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Gas chromatography–mass spectrometry of *Alternaria* mycotoxins

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Abstract

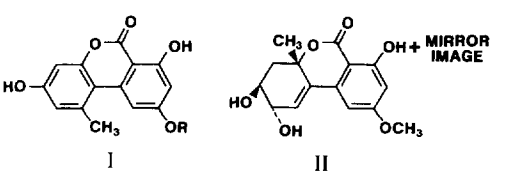
Heptafluorobutyrate (HFB) derivatives have not previously been used for GC of *Alternaria* mycotoxins. Capillary (0.5 μm film) GC–mass spectrometry (MS) showed that full and partial derivatives of alternariol (AOH), alternariol monomethyl ether (AME) and altenuene (ALT); a structurally uncharacterized derivative of altertoxin I (ALTX-I); and a tris-HFB derivative of tenuazonic acid (TA) were formed with heptafluorobutyric anhydride and a basic catalyst. Full and partial trimethylsilyl (TMS) ethers of these mycotoxins were formed with Tri-Sil TBT. Apple juice extracts caused increased response in GC–MS of AOH bis-HFB and bis-TMS derivatives. Natural occurrence of AOH in apple juice has been demonstrated.

Keywords: Apple juice; Derivatization, GC; Mycotoxins; Alternariol

1. Introduction

Alternaria species are ubiquitous in nature and include plant pathogens and post-harvest decay organisms [1]. *A. alternata* is of most interest to mycotoxicologists. Extracts of cultures of this species were tumorigenic in rats [2] and mutagenic in various microbial and cell systems [3,4]. The species produces a number of mycotoxins [1], some of which, including alternariol (AOH), alternariol monomethyl ether (AME) and altertoxins I (ALTX-I), II and III, are mutagenic [3,4]. *Alternaria* toxins have been found naturally occurring in grains [5–7], sunflower seeds [8], pecans [5] and various fruits, including apples, mandarins, raspberries and tomatoes [9–13]. Potential for occurrence in other fruits (oranges, lemons and blueberries) has been demonstrated by inoculation experiments [9,14]. The usual toxins determined are AOH, AME and altenuene (ALT), which are derivatives of dibenzo-α-pyrones (Fig. 1), ALTX-I (a perylene derivative, Fig. 2) and the tetramic acid tenuazonic acid (TA) (Fig. 3).

Before 1984, the most commonly used procedure for determining *Alternaria* toxins in food extracts was TLC [15] but this has now largely been replaced by HPLC. Both normal and reversed-phases have been used, primarily with UV detection [15]. Minimum detectable amounts of AOH and AME were 0.7



I II

Fig. 1. Structures of alternariol (AOH) (I, R=H), alternariol monomethyl ether (AME)(I, R=CH_3) and altenuene (ALT) (II).

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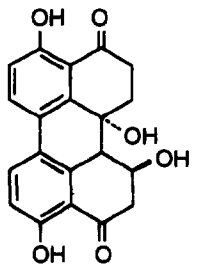

Fig. 2. Structure of altertoxin I (ALTX-I).

ng and 0.5 ng, respectively, at 256 nm [16] and 0.5 ng at 340 nm [17]. To include TA in the analysis, reversed-phase HPLC requires detection at 277–288 nm and addition of Zn(II) ion to the mobile phase [12] or use of a deactivated end-capped C_{18} silica with a high carbon load [18]; the latter allows detection of sub-ng amounts of TA. Fluorescence detection is not applicable to TA, but as little as 0.05–0.2 ng of AOH and AME could be detected using excitation at 278 nm [12]. Electrochemical detection is also a very sensitive technique for AOH and AME, as well as for altertoxins [19]. Particle beam HPLC–MS has been applied to detection of AOH and AME in *Alternaria* cultures [20,21].

A limited amount of previous work on GC of *Alternaria* toxins covered trimethylsilyl (TMS) derivatives of AOH, AME, ALT and TA, with detection by mass spectrometry (MS) or flame ionization [22]. The lower limit of quantitation for AOH, AME and ALT standards using flame ionization detection was 100 ng [23] while a limit of detection of 20 ng/g in fruit juices was achieved by GC–MS [24]. Limits of detection for TA and the isomeric D-*allo* TA in tomato paste were <6 ng/g by GC–MS [25]. There are no reports on the use of other derivatives for GC of *Alternaria* toxins; in particular, heptafluorobutyrate (HFB) derivatives appeared to be worth exploring in view of their successful use with other mycotoxins [22]. Furthermore, there has been no previous work at all on GC of ALTX-I or other altertoxins. Application of GC–MS, as well as HPLC, to analysis of apple juice for AOH has resulted in the first finding of AOH in this food.

