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Widespread Occurrence of Low Levels of Alternariol in Apple and Tomato Products, as Determined by Comparative Immunochemical Assessment using Monoclonal and Polyclonal Antibodies

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ABSTRACT: This study investigated the production of polyclonal (pAB) antibodies and the first time production of monoclonal (mAB) antibodies against the mycotoxin alternariol, and their implementation in enzyme immunoassay (EIA) for the rapid determination of alternariol in foods. Both EIAs were highly sensitive, with detection limits (IC_{20}) of 35 ± 6.9 pg/mL (mAb EIA) and 59 ± 16 pg/mL (pAb EIA). Food products (n = 109; apple and tomato products, white wine) from German retail shops were analyzed. At a detection limit of 1−2 μg/kg, alternariol at 1−13 μg/kg was found with high frequency in apple (67%) and tomato (93%) products. Tomatoes with visible signs of *Alternaria* infection, stored at room temperature for up to 4 weeks, contained alternariol at levels up to 50 mg/kg, as determined by EIA and HPLC-FLD. It is concluded that the alternariol immunoassays present a versatile screening tool which could facilitate food control for *Alternaria* toxins.

KEYWORDS: mycotoxin, antibodies, immunoassay, *Alternaria*, food

INTRODUCTION

Alternariol (3,7,9-trihydroxy-1-methyl-6*H*-dibenzo(*b*,*d*)pyran-6-one) is one of the major *Alternaria* mycotoxins.[1] The main producers of alternariol and some structurally related dibenzopyrone compounds (alternariol monomethyl ether, altenuisol, and altenuene, Figure 1) are *A. alternata* and some other species within the genus *Alternaria*.[2,3] However, species of other fungal genera, including the plant pathogen *Stagonospora* (syn. *Septoria*) *nodorum*,[4] *Phomopsis* spp.,[5] and *Pithomyces chartarum*[6] have also been reported as alternariol producers.

Alternariol-producing fungal species are ubiquitous in the environment, and natural occurrence of alternariol has been reported in many foods and feeds, including fruits, vegetables, cereals, and seeds.[7−12] Alternariol was found to be very stable in sunflower flour, in apple juice and in white wine, even at elevated temperatures up to 80−100 °C.[13,14] In a bread baking experiment, little degradation of alternariol was observed at temperatures as high as 230 °C.[15]

Current knowledge concerning adverse effects of alternariol, and other *Alternaria* toxins, in humans and in animals is still very limited. The acute toxicity of alternariol is low, but several authors reported genotoxic, estrogenic, and mutagenic properties in cell culture or in laboratory animals. More recently, alternariol was identified as a potent inhibitor of topoisomerase I and II, and as an inducer of DNA strand-breaks in different mammalian cell lines.[16−18]

Analytical methods developed for alternariol determination in foodstuff focused mainly on liquid chromatography and gas chromatography.[8,12,19,20] These methods usually are expensive, require extensive sample cleanup or have a low sample throughput.

Within an integrated analytical system, rapid and easy-to-perform methods for alternariol determination, such as EIA, would be helpful to assess its occurrence in foods and feeds. Immunochemical methods for *Alternaria* toxins have not been reported so far. However, after this manuscript was submitted, an enzyme immunassay of alternariol was published,[21] but this concerned grain and not fruits or vegetables.

Here we describe the development of monoclonal (mAb) and polyclonal (pAb) antibodies against alternariol, and their implementation in two highly sensitive enzyme immunoassays for alternariol. Both tests were used to analyze alternariol in a variety of foods from the German market, and some comparison of EIA results with that of a HPLC method was made.

MATERIALS AND METHODS

Chemicals, Buffers, and Equipment. Alternariol, alternariol monomethyl ether, altenuene, tenuazonic acid copper salt, bovine serum albumin (BSA, molecular weight: 66,000), Freund's complete adjuvant, 3,3′,5,5′-tetramethylbenzidine (TMB), dimethyl sulfoxide (DMSO), casein sodium salt and Tween 20 were supplied from Sigma-Aldrich (Taufkirchen, Germany). Keyhole limpet hemocyanin (KLH, molecular weight used for calculations: 3,000,000), formaldehyde solution (37%), methanol, and acetonitrile were obtained from Merck (Darmstadt, Germany). All chemicals used were at least of analytical grade. The alternariol standard solution (1.0 ± 0.01 mg/mL in methanol) was characterized by UV spectroscopy by full scan spectra (190−500 nm) of diluted solutions (1.5 to 5 μg/mL). The UV absorbance maximum for alternariol was at 256 nm (ε = 4.8 ± 0.3 × 10^4 L/mol/cm). For EIA,

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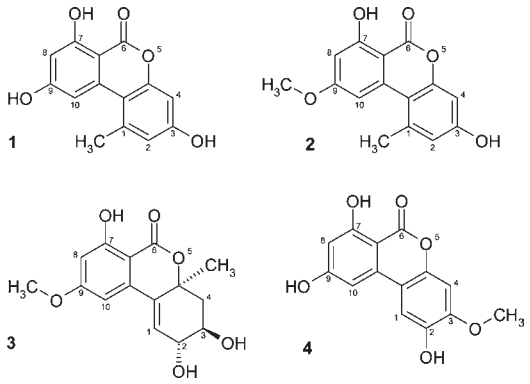

Figure 1. Structures of *Alternaria* dibenzopyrone metabolites: alternariol (**1**), alternariol monomethyl ether (**2**), altenuene (**3**), and altenuisol (**4**).

