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Short communication

# Natural occurrence of alternariol and alternariol methyl ether in Spanish apple juice concentrates

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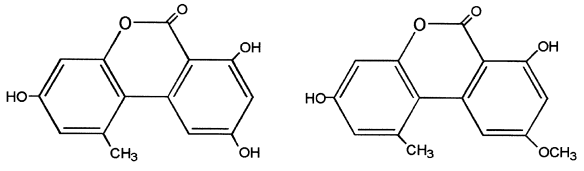
**Abstract**

A limited survey of 32 samples of apple juice concentrates, destined for the production of commercial juices, was carried out in order to evaluate the natural occurrence of the *Alternaria* metabolites alternariol and alternariol methyl ether. A high-performance liquid chromatographic method based on solid-phase extraction columns for extraction and purification of the toxins was used. Both mycotoxins were found as natural contaminants in 50% of the samples analyzed. Levels of alternariol were in the range 1.35–5.42 ng/ml. Alternariol methyl ether was present in most cases only at trace levels, and the highest amount detected was 1.71 ng/ml in one sample. 

*Keywords:* Apple juice; Fruit juice; Food analysis; Alternariol; Toxins

## 1. Introduction

*Alternaria* moulds are ubiquitous in fruits and vegetables at harvest, and they are able to grow during transport and storage under refrigerated conditions. They produce several toxic metabolites belonging to different structural groups, among them, the dibenzopyrone derivatives alternariol (AOH) and alternariol methyl ether (AME) (Fig. 1), the tetramic acid derivative tenuazonic acid (TA) and the perylene derivatives altertoxins (ATX I and II). Some of these compounds have been shown to be carcinogenic in rats [1] and mutagenic in various mammalian cell systems [2].




I II

Fig. 1. Structures of (I) alternariol (AOH) and (II) alternariol methyl ether (AME).

In the last two decades, these toxic metabolites have been found as natural contaminants in spoiled grains [3], sunflower seeds [4,5] and some visibly decayed fruits like mandarins, apples, olives and tomatoes [6–13]. Occurrence of these toxins has also been demonstrated by inoculation experiments [14–16]. Of the above mentioned mycotoxins, AOH and AME are the most important ones produced in apples and apple juices [7,16,17].

High-performance liquid chromatography (HPLC) has become the technique of choice for the analysis of *Alternaria* toxins in foodstuffs. UV detection at 254, 280 and 340 nm was mainly used; fluorescence detection was also reported using excitation at 278 nm [9] or 330 nm [13]. Recently electrochemical detection has been used as a new selective technique

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for the determination of AOH, AME and altertoxins in naturally contaminated foods [4,18]. Gas chromatography–mass spectrometry (GC–MS) is the most reliable technique to confirm the presence of these mycotoxins as their trimethylsilyl [6,13,19] and heptafluorobutyryl derivatives [13], although HPLC with diode array detection (DAD) allows their confirmation, without derivatization, by identification and purity assessment of the chromatographic peaks, with a reasonable degree of confidence [20].

Various sample preparation methods have been reported for the determination of *Alternaria* metabolites in foodstuffs. These methods required a wide variety of solvents for the extraction procedure with or without a further purification by silica columns or thin-layer chromatography. The detection limits reported were in the range 10–50 ppb for AOH and 20–30 ppb for AME [8,10]. With a new method based on solid-phase extraction (SPE) columns for extraction and purification of the toxins it was possible to reduce considerably the detection limits to 1.7 and 0.7 ppb for AOH and AME, respectively [21]. The natural occurrence of AOH was confirmed for the first time in two samples of commercial apple juice using SPE columns [13].

In the present work we try to establish the incidence of AOH and AME in a survey of 32 samples of apple juice concentrates intended for the production of reconstituted commercial apple juice destined for human consumption.

## 2. Experimental

### *2.1. Chemicals*

All solvents used for HPLC analysis were of HPLC grade. For SPE extraction, acetonitrile, ethyl acetate (Labscan, Dublin, Ireland), water (Milli-Q system, Millipore), formic acid and acetic acid (Scharlau, Spain) were of HPLC grade and acetone was of analytical reagent grade (Panreac, Spain).