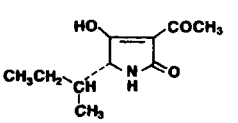

Fig. 3. Structure of tenuazonic acid (TA).

2. Experimental

2.1. Chemicals

AOH, AME, ALT, ALTX-I and TA copper salt were obtained from Sigma (St. Louis, MO, USA). TA was prepared from the copper salt by means of a cation-exchange resin [25]. Heptafluorobutyric anhydride was from Regis Technologies (Morton Grove, IL, USA); 4-dimethylaminopyridine was from Aldrich (Milwaukee, WI, USA); "dimethylaminopyridine on polystyrene" [polymer-bound 4-(*N*-benzyl-*N*-methylamino)pyridine] was from Fluka Chemie (Buchs, Switzerland); and Tri-SIL TBT [trimethylsilylimidazole+*N*,*O*-bis(trimethylsilylacetamide)+trimethylchlorosilane, 3:3:2] was from Pierce Chemical (Rockford, IL, USA). All solvents were HPLC grade.

2.2. Derivatization

Reactions were carried out in 4-ml vials with PTFE-lined screw-caps. Amounts of AOH, AME and ALT derivatized were generally in the range 0.25 to 5.0 μg, prepared by evaporation of aliquots of standard solutions in methanol. For ALTX-I, TA and TA Cu(II) salt, 10 μg amounts were derivatized. HFB derivatives were prepared by reaction with 30 μl heptafluorobutyric anhydride and 70 μl toluene–acetonitrile (1:1) containing 2 mg/ml of 4-dimethylaminopyridine [26] for 10 min at room temperature (23°C) or 65°C, mixing with 465 or 965 μl *n*-hexane and washing with 1 ml pH 6.0 phosphate buffer; 10 mg polystyrene-bound 4-(*N*-benzyl-*N*-methylamino)-pyridine was also used as catalyst and then separated from the *n*-hexane solution. TMS derivatives were formed by reaction with 50 μl Tri-Sil TBT for 10 min at 23°C, then mixing with 500 μl *n*-hexane and washing with 1 ml pH 6.0 phosphate buffer.

2.3. GC–MS

A VG Analytical (Manchester, UK) 7070 EQ mass

spectrometer, operating in the conventional double focusing configuration and incorporating a VG 11-250 data system, was interfaced with a Varian VISTA 6000 gas chromatograph equipped with a programmable on-column injector. A J&W DB-XLB capillary GC column (10 m×0.25 mm I.D., 0.5 μm film) was temperature programmed from 80°C (2 min hold) at 50°C/min to 180°C then at 10°C/min to 280°C. The carrier gas was helium (0.41 or 0.69 bar). The mass spectrometer was operating in the electron impact mode at a mass resolution of 1000.

2.4. HPLC

Equipment consisted of a Rheodyne (Berkeley, CA, USA) Model 7125 injector with 20 μl loop; Shimadzu (Kyoto, Japan) LC-10 AD pump, mixer and FCV-10AL gradient former; Shodex KT-27 degasser; Waters Associates (Milford, MA, USA) Model 440 UV detector; Shimadzu RF-551 fluorescence detector; and Varian 4270 integrator. An Ultratechsphere C_{18} (HPLC Technology, Macclesfield, UK) 5 μm particle size 25×3.2 mm I.D. stainless steel column was fitted with a Resolve C_{18} Guard-Pak (Waters). The mobile phase was usually methanol–acetonitrile–1% aqueous phosphoric acid (4:2:4) at a flow-rate of 1.5 ml/min, but other acidic mobile phases with different solvent proportions and a different acid (formic acid) were also used. Detection of AOH and AME was by UV (254 nm) and fluorescence (excitation λ 330 nm, emission λ 430 nm) in series. These optimum fluorescence parameters for AOH and AME were determined in prior experiments at 15 different combinations of excitation and emission wavelengths.

