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Chromatographic Method for *Alternaria* Toxins in Apple Juice

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1. Introduction

Fungi of the genus *Alternaria* are commonly parasitic on plants and other organic materials. Many are in fact plant pathogens of field crops whereas others infect foodstuffs after harvest ***(1)***. They can grow at low temperatures and so may cause spoilage of fruits and vegetables during refrigerated transport and storage. *Alternaria alternata* is a frequently occurring species of particular interest to mycotoxicologists because it produces a number of mycotoxins, including alternariol (AOH; 3,7,9-trihydroxy-1-methyl-6*H*-dibenzo[*b,d*]pyran-6-one; **Fig. 1**), alternariol monomethyl ether (AME; 3,7-dihydroxy-9-methoxy-1-methyl-6*H*-dibenzo[*b*,*d*]pyran-6-one; **Fig. 1**), altertoxins I, II, and III {[1*S*-(1α, 12aβ, 12bα)] 1,2,11,12,12a,12b-hexahydro-1,4,9,12a-tetrahydroxy-3,10-perylenedione; [7a*R*-(7aα,8aα,8b α,8cα)]-7a,8a,8b,8c,9,10-hexahydro-1,6,8c-trihydroxyperylo[1,2-*b*]oxirene-7,11-dione; and [la*R*-(laα,lbβ,5aα,6aα,6bβ,10aα)]-la,1b,5a,6a,6b,10a-hexahydro-4,9-dihydroxyperylo[1,2- *b*:7,8-*b*']bisoxirene-5,10-dione; respectively}, and L-tenuazonic acid {[5*S*-[5*R**(*R**)]]-3-acetyl-5-(1-methylpropyl)-2,4-pyrrolidinedione} ***(1-5)***. Isolation of AOH and AME was first reported in 1953 ***(2)***. A culture of *A. alternata* on corn flour has been found to be carcinogenic in rats, and culture extracts were mutagenic in various microbial and cell systems ***(6–8)***. *A. alternata* might be one of the etiological factors for human esophageal cancer in Linxian, China ***(8)***. AOH, AME and, in particular, the altertoxins are mutagenic ***(1,7,9–13)***. Although no long term cancer studies of these mycotoxins in laboratory animals have been carried out, there are reports of subcutaneous induction of squamous cell carcinoma in mice by human embryo

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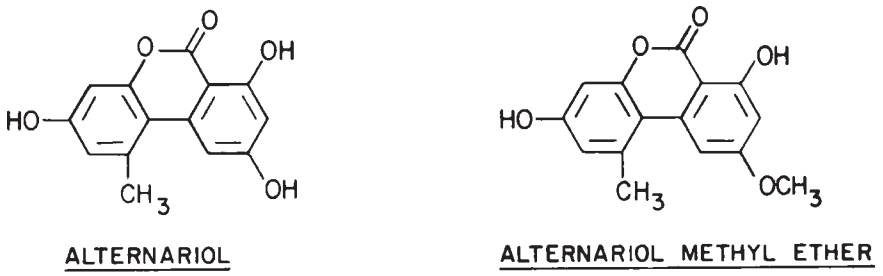

Fig. 1. Structures of alternariol (AOH) and alternariol monomethyl ether (AME).

esophageal tissue treated with AOH and of subcutaneous tumorigenicity with NIH/3T3 cells transformed by AME ***(9,14)***.

The natural occurrence of *Alternaria* toxins in grains ***(1,3,4,15,16)***, sunflower seeds ***(17,18)***, oilseed rape ***(18)***, pecans ***(3)***, and various fruits ***(4,19,20)***, including tomatoes, olives, mandarins, melons, peppers, apples, and raspberries, has been reported. As a result of inoculation experiments, the potential for their occurrence in other fruits (oranges, lemons, and blueberries) has also been demonstrated ***(4)***. The occurrence of AOH in a processed fruit product has only recently been reported—in apple juice ***(21–23)*** and in raspberry drinks ***(20)***. In addition, tenuazonic acid has been found occasionally in tomato products ***(4)*** and AME (mainly traces) has been detected in apple juice ***(22–24)***.