working standard solutions (5 pg/mL to 100 ng/mL) were prepared in phosphate buffered saline (PBS, NaCl 6.79 g, Na_2HPO_4 2.94 g, KH_2PO_4 0.86 g, H_2O 1 L, pH 7.3−7.4). The dilution buffer for coating type MaxiSorp microtiter plates (Nunc, Roskilde, Denmark) with solid-phase antigen was sodium bicarbonate buffer (0.05 M; pH 9.6). Blocking solution for microtiter plates (200 μL per well) was PBS containing 20 g/L (pAb EIA) or 30 g/L (mAb EIA) sodium caseinate. Wash solution was distilled water containing 8.5 g/L of NaCl and 0.25 mL/L of Tween 20. H_2O_2−citrate buffer solution for enyzme substrate/chromogen solution was $C_6H_7O_8$ 8.3 g, 1 M KOH 49 mL, H_2O 160 mL, 30% aqueous H_2O_2 72 μL, pH 3.95.

Enzyme substrate/chromogen solution was prepared as used earlier.[22] In brief, 50.4 mg of TMB was dissolved with 1 mL of acetone and 9 mL of methanol. Before use, 0.5 mL of TMB solution was mixed with 10 mL of H_2O_2−citrate buffer solution. EIA absorbance values were measured at 450 nm with a model Sunrise plate reader (Tecan, Crailsheim, Germany), and evaluated by Magellan EIA calculation software (Tecan) with parameters as described earlier.[22] In brief, absorbance was measured at 450 nm, with 620 nm reference filter. Seven standard concentrations (six serial dilutions of alternariol standard in buffer solution plus one buffer solution blank, B_0) were pipetted on each plate, and at four duplicate wells were analyzed for all standard and sample solutions. After transformation of absorbance values (absorbance B_0 = 100%), the 50% inhibition concentration (IC_{50}) and the 20% inhibition concentration (IC_{20}) values of the standard curves were recorded, to check stability and detection limit of the EIA over the period of analysis.

All animal manipulations were performed in compliance with the respective German laws and guidelines concerning animal welfare and with the formal allowance by the regional Bavarian and Hesse authorities.

Sample Materials. Food samples (n = 116) were purchased from retail shops in Hesse, Germany, in 2009/2010. This included seven samples of "plain fresh tomatoes" (in 250−500 g containers or bags). Of these, three packages contained one or more tomatoes showing visible black, concave spots, indicating fungal infestation with *Alternaria*, at the time of purchase. To further promote fungal growth, and to provide highly contaminated material, these samples were stored at ambient temperature for 1−4 weeks prior to analysis, allowing visible *Alternaria* decay to proceed. The results obtained for these samples were processed separately, and were not included in the food data evaluation.

Synthesis of Immunochemicals. The alternariol conjugates were prepared by the Mannich condensation reaction using conditions similar as described earlier for the mycotoxin citrinin.[23] For synthesis of the alternariol−KLH immunogen, solutions of alternariol (1.3 mg in 0.1 mL of DMSO), KLH (15 mg in 2 mL of 0.1 M sodium acetate buffer, pH 4.5), and aqueous formaldehyde (37%, 500 μL) were mixed and reacted for 16 h at 37 °C. For synthesis of the alternariol−BSA solid phase antigen, alternariol (3 mg) and BSA (39.8 mg) were dissolved with 3 mL of 0.1 M sodium acetate buffer (pH 4.5), aqueous formaldehyde solution (37%, 300 μL) was added and the mixture reacted for 16 h at 37 °C. Then the alternariol−KLH and the alternariol−BSA were each dialyzed against three changes (each 6 L) of PBS for 8 h. The UV spectra of both conjugates were qualitatively compared with those of nonconjugated proteins and with alternariol. The conjugates were stored in small portions at −18 °C.

Generation of Polyclonal Antialternariol Antibodies (pAb). For use as the immunogen, 0.3 mL of alternariol−KLH solution (containing approximately 0.75 mg of KLH) was mixed with 1.2 mL of PBS and emulsified with 4.5 mL of Freund's complete adjuvant. Three female chinchilla bastard rabbits (Charles River, Kisslegg, Germany) were each immunized with 2 mL portions of the emulsion by using multisite intradermal injections. Three booster injections, using the same composition and amount of immunogen, were given intramuscularly 7, 13, and 32 weeks after the primary injection. Blood was collected every two weeks, and the relative antibody titer was determined for each individual serum and rabbit by indirect EIA.

Generation of Monoclonal Antialternariol Antibodies (mAb). Twelve-week-old female mice (six each of BALB/c and of a BALB/c × NZW × NZB hybrid strain) were immunized by intraperitoneal injection of 60 μg of the immunogen, which was dissolved in PBS and emulsified in Freund's complete adjuvant (ratio 1:2). The booster injections, using the same composition and amount of antigen, were given intraperitoneally and subcutaneously. Three days before fusion, the selected animals received a final booster injection of 75 μg of antigen in PBS alone. mAb against alternariol were produced by fusion with myeloma cells using a standard protocol previously described.[24] For screening of antibody secreting hybridomas, a noncompetitive indirect EIA was used. One clone (mAb 4G4) was produced in cell culture, and culture supernatant was used to establish a competitive indirect EIA.