2.5. Extraction of apple juice

Apple juice was cleaned up with C-18 and aminopropyl solid-phase extraction columns essentially according to a recent method for AOH and AME determination [16]; 5 ml instead of 4 ml 1% formic acid in acetonitrile was used for elution of toxins from the aminopropyl column. Eight samples of retail apple juice were analyzed by HPLC and four of them by GC–MS. The method of standard additions was used for GC–MS of the two positive samples (equivalent to 0, 5 and 10 μg AOH/l added before TMS derivatization); linear curves were obtained for injections made within one day. The equivalent of 4 ml apple juice was derivatized for GC–MS after evaporation of the column eluate under nitrogen.

3. Results and discussion

3.1. GC–MS of HFB and TMS derivatives

Initially we used a 0.25 μm GC column film thickness to study derivatization of AOH, but precision for selected ion monitoring chromatography was poor and a 0.5 μm film thickness gave much better standard curves. HFB derivatives of AOH, AME and ALT could be formed at either room temperature or 65°C, but complete derivatization was not obtained. The partial derivatives were usually more readily detected than the fully derivatized mycotoxins, attributed to higher yields (shown by total ion current chromatography). Selected ion monitoring chromatograms of the bis- (M^+=650) and tris- (M^+=846) HFB derivatives of AOH are shown in Fig. 4. Mass spectra showed prominent ions at *m/z* 650 (base peak), 437 and 425 for AOH bis-HFB and at *m/z* 846 (base peak), 677, 650, 633 and 600 for AOH tris-HFB. Selected ion monitoring chromatograms of the mono- (M^+=468) and bis- (M^+=664) HFB derivatives of AME are shown in Fig. 5. AME

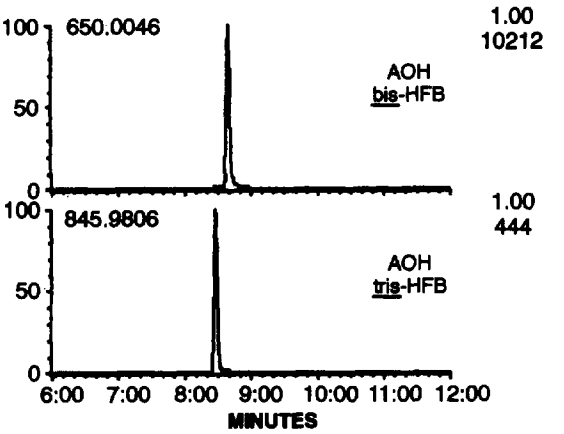

Fig. 4. GC–MS of AOH bis-HFB and AOH tris-HFB monitored at *m/z* 650 and 846, respectively. Derivatives were prepared at room temperature/10 min.

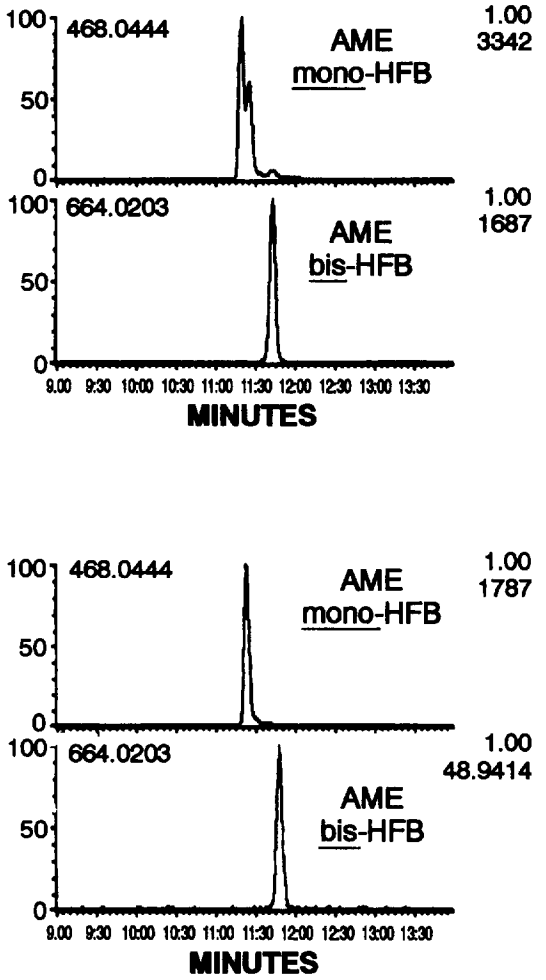

Fig. 5. GC–MS of AME mono-HFB and bis-HFB monitored at *m/z* 468 and 664, respectively. Upper two chromatograms: derivatives prepared at 65°C/10 min. Lower two chromatograms: derivatives were prepared at room temperature/10 min. 5 ng toxin was injected.

