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Determination of alternariol and alternariol methyl ether in apple juice using solid-phase extraction and high-performance liquid chromatography

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Abstract

The present work describes a new method for determination of alternariol (AOH) and alternariol methyl ether (AME) in apple juice using solid-phase extraction (SPE) columns for extraction and cleanup of samples for high-performance liquid chromatography (HPLC). Chromatograms of spiked samples show that both toxins can be easily detected without interferences, and good recoveries for AOH (82.8 ± 7.4%) and AME (91.9 ± 6.1%) with detection limits as low as 1.6 and 0.7 μg/l, respectively, were obtained.

Keywords: Apple; Fruit juices; Food analysis; Alternariol; Mycotoxins; Toxins

1. Introduction

Many species of the genus *Alternaria* are plant pathogens on a wide variety of crops in the field and produce post-harvest decay of fruits, grains and vegetables [1–12]. Because they can grow at low temperatures they are also responsible for spoilage during refrigerated transport and storage of these commodities [13,14], resulting in economic loss to growers and commercial marketers.

Alternaria spp. also produce a wide variety of toxic metabolites belonging to different structural classes. Among them, alternariol (AOH), alternariol methyl ether (AME) and altenuene (ALT), which are benzopyrone derivatives; altertoxin-I (ATX-I), a perylene derivative; and tenuazonic acid (TA), a tetramic acid derivative, are considered to be the most important *Alternaria* toxins. They are toxic in many biological systems, including animal and mammalian cells [15–21].

AOH and AME (Fig. 1) are the major mycotoxins produced in *Alternaria* infected apples, whereas TA, the main toxin in decayed tomatoes, as well as ALT

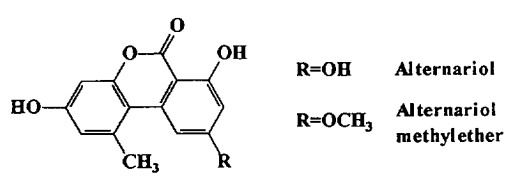

Fig. 1. Chemical structures of alternariol and alternariol methyl ether.

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and ATX-I, are produced in much smaller amounts [3,4,10,14,22].

HPLC is the technique of choice for detecting *Alternaria* mycotoxins in foodstuff extracts [23–27], although GC techniques [2,28] have also been used. Analysis of AOH, AME and ALT has been carried out mainly on mouldy and infected fruits and grains. Sample preparation involves a wide variety of solvents in the extraction procedure for AOH, AME and ALT with or without further purification by silica column or thin-layer chromatography [6,23,29]. For determination of *Alternaria* toxins in fruit juices a similar procedure is reported [25,28] consisting of extraction three times with 50 ml of dichloromethane and purification of the extracts by silica column chromatography using diethyl ether–hexane (1:1) in the washing step and ethyl acetate–methanol (9:1) for elution of mycotoxins.

In this work we have developed a new method for determination of AOH and AME in apple juice, including solid-phase extraction (SPE) of the toxins before HPLC determination.

2. Experimental

2.1. Standards

AOH and AME were obtained from Sigma (Deisenhofen, Germany). Standard solutions were prepared by dilution of the toxins in acetonitrile–water (3:1).

2.2. Solvents

For analytical HPLC, all the solvents were of HPLC grade (Labscan, Dublin, Ireland). For SPE column extraction, acetonitrile and ethyl acetate were of HPLC grade (Labscan) and dichloromethane (Merck, Darmstadt, Germany) acetone, acetic acid and formic acid (Sharlau, Spain) were of analytical-reagent grade.

2.3. Juice samples

Seven samples of apple juice were purchased at different local stores. The analyses were made in duplicate.

2.4. Statistical analysis

For calibration purposes, the data were subjected to statistical analysis utilizing the Statgraphics program (Statistical Graphics, Rockville, MD, USA).

2.5. Sample preparation

Cleanup of the sample was carried out in two steps using first a 3-ml, 500-mg sorbent polypropylene C_{18} not endcapped SPE column followed by a 3-ml aminopropyl SPE column (Chromabond, Macherey-Nagel).