Competitive Indirect EIA with pAb. A microtiter plate was coated with alternariol−BSA (1:8,000 in coating buffer, 100 μL per well) for 16 h in a water-saturated atmosphere at room temperature. Free protein-binding sites of the plates were blocked for 30 min, and then the plates were washed (each well filled four times with wash solution) and made semidry. To each well 50 μL of alternariol standard solution and 50 μL of pAb solution (1:3,000 in PBS) were added and incubated for 1 h at room temperature, and then the plates were washed again. Swine anti-rabbit IgG HRP conjugate solution (1:3,000 in PBS, 100 μL per well) was added and incubated for 1 h. After another wash step, enzyme substrate/chromogen solution (100 μL per well) was added. After 15 min, the color reaction was stopped and the absorbance was measured at 450 nm.

Competitive Indirect EIA with mAb. The competitive indirect EIA with mAb was performed similar to EIA using pAb. The alternariol−BSA conjugate was diluted 1:50,000 before coating. After coating, blocking, washing and semidrying 50 μL of alternariol standard solution and 50 μL of mAb solution (diluted 1:2,000 in PBS) were added to each well. After incubation for 1 h and an additional washing step, rabbit anti-mouse IgG HRP (1:2,000 in 1% sodium caseinate/PBS, 100 μL per well) was added. All further steps were performed as described at the competitive indirect EIA with pAb. The measuring range of the standard curve usually was from 30% to 80% relative binding.

EIA Sensitivity and Specificity. For each assay, standard curves (cubic spline) were established setting the absorbance of the blank value as 100% binding (B_0). The detection limit, defined as the concentration resulting in 20% binding inhibition (IC_{20}), and the 50% inhibition concentration (IC_{50}) were recorded for each test. The quasilinear range of the standard curve, usually from 30% to 80% relative binding ($B/B_0 \times 100$), was used for alternariol quantification in sample extracts. Test stability of the mAb and pAb EIA was checked by comparing

standard curve parameters from each 30−40 microtiter plates performed during routine analysis over 6 months.

Cross-reactivity was tested under the condition of the EIAs, using standard solutions of alternariol monomethyl ether, altenuene, and tenuazonic acid at concentrations up to 10 μg/mL. The IC_{50} value was used to calculate relative cross-reactivities, using alternariol as the reference substance.

Sample Extraction. Liquid foods (apple juice, tomato juice, white wine) were diluted 1:10 with PBS and directly analyzed by EIA (minimum sample dilution factor: 10).

Applesauce (5 g) was mixed with PBS (50 mL) by magnetic stirring for 10 min. An aliquot of the solution was transferred into a 2 mL Eppendorf vial, centrifuged (30000*g*, 5 min, 20 °C), and the supernatant was analyzed either directly (pAb EIA, minimum sample dilution factor 10) or after a further 1:2 dilution with PBS (mAb EIA).

To 5 g test portions of tomato paste or ketchup, 50 mL of methanol/PBS (70/30) was added and the apparent pH value adjusted to approximately 7.0 with 3 M NaOH. After magnetic stirring (400 rpm) for 30 min, the mixture was centrifuged (2000*g*, 15 min, 20 °C). Supernatants from tomato paste samples were filtered through a paper filter, and supernatants from ketchup samples were further processed directly. A 2 mL portion of the filtrate was mixed with 2 mL of distilled water, and then extracted twice with 3 mL of ethyl acetate each time. The organic solvent portions were pooled, the solvent was evaporated (40 °C, rotary evaporator), and the residue was dissolved with PBS (1 mL). The resulting solution was analyzed either directly (pAb EIA, sample dilution factor: 5) or after dilution with PBS (mAb EIA, minimum sample dilution factor: tomato ketchup, 25; tomato paste, 50).

Whole tomatoes were cut in small pieces, homogenized (Stomacher) and then extracted as described for tomato paste, except that the mixture was not centrifuged prior to filtration. The final solution was analyzed directly (minimum sample dilution factor: 5) in both EIAs.

For recovery experiments, sample materials were spiked with 50−100 μL of a methanolic alternariol solution, to yield the desired concentration. Then the sample was thoroughly mixed (liquid samples) or homogenized in a Stomacher (sample material containing solids). Spiked samples were then treated as described above. Spiked sample extracts were analyzed in parallel by mAb EIA and pAb EIA, and by HPLC if applicable.

Four replicate wells of each standard and extract solution, and at least three different dilutions per sample extract (minimum dilution plus two higher dilution steps), were analyzed. The arithmetic mean alternariol concentration of all dilutions yielding absorbance values within the standard curve measurement range (30−80% B/B_0) was used to calculate the alternariol concentration in the sample.

HPLC Analysis. For confirmatory purposes, whole tomato samples were reanalyzed by a HPLC method.[25] Sample extracts were prepared as described for EIA analysis of whole tomatoes, except that, after evaporation of the ethyl acetate solvent, the residue was dissolved with 500 μL of acetonitrile/water (15/85). A portion of this extract (50 μL) was injected onto a 250 mm × 4.6 mm i.d., 5 μm, Discovery HS RP-18 column (Sigma-Aldrich, Taufkirchen, Germany). The analysis was performed on a HPLC system consisting of a model ASI-100 autosampler, model P580 gradient pump, model STH 585 column oven set at 40 °C, model RF 2000 fluorescence detector, model PDA-100 photodiode array detector, and Chromeleon evaluation software (Dionex, Idstein, Germany). Mobile phase flow-rate was 1.0 mL/min with a linear water−acetonitrile gradient. Solvent A was acetonitrile/aqueous ammonium sulfate solution (0.1 g/L), 15/85 (v/v), and solvent B was acetonitrile. The mobile phase gradient was 100% A to 100% B within 40 min. Alternariol and alternariol monomethyl ether were detected by fluorescence detection (excitation wavelength 253 nm, emission wavelength 415 nm); additionally the UV spectrum was recorded (200−400 nm) by in-line UV detection using a diode-array detector. The retention times were typically about 15.9 min for alternariol and 22.3 min for alternariol monomethyl ether. For quantitation, an external calibration curve was used. Concentration of alternariol working standard solutions was 1 ng/mL to 100 ng/mL. The limit of detection (LOD) was defined as a signal-to-noise ratio (S/N) of 3:1, whereas the limit of quantitation (LOQ) was defined as a S/N of 9:1.

