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A METHOD FOR THE DETERMINATION OF TWO *ALTERNARIA* TOXINS, ALTERNARIOL AND ALTERNARIOL MONOMETHYL ETHER, IN TOMATO PRODUCTS

Silvana da Motta; Lucia M. Valente Soares*

Faculdade de Engenharia de Alimentos, Universidade Estadual de Campinas, Campinas, SP, Brasil

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ABSTRACT

Tomatoes, as any soft skinned fruit, are easily susceptible to fungal rot. *Alternaria* is the genus most frequently encountered in tomatoes. *A. alternata* and other spp. have been shown to produce the toxins alternariol monomethyl ether (AME) and alternariol (AOH) in tomatoes. A method for determining AME and AOH in tomato products was developed and evaluated. The method involves extraction with methanol, clarification with ammonium sulfate, and partition to chloroform. Quantification was conducted by high performance liquid chromatography with diode array detector (DAD). Average recoveries were 98.7% and 84.1% for AME and AOH, respectively. The quantification limits of the method, defined as the minimum amount that allowed quantification and confirmation by the DAD detector, were 2.0 ng/g for AME and 5.0 ng/g for AOH.

Key words: *Alternaria*, mycotoxins, alternariol monomethyl ether, alternariol, tomatoes

INTRODUCTION

The genus *Alternaria* contains many plant pathogens (31). It colonizes a wide number of agriculturally important plants. It can invade healthy as well as weakened or dead plant material before harvest and under certain conditions it will damage stored products (12). The genus produces 71 known mycotoxins and phytotoxins (15). Among the mycotoxins, alternariol (AOH) and alternariol monomethyl ether (AME) (Fig. 1) are reported to be toxins produced in large amounts by toxigenic *Alternaria* spp. (5,19,20). Among the known fungal sources for these toxins are *A. alternata*, *A. dauci*, *A. cucumerina*, *A. solani* and *A. tenuissima* (15).

Both toxins cause weakly acute toxic effects as it is shown by their LD_{50} which is higher than 400 mg/kg of b.w. for mice. AME is citotoxic and AOH and AME show sinergistic effects. AOH is lethal to unborn mice at levels of 100 mg/kg b.w. (19). Studies have indicated AME to be carcinogenic and a week mutagen by the AMES test utilizing *Salmonella typhimurium* (14). It acted as a strong mutagen in tests with *Escherichia coli* (2). No mutagenicity was observed for AOH (22).

The toxins AME and AOH have been found in sorghum (3), sunflower seeds (4), barley, wheat, oats (6), olives, tomatoes, mandarin oranges, peppers and melons (13) and pecans (21). *Alternaria* strains inoculated on apples, tomatoes, blueberries, oranges and lemons produced the toxins (24,25,29) demonstrating the potential for contamination.

Alternaria presents a special problem for tomatoes as it is their most frequent invader (8). This opens the possibility of the presence of mycotoxins in tomato products and their evaluation becomes advisable in order to assess if there exists a risk for consumers. No such data exists on Brazilian products. In order to accomplish that goal a search was conducted in the literature for a suitable analytical method for AME and AOH in tomato products.

The use of chloroform (23), acidified chloroform/ethanol (4:1) (17) and methanol/hexane (15:7) (13) has been described for the extraction of AME and AOH from tomatoes. The cleanup of the extract by partition (13,17) or open silica column (17) has been described and the final separation and quantification of the toxins from tomatoes and other foods has employed thin

* Corresponding author. Mailing address: Faculdade de Engenharia de Alimentos, Departamento de Ciências de Alimentos, Caixa Postal 6121, CEP 13081-970, Campinas, SP, Brasil. E-mail: valente@fea.unicamp.br

