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Effect of bromine oxidation on high-performance thin-layer chromatography multi-enzyme inhibition assay detection of organophosphates and carbamate insecticides

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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 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.

1. 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 use 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 anticholinesterase 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 oxidation step on the sensitivity of organophosphate thion pesticide detection (e.g. chlorpyrifos, chlorpyrifos methyl,

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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.

2. Experimental

2.1. Materials

Silica gel 60 F_{254} HPTLC glass plates (20 cm × 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 (>99.0%) 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).

2.2. Solutions

Pesticide stock solutions (1 g/L) were prepared in methanol and diluted with methanol to working concentrations 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 (2.5 g/L in water), both freshly prepared immediately before use. Primuline dipping solution (0.5 g/L) was prepared in acetone/water (4 + 1).

2.3. Planar chromatography

HPTLC plates were pre-washed with methanol, dried at 100 °C 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, 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. 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 of the 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 °C (20 min) using a TLC plate heater III (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 °C).

2.5. 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 °C, 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 °C for 5–7 min until dryness using a TLC plate heater III (CAMAG).

2.6. 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.

2.7. 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 °C; drying gas flow rate, 10 L min^{-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.

2.8. 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 min and then centrifuged for 5 min at 3500 × *g*. 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.

3. Results and discussion

3.1. Bromine oxidation versus iodine and UV irradiation oxidation

Ideally, oxidation of thiono phosphates would be performed only until the desired 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; 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 visible 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.

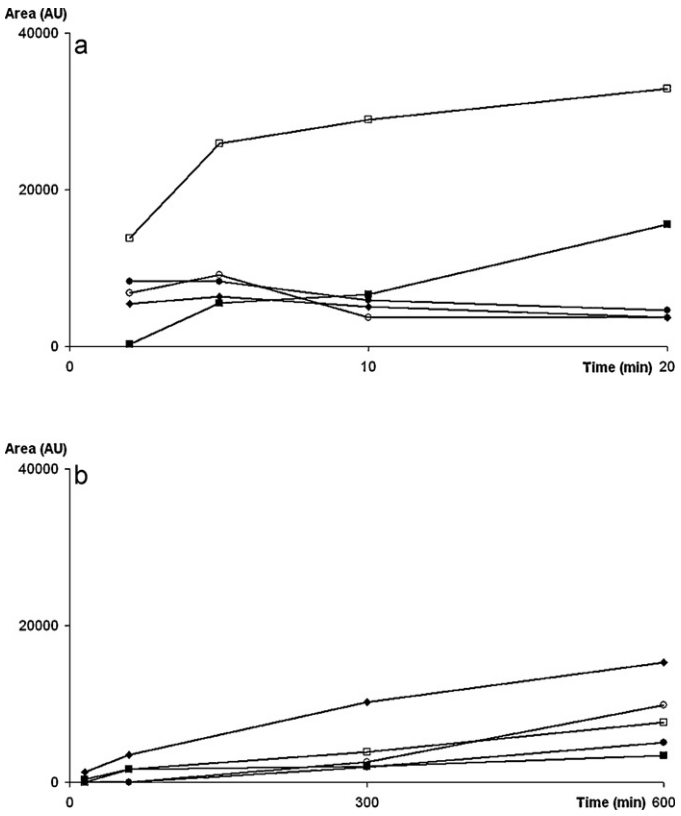


Fig. 1. Time course of organophosphorus oxons formation from chlorpyrifos methyl (□, 1000 ng), chlorpyrifos (■, 10 ng), malathion (♦, 500 ng), parathion (•, 0.2 ng), and parathion-methyl (○, 5 ng), determined by HPTLC-EI assay using BS2 esterase as the enzyme source. Oxidation was performed before chromatography,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

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 min of bromine treatment. During iodine vaporisation, oxon peak areas generally increased for up to 10 h 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 min bromine oxidation treatment was optimal; and, thus, was used for all remaining experiments to determine enzyme inhibition factors.

3.2. 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 (Table 1). 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-chlorimide 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 hydrolysis on the plate, followed by coupling the resulting phenols with diazotised 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 selectivity, 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 min 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.

