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Analysis of Alternariol and Alternariol Monomethyl Ether on Flavedo and Albedo Tissues of Tangerines (*Citrus reticulata*) with Symptoms of Alternaria Brown Spot

RODRIGO F. MAGNANI, GEZIMAR D. DE SOUZA, AND EDSON RODRIGUES-FILHO*

Laboratório de Espectrometria de Massas, Departamento de Química, Universidade Federal de São Carlos−UFSCar, Rodovia Washington Luiz, Km 235, postcode 676, São Carlos, São Paulo, Brazil

A method was developed for the quantification of alternariol and alternariol monomethyl ether on tangerines with and without symptoms of Alternaria brown spot disease. The method employs solid-phase extraction for cleanup, followed by high-performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS) for detection. This method was validated on flavedo (exocarp or epicarp, exterior yellow peel) and on albedo tissue (mesocarp, interior white peel). An excellent linearity over a range of 0.50−20.0 mg/kg was achieved, with $r^2 \geq 0.997$. The limits of detection (LOD) and quantification (LOQ) were fewer than 0.13 and 0.50 μg/kg, respectively. The relative standard deviations (RSDs) were ≤14.4% during the validation. The levels of these mycotoxins on flavedo of fruits with symptoms vary from 2.54 ± 0.24 to 17.40 ± 1.05 μg/kg. Surprisingly, neither alternariol nor altenariol monomethyl ether was detected on albedo tissues, suggesting that flavedo works as a barrier for such substances.

KEYWORDS: HPLC; MS; SRM; alternariol; mycotoxins; Alternaria brown spot; citrus; tangerine; albedo; flavedo; validation

INTRODUCTION

The presence of mycotoxins in natural environment as well as in foodstuffs has been reported as a problem for agriculture for several decades (*1*−*4*). In fact, according to the Food and Agriculture Organization (FAO), at least 25% of the world food crops are contaminated with mycotoxins (*5*). In the past few years, special attention has been given to the presence of *Alternaria* mycotoxins in grains, mainly because some of these secondary metabolites such as alternariol (**1**) and alternariol monomethyl ether (**2**) (**Figure 1**) are toxic for both humans and animals (*6*, *7*).

In addition, *Alternaria* mycotoxins are closely related with virulence symptoms on several plants such as wheat (*Triticum*) (*8*), tomatoes (*Lycopersicon*) (*9*), apples (*Malus*) (*10*), mangoes (*Mangifera*) (*11*), and others (*12*, *13*). Other important hosts for *Alternaria* species are plants from the *Citrus* genus (Rutaceae). According to Akimitsu et al. (*14*), there are four different diseases caused by *Alternaria* on these plants: "Alternaria brown spot" on tangerines (*Citrus reticulata*), "Alternaria leaf spot" on rough lemon (*Citrus limon*), "Alternaria black rot" on several *Citrus* species, and "mancha foliar" on Mexican lime (*Citrus aurantiifolia*).

Reported for the first time on emperor mandarins by Cobb in 1903 (*15*), Alternaria brown spot, which is now attributed to *Alternaria alternata* pv. *citri* (*16*), has created several problems for world citriculture. In Brazil, which is currently one of the main producers and exporters of both citrus fruits and juices worldwide (*17*), this disease has been responsible for serious damage on susceptible cultivars of tangerines, especially in São Paulo state (*18*). It promotes lesions on young leaves and branches and on fruits of tangerines, attacking almost the whole plant. On fruits, it boosts lesions that can vary from minute spots to large craters (*14*, *16*, *18*). Eruptions sometimes are formed and can be dislodged, generating irregular surfaces, reducing yields, and diminishing the marketability of fruits (*14*).

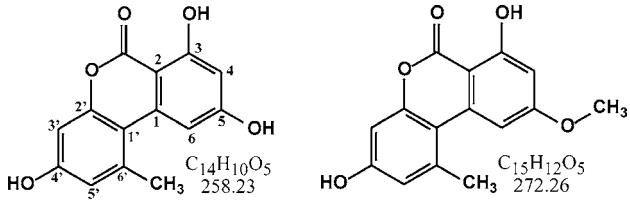

Figure 1. Chemical structures of alternariol (**1**) and alternariol monomethyl ether (**2**) mycotoxins.

Since the disease was discovered, many of its aspects have been reported, including the identification of some host-specific toxins (*19*−*23*). On the other hand, analytical methods which can be used for detection of *Alternaria* mycotoxins are currently in development. In fact, analytical approaches for detecting *Alternaria* compounds in different matrixes are still limited (*24*). Some methods have been developed for assessing such myco-

* Author to whom correspondence should be addressed. Tel.: +55 16 3351 8053. Fax: +55 16 3351 8350. E-mail: edson@dq.ufscar.br.

