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☆,☆☆

Rami Akkad, Wolfgang Schwack*

Institute of Food Chemistry, University of Hohenheim, Garbenstrasse 28, 70599 Stuttgart, Germany

ARTICLE INFO

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 coupling 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%.

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1. 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 (CUT) from *Fusarium solani pisi* was

introduced for rapid and sensitive detection of organophosphorus and carbamate insecticides [18–21]. In terms of 'bioauthography', this multi-enzyme 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.

2. Experimental

2.1. 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-naphthol 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) was 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 × 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).

2.2. 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-naphthol 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.

2.3. High-performance thin-layer chromatography (HPTLC)

HPTLC glass plates were pre-washed by development with methanol, followed by drying at 100 °C for 20 min and stored in a desiccator. 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 min including 5 min drying. Concerning 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/dichloromethane (75/10/15, v/v/v)).

2.4. 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 °C 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 °C for 5–7 min until dryness.

2.5. 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).

2.6. 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.

2.7. 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 $3500 \times g$. 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.

3. Results and discussion

3.1. Staining with Fast Blue Salt B

Following Weins and Jork [26], α-naphthol 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-naphthol 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 °C to prevent brightening of the violet background.

3.2. High-performance 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.

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.

3.2.1. 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
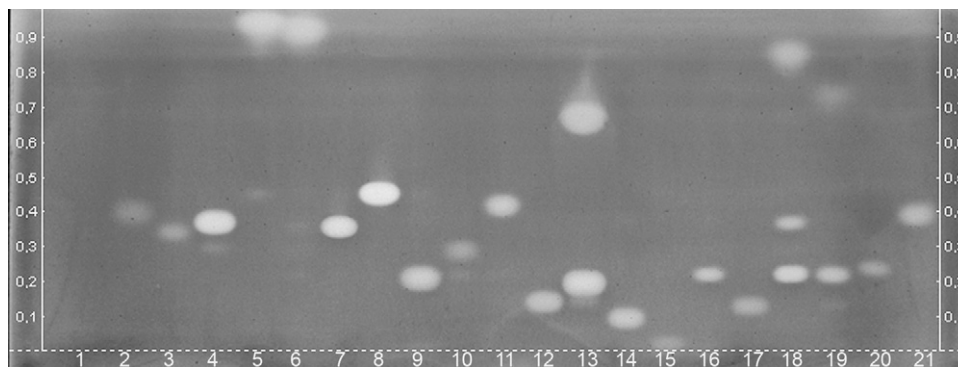

Fig. 1. HPTLC–EI assay of organophosporus and carbamate insecticides developed by n-hexane/ethyl acetate/dichloromethane (65:20:15) and detected by rabbit liver esterase inhibition: 1. acephate 1 μg, 2. carbaryl 10 ng, 3. carbofuran 100 ng, 4. chlorfenvinfos 100 pg, 5. chlorpyrifos 200 ng, 6. chlorpyrifos-methyl 1 μg, 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.
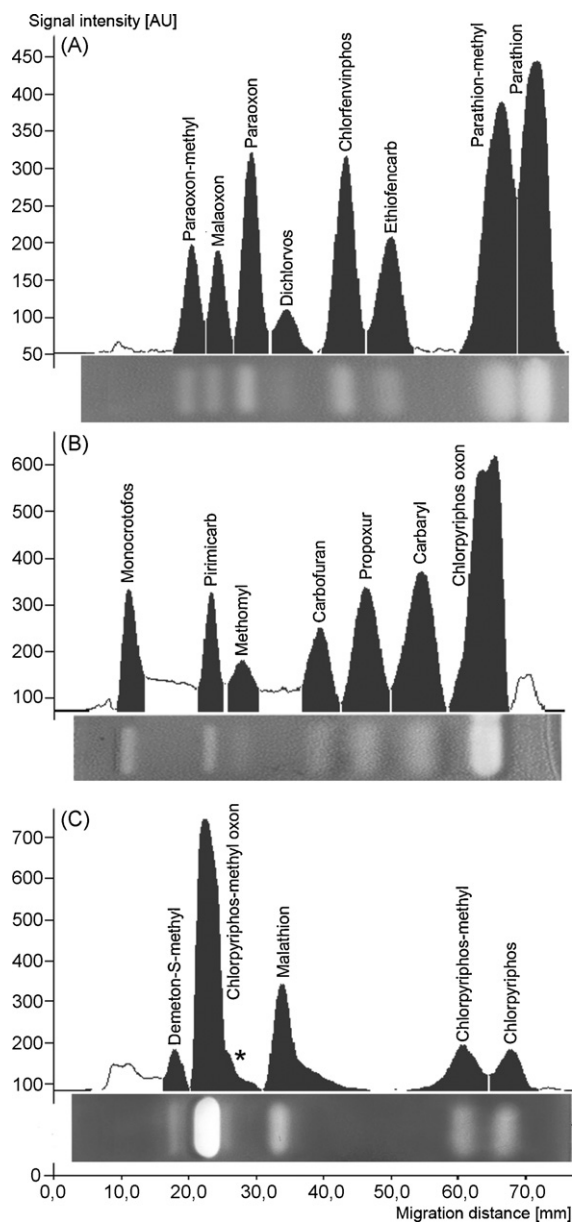
Fig. 2. HPTLC–EI assay of organophosphate and carbamate insecticides, divided into three groups. Solvent systems: (A) n-hexane/ethyl acetate (63:37), (B) chloroform/ethyl acetate (90:10), (C) n-hexane/acetone/dichloromethane (75:10:15) (* marks the oxon impurity of chlorpyrifos).

