Note

High-performance liquid chromatographic determination of major mycotoxins produced by *Alternaria* molds

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Molds of the genus *Alternaria* are widely distributed in the environment. The genus is indigenous to soil, and many species are plant pathogens that damage crops in the field or cause postharvest decay. Because the *Alternaria* grow well at low temperatures, they are associated with extensive spoilage of fruits and vegetables held under refrigeration.

Dibenzo-α-pyrones including alternariol (AOH), alternariol monomethyl ether (AME), and altenuene (ALT)[1,2], plus tenuazonic acid (TeA), a derivative of tetramic acid (Fig. 1), have been reported as major *Alternaria* metabolites and mycotoxins[3–7]. These compounds are of current interest because of their toxicity and because they have been isolated from a number of food and feed materials contaminated with various species of *Alternaria*. Adequate methods are needed to assess the amounts of these mycotoxins in fruits and vegetables, which frequently are infested with *Alternaria* but are uncharacterized as substrates for the production of mycotoxins by this genus. Seitz and Mohr[8] reported separation of ALT, AOH and AME with gradient elution of a normal-phase, high-performance liquid chromatography (HPLC) column with a solvent system of isooctane and tetrahydrofuran. The disadvantages of gradient elution and the use of tetrahydrofuran plus our need for a rapid method for determining ALT, AOH, AME and TeA in fruits and vegetables prompted us to seek alternate HPLC parameters.

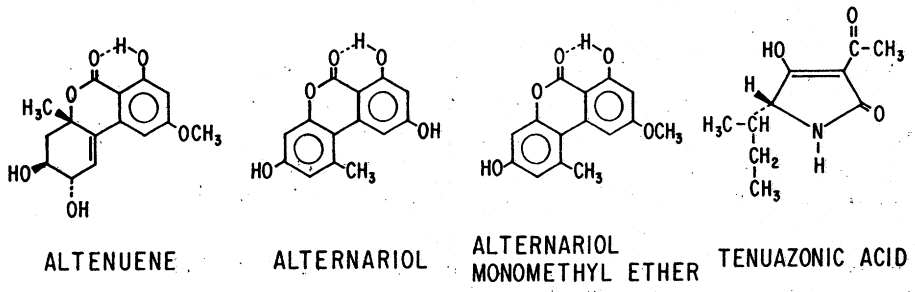

Fig. 1. Structures of four major mycotoxin metabolites produced by species of the genus *Alternaria*.

EXPERIMENTAL

Samples of AOH and AME were obtained from D. J. Harvan (National Institutes of Health, Research Triangle Park, N.C., U.S.A.). ALT was obtained from L. M. Seitz (U.S. Grain Marketing Research Laboratory, Manhattan, KS, U.S.A.). Additional quantities were prepared by fermentation of autoclaved rice with *Alternaria* strains obtained from the Northern Regional Research Center, Agricultural Research, Science and Education Administration, U.S. Department of Agriculture, Peoria, Il., U.S.A. (No. 5255; donated by C. E. Main as M-1) and the American Type Culture Collection, Rockville, MD, U.S.A. (No. 34457; donated by Rosemary Burroughs as RL8442-2).

AOH, AME and ALT were isolated and purified by column and thin-layer chromatography (TLC) and recrystallization[9]. The identities of AOH, AME and ALT were confirmed by mixed melting point, R_F on TLC plates (two solvent systems) and mass spectrometry[10]. TeA was isolated by extracting a concentrated chloroform–ethanol (4:1, v/v) extract of fermented rice with aqueous 5% $NaHCO_3$, acidifying the aqueous phase to pH 2.0 and extracting it with chloroform. The identity and purity of TeA were confirmed by TLC and mass spectrometry.

Standard solutions containing 0.1 μg of ALT, AOH, AME and TeA per μl of methanol were prepared and used to evaluate HPLC separation parameters. The HPLC system was assembled from Waters Assoc.* components (solvent delivery system, Model 6000 A; injection system, Model U6K; and variable wavelength UV detector, Model 450) and a column that was 30 cm $\times$ 3.9 mm I.D. and packed with 10 μm, reversed-phase, μBondapak C_{18} (monomolecular layer of organosilane bonded to porous silica particles). The system was operated isocratically with two experimental binary solvent systems consisting of methanol–water and acetone–water. The proportions of each organic solvent and water were varied over a range of 9:1 to 6:4 (v/v), respectively, to determine optimum proportions for separation of the mycotoxins. Detector wavelengths were 324 nm for AOH, ALT and AME and 278 nm for TeA. Since acetone absorbs strongly at 278 nm, the acetone–water solvent system was not useful for separations involving TeA.

Chloroform extracts of fruits and vegetables that were infected with *Alternaria* were prepared by homogenizing 200 g of tissue in a blender, adjusting the pH of the homogenate to 2.0 and extracting with two 500 ml portions of chloroform as described by Stinson *et al.*[11]. After drying over anhydrous Na_2SO_4 and concentrating to 25 or 50 ml under a nitrogen stream, extracts were ready for HPLC analysis.