RESULTS AND DISCUSSION

Antibodies against Alternariol. Alternariol was conjugated to proteins using the Mannich condensation reaction with formaldehyde. This reaction is very versatile and has successfully been used to couple mycotoxins and other haptens through various functional groups,[23,26] including hydroxyl groups which are present 3-fold in the alternariol molecule. From the specificity data shown below, we assume that formaldehyde conjugation of alternariol is achieved via hydroxyls at C3 or C7 but not C9 (Figure 1). However, we have no proof for this. Covalent binding of alternariol to KLH and BSA was qualitatively checked by comparing the UV spectra of conjugates with that of alternariol and proteins, and an increased absorption at 256 nm was found which corresponds to the UV maximum of alternariol. However, the ultimate proof of the success of this conjugation approach was the high immunogenicity of the alternariol−KLH−conjugate in rabbits and mice. In sera of all immunized mice, specific antibodies against alternariol were detected using alternariol−BSA (1:1,000) as solid phase antigen in an indirect EIA. Antibody titers of the sera taken ten weeks after primary immunization were in the range of 1:2,000 to 1:10,000. After booster injections, three mice showing high antibody titers (>1:10,000) and high affinity for free alternariol were selected as spleen donors for further procedure. After fusion with myeloma cells, hybridomas reacting positive in the indirect EIA were screened for competitive binding inhibition by free alternariol. One clone, named mAb 4G4 (subtype IgG_{2b}), exhibited a high affinity for free alternariol and was further used for EIA development.

Likewise, the alternariol−KLH conjugate induced a persistently high and specific immune response in all three immunized rabbits, with maximum relative serum titers of >1:100,000, as monitored by competitive indirect EIA. Competitive binding inhibition through free alternariol standard solution could be determined in all antisera from week four onward. A decision on further use was made after evaluation of alternariol standard curves established for all three sera. The sera of two rabbits (#2, #3) yielded similar IC_{50} values in the pg/mL range, while one rabbit (#1) provided antiserum which turned out to be 5−10 times less sensitive (IC_{50} >1 ng/mL). The sera of rabbit #3 had comparatively less nonspecific background color development at high alternariol concentrations, 5−10% B/B_0 vs 15−20% B/B_0 for sera of rabbit #2, and was therefore chosen for further EIA development.

EIA Sensitivity and Specificity. Long-term within-laboratory evaluation of EIA standard curves parameters from assays performed over a period of 6 months (Figure 2, Table 1) showed that both the pAb EIA and the mAb EIA enabled highly sensitive determination of alternariol; furthermore both tests revealed quite robust test performance characteristics. The intraassay coefficients of variation of alternariol standard concentrations (four replicates) were typically between 0.5% and 5%, but below 10% in all valid tests. The interassay coefficients of variation of the 50% inhibition concentration were 15% (mAb EIA) and 18% (pAb EIA).

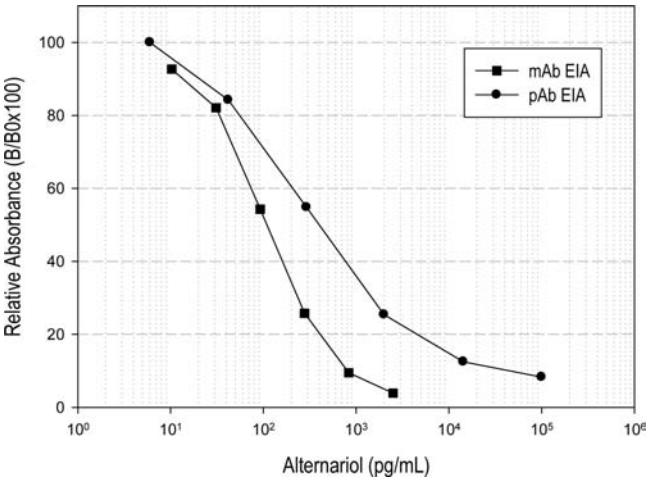

Figure 2. Typical standard curves of the competitive indirect EIAs for alternariol, using mAb or pAb, respectively. Four replicate wells of all standard concentrations were analyzed (RSD_r 0.5−10%).

Table 1. Long-Term Robustness of EIA Standard Curve Parameters[a]

	mAb EIA		pAb EIA	
param	50% inhibn concn	20% inhibn concn (detection limit)	50% inhibn concn	20% inhibn concn (detection limit)
mean, pg/mL	108	35	408	59
std dev, pg/mL	17	6,9	74	16
RSD,[b] %	15	20	18	26
min, pg/mL	75	22	280	30
max, pg/mL	139	48	581	96

[a] Evaluation of tests (mAb EIA: $n = 30$; pAb EIA: $n = 40$) performed over a period of 6 months. [b] Relative standard deviation.

