Determination of Cutin-Bound Residues of Chlorothalonil by Immunoassay

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An indirect competitive enzyme-linked immunosorbent assay (ELISA) was used to determine photochemically cutin-bound residues of chlorothalonil in enzymatically isolated tomato and apple fruit cuticles. The samples were spiked, irradiated, exhaustively extracted, and depolymerized with boron trifluoride complex resulting in a soluble depolymerisate. With this procedure, the ELISA could be calibrated with free target molecules for the quantification of originally bound chlorothalonil residues. In fruit cuticles that were irradiated for 8 h by simulated sunlight, 0.030 and 0.068 mg/g photoinduced cutin-bound residues of wax-free cuticles (calculated as chlorothalonil) were determined for tomatoes and apples, respectively. For the used antibody mAb chl. 4/11, cross-reactivities with derivatives of chlorothalonil simulating different types of cuticle-bound residues are given and discussed.

Keywords: *Chlorothalonil; ELISA; cuticle depolymerization; bound residues*

INTRODUCTION

Xenobiotic residues remaining in soil and plants even after exhaustive solvent extraction or digestion without significantly changing the nature of either the exocon or the associated endogenous macromolecules are termed "bound residues" (*1*).

In plants, bound pesticide residues are formed by various mechanisms. For example, pesticides are conjugated to amino acids or carbohydrates directly or after metabolic activation and incorporated into macromolecules such as proteins, lignin, or polysaccharides. Another mechanism for the formation of bound residues is the photochemical activation in plant surfaces and linking to components of the plant cuticle (wax, cutin). In 1984, Schwack was the first to report on the photoinduced formation of reaction products between organic pesticides and constituents of the plant cuticle (*2, 3*). Employing model systems, Schwack et al. (*4, 5*) demonstrated photoaddition reactions of different pesticides to cuticle components.

In the past, during metabolism studies bound residues were analyzed mainly by detection of nonextractable radioactivity after the application of ^{14}C-labeled pesticides. However, with this method it is impossible to study the chemical structure or binding sites of bound residues because metabolic incorporation of labeled carbons in macromolecules may also occur. Because fragments recycled through pathways leading to natural incorporation are not included by the definition for bound residues (*1*), analysis using radiolabeled pesticides leads to unsatisfactory information. Furthermore, this procedure cannot be employed for the investigation of commercial products. Alternatively, high-temperature distillation (HTD) and supercritical fluid extraction (SFE) were successfully used to release bound residues in plants and soils (*6*−*9*). For bound 2,4-D in citrus peels, with an alkaline pretreatment the total residues were extractable (*10*).

In recent years, immunochemical methods for the detection of bound residues have been established. Significant research work was done to detect bound residues of atrazin by Hahn et al. (*11*) and Ulrich et al. (*12*) for soil samples and by Sohn et al. (*13*) for plant tissue. For the determination of residues bound to macromolecules (solid or soluble), calibration requires reference material with known concentrations of the bound target compound. However, the composition of plant cuticles is very complex and changes continuously during maturation. For this reason, reference material for cuticle-bound residues can hardly be obtained. Because immunoassays can be calibrated much more easily with free target molecules, depolymerization of the insoluble cutin resulting in monomeric cutin acids using a procedure that does not destroy bound residues is the method of choice.

Chlorothalonil (**1**), a widely used nonsystemic fungicide with protective action, is classified as a potential human carcinogen (group B2) (*14*). It is often applied by aerial spraying to protect agricultural crops from blights, leaf spot, and mildews. After application, **1** remains in the plant cuticles of leaves and fruits, where it is exposed to environmental influences. Hence, it may be decomposed by sunlight irradiation and react with compounds of the plant cuticle. The chlorothalonil photochemistry was summarized by Zorn (*15*), who also has shown that bound residues of **1** are formed in plant cuticles predominately by sunlight exposure. Recently, thermally and photochemically bound residues were detected in isolated tomato fruit cuticles using monoclonal antibody mAb chl. 4/11 (*16*). Now we present a method for the selective determination of photochemically cutin-bound residues of chlorothalonil in plant cuticles.

