Determination of alternariol in tomato paste using solid phase extraction and high-performance liquid chromatography with fluorescence detection†

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Alternaria spp. produce a wide variety of toxic metabolites with different chemical structures. Tomato products have been considered a likely source of *Alternaria* toxins in the human diet because *Alternaria* is an important spoilage mold of tomatoes. A new method for the determination of these mycotoxins in tomato paste, involving solid phase cartridges for extraction before HPLC fluorescence detection with a reversed phase column and isocratic elution, was developed. The method was demonstrated to be linear in the range 5.2–196 ppb of alternariol (AOH) in tomato paste. Good recoveries were obtained for AOH at all levels assayed (minimum 77.2%). The detection limit of the AOH toxin in real samples of tomato paste was low, 1.93 ppb. The precision of the method was demonstrated with a good repeatability (RSD = 2.98%) and reproducibility (RSD = 9.35%).

Introduction

Alternaria spp. produce a wide variety of toxic metabolites with different chemical structures.[1] Dibenzo-α-pyrones, including alternariol (AOH), alternariol monomethyl ether (AME) and altenuene (ALT), have been reported as some of the major *Alternaria* mycotoxins[2] (Fig. 1). These secondary metabolites have been isolated from a large number of food and feed materials contaminated with various species of *Alternaria* and also have been implicated in animal and in human health disorders.[3–8] Tomato products have been considered a likely source of *Alternaria* toxins in the human diet because *Alternaria* is an important spoilage mold of tomatoes.[6,9]
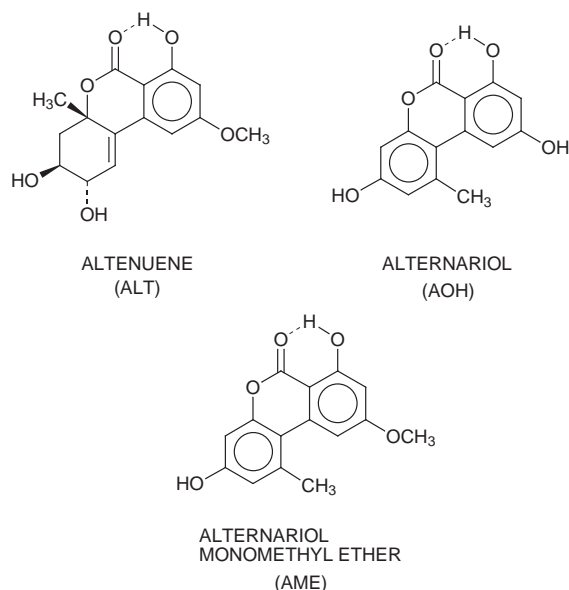

ALTENUENE
(ALT)

ALTERNARIOL
(AOH)

ALTERNARIOL
MONOMETHYL ETHER
(AME)

Fig. 1 Structural formulae of ALT, AOH and AME.

Tomatoes inoculated with strains of *Alternaria* spp. have been shown to produce AOH and AME.[9–11] Natural occurrence of *Alternaria* toxins in tomatoes has also been reported.[10,12–14]

Adequate methods are needed to assess the presence of these mycotoxins in fruits, vegetables and grains, which are frequently infested with *Alternaria*. These methods include extraction and detection techniques. Concerning the extraction step, reported methods involved liquid–liquid procedures with high consumption of organic solvents.[14–17] In 1996, Delgado *et al.*[18] developed a new method for the determination of AOH in apple juice involving solid phase extraction using two types of cartridges and subsequent purification of these extracts with organic solvents. HPLC is the most common technique for detecting *Alternaria* mycotoxins in foodstuff extracts and the methods reported for the separation of ALT, AOH and AME include gradient elution and normal or reversed phase HPLC.[2,11,17–21]

The aim of this work was to develop a method for the determination of these mycotoxins in foodstuffs including solid phase cartridges for the extraction before HPLC with a reversed phase column and isocratic elution and fluorescence detection.