RESULTS AND DISCUSSION

With both the methanol–water and the acetone–water solvent systems, baseline separations of authentic ALT, AOH and AME were obtained with resolution greater than 1.5. As expected for these three compounds, the capacity ratio (k') (based on the unretained solvent peak) for each compound increased as the amount of water, the more polar solvent in each binary system, was increased (Table I).

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* Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

TABLE I

VALUES OF k' FOR ALTENUENE (ALT), ALTERNARIOL (AOH), ALTERNARIOL MONOMETHYL ETHER (AME) AND TENUAZONIC ACID (TeA) ON A REVERSED-PHASE, μBONDAPAK C_{18} COLUMN WITH TWO BINARY SOLVENT SYSTEMS CONTAINING VARIED PORTIONS OF ORGANIC SOLVENT AND WATER

$k' = (R_{t_1} - R_{t_0})/R_{t_0}$; R_{t_0} = retention time of solvent front; R_{t_1} = retention time of compound.

Solvent system	*k' Values*			
	ALT	*AOH*	*AME*	*TeA*
Methanol–water				
90:10	0.09	0.18	0.92	3.7
80:20	0.26	0.60	1.46	2.5
65:35	0.66	1.72	5.34	1.4
Acetone–water				
65:35	0.30	0.59	1.38	
60:40	0.54	1.28	2.82	

In analytical practice, the methanol–water system was not satisfactory for the analysis of chloroform extracts of *Alternaria* infected tomatoes, apples, and blueberries because an interfering substance eluted concurrently with ALT; attempts to resolve the problem by adjusting flow-rate and the ratio of methanol–water in the solvent system were unsuccessful. Acetone–water (65:35, v/v) at a flow-rate of 0.4 ml/min was entirely satisfactory for separating and quantifying ALT, AOH and AME in such extracts. In addition to extracts being spiked with authentic compounds, the identities of ALT, AOH and AME separated from extracts were substantiated further in the HPLC eluate collected in fractions corresponding to the retention volumes of the authentic compounds, then concentrated, and spotted on TLC plates; TLC R_F

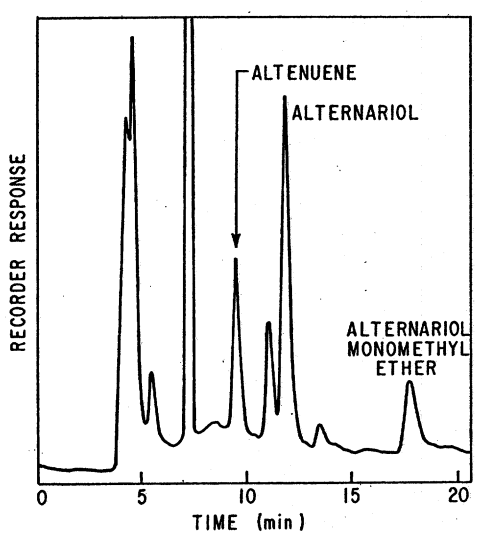


Fig. 2. HPLC separation of a chloroform extract of tomato tissue infected with *Alternaria*. HPLC parameters were: reversed-phase, μBondapak C_{18} column, 30 cm × 3.9 mm I.D.; acetone–water solvent (65:35, v/v), 0.4 ml/min; detection at 324 nm; sensitivity at 0.02 a.u.f.s.

values agreed with those of authentic compounds and no extraneous compounds were found. An example of a chromatogram obtained with a chloroform extract of tomato tissue infected with *Alternaria* is presented in Fig. 2. Standard curves were developed with known amounts of authentic ALT, AOH and AME (Fig. 3), and recorder response was linear throughout the range of weights used to establish the curve with the stated operating conditions.

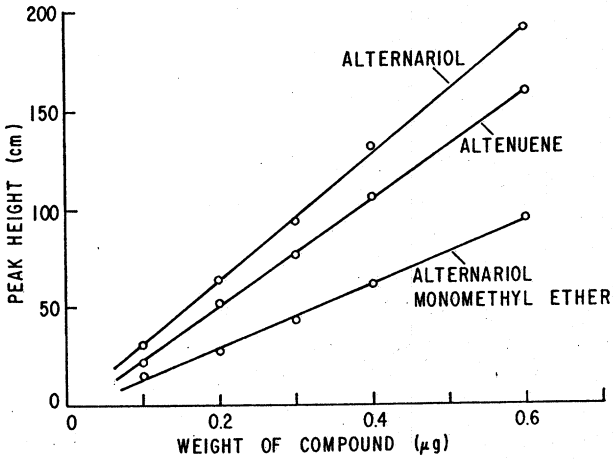
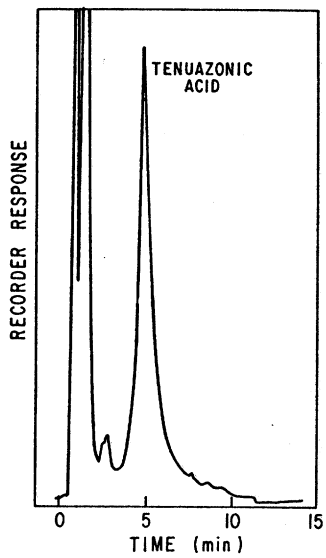


Fig. 3. Standard curves for weights of altenuene, alternariol and alternariol monomethyl ether *vs.* HPLC peak heights (measured peak height × attenuation factor).