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10.1021/jf000983j CCC: $20.00
Published on Web 02/15/2001

MATERIALS AND METHODS

Reagents. All reagents were of analytical grade unless otherwise specified. Ammonium formate and hexanoic acid were purchased from Fluka (Deisenhofen, Germany). Boron trifluoride/methanol complex (20% in methanol) and glacial acetic acid were purchased from Merck (Darmstadt, Germany). All solvents were distilled before use. Poly(ethylene glycol) 600 diacid was obtained from Sigma-Aldrich (Steinheim, Germany). Silica gel, 63−200 μm (Baker, Darmstadt, Germany), was used for column chromatography. All other reagents were obtained as described recently (*16*). 4-[(2-Aminoethyl)amino]-2,5,6-trichloroisophthalonitrile (**2**), 2,4,5-trichloro-6-cyclohexylisophthalonitrile (**3**), and 2,4,5-trichloro-6-(1-methylpentyl)isophthalonitrile (**4**) were synthesized as already published (*16*). For ELISA, coating conjugate, washing buffer, and ELISA solution were prepared as described recently (*16*).

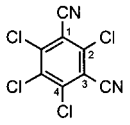
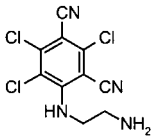
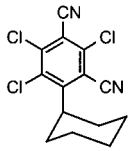
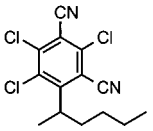
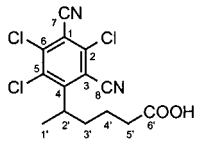
Instrumentation and Equipment. Melting points, UV spectra, and ^{1}H and ^{13}C NMR spectra were measured as published elsewhere (*16*). Chemical shifts (δ) are given in parts per million relative to internal Me_4Si (tetramethylsilane) (s = singlet, d = doublet, t = triplet, m = multiplet, br = broad, with dddt and dddd denoting a combination of the respective multiplicities).

ELISA analyses and irradiations were performed following the method of Jahn et al. (*16*). For HPLC-DAD a HP 1100 system was used: column, Eurospher 100-C18 (Knauer, Berlin, Germany) (250 × 4 mm), 5 μm; guard column, (10 × 4 mm), 5 μm; flow rate, 0.8 mL/min, MeOH/formate buffer (0.01 M, pH 4.0) gradient, 60 (0−5)−100 (25−35)−60 (40−45)% MeOH [*t* (min)]; DAD trace, 234 nm. LC-MS was run on an HP 1100 HPLC system, coupled to a Micromass (Manchester, U.K.) platform II quadrupole mass spectrometer equipped with an electrospray interface. Column, flow rate, and eluent were as described above. For LC analysis, the MS was operated in full-scan mode (*m*/*z* 200−1000). For accurate mass determination (*17*), data were collected in the multichannel acquisition (MCA) mode with 128 channels per *m*/*z* unit using 11 scans (5 s) with 0.1 s reset time. The resolution was 1010 (10% valley definition). The sample was dissolved for analysis in water/acetonitrile (1 + 1, v/v, c = 0.2 μg/mL) containing poly(ethylene glycol) 600 diacid (c = 0.2 μg/mL) as reference material. With an *m*/*z* 325−420 scan range, five reference peaks were used for calibration: 330.16023; 352.17333; 374.18644; 396.19955; and 418.21265.