Analytical standards of AOH and AME were purchased from Sigma (Deisenhofen, Germany). Standard solutions at concentrations of 0.5 and 2 μg/ml were prepared by dilution of the toxins in acetonitrile–water (3:1, v/v).

A 3-ml volume, 500 mg sorbent, polypropylene $C_{18}$ non-end-capped SPE columns and 3-ml aminopropyl SPE columns (Chromabond, Macherey-Nagel) were used for sample preparation. Conditioning of $C_{18}$ columns was carried out with 6 ml of methanol followed by 6 ml of water, and of aminopropyl columns, with 6 ml of ethyl acetate.

### *2.2. Apple juice concentrates*

Apple juice concentrates were supplied by a processing plant from Lerida (Spain). Variable percentages of different apple cultivars, grown within the province, were used in the production of concentrates. Thirty-two samples of two different harvest campaigns, 1993 and 1994, were taken at random for the determination of AOH and AME. Apple juice samples were prepared by diluting 20 g of concentrate with 120 ml of water [22], resulting in a final apple juice with a sugar content of around 10°Brix.

### *2.3. Sample preparation*

Extraction and purification of the samples was carried out according to a previously published method [21]. A 10-ml volume of reconstituted apple juice was passed through a $C_{18}$ column using 2 ml of water and 2 ml of acetonitrile–water (1:3, v/v) as the washing solvents, and 4 ml of 1% acetic acid in acetonitrile as the eluting solvent. The eluate was evaporated to dryness and the residue was dissolved in 1.5 ml of ethyl acetate. A further purification of the sample was carried out through the aminopropyl column. A 2-ml volume of acetone and 2 ml of acetonitrile were used in the washing steps, and the toxins were eluted with 5 ml of 1% formic acid in acetonitrile. The eluate was dried under a $N_2$ stream at 40°C, and the extract was then dissolved in 250 μl of acetonitrile–water (3:1, v/v) for HPLC analysis.

### *2.4. 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 NovaPak $C_{18}$ (300×3.9 mm I.D.). HPLC analysis was performed using a binary gradient system [21] composed of 0.02% aqueous

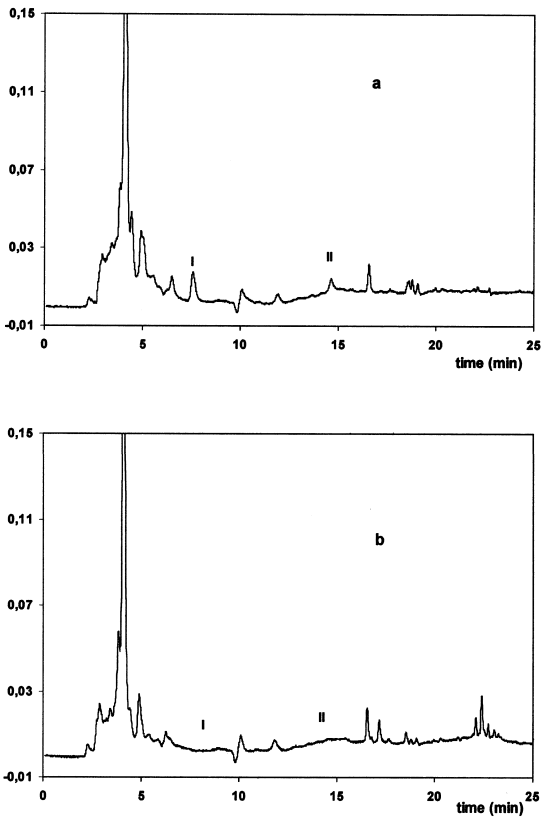


Fig. 2. HPLC of reconstituted apple juice from (a) a contaminated sample at a level of 2.99 ng/ml of AOH and traces of AME, and (b) a noncontaminated sample. Injection volume of 15 μl; flow-rate of 0.8 ml/min; detection wavelength set at 256 nm.

formic acid (pH 3) (solvent A) and methanol (solvent B), at a flow-rate of 0.8 ml/min. Detection wavelength was set at 256 nm, and 15 μl of sample was injected.