The separation and quantitation of TeA was possible with the same μBondapak C_{18} column, but a different solvent system, methanol–water, was necessary. Other solvent systems, acetonitrile–water, ethanol–water and chloroform–methanol, were unsatisfactory. Methanol–water acidified with 0.1–0.2% acetic acid gave sharper peaks for TeA, but reproducible results were not obtained. With methanol–water, the k' value for TeA decreased with increasing proportions of the more polar solvent, water (Table I), which suggests that TeA is subject to normal-phase chromatography on this column. The optimum proportions of methanol–water were 9:1 (v/v), with a flow-rate of 2.0 ml/min for separation and quantitation of TeA in chloroform extracts of fruits and vegetables; a chromatogram obtained under these conditions with an extract of tomato tissue infected with *Alternaria* is shown in Fig. 4. No substances other than TeA were found when this fraction of the eluate was collected from twelve HPLC separations of extracts, concentrated and spotted on TLC plates. Recorder response was linear for the range of weights of authentic TeA used to establish a standard curve (Fig. 5).

Calculated as peak height (cm) divided by weight of compound (μg), relative recorder responses for ALT, AOH, AME and TeA were 264, 320, 154 and 52, respectively, under the stated conditions for analyzing extracts.

The precision of the HPLC method was determined with a chloroform extract of tomato tissue that had been inoculated with spores of a wild strain of *Alternaria* and incubated for 20 days at 20 °C. A series of 10 or 15 determinations for ALT,



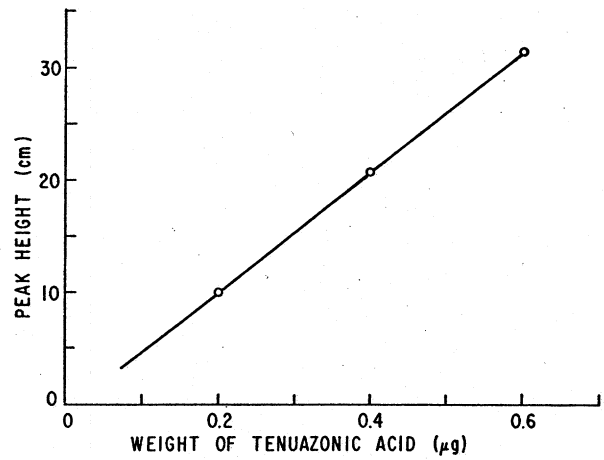


Fig. 4. HPLC separation of a chloroform extract of tomato tissue infected with *Alternaria*. HPLC parameters were: reversed-phase, μBondapak C_{18} column, 30 cm × 3.9 mm I.D.; methanol–water solvent (9:1, v/v), 2.0 ml/min; detection at 278 nm; sensitivity at 0.01 a.u.f.s.

Fig. 5. Standard curve for weight of tenuazonic acid *vs.* HPLC peak height (measured peak height × attenuation factor).

AOH, AME and TeA was made, and the standard deviation of the values obtained for the concentration of each compound was calculated (Table II).

As described herein, a reversed-phase, μBondapak C_{18} HPLC column eluted with acetone–water (65:35, v/v), for ALT, AOH and AME determinations or methanol–water (9:1, v/v), for TeA determinations now is used routinely in this laboratory to assess the contamination of a number of fruits and vegetables with the major mycotoxins produced by species of the genus *Alternaria*. The method requires only an isocratic HPLC system and readily available, relatively non-toxic solvents and has proven applicable to crude extracts of the plant materials we have analyzed thus far. These HPLC parameters should be useful for the determination of ALT, AOH, AME and TeA in extracts of other commodities, provided that interfering substances are not present in the extracts.

TABLE II

STANDARD DEVIATIONS OBTAINED FROM REPLICATE HPLC ANALYSES OF A CHLOROFORM EXTRACT OF TOMATO TISSUE INFECTED WITH *ALTERNARIA*

Compound	*n*	*Average concentration found (μg/μl)*	*S.D.*	*Relative S.D. (%)*
Altenuene	10	0.34	0.014	4.1
Alternariol	10	3.05	0.059	1.9
Alternariol monomethyl ether	10	1.01	0.048	4.7
Tenuazonic acid	15	1.32	0.071	5.4

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