Synthesis of 5-(2,3,5-Trichloro-4,6-dicyanophenyl)hexanoic acid (5). Compound **1** (3.76 mmol, 1 g) was dissolved in hexanoic acid (10 mL). The solution was heated under reflux for a total of 48 h with dibenzoyl peroxide (22 mmol, 22 g) in portions added in 2−3 h intervals. The solvent was removed in vacuo (80 °C, 5 Pa, nitrogen trap) and the residue dissolved in diethyl ether (4 mL). One milliliter of each was applied to a silica gel column (600 × 30 mm, 25 g silica gel, diethyl ether plus petroleum ether plus glacial acetic acid, 25 + 75 + 1, v/v/v), and the fraction between 70 and 130 mL was collected. The solvent was stripped off and the residue recrystallized from diethyl ether, yielding **5** (0.22 mmol, 76 mg, 5.9%) as colorless crystals: mp 166 °C; UV−vis (methanol), λ_{max} [nm] (log ϵ) 230 (4.8), 309 (3.2), 321 (3.4); LC-MS (ESI$^-$) *m*/*z* (relative intensity) 343 (28.8, [M − H]$^-$, isotope cluster for Cl_3), 345 (23.1), 347 (5.6); accurate mass (mean of 10 measurements ± standard deviation) *m*/*z* 342.9806 ± 0.0009 [M − H]$^-$ (342.9808 calcd for $C_{14}H_{10}Cl_3N_2O_2$); ^{1}H NMR (323 K, CD_3OD) δ 3.85 (s br, 1H, 2′−H), 2.30 [t, 3H, 5′-H, ^3J(5′-H; 4′-H_C/4′-H_D), 7.3 Hz], 2.13 (m, 1H, 3′-H_A), 1.93 [dddd, 1H, 3′-H_B, ^2J(3′-H_B; 3′-H_A) (−)13.8, ^3J(3′-H_B; 2′-H), 6.8, ^3J(3′-H_B; 4′-H_C)/^3J(3′-H_B; 4′-H_D), 5.5/10.7], 1.66/1.44 [dddt/dddt, 1H/1H, 4′-H_C/4′-H_D, ^2J(4′-H_C; 4′-H_D) (−)13.5], 1.52 [d, 3H, 1′-H, ^3J(1′-H; 2′-H), 7.1]; ^{13}C NMR (300 K, CD_3OD) δ 179.7 (C-6′), 156.9 (C-4), 142.4/141.8 (C-2/C-6), 135.3 (C-5), 118.2/116.3/114.6/113.2 (C-1/C-3/C-7/C-8), 39.3/36.8/35.5 (C-2′/C-3′/C-5′), 25.2 (C-4′), 18.6 (C-1′). The hydrogen/carbon numbering used for assignment of spectroscopic data is described in Table 1.

Determination of Cross-Reactivities. Cross-reactivities were determined as described recently (*16*).

Table 1. Cross-Reactivities (IC_{50}) of mAb chl. 4/11

		CR_{50} [%]
Chlorothalonil (**1**)		100
4-[(2-Aminoethyl)amino]-2,5,6-trichloroisophthalonitrile (**2**)		293.6
2,4,5-Trichloro-6-cyclohexylisophthalonitrile (**3**)		0.1
2,4,5-Trichloro-6-(1-methylpentyl)isophthalonitrile (**4**)		0.2
5-(2,3,5-Trichloro-4,6-dicyanophenyl)hexanoic acid (**5**)		3.8

Isolation of Fruit Cuticles. Tomato and apple fruit cuticles were enzymatically isolated as recently published (*16*).

Initial Content and Photodegradation. Isolated fruit cuticles were dipped into methanolic solutions of **1** (1 g/L), air-dried, and cut into two pieces of similar size. One part of the cuticles was irradiated by simulated sunlight or sunlight (irradiated samples, I). The other part was stored in the dark (samples spiked and stored in the dark, D). The procedure was repeated simultaneously without spiking (cuticles not spiked and irradiated, I_{ns}; cuticles not spiked and stored in the dark, D_{ns}). For chlorothalonil analyses (initial content and photodegradation), the cuticles were extracted with acetone (20−25 °C), the extract was filtered (0.45 μm membrane filter) and its content determined by HPLC-DAD. Afterward, exhaustive extraction was performed with hot acetone in a Soxhlett apparatus (twice, 4 h each). The wax content of the cuticles results from the difference of the cuticle mass of the native cuticle and the cuticle mass of the wax-free cuticle after hot solvent extraction.