To avoid overinterpretations of the standard curves, the detection limit was set at 80%, $B/B_0 \times 100$, which is a relatively conservative approach. Still, this resulted in highly sensitive assays, with mean detection limits of 35 ± 6.9 pg/mL (mAb EIA) and 59 ± 16 pg/mL (pAb EIA) determined from the alternariol standard curves. While the mAb EIA was nearly twice as sensitive as the pAb EIA, the quasilinear measurement range of the pAb standard curve was much wider (50−2000 pg/mL) than that of the mAb EIA (20−300 pg/mL). With these characteristics, both tests are far more sensitive than HPLC-FLD methods, and equally sensitive as HPLC−MS/MS methods using stable isotopes.[20]

Although only a limited number of structurally related *Alternaria* toxins was available for specificity tests, both EIAs seem to be specific for alternariol. In the pAb EIA, cross-reactivities were lower than 0.5% with altenuene and alternariol monomethyl ether. The mAb EIA weakly cross-reacted with alternariol monomethyl ether (0.9%) but not with altenuene. Both tests did not detect tenuazonic acid at levels as high as 10 μg/mL, which is not surprising considering the structural differences. The fact that both EIAs had no (pAb EIA) or very weak (mAb EIA) reactivity with alternariol monomethyl ether suggests that the conjugation of alternariol to the carrier protein is not achieved via the hydroxyl at C9, but more likely via hydroxyls at C3 or C7. Therefore it cannot be excluded that the mAb EIA and the pAb EIA may cross-react with some structurally related alternariol having an intact hydroxyl at C9 (such as altenuisol). Since very little is known concerning the natural occurrence of alternariol derivatives, further studies are desirable to fully establish the reactivity pattern of the EIAs.

Table 2. Recovery of Alternariol from Various Sample Materials, as Analyzed by pAb EIA and mAb EIA

			alternariol found		
sample type	EIA	alternariol added (ng/mL or ng/g)	mean recovery %	RSD[a] %	*n*
applesauce	pAb	2−10	60	16	23
	mAb	3−10	43	20	14
apple juice	pAb	5−10	82	23	16
	mAb	1−2	47	28	14
tomatoes	pAb	2−5	77	18	9
	mAb	2−5	75	19	8
tomato ketchup	pAb	2−5	92	20	15
	mAb	2−5	66	26	16
tomato paste	pAb	5−10	76	15	15
	mAb	5−10	56	22	10
tomato juice	pAb	1−10	78	27	17
	mAb	1−4	61	15	15
white wine	pAb	2−5	98	11	11
	mAb	1−2	80	15	10

[a] RSD, relative standard deviation.

Analysis of Alternariol in Foods. While most fruit and vegetable foods may be contaminated with alternariol, apple and tomato products, as well as wine, are particularly known as susceptible commodities. Therefore we selected these matrices to perform a first method application study and a comparison of pAb EIA and mAb EIA test performance. The high sensitivity of the EIAs offered the possibility to directly analyze alternariol in liquid food materials after dilution, without excessive extract cleanup necessary to overcome matrix interference. Liquid materials such as wine, apple juice, and tomato juice were analyzed after a 1:10 dilution with buffer solution. Alternariol in sample matrices containing more solid particles (applesauce, tomato) was purified from diluted samples by liquid−liquid extraction into ethyl acetate. This approach was also quite straightforward, and yielded very low detection limits of <1 ng/mL. Because the mAb EIA required higher sample dilution to overcome sample matrix interference, both the mAb EIA and the pAb EIA had nearly the same sensitivity for alternariol in foods. To compensate for day-to-day standard curve detection limit variability, and considering sample matrix variability and recovery studies, alternariol results were recorded as positive at ≥2 μg/kg in tomato paste, and ≥1 μg/kg in all other sample materials. Typically such weakly positive diluted samples or sample extracts yielded EIA absorbance values at approximately 80% B/B_0. Mean recoveries of alternariol at spiking levels of 1−10 μg/kg were 43−80% in mAb EIA, and 60−98% in pAb EIA, respectively (Table 2). In alternariol-negative samples (<1 ng/g) spiked with alternariol at levels of 2−10 ng/g, which is still relatively near to the detection limit of the EIA, minor differences concerning test robustness toward effects such as temperature and pH value may add up in

Table 3. Within-Laboratory Reproducibility of the pAb EIA and mAb EIA Methods for Naturally Contaminated Sample Material

		alternariol result (μg/kg or μg/L)[a]						
sample type	EIA	replicate analysis no.					mean ± SD	RSD[b] (%)
applesauce	pAb	2.0	2.1	1.9	2.1	2.2	2.0 ± 0.11	5.6
	mAb	7.6	8.3	8.2	8.3	9.3	8.3 ± 0.6	7.2
apple juice	pAb	2.1	2.1	2.3	3.1	2.7	2.5 ± 0.44	18
	mAb	2.3	4.6	2.5	3.2	4.3	3.4 ± 1.0	30
whole tomato, homogenate	pAb	9.8	8.3	6.7			8.3 ± 1.6	19
	mAb	11	7.5	8.6	9.0		9.0 ± 1.4	15
tomato ketchup	pAb	1.2	1.4	1.7	2.1	1.6	1.6 ± 0.34	21
	mAb	1.5	1.9	1.4	2.1	1.8	1.7 ± 0.26	15
tomato paste	pAb	5.6	5.6	6.7	5.5	6.5	6.0 ± 0.56	9.4
	mAb	7.3	5.6	8.6	6.8	6.2	6.9 ± 1.2	17
tomato juice	pAb	3.3	3.5	2.6	3.4	2.6	3.1 ± 0.4	14
	mAb	1.9	2.9	2.7	2.3	2.5	2.5 ± 0.38	15
white wine	pAb	1.3	1.5	1.0	1.3	1.1	1.2 ± 0.18	15
	mAb	1.3	1.1	1.0	1.3	1.2	1.2 ± 0.12	10

[a] Five (tomato: 3−4) independent extractions were made of all samples; each extract was analyzed by pAb EIA and mAb EIA in parallel. [b] RSD, relative standard deviation.

