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Multienzyme Inhibition Assay for Residue Analysis of Insecticidal Organophosphates and Carbamates

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A recently developed spectrophotometric assay for the detection of organophosphorus and carbamate insecticides by means of cutinase inhibition has been successfully extended to two esterases derived from *Bacillus subtilis* (BS2) and rabbit liver. These esterases were selected because of their high sensitivity to the examined insecticide classes and their pronounced inhibition profile. With inhibition constants (k_i) of 2.0×10^7 and 2.6×10^6 L/(mol·min) for rabbit liver esterase and BS2, respectively, chlorpyrifos oxon proved to be the strongest inhibitor directly followed by paraoxon. As compared to choline esterases and the recently studied cutinase, both esterases are surprisingly strongly inhibited by organophosphorus thions, showing k_i in the range of 5.3×10^2 to 2.3×10^4 L/(mol·min). All tested insecticidal carbamates were also inhibitors of BS2 and rabbit liver esterase, albeit in a rather uniform manner. Generally, both enzymes were found to be about 2 orders of magnitude more sensitive on the studied insecticides than cutinase even with an enhanced sensitivity against plant matrix effects. Plant extracts, obtained according to the QuEChERS method, were subjected to solid-phase extraction (SPE) using a mixed mode strong anion exchanger/primary secondary amine sorbent and $C18_{endcapped}$ cartridges for superior cleanup. With spiked samples of apple juice, best recoveries of 73% (±61%), 94% (±25%), and 134% (±17%) were obtained for chlorpyrifos, parathion–methyl, and paraoxon, respectively. Results of exemplarily performed liquid chromatography–mass spectrometry control measurements were well in accordance with measurements obtained by enzyme inhibition.

KEYWORDS: Multienzyme assay; enzyme inhibition; organophosphate insecticides; carbamate insecticides; *Bacillus subtilis* esterase BS2; rabbit liver esterase

INTRODUCTION

Despite the development of fast sample preparation and extraction methods (*1–3*), identification and quantification of pesticide residues and contaminations are most time consuming and cost intensive with respect to modern mass spectrometer coupled chromatography as GC–MS(/MS) or LC–MS(/MS). Thus, a prescreening test to separate positive from negative samples seemed to be a promising approach for saving time and costs, thereby enabling a considerable enhancement of sample throughput. Bioanalytical methods such as enzyme assays performed in microtiter plates offer the chance to simultaneously analyze many samples and to provide results within a few hours. As in the case of enzyme inhibition assays, effect-directed analysis represents a rather new approach for an enhanced throughput in food control by analyzing prescreened positive samples (*4, 5*); this strategy, so far, has been applied only in the field of ecotoxicology.

Insecticides—with a volume of ∼615 million kg—rank second in the amount of total world pesticide applied in 2000 and 2001, corresponding to 25% of the world pesticide market (*6*). For screening water samples with respect to insecticidal organophosphates and carbamates, two techniques by means of acetylcholine esterase (AChE) inhibition are in use (*7, 8*). However, an enzymatic assay for the screening of more complex matrices like plant or soil samples is in high demand. While our recently presented cutinase assay (*9–11*) fulfills these demands, the lack of qualitative information of an unknown sample is still a disadvantage. To overcome this drawback with the objective of a multienzyme test, the cutinase assay should be extended on further promising serine hydrolases, that is, enzymes sensitively inhibited by organophosphorus and carbamate insecticides but almost different from cutinase in terms of quality and quantity. Therefore, different esterases and proteases were tested for their inhibition profile to identify at least two further enzymes to be used in parallel with cutinase in a one-plate multienzyme assay.

MATERIALS AND METHODS

Materials. The esterase basic kit (containing esterases from *Bacillus* spp., *Bacillus stearothermophilus*, *Bacillus thermoglucosidarius*, *Candida lipolytica*, *Mucor miehei*, *Saccharomyces cerevisiae*, *Thermoanaerobium brockii*, hog liver, and horse liver (all EC 3.1.1.1)), esterase

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Table 1. Test of Various Enzymes for Applicability in the Multienzyme Assay

	enzyme	substrate turnover[a]	inhibition by the tested insecticides (cf. Figure 1)[b]
		p-nitrophenyl-butyrate	
esterase from	*Bacillus* spp.[c]	–	n.t.
	B. stearothermophilus[c]	+	+
	B. thermoglucosidarius[c]	–	n.t.
	C. lipolytica[c]	++	+
	M. miehei[c]	+ (no linear absorbance increase)	n.t.
	S. cerevisiae[c]	–	n.t.
	T. brockii[c]	–	n.t.
	hog liver[c]	+++	+
	horse liver[c]	+	+
	rabbit liver	+++	+
	BS2, recombinant from *E. coli*	+++	+
	S. diastatochromogenes, recombinant from *E. coli*	++	–
		p-nitrophenyl-butyrate	
other enzymes	proteinase K from *T. album*	++	–
	lipoprotein lipase from *P. fluorescens*	++	–
		N-α-carbobenzyloxy-L-lysine-4-nitrophenyl ester hydrochloride	
serine proteases	trypsin	+++	–
	α-chymotrypsin	+++	–

[a] Visually estimated intensity of the substrate turnover (i.e., yellow coloring of the batch within 5 min). [b] +, Inhibition; –, no inhibition; n.t., not testable because of lack of substrate turnover. [c] Esterases present in the esterase kit.