Cuticle Depolymerization. Exhaustively extracted (see above) cuticles (20 ± 0.1 mg) were depolymerized for 24 h at 70 °C in a sealed tube using boron trifluoride/methanol complex (4 mL). After cooling to room temperature, the solution was poured in water (20 mL). The tube was rinsed with water (5 mL). The aqueous phases (pH <1) were combined and extracted with diethyl ether (20 mL). The ether phase was washed with water (2 × 20 mL, neutral reaction of the final washing liquid). Phase separation was achieved by adding sodium chloride saturated water (1 mL). The solvent was stripped off and the residue dried under a gentle stream of nitrogen.

Recovery of Chlorothalonil and Stability Test for Chlorothalonil Derivatives. Isolated tomato fruit cuticles (20 ± 0.1 mg) were spiked with **1** (0.1 mL of methanolic stock solution, c = 10.5 μg/mL) and depolymerized as described above. The resulting residue was dissolved in methanol and analyzed by HPLC.

Compounds **2** (0.8 mg) and **3** (14.0 mg) were individually treated as described under Cuticle Depolymerization. The resulting residue was dissolved in methanol and the content determined by HPLC.

Sample Preparation for ELISA: Detection of Bound Chlorothalonil Residues. The resulting residues of the cuticle depolymerization (D, I, D_{ns}, and I_{ns} samples) were dissolved in methanol (1 mL for tomatoes, 2 mL for apples). These solutions (50 μL) were used for ELISA (final volume = 5.0 mL, 1% methanol).

ELISA Calibration and Evaluation. The ELISA test was calibrated with **1** in solutions of depolymerized D samples (spiked, not irradiated, and extracted). Evaluation of the ELISA was performed as described in a former study (*16*). For the determination of cross-reactivities and cutin-bound residues of chlorothalonil, linearization of the calibration curve was obtained between 10% and 80% B/B_0 using the logit−log transformation: $\text{logit}(\% B/B_0) = \ln[\% B/B_0/(100 - \% B/B_0)]$.

Sample Preparation for ELISA: Quantification of Bound Chlorothalonil Residues. The resulting residues of the cuticle depolymerization (D and I samples) were dissolved in methanol (1 mL for tomatoes, 2 mL for apples). Aliquots of these methanolic solutions (200 μL) were evaporated to dryness and redissolved in methanol (200 μL, I samples) or methanolic stock solutions of **1** (0.1−2 mg/L, 200 μL, D samples). These solutions (50 μL) were used for ELISA (final volume = 5.0 mL, 1% methanol).

RESULTS AND DISCUSSION

Determination of Cross-Reactivities (IC_{50}) and Capability of mAb chl. 4/11 for the Detection and Quantification of Bound Chlorothalonil Residues. Cross-reactivities (IC_{50}) of chlorothalonil (**1**) derivatives were determined in relation to **1** and calculated according to the formula

$$\%\ \text{cross-reactivity}\ (IC_{50}) = (A/B) \times 100$$

(*18*), with *A* denoting the concentration of **1** in mol/L at $\text{logit}(\% B/B_0) = 0$ ($\% B/B_0 = 50\%$) and *B* denoting the concentration in mol/L of the cross-reacting hapten at $\text{logit}(\% B/B_0) = 0$ ($\% B/B_0 = 50\%$). Highest cross-reactivities were obtained for chlorothalonil derivatives substituted by amines at C-4 as is shown for compound **2** in Table 1 (for further cross-reactivities see ref *16*). As compared to its mode of action, chlorothalonil is expected to thermally bind to proteins known to be present in plant cuticles (*19*). Therefore, it can be assumed that protein-bound residues of chlorothalonil will be detected very sensitively by mAb Chl. 4/11.