Monitoring of fruit juices for *Alternaria* toxins is necessary to give impetus for further toxicological studies should the level of human exposure from these foods prove to be a concern. Apple juice was chosen as the matrix to be analyzed initially, since interferences in liquid chromatography (LC) for AOH and AME are fewer than in other fruit juices such as citrus and grape juices. As shown by the natural occurrence ***(4)*** and inoculation studies ***(25–28)***, AOH and AME are the main mycotoxins produced in *Alternaria* infected apples and hence would serve as indicators of *Alternaria* contamination of the fruit before processing.

These mycotoxins have been determined by gas chromatography ***(21,29)*** and by LC, mainly with ultraviolet detection ***(24,28,30–38)***, although fluorescence ***(15,31,35,39)*** and mass spectrometry ***(23,40)*** have also been used for detection. A detection limit of 0.05 ng for AOH and AME by fluorescence has been reported ***(35)***. Another very sensitive LC determination procedure for AOH and AME (as well as altertoxins I and II) is electrochemical; 0.05 ng AOH was the detection limit ***(41)***.

A sensitive LC method for AOH and AME in apple juice was developed by Delgado *et al.* ***(24)***, who used two solid phase extraction columns in series for cleanup followed by LC with UV detection at 256 nm. Detection limits were

reported to be 1.6 and 0.7 µg/L apple juice, respectively. A previous LC method for AOH and AME in apple juice had detection limits of 10 and 25 µg/L respectively, by UV detection at 340 nm; extraction was with dichloromethane followed by silica gel column cleanup ***(34)***. With modifications to the cleanup procedure consisting of increased volumes of the wash solvents on both solid phase extraction columns, a change in composition of the acetonitrile-water wash solvent on the C_{18} column, and an increase in volume of the acetonitrile-formic acid (100:1) eluting solvent on the aminopropyl column, the newer method ***(24)*** is described below, together with two further variations (A and B) to the cleanup procedure.

2. Materials

1. Standard solutions of AOH and AME: Weigh crystalline AOH and AME (available from Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178, USA, catalog nos. A 1312 and A 3171, respectively) and dissolve each separately in methanol to make 250 µg/mL stock solutions (*see* **Note 1**). Store stock solutions in a freezer at –12°C (*see* **Note 2**). Evaporate 100 µL AOH stock solution and 100 or 200 µL AME stock solution in a 4-mL screwcap vial under a gentle stream of nitrogen and dissolve in 1 mL methanol to prepare mixed spiking solution containing 2.5 µg AOH/mL and 2.5 or 5 µg AME/mL (*see* **Note 3**). Evaporate aliquot of spiking standard under nitrogen and dissolve in 500 µL of LC mobile phase to give LC standards containing 0.2 µg AOH/mL and 0.2 or 0.4 µg AME/mL. Store spiking and LC standard solutions in a refrigerator (4°C).
2. Pectinase: *Aspergillus niger* solution in 40% glycerol, 445 units/mL (Sigma Chemical Co., catalog no. P4176).
3. Ultrasonic bath: Branson model 1210.
   a. C_{18}: Chromabond, 3-mL, 500 mg, not endcapped (Macherey-Nagel, Neuman Neander Strasse, D-52355 Düren, Germany, catalog no. 730 003) (*see* **Note 4**).
   b. NH_2: Chromabond, 500 mg (Macherey-Nagel, catalog no. 730 033) (*see* **Note 5**).
5. Adaptors: Bond Elut, fits 1, 3, and 6-mL tubes (Varian Sample Preparation Products, 24201 Frampton Avenue, Harbor City, CA 90710, USA, catalog no. 1213 1001).
6. Reservoirs: empty SPE tubes.
7. Stopcocks: Luer (Varian Sample Preparation Products, catalog no. 1213 1005).
8. Reverse phase LC column: Inertsil 5 µ*M* ODS-2, 250 × 4.6 mm (MetaChem Technologies Inc., 3547 Voyager Street, Bldg. 102, Torrance, CA 90503, USA) (*see* **Note 6**).
9. LC mobile phase: Methanol-acetonitrile-1% aqueous *ortho*-phosphoric acid (50:20:30, v/v/v) (*see* **Note 7**).

3. Methods

3.1. C_{18} Cleanup

1. Attach adaptor with reservoir to the top of the C_{18} SPE column and attach the tip of the column to a stopcock inserted into the port on the lid of the vacuum manifold.