Bacillus subtilis (BS2), recombinant from *Escherichia coli*, esterase *Streptomyces diastatochromogenes*, recombinant from *E. coli* (EC 3.1.1.1), lipoprotein lipase from *Pseudomonas fluorescens* (EC 3.1.1.34), α-chymotrypsin from bovine pancreas (EC 3.4.21.1), and trypsin from hog pancreas (EC 3.4.21.4) were all obtained from Fluka (Taufkirchen, Germany). Esterase from rabbit liver (lyophilized powder containing ≥85% protein) (EC 3.1.1.1) and proteinase K from *Tritirachium album* (EC 3.4.21.64) were purchased from Sigma (Taufkirchen, Germany). Cutinase (EC 3.1.1.74) from *Fusarium solani pisi*, lyophilized (molecular mass, 20 605 Da), 78% protein content, 22% NaCl, was kindly provided by Unilever Research Laboratory, Vlaardingen, The Netherlands. *N*-α-Carbobenzyloxy-L-lysine-4-nitrophenyl ester hydrochloride (98%) was obtained from Acros (Geel, Belgium). Resorufin butyrate (>95%) was obtained from Fluka and resorufin (95%) from Sigma-Aldrich. Pesticide reference standards (cf. ref *10*) were obtained from Riedel-de-Haën (Pestanal, Taufkirchen, Germany) or Dr. Ehrenstorfer GmbH (Augsburg, Germany). Supelclean solid-phase extraction (SPE) dual layer cartridges (6 mL) containing 500 mg of strong anion exchanger (SAX, upper layer) and 500 mg of primary secondary amine sorbent (PSA, lower layer) and Discovery DSC-18 SPE cartridges (6 mL, 1 g) were obtained from Supelco (Taufkirchen, Germany). Further SPE columns tested were Supelclean ENVI-Carb (graphitized carbon black, 6 mL, 500 mg), PSA bonded silica (6 mL, 500 mg), Discovery DSC-NH_2 (6 mL, 1 g), and Discovery DSC-SAX (6 mL, 500 mg), all obtained from Supelco. Magnesium sulfate (≥99.5%) was obtained from Sigma-Aldrich and sodium chloride (p.a.) from Merck (Darmstadt, Germany). All solvents used were of analytical grade, and water was prepared with a Milli-Q 185 Plus water purification system (Millipore, Eschborn, Germany). All reagents of the spectrophotometric assay (*9*) and the oxidation-step (*11*) have been reported previously.

Juice and blank samples of fruits and vegetables were purchased from a local organic produce store.

Enzyme Assay. The enzyme assay was performed by means of a 96-well automated microplate spectrophotometer as described by Walz and Schwack, ref *9*, thereby replacing cutinase solution by other enzymes solutions (see below). To enhance the inhibition strength of phosphorothionates, a preceding oxidation step by means of chloroperoxidase and hydrogen peroxide was performed as described by Walz and Schwack, ref *11*. Measurements were performed against solvent blanks.

Enzyme Solutions. With the exception of hog liver esterase, the tested enzymes were dissolved in Tris/HCl buffer (pH 7.0; 0.5 mol/L) to obtain the following concentrations: rabbit liver esterase (3.6 mg/L), BS2 (13.6 mg/L), *B. stearothermophilus* esterase (1000 mg/L), *C. lipolytica* esterase (520 mg/L), horse liver esterase (1650 mg/L), *Bacillus* spp. (60 mg/L), *B. thermoglucosidarius* esterase (900 mg/L), *M. miehei* esterase (300 mg/L), *T. brockii* esterase (200 mg/L), *S. diastatochromogenes* esterase (15 mg/L), α-chymotrypsin (250 mg/L), trypsin (15 mg/L), proteinase K (150 mg/L), and lipoprotein lipase (1250 mg/L). Because of flocculation at pH 7.0, hog liver esterase was dissolved in Tris/HCl buffer of pH = 7.4 (7.6 mg/L), and the solution had yet to be filtered.

Pesticide Standard Solutions. Stock solutions of 50–1000 mg/L were prepared in methanol, and working standards of 0.1 μg/L to 100 mg/L were subsequently diluted by water or water/methanol mixtures, respectively, to a methanol content of 10%.

Substrate Solutions. The substrate solution of *N*-α-carbobenzyloxy-L-lysine 4-nitrophenyl ester hydrochloride for the proteases trypsin and α-chymotrypsin was prepared by dissolving 6 mg in 1 mL of methanol and filling up to a volume of 10 mL by phosphate buffer (66.67 mmol/L; pH = 6.6). The *p*-nitrophenyl-butyrate solution was prepared as described in ref *9*.

Extract Preparation of Plant Samples and Fruit Juice. According to the QuECHERS method (*1*), a 10 g aliquot of a previously homogenized and spiked sample or 10 mL of fruit juice, respectively, was extracted with 10 mL of acetonitrile (MeCN) in the presence of 4 g of anhydrous $MgSO_4$ and 1 g of NaCl by vigorous shaking. Sample extracts were cleaned by means of SPE (*11*), and a combination of SAX/PSA and $C18_{endcapped}$ material (Discovery DSC-18) proved to be optimum. To avoid SPE material-caused enzyme inhibition, the cartridges were preconditioned as follows: SAX/PSA, 6 × 6 mL MeCN; DSC-18, 2 × 6 mL MeCN, 2 × 6 mL H_2O, 6 × 6 mL MeCN.

The colorless extract solutions were subjected to the oxidation step (*11*) and subsequent enzyme assay. Measurements were performed against the oxidized solvent blanks and evaluated against the corresponding matrix blanks.

High-Performance Liquid Chromatography–Electrospray Mass Spectrometry (LC/MS). LC/MS analyses were performed by means of an HP1100 (Hewlett-Packard, Waldbronn, Germany) system consisting of an autosampler, a gradient pump, and a diode array detector (DAD) module coupled to a VG platform II quadrupole mass spectrometer (Micromass, Manchester, U.K.) equipped with an electrospray interface (ESI). MS parameters: ESI+, source temperature 120 °C; capillary voltage, 3.5 kV; HV lens, 0.5 kV; and cone voltage, 30 V. For LC/MS analyses, the MS system was operated in the selected ion monitoring mode (SIM) at *m/z* 350 ($[M + H]^+$ of chlorpyrifos). The MassLynx 3.2. software was used for data acquisition and processing.

The separation was performed at 25 °C using a reversed phase analytical column (5 μm, C_{18} Luna, 250 × 3 mm, Phenomenex, Aschaffenburg, Germany). Gradient elution was achieved by means of (A) methanol and (B) ammonium formate buffer (10 mmol/L, pH 4.0); the gradient chosen to %A (*t* [min]), 50 (0)–100 (8–11)–50 (12–18); flow rate, 0.5 mL/min; injection volume, 20 μL.