As Zorn (*15*) has shown, formation of bound residues of **1** in cuticles is predominately caused by photoaddition processes to olefinic compounds of the plant cuticle (e.g., cutin acids, terpenes), resulting in derivatives such as **A** (Figure 1). Cross-reactivities of mAb chl. 4/11 to model substances for derivatives such as **A** range from 0.1 to 3.8% (**3**−**5**, Table 1), which should be sufficient for the analysis of such residues with this mAb. However, as recently reported, binding to cuticles without photoactivation actually takes place (*16*). Therefore, the high affinity of mAb chl. 4/11 to protein-bound residues such as **B** and **C** (Figure 1) of chlorothalonil will disturb the quantification of cutin-bound residues such as **A**. Consequently, accurate analysis of photoinduced cutin-bound residues requires the removal of protein-bound residues.

As a nonsystemic fungicide, chlorothalonil remains in plant cuticles. Consequently, thermal binding reactions are usually restricted to cuticular peptides. However, after homogenization of crops during food production or residue analysis chlorothalonil may also react with cellular proteins. This topic is currently under investigation.

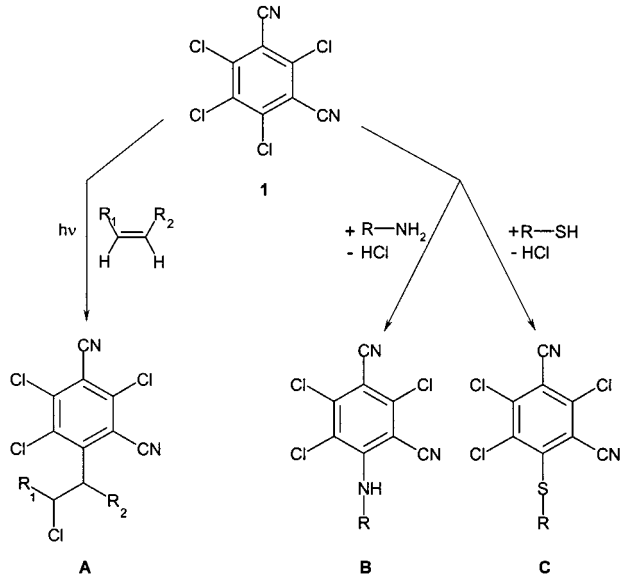


Figure 1. Hypothetic structures of cutin-bound (A) and peptide-bound (B, C) residues of chlorothalonil in plant cuticles.

Table 2. Residues of Chlorothalonil in Fruit Cuticles and Photodegradation (Acetone Extraction, 20−25 °C)

sample (irradiation conditions)	initial residue [mg/g]	resulting residue after irradiation [mg/g]	photo-degradation [mg/g] (%)
tomato fruit cuticle (8 h of simulated sunlight)	5.77	4.04	1.73 (30.0)
apple fruit cuticle (8 h of simulated sunlight)	4.24	1.78	2.46 (58.0)
apple fruit cuticle (22 h of natural sunlight)	3.33	0.68	2.65 (79.6)

Determination of Initial Residue Contents and Photodegradation. The use of isolated fruit cuticles allows work with authentic material but avoids disturbances by metabolic processes. By dipping isolated fruit cuticles in methanolic solutions of **1**, initial extractable residue levels (acetone extraction, 20−25 °C) in cuticles of ∼6 mg/g (tomato) and ∼4 mg/g (apple) were obtained (Table 2). As the enzymatic isolation procedure yields about 1.5 and 3.3 g of cuticle material from 1 kg of tomatoes and apples, respectively, the initial residue level of isolated and spiked cuticles corresponded to residue levels of about 9 and 13 mg/kg in tomato and apple fruits, respectively. By irradiation, the extractable residue level was reduced to ∼70% (tomatoes) and ∼20−40% (apples), respectively (Table 2). Of the known extractable photodegradates (*15*), only the 4-dechloro derivative was found in the range of 1−3% of initial chlorothalonil [data not shown (*20*)]. The difference of nearly 30% (tomatoes) and 60−80% (apples) is supposed to be bound to the cuticle.