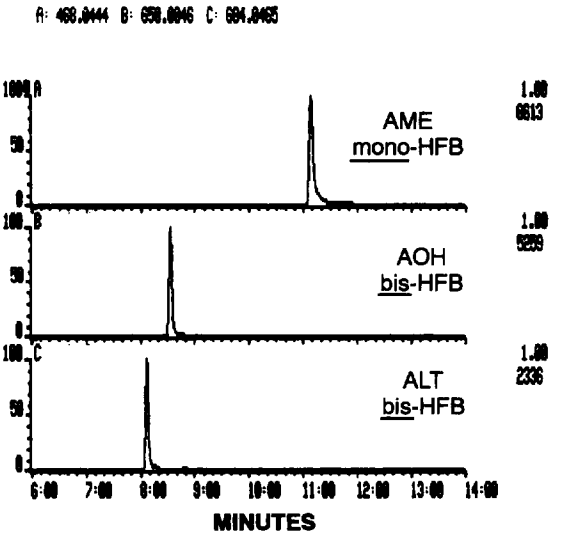

Fig. 6. GC–MS of a mixture of AME mono-HFB, AOH bis-HFB and ALT bis- HFB monitored at *m/z* 468, 650 and 684, respectively. Derivatives were prepared at room temperature.

mono- HFB was unusual in that two isomers with very similar mass spectra (*m/z* 468 (base peak), 439, 395, 243) were formed at 65°C but not at 23°C. AME bis-HFB had prominent high mass ions at *m/z* 664, 495, 451, 439, 423 and 411; ALT bis-HFB at *m/z* 684 and 452; and ALT tris-HFB at *m/z* 651 and 452, with only a weak molecular ion at *m/z* 880. Separation of the partial HFB derivatives of AME, AOH and ALT, monitored at *m/z* 468, 650 and 684, respectively, is illustrated in Fig. 6. A problem observed with the AOH bis- and tris-HFB derivatives was that the standard curves were not reproducible on different days; they could also be non-linear and described by a quadratic equation. Sensitivity to the condition of the GC column was indicated as a possible cause of these variations.

ALTX-I formed an HFB derivative at room temperature. The major peak in the total ion current chromatogram had a highest mass ion at *m/z* 1100 (Fig. 7), which corresponded to a tetrakis-derivative (expected molecular mass 1136) but with loss of $2H_2O$. The mechanism for formation of this ion is not apparent.

When TA or its Cu(II) salt was heptafluorobutyrylated at room temperature, the tris-HFB derivative was mainly formed together with a trace of isomer detected by monitoring at *m/z* 785, the molecular ion. Retention times were approx. 3.4 and 3.2 min, respectively. Mass spectra showed higher mass ions at *m/z* 785, 571 (base peak), 542, 402 and 374 (base peak with Cu salt) for TA HFB and 785, 756, 588, 571, 542, 514 and 374 (base peak) for TA isomer HFB.

Trimethylsilylation of the phenolic *Alternaria* toxins AOH, AME and ALT also gave full and partial derivatives (Fig. 8). Previously Kellert et al. [24] reported GC–MS of the fully TMS derivatized toxins and published their mass spectra. These