A 10-ml sample of apple juice was passed directly, without any preliminary treatment, through the C_{18} column, previously conditioned with 6 ml of methanol and 6 ml of water, by gentle syringe pressure at a flow-rate of 1 drop/s. The column was washed with 2 ml of water and 2 ml of acetonitrile–water (1:3), and the toxins were eluted with 4 ml of 1% acetic acid in acetonitrile into a round-bottom flask. The eluate was evaporated to dryness in a rotary evaporator at 40°C. The residue was extracted three times with 500 μl ethyl acetate, holding for 30 s each time in an ultrasonic bath. The extract solution was added to the aminopropyl column, previously conditioned with 6 ml of dichloromethane, and allowed to drain by gravity. After washing with 2 ml of acetone followed by 2 ml of acetonitrile, elution was carried out with 4 ml of 1% formic acid in acetonitrile into a 4-ml vial. The eluate was carefully evaporated under a nitrogen stream at 40°C, and the residue dissolved in 250 μl of acetonitrile–water (3:1) for HPLC determination.

2.6. HPLC conditions

A Model 600-MS pump system controller, U6K universal injector, Model 996 photodiode-array detector and a Millenium 2010 software data system from Waters (Milford, MA, USA) were used. The column was a 4-μm NovaPack C_{18} (300 × 3.9 mm I.D.). HPLC analysis was performed using a binary gradient system composed of 0.02% aqueous formic acid (pH 3) (solvent A) and methanol (solvent B). The elution programme is given in Table 1. The equilibration time was 15 min and the flow-rate was 0.8 ml/min. Detection wavelength was 256 nm. Injection volume was 25 μl. For peak identity

Table 1
Mobile phase gradient composition (gradient curve No. 6)

Time (min)	% Solvent A[a]	% Solvent B[b]
0	40	60
3	40	60
15	0	100
17	0	100
18	40	60

[a]Solvent A: 0.02% aqueous formic acid (pH 3).
[b]Solvent B: methanol.

purposes, UV spectra were recorded from 210 to 350 nm. Quantification of AOH and AME was performed by the external standard method.

3. Results and discussion

3.1. HPLC method

This method allowed us to resolve the major toxins of *Alternaria*, AOH and AME, in less than 15 min with retention times of 7.5 and 14.5 min, respectively (Fig. 2). Calibration curves for pure AOH and AME were developed, and a linear response was observed in the range of 1–50 ng on column of both standards. High correlation coefficients of 0.9998 for AOH and 0.9999 for AME were obtained. Detection limits of pure AOH and AME, defined as twice the standard error, were 0.7 and 0.5 ng, respectively, amounts lower than those reported so far in the literature for the use of a UV detector [27].

3.2. Recovery

This is the first report on the use of SPE columns in the sample preparation step prior to HPLC determination of AOH and AME. Because of the complexity of apple juice a purification of samples in two stages was needed in order to obtain clean extracts for HPLC analysis. The use of C_{18} columns in the first step followed by aminopropyl columns gave the best results relating to recovery and cleanup

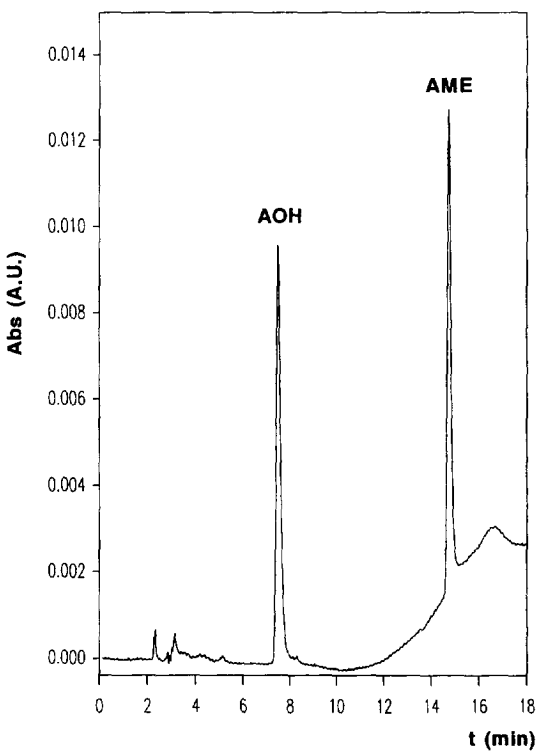

Fig. 2. HPLC of standard AOH and AME (10 ng of each injected).

