogenic substrate systems have been reported. In the presence of enzymatically released thiocholine, 2,6-dichloroindophenol is reduced, resulting in a white background, whereas the inhibitor zones remain blue [17]. α-Naphthol, the product of enzymatic hydrolysis of the respective acetate, yields an orange background after coupling with 4-nitrobenzenediazonium tetrafluoroborate, and the location of inhibitors appears as

white spots [14]. *Weins* and *Jork* [20] also used α-naphthol acetate as substrate, but performed the coupling with fast blue salt B, resulting in colorless inhibitory zones on a violet background.

The objective of our current work was to transfer our highly effective multi-enzyme assay [6] to HPTLC. As an advantage, the multi–esterase system used does not require an oxidation step to transform thionophosphates into the corresponding oxons, although in some cases the sensitivity can be improved by oxidation. In the first step of method development, three pesticides (paraoxon, malaoxon, and carbofuran) were chosen, representing compounds with high, medium, and low enzyme inhibitory power (**Figure 1**).

Enzyme inhibitory power can be described by the inhibition constant K_i, an equilibrium constant for dissociation of the inhibitor from the enzyme binding site (K_i = [E][I]/[EI]). This is why the inhibitory power of an agent results only from combination of the inhibitor and the appropriate enzyme.

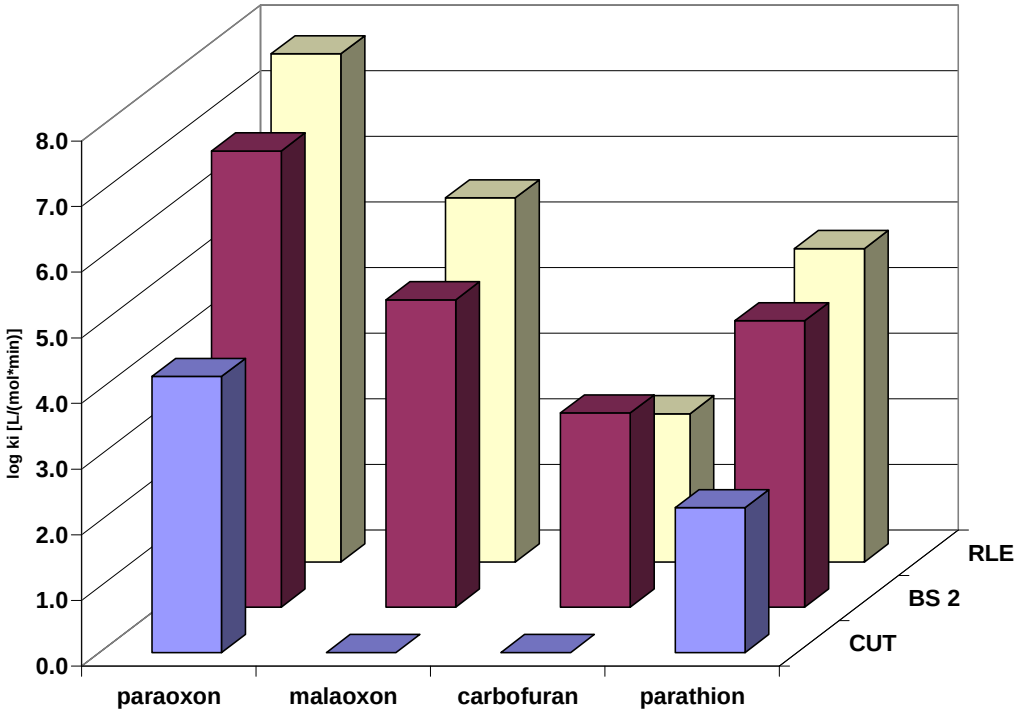

Figure 1 Inhibition constants (K_i) of paraoxon, malaoxon, carbofuran, and parathion for inhibition of cutinase (CUT), *Bacillus subtilis* esterase (BS2), and rabbit liver esterase (RLE) using a microtiter–plate assay (data taken from Ref.9).

3 Experimental

3.1 Materials

Cutinase (EC 3.1.1.74) from *Fusarium solani pisi* (lyophilized, protein content 75%, 356 U mg^{-1} protein [9]) was kindly provided by Unilever Research Laboratory (Vlaardingen, The Netherlands). *Bacillus subtilis* (BS2) esterase (14.1 U mg^{-1}) was purchased from Julich Chiral Solutions (Jülich, Germany), rabbit liver esterase (lyophilized, 80 U mg^{-1} protein), bovine albumin (BSA, >98%), and α-naphthyl acetate (≥98%) were from Sigma-Aldrich (Taufkirchen, Germany). Fast blue salt B and silica gel 60 F_{254} HPTLC plates (20 cm x 10 cm) were supplied by Merck (Darmstadt, Germany). Pesticide standards (paraoxon, malaoxon, ethiofencarb, and carbofuran) were purchased from Riedel-de Haën (Taufkirchen, Germany) and parathion from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Tris-(hydroxymethyl) aminomethane (TRIS, ≥99.9%) and dichloromethane (≥99.9%) were from Carl Roth (Karlsruhe, Germany). Methanol, ethanol, *n*-hexane, and ethyl acetate (analytical grade) were obtained from Merck and were distilled before use. Ultra pure water was obtained from a Millipore (Schwalbach, Germany) Synergy system.

3.2 Solutions

To prepare enzyme solutions 0.5 mg cutinase, 5 mg BS2 esterase, and 0.9 mg rabbit liver esterase were each dissolved in 50 mL Tris–HCl buffer (0.05 M, pH 7.8) containing 0.1% BSA. Pesticide stock solutions (1 g L^{-1}) were prepared in methanol. Working standards (1, 10, and 100 µg L^{-1} were obtained by appropriate dilution. Substrate solution was prepared by mixing one volume α-naphthyl acetate solution (2.5 g L^{-1} ethanol) and four volumes of fast blue salt B (2.5 g L^{-1} water). Both solutions were freshly prepared directly before use.

3.3 Chromatography

HPTLC plates were pre–washed by development with methanol, followed by drying at 100℃ for 20 min. Standard solutions were applied as 5–mm bands by means of an automatic TLC sampler (ATS4; CAMAG, Muttenz, Switzerland). Distances from the lower edge, from the left side, and between tracks were 10 mm. Plates were developed in an automatic development Chamber (ADC2, CAMAG) with ethyl acetate-dichloromethane-*n*-hexane 2:1.5:6.5 (*v*/*v*) as mobile phase to a

distance of 85 mm from the lower edge (the migration time was approximately 35 min including 5 min drying). Plate activity was adjusted to 33% relative humidity by use of saturated aqueous magnesium chloride ($MgCl_2$.6 H_2O) solution.

3.4 Detection

The developed and dried plate was dipped in the enzyme solution for 2 s by use of the TLC Immersion Device III (CAMAG) at a dipping speed of 1 cm s^{-1}, followed by horizontal incubation for 60 min at 37℃ in a humid chamber containing water. Thereafter, the plate was dipped in substrate solution for 30 s at a dipping speed of 1 cm s^{-1}, followed by 3 min reaction time (laying the plate horizontally). The reaction was finally stopped by heating the plate on a TLC Plate Heater III (CAMAG) at 50℃ for 5–7 min (until dryness).

3.5 Documentation and evaluation

Plate images were documented by use of the DigiStore 2 documentation system (CAMAG) with illumination in the visible range and in reflectance mode. Densitometric evaluation with the TLC Scanner 3 (CAMAG) was by absorbance measurement at 533 nm (inverse scan using fluorescence measurement mode without edge filter). The peak area data obtained were processed with winCATS software, version 1.3.2 (CAMAG).

4 Results and Discussion

4.1 Method Comparison

First, the three esterases used for the microtiter–plate assay [6] were transferred to conditions of planar chromatography. Five organophosphorus and carbamate insecticides were separated by HPTLC and subjected to enzyme inhibition assays according to the procedure described by *Weins* and *Jork* [20], who used butyryl cholinesterase and α-naphthyl acetate-fast blue salt B as substrate for detection. The results showed there was not usually much difference between butyryl cholinesterase and *Bacillus subtilis* (BS2) esterase, as shown in **Figure 2**. BS2 esterase, however, enables sensitive detection of parathion without an oxidation step, which clearly is an advantage over cholinesterases, but Figure 2 also shows that the parathion standard used contains paraoxon as impurity. Cutinase and rabbit

liver esterase (RLE) gave similar results (data not shown), but both malaoxon and carbofuran, surprisingly, were detected by cutinase on HPTLC plates, whereas both insecticides were not identified as inhibitors of cutinase using the microtiter–plate format (Figure 1). We cannot yet explain this discrepancy.

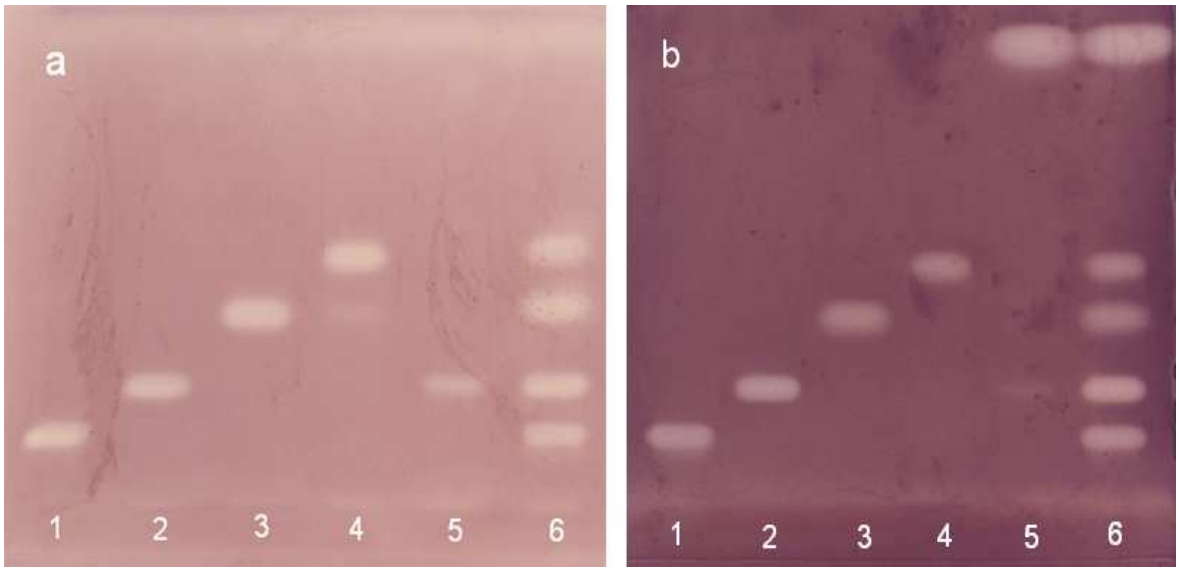

Figure 2 HPTLC–enzyme inhibition assay using (a) butyryl cholinesterase and (b) *Bacillus subtilis* (BS2) esterase. 1, malaoxon (125 ng per band); 2, paraoxon (0.5 ng per band); 3, carbofuran (500 ng perband); 4, ethiofencarb (500 ng per band); 5, parathion (250 ng per band); 6, mixture of 1-5. The assay conditions used were those of *Weins* and *Jork* [20].

4.2 Determination of Optimum Assay Conditions

To determine the optimum conditions for sensitive HPTLC esterase inhibition assay, we studied:

- the concentration of the enzyme solution;
- the time of incubation with the enzyme; and
- immersion time in the substrate solution.

Paraoxon was chosen for these experiments, because it is the insecticide with the highest inhibition constant.

Enzyme concentration is a critical condition to be optimized for sensitivity. From theoretical considerations, small amounts of inhibitors should be detected by small amounts of enzyme, but the background will only be rather pale if the enzyme concentration is chosen too low, and so the bands of inhibitors will be almost

invisible. A sufficient enzyme concentration is, therefore, necessary to obtain a deeply colored background within a reasonable incubation time. If enzyme concentration is too high, however, the fraction of enzyme inhibited will be too low to be detected (**Figure 3**). From the results obtained, a concentration of 0.5x, equivalent to 1.4 U mL^{-1}, was identified as optimum for BS2 and RLE whereas cutinase worked best at 0.25x, i.e. 2.7 U mL^{-1}.

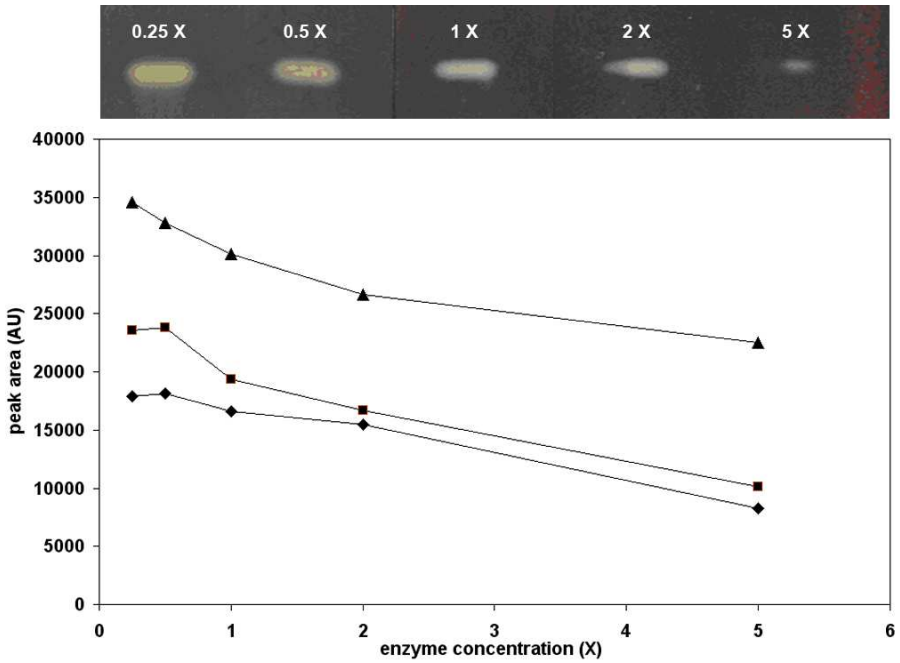

Figure 3 Effect of enzyme concentration on signal intensity for inhibition of cutinase (▲, *X* = 1.5 mg/100 mL), rabbit liver esterase (■, *X* = 0.9 mg/100 mL), and *Bacillus subtilis* (BS2) esterase (♦, *X* = 10 mg/100 mL) by paraoxon. Plate images show BS2 inhibition by 100 pg per band.

Incubation of the enzyme-loaded HPTLC plate must be performed under controlled environmental temperature and humidity conditions. During incubation, the almost lipophilic inhibitors adsorbed on the silica gel must have a chance to react with the applied enzyme dissolved in aqueous buffer. This step is, therefore, obviously time-dependent and not fully optimum after a few minutes (**Figure 4**). Although a long incubation time is associated with band broadening, signal intensity clearly increases. As the best compromise an incubation time of 60 min was identified for all three esterases.

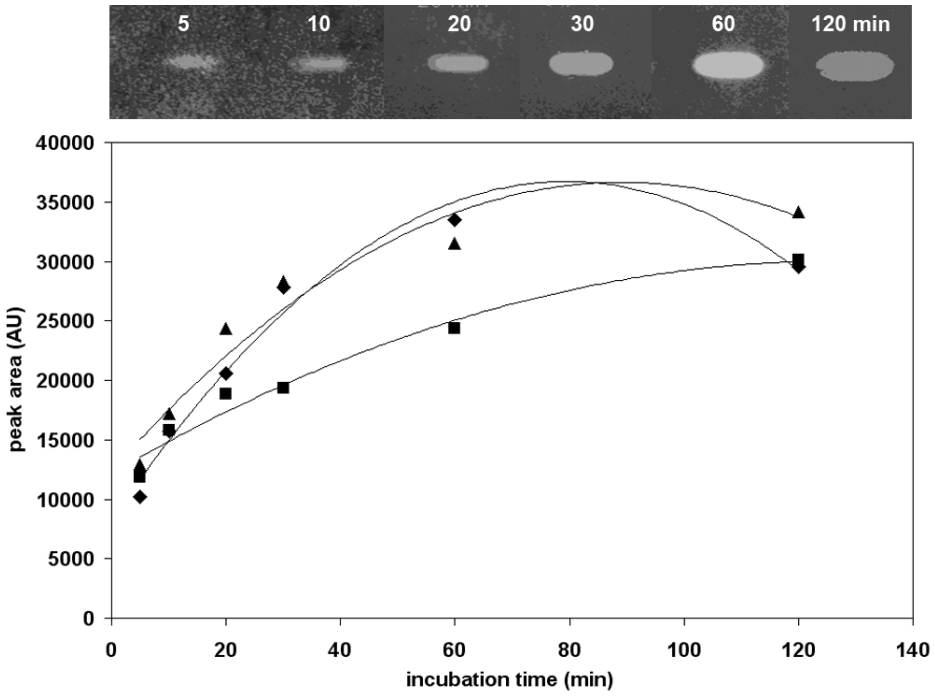

Figure 4 Effect of incubation time on signal intensity for inhibition of cutinase (▲), rabbit liver esterase (■), and *Bacillus subtilis* (BS2) esterase (♦) by paraoxon. Plate images show BS2 inhibition by 100 pg per band.

After incubation, the plate is dipped into substrate solution. Originally this was not thought to be a critical step. Immersion time has, however, been shown to strongly effect the result. Obviously, the plate needs some time to sufficiently adsorb the substrate reagent and to yield a dark background, against which a high signal intensity of nearly colorless bands can be measured (**Figure 5**). If the immersion time is only brief, the background is rather light; if immersion times were too long, however, darkening of inhibitory bands occurred. The optimum immersion time was 30 s.

The detection stability of assayed plates was monitored for six days. When the plates were stored protected from light, for example covered by a glass plate and wrapped by aluminium foil, the signal was rather stable for at least four days with intensities remaining >99%.

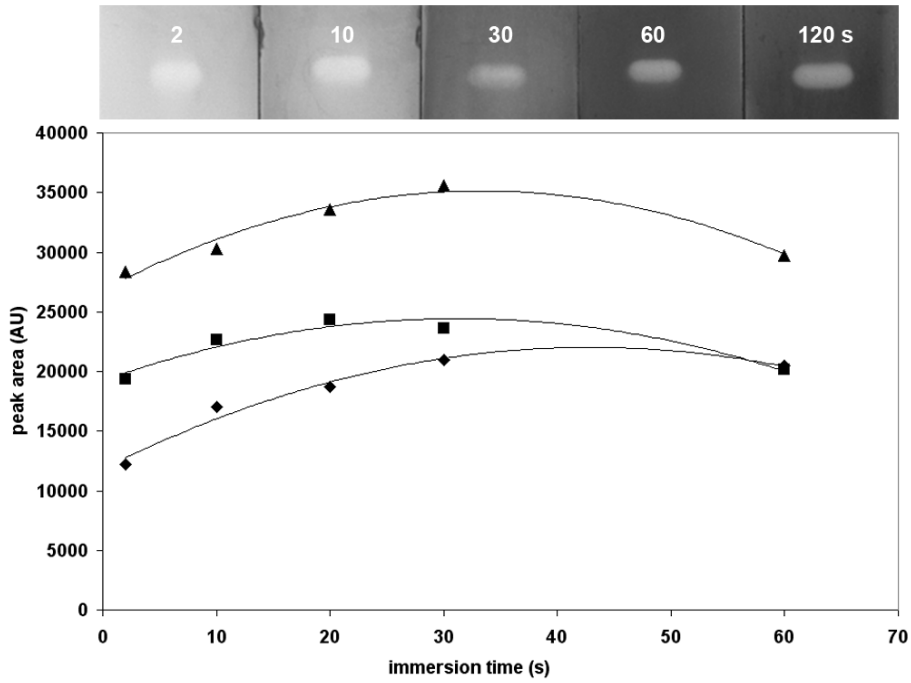

Figure 5 Effect of substrate immersion time on signal intensity for inhibition of cutinase (▲), rabbit liver esterase (■), and *Bacillus subtilis* (BS2) esterase (♦) by paraoxon. Plate images show BS2 inhibition by 100 pg per band.

4.3 Calibration and Detectability

Under the optimized assay conditions, calibration can be performed over a range of at least a factor of ten (**Figure 6**). The upper limit is that which leads to serve band broadening resulting in large oval spots. In the lower range of calibration, the plots were absolutely linear with high coefficients of determination (**Table 1**). Limits of detection (LOD) and quantification (LOQ) are expected to depend both on the insecticide and on the enzyme used (**Table 2**). LODs were usually lowest for BS2 and RLE, proving the high sensitivity of these esterases to organophosphorus and carbamate insecticides. The values found – down to 1 pg per band for strong inhibitors, for example paraoxon – are clearly superior to previously published LODs, obtained by use of cholinesterases. For insecticides with medium or low inhibitory strength, for example malaoxon and carbofuran, LOD and LOQ were several orders of magnitude higher - nanogram rather than picogram. Compared with BS2 and RLE, cutinase is noticeably more resistant to inhibition, which can be an advantage for rapid screening if highly concentrated samples are to be analyzed without dilution. With regard to inhibition of cutinase, the differences between paraoxon, malaoxon, and carbofuran as strong, medium, and weak inhibitors, respectively, are, again,

much lower than for RLE and BS2. Thus, the concentration range of detectability is favourably reduced when cutinase is used (0.5–1420 ng per band compared with approximately 1–33,000 and 1–54,000 pg per band for RLE and BS2, respectively).