For the quantification of olefinic constituents of plant cuticles, Schynowski (*21*) determined the iodine numbers of apple fruit cuticles and tomato fruit cuticles. The values of 46 and 26, respectively, clearly demonstrate that apple fruit cuticles have higher contents of olefinic structures than do tomato fruit cuticles. With regard to irradiation studies by Zorn (*15*), photodegradation of chlorothalonil (**1**) in apple fruit cuticles is faster than in tomato fruit cuticles, when samples are spiked and

irradiated in the same way. After irradiation under standardized conditions in a sunlight simulator, the results obtained are in accordance with these findings (Table 2). Photodegradation of chlorothalonil (**1**) in apple fruit cuticles was nearly double compared to that in tomato fruit cuticles.

As the wax portion of cuticles is removed by exhaustive extraction, residues bound to extractable wax are removed, too. Because these wax-bound residues are extractable, they are not included in the IUPAC definition for bound residues. In this context, Zorn (*15*) could not detect bound residues of chlorothalonil in the cuticular wax of grapes.

Depolymerization of Cuticles. Usually, the dominant polymer structure in the plant cuticle is the lipid polyester cutin, a polar cross-linked polymer of high molecular weight (*22*). It consists of a complex network of interesterified aliphatic hydroxy acids with chain lengths of C_{16} and C_{18}. This network can be depolymerized by saponification. However, an insoluble residue of the cuticle often remains, the nonsaponifiable highly aliphatic and chemically resistant biopolymer cutan. The two polymers cutin and cutan may occur in any ratio and differ in their relative abundance at different stages of cuticle development (*22*). The cuticles of some species appear completely to lack cutan, notably those of tomato fruits (*23*). As an example, the cutin content of mature tomato fruit cuticles is reported to be ~80% (*24*). By boron trifluoride catalyzed methanolysis cutin is readily solubilized, resulting in monomeric cutin acid methyl esters. Cutin-bound residues of chlorothalonil are solubilized by this depolymerization procedure, as well.

Stability and Recovery of Chlorothalonil (1) and Its Derivatives 2 and 3 in the Depolymerization Procedure. The recovery of **1** in the depolymerisate of tomato cuticles was 95.2% ($n = 6$, CV = 7.2%) in the ether phase of the employed liquid−liquid partition procedure. Recoveries of **2** and **3** (without cuticle matrix) were 0% (**2**) and 89% (**3**) in the ether layer. However, 94% of **2** was recovered in the aqueous phase. Therefore, chlorothalonil and its derivatives **2** and **3**, simulating cuticle-bound residues, are stable under the selected conditions of depolymerization. Furthermore, compounds such as **2** bearing basic amino groups are protonated during extraction (pH of the aqueous phase <1) and removed from the organic layer by extraction. Although plant cuticles mainly consist of cross-linked cutin acids, nitrogen-containing compounds (for example, peptides) are also present. Walker (*25*) and Huelin (19) determined the nitrogen contents of isolated apple fruit cuticles to be 5.5 mg/g (5.2−5.9 mg/g, cv. Jonathan) and 5 mg/g (cv. Granny Smith), respectively. Calculated as protein, these values correspond to a protein content of ~3% for the apple fruit cuticle. In this context, Pyee et al. (*26*) identified a lipid transfer protein as the major protein in the surface wax of broccoli leaves. Therefore, caused by nucleophilic substitution reactions, peptide-bound residues may likewise be formed in plant cuticles without photoactivation. As mentioned above, thermally bound residues of chlorothalonil were detected in spiked but nonirradiated tomato fruit cuticles recently (*16*). If these thermally bound residues can be removed by extraction after depolymerization, a polar character of the binding partner of chlorothalonil has to be assumed. Furthermore, the determination of cutin-bound residues would be possible without disturbances of the analysis by thermally bound residues (Figure 2)