2. Condition the C_{18} SPE column with 6 mL methanol followed by 6 mL of water. Adjust the flow rate to about 1 drop/s using the flow control valve of the vacuum manifold and continue with this flow rate for subsequent elutions of this column.
3. Pass a 10-mL test sample of apple juice through the column (*see* **Subheading 3.3.** for cloudy apple juice procedure).
4. Wash the column with 6 mL of distilled, deionized, water followed by 2.5 mL of acetonitrile-water (35:65, v/v). Discard all washings.
5. Elute the toxins with 4 mL acetonitrile-acetic acid (100:1, v/v) into a 50-mL round-bottomed flask or a 4 mL vial.
6. Evaporate the eluate to dryness in a round bottom flask using a rotary evaporator with a water bath temperature of 40°C; or evaporate the eluate in a vial under a stream of N_2 with minimum heating.
7. Dissolve the residue with three 500 µL portions of ethyl acetate (*see also* **Subheading 3.4.**), holding each for 5 min in an ultrasonic bath. Proceed to **Subheading 3.2.**

3.2. Aminopropyl Column

1. Attach the adaptor with reservoir (if required) to the top of the amino SPE column.
2. Condition the column with 6 mL dichloromethane, using gravity flow for this and subsequent elutions from this column.
3. Add the combined extract from **Subheading 3.1.** to the top of the column and wash with 3 mL of acetone followed by 3 mL of acetonitrile (*see also* **Subheading 3.4.**). Discard washings.
4. Elute the toxins with 5 mL acetonitrile-formic acid (100:1, v/v) into a 4-mL vial (capacity 5 mL).
5. Evaporate the eluate carefully to dryness under a stream of nitrogen at 40°C and dissolve residue in 500 µL of LC mobile phase.

3.3. Cleanup Variation A (for Cloudy Apple Juice)

1. In a 15-mL centrifuge tube, mix 10 mL of cloudy apple juice with 25 µL of pectinase on a vortex mixer for 30 s.
2. Heat in a water bath at 40°C for 1 h.
3. Centrifuge at 830 × g for 20 min.
4. Using a Pasteur pipet add the clear supernatant to the conditioned C_{18} SPE column (*see* **Subheading 3.1.**, **steps 1** and **2**).
5. After draining, wash the column with 5 mL of distilled, deionized, water and discard washing.
6. Add 250 µL of acetonitrile-acetic acid (100:1) to the residue in the centrifuge tube with vortex mixing, then place in an ultrasonic bath for 10 min. Add 2.25 mL of distilled, deionized water, vortex mix 30 s, then centrifuge for 10 min.
7. Transfer the clear supernatant to a C_{18} column.
8. Repeat **steps 6** and **7** two more times.
9. Wash the column with 1.5 mL of acetonitrile-water (35:65, v/v). Discard all of the washings.

10. Elute the toxins with 4 mL acetonitrile-acetic acid (100:1, v/v) into a 4-mL vial and evaporate to dryness under nitrogen.
11. Dissolve the residue with three 500 µL portions of ethyl acetate, holding each for 10 min in an ultrasonic bath.
12. Add the ethyl acetate extracts to a conditioned aminopropyl SPE column and carry out the cleanup on this column (*see* **Subheading 3.2.**).

3.4. Cleanup Variation B

Improved recoveries may be obtained by dissolving the residue from **Subheading 3.1.** with 100 µL of methanol plus 400 (or 500) µL of ethyl acetate twice, then 500 µL of ethyl acetate (with ultrasound) (cf. **Subheading 3.1.**, **step 7**). Proceed to **Subheading 3.2.** where the results of the acetone and acetonitrile washes of the aminopropyl column are then omitted.