### *2.5. Statistical analysis*

The data were subjected to statistical analysis using the STATGRAPHICS program v. 5.0 (Statistical Graphics, Rockville, MD, USA) for quantification.

## 3. Results and discussion

A standard mixture of AOH and AME was injected into the column at a range of 1–10 ng, and a linear response was obtained. The calibration curves were used for quantitation of the toxins. Mean recoveries of 83 and 92% for AOH and AME, respectively [21] were confirmed by injection of a blank sample spiked at a level of 5 ppb. Typical chromatograms for contaminated (2.99 ng/ml of AOH and traces of AME) and noncontaminated apple juice concentrates are shown in Fig. 2.

Results from the analysis of 32 apple juice concentrates, previously diluted to obtain their corresponding reconstituted juices, are summarized in Table 1. The results indicated that a relatively high percentage of the samples (50%) were contaminated with AOH and AME. The amounts of both mycotox-

Table 1
Incidence of alternariol (AOH) and alternariol methyl ether (AME) in commercial apple juice concentrates of two different harvest campaigns

| Harvest campaign | Number of samples | | | | | |
|---|---|---|---|---|---|---|
| | AOH | | | AME | | |
| | Nd[a] | Trace[b] | >0.8 ng/ml | Nd | Trace | >0.8 ng/ml |
| 1993 | 5 | 1 | 12[c] | 5 | 13 | – |
| 1994 | 10 | – | 4[d] | 10 | 3 | 1[e] |

[a] Nd, not detected.
[b] Trace, <0.8 ng/ml.
[c] Concentration range, 1.35–3.56 ng/ml.
[d] Concentration range, 2.99–5.42 ng/ml.
[e] Concentration of 1.71 ng/ml.