RESULTS AND DISCUSSION

Selection of Suitable Enzymes. A major aim for the development of a multienzyme inhibition assay was the identification of suitable enzymes. Therefore, various enzymes, especially esterases, were tested with respect to both turnover

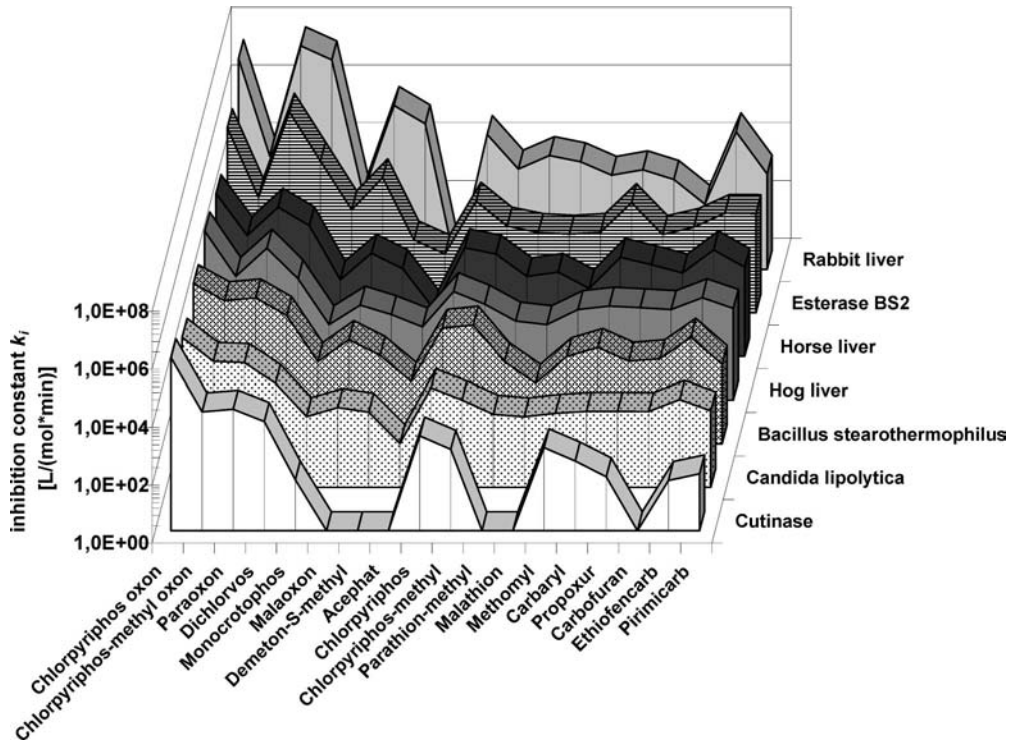


Figure 1. Inhibition profiles of the tested esterases.

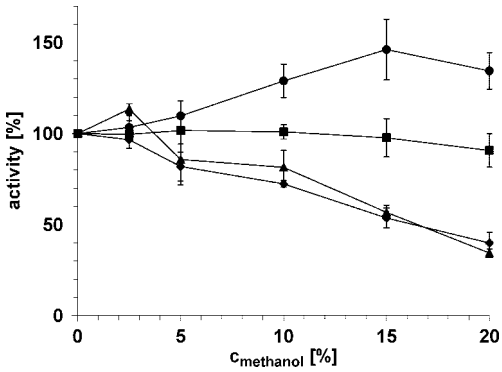


Figure 2. Influence of varying methanol concentrations (in the inhibition batch) on the activity of esterases; incubation time 30 min, hog liver esterase (●, $n = 4$); *C. lipolytica* esterase (■, $n = 5$); rabbit liver esterase (▲, $n = 4$); BS2 (♦, $n = 5$).

of a *p*-nitrophenol releasing substrate (*9*) and insecticide-caused inhibition. For this purpose, insecticide standards diluted so as to fall within a range of 10–90% inhibition were used for screening. Enzymes that proved to be promising were the esterases from rabbit liver, horse liver, hog liver, BS2, *B. stearothermophilus*, and *C. lipolytica* (**Table 1**), whose inhibition profiles are visualized in **Figure 1** as compared to the previously studied cutinase (*9*, *10*). Obviously, the aryl phosphates chlorpyrifos oxon, chlorpyrifos–methyl oxon, and paraoxon exerted the strongest inhibition effects on all tested esterases. Esterase from *B. stearothermophilus* exhibited a reduced substrate turnover so that measurements had to be continued over 15 min instead of the 2 min typically used for the former cutinase assay.

Interestingly, a rough similarity between the inhibition profiles of hog liver and those of *C. lipolytica* esterases was revealed, while hog liver and horse liver esterase profiles differ only under the influence of carbamates (**Figure 1**). With respect to organophosphorus insecticides, the inhibition profiles of BS2, horse liver, hog liver, and *C. lipolytica* esterases were found to be nearly identical (**Figure 1**). However, horse liver esterase and hog liver esterase failed the screening because of the brown color of the enzyme solution and the almost reversible enzyme inhibitions, respectively. In conclusion, BS2 and rabbit liver esterase were identified as optimal enzymes for the development of a multienzyme assay, especially with respect to combining enzymes with significantly different inhibition profiles as compared to cutinase.

Proteinase K and lipoprotein lipase both were able to hydrolyze well the substrate *p*-nitrophenyl butyrate; however, they were not inhibited by either organophosphorus or carbamate insecticides (**Table 1**). The serine proteases trypsin and α-chymotrypsin were further regarded as interesting test candidates in view of their reported inhibition by organophosphates (*12*). An appropriate substrate with which to study these enzymes was *N*-α-carbobenzyloxy-L-lysine-4-nitrophenyl ester hydrochloride (*13*,*14*), which also releases *p*-nitrophenol. Optimum pH values are reported to be 6.5–8.5 for trypsin and 7.8 for α-chymotrypsin, respectively (*13*). Because the substrate was not stable in the tris(hydroxymethyl)aminomethane (Tris) buffer at pH 7.4 (*9*), an alternative phosphate buffer of pH 6.6 was used. In performing the inhibition assays with the tested insecticides, only acephate was found to exhibit a weak inhibition effect on both proteases, whereas α-chymotrypsin was additionally inhibited by paraoxon and chlorpyrifos–methyl oxon but to a rather lesser extent. Therefore, trypsin and α-chymotrypsin proved to be absolutely inappropriate for application in the multienzyme assay.