3.5. Liquid Chromatography (LC)

1. Carry out the determination of AOH and AME by isocratic reverse phase LC on a C_{18} column (*see* **Subheading 2., step 8**) with acetonitrile-methanol-1% aqueous *ortho*-phosphoric acid mobile phase (*see* **Subheading 2., step 9**) at a flow rate of 1 mL/min. The following additional equipment comprised the LC system, but any suitable equivalent apparatus may be substituted:
   a. Pump: Shimadzu model LC-10AD.
   b. Injector: Rheodyne model 7125 with 20 µL loop.
   c. Guard column: Guard-Pak precolumn module (Waters) with Resolve C18 precolumn insert.
   d. In-line degasser (Shodex).
   e. Absorbance detector, 254 nm: Waters model 440 or Thermo Separation Products UV 2000 dual wavelength detector.
   f. Integrator: Varian (Spectra-Physics) 4270.
2. Inject 10 µL of extract solution (20 mL of apple juice equivalent/mL mobile phase) and 10 µL of mixed standard solution of AOH and AME (0.2 µg/mL and 0.2 or 0.4 µg/mL, respectively). The given amounts of AOH and AME injected are for the UV 2000 detector but will depend on the sensitivity of the UV detector. Make two injections of sample extract and compare average peak areas for AOH and AME with those of standards at the same retention time. Typical retention times were 6 and 13 min for AOH and AME, respectively (**Fig. 2**). The standard curve was linear in the range 0.25–12.5 ng AOH or AME injected (0.025–1.25 µg/mL). Detection limits (UV 2000 detector) for standards were 0.05 ng AOH and 0.1 ng AME (S/N 3:1).

3.6. Method Performance

Added at 10 and 20 ng/mL, respectively, percent recoveries of AOH and AME from apple juices averaged 79 ± 14 (n = 9) and 87 ± 16 (n = 13) by the procedure presented here. By the pectinase modification (variation A) (*see*

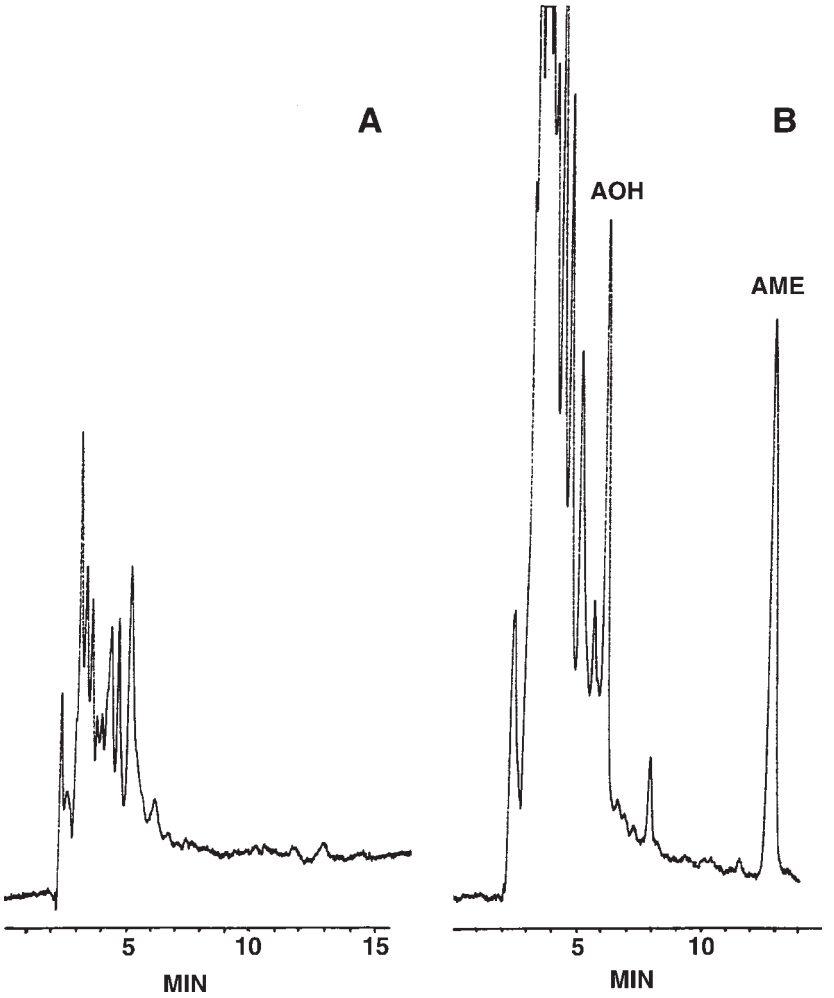

Fig. 2. Chromatograms of apple juice (containing an unconfirmed trace—approx 0.5 ng/mL—of AOH) **(A)** and the same juice spiked with 10 ng AOH/mL and 20 ng AME/mL **(B)**, analysed by method variation B.