Table 4. Alternariol in Food Samples from the German Market: Comparison of pAb and mAb EIA Results

			alternariol μg/kg or μg/L						
commodity	EIA	% positive	mean ± SD	min	p25[a]	median	p75[b]	max	r
applesauce (n = 10)	pAb	60	1.5 ± 0.3	1.2	1.2	1.4	1.8	2.0	0.658
	mAb	100	4.3 ± 2.4	1.8	2.7	3.6	5.8	8.7	
apple juice (n = 44)	pAb	20	2.4 ± 0.62	1.7	2.1	2.2	2.5	3.5	0.606
	mAb	59	1.9 ± 0.89	1.1	1.4	1.6	2.0	4.2	
tomato ketchup (n = 18)	pAb	100	2.5 ± 1.2	1.0	1.8	2.2	3.1	5.0	0.495
	mAb	100	2.9 ± 1.1	1.4	1.8	2.9	3.5	4.6	
tomato paste (n = 10)	pAb	100	6.6 ± 3.1	3.1	4.6	5.7	8.1	13	0.925
	mAb	100	6.7 ± 3.5	2.6	4.4	5.8	7.8	13	
tomato juice (n = 16)	pAb	56	1.9 ± 0.84	1.1	1.2	1.6	2.9	3.1	0.641
	mAb	81	1.5 ± 0.44	1.0	1.1	1.2	1.6	2.4	
white wine (n = 11)	pAb	18	1.6 ± 0.58	1.2	1.4	1.6	1.8	2.0	0.984
	mAb	18	1.3 ± 0.17	1.2	1.3	1.3	1.4	1.4	

[a] 25th percentile. [b] 75th percentile.

differing recoveries, as seen for the mAb EIA and the pAb EIA in this study. Repeated analyses (n = 5) of each individual sample of various naturally contaminated sample matrices showed (Table 3) that the mAb EIA and the pAb EIA procedures yielded highly reproducible results (RSD: mAb EIA 7−30%, pAb EIA 6−21%).

The results of the alternariol analyses by pAb EIA and mAb EIA in various foods are summarized in Table 4. Alternariol contamination was 100% in tomato paste (3−13 μg/kg) and in tomato ketchup (1−5 μg/kg); contamination frequency of tomato juice was slightly lower but still exceeded 50%. These findings are in agreement with the fact that *Alternaria* is the major postharvest spoilage fungus in fresh tomato under humid and temperate climate conditions.[27] Our results are also consistent with those reported by Asam et al.,[12] who found alternariol in 2/2 samples of tomato juice (0.52 and 1.99 μg/kg). The fact that da Motta and Soares[28] did not find alternariol contamination in tomato samples can be explained by the relatively high detection limit (5 μg/kg) of the method used by these authors.

The finding that one of the tomato samples, which showed a typical decay due to *Alternaria* spoilage, contained exceedingly high levels of alternariol (53 mg/kg, Table 5) clearly illustrates the risk that a single *Alternaria*-infested tomato within a large batch of tomatos may be enough to measurably contaminate a certain tomato product. Such a high contamination of an *Alternaria*-infested tomato is well in agreement with results reported by other groups.[3,8] Alternariol monomethyl ether might co-occur in such samples at similarly high levels, which is again consistent with our HPLC data.

Apples are frequently infected with toxinogenic *A. tenuissima* and *A. arborescens* species, and consequently a contamination of apples and apple products with alternariol and its monomethyl ether has been reported earlier.[2,8,29] In our study, alternariol was found by EIA in the majority of applesauce products (60−100%), and in many apple juice products (20−59%). Again, alternariol levels in positive samples were consistently low (1−9 μg/kg). Using the mAb EIA, a higher percentage of positive samples was

Table 5. Comparison of EIA and HPLC Results for Alternariol in Tomato Samples

	alternariol (μg/kg)		
	EIA		
(sample no.) visible *Alternaria* decay	mAb	pAb	HPLC[a]
(1) none	<1	<1	<15
(1) spiked with alternariol 25 ng/g	19.6	20.8	22.9[b]
(1) spiked with alternariol 50 ng/g	38.8	41.4	42.8
(1) spiked with alternariol 100 ng/g	73.5	88.0	93.5
(2) none	<1.0	<1.0	<15.0
(3) none	<1.0	<1.0	<15.0
(4) none	<1.0	<1.0	<15.0
(5) mild	<1.0	<1.0	<15.0
(6) heavy	9.0	8.3	<15.0
(7) heavy	15	11	<15.0
(8) very heavy	33,000	41,000	53,000[c]

[a] LOD 15 μg/kg; LOQ 45 μg/kg. [b] Peak area was < LOQ, but peak could be integrated. [c] This sample also contained alternariol monomethyl ether (5,200 μg/kg).

found compared with the pAb EIA, and quantitative alternariol results were slightly higher in most positive samples. Although it cannot be excluded that some remaining sample matrix had a stronger effect on the mAb EIA, we think it is more likely that the presence of an alternariol analogue, which may be exclusively cross-reactive in the mAb EIA but not in the pAb EIA, could have caused the discrepancies betweeen both EIAs in some of these samples.