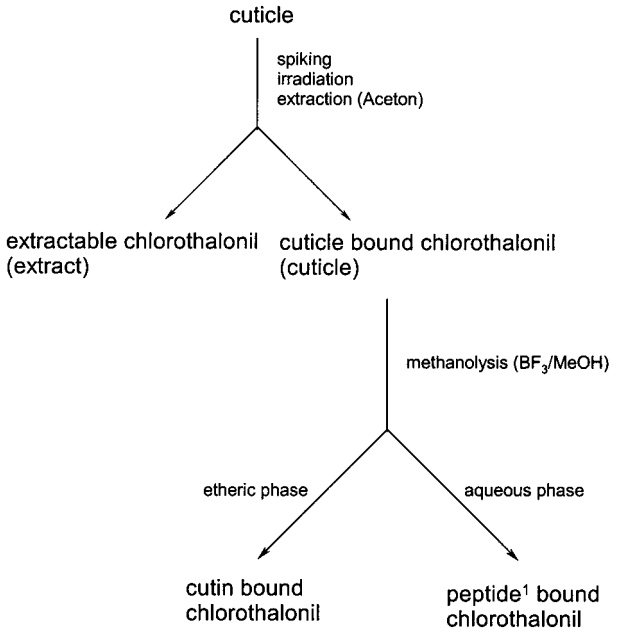


Figure 2. Scheme of cuticle treatment and depolymerization ([1]hypothesis).

Table 3. ELISA Results in Apple and Tomato Fruit Cuticles for the Detection of Cutin-Bound Chlorothalonil

	sample (irradiation conditions)	
	tomato fruit cuticle (8 h of simulated sunlight)	apple fruit cuticle (8 h of simulated sunlight)
sample wt [mg][a]	1.0	0.5
I	54.8	32.3
[% B/B_0]	(45.2−63.8)	(29.6−34.1)
	$n = 5$	$n = 4$
D	92.6	92.0
[% B/B_0]	(85.3−98.6)	(83.1−98.1)
	$n = 6$	$n = 5$
I_{ns}	97.9	91.0
[% B/B_0]	(98.0−97.7)	(87.4−94.6)
	$n = 2$	$n = 2$
D_{ns}	99.9	92.8
[% B/B_0]	(97.4−102.4)	(92.5−93.0)
	$n = 2$	$n = 2$

[a] Aliquot of depolymerized cuticle.

Detection of Bound Residues in Depolymerized Plant Cuticles. Values of % B/B_0 of depolymerized I_{ns} and D_{ns} samples demonstrate that nonspecific binding of mAb chl. 4/11 to cuticle depolymerisate does not occur (Table 3). For depolymerized dipped cuticle samples, % B/B_0 values of irradiated samples (I) ranged between 32% B/B_0 (apple, aliquot of 0.5 mg of depolymerized apple fruit cuticle) and 55% B/B_0 (tomato, aliquot of 1 mg of depolymerized tomato fruit cuticle). Bound chlorothalonil residues were not detectable in the depolymerisates of dipped and dark-stored cuticles (limit of detection = 90% B/B_0) (Table 3). This result is in contrast to the analysis of non-depolymerized cuticles, where thermally bound residues were detected (*16*). Therefore, it can be assumed that thermally bound residues are removed by the depolymerization procedure, supporting the idea of the polar and basic character of these residues.