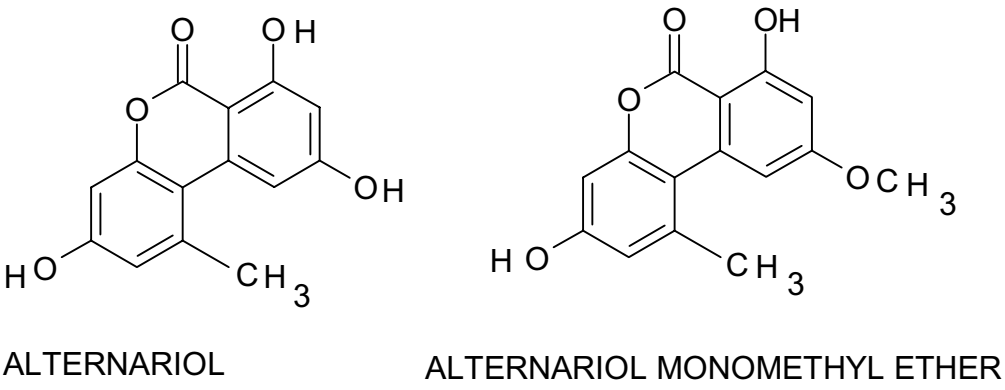

Figure 1. Structures of alternariol and alternariol monomethyl ether.

layer chromatography (6,7,29) or high performance liquid chromatography with detection by absorbance in the ultraviolet, by fluorescence or by electrochemistry (6,18,23,29). Surprisingly, the diode array detector (DAD) has been little used for mycotoxins (26,27, 28) and has not been used for AME and AOH. Yet it can be an useful tool for mycotoxins determinations (16). The DAD detector, besides working as the usual UV detector in the quantification process, can also provide the analyst with the UV spectra of the compound of interest and of the standard employed. If the compound of interest elutes without interferences, the analyst may be able to confirm the identity of the toxin by comparing the spectra of the suspected peak and of the standard. This will free the analyst from having to use other techniques for confirmation of the identity of the analyte. Such techniques may involve chemical reactions, mass spectrometry, infrared or ultraviolet spectrometry or immunoaffinity methods and may be tedious and expensive or, in the case of mass spectrometry, simply not available at the institution where the work is being performed.

The present paper describes a method for the determination of AME and AOH in tomato products. The extraction and cleanup steps are simple to perform and use easily available solvents and reagents. High performance liquid chromatography and diode array detection (DAD) were used for quantification and confirmation of identity. The intralaboratory evaluation of the method performance is also described.

MATERIALS AND METHODS

Reagents

Analytical grade methanol, chloroform, anhydrous sodium sulfate, and hepta hydrate zinc sulfate and HPLC grade methanol were obtained from Merck (Darmstadt, Germany). The alternariol and alternariol monomethyl ether standards were purchased from Sigma (St. Louis, MO, USA). Solutions containing 0.133 mg/ml AME and 0.500 mg/ml AOH were prepared in methanol. Working standards were then prepared by dilution according with the need. Standard solutions were sonicated before use.

Sample preparation

Tomato products such as tomato juice, pulp, paste, purée, and whole stewed tomatoes were blended or shaken for homogeneity. A 50 g portion of the product was weighed and transferred to blender cup with the help of 150 ml methanol. It was blended at low speed for 3 minutes and transferred to a glass funnel fitted with a fluted filter paper. An additional 50 ml methanol was used for washing the residues left in the blender cup into the filter paper. An aliquot of 200 ml of the filtrate was collected into a beaker and 60 ml of a 10% ammonium sulfate solution was added. The mixture was filtered through fluted filter paper. An aliquot of 200 ml of the filtrate, or less, was then transferred to a separating funnel and 50 ml of water at 8°C or below were added. Two extractions with 40 ml chloroform, shaking for 2 minutes each time, were conducted. All the chloroform was collected in a separating funnel and washed with 30 ml ultra pure water at 5 - 8°C. The chloroform was then transferred to a graduated cylinder and the volume noted for future calculations. The chloroform extract was evaporated in a rotary evaporator at 35°C. The residue was dissolved in 2 ml methanol and filtered through anhydrous sodium sulfate.