In general, however, our results for apple products are largely in agreement with previous reports. Scott et al.[8,10] reviewed literature data and concluded that apple and apple products are frequently contaminated with alternariol and other *Alternaria* toxins. Delgado and Gómez-Cordovés[7] analyzed 32 samples of apple juice concentrate and detected alternariol in 16 samples, at levels of 1.4−5.4 μg/L. Recently, trace levels of alternariol (0.16−0.22 μg/L) were detected in 3 out of 4 apple juice samples from Germany.[12] These data, obtained by different chromatographic methods, as well as our EIA data support the conclusion that apple products (juice and sauce) are regularly contaminated with alternariol, and that the average concentration is in the low μg/kg range. With a total of 54 apple products analyzed, our study also presents the largest data set on alternariol in apple products published so far.

Although the number of wine samples was limited, the fact that two out of eleven samples were positive for alternariol indicates that contamination of white wine with trace alternariol levels may be very common. Again, this result is in agreement with previous studies.[10,12] In general, grapes are good substrates for production of alternariol and alternariol monomethyl ether by *A. alternata*. Interestingly, *A. alternata* is capable of growing and producing alternariol and its monomethyl ether on inoculated grapes even at refrigeration temperatures, and both toxins are stable in white wine.[10,14] Scott et al.[10] reported a very similar contamination situation as found in our study: these authors analyzed white wine (23 samples) and white grape juices (4 samples) and found alternariol at levels of about 1.5 μg/L in two samples. A slightly worse situation was reported by Asam et al., who analyzed six samples of white wine, and found alternariol in all samples, at levels of up to 8 μg/L.[12]

The overall agreement of the pAb EIA and the mAb EIA for alternariol in apple and tomato products was acceptable, with the exception of applesauce. However, as shown in Figure 3, the correlation between both tests was only moderate in most cases. This may largely be attributable to the fact that most alternariol results were close to the method detection limits, at which levels discrepancies between methods occur more frequently. However, samples with slightly higher alternariol levels were in most cases clearly positive in both tests, with the exception of applesauce. From the results for applesauce shown in Figure 3B, it may be speculated that the mAb EIA detects not only alternariol but also another alternariol analogue in some samples, because of the consistently higher numerical results. From the fact that the mAb EIA cross-reacts with alternariol monomethyl ether, albeit weakly, while the pAb EIA has no detectable cross-reactivity with the monomethyl ether, it becomes clear that the two EIAs have slightly different recognition preferences with regard to alternariol and closely related molecules.

Comparative Determination of Alternariol in Tomatoes after Experimental Spoilage. Since all regular food samples analyzed within this study resulted in very low alternariol levels, below the detection limit of our HPLC-FLD method (15 ng/mL), validation of the EIA results by comparison with HPLC analysis was not possible. We therefore sought to generate highly contaminated samples during natural fungal spoilage. At the retail level, whole tomatoes were found to be frequently dotted with black spots, which may be indicative of an infection with *Alternaria* spp. We tried to provoke toxin production by simply storing such tomatoes at room temperature in the office for up to 4 weeks. Indeed, moderate or even massive fungal decay was observed in three samples. In one sample, excessively high levels of alternariol and its monomethyl ether were found by HPLC (Figure 4). For this sample highly diluted extracts (>1:1000) had to be analyzed to shift the measurement signals within the calibration range, thus the composition of these extracts was almost like that of standard solutions. The HPLC results agreed very well with those obtained by the EIAs (Table 5). Two other, visibly spoiled tomatoes contained only moderate amounts (8−15 μg/kg) of alternariol, below the HPLC detection limit. Nevertheless, these experiments indicate that a single fruit could contain very high levels of *Alternaria* toxins, a fact which may be of importance in the food industry.

Dietary Exposure to Alternariol. The total daily per capita consumption of fruits and fruit products (including fruit juices) in Germany is at approximately 500 g, and vegetables and products thereof (including processed vegetables) account for approximately 235 g per person.[30] Apple and tomato products represent a significant part of these foods. Therefore, alternariol contaminated products are consumed regularly by the majority of the population. This results in a long-term, low-level daily dietary exposure via tomato and apple products in the ng/kg body weight range. Since most other fruits and vegetables, as well as cereals, may be contaminated with alternariol, a larger and broader food survey is advisible to improve the knowledge about other sources of exposure.