Solvent Tolerance of Esterases. Because most organophosphorus and carbamate insecticides possess only limited water solubility (*14*), water-miscible organic modifiers have to be used for both standard solutions and sample extracts. As observed for cutinase (*9*), organic solvents impede enzyme activity, especially at higher concentrations. Because acetonitrile, as a most effective solvent for sample extractions (*1*), had the strongest effect on cutinase activity, an evaporation step for sample extracts was necessary, which considerably

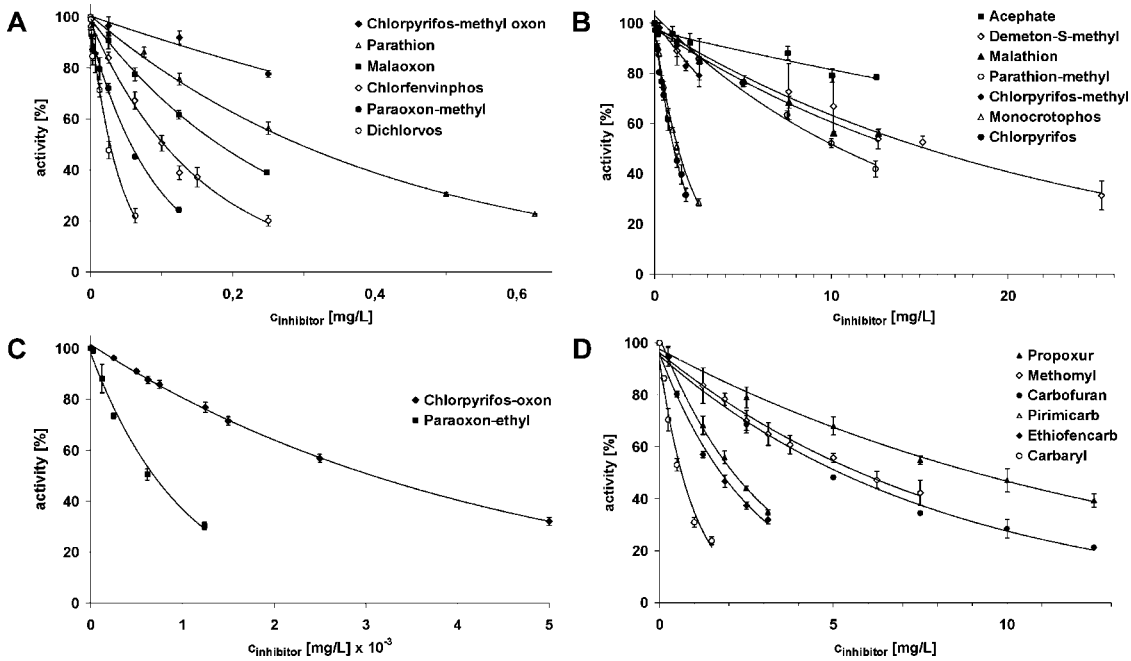


Figure 3. Inhibition of BS2 as a function of pesticide concentrations (mg/L in the inhibition batch) at constant incubation time (30 min) and temperature (25 °C). **A**, **B**, **C**: organophosphorus insecticides. **D**, carbamates ($n \geq 3$ for each concentration).

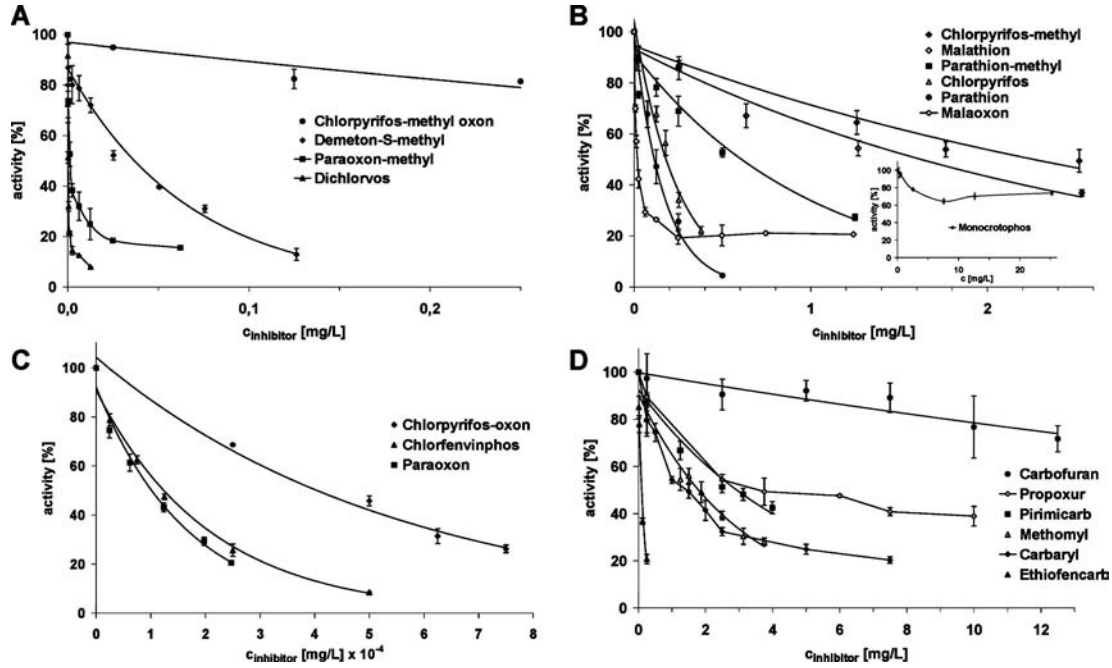


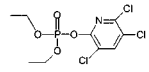
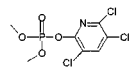
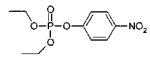
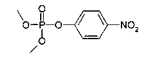
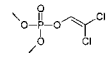
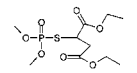
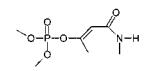
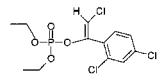
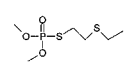
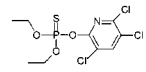
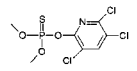
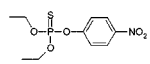
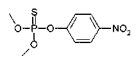
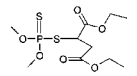
Figure 4. Inhibition of rabbit liver esterase as a function of pesticide concentrations (mg/L in the inhibition batch) at constant incubation time (30 min) and temperature (25 °C). **A**, **B**, **C**: organophosphorus insecticides. **D**: carbamates ($n \geq 3$ for each concentration).

increased the detection limits (*9*). The presence of methanol, as also studied for the cutinase assay (*9*), affected the investigated esterases differently. While BS2 and rabbit liver esterase activities were found to decrease with higher methanol concentrations (>5%) in the assay, there was nearly no effect on *C. lipolytica* esterase. On the contrary, the activity of hog liver esterase significantly increased with methanol concentrations of 5–15% (**Figure 2**). However, a methanol concentration of 2.5% in the inhibition batch of the assay was tolerable for all tested enzymes, similar to the case of cutinase (*9*).

Inhibition Effects of Organophosphate and Carbamate Insecticides. To characterize the inhibition profile of the elected esterases of rabbit liver and BS2 in more detail, the calibration curves of the selected insecticidal organophosphates and carbamates (*9–11*) were measured (**Figures 3** and **4**) and the respective inhibition constants calculated (*9, 10*) (**Tables 2** and **3**).