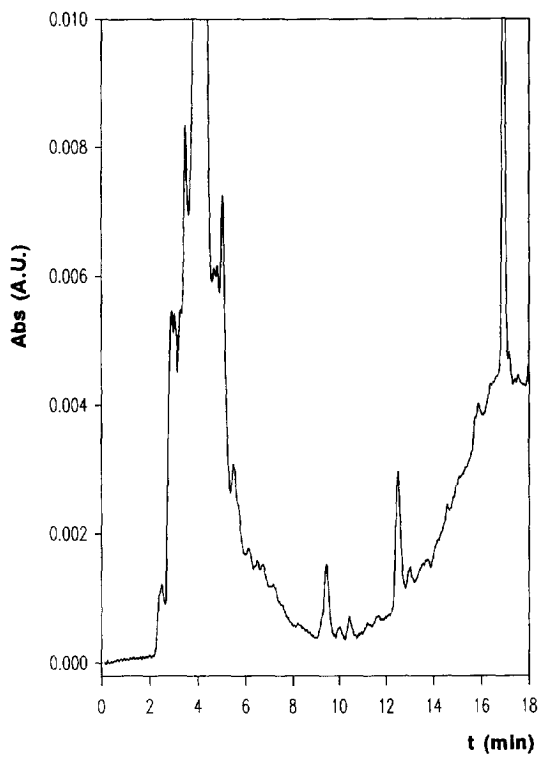

Fig. 3. HPLC of blank apple juice.

Table 2
Recoveries for alternariol (AOH) and alternariol methyl ether (AME) in apple juice samples at different spiking levels

	Amount added (μg/l)	Recovery (%)	n
Alternariol	3.5	82; 77.1, 70.3	3
	7.35	77.6, 92.5, 84.3	3
	14	91.9, 80.7, 75.4	3
	32	89.1, 89.7	2
Mean ± standard deviation= 82.8 ± 7.4			
Alternariol methyl ether	3.6	93.3, 83.8, 80.8	3
	6.3	90.5, 98.4, 96.8	3
	7.2	95.8, 84.4	2
	12.6	95.8	1
	32	95.3, 95.7	2
Mean ±standard deviation = 91.9 ± 6.1			

of samples. According to the chemical composition of C_{18} column extracts, aminopropyl columns were chosen instead of silica columns [25]. The higher polarity of aminopropyl solid phase causes the toxins to be more strongly retained and therefore more polar solvents can be used to enhance the washing performance without any loss in the recovery of AOH and AME.

The use of gradient elution was necessary to obtain a good resolution between mycotoxins and some interfering peaks, although it produces some drift in baseline. In a blank chromatogram corresponding to an apple juice sample, no peaks at the same retention time of AOH and AME were detected (Fig. 3).

The recoveries calculated at different spiking levels of AOH (3.5, 7.35, 14 and 32 μg/l) and AME (3.6, 6.3, 7.2, 12.6 and 32 μg/l) are given in Table