Liquid chromatography

The HPLC system consisted of a Hewlett-Packard HP 1050 liquid chromatograph (Hewlett-Packard, Palo Alto, CA, USA) equipped with a Rheodyne sample valve fitted with a 20 ml loop and an HP diode array detector (model 1050, Phoenix and Macro Spectro softwares). The analytical column was Spherisorb ODS-2, 5 mm, 250 mm (Phase Separations, Deeside, Chwyd, UK). The sample and standards solutions were sonicated for 30 seconds before injection into the chromatograph. The mobile phase was methanol/water (80:20)

containing 300 mg $ZnSO_4$. H_2O/L, 0.7 ml/min. The wavelength for recording chromatograms was 250 nm. A calibration curve was constructed for quantification purposes using the toxin standards and correlating peak-area versus concentration. The peak identity was confirmed by means of comparing the spectrum of the standard with the presumptive positive peak in the sample after normalization. Quantification limits of the method were taken as the minimum amount of the toxin detected in the product that allowed for confirmation by the multiple wavelength detector. The detection limits of the pure toxins by the DAD detector were measured as three times the baseline standard variation under the same conditions employed for the tomato products.

RESULTS AND DISCUSSION

Several techniques for extraction and cleanup of AME and AOH described in the literature were tried, either isolated or in combination, with unsatisfactory results in terms of recovery, time involved in performing the analysis, or use of large quantities of solvents, some highly toxic. All this led to the development of the method described in the present paper.

The intralaboratorial evaluation of the proposed method involved the following tests: recovery, precision, and limit of detection and ruggedness. The average recoveries for seven levels of addition of pure standards to tomato paste were 98.7% and 84.1% for AME and AOH, respectively. Recoveries of 70% and above are considered acceptable for trace analyses at ung/g levels (11). The average RSDs between duplicates for 14 sample preparations, for spiked tomato product samples (Fig. 2), were 0.8% for AME and 5.4% for AOH, indicating an excellent precision for proposed method. Horwitz *et al.* (10,11) demonstrated that at levels of 1 ng/g interlaboratory precision of well conducted tests with blind samples can reach 45%. Duplicates of the same sample analyzed in the same laboratory can be within 2/3 or 1/2 of this value. These apparently high RSD values are a consequence of the difficulties involved in quantifying substances at trace levels. Horwitz (9) also showed that at the 20 ng/g level most interlaboratory results for the same sample have RSD values of 30% and within-laboratory RSDs of 20%.

The detection limits of the DAD detector for pure standards were taken as three times the baseline standard deviation according to the guidelines of the American Chemical Society Subcommittee on Environmental Analytical Chemistry (1). The wavelength was set at 250 nm. The detection limits were 0.6 ng and 1.0 ng for AME and AOH, respectively. The method quantification limits for the same toxins were taken as the minimum amount of the toxin detected in a tomato product that allowed quantification and confirmation by the DAD detector. They were 2.0 ng/g and 5.0 ng/g for AME and AOH, respectively. The calibration curves were linear in the range of use, 0.57-12.04 ng/ml, with a correlation coefficient of 0.9932, for AME and 0.37-15.0 ng/ml, with a correlation coefficient of 0.9995, for AOH. The comparison between the spectra of the pure standards and of the toxins added to the tomato matrix (Fig. 3) allows the conclusion that the separation of the toxin was complete and that there was no interfering compounds.