The given results indicate that photoinduced cutin-bound residues of **1** can be detected in isolated tomato and apple fruit cuticles by a structure-specific antibody

Table 4. Determination of Cutin-Bound Residues of Chlorothalonil by ELISA

sample (irradiation conditions)	bound residue calcd as chlorothalonil [mg/g][a]	% of initial residue	% of photodegradation
tomato fruit cuticle (8 h of simulated sunlight)	0.030, $n = 3$ (0.025−0.035)	0.5	1.7
apple fruit cuticle (8 h of simulated sunlight)	0.068, $n = 4$ (0.066−0.073)	1.6	2.8
apple fruit cuticle (22 h of sunlight)	0.055, $n = 2$ (0.050−0.060)	1.7	2.1

[a] Related to wax-free cuticles after exhaustive solvent extraction.

method without disturbances by thermally bound residues. Insufficient solvent extraction of cuticles would cause residues of nonbound (free) analytes, leading to signal inhibition in the ELISA analysis. Because signal inhibition of D samples in ELISA analysis of depolymerized cuticles did not occur, false positive results caused by insufficient extraction were definitely ruled out for our experiments.

ELISA Calibration. Because depolymerization of cuticles yields free monomers, the ELISA can be calibrated by monomeric target compounds such as **1**.

To exclude the influence of matrix effects on the analysis, calibration was performed in the presence of depolymerized D samples. Additionally, this procedure ensures exhaustive solvent extraction of nonbound analytes, because D samples are spiked and extracted the same way as I samples.

Quantification of Cutin-Bound Residues in Depolymerized Fruit Cuticles. The precision of ELISA analysis is best at the center point of the calibration curve. Thus, the analyzed amount of depolymerized D and I cuticles was selected in such a manner that the signals of I samples were in the size of 50% B/B_0.

Because all cuticles (I, D, I_{ns}, and D_{ns}) were exhaustively extracted before depolymerization, the wax of the fruit cuticles was removed and the amount of cutin-bound residues determined is related to the wax-free cuticle. The wax contents of the fruit cuticles were 3.5 ± 0.2 g/100 g (tomato, $n = 3$) and 37.5 ± 0.8 g/100 g (apple, $n = 4$).

In fruit cuticles that had been irradiated for 8 h with simulated sunlight, 0.030 and 0.068 mg/g cutin-bound residues (calculated as chlorothalonil) were determined for tomatoes and apples, respectively (Table 4). Good repeatabilities were obtained. The amount of cutin-bound chlorothalonil residues was 2 times higher in apple fruit cuticles than in tomato fruit cuticles, corresponding to the enhanced photodegradation of **1** in apple fruit cuticles.

Because the cutin-bound chlorothalonil residues of sunlight-irradiated samples were in the same order of magnitude (0.058 mg/g, Table 4) as compared to samples irradiated by simulated sunlight, the use of simulated sunlight seems to be a proper tool, allowing laboratory work during all seasons under constant conditions.

Taking into account the low cross-reactivity of mAb chl. 4/11 to model substances for cutin-bound chlorothalonil (**3**−**5**, 0.1−3.8%), cutin-bound residues of chlorothalonil found can only give an idea of the true amount of bound residues. By the use of **5** for calibration, the amount of cutin-bound residues would rise by a factor of ∼20.

Conclusions. Cutin-bound residues of chlorothalonil in plant cuticles were selectively determined by ELISA as a structure-specific method, for the first time. It was shown that these residues are formed in fruit cuticles only after photochemical activation. Besides this, the developed depolymerization is a powerful tool for ensuring representative sampling, because analysis is performed with an aliquot of a larger sample size. In further studies, specific antibodies with higher affinities for photochemically bound residues have to be produced.

ABBREVIATIONS USED

ELISA, enzyme-linked immunosorbent assay; I, spiked, irradiated sample; D, spiked, dark-stored sample; I_{ns}, nonspiked, irradiated sample; D_{ns}, nonspiked, dark-stored sample; CR_{50}, cross-reactivity at 50% B/B_0.

ACKNOWLEDGMENT

We thank Dr. Arnd Petersen (Forschungszentrum Borstel, Abteilung Biochemische und Molekulare Allergologie, Germany) for providing mAb chl. 4/11.

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Received for review August 8, 2000. Revised manuscript received November 29, 2000. Accepted November 29, 2000. This work was supported by the Deutsche Forschungsgemeinschaft.

JF000983J