Subheading 3.3.), recoveries of AOH and AME added at these concentrations averaged 84% and 76%, respectively ($n = 2$). By the modification described in **Subheading 3.4.** (variation B), recoveries were 87 ± 8 and 84 ± 6% ($n = 5$) when AOH and AME were added together at 10 and 20 ng/mL, respectively, and 94 ± 8 and 102 ± 13% ($n = 12$) in experiments where both toxins were added together at 10 ng/mL. Detection limits for AOH and AME were dependent on the sample but were of the order of 0.5–1 ng/mL.

4. Notes

1. Check the weights of AOH and AME by UV analysis. Prepare dilutions of each at 5 µg/mL in 95% ethanol. For AOH the extinction coefficient at 257 nm is 53700 ***(42)*** and for AME at 259 nm the coefficient of extinction is 47900 ***(43)***.
2. It may be necessary to place the flask containing AME in an ultrasonic bath for 20–30 min to dissolve the AME if it crystallizes out of solution upon storage.
3. This solution can be used to determine by LC the concentration of AME present as an impurity in AOH and vice versa. The standards used here contained 2.5% AME in AOH and 0.1% AOH in AME.
4. Bond Elut 500 mg, 3 mL columns (Varian) also performed satisfactorily, with gravity flow. However, other C_{18} SPE columns may not be suitable.
5. Other aminopropyl columns may not be suitable.
6. The Inertsil column gave a better separation of AOH from an interference compared to two other brands of columns.
7. Mobile phase composition may be varied if not detrimental to resolution. For example, methanol-acetonitrile-1% aqueous *ortho*-phosphoric acid (45:32:23) can also be used.

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References

1. Chekowski, J. and Visconti, A., eds. (1992) *Alternaria. Biology, Plant Diseases and Metabolites*. Elsevier, Amsterdam.
2. Raistrick, H., Stickings, C. E., and Thomas, R. (1953) Studies in the biochemistry of micro-organisms. 90. Alternariol and alternariol methyl ether, metabolic products of *Alternaria tenuis. Biochem. J.* **55,** 421–433.
3. King, A. D., Jr. and Schade, J. E. (1984) *Alternaria* toxins and their importance in food. *J. Food Prot.* **47,** 886–901.
4. Visconti, A. and Sibilia, A. (1994) *Alternaria* toxins, in *Mycotoxins in Grain. Compounds Other Than Aflatoxins* (Miller, J. D. and Trenholm, H. L., eds.), Eagan, St. Paul, MN, pp. 315–336.
5. Panigrahi, S. (1997) *Alternaria* toxins, in *Handbook of Plant and Fungal Toxicants* (D'Mello, J. P. F., ed.), CRC, Boca Raton, FL, pp. 319–337.
6. Liu, G., Miao, J., Liu, Z., Yu, G., Zhen, Y., Li, X., Zhang, J., Zhao, C., and Hao, H. (1982) The experimental study of the papillomas of the forestomach and esophagus induced by *Alternaria alternata* in rats. *Acta Acad. Med. Henan* **17,** 5–7 (in Chinese).