Considering the synergistic effects between alternariol and alternariol monomethyl ether,[17] and the likely presence of other *Alternaria* toxins such as tenuazonic acid and altertoxins in

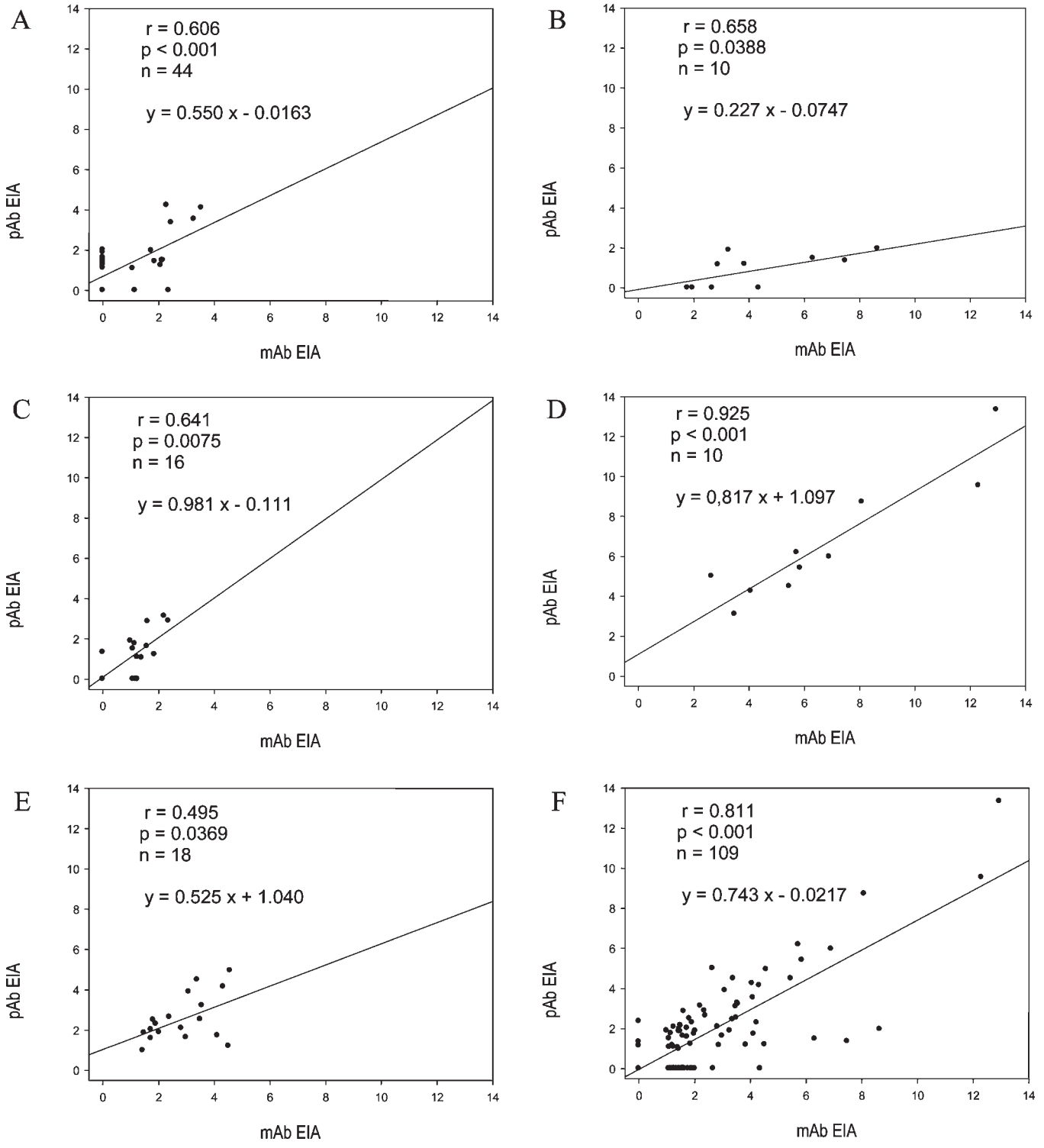

Figure 3. Comparison of pAb and mAb EIA for the detection of alternariol (μg/kg or μg/L) in apple juice (A), applesauce (B), tomato juice (C), tomato paste (D), tomato ketchup (E), and white wine + A−E (F).

contaminated samples,[8] the overall effect of *Alternaria*-infested foods on human health is still largely unknown. Therefore, more toxicological data are necessary to estimate the risk of the low-level long-term alternariol exposure to human health.

In conclusion, both immunochemical tests for alternariol described here are novel analytical tools, which enable sensitive and easy detection of this toxin in food. Complementary to existing EIAs for other mycotoxins, these tests could be implemented in screening programs, or for rapid on-site control in the food industry. Further evaluation studies are under way to enhance the spectrum of food matrices. The monoclonal antibodies against alternariol could also serve as key reagent for the

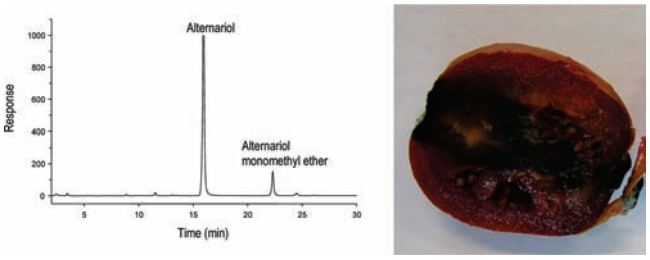

Figure 4. HPLC-FLD chromatogram (alternariol 53 mg/kg; alternariol monomethyl ether 5.2 mg/kg) of a naturally infected tomato stored at ambient temperature for 4 weeks after purchase. The corresponding EIA results were 33 mg/kg (mAb EIA) and 41 mg/kg (pAb EIA).

development of an immunoaffinity cleanup system, enhancing, for example, the detection limit of HPLC-FLD methods.

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ABBREVIATIONS USED

EIA, enzyme immunoassay; mAb, monoclonal antibodies; pAb, polyclonal antibodies; HPLC-FLD, high-performance liquid chromatography with fluorescence detection; BSA, bovine serumalbumin; HRP, horseradish peroxidase; KLH, keyhole limpet hemocyanin

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