Both enzymes were found to be almost very sensitive against the tested insecticides, resulting in low limits of detection (LOD) extending even into the ng/L region for the strongest inhibitor paraoxon (**Tables 2** and **3**), whereas LOD is defined as an inhibitor concentration causing at least 10% inhibition under assay conditions (*9*). Organophosphates, that is, oxons, proved to be more inhibitory by about 2 to 3 orders of magnitude than the corresponding thions (**Table 2**). As compared to cutinase (*10*), thions are more inhibitory against the two esterases (**Figure**

Table 2. Inhibition Constants k_i of Organophosphorus Insecticides[d]

pesticide	structure formula		esterase BS2 k_i (RSD, n) (R^2)[a]	esterase BS2 LOD[b] [mg/L]	rabbit liver esterase k_i (RSD, n) (R^2)[a]	rabbit liver esterase LOD[b] [mg/L]
chlorpyrifos-oxon		c	2.4×10^{6} (±10%, 25)	5.2×10^{-4}	2.0×10^{7} (±11%, 21)	8.1×10^{-5}
		s	2.6×10^{6} (0.9996)		2.0×10^{7} (0.9880)	
chlorpyrifos-methyl oxon		c	9.7×10^{3} (±37%, 7)	0.11	$1.6\cdot10^{4}$ (±37%, 9)	0.09
		s	9.9×10^{3} (0.9595)		8.4×10^{3} (0.8250)	
paraoxon		c	1.0×10^{7} (±12%, 14)	8.7×10^{-5}	6.4×10^{7} (±14%, 28)	4.0×10^{-6}
		s	8.9×10^{6} (0.9918)		5.5×10^{7} (0.9920)	
paraoxon-methyl		c	1.1×10^{5} (±26%, 15)	2.0×10^{-3}	4.1×10^{6} (±37%, 21)[c, 1)]	~1×10^{-4}
		s	9.1×10^{4} (0.9940)		2.9×10^{6} (0.9377)[c, 1)]	
dichlorvos		c	2.0×10^{5} (±12%, 9)	2.8×10^{-3}	1.8×10^{7} (±23%, 31)[c, 2)]	3.2×10^{-5}
		s	1.8×10^{5} (0.9913)		1.5×10^{7} (0.9837)[c, 2)]	
malaoxon		c	4.2×10^{4} (±11%, 18)	0.03	4.7×10^{5} (±23%, 17)[c, 3)]	6.7×10^{-4}
		s	4.8×10^{4} (0.9995)		3.5×10^{5} (0.9538)[c, 3)]	
monocrotophos		c	4.1×10^{3} (±9%, 24)	0.15	5.1×10^{2} (±32%, 17)[c, 4)]	1.60
		s	3.7×10^{3} (0.9945)		4.3×10^{2} (0.9333)[c, 4)]	
chlorfenvinphos (mixture of Z- and E-isomers)		c	8.2×10^{4} (±11%, 37)	0.01	8.4×10^{7} (±27%, 19)	2.0×10^{-6}
		s	7.8×10^{4} (0.9918)		5.8×10^{7} (0.9958)	
demeton-S-methyl		c	3.5×10^{2} (±26%, 23)	2.06	2.0×10^{5} (±39%, 32)	2.5×10^{-4}
		s	3.3×10^{2} (0.9889)		1.1×10^{5} (0.9856)	
acephat		c	1.3×10^{2} (±24%, 13)	4.25	no inhibition	
		s	1.1×10^{2} (0.9268)			
chlorpyrifos		c	7.9×10^{3} (±15%, 32)	0.14	4.3×10^{4} (±17%, 22)	0.03
		s	7.3×10^{3} (0.9964)		4.7×10^{4} (0.9818)	
chlorpyrifos-methyl		c	9.5×10^{2} (±36%, 26)	1.15	3.5×10^{3} (±17%, 31)	0.17
		s	1.0×10^{3} (0.9764)		3.0×10^{3} (0.9656)	
parathion		c	2.2×10^{4} (±10%, 22)	4.6×10^{-2}	6.5×10^{4} (±37%, 26)	1.6×10^{-2}
		s	2.3×10^{4} (0.9992)		5.9×10^{4} (0.9926)	
parathion-methyl		c	5.5×10^{2} (±9%, 35)	1.97	1.3×10^{4} (±30%, 27)	0.02
		s	6.0×10^{2} (0.9898)		8.7×10^{3} (0.9855)	
malathion		c	5.7×10^{2} (±14%, 21)	1.74	5.3×10^{3} (±28%, 28)	0.09
		s	5.3×10^{2} (0.9699)		5.3×10^{3} (0.9612)	

[a] RSD [%], relative standard deviation; *n*, number of determinations; R^2, statistical correlation factor of the calibration curves. [b] LOD, limit of detection [batch concentration (mg/L)]. Multiplication by 4 gives the concentration of the pesticide standard solution/sample extract. [c] Reaction of pseudo first order kinetics only up to standard concentrations of (1) 0.01 mg/L, (2) 3×10^{-3}mg/L, (3) 0.1 mg/L, (4) 30 mg/L; at higher concentrations reversible enzyme inhibition. [d] k_i, Inhibition constant (L/(mol · min)); c, calculated; cf. refs *9* and *10*; s, slope; quotient of slope of calibration curve [ln(activity) vs inhibitor concentration (mol/L, in the inhibition batch)] and incubation time (30 min); cf. ref *9*.

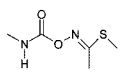
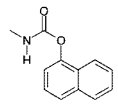
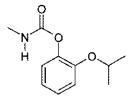
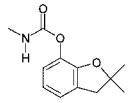
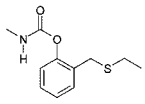
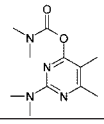
1), showing proof of the advantageous combination of cutinase, BS2, and rabbit liver esterase for a multienzyme assay although inhibition effects on rabbit liver esterase obeyed reversible reaction kinetics for some insecticides resulting in constant levels of residual enzyme activities independent of increasing concentrations (**Figure 4**).

As compared to organophosphorus insecticides, inhibition effects of the carbamates on BS2 and rabbit liver esterase were significantly reduced and revealed only minor variations in inhibition strength. No really outstanding inhibitors were identified as was the case of methomyl and carbaryl for cutinase inhibition (*10*).