7. Zhen, Y. Z., Xu, Y. M., Liu, G. T., Miao, J., Xing, Y. D., Zheng, Q. L., Ma, Y. F., Su, T., Wang, X. L., Ruan, L. R., Tian, J. F., Zhou, G., and Yang, S. L. (1991) Mutagenicity of *Alternaria alternata* and *Penicillium cyclopium* isolated from grains in an area of high incidence of oesophageal cancer—Linxian, China, in *Relevance to Human Cancer of N-Nitroso Compounds, Tobacco Smoke and Mycotoxins* (O'Neil, I. K, Chen, J., and Bartsch, H., eds.), International Agency for Research on Cancer, Lyon, France, pp. 253–257.
8. Dong, Z., Liu, G., Dong, Z., Qian, Y., An, Y., Miao, J**.,** and Zhen, Y. (1987) Induction of mutagenesis and transformation by the extract of *Alternaria alternata* isolated from grains in Linxian, China. *Carcinogenesis* **8,** 989–991.
9. Liu, G., Qian, Y., Peng, Z., Dong, W., Qi, Y., and Gou, H. (1992) Etiological role of *Alternaria alternata* in human esophageal cancer. *Chinese Med. J.* **105,** 394–400.
10. An, Y.-H., Zhao, T.-Z., Miao, J., Liu, G.-T., Zheng,Y.-Z., Xu, Y.-M., and Van Etten, R. L. (1989) Isolation, identification, and mutagenicity of alternariol monomethyl ether. *J. Agr. Food Chem.* **37,** 1341–1343.
11. Scott, P. M. and Stoltz, D. R. (1980) Mutagens produced by *Alternaria alternata. Mutat. Res.* **78,** 33–40.
12. Stack, M. E. and Prival, M. J. (1986) Mutagenicity of the *Alternaria* metabolites altertoxins I, II, and III. *Appl. Environ. Microbiol.* **52,** 718–722.
13. Davis, V. M. and Stack, E. (1994) Evaluation of alternariol and alternariol methyl ether for mutagenic activity in *Salmonella typhimurium. Appl. Environ. Microbiol.* **60,** 3901–3902.
14. Liu, G. T., Qian, Y. Z., Zhang, P., Dong, Z. M., Shi, Z. Y., Zhen, Y. Z., Miao, J., and Xu, Y. M. (1991) Relationship between *Alternaria alternata* and oesophageal cancer, in *Relevance to Human Cancer of N-Nitroso Compounds, Tobacco Smoke and Mycotoxins* (O'Neill, I. K., Chen, J., and Bartsch, H., eds.), International Agency for Research on Cancer, Lyon, France, pp. 258–262.
15. Gruber-Schley, S. and Thalmann, A. (1988) Zum Vorkommen von *Alternaria* spp. und deren Toxine in Getreide und mögliche Zusammenhänge mit Leistungsminderungen landwirtschaftlicher Nutztiere. *Landwirtsch. Forschung* **41,** 11–29.
16. Webley, D. J., Jackson, K. L., Mullins, J. D., Hocking, A. D., and Pitt, J. I. (1997) *Alternaria* toxins in weather-damaged wheat and sorghum in the 1995–1996 Australian harvest. *Aust. J. Agr. Res.* **48,** 1249–1255.
17. Chulze, S. N., Torres, A. M., Dalcero, A. M., Etcheverry, M. G., Ramirez, M. L., and Farnochi, M. C. (1995) *Alternaria* mycotoxins in sunflower seeds: incidence and distribution of the toxins in oil and meal. *J. Food Prot.* **58,** 1133–1135.
18. Nawaz, S., Scudamore, K. A., and Rainbird, S. C. (1997) Mycotoxins in ingredients of animal feeding stuffs: 1. Determination of *Alternaria* toxins in oilseed rape meal and sunflower seed meal. *Food Addit. Contam*. **14,** 249–262.
19. Hasan, H. A. H. (1995) *Alternaria* mycotoxins in black rot lesion of tomato fruit: conditions and regulation of their production. *Mycopathologia* **130,** 171– 0177.