Experimental

Chemicals and materials

Glacial acetic acid was of AnalaR grade from BDH (Poole, Dorset, UK). Methanol of HPLC grade and orthophosphoric acid of analytical-reagent grade were supplied by Merck (Darmstadt, Germany). Ultra-pure water was of milli-Q quality (Millipore, Bedford, MA, USA). Oasis HLB 3 cc (60 mg) extraction cartridges were obtained from Waters (Milford, MA, USA).

Standards

AOH, AME and ALT were obtained from Sigma (Steinheim, Germany). Stock standard solutions (1 mg ml^{-1}) were prepared

by dissolution of the individual toxins in methanol. Working standard solutions were prepared by dilution with methanol.

Sample preparation

Commercial tomato paste was cleaned up in one step using Oasis cartridges. A 10 g amount of sample was spiked with 200 µl of each working solution of AOH (1×10^{-6}, 3.8×10^{-6}, 1×10^{-5} and 3.8×10^{-5} M), stirred and centrifuged in a Kokusan (Tokyo, Japan) H-103N centrifuge at 3500 rpm for 20 min. The upper layer was used for the extraction procedure. The Oasis cartridges were placed in a vacuum manifold (Waters), which was set to 5 in Hg vacuum. The column was conditioned with 2 ml of methanol and equilibrated with 2 ml of water. The supernatant was added to and drawn through each cartridge. The cartridge was washed with 2 ml of water and 2 ml of methanol–water (1 + 3). The toxins were eluted into an injection vial (Supelco, Bellfonte, PA, USA) by gentle syringe pressure at a flow rate of 1 drop s^{-1} with 5 ml of 1% acetic acid in methanol. The first 25 drops were discarded. The eluate was carefully evaporated to dryness under a stream of nitrogen at 45 °C in an evaporation system with a thermostated heating plate (Model 18780, Pierce, Rockford, IL, USA) and the residue was dissolved in 200 µl of mobile phase. Volumes of 25 µl of extract were injected into the HPLC column.

HPLC conditions

A Model 2690 separation module with a universal injector, a Model 996 photodiode-array detector (Waters), a Shimadzu (Kyoto, Japan) RF-535 fluorescence detector conected in series and a Millennium v. 2.01 software data system from Waters were used. The column was a Spherisorb ODS-2 (250 × 4.6 mm id, 5 µm) (Quimica Analitica, Barcelona, Spain).

The mobile phase was methanol–water (32 + 68) acidified to pH 3 with orthophosphoric acid. The eluent was carefully degassed with helium and filtered prior to use and a flow rate of 0.8 ml min^{-1} was applied. Detection of AOH, AME and ALT was carried out with UV (254 nm) and fluorescence (λ_{ex} = 330 nm, λ_{em} = 430 nm) detectors in series.

Statistical analysis

Calibration curves for pure AOH, AME and ALT were constructed. The amounts injected on to the column were ALT 7.1, 19.4, 71.5 and 194 ng, AOH 4.9, 12, 49, 120 and 490 ng and AME 6.2, 18.1, 61.7 and 181 ng of standard.

For validation of the proposed method for the determination of AOH in tomato paste, the experimental design (total of processed samples) was five blanks of tomato paste without AOH, five samples of tomato paste spiked with 5.2 ppb of AOH, five samples of tomato paste spiked with 19.6 ppb of AOH, five samples of tomato paste spiked with 51.6 ppb of AOH and five samples of tomato paste spiked with 196 ppb of AOH.

Using the results obtained with the above samples, we calculated the calibration curve for the AOH in tomato paste, accuracy (bias), recovery, detection limit, repeatability (results obtained with the method in one day by the same analyst using the same reagents) and reproducibility (results obtained during 5 days by two analysts using different reagents).