Oxidation of Organophosphorothionates. The recently introduced preoxidation step by means of chloroperoxidase to enhance the inhibition strength of organophosphorothionates (*11*) proved to be also applicable for the BS2 and rabbit liver esterases. The inhibition constants of oxidized thions were well in accordance with those determined for the corresponding oxon standards (**Table 4**) with the sole exception, however, of oxidized chlorpyrifos–methyl, already known from the previous cutinase studies (*11*). As in the case of cutinase, the enhanced inhibition strength cannot be explained by the formation of the decomposition products 3,5,6-trichloro-2-pyridinol and dimethyl phosphate (**Table 5**).

Sample Extraction and Cleanup. Plant samples were extracted with acetonitrile according to the QuEChERS method (*1*). However, careful cleanup of extracts yielding completely colorless extracts is necessary to avoid enzyme inhibition caused

Table 3. Inhibition Constants k_i of Insecticidal Carbamates[d]

pesticide	structure formula		esterase BS2 k_i (RSD, n) (R^2)[a]	esterase BS2 LOD[b] [mg/L]	rabbit liver esterase k_i (RSD, n) (R^2)[a]	rabbit liver esterase LOD[b] [mg/L]
methomyl		c	6.9×10^2 (±15%, 43)	0.59	2.2×10^3 (±17%, 28)	2.2×10^{-2}
		s	6.1×10^2 (0.9907)		1.8×10^3 (0.9786)	
carbaryl		c	8.0×10^3 (±13%, 21)	0.03	3.7×10^3 (±16%, 28)[c, 1)]	0.05
		s	6.6×10^3 (0.9755)		2.8×10^3 (0.9769)[c, 1)]	
propoxur		c	5.6×10^2 (±16%, 19)	1.10	1.5×10^3 (±15%, 15)[c, 2)]	0.29
		s	5.1×10^2 (0.9964)		1.3×10^3 (0.9785)[c, 2)]	
carbofuran		c	1.0×10^3 (±11%, 22)	0.45	1.6×10^2 (±56%, 30)	4.31
		s	9.1×10^2 (0.9914)		1.8×10^2 (0.8965)	
ethiofencarb		c	3.1×10^3 (±9%, 18)	0.17	6.1×10^4 (±24%, 18)	8.7×10^{-3}
		s	2.8×10^3 (0.9904)		5.8×10^4 (0.9829)	
pirimicarb		c	2.5×10^3 (±9%, 17)	0.38	2.1×10^3 (±16%, 16)	0.24
		s	2.7×10^3 (0.9970)		2.0×10^3 (0.9821)	

[a] RSD [%], relative standard deviation; *n*, number of determinations; R^2, statistical correlation factor of the calibration curves. [b] LOD, limit of detection [batch concentration (mg/L)]. Multiplication by 4 gives concentration of the pesticide standard solution/sample extract. [c] Reaction of pseudo first order kinetics only up to the standard concentrations of (1) 10 mg/L, (2) 20 mg/L; at higher concentrations reversible enzyme inhibition. [d] k_i, Inhibition constant (L/(mol·min)); c, calculated; cf. refs *9* and *10*; s, slope; quotient of slope of calibration curve [ln(activity) vs inhibitor concentration (mol/L; in the inhibition batch)] and incubation time (30 min); cf. ref *9*.

by matrix compounds. To this purpose, SPE seemed to be most promising. In view of the preconditioning necessary to prevent SPE material-originated enzyme inhibition, cartridges in terms of handling proved superior over dispersive SPE of the QuEChERS method, avoiding multiple centrifugation steps. Different SPE materials were tested for their suitability concerning matrix elimination. Graphitized carbon black used in combination with the PSA in the modified QuEChERS version for matrices with high chlorophyll or carotene contents (*2*) resulted in a significant inhibition of all three esterases, especially of the most sensitive rabbit liver esterase, even after extended and repeated prerinsing with solvents of different polarities. For removing carotenes, C18 silica, especially in the endcapped form, proved to be the optimal sorbent. Moreover, $C18_{endcapped}$ retained chlorophyll, which is also retained to some extend by PSA. A polymer bonded amino phase (DSC–NH_2) was tested as an alternative to the PSA, but the PSA was more efficient in plant matrix removal, especially with respect to anthocyans. However, a polymerically bonded quarternary amine as SAX was most effective for the retention of anthocyans. Therefore, it was decided that a SAX/PSA mixed-mode column combined with a $C18_{endcapped}$ cartridge would be a promising approach for sample extract cleanup. These adsorbents were also applied in the "Luke method" for residue analysis in plant matrices but in reversed order followed by different solvent extraction and evaporation steps (*15, 16*).

Despite the colorlessness of the sample extracts, measurement had to be performed against matrix blanks because of the high matrix sensitivity of the BS2 and rabbit liver esterases, expressed in reduced enzyme activities as compared to solvent blanks (**Table 6**). In contrast, cutinase proved to be more robust, retaining activity of about 100% in the blank samples (**Table 6**). This tolerance, however, is obtained at the expense of a reduced sensitivity against insecticides as compared to the latter esterases. The influence of plant substances on enzyme activities has not been examined well so far. However, Rohn et al. (*17–20*) studied the influence of plant phenols on enzymes, for example, the serine proteases α-chymotrypsin and trypsin, and found that these substances affect the in vitro enzyme activity under relatively mild conditions (pH 5–9, room temperature) by derivatization of nucleophilic side chains like lysine, tryptophan, and cysteine. In a recent publication, Djeridane et al. (*21*) showed that extracts of plant phenols, that is, phenolic acids and flavonoids, significantly exhibit inhibitory effects on porcine liver carboxylesterase (EC 3.1.1.1), which is a serine esterase related to the enzymes studied here.

However, matrix influence is not a specific drawback of enzymatic measurements. It is a common practice also to perform GC/MS and LC/MS measurements against matrix-matched calibration standards (*1, 3, 22*) to warrant exclusion of matrix-induced enhancement or suppression of signal intensity.