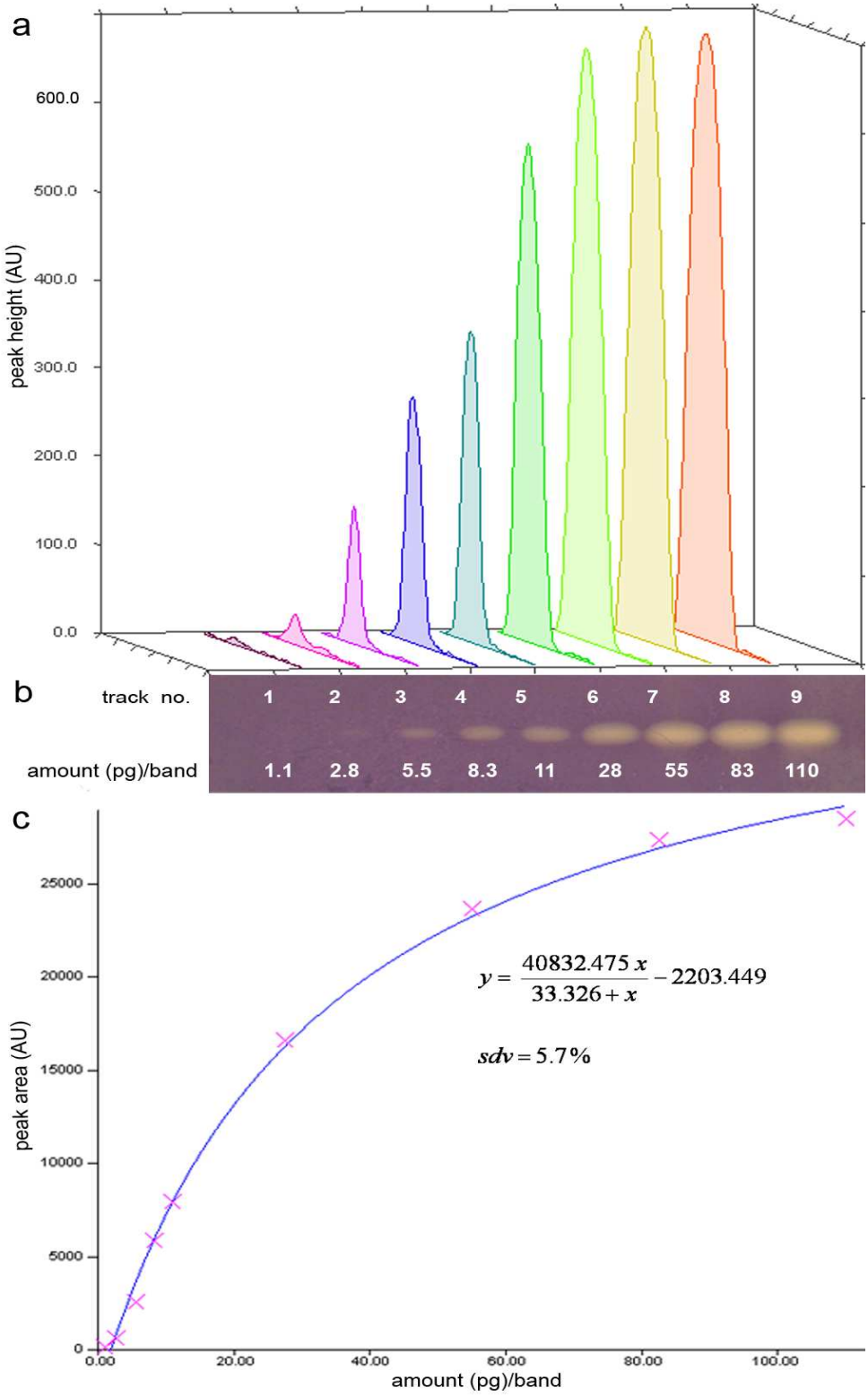

Figure 6 Set of paraoxon calibration standards in the range 1–110 pg per band: (a) inverse scan at 533 nm, (b) plate image, (c) calibration curve.

Table 1 Calibration data for HPTLC–enzyme inhibition assay using rabbit liver esterase (RLE), *Bacillus subtilis* esterase (BS2) and cutinase (CUT).

Insecticide	Calibration range per band (determination coefficient, R^2)		
	RLE	BS2	CUT
Paraoxon	1–5 pg (0.9928)	1–5 pg (0.9944)	0.4–2 ng (0.9924)
Malaoxon	5–25 ng (0.9900)	5–25 ng (0.9909)	0.5–2.5 µg (0.9902)
Carbofuran	50–250 ng (0.9983)	50–250 ng (0.9952)	1–5 µg (0.9916)

Table 2 Limits of detection (LOD) and quantification (LOQ) obtained by use of the calibration plot concept [23].

Insecticide	Enzyme	LOD	LOQ
Paraoxon (pg per band)	RLE	1.3	1.9
	BS2	1.2	1.7
	CUT	540	790
Malaoxon (ng per band)	RLE	7.9	11.4
	BS2	7.4	10.7
	CUT	760	1100
Carbofuran (ng per band)	RLE	33	49
	BS2	54	79
	CUT	1420	2070

5 Conclusion

Use of selected esterases, introduced for microtiter–plate assays, in HPTLC bioassays was successful for the three insecticides studied. The optimized enzyme inhibition assay enables very sensitive detection, especially when rabbit liver esterase or *Bacillus subtilis* esterase are used. For the next step, extensive screening of the most important insecticides must be undertaken, followed by application to plant samples. For the microtiter–plate assay, a sophisticated clean–up procedure for plant extracts was essential, because of enzyme inhibition by natural matrix compounds, especially for the two highly sensitive esterases [6, 7]. As a result of chromatographic separation (HPTLC) clean–up may be simplified and reduced. It must, nevertheless, be remembered that matrix compounds present in plant extracts

and separated by HPTLC may also result in inhibition, as already pointed out by *Ambrus* et al. [18].

Acknowledgments

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CHAPTER III

MULTI-ENZYME INHIBITION ASSAY FOR THE DETECTION OF INSECTICIDAL ORGANOPHOSPHATES AND CARBAMATES BY HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY APPLIED TO DETERMINE ENZYME INHIBITION FACTORS AND RESIDUES IN JUICE AND WATER SAMPLES

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Key words:

High-performance thin-layer chromatography, enzyme inhibition, effect-directed analysis, cutinase, rabbit liver esterase, *Bacillus subtilis* (BS2) esterase, inhibition factor, organophosphorus insecticides, carbamate insecticides.

1 Abstract

Esterase inhibition assays provide an effect-directed tool of rapid screening for inhibitors in environmental and food samples. According to a multi-enzyme microtiter-plate assay, rabbit liver esterase (RLE), *Bacillus subtilis* esterase (BS2), and cutinase from *Fusarium solani pisi* (CUT) were used for the detection of 21 organophosphorus and carbamate pesticides by high-performance thin-layer chromatography–enzyme inhibition assays (HPTLC–EI). Staining was performed with Fast Blue Salt B coupled to α-naphthol enzymatically released from the respective acetate used as substrate. Quantitative analysis was achieved by densitometric evaluation at 533 nm. Enzyme inhibition factors derived from HPTLC-EI were calculated from the slopes of the linear calibration curves, which allowed comparisons to published inhibition constants and well correlated to sensitivity parameters. Limits of detection ranged from a few pg/zone for organophosphates as strongest inhibitors to a few ng/zone for most carbamates, when RLE and BS2 were used. Without oxidation, chlorpyrifos and parathion were directly detectable at approximately 60 and 14 ng/zone, respectively. As the enzyme of lowest sensitivity, CUT was able to detect insecticides of high and low inhibitory power from the ng to µg range per zone. Due to high selectivity of enzyme inhibition, oxon impurities of thionophosphate standards were strongly detected, although only present in low traces. The exemplary application of HPTLC-EI (RLE) to apple juice and drinking water samples spiked with paraoxon (0.001 mg/L), parathion (0.05 mg/L) and chlorpyrifos (0.5 mg/L) resulted in mean recoveries between 71 and 112% with standard deviations of 2.0-18.3%.

2 Introduction

Organophosphorus and carbamate insecticides still represent important pesticides, which are used worldwide in agriculture to protect plants and animals and to prevent crop damages due to insects. Additionally, they are used against storage and domestic pests, and to control insect-borne diseases. As compounds of high acute toxicity, organophosphates were occasionally involved in food extortion threats and formerly used as neurotoxins in chemical warfare. The international destruction of military arsenals supervised by the Organization for the Prohibition of Chemical Weapons is still in progress [1]. Consequently, there is a great interest in rapid and sensitive analytical systems for the detection of contaminants and residues.

In routine pesticide residue analysis, rapid methods of sample extraction and clean-up have been developed (e.g. [2-6]). For the identification and quantification of pesticides, both gas and liquid chromatography coupled to mass spectrometry (GC-MS(/MS) or LC-MS(/MS)) are generally used [7]. With special emphasis on organophosphorus compounds, LC-MS based procedures for the analysis of food, environmental and biological samples were recently reviewed by John et al. [8]. Apart from target-oriented analysis, there is an increasing interest in effect-directed analysis for official food control, which offers an efficient tool to identify positive samples to be subjected to further instrumental analysis [9]. Since organophosphorus and carbamate pesticides share a common effect of the inhibition of choline esterases [10], there is a great chance of effect-directed analysis by using acetylcholine esterases (AChE) from different sources for cuvette or microtiter-plate assays [11-14], which also led to a norm method for the analysis of water samples [15]. During the last two decades, choline esterase biosensor development was of great interest, recently reviewed by Andreescu and Marty [16] and Pohanka et al. [17]. Differing from choline esterases, a microtiter-plate multi-enzyme inhibition assay using rabbit liver esterase (RLE), *Bacillus subtilis* (BS2) esterase, and cutinase from *Fusarium solani pisi* was introduced for rapid and sensitive detection of organophosphorus and carbamate insecticides [18-21]. In terms of 'bioauthography', this multi-enzym assay recently was successfully transferred to high-performance thin-layer chromatography (HPTLC) [22], which is unrivaled in rapid and matrix robust screening of many samples in parallel [23]. (HP)TLC–choline esterase assays have differently been published since more than four decades, briefly reviewed in [22], and also were reported for the screening for inhibitors in plant extracts to identify potent candidates for the treatment of Alzheimer's disease [24, 25].

The aim of the present work was to apply the HPTLC–enzyme inhibition assay (HPTLC–EI) to 21 representative insecticides, which involve the organophosphorus compounds acephate, chlorfenvinfos, chlorpyrifos, chlorpyrifos-methyl, chlorpyrifos oxon, chlorpyrifos-methyl oxon, demeton-S-methyl, dichlorvos, malathion, monocrotofos, parathion, parathion-methyl, paraoxon, and paraoxon-methyl, and the carbamates carbaryl, carbofuran, ethiofencarb, methomyl, pirimicarb, and propoxur. Additionally, a trial was undertaken to determine HPTLC-EI related enzyme inhibition constants for the insecticides under study.

3 Experimental

3.1 Chemicals

B. subtilis (BS2) esterase (14.1 U/mg) was purchased from Julich Chiral Solutions (Julich, Germany). Cutinase (EC 3.1.1.74) from *Fusarium solani pisi* (lyophilized, protein content 75%, 356 U/mg protein) [19] was provided by Unilever Research Laboratory (Vlaardingen, The Netherlands). Rabbit liver esterase (lyophilized, 80 U/mg protein), bovine albumin (BSA, >98%), Fast Blue Salt B (dye content, ~95%), and α-naphthyl acetate (≥98%) were obtained from Sigma-Aldrich (Taufkirchen, Germany). Pesticide standards, carbofuran, chlorfenvinfos, demeton-S-methyl, dichlorvos, methomyl, monocrotofos, paraoxon, and paraoxon-methyl, were purchased from Riedel-de Haën (Taufkirchen, Germany), acephate, carbaryl, chlorpyrifos, chlorpyrifos-methyl, chlorpyrifos-methyl oxon, chlorpyrifos oxon, pirimicarb, and propoxur from Dr. Ehrenstorfer GmbH (Augsburg, Germany), and ethiofencarb, malaoxon, malathion, parathion, and parathion-methyl from Sigma-Aldrich (Taufkirchen, Germany). Chloroform (>99%) and acetonitrile (HPLC grade) were purchased from Fisher Scientific (Schwerte, Germany). Tris(hydroxymethyl)aminomethane (TRIS, ≥99.9%) and dichloromethane (≥99.9%) were provided by Carl Roth GmbH & Co. (Karlsruhe, Germany). Methanol, ethanol, n-hexane, acetone, ethyl acetate (analytical grade and distilled before use), and Silica gel 60 F_{254} HPTLC glass plates (20 cm x 10 cm) were supplied by Merck (Darmstadt, Germany). Ultra pure water was purchased by a Synergy system (Millipore, Schwalbach, Germany). BONDESIL-PSA (40 µm) was obtained from Varian (Darmstadt, Germany).

3.2 Enzyme and pesticide solutions

Cutinase (5 mg), BS2 esterase (50 mg), and rabbit liver esterase (9 mg) were individually dissolved in 10 mL Tris–HCl buffer (0.05 M, pH 7.8) containing 0.1% BSA and stored in a freezer (enzyme stock solutions). Working solutions were prepared by diluting 1 mL stock solution to 50 mL with the same buffer. Pesticide stock solutions (1 g/L) were prepared in methanol and diluted by methanol to working standards of 10 mg/L, 100 µg/L, and 1 µg/L. Substrate solution was prepared by mixing 1 volume α-naphthyl acetate solution (2.5 g/L in ethanol) and 2 volumes of

Fast Blue Salt B (2.5 g/L in water). Both solutions were freshly prepared directly before use.

3.3 High-performance thin-layer chromatography (HPTLC)

HPTLC glass plates were pre–washed by development with methanol, followed by drying at 100℃ for 20 min and stored in a desic cator. Appropriate volumes of pesticide working standard solutions were applied by the Automatic TLC Sampler 4 (ATS4, CAMAG, Muttenz, Switzerland) as 5–mm bands with 10 mm distances from the lower edge, the left side, and between tracks. Chromatographic development was done using the Automatic Developing Chamber 2 (ADC2, CAMAG) with the n-hexane/ethyl acetate/dichloromethane (65:20:15) without tank saturation to a migration distance of 80 mm from the lower edge; the developing time was approximately 35 minutes including 5 min drying. Concerning the solvent systems for chromatography adjusted to polarity of insecticides, they were divided into three groups; group 1: paraoxon, paraoxon-methyl, malaoxon, dichlorvos, chlorfenvinfos, ethiofencarb, parathion and parathion-methyl (separated with ethyl acetate/n-hexane (37/63, v/v); group 2: monocrotofos, pirimicarb, methomyl, carbofuran, propoxur, carbaryl, and chlorpyrifos oxon (separated with ethyl acetate/chloroform (10/90, v/v); group 3: acephate, demeton-S-methyl, chlorpyrifos-methyl oxon, malathion, chlorpyrifos-methyl, and chlorpyrifos (separated with n-hexane/acetone/dichloro-methane (75/10/15, v/v/v).

3.4 Detection

Using the TLC Immersion Device III (CAMAG), the developed and dried plate was dipped into the enzyme solution for 2 s at a dipping speed of 1 cm/s. The following horizontal incubation for 30 min at 37℃ was performed in a humid chamber containing water. Then, the plate was dipped into the freshly prepared substrate solution for 1 s at the same dipping speed and left 3 min for reaction (laying the plate horizontally). To stop the reaction, the plate was finally heated on a TLC Plate Heater III (CAMAG) at 50℃ for 5–7 min until dryness.

3.5 Evaluation and documentation

Densitometric evaluation was performed via peak area by absorbance measurement at 533 nm (inverse scan using fluorescence measurement mode without edge filter) using TLC Scanner 3 (CAMAG). Plate documentation was done under illumination in the visible range and in the reflectance mode using a DigiStore 2 documentation system (CAMAG). All data obtained were processed with winCATS software, version 1.3.2 (CAMAG).

3.6 HPTLC–mass spectrometry

For HPTLC/MS, the zones of interest were previously detected in DigiStore 2 at 254 nm and marked with a pencil. Zone extraction was performed by the TLC–MS Interface (CAMAG) with methanol/formic acid (0.1%) at a flow rate of 0.1 mL min^{-1} provided by an HPLC 1100 pump (Agilent Technologies, Waldbronn, Germany). A G1956B MSD single quadrupole mass spectrometer equipped with an electrospray interface (ESI) and ChemStation B.02.01 SR2 software (Agilent Technologies) was used. The mass spectrometer operated under the following parameters for positive electrospray ionization: capillary voltage 4.0 kV, drying gas temperature 300 °C, drying gas flow rate 10 L min^{-1}, nebulizer gas pressure 30 psi (207 kPa), fragmentator voltage 100 V, gain 1, threshold 1, step-size *m*/*z* 0.05, time filter off, scan data storage full.

Exact masses and spectral accuracies were determined by MassWorks software (Cerno Bioscience, Danbury, CT, USA) using parathion or paraoxon as mass calibration standards.

3.7 Sample extraction

Apple juice samples (10 mL) obtained from the local market and tap water samples (10 mL) were individually spiked with a methanolic solution of paraoxon, parathion or chlorpyrifos and extracted following the so-called QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) procedure [2, 3] without the addition of buffer salts, but including the dispersive PSA (primary secondary amine) clean-up for apple juice extracts. Briefly, 10 mL sample and 10 mL acetonitrile were vigorously shaken in a 50-mL centrifuge tube for 1 min. After the addition of a mixture of 1 g

sodium chloride and 4 g magnesium sulfate, the tube was shaken for another minute and centrifuged for 5 min at 3500 x g. In the case of apple juice, 10 mL of the upper organic layer were shaken with 250 mg PSA and 1.5 g magnesium sulfate for 30 s and centrifuged. The extractions were performed in triplicates, and the extracts applied (10 µL) onto the HPTLC plate together with a set of calibration standards. For the determination of parathion and chlorpyrifos, the extracts were 10-fold concentrated before application.

4 Results and Discussion

4.1 Staining with Fast Blue Salt B

Following Weins and Jork [26], α-naphthyl acetate was used as enzyme substrate on HPTLC plates after development and incubation in the presence of an esterase. Depending on enzyme activity, α-naphthol is formed immediately coupling with Fast Blue Salt B (3,3'-dimethoxy-4,4'-biphenylbis(diazonium) chloride), thus resulting in a violet background while zones of inhibitors remain colorless due to lack of substrate conversion. During the previous study [22], a Fast Blue Salt B from Merck was used, that is not offered any more. However, the same product supplied by Sigma-Aldrich surprisingly failed following the formerly optimized detection protocol. Instead of a dark violet plate background a rather pale background was obtained, which made zone identification very difficult and resulted in a bathochrome shift of nearly 100 nm for the maximum wavelength (λ_{max}) of the background. When the reaction of α-naphthol with Fast Blue Salt B was performed in aqueous solution, a violet product was immediately formed with λ_{max} at 530 nm, but within minutes the color decreased and λ_{max} changed to 620 nm. The differences between the old and new reagent are difficult to explain, but could be managed by empirically reconditioning the detection protocol. The former situation of colorless inhibitory zones on a dark violet background was recovered (**Fig. 1**) by (a) reducing the enzyme incubation time from 60 to 30 min, (b) changing the mixing ratio of Fast Blue Salt B and α-naphthyl acetate reagents for the substrate solution from 4+1 to 2+1 volumes, and (c) strongly reducing the substrate solution dipping time from 30 to 1 s. Finally, the temperature of plate drying must not exceed 50 ℃ to prevent brightening of the violet background.

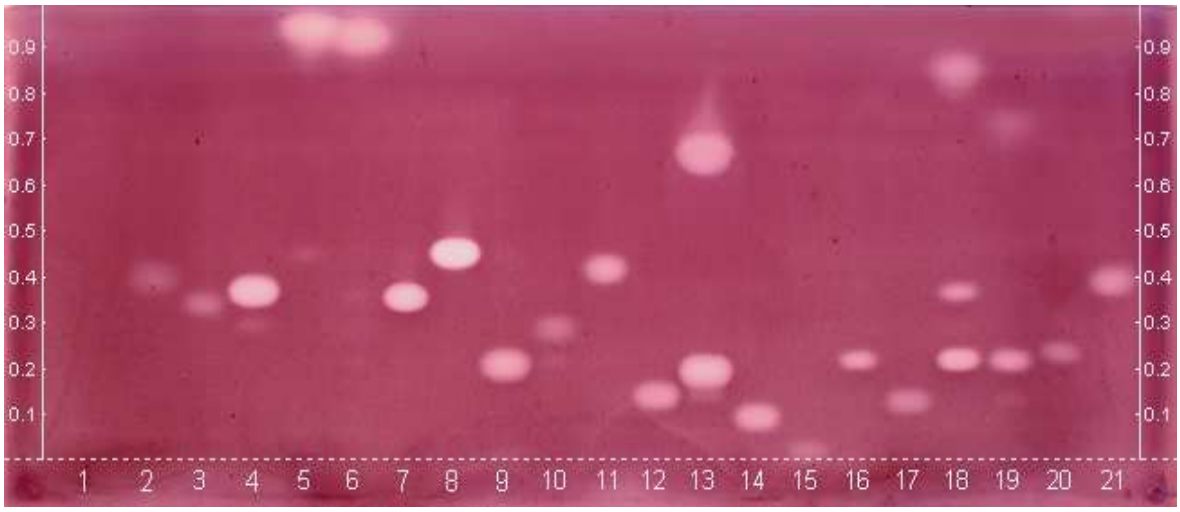


Fig. 1. HPTLC–EI assay of organophosporus and carbamate insecticides developed by n-hexane/ethyl acetate/dichlormethane (65:20:15) and detected by rabbit liver esterase inhibition: 1. acephate 1 µg, 2. carbaryl 10 ng, 3. carbofuran 100 ng, 4. chlorfenvinfos 100 pg, 5, chlorpyrifos 200 ng, 6. chlorpyrifos-methyl 1µg, 7. chlorpyrifos-methyl oxon 1 ng, 8. chlorpyrifos oxon 100 pg, 9. demeton-S-methyl 50 ng, 10. dichlorvos 10 pg, 11. ethiofencarb 50 ng, 12. malaoxon 10 ng, 13. malathion 2 µg, 14. methomyl 50 ng, 15. monocrotofos 50 ng, 16. paraoxon 10 pg, 17. paraoxon-methyl 100 pg, 18. parathion 20 ng, 19. parathion-methyl 50 ng, 20. pirimicarb 50 ng, 21. propoxur 70 ng.