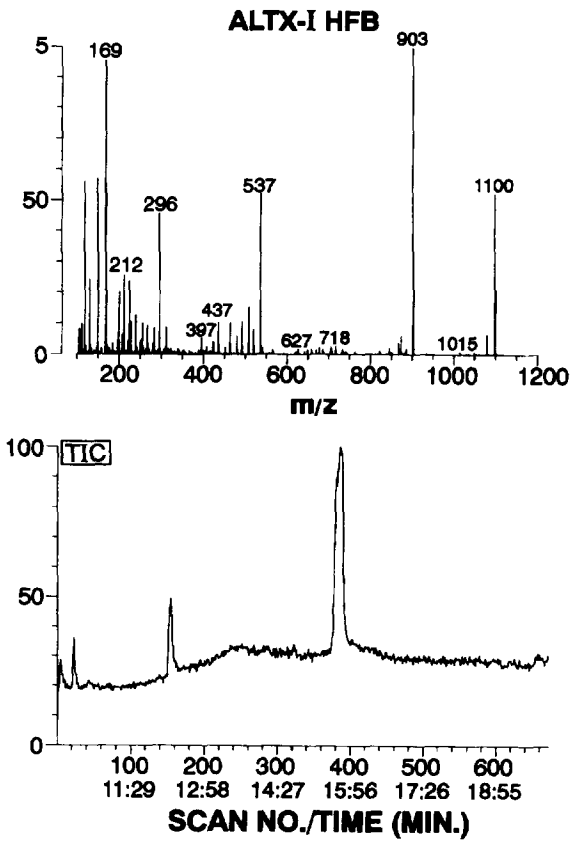

Fig. 7. Mass spectrum of ALTX-I HFB derivative with retention time 15.4 min in the total ion current chromatogram (lower trace); 20 ng toxin was injected.

agreed with our data except we found additional peaks at m/z 208 and 207 in the mass spectrum of AOH tris-TMS ether. We found that for AOH the bis-TMS derivative was usually the most useful for GC–MS, with single ion monitoring at m/z 402 (base peak; an additional prominent ion was at m/z 387). The standard curve was linear in the range 250–1000 pg (4 injections from dilution of derivative). Sensitivity was better than for the HFB derivative. It was the TMS derivative that was eventually applied for the determination of AOH in apple juice (see Section 3.2). Characterizing the partial TMS derivatives of the two other phenolic *Alternaria* toxins, the mass spectrum of AME mono-TMS had prominent high mass ions at m/z 344 (base peak) and 329 while that of ALT bis-TMS had a weak M−15 peak at m/z 421 and a base peak at m/z 320. However, neither of these partial derivatives were formed in greater quantities than fully derivatized AME and ALT [24] so were not preferable for GC–MS.

ALTX-I showed four peaks in the total ion current chromatogram after TMS derivatization, of which the largest was the tetrakis-derivative with retention time 17.9 min (m/z 640, 625, 535, and 445). there were two isomeric tris-TMS ethers at retention times 16.8 min (m/z 568, 553, 496, 462 and 373) and 17.1 min (m/z 568, 463 and 373), as well as a bis-TMS ether at 16.2 min (m/z 496, 406, 373, 316 and 291).

TA tris-TMS derivative could be monitored at m/z 413, as previously reported [25], or with better sensitivity at m/z 398. This derivative was formed from both the free acid and the Cu(II) salt. The complete mass spectrum differed slightly from the one recorded previously [25], with an additional peak present at m/z 270. Reconstructed ion chromatograms showed a small amount of the bis-derivative, which eluted about a minute later and was particularly evident using the Cu(II) salt and by monitoring at m/z 326 (base peak).

Table 1 summarizes the molecular or other high mass ions of HFB and TMS derivatives of AOH, AME, ALT, ALTX-I and TA, indicating which derivatives are preferred for GC–MS and, where the molecular ion is weak or absent, the ion of next highest mass.

3.2. Analysis of apple juice extracts for AOH

Apple juice extracts caused increased GC–MS responses for bis-HFB and bis-TMS derivatives. Using one blank apple juice sample, mean increases for derivatized spiked extracts containing the equivalents of 5, 15, 30 and 50 μg AOH/l apple juice were 2.0- and 2.9-fold for the two derivatives, respectively. Therefore to determine AOH in naturally contaminated apple juice, it was necessary to use the method of standard additions and make all injections in one day. GC–MS results for AOH in two naturally contaminated commercial samples are included in Table 2. Selected ion chromatograms after trimethylsilylation are shown for one of these samples in Fig. 9. Confirmation of identity of AOH in the sample containing 2 μg/l was obtained from the mass spectrum of the bis-HFB derivative, which