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 elemen-

tal 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 (calculated 358.9774) 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 are comparably sensitive for both the main compounds and 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 a 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.

3.2.2. 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:

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

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]:

$$k_i = -\frac{\ln(Ac)}{P_0} \times t\,[\mathrm{L\,mol^{-1}\,min^{-1}}] \tag{2}$$

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*)).
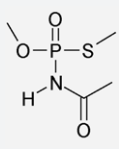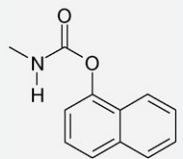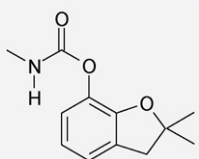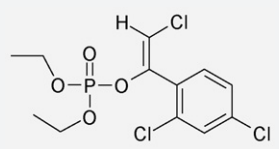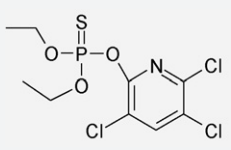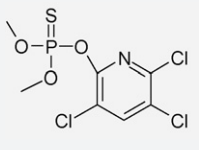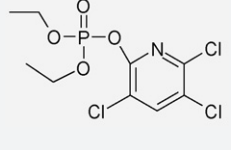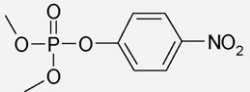
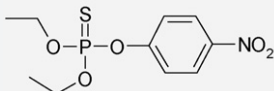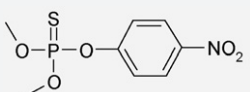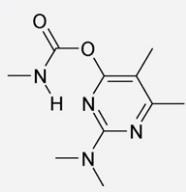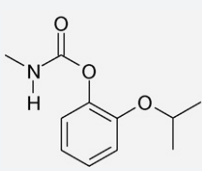| Insecticide	Structure formula	Enzyme	Calibration range [amount/zone]	R^2	LOD [ng/zone]	LOQ [ng/zone]	f_i [mol^{-1} min^{-1}]	RSD [%] (n)
Acephate		RLE	N.I.	–	–	–	–	
		BS2	N.I.	–	–	–	–	
		CUT	N.I.	–	–	–	–	
Carbaryl		RLE	3–15 ng	0.9940	3.6	5.3	2.1×10^{12}	12.2 (4)
		BS2	2–10 ng	0.9922	2.7	4.0	5.8×10^{12}	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	$1,3 \times 10^{11}$	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	0.9904	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)
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)

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)
		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)

3.2.3. 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.

3.2.4. 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).

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

Organophosphorus thions will also be detectable at the same low level, if an oxidation step by bromine vapor 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).

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.

4. 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.

Acknowledgements

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

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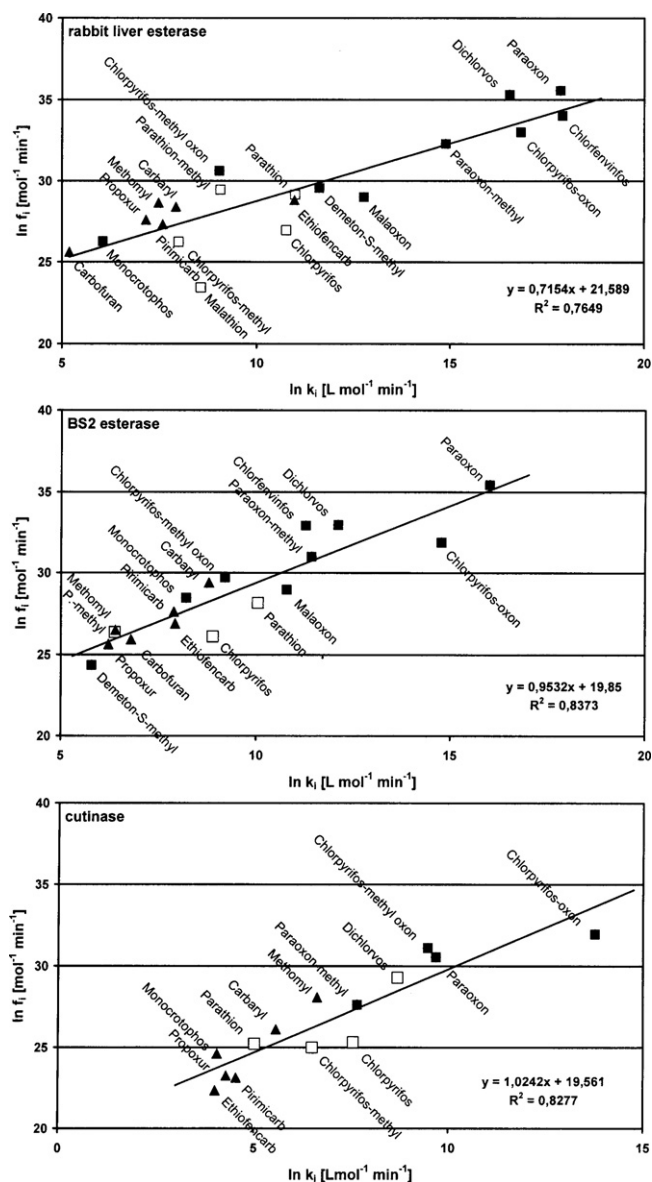[14] P. Herzsprung, L. Weil, R. Niessner, Vom Wasser 76 (1991) 51.

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