4.2 High-performace thin-layer chromatography–enzyme inhibition assay (HPTLC-EI)

As to be expected, it is difficult to completely separate all 21 insecticides under study with a wide range of polarity in a single planar chromatographic run (**Fig. 1**). However, the chosen solvent system is quite suitable for rapid screening objects and to find out if any inhibitor is present in a sample, even though an insecticide remains on the start, while another one is eluted near to the solvent front. By subdividing the insecticides into three groups and adjusting the solvent composition for plate development, a clear separation was obtained within each group (**Fig. 2**), which is used for conformation purposes.

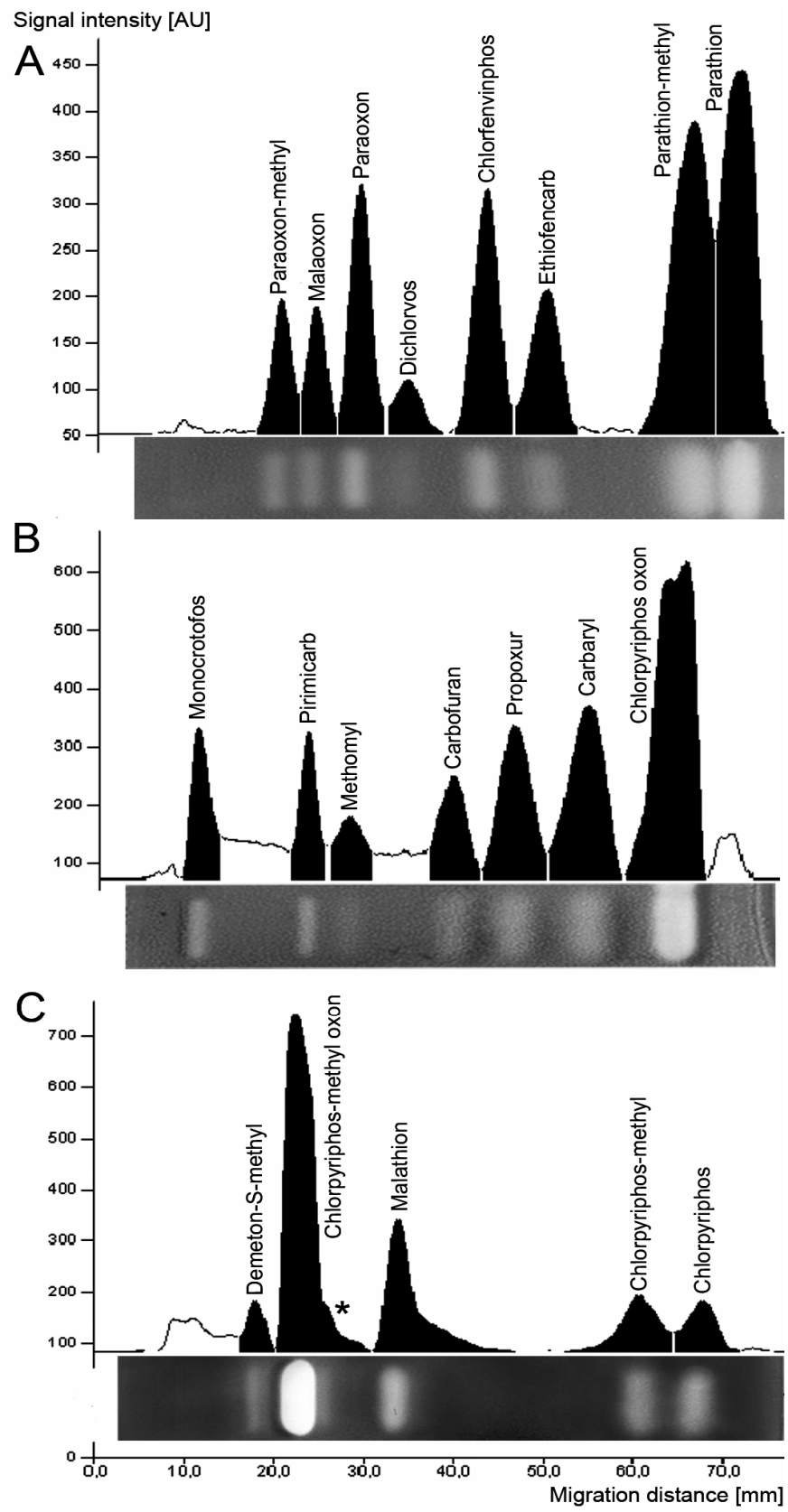


Fig. 2. HPTLC-EI assay of organophosphate and carbamate insecticides, divided into three groups. Solvent systems: (A) n-hexane/ethyl acetate (63:37), (B) chloroform/ethyl acetate (90:10), (C) n-hexane/acetone/dichloromethane (75:10:15) (* marks the oxon impurity of chlorpyrifos).

The automated multiple development chamber (AMD) enabling gradient elution clearly improved plate selectivity (data not shown), but will not necessarily facilitate insecticide identification for two reasons. Enzyme inhibition detection may result in rather big and oval-shaped zones instead the line-shaped zone applied onto the plate, which intentionally is shown for some insecticides in **Fig. 2**. This effect is influenced by both the absolute amount applied onto the plate and the incubation time, and is obviously caused by diffusion processes happening during the dipping and incubation steps. The second problem concerning identification arises from impurities or transformation products like products of hydrolyses oxidations, or rearrangements. Since they may already be present in commercial standards, they even more have to be expected in environmental samples. Such by-products in low amounts are almost not visible by UV detection, but will be clearly detectable by enzyme inhibition in the case of strong inhibitors.

4.2.1 Detectability of impurities in analytical standards

Impurities of paraoxon in the parathion standard resulted in an intensive zone of inhibition (**Fig. 1**), although invisible under UV light illumination, when about 1 µg parathion per zone was applied. An identical observation was made for a second parathion impurity eluting above paraoxon. After chromatography of 10 µg parathion, HPTLC–MS experiments proved the presence of paraoxon by the protonated molecule at *m/z* 276 with an exact mass of 276.0873 Da (calculated 276.0637 Da) and spectral purity >98 %. For the second impurity, the protonated molecule was found at *m/z* 292 with the exact mass of 292.0600 Da and the elemental composition $C_{10}H_{15}NO_5PS$. The findings perfectly match parathion itself, why the impurity must be the known thiono-thiolo parathion rearrangement product O,S-diethyl-O-(4-nitrophenyl) phosphorothioate, also called iso-parathion [27], which already may be formed during parathion distillation. As oxon, iso-parathion probably is a strong esterase inhibitor explaining the intensive inhibition zone, although only present in traces. Identical impurities were found in the standard of parathion-methyl. In the cases of chlorpyriphos and chlorpyriphos-methyl, traces of the respective oxons were also detectable by HPTLC-EI (**Fig. 1**).

Besides traces of malaoxon, the malathion standard exhibited an additional inhibition zone, when RLE was used as enzyme source, which both could not be

detected under UV. After chromatography of 100 µg malathion, the two impurities could be located under UV illumination and subjected to HPTLC-MS experiments. The presence of malaoxon was proven by the protonated molecule at *m/z* 315, while the second impurity showed the protonated molecule at *m/z* 331 with the exact mass of 331.0412 Da and an elemental composition of $C_{10}H_{20}O_6PS_2$. This is a best fit to malathion, why this impurity also should represent the thiono-thiolo rearrangement product iso-malathion [27], revealing strong esterase inhibition properties.

The additional small zone detectable in the chlorfenvinphos standard provided identical MS data as the compound of the main zone, i.e., the protonated molecule at *m/z* 358.9775 Da (calculated 358.9774 Da) with the typical isotope pattern for three chlorines. Therefore, the by-product will be the E-isomer, which is described to be present at about 10 % in the technical product [27].

Composition of insecticide standards is depending upon their source and both storage time and storage conditions of stock solutions. It should be pointed out, that the standards' purity declared by the manufacturers and determined by HPLC-UV was generally given. Since an UV detector as well as a mass spectrometer is comparably sensitive for the main compounds and the impurities, trace impurities may be overlooked. The enzyme inhibition assay, however, preferably detects the impurities in case the inhibition constants of the main component and impurities differ by some orders of magnitude. For example, the application of 10 ng of parathion standard having an impurity of only 1 ‰ paraoxon, i.e., 10 pg, will result in two separated inhibition zones of identical intensity. Such situations are not only to be respected for HPTLC–EI, but also for HPLC coupled enzyme inhibition assays [28]. Trace impurities, however, may also be understood as additional markers proving the presence of an insecticide, sensitively detected by the respected esterases.

4.2.2 Calculation of enzyme inhibition factors

During esterase cuvette assays, the residual enzyme activity (% Ac) in the presence of an inhibitor as compared to a blank sample (100 %) is determined, when the initial slope of the kinetic curve (after substrate addition) is taken as the measure:

$$Ac(\%) = \frac{\Delta A_i}{\Delta A_c} \times 100\% \quad (1)$$

where ΔA_i and ΔA_c are the slopes of the kinetic curves for the sample and the blank control, respectively, observed during 2 min [11, 13, 19]. Inhibitions constants (k_i) are then calculated from the slope of the linear calibration curve obtained by plotting ln(Ac) [%] against the inhibitor concentration [mol L^{-1}], divided by the incubation time [min]:

$$k_i = -\frac{\ln(Ac)}{P_0} \times t[Lmol^{-1}\min^{-1}] \quad (2)$$

where P_0 is the initial inhibitor concentration [19].

During HPTLC-EI, however, the reaction kinetics of substrate conversion are not accessible, just the final situation. Additionally, there is only the peak area or the peak height of an inhibition zone available instead of %Ac. Therefore, inhibition constants derived from HPTLC analysis were calculated from the slope of the calibration curves using up to five different amounts per zone in the linear calibration range. Each value was determined as the average of at least three repeated plates, and the outliers test was performed according to Nalimov [29] for outliers on the level of *P* = 95%. Since the signal intensity (arbitrary units, AU) is dimensionless, the determined inhibition constants are based on the molar inhibitor amount per zone and the incubation time, expressed as mol^{-1} min^{-1} (**Table 1**), (which were named inhibition factors f_i to avoid confusions with the published inhibition constants k_i. The obtained data well reflect the inhibition power of the respective insecticides, as known from previous studies [11, 20, 21], and presented good correlations between inhibition factors and inhibition constants obtained from HPTLC and microtiter-plate enzyme inhibition assays, respectively (**Fig. 3**).

Table 1 Sensitivity data of HPTLC-EI assays for the studied insecticides (LOD and LOQ, limit of detection and quantification determined according to [30]; f_i, enzyme inhibition factor calculated from the slope of calibration curves divided by incubation time (30 min); N.I., no inhibition; RLE (rabbit liver esterase), BS2 (Bacillus subtilis esterase), CUT (cutinase from Fusarium solani pisi)).

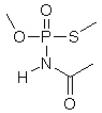
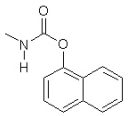
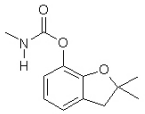
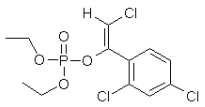
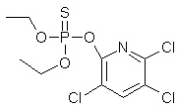
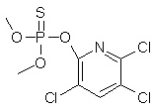
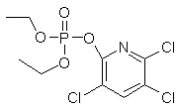
Insecticide	Structure formula	Enzyme	Calibration range [amount/zone]	R^2	LOD [ng/zone]	LOQ [ng/zone]	f_i [$mol^{-1}.min^{-1}$]	RSD [%] (n)
Acephate		RLE	N.I.	-	-	-	-	-
		BS2	N.I.	-	-	-	-	-
		CUT	N.I.	-	-	-	-	-
Carbaryl		RLE	3-15 ng	0.9940	3.6	5.3	2.1×10^{12}	12.2 (4)
		BS2	2-10 ng	0.9922	2.7	4.0	5.8×10^{12}	2.0 (3)
		CUT	20-100 ng	0.9946	22.9	33.4	2.2×10^{11}	11.3 (4)
Carbofuran		RLE	50-250 ng	0.9920	69.6	101	1.3×10^{11}	3.2 (3)
		BS2	20-100 ng	0.9906	29.9	43.5	1.8×10^{11}	11.4 (4)
		CUT	1-5 µg	0.9887	1632	2363	1.1×10^{10}	11.6 (3)
Chlorfenvinfos		RLE	10-50 pg	0.9914	0.014	0.021	6.0×10^{14}	13.8 (4)
		BS2	100-500 pg	0.9932	0.128	0.201	2.0×10^{14}	14.7 (4)
		CUT	60-300 ng	0.9952	65.1	95.2	3.2×10^{11}	14.7 (4)
Chlorpyrifos		RLE	50-250 ng	0.9942	59.8	89.1	5.0×10^{11}	9.9 (4)
		BS2	100-500 ng	0.9958	102	150	2.2×10^{11}	12.2 (4)
		CUT	200-1000 ng	.09904	301	436	1.0×10^{11}	14.4 (4)
Chlorpyrifos-methyl		RLE	200-1000 ng	0.9958	203	337	2.4×10^{11}	15.4 (4)
		BS2	N.I.	-	-	-	-	-
		CUT	0.5-2.5 µg	0.9918	697	1014	7.2×10^{10}	5.0 (3)
Chlorpyrifos oxon		RLE	10-50 pg	0.9956	0.010	0.015	2.2×10^{14}	11.9 (3)
		BS2	200-1000 pg	0.9958	0.206	0.330	7.0×10^{13}	8.1 (3)
		CUT	100-500 pg	0.9952	0.110	0.170	7.5×10^{13}	9.1 (3)

Table 1 (continued)

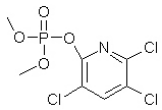
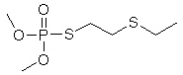
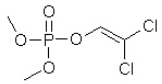
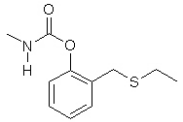
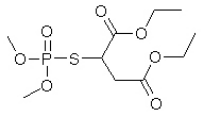
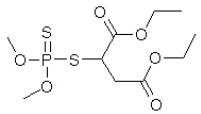
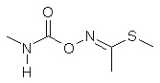
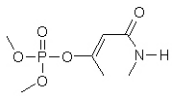
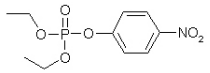
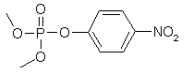
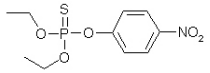
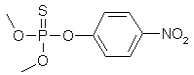
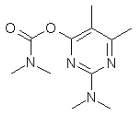
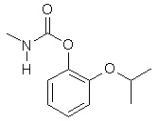
Insecticide	Structure formula	Enzyme	Calibration range [amount/zone]	R^2	LOD [ng/zone]	LOQ [ng/zone]	f_i [$mol^{-1}.min^{-1}$]	RSD [%] (n)
Chlorpyrifos-methyl oxon		RLE	200-1000 pg	0.9946	0.231	0.340	2.0×10^{13}	8.5 (3)
		BS2	1-5 ng	0.9958	1.0	1.5	8.0×10^{12}	9.9 (4)
		CUT	200-1000 pg	0.9908	0.295	0.502	3.2×10^{13}	7.2 (4)
Demeton-S-methyl		RLE	1-5 ng	0.9958	1.0	1.5	6.9×10^{12}	12.7 (4)
		BS2	0.5-2.5 µg	0.9944	588	910	3.8×10^{10}	10.4 (4)
		CUT	N.I.	-	-	-	-	-
Dichlorvos		RLE	1-5 pg	0.9924	0.0014	0.0020	2.2×10^{15}	16.0 (3)
		BS2	40-200 pg	0.9920	0.056	0.081	2.1×10^{14}	10.6 (4)
		CUT	1-5 ng	0.9908	1.5	2.2	5.1×10^{12}	6.7 (4)
Ethiofencarb		RLE	2-10 ng	0.9942	2.4	3.8	3.2×10^{12}	4.9 (3)
		BS2	10-50 ng	0.9940	12.0	17.6	4.8×10^{11}	9.8 (4)
		CUT	1-5 µg	0.9938	1220	1780	5.0×10^{9}	10.9 (4)
Malaoxon		RLE	1-5 ng	0.9912	1.5	2.1	3.9×10^{12}	3.5 (3)
		BS2	5-25 ng	0.9932	6.4	9.4	3.8×10^{12}	4.8 (3)
		CUT	0.5-2.5 µg	0.9849	936	1350	5.1×10^{10}	6.7 (3)
Malathion		RLE	0.5-2.5 µg	0.9914	714	1038	1.5×10^{10}	9.2 (3)
		BS2	N.I.	-	-	-	-	-
		CUT	N.I.	-	-	-	-	-
Methomyl		RLE	1-5 ng	0.9938	1.2	1.8	2.7×10^{12}	13.1 (3)
		BS2	10-50 ng	0.9950	11.2	16.4	3.2×10^{11}	14.8 (4)
		CUT	5-25 ng	0.9936	6.2	9.1	1.6×10^{12}	10.0 (4)

Table 1 (continued)

Insecticide	Structure formula	Enzyme	Calibration range [amount/zone]	R^2	LOD [ng/zone]	LOQ [ng/zone]	f_i [$mol^{-1}.min^{-1}$]	RSD [%] (n)
Monocrotofos		RLE	10-50 ng	0.9958	10.2	15.0	2.5×10^{11}	9.7 (4)
		BS2	3-15 ng	0.9954	3.2	4.7	2.3×10^{12}	5.1 (3)
		CUT	200-1000 ng	0.9914	296	430	4.9×10^{10}	13.6 (4)
Paraoxon		RLE	1-5 pg	0.9933	0.0013	0.0019	2.8×10^{15}	8.5 (4)
		BS2	3-15 pg	0.9924	0.004	0.007	2.5×10^{15}	5.8 (4)
		CUT	0.4-2.0 ng	0.9908	0.590	0.860	1.8×10^{13}	9.8 (4)
Paraoxon-methyl		RLE	30-150 pg	0.9946	0.035	0.051	1.1×10^{14}	5.5 (3)
		BS2	200-1000 pg	0.9956	0.210	0.338	2.9×10^{13}	12.8 (4)
		CUT	10-50 ng	0.9930	12.9	18.9	1.0×10^{12}	1.1 (3)
Parathion		RLE	10-50 ng	0.9922	13.7	19.9	4.5×10^{12}	5.8 (3)
		BS2	10-50 ng	0.9912	14.5	21.1	1.7×10^{12}	3.4 (3)
		CUT	170-850 ng	0.9950	188	274	9.1×10^{10}	5.8 (3)
Parathion-methyl		RLE	2-10 ng	0.9918	2.8	4.4	6.1×10^{12}	15.4 (3)
		BS2	100-500 ng	0.9948	113	166	2.8×10^{11}	7.3 (4)
		CUT	N.I.	-	-	-	-	-
Pirimicarb		RLE	10-50 ng	0.9951	11.0	16.1	7.1×10^{11}	13.1 (4)
		BS2	10-50 ng	0.9952	10.9	15.9	1.0×10^{12}	12.5 (4)
		CUT	1-5 µg	0.9916	1413	2054	1.1×10^{10}	12.7 (4)
Propoxur		RLE	15-75 ng	0.9956	15.5	22.8	9.4×10^{11}	6.0 (3)
		BS2	100-500 ng	0.9958	102	149	1.4×10^{11}	2.9 (3)
		CUT	1-5 µg	0.9807	2105	3027	1.3×10^{10}	8.9 (4)

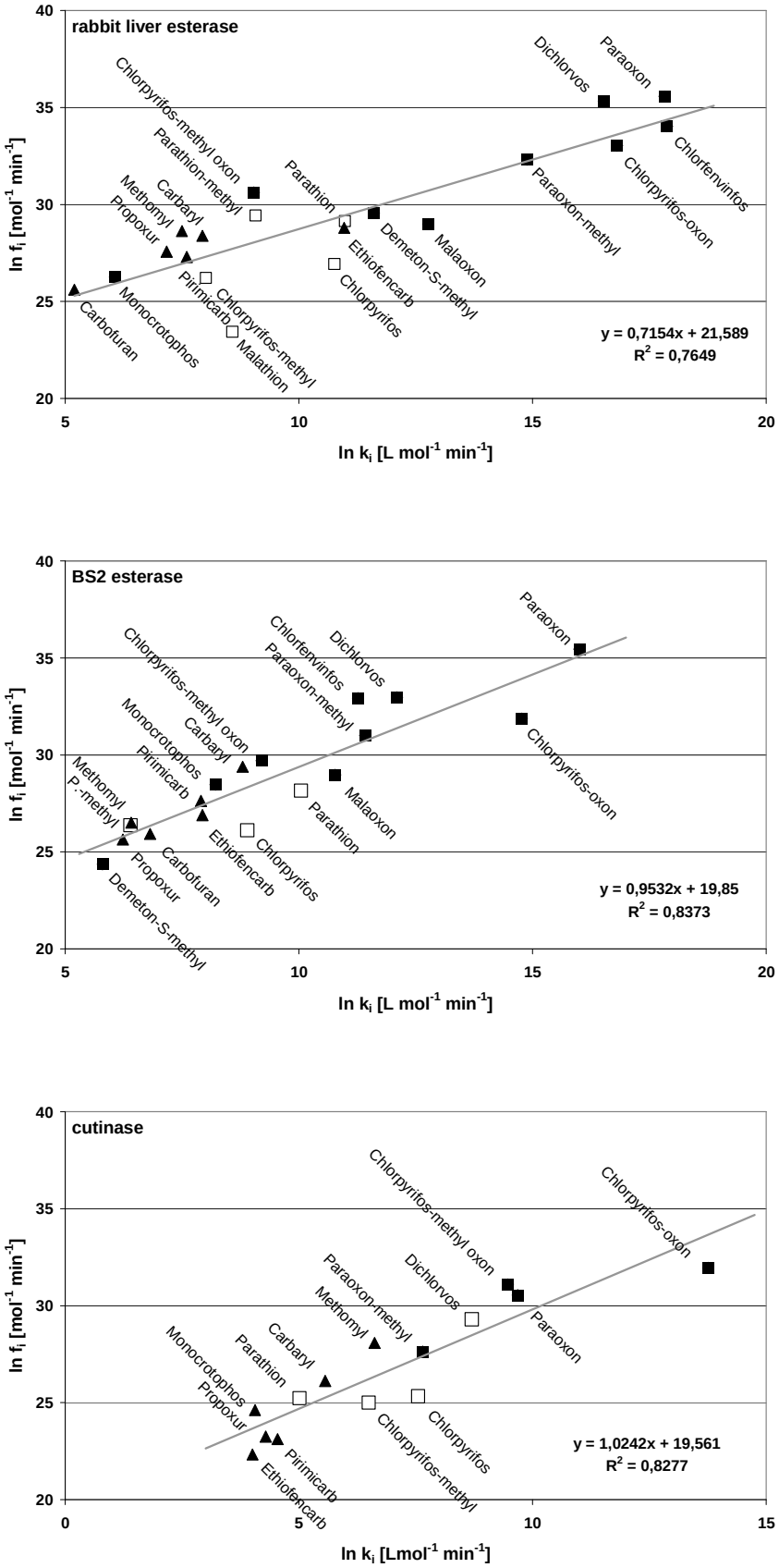


Fig. 3. Comparison of enzyme inhibition constants (ki) (data from [20-21]) and HPTLC enzyme inhibition factors (fi) of insecticidal carbamates (▲), phosphates (■) and thiophosphates (□).