Pesticide Residue Analysis by Means of Multienzyme Assay in Plant Samples. In our present study BS2, rabbit liver

Table 4. Inhibition Constants k_i of Organophosphorus Thions after Chloroperoxidase Oxidation (*11*) As Compared to the Corresponding Oxon Standards[b]

	esterase BS2 k_i [L/(mol·min)]			rabbit liver esterase k_i [L/(mol·min)]		
	after oxidation			after oxidation		
pesticide	calculated (RSD, *n*)	slope	oxon standard	calculated (RSD, *n*)	slope	oxon standard
chlorpyrifos	1.7×10^6 (±29%, 35)	1.8×10^6	2.6×10^6	7.3×10^6 (±34%, 42)	5.0×10^6	2.0×10^7
chlorpyrifos–methyl	1.3×10^5 (±19%, 21)	1.2×10^5	9.9×10^3	5.2×10^6 (±48%, 42)	5.5×10^6	8.4×10^3
parathion	3.6×10^6 (±37%, 20)	2.6×10^6	8.9×10^6	2.4×10^7 (±26%, 9)	1.7×10^7	5.5×10^7
parathion–methyl	6.1×10^4 (±14%, 12)	7.2×10^4	9.1×10^4	reversible enzyme inhibition		2.9×10^6 (up to 0.1 mg/L)[a]
malathion	1.2×10^4 (±37%, 38)	1.7×10^4	4.8×10^4	not measured		3.5×10^5 (up to 0.1 mg/L)[a]

[a] Reaction of pseudo first order kinetics only up to the standard concentration given in brackets; at higher concentrations reversible enzyme inhibition. [b] k_i, inhibition constant (L/(mol·min)); calculated, cf. refs *9* and *10*; slope, quotient of slope of calibration curve [ln(activity) vs inhibitor concentration (mol/L, in the inhibition batch)] and incubation time (30 min); cf. ref *9*; RSD [%], relative standard deviation; *n*, number of determinations.

Table 5. Inhibition Constants k_i of the Decomposition Products of Chlorpyrifos−Methyl, Formed by Chloroperoxidase Oxidation[a]

	esterase BS2		rabbit liver esterase	
	k_i [L/(mol · min)]			
degradation products	calculated (RSD, *n*)	slope	calculated (RSD, *n*)	slope
3,5,6-trichloro-2-pyridinol	1.3×10^3 (±33%, 6)	1.0×10^3	5.4×10^2 (±33%, 15)	6.4×10^2
dimethyl phosphate	7.2×10^1 (±58%, 7)	8.4×10^1	no inhibition	

[a] k_i, inhibition constant; calculated, cf. refs *9* and *10*; slope, quotient of slope of calibration curve [ln(activity) vs inhibitor concentration (mol/L, in the inhibition batch)] and incubation time (30 min); cf. ref *9*; RSD [%], relative standard deviation; *n*, number of determinations.

esterase, and cutinase have been used for the detection of selected insecticides in exemplarily spiked samples, thereby considering representatives of the three classes of studied insecticides, that is, organophosphorus thions, oxons, and carbamates. Pesticides were chosen according to their relevance in application (*11*), that is, chorpyrifos, paraoxon, parathion–methyl, dichlorvos, and carbaryl, and spiked with regard to the MRL. The optimized sample preparation method was tested with respect to accuracy, sensitivity, and practicability by analyzing the spiked samples and sample blanks of each matrix in triplicate, at least. Accuracy was assessed by calculating the attained recovery rates, which were found to vary significantly from matrix to matrix ranging from 3% for chlorpyrifos in grapefruit to 134% for paraoxon in apple juice (**Table 7**). Obviously, best recoveries were generally obtained for juice samples, in accordance with the results obtained by means of an AChE biosensor assay, reporting paraoxon recoveries of 84% in orange juice while only 44% and 52% were recovered from apple and peach pulp, respectively (*25*). Cucumber, zucchini, grapefruit, and figs seem to represent problematic matrices, going along with low recoveries (**Table 7**), but also were rather low spiked. The comparison with exemplarily analyzed chlorpyrifos spiked samples by means of LC/MS showed, however, that low recoveries are not a specific drawback of the enzymatic measurement.

The repeatability, that is, the precision, was assessed by calculating the relative standard deviations (RSD), which varied between 17% for paraoxon in apple juice and 117% for chlorpyrifos in grapefruit (**Table 7**). It comes as no surprise that low recovery rates generally correlated with higher RSD values; thus, for example, for chlorpyrifos in grapefruit (RSD, 117%) a recovery of only 3% was obtained in contrast to that for parathion–methyl in apple juice (RSD, 25%) with a recovery of 94%. However, it has to be considered that according to Gilsbach (*26*) deviations of 60% have to be faced for a concentration interval of 0.01–3 mg/kg in plant materials.

The sensitivity of the assay is given by the LOD for each insecticide and corresponding enzyme. With respect to the LODs for pesticide standards given in **Tables 2** and **3**, it can be seen that the assay itself is almost rather sensitive. Considering the obtained recoveries in different matrices, the detectability is respectively reduced depending on the sample type. Nevertheless, the concentrations of spiked samples determined demonstrate well that the assay is able to identify samples violating the maximum residue limits (MRL). EU requirements, however, for validated methods and routine analyses (*27*), that is, recoveries of 70–110% and 60–140%, respectively, could not be verified for all analyzed samples. So far, a drawback of enzyme assays is the fact that an internal standard usually applied in LC and GC analyses (*1*, *3*) is not applicable because of mixed mode inhibition. Thus, there is no possibility to compensate for losses during sample extraction and cleanup by calculation.

Table 6. Influence of Sample Matrix on Enzyme Activities[a]

	enzyme activity in sample blanks		
sample matrix	cutinase (RSD, *n*)	esterase BS2 (RSD, *n*)	rabbit liver esterase (RSD, *n*)
apple juice	100% (±1%, 4) oxidized, undiluted	74% (±7%, 4) oxidized, 1:5 diluted	53% (±13%, 4) oxidized, undiluted
	110% (4%, 3) not oxidized, 1:10 diluted	102% (±9%, 3) not oxidized, 1:10 diluted	46% (±9%, 3) not oxidized, undiluted
apple	100% (±3%, 4) oxidized, 1:10 diluted	91% (±3%, 4) oxidized, 1:10 diluted	93% (±3%, 4) oxidized, 1:50 diluted
		57% (±12%, 4) not oxidized, undiluted	44% (±6%, 4) not oxidized, undiluted
cherry juice	96% (±3%, 3) oxidized, 1:10 diluted	88% (±9%, 3) oxidized, 1:50 diluted	72% (±33%, 3) oxidized, 1:50 diluted
cherry	97% (±3%, 4) oxidized, 1:10 diluted	96% (±2%, 4) oxidized, 1:50 diluted	88% (±4%, 4) oxidized, 1:50 diluted
		75% (±5%, 4) not oxidized, undiluted	63% (±10%, 4) not oxidized, undiluted
peach	104% (±3%, 3) oxidized, 1:10 diluted	95% (±3%, 3) oxidized, 1:50 diluted	94% (±6%, 3) oxidized, 1:100 diluted
kiwi	93% (±4%, 4) oxidized, undiluted	81% (±9%, 7) oxidized, 1:5 diluted	81% (±7%, 4) oxidized, undiluted
	117% (±1%, 3) not oxidized, undiluted	86% (±5%, 3) not oxidized, 1:5 diluted	71% (±15%, 4) not oxidized, 1:5 diluted
grapefruit	98% (±2%, 4) oxidized, undiluted	61% (±2%, 3) oxidized, 1:5 diluted	64% (±16%, 3) oxidized, 1:5 diluted
			37% (±13%, 3) oxidized, undiluted
zucchini	96% (±1%, 4) oxidized, undiluted	~0% oxidized, 1:5 diluted	~ 20% oxidized, 1:5 diluted
cucumber	99% (±3%, 4) oxidized, undiluted	52% (±31%, 3) oxidized, undiluted	88% (±3%, 4) oxidized, 1:10 diluted
		85% (±8%, 4) oxidized, 1:10 diluted	
fig	140% (±9%, 4) not oxidized, undiluted	47% (±4%, 4) not oxidized, undiluted	36% (±9%, 4) not oxidized, undiluted
		68% (±7%, 4) not oxidized, 1:5 diluted	60% (±11%, 3) not oxidized, 1:5 diluted
peach	151% (±2%, 3) not oxidized, undiluted	70% (±0.4%, 3) not oxidized, 1:10 diluted	50% (±8%, 3) not oxidized, 1:10 diluted