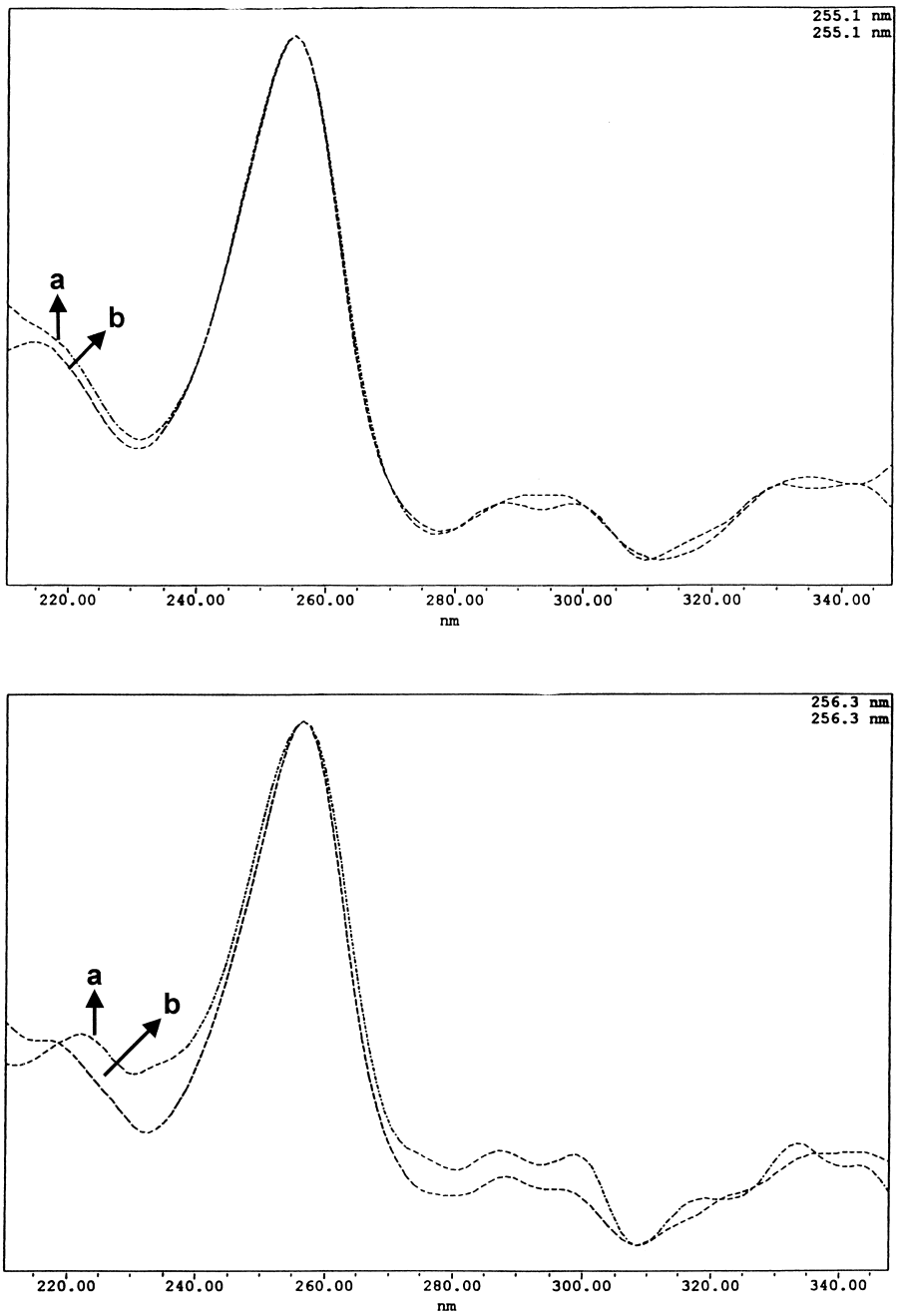


Fig. 3. Peak identity test for AOH and AME in a sample containing 4.92 ng/ml of AOH and 1.71 ng/ml of AME: top, confirmation of AOH (a) sample spectrum, (b) reference spectrum, maximum absorbance at 255.1 nm; bottom, confirmation of AME (a) sample spectrum, (b) reference spectrum, maximum absorbance at 256.3 nm.

ins detected were lower than those reported to date in mouldy apples [7,8]. These results agree with the levels of AOH found for the first time in two out of eight samples of commercial apple juice [13] using SPE columns for extraction of the toxins. When some other methods were used in sample prepara-

tion, no mycotoxins were found in the analysis of several commercial fruit juices [8], and only traces of AOH were detected in Polish raspberry drinks [12].

A different rate of contamination was found between samples processed from apples of two different harvests. Concentrates from apples harvested in 1993 showed a higher percentage of contamination (72%) with concentration levels of AOH ranging from 1.35 to 3.56 ng/ml in 14 out of 18 samples analyzed. Concentrates from apples harvested in 1994 showed higher levels of AOH ranging from 2.99 to 5.42 ng/ml, but in only four out of fourteen samples (28%). We supposed the reason to be the variation in the climatic conditions between the two years. In fact, the smaller volume of rainwater during the 2 months before harvesting and the high percentage of insolation (i.e. the percentage of sun hours in a month) in 1993, caused a severe drought period during the ripening of the fruits, making them more easily attacked by fungi.

Confirmation of AOH in the samples was carried out by comparing the UV spectrum of the peak with a reference spectrum obtained by injection of the standard. A good correspondence was observed, as is shown in Fig. 3.

AME was also found in all the samples positive for AOH, but only at trace levels. Confirmation of the toxin was difficult because of the low amounts detected, and a correspondence with the reference spectrum was only possible in the sample containing 1.71 ng/ml (Fig. 3).

It can be concluded that AOH, and to a lesser extent AME, naturally occur in apple juice concentrates used in the production of reconstituted commercial apple juices. Further studies should be undertaken in order to establish the influence of heat and processing treatments in the degradation of these toxins to evaluate their final presence in apple juices and other products obtained from apples, like jam and infant foods.

The new method based on the use of SPE columns for extraction and purification of the toxins has shown to be quite useful in a rapid and reliable determination of low levels of AOH and AME in apple juices and concentrates.

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