4.2.3 Limits of detection and quantification

Limits of detection (LOD) and limits of quantification (LOQ) were calculated from the calibration curves according to [30] and are expectedly related to inhibition factors. Acephate had generally no inhibitory effect on RLE, BS2 or CUT, but RLE was inhibited by all other studied insecticides and almost recorded best results in terms of sensitivity as compared to BS2 and CUT. For the carbamates carbaryl and carbofuran, the BS2 esterase reacted slightly more sensitive than RLE. As known from choline esterases [11], organophosphate oxons showed the strongest inhibition toward all three esterases, while thions in general were also effective inhibitors, but at increased amounts per zone. This is a great advantage over choline esterases, which are generally not inhibited by thions, since they can be directly identified without former oxidation into the respective oxons. As compared to RLE and BS2, cutinase is the enzyme of lowest sensitivity.

Against this background, LODs and LOQs at the low picogram range were obtained for strongest inhibitors as represented by the most organophosphorus oxons in combination with the most sensitive esterases, RLE and BS2 (**Table 1**). Thiono phosphates were detectable by RLE in the nanogram range, except malathion, which only is a weak inhibitor for RLE and was even ineffective on BS2 and cutinase. Insecticidal carbamates generally were detectable by RLE and BS2 in the low nanogram range. For cutinase as the esterase of highest stability against the studied insecticides, amounts of approximately 1 µg/zone are needed to be detected. This may be taken as an advantage, since typically it is unknown to which extend residues or contaminants are present in a sample, thus choosing two enzymes of high (RLE) and low (CUT) sensitivity for a first rapid screening.

4.2.4 Application to apple juice and tap water analysis

Following the QuEChERS method [2, 3] for the extraction of fruits and vegetables, an extract of 1 g sample in 1 mL acetonitrile is obtained. In consideration of the lowest residue limit of 0.01 mg/kg generally being effective for non-registered pesticides and for baby food or organic food, a pesticide concentration of 10 ng/mL is obtained. Such a concentration is quite sufficient without any concentration step to detect strong inhibitors like organophosphorus oxons, when 10 µL extract are applied

onto the HPTLC plate. This was shown by spiking an apple juice with paraoxon at a level of even 0.001 mg/L and resulting in a mean recovery of 103% (**Table 2**).

Organophosphorus thions will also be detectable at the same low level, if an oxidation step by bromine vapour is applied after chromatography, which is presently under study. On the other hand, detectability of thions and also carbamates at a level of 0.01 mg/L requires concentration of the extract or application of volumes >10 µL to obtain amounts of about 10 ng/zone (**Table 2**).

Table 2 Recoveries of organophosphorus pesticides from spiked apple juice and drinking water. For the determination of parathion and chlorpyrifos, the acetonitrile extracts were 10-fold concentrated. Rabbit liver esterase was exemplarily used as enzyme source.

Sample	Pesticide	Spiking level (mg/L)	Recovery (%)	RSD (%) (n=3)
Apple juice	Paraoxon	0.001	103	3.7
	Parathion	0.05	71	5.9
	Chlorpyrifos	0.5	95	12.6
Water	Paraoxon	0.001	99	10.9
	Parathion	0.05	112	2.0
	Chlorpyrifos	0.5	106	18.3

Taking the same complications into account, HPTLC-EI can also be applied to the determination of respective contaminants in drinking water (**Table 2**). However, regarding the general European limit of 0.1 µg/L for any pesticide, a solid phase extraction typically applied for the analysis of contaminants in drinking water is essential and results in enrichment factors of up to 1000, i.e., 100 ng/100 µL, which is a quite sufficient concentration to detect all organophosphorus and carbamate insecticides under study except the non-inhibiting Acephate.

5 Conclusions

The newly developed HPTLC–EI assay with rabbit liver esterase, BS2 esterase and cutinase was successfully applied to a selection of 20 representative organophosphorus and carbamate insecticides, while acephate generally was not able to inhibit the used esterases. It provides a very sensitive system of effect-directed analysis [31] coupled to planar chromatography for rapid screening of many samples in parallel, including quantification at trace levels. Using RLE and BS2, limits of detection were lower than reached before by HPTLC–choline esterase assays

[26]. While thiono phosphates are also directly detectable, sensitivity can be further improved by a simple oxidation step with bromine vapor on the plate [26].

Chromatographic separation partly showed the presence of trace by-products of strong inhibitory power in commercial standards. Therefore, enzyme inhibition factors determined after HPTLC separation refer to the insecticide itself in contrast to the mixed-mode inhibition obtained in cuvette assays, unless a specific standard purification is performed.

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CHAPTER IV

EFFECT OF BROMINE OXIDATION ON HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY MULTI-ENZYME INHIBITION ASSAY OF ORGANOPHOSPHATES AND CARBAMATE INSECTICIDES

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Key words:

Bromine oxidation, high-performance thin-layer chromatography, enzyme inhibition, effect-directed analysis, cutinase, rabbit liver esterase, *Bacillus subtilis* (BS2) esterase, inhibition factor, organophosphorus insecticides, carbamate insecticides.

1 Abstract

Following high-performance thin-layer chromatography, thiophosphate pesticides, which inhibit choline esterases, are detectable using a multi-enzyme inhibition assay (HPTLC-EI) based on rabbit liver esterase (RLE), *Bacillus subtilis* (BS2) esterase, or cutinase (from *Fusarium solani pisi*). Because choline esterase inhibition is more effective after conversion of thiophosphate thions into their corresponding oxons, a pre-oxidation step was added to the HPTLC-EI assay. Bromine vapour was found to be more effective than iodine or UV irradiation for oxidation. Following oxidation, the inhibitory strength of parathion, parathion-methyl, chlorpyrifos, chlorpyrifos-methyl, and malathion, expressed as HPTLC enzyme inhibition factors (f_i), increased by approximately 2 orders of magnitude. In contrast, bromine oxidation of organophosphate and carbamate insecticides resulted in a slight reduction in their inhibition factors, due to partial bromination and degradation of the parent compounds, while bromine oxidation increased the inhibition factors for demeton-S-methyl and propoxur. Apple juice and drink water samples spiked with paraoxon (0.001 mg/L), parathion (0.05 mg/L), and chlorpyrifos (0.5 mg/L) were used to test the HPTLC-EI system, resulting in mean recoveries of 95-106% and 91-102% for RLE and cutinase, respectively.

2 Introduction

Although several different validated methods for rapid sample extraction and clean-up are currently available for routine determination of pesticide residues in food and feed (see for example [1-5]), the so-called QuEChERS methods [6] are generally preferred. For target-oriented analysis, both gas and liquid chromatography, coupled to mass spectrometry are generally used [7]. However, effect-directed analysis approaches, which are high-throughput tools to separate positive from negative samples for further instrumental analyses [8], are attractive for food control.

Because organophosphate and carbamate based insecticides are both choline esterase inhibitors [9], they represent ideal targets for effect-directed analysis using enzyme inhibition assays. Choline esterases from different animal sources have been used in cuvette, microtiterplate [10-13], biosensor [14-15], and thin-layer chromatography (TLC) based assays [16]. In addition to choline esterases,

microtiterplate multi-enzyme inhibition assays using rabbit liver esterase (RLE), Bacillus subtilis (BS2) esterase, or cutinase (from Fusarium solani pisi) have been previously reported [17-20], and recently have been successfully incorporated in high-performance thin-layer chromatography-enzyme inhibition assays (HPTLC-EI) [21-22].

Organophosphate thion containing pesticides, in which a sulphur atom is directly attached to a phosphorus atom (P=S), generally have lower mammalian toxicities and negligible anti-cholinesterase activities [23]. The inhibitory strength and toxicity of these compounds can be increased by conversion of the thion into the corresponding oxon (P=O), which can occur: biologically, in insects and mammals [23]; through the action of microorganisms [24]; photochemically [25]; or chemically, using suitable oxidizing agents [16, 26-28].

Although N-bromosuccinimide has been used for water sample testing in choline esterase inhibition assays [29], this reagent was not effective in tests of organic matrices, such as plant food [17]. In food sample extracts, enzymatic oxidation by chloroperoxidase has been shown to be a suitable alternative [17], which recently was directly applied for testing fruit juice samples, coupled with biosensor detection [30]. For TLC-HPTLC based assays, oxidation by both bromine vapour and UV irradiation were the most commonly used procedures, although bromine was described to be more efficient than UV irradiation in converting pesticides to more potent inhibitors [31].

The aim of the present study was to test the effect of adding an additional step on the sensitivity of organophosphate thion pesticide detection (e.g. chlorpyrifos, chlorpyrifos methyl, malathion, parathion, and parathion methyl) using a recently published HPTLC-EI assay [22]. In addition, the effect of this additional oxidation step on other organophosphorus and carbamate insecticides was examined. Finally, organophosphate thion pesticide spiked apple juice and drinking water samples were used as test cases for our optimised HPTLC-EI assay.

3 Experimental

3.1 Materials

Silica gel 60 F_{254} HPTLC glass plates (20 cm x 10 cm) and sodium chloride (≥99.5%) were obtained from Merck (Darmstadt, Germany). Pesticide standards (carbofuran, chlorfenvinfos, demeton-S-methyl, dichlorvos, methomyl, monocrotofos, paraoxon, and paraoxon methyl) were purchased from Riedel-de Haën (Taufkirchen, Germany), (acephate, carbaryl, chlorpyrifos, chlorpyrifos methyl, chlorpyrifos methyl oxon, chlorpyrifos oxon, pirimicarb, and propoxur) Dr. Ehrenstorfer GmbH (Augsburg, Germany), and (ethiofencarb, malaoxon, malathion, parathion, and parathion methyl) from Sigma-Aldrich (Taufkirchen, Germany). Cutinase (EC 3.1.1.74) from F. solani pisi (lyophilised, protein content 75%, 356 U/mg protein [18]) was kindly provided by Unilever Research Laboratory (Vlaardingen, The Netherlands). *B. subtilis* (BS2) esterase (14.1 U/mg) was purchased from Julich Chiral Solutions (Julich, Germany). Rabbit liver esterase (lyophilised, 80 U/mg) protein), bovine serum albumin (BSA, >98% pure), fast blue salt B (dye content, ~95%), α-naphthyl acetate (≥98%), anhydrous magnesium sulphate (reagent grade, ≥97%), and bromine (>98.0%) were purchased from Sigma-Aldrich (Taufkirchen, Germany). Ultra pure water was obtained using a Synergy system (Millipore, Schwalbach, Germany). Tris-(hydroxymethyl)-aminomethane (TRIS, ≥99.9%) and dichloromethane (≥99.9%) were obtained from Carl Roth GmbH & Co. (Karlsruhe, Germany), primuline was obtained from Division Chroma (Muenster, Germany). Formic acid (reagent grade, 98%), chloroform (>99%) and acetonitrile (HPLC grade) were purchased from Fisher Scientific (Schwerte, Germany). Methanol, ethanol, n-hexane, acetone, and ethyl acetate (analytical grade) were obtained from Merck and distilled before use. BONDESIL-PSA (40 µm) was obtained from Varian (Darmstadt, Germany).

3.2 Solutions

Pesticide stock solutions (1 g/L) were prepared in methanol and diluted with methanol to working concentration of 10 mg/L, 100 µg/L, and 1 µg/L. Enzyme stock solutions were prepared by individually dissolving 5 mg cutinase, 50 mg BS2 esterase, or 9 mg rabbit liver esterase in 10 mL Tris-HCl buffer (0.05 M, pH 7.8) containing 0.1% BSA, and stored in a freezer. Working solutions were prepared by diluting 1 mL of each stock solution in 50 mL of the same buffer. Substrate solutions

were prepared by mixing 30 mL α-naphthyl acetate solution (2.5 g/L in ethanol) and 60 mL fast blue salt B solution, both freshly prepared immediately before use. Primuline dipping solution (0.5 g/L) was prepared in acetone/water (4+1).

3.3 Planar chromatography

HPTLC plates were pre-washed with methanol, dried at 100℃ for 20 min, and stored in a desiccator. Pesticide working standard solutions were applied at desired volumes onto HPTLC plates using an automatic TLC Sampler 4 (ATS4, CAMAG, Muttenz, Switzerland), as 5 mm bands, 10 mm from the lower edge and the left side, with 10 mm spacing between tracks. After drying for 5 min with hot air, plates were developed in an Automatic Developing Chamber 2 (ADC2, CAMAG), to a distance of 80 mm from the lower edge, using n-hexane/ethyl acetate/dichloromethane (65:20:15) as the mobile phase, without tank saturation. The migration time was approximately 35 min, including 5 min drying. Three chromatography solvent systems were used: group 1 (paraoxon, paraoxon-methyl, malaoxon, dichlorvos, chlorfenvinfos, ethiofencarb, parathion and parathion-methyl), separated with ethyl acetate/n-hexane (37/63, v/v); group 2 (monocrotofos, pirimicarb, methomyl, carbofuran, carbaryl, and chlorpyrifos oxon), separated with ethyl acetate/chloroform (10/90, v/v); group 3 (acephate, demeton-S-methyl, chlorpyrifos-methyl oxon, malathion, chlorpyrifos-methyl, and chlorpyrifos), separated with n-hexane/acetone/dichloromethane 75/10/15, v/v/v).

3.4 Oxidation

HPTLC plates were oxidised in a twin-trough chamber, by placing plates vertically in one trough and adding two drops of bromine to the second trough. The top cover chamber was tightly closed and oxidation was performed for 5 min. Excess adsorbed bromine was removed according to the method of Ackermann [25], by heating at 60℃ (20 min) using a TLC plate heater I II (CAMAG, Muttenz, Switzerland) in a well-ventilated fume cupboard.

For iodine oxidation, iodine (100 mg) was placed in one trough, and the covered chamber was equilibrated overnight to allow formation of a homogenous iodine climate. The HPTLC plate was then placed into the second trough. UV irradiation was performed using a Suntest CPS+ system (Atlas Material Testing Technology

GmbH, Linsengericht, Germany) at 350 W/m^2 (xenon lamp, equipped with a combination of coated quartz and standard solar glass, air cooling, and a standard black temperature of 35℃).

3.5 Detection

The developed, oxidised, and heated plates were cooled to room temperature for 1 min and then dipped into enzyme solution for 2 s at a dipping speed of 1 cm/s, using a TLC Immersion Device III (CAMAG), followed by horizontal incubation for 30 min at 37℃, in a humid chamber containing water. The plate was then immersed in freshly prepared substrate solution for 1 s, at a dipping speed of 1 cm/s, followed by 3 min reaction time (plates were incubated horizontally). Reactions were stopped by heating at 50℃ for 5-7 min until dryness using a TLC Plate Heater III (CAMAG).

3.6 Documentation and evaluation

Images of developed plates were captured using a DigiStore 2 documentation system (CAMAG), in reflectance mode under visible light illumination. Plate peak areas were quantitated by densitometry using a TLC Scanner 3 (CAMAG), via measurements at 533 nm in fluorescence mode without edge filtering (instrument setting to obtain positive peaks from light zones on a dark background). Obtained data were processed using winCATS software, version 1.4.4 (CAMAG). For oxidation experiments, plate images were captured under UV illumination at 254 nm, and, after dipping in primuline solution, at 366 nm.

3.7 High-performance thin-layer chromatography–mass spectrometry (HPTLC/MS)

Standards were applied on two plates and developed under the same conditions. One plate was subjected to EI assay, and the detected zones of inhibition were marked with a pencil on the second plate. A mixture of methanol/formic acid (0.1% [95:5 vol. %] was used for zone extraction via a TLC-MS interface (CAMAG), at a flow rate of 0.1 mL min^{-1} (provided by an HPLC 1100 pump, Agilent Technologies, Waldbronn, Germany). A G1956B MSD single quadrupole mass spectrometer, equipped with an electrospray interface (ESI) and ChemStation B.02.01 SR2 software (Agilent Technologies) was used. For positive electrospray

ionisation, the mass spectrometer was operated using the following parameters: drying gas temperature, 300 ℃; drying gas flow rate, 10 $Lmin^{-1}$; capillary voltage, 4.0 kV; nebuliser gas pressure, 30 psi (207 kPa); fragmentor voltage, 100 V; gain, 1; threshold, 1; step-size, *m*/*z* 0.05; time filter, off; scan data storage, full.

3.8 Sample extraction

Apple juice (obtained from the local market) and tap water samples were individually spiked with a methanol solution containing paraoxon, parathion, or chlorpyrifos, and extracted using the QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) procedure [1-2], without the addition of buffer salts. Apple juice extracts were cleaned-up using primary secondary amine (PSA). Briefly, 10 mL of sample was vigorously shaken with 10 mL acetonitrile in a 50-mL centrifuge tube for 1 min. After addition of a mixture of 1 g sodium chloride and 4 g anhydrous magnesium sulphate, the tube was shaken for 1 minute and then centrifuged for 5 min at 3500 xg. For detection of cutinase, 10 mL of the resulting supernatant was concentrated to 1 mL under a gentle stream of nitrogen. For apple juice samples, 1 mL of acetonitrile extract, obtained after dilution or concentration, was shaken with 25 mg PSA and 150 mg magnesium sulphate for 30 s and centrifuged; extracts were then acidified with 5% formic acid in acetonitrile (10 µL added to each 1 mL acetonitrile extract). Finally, extracts (10 µL) were applied in triplicate onto a HPTLC plate, along with a set of calibration standards.

4 Results and discussion

4.1 Bromine oxidation versus iodine and UV irradiation oxidation

Ideally, oxidation of thiono phosphates would be performed only until the described oxon is obtained, while avoiding formation of by-products. However, Mendoza et al. observed formation of products other than oxons during oxidation of different insecticides, both by UV irradiation and bromine oxidation [31]. In addition to strong oxidation properties, bromine is an effective halogenation reagent of olefinic and aromatic systems; and these side-reactions were expected on the HPTLC plates following oxidation of the insecticides under study. Thus, in an attempt to minimise by-product formation, iodine (which is the least reactive halogenating agent, but also a weaker oxidant than bromine) was tested. However, iodine vapour treatment of

start zones for up to 60 min failed to yield any P=S/P=O conversion for parathion and parathion-methyl. Even when the plate was incubated in the iodine chamber overnight, only small amounts of the corresponding oxons could be detected. In contrast, application of bromine vapour for 5 min completely transformed all five thions into the corresponding oxons (**Fig. 1S**). The same poor oxidation results were obtained with UV irradiation. UV irradiation stronger than provided by the Suntest system was not tested, because organophosphorus insecticides are easily photodegraded [32].

In addition, the present study revealed impurities in commercial standards of paraoxon, paraoxon-methyl, chlorpyrifos oxon, and chlorpyrifos-methyl oxon, by corresponding phenol constituents (4-nitrophenol, Rf 0.36, and trichloropyridinol, Rf 0.25), which were only visibly after more sensitive detection using primuline (**Fig. 1S**). During iodine and bromine treatment, 4-nitrophenol was completely halogenated into compounds with Rf < 0.1, while bromine treatment of trichloropyridinol resulted in reaction products with Rf 0.2-0.7 that were not detectable by HPTLC-EI. Although bromine treatment yielded a single side-product from chlorpyrifos and chlorpyrifos methyl standards (at Rf 0.35), which was easily detectable using primuline, this by-product was not an esterase inhibitor. Compared to plate images of untreated standards, bromine oxidation, and to a lesser extent iodine and UV irradiation-mediated oxidation, resulted in some compounds remaining at the start zone (**Fig. 1S**). In addition to oxidation, this effect may be due to heat treatment used to evaporate bromine and iodine from the plates.