[a] Matrix blanks were measured against equally treated and diluted aqueous 10% methanol solutions. RSD [%], relative standard deviation; *n*, number of determinations.

Regarding the high sensitivity, the developed multienzyme assay will at least raise an alarm if there is any inhibitor present. Additionally, as the three elected esterases used in the present assay exhibit different selectivities toward organophosporus and carbamate insecticides, there is a great chance to use the obtained

Table 7. Analyses of Various Spiked Samples by Means of the Multienzyme Assay As Compared to LC/MS Measurements[a]

					enzymatic measurements								
		spiking level	extr.[b]	MRL[c]			AV[e]	RSD[f]	AV[e]	RSD[f]	recov.[g]	LC/MS[h]	
pesticide	sample matrix	[mg/kg]	(juice: [mg/L])		enzyme	ox.[d]	[mg/kg] [mg/L]	[%]	[mg/kg] [mg /L]	[%]	[%]	[mg/kg] [mg/L]	recov.[g]
chlorpyrifos	apple juice	0.4	4.0		cutinase	+	0.22	61	0.29	61	73	0.30	75%
					BS2	+	0.36	60				0.43	86%
					rabbit	+	0.31	65					
chlorpyrifos	apple	0.5	5.0	0.5	cutinase	+	0.01	150	0.04	83	8	0.04	8%
					BS2	+	0.07	50				0.03	6%
					rabbit	+	0.04	83					
					rabbit	–	0.05	49					
chlorpyrifos	cherry juice	0.25	2.5		cutinase	+	0.10	47	0.10	48	40	0.19	76%
					BS2	+	0.13	4					
					rabbit	+	0.09	30					
chlorpyrifos	cherry	0.3	3.0	0.3	cutinase	+	0.09	92	0.12	73	40	0.15	50%
					BS2	+	0.17	68					
					rabbit	+	0.06	90					
					cutinase	–							
					BS2	–	0.18	58					
					rabbit	–	0.11	39					
chlorpyrifos	peach	0.2	2.0	0.2	cutinase	+	0.08	13	0.09	63	45	0.11	55%
					BS2	+	0.15	31					
					rabbit	+	0.03	0					
chlorpyrifos	kiwi	0.5	5.0	2.0	cutinase	+	0.27	39	0.34	51	68	0.04	8%
					BS2	+	0.57	17					
					rabbit	+	0.12	19					
					cutinase	–	0.46	4					
					BS2	–	0.44	22					
					rabbit	–	0.20	20					
chlorpyrifos	grapefruit	0.3	3.0	0.3	cutinase	+	0.01	115	0.01	117	3	0.03	10%
					BS2	+	0.02	41					
					rabbit	+	0.001	102					
chlorpyrifos	zucchini	0.05	0.5	0.05	cutinase	+	0.004	91				0.02	40%
					BS2	+		n.e.					
					rabbit	+		n.e.					
chlorpyrifos	cucumber	0.05	0.5	0.05	cutinase	+	0.003	85	0.004	59	8	0.004	8%
					BS2	+	0.006	57					
					rabbit	+	0.005	38					
paraoxon	apple juice	0.5	5.0		cutinase	–	0.56	16	0.67	17	134		
					BS2	–	0.66	4					
					rabbit	–	0.79	7					
parathion–methyl	apple juice	0.5	5.0		cutinase	+	0.42	36	0.47	25	94		
					BS2	+	0.52	11					
					rabbit	+	significant inhibition, but reversible reaction kinetics						
dichlorvos	fig	0.1	1.0	0.1	cutinase	–			0.02	55	20		
					BS2	–	0.01	0					
					rabbit	–	0.03	0					
carbaryl	apple	3.0	30.0	3.0	cutinase	–	3.12	18	2.47	27	82		
					BS2	–	2.31	22					
					rabbit	–	1.96	12					
carbaryl	peach	3.0	30.0	3.0	cutinase	–	2.85	25	2.72	20	91		
					BS2	–	2.64	9					
					rabbit	–	2.68	25					

[a] Results were tested for outliers after Grubbs on the level of 95% (*24*); n.e., not evaluable because of complete or significant, respectively, enzyme inhibition by means of oxidized matrix. [b] Concentration in the obtained sample extract. [c] MRL, Maximum residue limit (*23*). [d] Oxidation step; +, performed, –, not performed. [e] Average. [f] Relative standard deviation ($n = 4$; chlorpyrifos in apple, $n = 8$). [g] Recovery. [h] Single samples exemplarily measured by means of LC/MS.

inhibition results of a sample as a key to qualitative information, that is, identification. Therefore, the development of a proper software tool that automatically transforms residual enzyme activities into both identification and concentration information is a promising challenge.

For the first time, reliable inhibition data on BS2 and rabbit liver esterases are presented. The developed multienzyme assay proved to be highly sensitive and most appropriate for the identification of positive samples. It was observed that with increasing sensitivity the enzymes also become more susceptible to matrix interferences. To deal with this disadvantage, a careful extract cleanup is indispensable, although it means a corresponding loss of the inhibitor. Nevertheless, the multienzyme assay proved to be a convenient, rapid, and most practicable screening tool for the detection of insecticide residues in food commodities and, no doubt, will also work with water or air samples after adequate preparation.

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