Results and discussion

HPLC method for the major toxins of *Alternaria*

The developed HPLC method with fluorescence detection was able to resolve the major toxins of *Alternaria* (ALT, AOH and

AME) in less than 19 min, with retention times of 4.7, 8.3 and 18.4, respectively. With the use of an acidified mobile phase and a Spherisorb ODS-2 column, gradient elution was not necessary to obtain good resolution between these three mycotoxins (Fig. 2). Using tomato paste, the peak corresponding to ALT was overlapped by the solvent front (see Fig. 3), so this HPLC method is useful only for AOH and AME. Calibration curves for AOH ($y = 714.16 + 809.41x$), AME ($y = 276.54 + 714.72x$) and ALT ($y = -23737 + 5982.3x$) were constructed (y = peak area in arbitrary units and x = concentration in ppb), and the HPLC method was determined to be linear with high correlation coefficients for ALT ($r = 0.9989$), AOH ($r = 0.9997$) and AME ($r = 0.9978$). The detection limit for pure toxins, defined as the blank response plus three times the standard error of the blank response, was 1 ng using fluorescence detection. This detection limit is lower than[16] or similar to[18] those found in the literature.

Method for determination of AOH in tomato paste

The first report of the use of SPE columns in sample preparation for the determination of AOH was by Delgado *et al.*[18] They reported a method for the determination of AOH and AME in apple juice in which two steps were needed in order to obtain clean extracts for HPLC analysis. We have developed the first method for determination of AOH in tomato paste using only one step for extraction and purification of samples with the use of the new Oasis cartridges. With this procedure, the consumption of organic solvent is minimized and only methanol is used. The first 25 drops of eluate were discarded after evaluating that in this portion only pigments of tomato samples were eluted and the presence of AOH was not detected. We observed that the samples for which this portion was discarded gave the same recoveries of mycotoxin AOH as others with this portion retained but the chromatograms were cleaner, and therefore the HPLC column durability was improved. The recovery obtained was good only for AOH, hence the validation of the method for tomato paste was performed only for this mycotoxin.

The results of the experimental design are given in Tables 1 and 2.

The method was demonstrated to be linear in the range 5.2–196 ppb of AOH in tomato paste. The calibration curve with the mean of the results obtained with the experimental
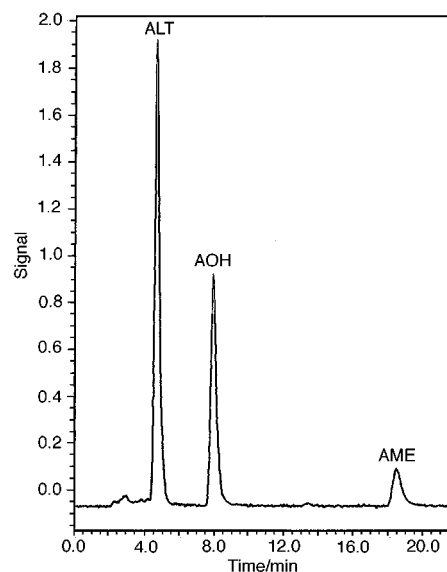

Fig. 2 Liquid chromatogram of standards of ALT, AOH and AME. Fluorescence detection at λ_{ex} = 330 nm and λ_{em} = 430 nm. Retention times were ALT 4.7, AOH 8.3 and AME 18.4 min.

Table 1 Results of experimental design for validation of the proposed method for the determination of AOH in tomato paste (repeatability)

Sample concentration/ng injected on-column (S_i)	Mean[a] result (R_i) ($n = 5$)	Repeatability[b]		Bias ($R_i - S_i$)	Recovery, (R_i/S_i) × 100 (%)
		SD_{R_i}	RSD_{R_i}(%)		
0	1.0062	0.31			
196.2	172.7	2.55	1.48	23.49	88.03
51.6	50.59	1.16	2.29	0.80	98.43
19.6	15.14	0.42	2.81	4.45	77.26
5.2	5.11	0.27	5.33	0.028	99.45

[a] Results obtained with the calibration curve for pure AOH solutions.
[b] Results obtained with the experiments carried out in one day by the same analyst using the same reagents.