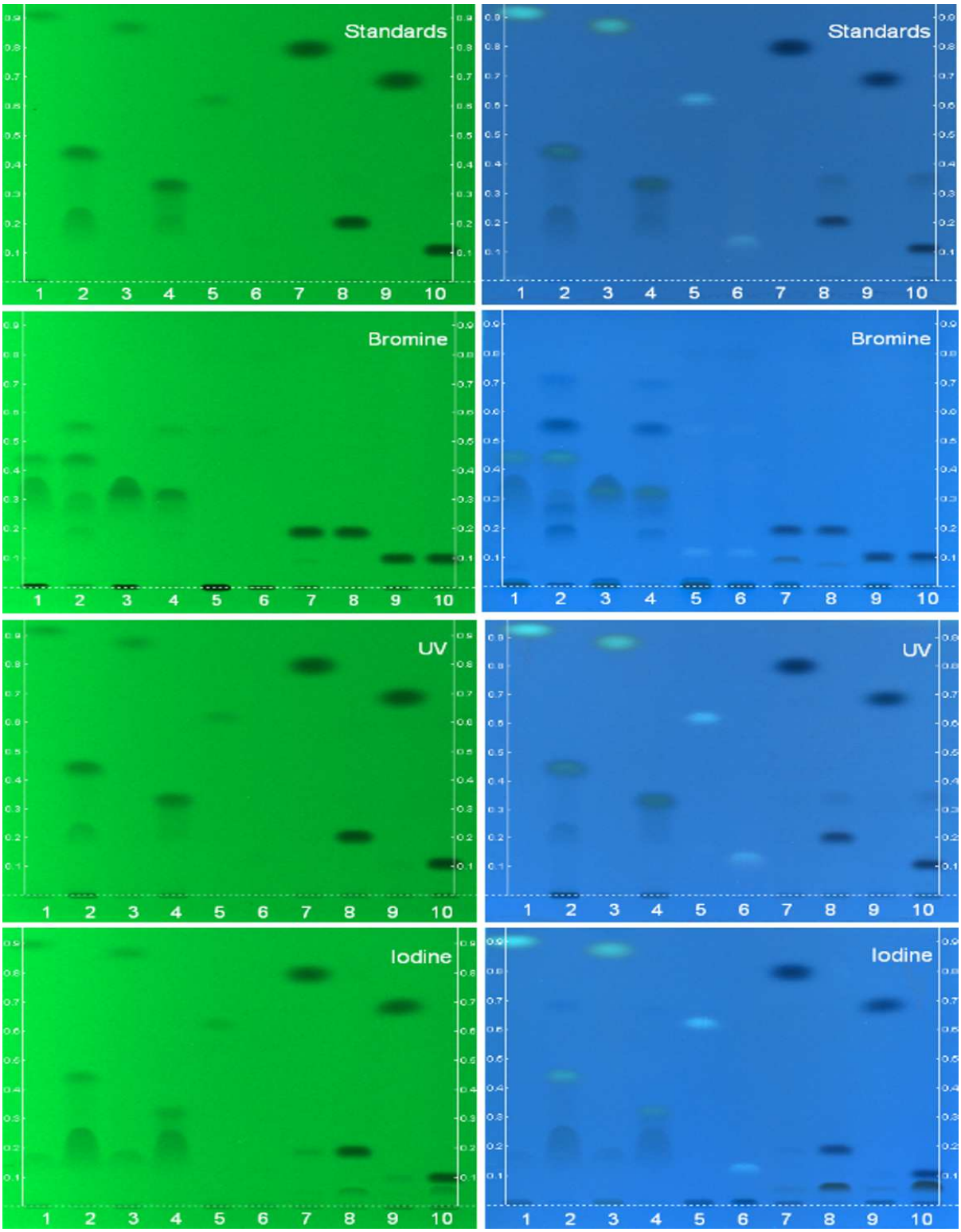


Fig. 1S. Plate images of organophosphates treated with bromine, UV irradiation, or iodine after sample application at the start zones, followed by development (n-hexane/ethyl acetate/dichloromethane 65/20/15). Treated plates are compared to untreated standards (top): 1. chlorpyrifos (20 µg), 2. chlorpyrifos oxon (20 µg), 3. chlorpyrifos-methyl (20 µg), 4. chlorpyrifos-methyl oxon (20 µg), 5. malathion (20 µg), 6. malaoxon (20 µg), 7. parathion (10 µg), 8. paraoxon (10 µg), 9. parathion-methyl (10 µg), 10. paraoxon-methyl (10 µg). Detection was performed using UV illumination at 254 nm (left) and, after dipping into primuline reagent, at 366 nm (right).

Although rather large amounts of sample (10 µg or 20 µg) were applied onto the plates for initial experiments, to enable detection under UV illumination, the oxidation experiments were repeated with insecticides applied in smaller quantities, and detection was possible in the nanogram range by enzyme inhibition assay. Bromine or iodine treatment of the start zones was performed for different time intervals, samples were assayed HPTLC-EI, and zones containing the desired oxons were scanned. For malathion, parathion and parathion-methyl, bromine vaporisation yielded maximum peak areas within a few minutes, after which the oxon peak areas started to decrease (**Fig. 1a**). In contrast, yields of chlorpyrifos oxon and chlorpyrifos-methyl oxon continued to increase even after up to 20 minutes of bromine treatment. During iodine vaporisation, oxon peak areas generally increased for up to 10 hours without reaching a maximum (**Fig. 1b**). In conclusion, UV irradiation and iodine do not appear to be mild alternatives for bromine oxidation. In fact, based on our results, a 5 minutes bromine oxidation treatment was optimal; and, thus, was used for all remaining experiments to determine enzyme inhibition factors.

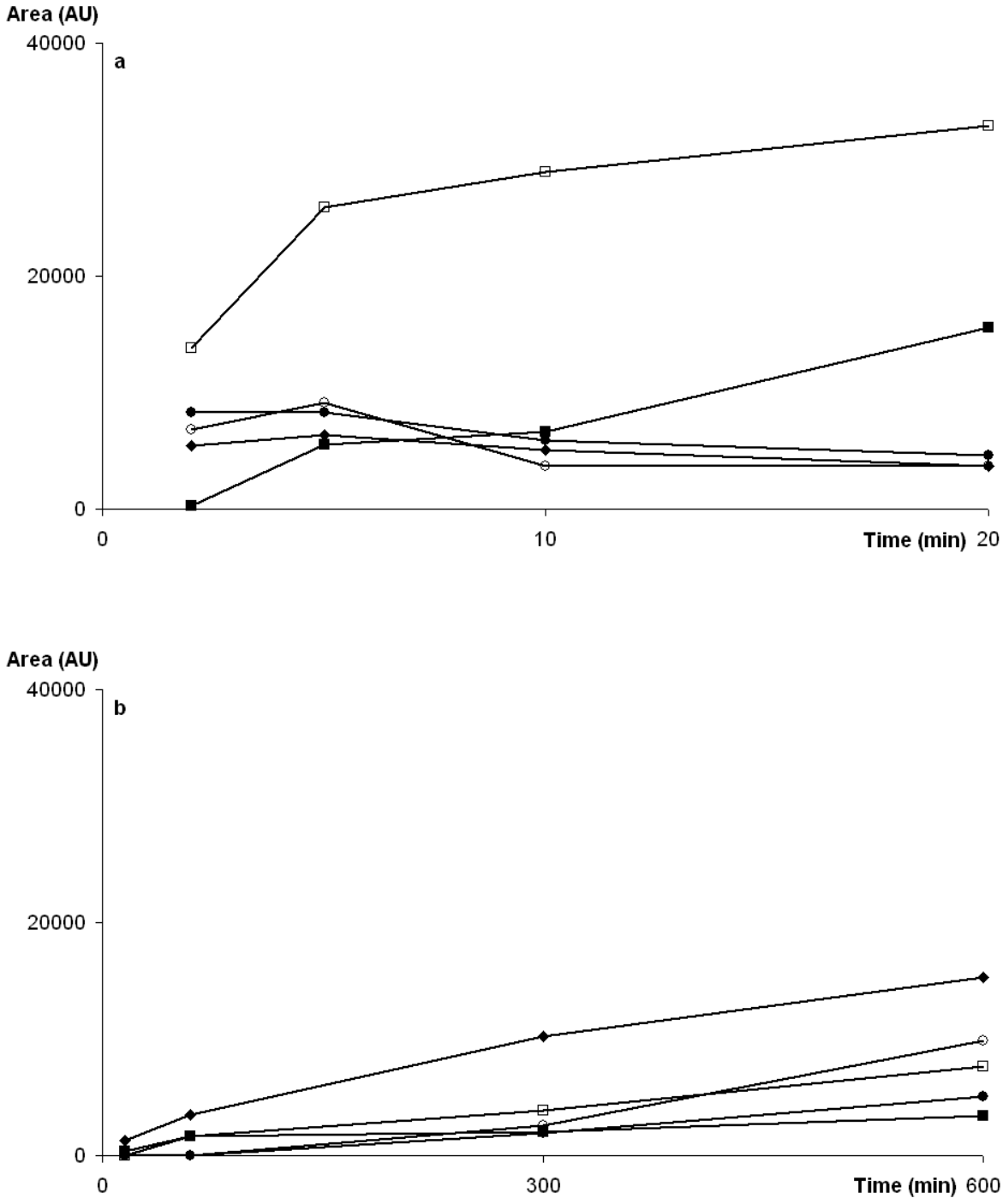


Fig. 1. Time course of organophosphorus oxons formation from chlorpyrifos methyl (□, 1000 ng), chlorpyrifos (■, 10 ng), malathion (♦, 500 ng), parathion (●, 0.2 ng), and parathion-methyl (○, 5 ng), determined by HPTLC-EI assay using BS2 esterase as the enzyme source. Oxidation was performed before chromatography, by bromine (a) and iodine (b) treatment.

4.2 Enzyme inhibition factors

The effects of bromine oxidation on all insecticides tested in our previous study [22] were evaluated by comparing HPTLC enzyme inhibition factors between oxidised and non-oxidised insecticides (**Table 1**). Each value represents the average of at least three repeated plates, and outliers were identified using Nalimov's outlier test [33]. As expected, the sensitivity of the assay for the five thiono phosphates tested was significantly improved by oxidation for all three esterases used. In contrast, with the exception of a few insecticides, all others (including the corresponding oxons) were more or less negatively affected. Interestingly, both demeton-S-methyl and propoxur became stronger inhibitors after bromine treatment, while the same effect was observed for methomyl, but only in the presence of cutinase. Bromine treatment also improved the sensitivity of the assay for carbofuran against RLE and BS2, but prevented detection by cutinase. Bromine treated acephate, which is not an esterase inhibitor [22], had no inhibitory effects on any of the esterases.

Table 1 shows changes to the limits of detection (LOD) and quantification (LOQ) of the assay upon bromine treatment. Using the most sensitive esterase (RLE), LOD/LOQ ranged between 0.01 and 100 ng/zone for the strongest to the weakest inhibitors. Such sensitivity levels have never before been achieved by (HP)TLC-EI using choline esterases [16, 35, 36]. Based on our own experiences, the simplest UV detection requires micrograms amounts per zone (data not shown), if a chromophore absorbing at 254 nm is present at all in the pesticide to be detected. Rather sensitive detection of approximately 20 ng/zone was achieved for some thiophosphates using palladium chloride or 2,6-dibromoquinone-4-chloroimide [37]. In addition, Sherma and Bretschneider used 2,6-dichloroquinone-4-chloroimide and reported an LOQ of 200 ng/zone [38], while detection of sulphur-free compounds was also possible at approximately 20 ng/zone, following derivatisation with 4-(4-nitrobenzyl)pyridine. Lower detection limits of 0.1-10 µg/zone have been reported for 15 organophosphorus pesticides, following derivatisation with 9-methylacridine [39].

Unfortunately, for carbamate insecticides, no generic derivatisation procedure has been reported, while for aryl carbamates, typical derivatisation methods involve alkaline hydrolsis on the plate, followed by coupling the resulting phenols with

diazotized reagents. The resulting coloured zones enabled an LOD of 100 ng/zone [40]. In any case, the high sensitivity of the HPTLC-EI assay demonstrated in the present study is not currently possible using other detection techniques in planar chromatography. Importantly, our optimised assay also displays high, effect-directed sensitivity, while more general derivatisation reagents may also detect co-extracts, such phenols from the food sample.

Of course, HPTLC-EI cannot compete with the sensitivity and selectivity of modern GC/MS or LC/MS instruments, although the absolute amounts injected onto the columns are quite comparable [7]. However, HPTLC-EI does offer a selective, rapid and low-price screening approach. The analysis of 20 sample extracts on a plate requires a chromatographic run time of less than 5 minutes per sample, and only small volumes of solvents and reagents are consumed. Additionally, unknown inhibitors can be detected, which may not be included in the calibration set of MS methods.

Table 1 HPTLC-EI assay sensitivities for the studied insecticides following bromine treatment of the developed plates.

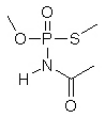
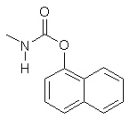
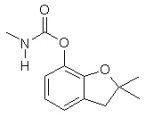
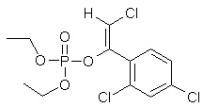
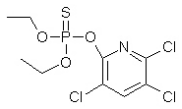
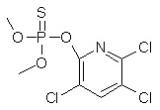
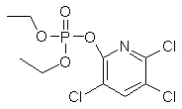
Insecticide	Structure formula	Enzyme	Calibration range [ng/zone]	R^2	LOD [ng/zone]	LOQ [ng/zone]	Enzyme inhibition factor f_i [$mol^{-1}.min^{-1}$]			
							After oxidation		Non-oxidized standard [22]	Dev.%
							f_i	RSD [%] (n)		
Acephate		RLE	N.I.	-	-	-	-	-	-	-
		BS2	N.I.	-	-	-	-	-	-	-
		CUT	N.I.	-	-	-	-	-	-	-
Carbaryl		RLE	10-50	0.9939	12	18	1.1×10^{12}	11.7 (3)	2.1×10^{12}	-47.6
		BS2	10-50	0.9901	15	20	1.0×10^{12}	11.7 (4)	5.8×10^{12}	-82.8
		CUT	100-500	0.9899	15	250	1.4×10^{11}	12.4 (4)	2.2×10^{11}	-36.4
Carbofuran		RLE	50-250	0.9941	60	90	1.9×10^{11}	10.3 (3)	1.3×10^{11}	+46.2
		BS2	20-100	0.9927	25	40	3.5×10^{11}	5.2 (3)	1.8×10^{11}	+94.4
		CUT	N.I.	-	-	-	-	-	1.1×10^{10}	0
Chlorfenvinfos		RLE	0.05-0.25	0.9952	0.05	0.08	1.3×10^{14}	12.2 (3)	6.0×10^{14}	-78.3
		BS2	5-25	0.9922	7	10	1.4×10^{12}	24.6 (4)	2.0×10^{14}	-99.3
		CUT	60-300	0.9914	85	140	1.2×10^{11}	10.0 (4)	3.2×10^{11}	-62.5
Chlorpyrifos		RLE	1-5	0.9935	1.3	1.9	1.8×10^{13}	1.5 (4)	5.0×10^{11}	+3500
		BS2	2-10	0.9937	2.5	3.7	1.6×10^{13}	11.2 (4)	2.2×10^{11}	+7173
		CUT	2-10	0.9935	2.5	4.5	1.6×10^{13}	20.6 (4)	1.0×10^{11}	+15900
Chlorpyrifos-methyl		RLE	5-25	0.9905	7	13	3.6×10^{12}	2.8 (4)	2.4×10^{11}	+1400
		BS2	200-1000	0.9903	304	440	1.5×10^{11}	25.5 (4)	-	(∞)
		CUT	100-500	0.9914	144	209	2.9×10^{11}	8.7 (3)	7.2×10^{10}	+303
Chlorpyrifos oxon		RLE	0.3-1.5	0.9854	0.5	0.9	3.0×10^{13}	8.0 (3)	2.2×10^{14}	-86.4
		BS2	0.5-2.5	0.9958	0.5	0.7	8.1×10^{12}	3.1 (3)	7.0×10^{13}	-88.4
		CUT	1-5	0.9946	1.2	1.7	5.5×10^{12}	20.0 (3)	7.5×10^{13}	-92.7

Table 1 (continued)

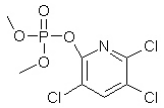
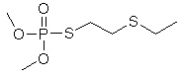
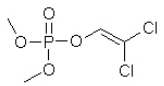
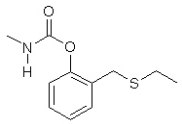
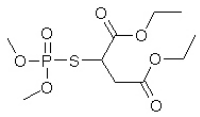
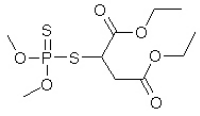
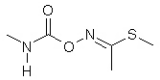
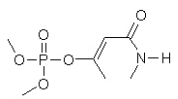
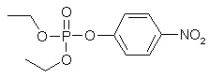
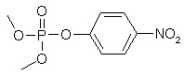
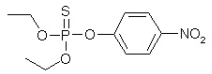
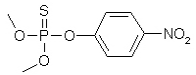
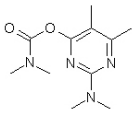
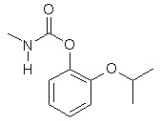
Insecticide	Structure formula	Enzyme	Calibration range [ng/zone]	R^2	LOD [ng/zone]	LOQ [ng/zone]	Enzyme inhibition factor f_i [$mol^{-1}.min^{-1}$]			
							After oxidation		Non-oxidized standard [22]	Dev.%
							f_i	RSD [%] (n)		
Chlorpyrifos-methyl oxon		RLE	1-5	0.9935	1.3	1.8	5.1×10^{12}	18.4 (4)	2.0×10^{13}	-74.5
		BS2	100-500	0.9937	124	210	1.9×10^{11}	14.0 (4)	8.0×10^{12}	-97.6
		CUT	20-100	0.9823	40	60	4.7×10^{11}	6.0 (4)	3.2×10^{13}	-98.5
Demeton-S-methyl		RLE	1-5	0.9947	1.1	1.9	9.6×10^{12}	3.3 (4)	6.9×10^{12}	+39.1
		BS2	1-5	0.9943	1.2	1.7	3.4×10^{12}	16.7 (4)	3.8×10^{10}	+8847
		CUT	500-2500	0.9900	770	1330	2.6×10^{10}	7.7 (4)	-	(∞)
Dichlorvos		RLE	0.05-0.25	0.9935	0.06	0.1	1.7×10^{14}	6.4 (3)	2.2×10^{15}	-92.3
		BS2	2-10	0.9956	2.1	3.0	2.1×10^{12}	17.7 (4)	2.1×10^{14}	-99.0
		CUT	50-250	0.9925	70	100	1.1×10^{11}	11.2 (4)	5.1×10^{12}	-97.8
Ethiofencarb		RLE	10-50	0.9919	15	20	6.7×10^{11}	22.6 (4)	3.2×10^{12}	-79.1
		BS2	50-250	0.9920	69	100	1.1×10^{11}	17.7 (4)	4.8×10^{11}	-77.1
		CUT	2000-10000	0.9926	2670	3880	4.0×10^{9}	7.6 (3)	5.0×10^{9}	-20.0
Malaoxon		RLE	10-50	0.9914	15	20	8.3×10^{11}	4.2 (4)	3.9×10^{12}	-78.7
		BS2	10-50	0.9923	15	20	6.0×10^{11}	18.9 (4)	3.8×10^{12}	-84.2
		CUT	1000-5000	0.9907	1480	2330	2.4×10^{10}	10.8 (4)	51×10^{10}	-52.9
Malathion		RLE	50-250	0.9917	70	120	4.7×10^{11}	6.3 (4)	1.5×10^{10}	+3033
		BS2	50-250	0.9873	85	125	3.6×10^{11}	14.0 (4)	-	(∞)
		CUT	1000-5000	0.9950	1110	1810	2.3×10^{10}	8.8 (4)	-	(∞)
Methomyl		RLE	5-25	0.9957	5.2	7.6	4.4×10^{11}	3.4 (4)	2.7×10^{12}	-83.7
		BS2	50-250	0.9884	80	140	8.6×10^{10}	26.6 (4)	3.2×10^{11}	-73.1
		CUT	1-5	0.9931	1.3	1.9	1.1×10^{13}	9.5 (3)	1.6×10^{12}	+588

Table 1 (continued)

Insecticide	Structure formula	Enzyme	Calibration range [ng/zone]	R^2	LOD [ng/zone]	LOQ [ng/zone]	Enzyme inhibition factor f_i [$mol^{-1}.min^{-1}$]			
							After oxidation		Non-oxidized standard [22]	Dev.%
							f_i	RSD [%] (n)		
Monocrotofos		RLE	50-250	0.9832	100	140	1.6×10^{11}	19.0 (4)	2.5×10^{11}	-36.0
		BS2	200-1000	0.9959	200	320	4.0×10^{10}	21.8 (4)	2.3×10^{12}	-98.3
		CUT	1000-5000	0.9932	1280	1870	1.4×10^{10}	5.9 (4)	4.9×10^{10}	-71.4
Paraoxon		RLE	0.005-0.025	0.9934	0.006	0.009	1.2×10^{15}	3.9 (4)	2.8×10^{15}	-57.1
		BS2	0.01-0.05	0.9900	0.015	0.02	1.1×10^{15}	10.8 (3)	2.5×10^{15}	-56.0
		CUT	0.4-2	0.9950	0.4	0.7	1.7×10^{13}	1.6 (4)	1.8×10^{13}	-5.6
Paraoxon-methyl		RLE	0.05-0.25	0.9958	0.05	0.07	6.2×10^{13}	12.6 (4)	1.1×10^{14}	-43.6
		BS2	0.3-1.5	0.9927	0.4	0.6	2.2×10^{13}	8.4 (4)	2.9×10^{13}	-24.1
		CUT	10-50	0.9947	15	20	3.7×10^{11}	1.3 (3)	1.0×10^{12}	-63.0
Parathion		RLE	0.05-025	0.9957	0.05	0.07	4.3×10^{14}	21.7 (4)	4.5×10^{12}	+9456
		BS2	0.05-0.25	0.9896	0.08	0.1	6.8×10^{14}	10.9 (4)	1.7×10^{12}	+39900
		CUT	1-5	0.9929	1.3	2.1	8.0×10^{12}	10.6 (4)	9.1×10^{10}	+8691
Parathion-methyl		RLE	0.1-0.5	0.9959	0.1	0.15	5.1×10^{13}	7.7 (3)	6.1×10^{12}	+736
		BS2	0.5-2.5	0.9944	0.6	0.9	1.9×10^{13}	12.1 (4)	2.8×10^{11}	+6686
		CUT	20-100	0.9923	25	40	2.9×10^{11}	24.5 (4)	-	(∞)
Pirimicarb		RLE	50-250	0.9944	60	100	9.1×10^{10}	5.1 (3)	7.1×10^{11}	-87.2
		BS2	50-250	0.9925	65	100	1.1×10^{11}	11.2 (3)	1.0×10^{12}	-89.0
		CUT	1000-5000	0.9907	1490	2160	7.0×10^{9}	23.9 (4)	1.1×10^{10}	-36.4
Propoxur		RLE	5-25	0.9944	6	9	9.7×10^{11}	2.6 (4)	9.4×10^{11}	+3.2
		BS2	10-50	0.9931	15	20	6.8×10^{11}	13.5 (4)	1.4×10^{11}	+386
		CUT	100-500	0.9951	110	170	2.2×10^{11}	3.0 (4)	1.3×10^{10}	+1592

R^2 (correlation coefficient of the linear calibration curve);LOD and LOQ (limits of detection and quantification determined according to DFG [34]); f_i (enzyme inhibition factor calculated from the slope of calibration curves divided by incubation time (30 min)); N.I. (no inhibition); RLE (rabbit liver esterase); BS2 (*Bacillus subtilis* esterase); CUT (cutinase from *Fusarium solani pisi*); Dev.% (relative deviation between enzyme inhibition factors of oxidised and non-oxidised insecticides under study ($100x(f_{i\text{-}ox}\text{-}f_i)/f_i$)); and n (number of determinations).