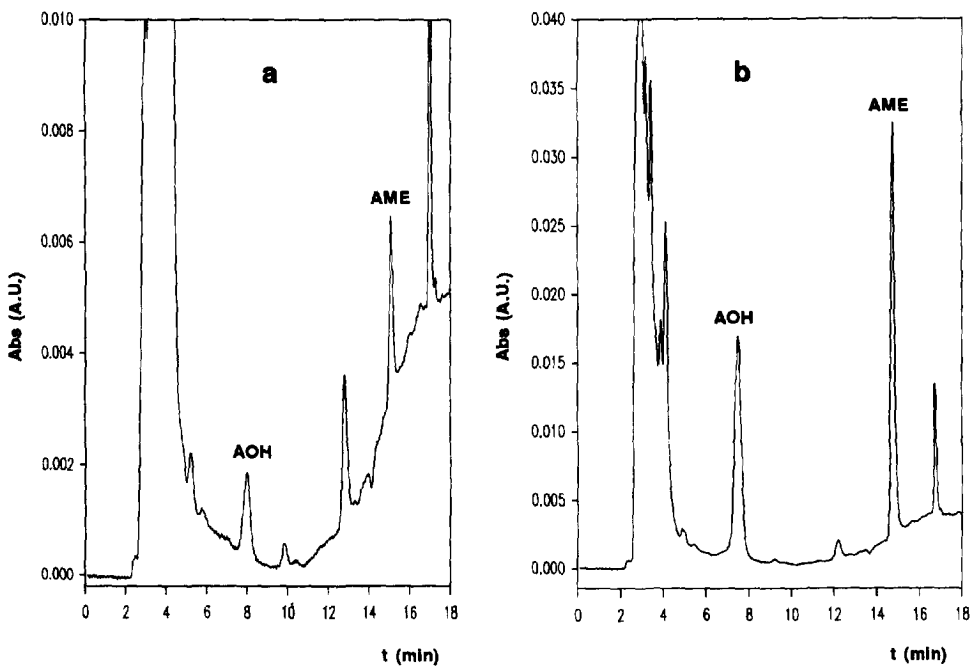

Fig. 4. HPLC of apple juice spiked samples. (a) 3.5 μg AOH/l and 3.6 μg AME/l. (b) 32 μg/l AOH and AME.

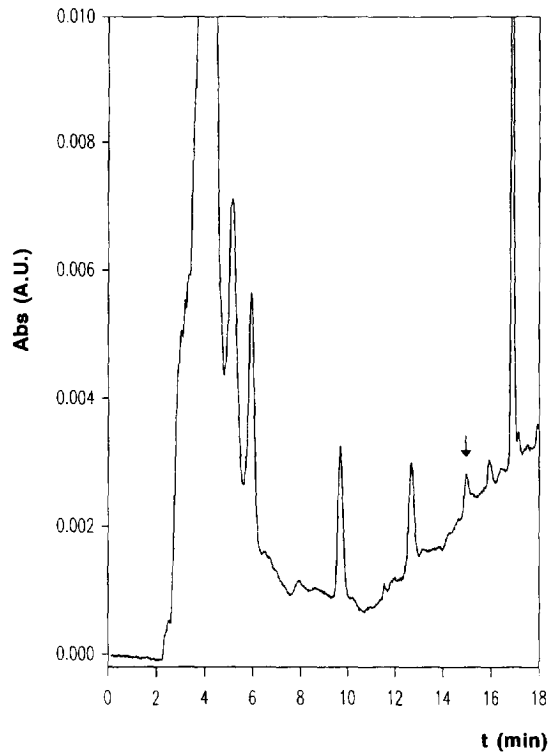

Fig. 5. HPLC of apple juice containing a peak (shown by arrow) corresponding to AME (ca. 0.85 μg/l).

2. Identity of both mycotoxins in the chromatograms of spiked samples was confirmed by comparing their UV spectra with those of standards.

Good results were obtained for AME with recoveries ranging from 80.8 to 98.4% (R.S.D. = 6.6%; $n = 11$) for all the concentrations tested. In the case of AOH, the recoveries were slightly lower, ranging from 70.3 to 92.5% (R.S.D. = 8.9%; $n = 11$), most probably due to the higher polarity of the molecule (Fig. 4). The variation in the recoveries is mainly due to the low spiking levels assayed, as for spiked samples at 32 μg/l a small variation was observed.

In the calibration study of the spiked samples, standard curves for both mycotoxins showed a linear response at the different amounts added. High correlation coefficients for AOH ($r = 0.998$) and AME ($r = 0.999$) were also obtained. It was possible, therefore, to establish detection limits of the toxins in real apple juice samples, as low as 1.6 and 0.7 μg/l for AOH and AME respectively, defined as twice the standard error.

In previous work [25,28], determination of *Alternaria* toxins in fruit juices required more than 2 h in the sample preparation step. Detection limits for AOH and AME in apple juice samples were 10 and 25 μg/l with recoveries of 73 and 82%, respectively [25].

3.3. Commercial apple juices

Among seven samples of commercial apple juice, AME was detected in only one sample at a concentration level of 0.85 μg/l, very close to the detection limit calculated for this toxin (Fig. 5).

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