Table 2 Results of experimental design for validation of the proposed method for the determination of AOH in tomato paste (reproducibility)

Sample concentration/ng injected on-column(S_i)	Mean result[a] (R_i) ($n = 5$)	Reproducibility[b]	
		SD_{R_i}	RSD_{R_i}(%)
196.2	184.5	13.28	7.21
51.6	49.32	3.00	6.11
19.6	16.20	1.701	10.50
5.2	4.99	0.67	13.61

[a] Results obtained with the calibration curve for pure AOH solutions.
[b] Results obtained with the experiments carried out during five days (one sample of each concentration per day) by two analysts using different reagents.
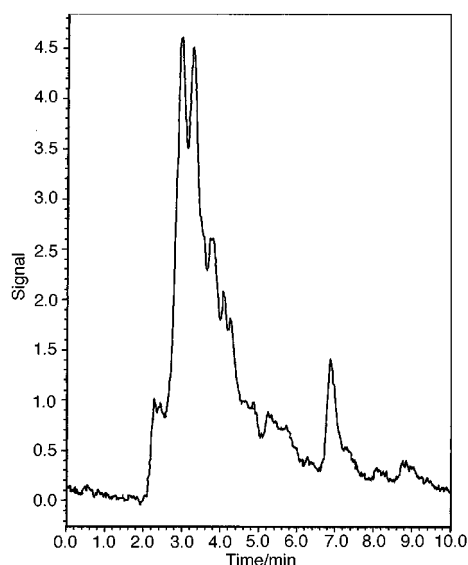

Fig. 3 HPLC of a blank of tomato paste processed with Oasis SPE extraction cartridges.

design was $y = 1544.4 + 711.68x$ with a correlation coefficient of 0.9991.

The recoveries calculated at different spiking levels of AOH in tomato paste (5.2, 19.6, 51.6 and 196 ppb) are given in Table 1. Good recoveries were obtained for AOH at all levels assayed (ranging from 77.2 to 99.4%) and additionally the present process is less contaminating, faster and cheaper than those reported previously.[15,18,19]

The accuracy was acceptable. The detection limit of AOH in real samples of tomato paste was low, 1.93 ppb. Stinson *et al.*[12] and Ozcelik *et al.*[13] found AOH in tomatoes artificially infected with *Alternaria* at a minimum level of 300 ppb. Logrieco *et al.*[10]
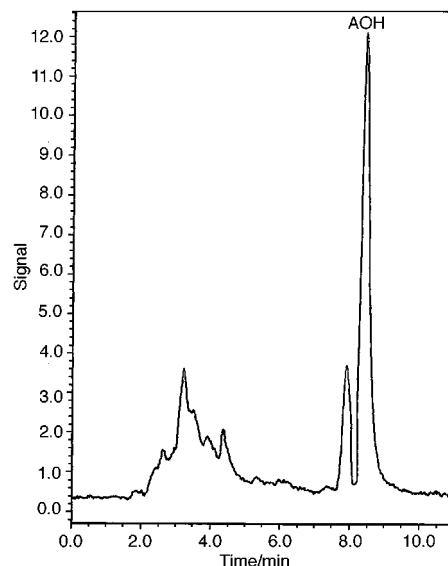

Fig. 4 Chromatogram of tomato paste spiked with 51.6 ppb of AOH and processed with Oasis SPE extraction cartridges. Fluorescence detection at $\lambda_{ex} = 330$ nm and $\lambda_{em} = 430$ nm. Retention time of AOH 8.3 min.

detected AOH in naturally infected tomatoes at a level of 5.2 ppm. With the method developed here, such levels could be easily detected. The selectivity of the method was demonstrated using a diode-array detector and a fluorescence detector connected in series. The spectrum of the peak corresponding to the retention time of AOH confirmed its identity (characteristic maxima of 257.0, 299.6 and 337.6 nm). Fig. 3 shows a chromatogram of a tomato paste blank. No interfering peaks were found at the retention time of AOH.

The precision of the method is demonstrated by the repeatability obtained in one day by the same analyst using the same reagent solutions ($n = 5$) (RSD = 2.98%) and the reproducibility obtained by two analysts during 5 days (one sample of each concentration per day) using different reagent solutions ($n = 5$) (RSD = 9.35%). The results are given in Tables 1 and 2.

Fig. 4 shows a chromatogram of tomato paste spiked with 51.6 ppb of AOH and processed with the method reported here.

In conclusion, the routine screening of tomato paste for AOH could be carried out by SPE extraction followed by HPLC with fluorescence detection in less than 9 min.

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