4.3 HPTLC/MS

In an attempt to understand some of the unexpected differences uncovered in the present study, the detectable zones of enzyme inhibition were analysed by HPTLC/MS. For these experiments, a set of two plates containing all insecticides was prepared, treated by bromine vapour before chromatography, and developed. One plate was subjected to an inhibition assay using BS2 esterase (**Fig. 2**). The detected inhibition zones were then marked on the second, enzyme-free plate, and extracted by the TLC-MS interface.

For the five thiophosphates and their corresponding oxons, only the oxon zones could be identified (**Fig. 2a**), providing the correct mass signals for the protonated, ammoniated or sodiated molecules (**Table 2**). Chlorfenvinfos resulted in an additional zone of equal intensity (**Fig. 2b**), with mass signals clearly indicating a monobrominated derivative. In the case of demeton-S-methyl, an additional zone near the start was detected, resulting from the corresponding sulphoxide, oxydemeton-methyl. The track of dichlorvos showed traces of a dibromo derivative, while monocrotofos was nearly completely transformed into brominated species, with loss of the phosphate group.

Of the carbamate insecticides, only pirimicarb survived bromine treatment. The track of propoxur most positively affected by bromine only showed one zone with a retention factor different from the parent sample (**Fig. 2c**), which yielded mass signals consistent with a monobromo derivative (**Table 2**). Interestingly, this monobromo propoxur derivative is clearly a stronger inhibitor than the parent propoxur. The enhanced inhibition activity of carbofuran toward RLE and BS2 may also be attributed to bromination reactions. Two roughly separated zones clearly provided mass signals corresponding to singly and doubly brominated carbofuran, while the parent compound was not detectable. A similar effect was found for carbaryl, where bromination also resulted in mono- and dibromo derivatives. In the case of Methomyl, a zone near the start was detected, corresponding to a sulphoxide derivative, which is apparently responsible for the significantly improved inhibition of cutinase following bromine treatment. Although some sulphoxidation was also observed for ethiofencarb (Rf 0.15), the most intensive zone (Rf 0.41) contained a mixture of singly and doubly brominated compounds which were strong inhibitors.

However, mass spectrometry only detected signals corresponding to brominated ethiofencarb derivatives which have lost 60 amu (Table 2).

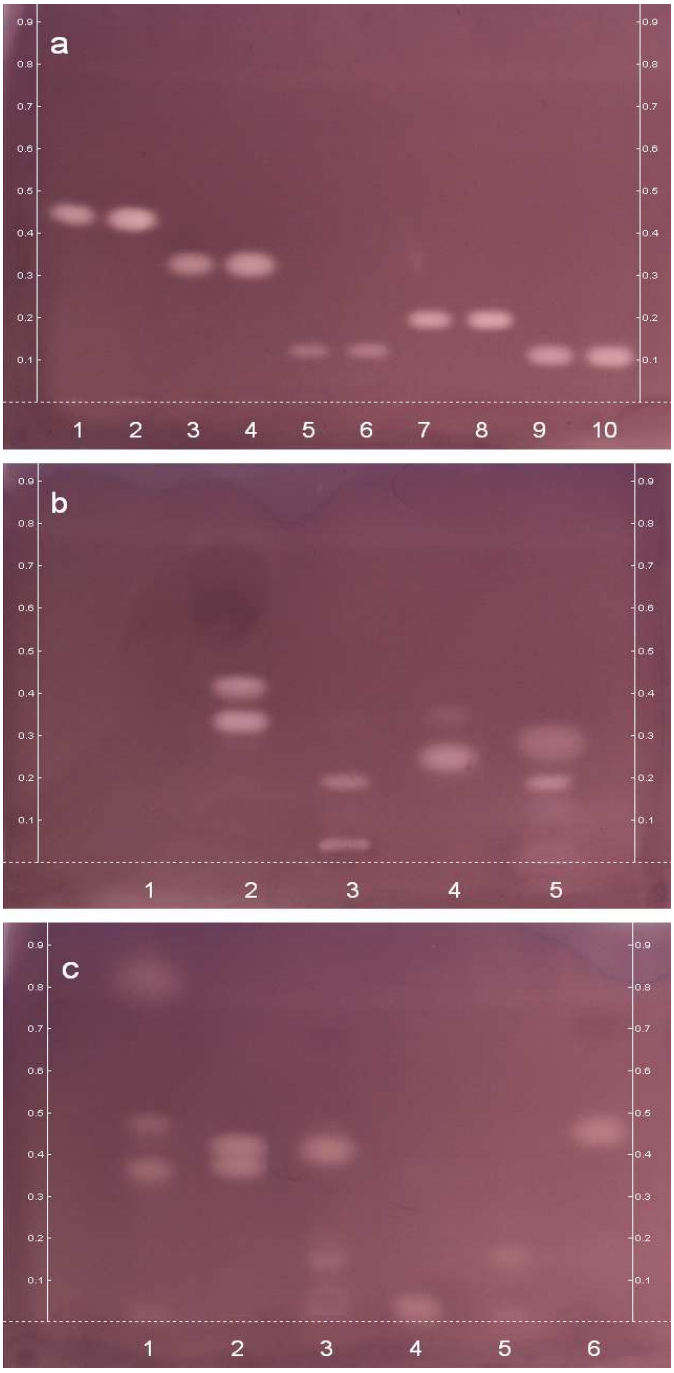


Fig. 2. Plate images of HPTLC-EI assays using BS2 esterase as an enzyme source, with bromine oxidation performed before chromatography: (a) chlorpyrifos, 20 ng (1), chlorpyrifos oxon, 20 ng (2), chlorpyrifos-methyl, 500 ng (3), chlorpyrifos-methyl oxon, 500 ng (4), malathion, 100 ng (5), malaoxon, 100 ng (6), parathion, 500 pg (7), paraoxon, 500 pg (8), parathion-methyl, 20 ng (9), paraoxon-methyl, 20 ng (10); (b) acephate, 1 µg (1), chlorfenvinfos, 200 ng (2), demeton-S-methyl, 200 ng (3), dichlorvos, 50 ng (4), monocrotofos, 500 ng (5); (c) carbaryl, 200 ng (1), carbofuran, 100 ng (2), ethiofencarb, 500 ng (3), methomyl, 1000 ng (4), pirimicarb, 500 ng (5), propoxur, 250 ng (6).

Table 2 TLC-MS data for bromine oxidation products of the studied insecticides, detected by the HPTLC-EI assay (see Fig. 2)

Insecticide applied	Track number[a)]	Rf	$M+H^+$	$M+NH_4^+$	$M+Na^+$	Attributed to
Acephate	b1	N.I.[b)]				
Carbaryl	c1	0.36	280	297	302	Bromocarbaryl
		0.47	358	375	380	Dibromocarbaryl
		0.82	237	254	259	(Bromocarbaryl - 43)
Carbofuran	c2	0.37	300		322	Bromocarbofuran
		0.42	378		400	Dibromocarbofuran
Chlorfenvinfos	b2	0.33	359		381	Chlorfenvinfos
		0.42	437		459	Bromochlorfenvinfos
Chlorpyrifos	a1	0.44	334	351	356	Chlorpyrifos oxon
Chlorpyrifos-methyl	a3	0.33	306	323	328	Chlorpyrifos-methyl oxon
Chlorpyrifos-methyl oxon	a4	0.33	306	323	328	Chlorpyrifos-methyl oxon
Chlorpyrifos oxon	a2	0.44	334	351	356	Chlorpyrifos oxon
Demeton-S-methyl	b3	0.04	247		269	Oxydemeton-methyl
		0.19	231		253	Demeton-S-methyl
Dichlorvos	b4	0.25	221	238	243	Dichlorvos
		0.35			401	Dibromodichlorvos
Ethiofencarb	c3	0.15	242		264	Ethiofencarb sulfoxide
		0.41	244	261	266	(Bromoethiofencarb - 60)
		0.41	322	339	344	(Dibromoethiofencarb - 60)
Malaoxon	a6	0.13	315		337	Malaoxon
Malathion	a5	0.13	315		337	Malaoxon
Methomyl	c4	0.03		196	201	Methomyl sulfoxide
Monocrotofos	b5	0.02	302		324	Bromomonocrotofos
		0.05	382		404	Dibromomonocrotofos
		0.19	194		216	Bromo-N-methylacetoacetamide
		0.28	272	289	294	Dibromo-N-methylacetoacetamide
Paraoxon	a8	0.20	276	293	298	Paraoxon
Paraoxon-methyl	a10	0.11	248	265	270	Paraoxon-methyl
Parathion	a7	0.20	276	293	298	Paraoxon
Parathion-methyl	a9	0.11	248	265	270	Paraoxon-methyl
Pirimicarb	c5	0.16	239		261	Pirimicarb
Propoxur	c6	0.46	288		310	Bromopropoxur

[a] Refer to Fig.2
[b] No inhibition

Because different compounds were detected in these experiments as a result of bromine treatment, it should be kept in mind that all possible products, including the parent compounds, will be located in a single zone if the bromine treatment is performed *after* chromatography, resulting in mixed mode inhibition effects.

4.4 Application to spiked samples

To validate our optimised HPTLC-EI assay with an additional pre-oxidation step, apple juice and tap water samples were used as test samples, following the QuEChERS method for the extraction of fruits and vegetables [1-2]. Results were compared to our previous results obtained with RLE [22]. The additional bromine pre-oxidation step eliminated the need for a 10-fold concentration step of sample extracts spiked with chlorpyrifos and parathion. In fact, extracts could even be diluted 2-fold before performing the HPTLC-EI assay, which has the added benefit of reducing interfering matrix components if present. For the less sensitive cutinase based assays, however, sample extracts still have to be concentrated somewhat, depending on the residue level expected. Generally, good recoveries, in the range 91-106 %, with acceptable standard deviations, were obtained for the spiked apple juice and water samples (**Table 3**).

Table 3 Recoveries of organophosphorus pesticides from spiked apple juice and drinking water by HPTLC-EI assay after bromine oxidation, using rabbit liver esterase (RLE) or cutinase from *F. solani pisi* (CUT) as enzyme sources.

				RLE			CUT	
Sample	Insecticide	Spiking level (mg/L)	Dilution/ concentration	Recovery %	RSD % (n=3)	Dilution/ concentration	Recovery %	RSD % (n=3)
Apple juice	Paraoxon	0.001	-	99.3	26.0	100 → 1	91.3	19.7
	Parathion	0.05	1 → 2	94.7	9.3	10 → 1	98.9	1.0
	Chlorpyrifos	0.5	-	100.9	15.0	2 → 1	102.0	4.3
Water	Paraoxon	0.001	-	96.0	3.6	100 → 1	97.7	4.1
	Parathion	0.05	1 → 2	104.2	12.6	10 → 1	99.3	7.1
	Chlorpyrifos	0.5	-	105.9	6.8	2 → 1	101.7	1.2

5 Conclusions

Bromine vapour treatment of the developed HPTLC plates strongly increased the detection sensitivity for the following organothiophosphate pesticides: chlorpyrifos, chlorpyrifos-methyl malathion, parathion, and parathion-methyl, by transformation of thions into their corresponding oxons, which are more potent esterase inhibitors. This improved sensitivity was demonstrated for all three esterases tested: rabbit liver esterase, *B. subtilis* esterase, and cutinase from *F. solani pisi*. Although a slight improvement in sensitivity was noticed for both demeton-S-methyl and propoxur with RLE, the pre-oxidation step does not appear useful for detection of the rest of the studied pesticides, because sensitivities were reduced after bromine treatment, resulting from degradation or bromination reactions. HPTLC-EI assays, in combination with QuEChERS extraction methods, resulted in very good recoveries without notable losses, validating our effect-directed, optimised method for highly sensitive high-throughput screening of esterase inhibitors.

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CHAPTER V

DETERMINATION OF ORGANOPHOSPHORUS AND CARBAMATE INSECTICIDES IN FRESH FRUITS AND VEGETABLES BY HIGH-PERFORMANCE THIN-LAYER CHROMATOGRAPHY MULTI-ENZYME INHIBITION ASSAY

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Key words:

High-performance thin-layer chromatography, enzyme inhibition assay, effect-directed analysis, cutinase, rabbit liver esterase, organophosphorus insecticides, carbamate insecticides, fruits and vegetables.

1 Abstract

High-performance thin-layer chromatography enzyme inhibition assay (HPTLC-EI) was applied to different fruit and vegetable samples after individual spiking with organophosphate and carbamate pesticides at their maximum residue limits documented by the European Commission. Samples were extracted according QuEChERS method including clean-up by PSA (primary secondary amine). Additional clean-up was performed on the HPTLC plate by a pre-chromatographic step to separate most co-extracted matrix compounds from the pesticides. Good results were obtained for both rabbit liver esterase (RLE) and cutinase from *Fusarium solani pisi* (CUT) as enzyme sources. Recoveries were in the range 98-109%, 95-114%, 96-114%, and 90-111% for chlorpyrifos, paraoxon, parathion, and pirimicarb, respectively with acceptable standard deviations.

2 Introduction

Consumers can be exposed to pesticides by eating or drinking contaminated foods or water leading to different health risks and diseases [1-10]. Therefore, governments set limits on allowable levels of pesticide residues in food and animal feed. Different analytical methods are applied for regular monitoring of food and plant residues of pesticides. Multi-residue methods using GC/MS or LC/MS are currently preferred to determine pesticides in plant samples [11-13]. Combination of thin-layer chromatography (TLC) with cholinesterase inhibition was very effective in determination of organophosphate and carbamate pesticides [14-23]. Rabbit liver esterase (RLE), *Bacillus subtilis* (BS2) esterase, and cutinase (CUT) from *Fusarium solani pisi* were successfully used for a multi-enzyme inhibition assay by high-performance thin-layer chromatography (HPTLC) for rapid and sensitive screening of organophosphates and carbamate [24-26]. With the so-called QuEChERS method (**Qu**ick, **E**asy, **Ch**eap, **E**ffective, **R**ugged, and **S**afe), a relatively simple extraction procedure was introduced for the determination of pesticide residues in fruits and vegetables [27-28]. Extracts containing matrix compounds interfering with pesticide quantification must be cleaned-up before analysis, but the more purification steps the more loss in the residues. Many clean-up techniques and materials were used in combination with multi-residue methods for determination of pesticides [e.g. 29-33].

In this study, QuEChERS extracts of fresh fruit and vegetable samples (apple, cucumber, grape, nectarine, plum, and tomato) were spiked individually with chlorpyrifos, paraoxon, parathion, and pirimicarb at their maximum residue limits (MRL), cleaned-up with PSA (primary secondary amine), and then transferred to be analysed with HPTLC multi-enzyme inhibition assay (HPTLC-EI). Since RLE and CUT proved to be enzymes of highest and lowest sensitivity towards organophosphate and carbamate pesticides under study [25], both enzymes were chosen to compare the detection of selected pesticides in different sample matrices.

3 Experimental

3.1 Materials

Insecticidal standards (acephate, carbaryl, chlorpyrifos, chlorpyrifos methyl, chlorpyrifos methyl oxon, chlorpyrifos oxon, pirimicarb, and propoxur) were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany), (ethiofencarb, malaoxon, malathion, parathion, and parathion methyl) from Sigma-Aldrich (Taufkirchen, Germany), and (carbofuran, chlorfenvinfos, demeton-S-methyl, dichlorvos, methomyl, monocrotofos, paraoxon, and paraoxon methyl) from Riedel-de Haën (Taufkirchen, Germany). Silica gel 60 F_{254} HPTLC glass plates (20 cm x 10 cm) and sodium chloride (≥99.5%) were supplied by Merck (Darmstadt, Germany). Cutinase (EC 3.1.1.74) from *Fusarium solani pisi* (lyophilized, protein content 75%, 356 U/mg proteins [34]) was kindly provided by Unilever Research Laboratory (Vlaardingen, The Netherlands). *Bacillus subtilis* (BS2) esterase (14.1 U/mg) was purchased from Julich Chiral Solutions (Julich, Germany). Rabbit liver esterase (lyophilized, 80 U/mg protein), bovine albumin (BSA, >98%), fast blue salt B (dye content, ~95%), α-naphthyl acetate (≥98%), anhydrous magnesium sulphate (reagent grade, ≥97%), and Bromine (>99.0%) were purchased from Sigma-Aldrich (Taufkirchen, Germany). Tris-(hydroxymethyl)-aminomethane (TRIS, ≥99.9%) and dichloromethane (≥99.9%) were provided by Carl Roth GmbH & Co. (Karlsruhe, Germany). Ultra pure water was purchased by a Synergy system (Millipore, Schwalbach, Germany). Formic acid (reagent grade, 98%), chloroform (>99%) and acetonitrile (HPLC grade) were purchased from Fisher scientific (Schwerte, Germany). BONDESIL-PSA (40 µm) was obtained from Varian (Darmstadt, Germany). Fruits and vegetables samples were obtained from local shops and checked by GC/MS-MS to be free of organophosphorus and carbamate insecticides.

Methanol, ethanol, n-hexane, acetone, and ethyl acetate (analytical grade) were obtained from Merck and distilled before use.

3.2 Solutions

Enzymes solutions were prepared by dissolving 0.9 mg rabbit liver esterase, 5 mg BS2 esterase, 0.5 mg Cutinase, individually, in 50 mL Tris–HCl buffer (0.05 M, pH 7.8) containing 0.1% BSA and stored in a cooler. Pesticide stock solutions were prepared in methanol at (1 g/L) followed diluting by methanol to working standards of 10 mg/L, 100 µg/L, and 1 µg/L, as need. Substrate solution was prepared by mixing 60 mL Fast Blue Salt B (2.5 g/L in water) and 30 mL α-naphthyl acetate solution (2.5 g/L in ethanol), both freshly prepared directly before use. Primuline solution was prepared at 0.5 g/L in acetone/water (4+1).

3.3 High-performance thin-layer chromatography

After pre-washing with methanol, HPTLC plates were dried at 100℃ for 20 min then stored in a desiccator. Pesticide working standard solutions as well spiked fruit extracts were applied as desired volumes onto plates by the automatic TLC Sampler 4 (ATS4, CAMAG, Muttenz, Switzerland) as 4–mm bands and 5 mm distances from the lower edge, from the left side, and between tracks were 10 mm. Plates, after 5 min hot air drying, were developed by the Automatic Developing Chamber 2 (ADC2, CAMAG) to a distance of 30 mm from the lower edge with a mobile phase of methanol/dichloromethane (10:90) without tank saturation and the migration time was approximately 7 min including 2 min drying. The plate was cut at a distance of 25 mm from the lower edge using CAMAG SmartCUT system and then focused with acetone up to 10 mm from the new lower edge. Thereafter, the plate was developed again with a mobile phase of n-hexane/ethyl acetate/dichloromethane (65:20:15) up to 60 mm in normal developing chamber without saturation and the migration time was approximately 27 min including 5 min drying. In case of chlorpyrifos, the plate was developed with n-hexane/acetone/dichloromethane (75/10/15) with a migration of 24 min including 5 min drying.

3.4 Oxidation

For determination of chlorpyrifos and parathion, the HPTLC plates were oxidized in a twin-trough chamber and oxidized by two drops of bromine added into the second trough. The top cover of the chamber was applied tightly and the oxidation was performed in 5 min. Following Ackermann [16], the excess adsorbed bromine was removed during heating at 60℃ (20 min) on a TLC plate heater III (CAMAG, Muttenz, Switzerland) under a well ventilated fume cupboard. Thereafter, the plate was cooled to room temperature for 1 min.

3.5 Detection

The prepared HPTLC plate was dipped into the enzyme solution for 2 s at a dipping speed of 1 cm/s using the TLC Immersion Device III (CAMAG), followed by horizontal incubation for 30 min at 37℃ in a humid chamber containing water. Thereafter, the plate was immersed into a freshly prepared directly before use substrate solution for 1 s at a dipping speed of 1 cm/s, followed by 3 min reaction time (laying the plate horizontally). To end the reaction, the plate was heated on a TLC Plate Heater III (CAMAG) at 50℃ for 5–7 min un til dryness.