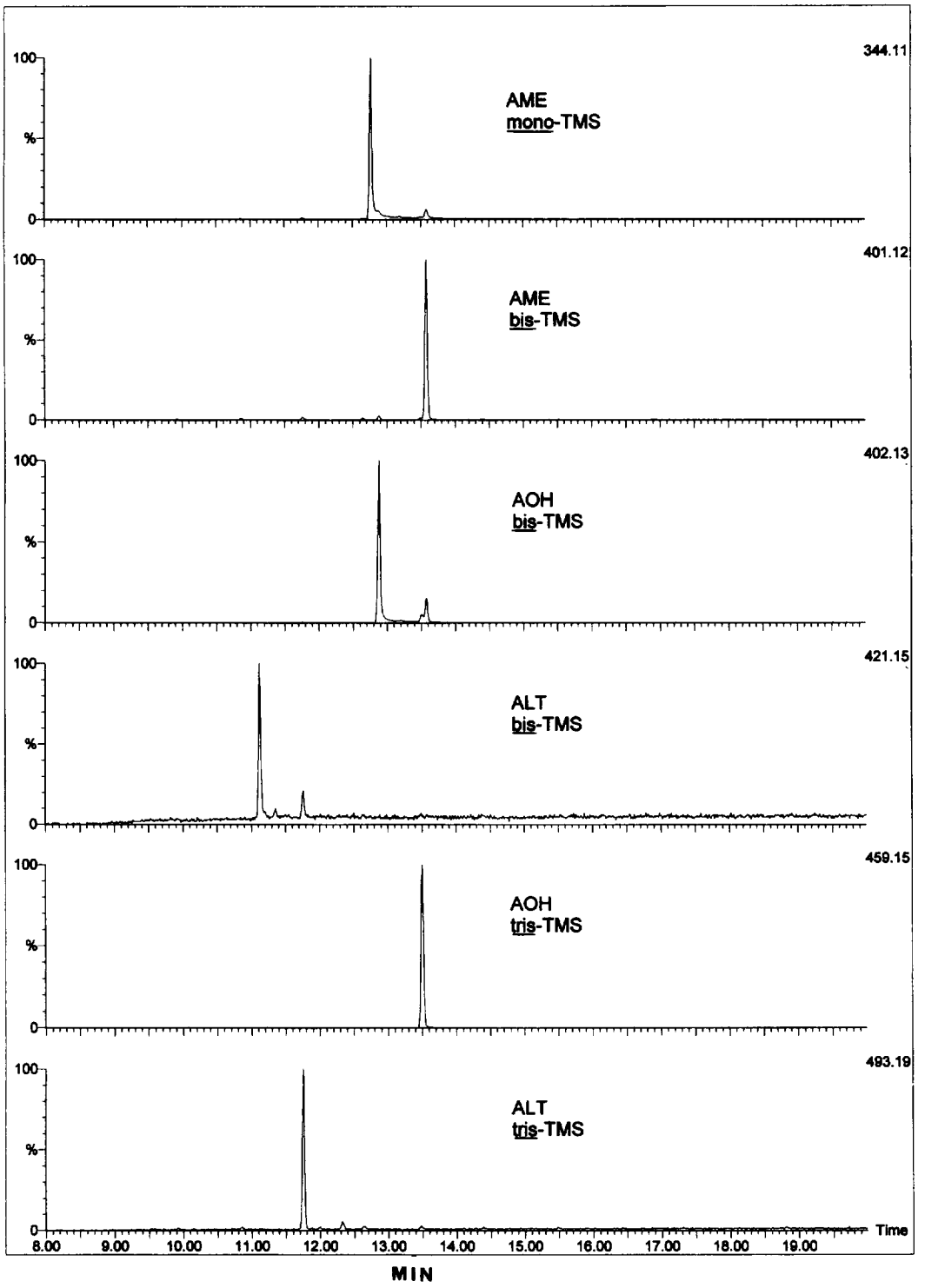

Fig. 8. GC–MS of a mixture of AME mono-TMS; AME, AOH and ALT bis-TMS; and AOH and ALT tris-TMS derivatives; monitored at *m/z* 344, 401, 402, 421, 459 and 493, respectively.

Table 1
MS molecular or other high mass ions (m/z) of full and partial derivatives of *Alternaria* toxins

Toxin	Derivatization level[a]	HFB	TMS
AOH	3	846	459[f]
	2	650	402
AME	2[b]	664	401[f]
	1[c]	468[d]	344
ALT	3[b]	880	493[f]
	2[c]	684	421[f]
ALTX-I	4	1100[e]	640, 625[f]
	3	–	568[d]
	2	–	496
TA (CuTA)	3	785[d]	413, 398[f]
	2	589	341, 326[f]

[a] Preferred derivative underlined: 4=tetrakis, 3=tris, 2=bis, 1=mono.
[b] Preferred for TMS.
[c] Preferred for HFB.
[d] Two isomers.
[e] M−36.
[f] M−15.

showed the same prominent high mass ions at m/z 650, 437 and 425 as in the standard. This is the first report of natural occurrence of AOH in processed apple products and the first confirmed finding of AOH in any processed fruit product, although traces of AOH were recently detected in Polish raspberry drinks [13,27]. Other *Alternaria* toxins have occasionally been found in commercial processed fruit products, i.e., TA in tomato products [13,25,27] and an unconfirmed trace of AME in apple juice [16].