20. Giryn, H. and Szteke, B. (1996) *Alternaria* mycotoxins in food. *Abstr. IX IUPAC Symposium on Mycotoxins and Phycotoxins,* May 27–31, 1996, Rome, Italy, p. 165.
21. Scott, P. M., Weber, D., and Kanhere, S. R. (1997) Gas chromatography-mass spectrometry of *Alternaria* toxins. *J. Chromatogr. A* **765,** 255–263.
22. Delgado, T. and Gómez-Cordovés, C. (1998) Natural occurrence of alternariol and alternariol methyl ether in Spanish apple juice concentrates. *J. Chromatogr. A* **815,** 93–97.
23. Scott, P. M., Kanhere, S. R., Cléroux, C., Lau, B. P.-Y., Lewis, D. A., and Roscoe, V. A. (1998) Determination of *Alternaria* mycotoxins in fruit beverages. *Abstr. 112th AOAC Int. Ann. Mtg.*, September 13–17, 1998, Montreal, Canada, p. 62.
24. Delgado, T., Gómez-Cordovés, C., and Scott, P. M. (1996) Determination of alternariol and alternariol methyl ether in apple juice using solid-phase extraction and high performance liquid chromatography. *J. Chromatogr. A* **731,** 109–114.
25. Stinson, E. E., Bills, D. D., Osman, S. F., Siciliano, J., Ceponis, M. J., and Heisler, E. G. (1980) Mycotoxin production by *Alternaria* species grown on apples, tomatoes, and blueberries. *J. Agr. Food Chem.* **28,** 960–963.
26. Viñas, I., Bonet, J., and Sanchis, V. (1992) Incidence and mycotoxin production by *Alternaria tenuis* in decayed apples. *Lett. Appl. Microbiol.* **14,** 284–287.
27. Robiglio, A. L. and Lopez, S. E. (1995) Mycotoxin production by *Alternaria alternata* strains isolated from red delicious apples in Argentina. *Int. J. Food Microbiol.* **24,** 413–417.
28. Ozcelik, S., Ozcelik, N., and Beuchat, L. R. (1990) Toxin production by *Alternaria alternata* in tomatoes and apples stored under various conditions and quantitation of the toxins by high performance liquid chromatography. *Int. J. Food Microbiol.* **11,** 187–194.
29. Scott, P. M. (1993) Gas chromatography of mycotoxins, in *Chromatography of Mycotoxins. Techniques and Applications* (Betina, V., ed.), Elsevier, Amsterdam, pp. 373–425.
30. Schade, J. E. and King, A. D., Jr. (1984) Analysis of the major *Alternaria* toxins. *J. Food Prot.* **47,** 978–995.
31. Seitz, L. M. and Mohr, H. E. (1976) Analysis of *Alternaria* metabolites by high-pressure liquid chromatography. *Anal. Biochem.* **70,** 224–230.
32. Heisler, E. G., Siciliano, J**.,** Stinson, E. E., Osman, S. F., and Bills, D. D. (1980) High performance liquid chromatographic determination of major mycotoxins produced by *Alternaria* molds. *J. Chromatogr.* **194,** 89–94.
33. Maas, M. R., Woody, M. A., and Chu, F. S. (1981) Production of alternariol and alternariol methyl ether by *Alternaria* spp. *J. Food Safety* **3,** 39–47.
34. Wittkowski, M., Baltes, W., Krönert, W., and Weber, R. (1983) Bestimmung von *Alternaria*-toxine in Obst- und Gemüseerzeugnissen. *Z. Lebensm. Unters. Forsch.* **177,** 447–453.
35. Stack, M. E., Mislivec, P. B., Roach, J. A. G., and Pohland, A. E. (1985) Liquid chromatographic determination of tenuazonic acid and alternariol methyl ether in tomatoes and tomato products. *J. Assoc. Off. Anal. Chem*. **68,** 640–642.

36. Palmisano, F., Zambonin, P. G., Visconti, A., and Bottalico, A. (1989) Profiling of *Alternaria* mycotoxins in foodstuffs by high-performance liquid chromatography with diode-array ultraviolet detection. *J. Chromatogr.* **465,** 305–313.
37. Visconti, A., Logrieco, A., and Bottalico, A. (1986) Natural occurrence of *Alternaria* mycotoxins in olives- their production and possible transfer into the oil. *Food Addit. Contamin.* **3,** 323–330.
38. Müller, M. and Lepom, P. (1992) Nachweis von *Alternaria*-Mycotoxinen in Laborkulturen. *Zentralbl. Mikrobiol.* **147,** 197–206.
39. Sydenham, E. W., Thiel, P. G., and Marasas, W. F. O. (1988) Occurrence and chemical determination of zearalenone and alternariol monomethyl ether in sorghum-based mixed feeds associated with an outbreak of suspected hyperestrogenism in swine. *J. Agr. Food Chem.* **36,** 621–625.
40. Liu, F., Luo, Y., Zhang, L. H., Kuang, K. Y., and Shi, S. Y. (1993) Identification of alternariol, alternariol monomethyl ether and zearalenone by particle beam LC/MS. *Chin. Chem. Lett.* **4,** 975–978.
41. Palmisano, F., Zambonin, P. G., Visconti, A., and Bottalico, A. (1989) Determination of *Alternaria* mycotoxins in foodstuffs by gradient elution liquid chromatography with electrochemical detection. *Chromatographia* **27,** 425–430.
42. Thomas, R. (1961) Studies in the biosynthesis of fungal metabolites. 4. Alternariol methyl ether and its relation to other phenolic metabolites of *Alternaria tenuis*. *Biochem. J.* **80,** 234–240.
43. Coombe, R. G., Jacobs, J. J., and Watson, T. R. (1970) Metabolites of some *Alternaria* species. The structures of altenusin and dehydroaltenusin. *Aust. J. Chem.* **23,** 2343–2351.