3.6 Documentation and evaluation

Plate images were captured by the DigiStore 2 documentation system (CAMAG) under illumination in the visible range and in the reflectance mode. Plate's evaluation was done densitometrically using the TLC Scanner 3 (CAMAG) via peak area by absorbance measurement at 533 nm (inverse scan using fluorescence measurement mode without edge filter). The data obtained were processed with winCATS software, version 1.4.4 (CAMAG). Images of pesticide standards as well fresh fruit and vegetable extracts matrices were captured under UV illumination at 366 nm after dipping in primuline solution.

3.7 Sample extraction

Following QuEChERS extraction method [27-28], 10 g of a previously homogenized sample were spiked with a pesticide at its EU-MRL then vigorously shaken with 10 mL acetonitrile for 1 min. Without addition of buffer salts, 4 g magnesium sulphate and 1 g sodium chloride were added onto acetonitrile extracts

and the tube was shaken for another minute followed by centrifugation for 5 min at 3500 x g. Native, concentrated, or diluted extracts were cleaned up with PSA by shaking 1 mL extract with 25 mg PSA and 150 mg magnesium sulphate for 30 s followed by centrifugation. The cleaned-up extracts were applied (10 µL) onto the HPTLC plate together with a set of calibration standards.

4 Results and discussion

4.1 Modified development

In addition to water, the chosen samples contain other matrix compounds, like proteins, vitamins, carbohydrates, pigments, oils and wax. They can be partly or mostly co-extracted with the pesticide residue and, therefore, may interfere with its quantification. During GC/MS or LC/MS, co-extracted matrix compounds are responsible for both signal suppression and enhancement, but with enzyme inhibition methods the situation is a little bit different. There are some natural enzyme inhibitors or even some components just reaction with the used substrate forming coloured complexes interfering with pesticide detection. Besides PSA, GCB [35-36] and activated carbon [37] are very useful and effective adsorbents for the co-extracted pigments of different matrices. Application of activated carbon as adsorbent to our samples resulted in very clean background, but also free of the spiked pesticides (data not shown). Therefore, PSA (primary secondary amine) was used for clean-up of acetonitrile QuEChERS extracts even though that extracts leaving them partly still coloured and providing a background of interfering matrix compounds after enzyme detection on the plate.

Benefits of planar chromatography, however, can help to perform another clean-up directly on the HPTLC plate. This was successfully applied by a pre-development step with dichloromethane/ methanol (90/10) for 30 mm, when the desired pesticides migrate to the front leaving most interfering components behind. Thus, by cutting the plate at 25 mm from the lower edge, all pesticides except monocrotofos were separated from the interfering matrix as shown in **Figure 1**. To include the most polar monocrotofos, the plate should be cut at 20 mm, but more matrix compounds will be included partially resulting in interfering of enzyme detection (plates not shown).

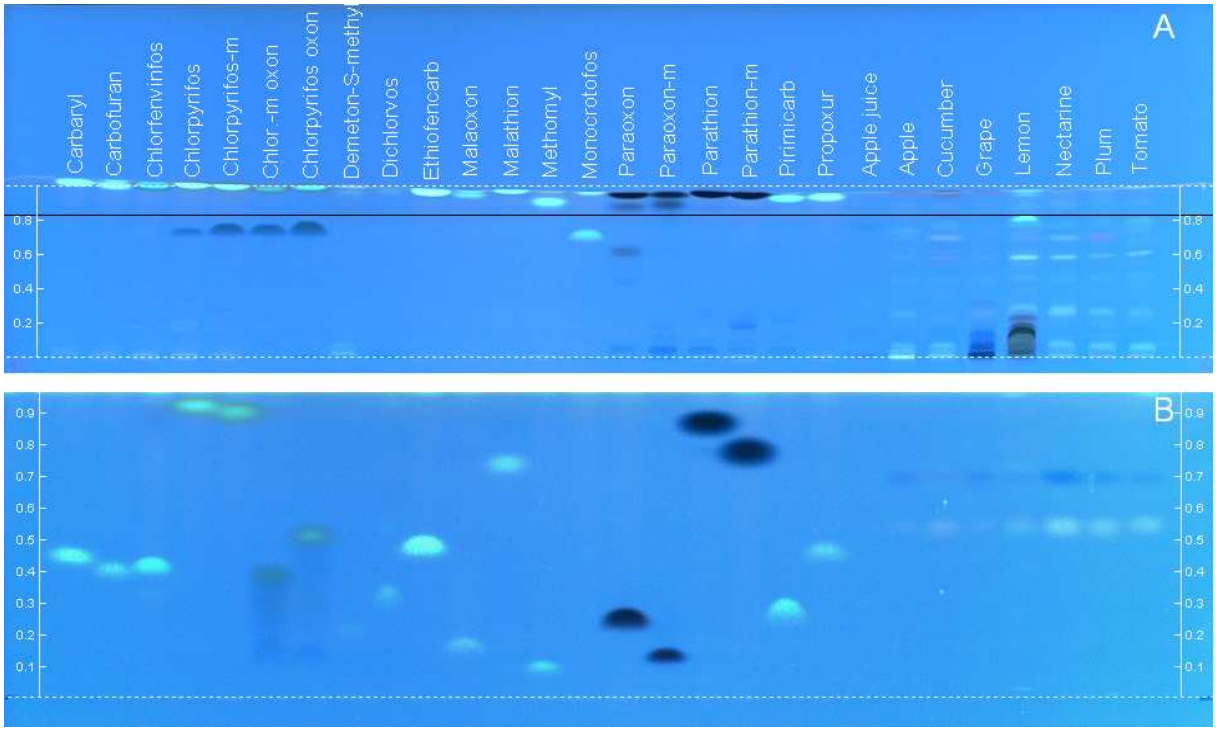

Figure 1 Modified HPTLC separation of organophosphate and carbamate insecticides from fruit and vegetable samples under study. Pesticides (10 µg/zone) and QuEChERS extracts (10 µL) were applied and developed firstly with A) 10% methanol in dichloromethane. After cutting the plate and focusing with acetone, the plate was developed secondly with B) n-hexane/ethyl acetate/ dichloromethane (65:20:15). Both plates were detected after dipping in primuline reagent.

All sample extracts except apple juice have two substances left after first development (figure 1), which do not interfere with pesticide standards (according to Rf values). They were appeared as blue bands after enzyme inhibition assay at Rf 0.54 and Rf 0.70, assumedly resulting from reaction with the substrate forming a coloured complex), but probably not being inhibitors (**Figure 2**). During a search for a common source of contamination, only the used mill came into question, since only the apple juice was not in contact with it. However, pesticides under study (except monocrotofos) are located either above the Rf 0.70 (thiophosphate thions) or below Rf 0.54. Thus, all inhibition bands (white zones) found in a sample inside this range (0.54-0.70) do not relate to the organophosphate and carbamate insecticides under study.

Matrix components still present after modified clean-up appeared as inhibitors (colourless zones) by detection with cutinase, but in concentrated extracts they appeared as blue bands. Lemon extracts are most rich in matrix compounds, which unfortunately do interfere with insecticides above Rf 0.70 (thions if present) as shown

in **figure 2**. PSA was very effective to clean grape samples from their co-extractives, which were almost not noticeable with RLE, appeared as light blue bands with BS2, but as white bands with CUT.

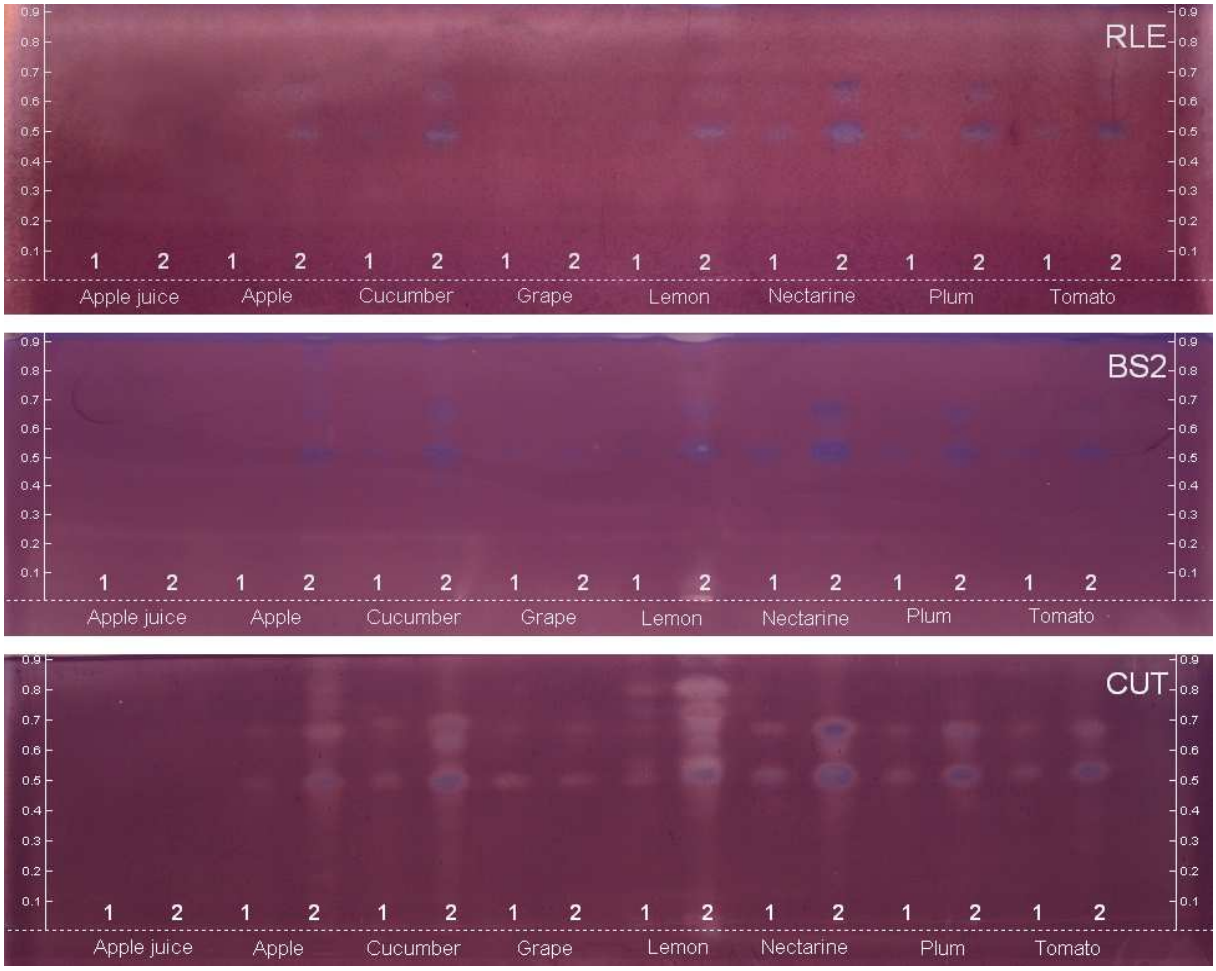

Figure 2 HPTLC-EI assay of 10 µL native (1) and 10:1 concentrated (2) QuEChERS extracts of different fruit and vegetable samples free of inhibitory residues.

4.2 Sample screening concept

After sample extract preparation and clean-up, 10 µL of native and concentrated (10:1) extracts were applied onto the HPTLC plate and developed. **Figure 3** shows steps of pesticide determination in fruit and vegetable samples. After enzyme detection we have two possibilities according to the chromatogram. If there are any inhibitor bands detected outside the range of Rf 0.54-0.70, then we can compare them with our pesticide group. If there are no inhibitors, extracts can be then either oxidized after chromatography or concentrated and then detected again with enzyme.

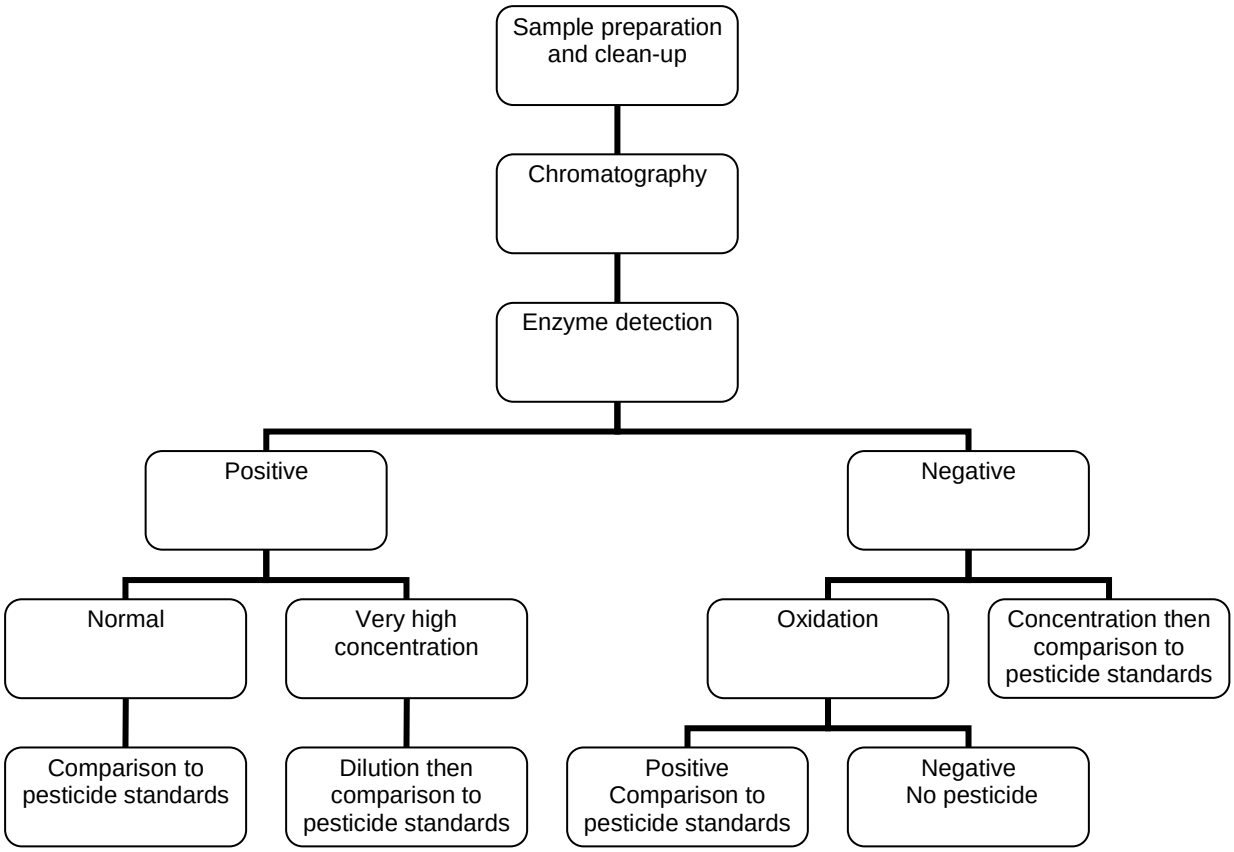

Figure 3 Schematic diagram of sample screening with HPTLC-EI assay

Table 1 shows the content of each pesticide in food samples under study spiked at its MRL according to the European Commission [38] in native and concentrated extracts. For non-listed pesticides, MRLs were considered 0.01 mg/kg. Organophosphate and carbamate insecticides can be classified under two main groups according to the modified method and their detection sensitivity with RLE.

Chlorfenvinfos, chlorpyrifos oxon, chlorpyrifos-methyl oxon, demeton-S-methyl, malaoxon, dichlorvos, paraoxon, and paraoxon methyl belong to the first group. They required extract dilution before application onto plates which reduces matrix problems and gives matrix-free background after enzyme detection. Chlorpyrifos, chlorpyrifos methyl, parathion, and parathion methyl are running with the systemic mobile phase far from co-extractive matrix and can be detected at very nice quantities after bromine oxidation. All six carbamates under study can be determined easily without any interfering with matrices. Ethiofencarb (Rf 0.48) as well as propoxur (0.46) may be affected by matrices because of diffusion effect happening during incubation [25].

To the second group belong malathion and monocrotofos. RLE is not very sensitive to malathion, even after bromine oxidation (LOQ > 100 ng/zone). In addition, samples need more concentration to be applied in the calibration range of malathion and because of the oxidation effect on some matrices resulting in more interfering components, malathion determination may suffer some problems. Monocrotofos could not be detected because it is under cutting line after first chromatography step. Pesticides can be actually found at quantities over their MRL and therefore sample extracts have to be diluted before application which is an additional method's benefit concerning matrix interferences.

Table 1 Content of organophosphate and carbamate pesticides

Insecticide	Calibration range (ng/band)		Apple			Cucumber			Grape			Nectarine			Plum			Tomato		
	RLE	CUT	MRL	A	B	MRL	A	B	MRL	A	B	MRL	A	B	MRL	A	B	MRL	A	B
Carbaryl	3-15	20-100	0.05	0.5	5	0.05	0.5	5	0.05	0.5	5	0.05	0.5	5	0.05	0.5	5	0.5	5	50
Carbofuran	50-250	1000-5000	0.02	0.2	2	0.02	0.2	2	0.02	0.2	2	0.03	0.3	3	0.02	0.2	2	0.02	0.2	2
Chlorfenvinfos	0.01-0.05	60-300	0.02	0.2	2	0.02	0.2	2	0.02	0.2	2	0.02	0.2	2	0.02	0.2	2	0.02	0.2	2
Chlorpyrifos[1]	1-5	2-10	0.5	5	50	0.05	0.5	5	0.5	5	50	0.2	2	20	0.2	2	20	0.5	5	50
Chlorpyrifos-m[1]	5-25	100-500	0.5	5	50	0.05	0.5	5	0.2	2	20	0.5	5	50	0.05	0.5	5	0.5	5	50
Chlorpyrifos-m oxon[2]	0.2-1	0.2-1	0.01	0.1	1	0.01	0.1	1	0.01	0.1	1	0.01	0.1	1	0.01	0.1	1	0.01	0.1	1
Chlorpyrifos oxon[2]	0.01-0.05	0.1-0.5	0.01	0.1	1	0.01	0.1	1	0.01	0.1	1	0.01	0.1	1	0.01	0.1	1	0.01	0.1	1
Demeton-S-methyl	1-5	500-2500[1]	0.01	0.1	1	0.01	0.1	1	0.01	0.1	1	0.01	0.1	1	0.01	0.1	1	0.01	0.1	1
Dichlorvos	0.001-0.005	1-5	0.01	0.1	1	0.01	0.1	1	0.01	0.1	1	0.01	0.1	1	0.01	0.1	1	0.01	0.1	1
Ethiofencarb[2]	2-10	1000-5000	0.01	0.1	1	0.01	0.1	1	0.01	0.1	1	0.01	0.1	1	0.01	0.1	1	0.01	0.1	1
Malaoxon[2]	1-5	500-2500	0.01	0.1	1	0.01	0.1	1	0.01	0.1	1	0.01	0.1	1	0.01	0.1	1	0.01	0.1	1
Malathion1	50-250	1000-5000	0.5	5	50	0.2	2	20	5	50	500	0.02	0.2	2	0.02	0.2	2	0.5	5	50
Methomyl	1-5	5-25	0.02	0.2	2	0.02	0.2	2	0.02	0.2	2	0.02	0.2	2	0.02	0.2	2	0.02	0.2	2
Monocrotofos[2]	10-50	207-1035	0.01	0.1	1	0.01	0.1	1	0.01	0.1	1	0.01	0.1	1	0.01	0.1	1	0.01	0.1	1
Paraoxon[2]	0.001-0.005	0.4-2	0.01	0.1	1	0.01	0.1	1	0.01	0.1	1	0.01	0.1	1	0.01	0.1	1	0.01	0.1	1
Paraoxon-m[2]	0.03-0.15	10-50	0.01	0.1	1	0.01	0.1	1	0.01	0.1	1	0.01	0.1	1	0.01	0.1	1	0.01	0.1	1
Parathion[1]	0.05-0.025	1-5	0.05	0.5	5	0.05	0.5	5	0.05	0.5	5	0.05	0.5	5	0.05	0.5	5	0.05	0.5	5
Parathion-m[1]	0.1-0.5	20-100	0.02	0.2	2	0.02	0.2	2	0.02	0.2	2	0.02	0.2	2	0.02	0.2	2	0.02	0.2	2
Pirimicarb	10-50	1000-5000	2	20	200	1	10	100	1	10	100	2	20	200	1	10	100	1	10	100
Propoxur[1]	5-25	100-500	0.05	0.5	5	0.05	0.5	5	0.05	0.5	5	0.05	0.5	5	0.05	0.5	5	0.05	0.5	5

1 calibration range after bromine oxidation [data not published yet]. 2 maximum residue levels were not listed by EU [34] and considered as 0.01 mg/kg. MRL maximum residue level (mg/kg). A refers to the content of pesticide in ng per 10 µL native acetonitrile extract. B refers to the content of pesticide in ng per 10 µL concentrated acetonitrile extract (10:1). RLE rabbit liver esterase. CUT cutinase from Fusarium solani pisi. Calibration ranges of non-oxidized pesticides were listed from previous work [25].

4.3 Recovery studies

Four pesticides (chlorpyrifos, paraoxon, parathion, and pirimicarb) were chosen for method comparison between RLE and cutinase and to see the effect of dilution and concentration of extracts on detection. Pirimicarb was only studied with RLE, because determination with CUT requires at least 100-folds extract concentration. There was no loss in pesticide quantities after QuEChERS extraction method and HPTLC-EI assay as shown in **Table 2**. HPTLC-EI of Lemon samples showed matrix problems especially after bromine oxidation in case of concentrated extracts (**Figure 4**).