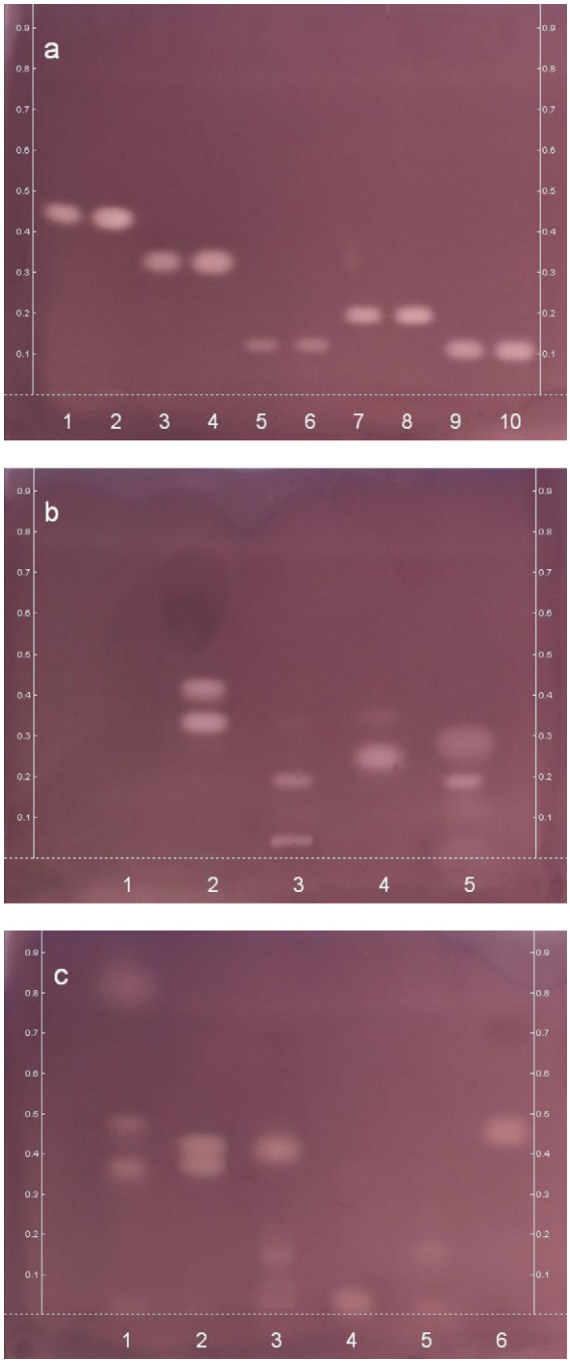


Fig. 2. Plate images of HPTLC-EI assays using BS2 esterase as an enzyme source, with bromine oxidation performed before chromatography: (a) chlorpyrifos, 20 ng (1), chlorpyrifos oxon, 20 ng (2), chlorpyrifos-methyl, 500 ng (3), chlorpyrifos-methyl oxon, 500 ng (4), malathion, 100 ng (5), malaoxon, 100 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).

3.3. 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

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

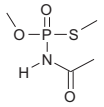
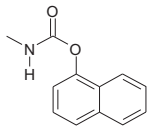
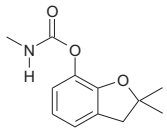
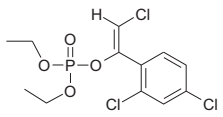
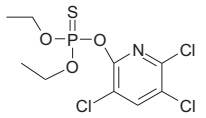
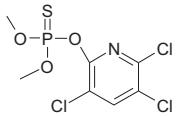
Insecticide	Structure formula	Enzyme	Calibration range [ng/zone]	R^2	LOD [ng/zone]	LOQ [ng/zone]	Enzyme inhibition factor f_i [mol^{-1} min^{-1}]			
							After oxidation		Non-oxidised standard [22]	Dev. %
							f_i	RSD [%] (n)		
Acephate		RLE	N.I.	–	–	–	–	–	–	–
		BS2	N.I.	–	–	–	–	–	–	–
		CUT	N.I.	–	–	–	–	–	–	–
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

Table 1 (*Continued*)

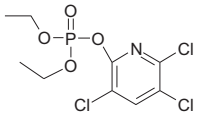
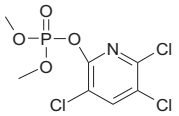
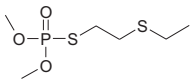
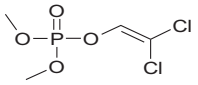
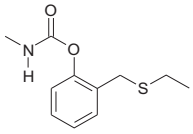
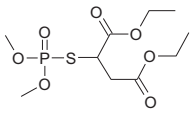
Insecticide	Structure formula	Enzyme	Calibration range [ng/zone]	R^2	LOD [ng/zone]	LOQ [ng/zone]	Enzyme inhibition factor f_i [mol^{-1} min^{-1}]			
							After oxidation		Non-oxidised standard [22]	Dev. %
							f_i	RSD [%] (n)		
Chlorpyrifos oxon		RLE	0.3–1.5	0.9854	0.5	0.9	3.0×10^{13}	8.0 (3)	2.2×10^{14}	−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
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	2000–10,000	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)	5.1×10^{10}	−52.9

Table 1 (*Continued*)

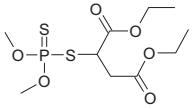
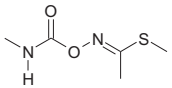
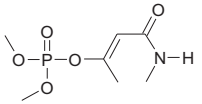
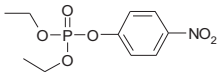
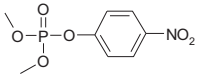
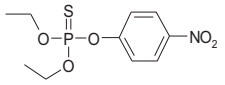
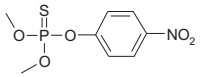
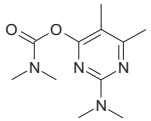
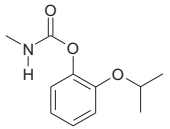
Insecticide	Structure formula	Enzyme	Calibration range [ng/zone]	R^2	LOD [ng/zone]	LOQ [ng/zone]	Enzyme inhibition factor f_i [mol^{-1} min^{-1}]			
							After oxidation		Non-oxidised standard [22]	Dev. %
							f_i	RSD [%] (n)		
Malathion		RLE	50–250	0.9917	70	120	4.7×10^{11}	6.3 (4)	1.5×10^{10}	+3033
		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
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

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-oxidised standard [22]	Dev. %
							f_i	RSD [%] (n)		
Parathion		RLE	0.05–0.25	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	0.1–05	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 ($100 \times (f_{i-ox} - f_i)/f_i$)); n (number of determinations).

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.

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).

Because different compounds were detected in these experiments as a result of bromine treatment, it should be kept in

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.

Sample	Insecticide	Spiking level (mg/L)	Dilution/concentration	RLE		Dilution/concentration	CUT	
				Recovery %	RSD % ($n=3$)		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

mind that all possible reaction 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.

3.4. 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).

4. 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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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.02.029.

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