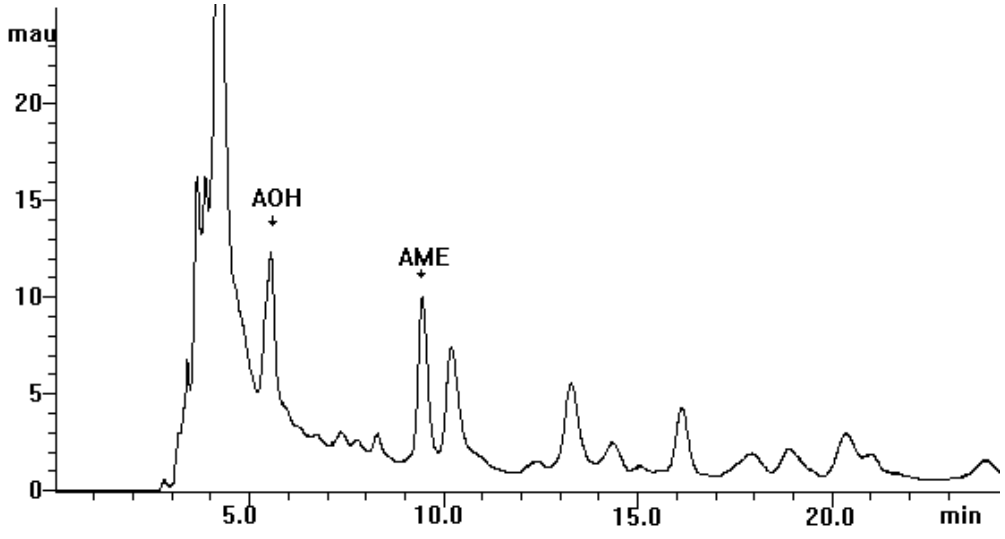

Figure 2. Chromatogram of an uncontaminated tomato paste sample spiked with AME and AOH. Chromatographic conditions: C18 Spherisorb ODS-2 250x4,6 mm, 5 mm column, methanol/water (80+20) with 300 mg $ZnSO_4.7H_2O$/L, 0.7 ml/min as mobile phase. DAD detector at 250 nm, response in terms of absorvance units X 10^{-3} (mau). Volume injected: 20 ml.