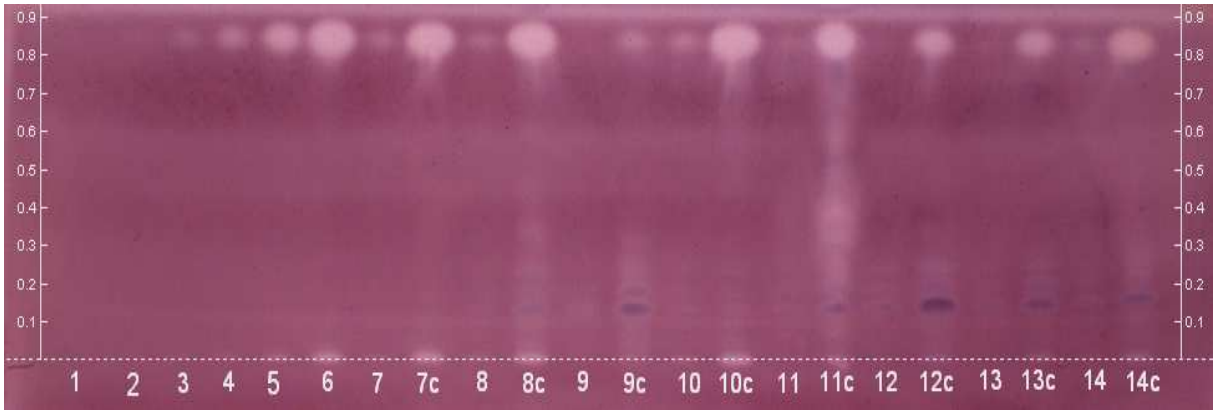

Figure 4 HPTLC-EI determination of chlorpyrifos (1-6) in apple juice (7-7c), apple (8-8c), cucumber (9-9c), grape (10-10c), lemon (11-11c), nectarine (12-12c), plum (13-13c), and tomato (14-14c). Where c refers to the concentrated (10:1) extracts and RLE was used after development with n-hexane/ acetone/dichloromethane (75:10:15) and bromine oxidation.

RLE is highly sensitive towards paraoxon at quantities down to pg/zone. Therefore, 100-folds dilution of extracts before application is needed, when the background of all matrices under study is completely free of interfering compounds. By using CUT, paraoxon can also be detected without any matrix problems, although extracts must be concentrated 10-folds before application onto HPTLC.

Table 1 Recovery data of HPTLC-EI assay of various spiked matrices

Insecticide	Matrix	MRL [mg/kg] (34)	Spiking Level [mg/kg]	Enzyme	Oxidation	Sample	Rec. [%]	RSD [%]
Chlorpyrifos	Apple	0.5	0.5	RLE	+	-	101	7.8
				CUT	+	-	100	6.3
	Cucumber	0.05	0.05	RLE	+	10:1	99	4.1
				CUT	+	10:1	102	9.4
	Grape	0.5	0.5	RLE	+	-	101	9.0
				CUT	+	-	106	8.8
	Nectarine	0.2	0.2	RLE	+	-	109	14.4
				CUT	+	10:1	103	10.5
	Plum	0.2	0.2	RLE	+	-	98	23.1
				CUT	+	10:1	99	33.0
	Tomato	0.5	0.5	RLE	+	-	101	14.6
				CUT	+	-	98	6.5
Paraoxon	Apple	-	0.05	RLE	-	1:100	95	3.7
				CUT	-	10:1	102	10.5
	Cucumber	-	0.05	RLE	-	1:100	100	3.6
				CUT	-	10:1	108	10.0
	Grape	-	0.05	RLE	-	1:100	100	4.3
				CUT	-	10:1	99	12.9
	Nectarine	-	0.05	RLE	-	1:100	99	3.2
				CUT	-	10:1	106	14.5
	Plum	-	0.05	RLE	-	1:100	95	0.7
				CUT	-	10:1	114	4.4
	Tomato	-	0.05	RLE	-	1:100	100	8.4
				CUT	-	10:1	107	2.0
Parathion	Apple	0.05	0.05	RLE	+	-	104	3.1
				CUT	+	10:1	98	16.0
	Cucumber	0.05	0.05	RLE	+	-	100	8.9
				CUT	+	10:1	99	3.4
	Grape	0.05	0.05	RLE	+	-	96	0.6
				CUT	+	10:1	104	3.2
	Nectarine	0.05	0.05	RLE	+	-	99	2.1
				CUT	+	10:1	102	17.8
	Plum	0.05	0.05	RLE	+	-	96	11.1
				CUT	+	10:1	114	1.8
	Tomato	0.05	0.05	RLE	+	-	101	4.7
				CUT	+	10:1	107	21.0
Pirimicarb	Apple	2	2	RLE	-	-	111	4.2
	Cucumber	1	1	RLE	-	10:1	94	13.1
	Grape	1	1	RLE	-	10:1	102	12.8
	Nectarine	2	2	RLE	-	-	96	5.2
	Plum	1	1	RLE	-	10:1	93	6.2
	Tomato	1	1	RLE	-	10:1	90	10.3

MRL: Maximum residue limit. Rec.: Recovery. RSD: Relative standard deviation

5 Conclusions

Multi-enzyme inhibition assay combined to planar chromatography enabled the quantification of a wide group of organophosphate and carbamate pesticides in different fruit and vegetable samples down to their MRL recorded by the European Union resulting in very nice recoveries which are comparable to other analysis methods like LC-MS and GC-MS. HPTLC-EI can be enhanced after an additional pre-chromatographic step resulted in separation of most co-extractive matrices from desired insecticides. RLE still represent the highest sensitive enzyme toward organophosphate and carbamate pesticides under study, whereas CUT, the lowest sensitive enzyme, can be used well in few cases and even for first sample screening.

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CHAPTER VI

SUMMARY

Summary

In terms of effect-directed analysis, esterase inhibitor assays allow a rapid and selective detection of insecticidal organophosphates and carbamates in food and environmental samples. With consideration to the toxicological mechanism of action of these insecticides, cholinesterases of different origin were used in different test formats, as microtiterplate assays, in test strip formats, as biosensors or coupled to thin-layer chromatography (bio-autography). Instead of cholinesterases, Ingrid Walz (PhD thesis, University of Hohenheim, 2008) introduced rabbit liver esterase (RLE), *Bacillus subtilis* (BS2)-esterase and cutinase (CUT) from *Fusarium solani pisi* for a multi-enzyme microtiterplate assay. In particular, RLE and BS2 proved to be much more sensitive than chloinesterases for the detection of inhibitors, while CUT displayed oneself by special tolerance for matrix components from fruits.

This multi-enzyme assay was successfully transferred onto high-performance thin-layer chromatography (HPTLC) (**Chapter II**). With the insecticide examples of carbofuran, malaoxon and paraoxon as weak, medium and strong inhibitors, HPTLC-enzyme inhibition (HPTLC-EI) assay conditions were optimized concerning enzyme concentrations, incubation times and substrate reactions. In the presence of the substrate α-naphthyl acetate/Fast Blue Salt B leads to colourless inhibitor zones are obtained on a purple background, which can be sensitively quantified by scanning at 533 nm. The limits of detection for paraoxon were determined to 1.3, 1.2, and 540 pg/zone for RLE, BS2, and CUT, respectively. Malaoxon was detectable up to 7.9, 7.4 and 760 ng/zone, while the limits of detection for carbofuran were at 33, 54 and 1420 ng/zone.

After this initial success, HPTLC-EI assay was extended on all important organophosphates and carbamates (**Chapter III**). The mandatory substrate substitution of Fast Blue Salt B from another supplier first made re-optimization of the method necessary with regard to incubation time and reagent composition. During the subsequent insecticides' screening, acephate proved itself as well as in microtiterplate assay as non-inhibitor for all three enzymes. All other 20 representative organophosphate and carbamate insecticides could be successfully detected using HPTLC-EI assay. The enzymes under study have the advantage over cholinesterases that thionophosphate pesticides are denoted directly as inhibitors,

without pre-transferring into the corresponding oxons. Impurities in many standards were also visible after the chromatographic separation. The high sensitive detectable oxons were found in most thionophosphate standards. Malathion, parathion, and parathion methyl had further contaminations of iso-malathion, iso-parathion and iso-parathion-methyl, respectively, which are products of a thiono-thiolo rearrangement. Chlorfenvinfos was contaminated to about 10% with the E-isomer. Carbofuran, chlorfenvinfos, and malaoxon that were no CUT inhibitors by microtiterplate assay, were surprisingly detected on the HPTLC plate with CUT. For optimum chromatographic separation, the insecticides were divided into three groups and each developed with a customized mobile phase. Since on an HPTLC plate, no concentrations (mol/L) can be given, enzyme inhibition factors were defined as new index and calculated from the slope of the linear calibration curves. They are a measure of inhibition strength of the respective insecticide and showed good correlation to the inhibition constants of the microtiterplate format and also to limits of detection in HPTLC-EI assay. They varied from few pg/zone of oxons as the strongest inhibitors to few ng/zone for most carbamates when the high sensitivity enzymes RLE and BS2 were used. The less sensitive cutinase requires some micrograms/zone, which can be taken as an advantage when samples with correspondingly high residues are under study. Both enzymes of the highest (RLE) and the lowest (CUT) sensitivity can thus be selected for a first rapid screening. HPTLC-EI assay was exemplary applied on drinking water and apple juice samples which were spiked with paraoxon (0.001 mg/L), parathion (0.05 mg/L) and chlorpyrifos (0.5 mg/L). Mean recoveries of 71-112% with relative standard deviations of 2-18% were achieved.

While organophosphate thions could be satisfactorily detected with the developed HPTLC-EI assay, the sensitivities expressed as enzyme inhibition factors could be increased after bromine oxidation by about two orders of magnitude (**Chapter IV**). As to be expected, HPTLC plates exposed to bromine vapor showed besides thion-oxon reaction a series of side-reactions of the insecticides. Bromination and oxidation of thioethers were observed through HPTLC-MS. They proved themselves even as an advantageous as in case of demeton-S-methyl since it is itself no CUT inhibitor but however oxydemeton-methyl is well. The corresponding phenols could be also detected by these investigations as further impurities in the commercial standards. Therefore, an alternative iodine-vapor and UV-irradiation of

the HPTLC plate were tested. Both procedures were not able to perform the desired oxidation of thionophosphates in an acceptable time. Enzyme inhibition factors of all insecticides were therefore determined after bromine oxidation, which were as expected clearly higher for thiophosphate, but almost slightly lower for the other pesticides. As before, the extended HPTLC-EI assay, was exemplarily applied on drinking water and apple juice samples, which were spiked with paraoxon (0.001 mg/L), parathion (0.05 mg/L) and chlorpyrifos (0.5 mg / L). The mean recoveries were at 95-106% for RLE and 91-102% for CUT as enzyme sources with standard deviations from 3.6 to 26% and from 1.2 to 19.7%, respectively.

The broader application on fruit and vegetable samples (apples, grapes, nectarines, lemons, plums, tomatoes and cucumbers) made matrix interferences noticeable that already were described in the literature. Many plant compounds are obviously esterase inhibitors. Therefore, Ingrid Walz carried out a costly SPE clean-up to avoid interferences in the microtiterplate assay, which should be avoided in the present work in the sense of rapid screening. For extraction the rapid QuEChERS method was used (acetonitrile extraction) including a clean-up by PSA (primary secondary amine). Further clean-up was performed elegantly on the HPTLC plate by a pre-development with methanol / dichloromethane (30 mm), when all insecticides run near to the front leaving matrix components mainly behind. The plate was cut at 25 mm and subjected to the actual development for pesticides separation. This preserved largely matrix-free tracks, even at 10-fold extract concentration (**Chapter V**). The selected fruit and vegetable samples were then spiked with chlorpyrifos, paraoxon, parathion and pirimicarb at level of maximum residue limits, worked up and analyzed by HPTLC-EI assay. They resulted in very good recoveries of 98-109%, 95-114% and 96-114% for chlorpyrifos, parathion and paraoxon using the enzymes RLE and CUT. Pirimicarb was recovered to 90-111%, which for reasons of detectability only RLE was used.

Overall, the developed HPTLC-EI assay can be presented as a very sensitive and rapid method for screening of organophosphate and carbamate insecticides including active inhibition metabolites or generally esterase inhibitors in environmental and food samples.

CHAPTER VII

ZUSAMMENFASSUNG

Zusammenfassung

Im Sinne einer wirkungsbezogenen Analytik erlauben Esterase-Hemmstoff-Assays einen schnellen und selektiven Nachweis von insektiziden Organophosphaten und Carbamaten in Lebensmitteln und Umweltproben. Mit Rücksicht auf den toxikologischen Wirkungsmechanismus dieser Insektizide kommen Cholinesterasen verschiedener Herkunft in diversen Testformaten zum Einsatz, als Küvetten-/Titerplatten-Assays, in Teststreifenformaten, als Biosensoren oder auch gekoppelt mit der Planarchromatographie (Bioautographie). Anstelle von Cholinesterasen führte Ingrid Walz (Dissertation Universität Hohenheim, 2008) Kaninchenleber-Esterase (RLE), *Bacillus subtilis* (BS2)-Esterase und Cutinase (CUT) aus *Fusarium solani pisi* für einen Multienzym-Titerplattenassay ein. Insbesondere RLE und BS2 erwiesen sich dabei als wesentlich empfindlicher als Chloinesterasen zum Nachweis von Hemmstoffen, während CUT sich durch besondere Toleranz gegenüber Matrixkomponenten aus Früchten auszeichnete.

Dieser Multienzym-Assay wurde erfolgreich auf die Hochleistungs-Dünnschicht-chromatographie (High-performance thin-layer chromatography, HPTLC) übertragen (**Kapitel II**). Mit den Wirkstoffbeispielen Carbofuran, Malaoxon und Paraoxon als schwache, mittlere und starke Hemmstoffe wurden die Bedingungen für den HPTLC–Enzyme Inhibition (HPTLC–EI) Assay hinsichtlich Enzymkonzentrationen, Inkubationszeiten sowie Substratreaktionen optimiert. In Gegenwart des Substrates α-Naphthylacetat/Echtblausalz B kommt es zu farblosen Hemmstoffzonen auf einem violetten Hintergrund, die sich mittels Scan bei 533 nm empfindlich quantifizieren lassen. Die Nachweisgrenzen für Paraoxon wurden zu 1,3, 1,2, und 540 pg/Zone für die Enzyme RLE, BS2, und CUT bestimmt. Malaoxon war nachweisbar bis zu 7,9, 7,4 und 760 ng/Zone, während die Nachweisgrenzen für Carbofuran bei 33, 54 und 1420 ng/Zone lagen.

Nach diesem ersten Erfolg ging es darum, den HPTLC-EI Assay auf alle bedeutenden Organophosphate und Carbamate auszudehnen (**Kapitel III**). Der zwingende Wechsel zum Substratreagenz Echtblausalz B eines anderen Lieferanten machte zunächst erneute Methodenoptimierungen hinsichtlich Inkubationszeiten und Reagenzzusammensetzung notwendig. Beim nachfolgenden Wirkstoffscreening erwies sich Acephate wie auch im Titerplatten-Assay als nicht hemmend für alle drei

Enzyme. Alle anderen 20 repräsentativen Organophosphat- und Carbamat-Insektizide konnten erfolgreich mittels HPTLC-EI Assay detektiert werden. Die eingesetzten Enyzme haben gegenüber Cholinesterasen den Vorteil, dass Thionophosphate direkt als Hemmstoffe angezeigt werden, ohne sie vorher in die korrespondierenden Oxone zu überführen. Die chromatographische Trennung machte darüber hinaus Verunreinigungen in vielen Standardsubstanzen sichtbar. So waren in den meisten Standards der Thionophosphate die hoch empfindlich detektierbaren Oxone nachweisbar. Malathion, Parathion, und Parathion-methyl hatten zudem weitere Verunreinigungen iso-Malathion, iso-Parathion, und iso-Parathion-methyl, die Produkte einer Thiono-Thiolo-Umlagerung sind. Chlorfenvinfos war zu etwa 10 % mit dem E-Isomer verunreinigt. Carbofuran, Chlorfenvinfos und Malaoxon, die keine CUT-Inhibitoren beim Mikrotiterplatten-Assay waren, waren überraschenderweise auf der HPTLC-Platte mit CUT detektierbar. Zur optimalen chromatographischen Trennung wurden die Insektizide in drei Gruppen eingeteilt und mit einem jeweils angepassten Fließmittel entwickelt.

Da in einer HPTLC-Zone keine Konzentrationsangaben (mol/L) möglich sind, wurden Enzymhemmfaktoren als neue Größe definiert und aus der Steigung der linearen Kalibrierfunktionen berechnet. Sie sind ein Maß für die Hemmstärke des jeweiligen Insektizids und zeigten gute Korrelationen zu den Hemmkonstanten des Titerplattenformates sowie auch zu den Nachweisgrenzen im HPTLC-EI Assay. Diese bewegten von wenigen pg/Zone für Oxone als stärkste Hemmstoffe bis zu wenigen ng/Zone für die meisten Carbamate, wenn die nachweisstarken Enzyme RLE und BS2 eingesetzt wurden. Die weniger sensitive Cutinase erfordert einige µg/Zone, was als ein Vorteil angenommen werden kann, wenn es darum geht, Proben mit entsprechend hohen Rückständen zu untersuchen. Beide Enzyme der höchsten (RLE) und der niedrigsten (CUT) Empfindlichkeit können somit zu einem ersten schnellen Screening gewählt werden. Beispielhaft wurde der HPTLC-EI Assay auf Trinkwasser- und Apfelsaft-Proben angewandt, die mit Paraoxon (0,001 mg/L), Parathion (0,05 mg/L) sowie Chlorpyrifos (0,5 mg/L) dotiert wurden. Dabei wurden mittlere Wiederfindungen von 71-112 % bei relativen Standardabweichungen von 2-18 % erreicht.

Während Organophosphat-Thione bereits befriedigend mit dem entwickelten HPTLC-EI Assay nachgewiesen werden konnten, ließen sich die Empfindlichkeiten,

ausgedrückt als Enzymhemmfaktoren, nach Brom-Oxidation um ungefähr zwei Zehnerpotenzen erhöhen (**Kapitel IV**). Wie zu erwarten, zeigte aber die Brom-Bedampfung der HPTLC-Platten neben der gewünschten Thion-Oxon-Reaktion einen Reihe von Nebenreaktionen an den Wirkstoffen. Mittels HPTLC-MS wurden hier Bromierungen sowie Oxidationen an Thioethern beobachtet. Letztere erwiesen sich im Falle von Demeton-S-Methyl sogar als vorteilhaft, da es selbst kein CUT-Inhibitor ist, Oxydemeton-methyl jedoch sehr wohl. Bei diesen Untersuchungen konnten auch die entsprechenden Phenole als weitere Verunreinigungen in kommerziellen Standards nachgewiesen werden. Daher wurden alternativ eine Jod-Bedampfung sowie eine UV-Bestrahlung der HPTLC-Platte getestet. Beide Verfahren waren aber nicht in der Lage, die angestrebte Oxidation der Thiono-Phosphate in einer akzeptablen Zeit durchzuführen. Daher wurden für alle Insektizide die Enzymhemmfaktoren nach Brom-Oxidation bestimmt, die erwartungsgemäß für Thionophosphate deutlich höher ausfielen, für andere Wirkstoffe dagegen in der Regel etwas geringer.

Wie zuvor wurde der um die Brom-Oxidation erweiterte HPTLC-EI Assay beispielhaft auf Trinkwasser- und Apfelsaft-Proben angewandt, die mit Paraoxon (0,001 mg/L), Parathion (0,05 mg/L) sowie Chlorpyrifos (0,5 mg/L) dotiert wurden. Die durchschnittlichen Wiederfindungen lagen bei 95-106 % für RLE bzw. 91-102 % für CUT als Enzymquellen bei Standardabweichungen von 3,6-26 % bzw. 1,2-19,7 %.

Bei einer breiteren Anwendung auf Obst- und Gemüseproben (Äpfel, Weintrauben, Nectarinen, Zitronen, Pflaumen, Tomaten und Gurken) machten sich die schon in der Literatur beschriebenen Matrixstörungen bemerkbar. Zahlreiche Pflanzeninhaltsstoffe sind offensichtlich auch Esterase-Hemmstoffe. Ingrid Walz hatte daher ein aufwändiges SPE-Clean-up vorgenommen, um die Störungen im Titerplattenassay zu umgehen, was jedoch im Rahmen der vorliegenden Arbeit im Sinne eines schnellen Screenings vermieden werden sollte. Zur Extraktion kam das schnelle QuEChERS-Verfahren zum Einsatz (Acetonitril-Extraktion) einschließlich eines Clean-up mittels PSA (primary secondary amine). Das weitere Clean-up wurde elegant auf der HPTLC-Platte durch eine Vorentwicklung mit Methanol/Dichlormethan (30 mm) vorgenommen, bei der die Insektizide nahezu in die Front laufen und Matrixkomponenten hauptsächlich hinter sich lassen. Die Platte

wurde bei 25 mm abgeschnitten und der eigentlichen Entwicklung zur Auftrennung der Wirkstoffe unterworfen. Dadurch erhielt man weitgehend matrixfreie Bahnen, selbst bei einer zehnfachen Konzentrierung der Extrakte (**Kapitel V**).

Die ausgewählten Obst- und Gemüseproben wurden anschließend mit Chlorpyrifos, Paraoxon, Parathion und Pirimicarb auf dem Niveau der Rückstandshöchstgehalte dotiert, aufgearbeitet und mittels HPTLC-EI Assay analysiert. Es ergaben sich sehr gute Wiederfindungen von 98-109 %, 95-114 % und 96-114 % für Chlorpyrifos, Paraoxon und Parathion unter Einsatz der Enyzme RLE und CUT. Pirimicarb wurde zu 90-111 % wiedergefunden, wobei aus Gründen der Detektierbarkeit nur RLE zum Einsatz kam.

Insgesamt kann der entwickelte HPTLC-EI Assay als ein sehr empfindliches und schnelles Instrument zum Screening von Organophosphat- und Carbamat-Insektiziden einschließlich hemmaktiver Metaboliten bzw. ganz allgemein von Esterase-Hemmstoffen in Umwelt- und Lebensmittelproben präsentiert.