Table 2
Analysis of apple juice for AOH and AME by HPLC and GC–MS

Sample	AOH (ng/ml)		
	HPLC		GC–MS[a]
	UV	Fluorescence	
1	2.2	6.8	2.0 (bis)[b]
2	5.0	14.8	4.1 (bis) 5.0 (tris)
3	ND[c]	ND	Trace (<1)
4	ND	Int.[d]	ND
5	ND	ND	–
6	0.8	ND	–
7	ND	ND	–
8	ND	ND	–

[a] TMS ether.
[b] Identity of AOH also confirmed by mass spectrum of bis-HFB derivative.
[c] ND, not detected (<0.7–1.5 ng/ml) by UV; AME was not detected in any sample.
[d] Int., interference.

A total of eight apple juice samples were analyzed for AOH and AME by HPLC after cleanup by two solid-phase extraction columns. The method gave recoveries of AOH spiked at a level of 5 μg/l into blank apple juice of 82.3% (S.D. 12.4, $n=10$), determined by HPLC with fluorescence detection. Previously, mean recoveries of 83 and 92% were reported for AOH and AME, respectively, added to apple juice at concentrations of 3.5 to 32 μg/l and determined by HPLC with UV detection [16]. The two apple juice samples that we found positive for AOH by GC–MS were also positive by HPLC (Table 2). However, only results obtained by UV detection agreed with the GC–MS determinations and parallel measurements by fluorescence were higher for these two samples, possibly due to an interference.

In conclusion, while routine screening of apple juice for AOH and AME should be carried out by HPLC (with UV detection [16]), GC–MS of HFB and TMS derivatives has considerable value for confirmation of identity of these and other *Alternaria* toxins. Future work in this area will focus on HPLC–

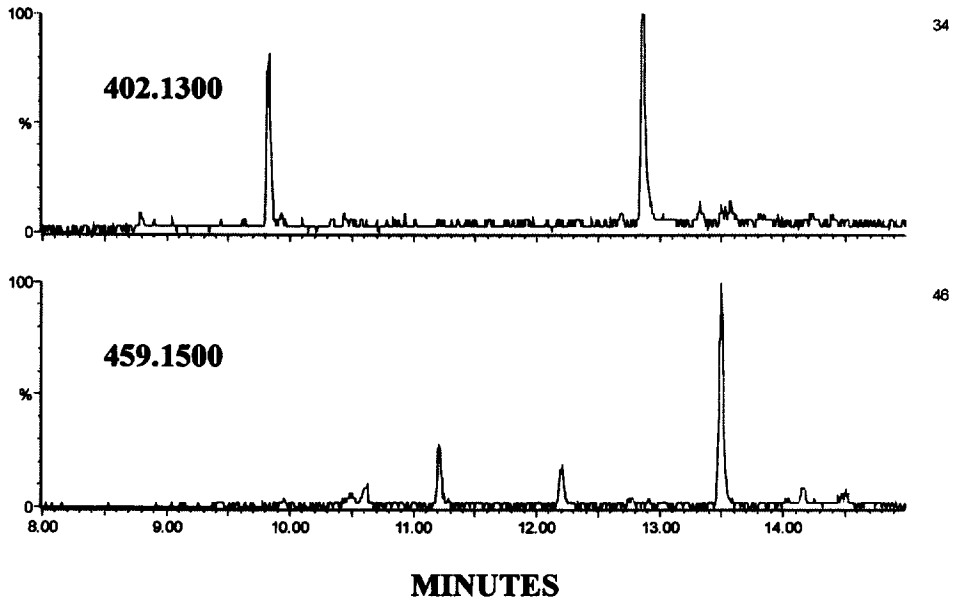

Fig. 9. GC–MS of AOH in naturally contaminated apple juice (2 μg/l). Upper chromatogram AOH bis-TMS (12.9 min); lower chromatogram AOH tris-TMS (13.5 min).

MS and HPLC–MS/MS for confirmation of *Alternaria* toxins without derivatization.

Acknowledgments

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