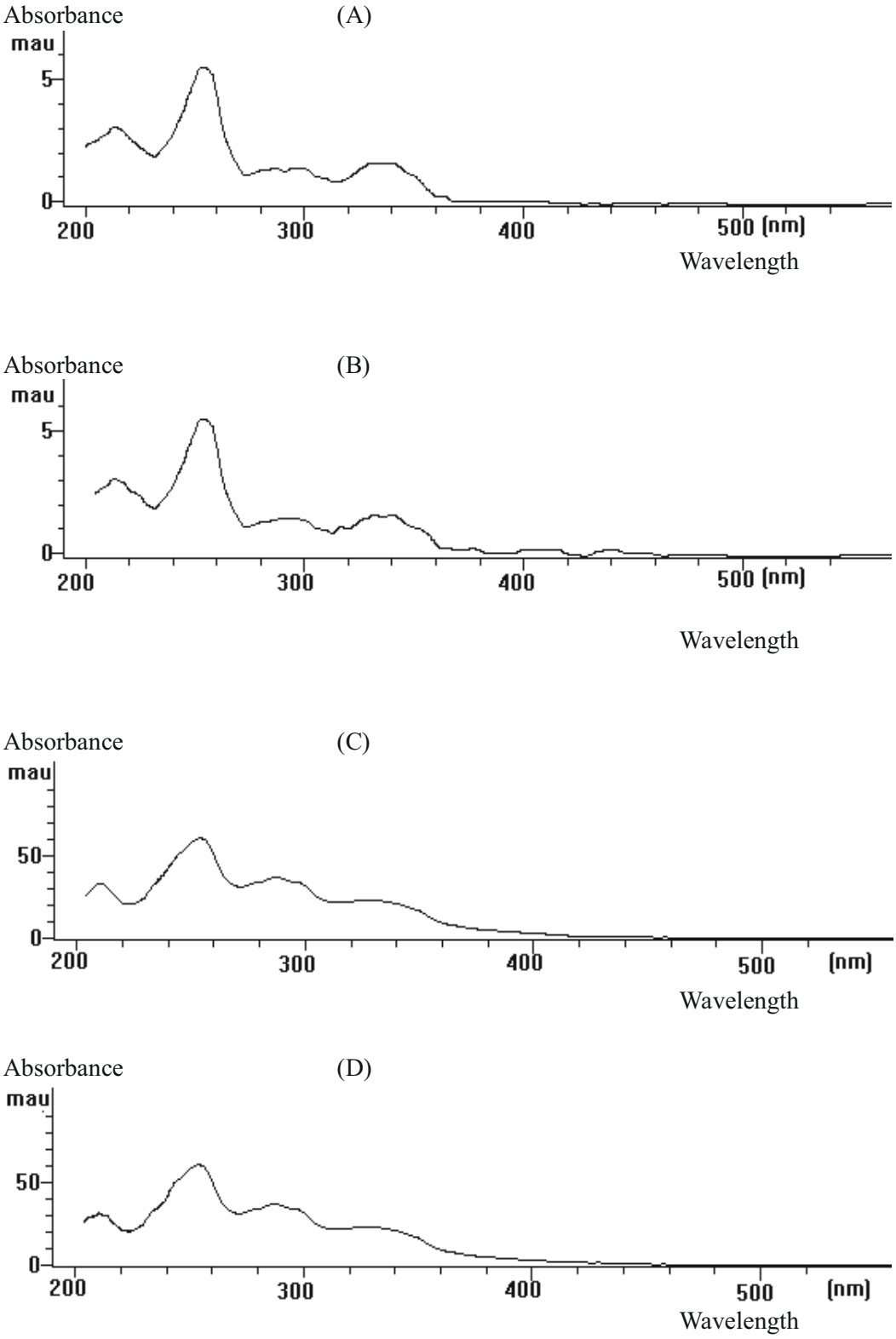

Figure 3. Spectra of AME by a pure standard (A) and in spiked tomato paste (B) and of AOH by a pure standard (C) and in a spiked tomato paste (D) (see chromatogram in Fig. 2).

A ruggedness test was conducted according to Wernimont (30). It evaluates the behavior of the method in the face of small changes in the working conditions. The conditions evaluated were solvents quality and volumes, concentration of the solution of ammonium sulfate used as clarifying agent, and temperature used for final drying of the extract. The recoveries ranged from 85.9 to 88.6% and from 76.3 to 92.8% for AME and for AOH, respectively. They indicate a good degree of ruggedness in the procedure being tested. The isolated factors show the importance of some of the factors studied in the recoveries of the toxins. It showed also that AME was less affected by changes in the analytical procedure. The quality of the chloroform followed by that of the methanol were the most important variables for the recovery of AOH. Different brands of solvents may contain different amounts and types of impurities. These impurities, usually other organic compounds, change the polarity of the solvent and may affect the recovery of any compound sensitive to small changes in polarity of an extracting or eluting solvent.

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RESUMO

Um método para determinação de duas toxinas de *Alternaria*, alternariol monometil éter e alternariol, em produtos de tomate

Tomates são frutas de pele fina e assim facilmente susceptíveis a deterioração por fungos. *Alternaria* é o gênero que mais freqüentemente invade tomates. *A. alternata* e outras espécies deste gênero produzem as toxinas alternariol (AOH) e alternariol monometil (AME). Um método analítico para determinação de AME e AOH em produtos de tomate foi desenvolvido e avaliado. O método consiste em uma extração com metanol, clarificação com sulfato de amônio e partição para clorofórmio. Quantificação foi executada por cromatografia líquida de alta eficiência com detetor de arranjo de diodos (DAD). Recuperações médias foram 98,7% e 84,1% para AME e AOH, respectivamente. Os limites de detecção do método, definidos como a menor quantidade das toxinas que permitiu quantificação e confirmação pelo DAD, foram 2,0 ng/g para AME e 5,0 ng/g para AOH.

Palavras-chave: *Alternaria*, micotoxinas, alternariol monometil éter, alternariol, tomate

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