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toxins in juices from different kinds of fruits (*25*), but there are not many reports concerning the quantity of these mycotoxins on citrus fruits. In addition, there is a lack of information concerning on which part of fruits these mycotoxins are accumulated. Nevertheless, it is very interesting to check if *Alternaria* mycotoxins, produced during the symptoms of Alternaria brown spot, are able to cross through flavedo tissues (epicarp or exocarp) directly into albedo tissues (mesocarp). Flavedo is the outermost layer and is usually the first protection of the fruit, while albedo is the white spongy part of citrus fruits (*26*). Furthermore, analyses of these tissues are very important for their quality control, since they have been widely used by the food and pharmaceutical industries during the manufacture of many useful products (*27*).

Therefore, we have used high-performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS) to develop a rapid and sensitive method for detecting alternariol (**1**) and alternariol monomethyl ether (**2**) on tangerine fruits. The goal of this technique is to achieve both high sensitivity and specificity, especially when using the selected reaction monitoring (SRM) mode. We also report the difference between the amounts of these mycotoxins on flavedo and on albedo of tangerines with and without symptoms of Alternaria brown spot, in an attempt to verify the accumulation of these compounds inside fruits or even if they are restricted to the external surface.

MATERIALS AND METHODS

Chemicals. Both alternariol (**1**) and alternariol monomethyl ether (**2**) were produced by a Brazilian strain of *Alternaria alternata* pv. *citri*, isolated from fruits of tangerines with symptoms of Alternaria brown spots. This strain was grown in autoclaved corn (Yoki, São Paulo, Brazil) containing 50% of water (m/v) at 25 °C for 20 days. Therefore, compounds **1** and **2** were isolated as previously reported by Schroeder and Cole (*28*), and they were completely characterized by 1D and 2D NMR spectroscopy as well as by mass spectrometry. The levels of purity were higher than 98%.

Plant Material. Tangerines (*Citrus reticulata*) with and without symptoms of Alternaria brown spot disease were collected in the city of Aguaí, in the center of São Paulo State, on April 2004. Healthy tangerines were also acquired in a local market in Recife, the major city of Pernambuco state, in which this disease has not yet been reported. Flavedo was manually separated from albedo using a small knife. These materials were then dried at 45 °C for 3 days and were ground to a powder.

Calibration Standard and Analytical Curves. Stock solutions (100 μg/mL) of both **1** and **2** were prepared in HPLC grade methanol (JT Baker, HPLC grade, Ecatepec, Mexico). Working solutions were prepared at 0.1, 0.2, 0.5, 1.0, 2.0, and 4.0 ng/mL by diluting stock solutions. An aliquot (1 mL) of working solutions was added to 9 mL of HPLC grade acetonitrile (JT Baker, Ecatepec, Mexico), and then these solutions were spread over 200 mg of dried and powered flavedo. The same procedure was employed with albedo. Calibration standards curves were obtained in sextuplicates. For each point of the analytical curves, the worst value in comparison with the average was discarded. The dynamic range used was from 0.5 to 20 μg/kg in all analyzed curves. Calibration curves were obtained using weighting factor of $1/x$ and by plotting concentration (μg/kg) in axis x per area below SRM peaks (arbitrary units) in axis y.

Extraction and Cleanup. The procedure employed was adapted from Lau et al. (*25*). Both flavedo and albedo tissues (200 mg) were extracted with 10 mL of acetonitrile (JT Baker, Ecatepec, Mexico) containing 1% acetic acid (JT Baker, Ecatepec, Mexico). This mixture was left in an ultrasonic bath for 5 min and then was filtered through JP40 paper (JProlab, São José dos Pinhais, Brazil). Both filter paper and the flask used during the extraction were washed out three times with 2 mL of the same solvent composition, and the solvent was evaporated under a continuous flow of nitrogen with a TurboVap II (Zymark, Hessen, Germany), and the residue was reconstituted in 1.5 mL of ethyl acetate (JT Baker). The cleanup was performed by applying this solution onto a 3 mL/500 mg aminopropyl SPE cartridge (Varian, Palo Alto, CA) previously conditioned with 6 mL of dichoromethane (JT Baker), and the cartridge was then washed with 2 mL of acetone (JT Baker) and with 2 mL of acetonitrile. For elution of alternariols, 4 mL of acetonitrile with 1% acetic acid was used. This fraction was concentrated under nitrogen flow and was reconstituted in 1 mL of acetonitrile:water (4:1, v/v).

HPLC and MS Parameters. All analyses were carried out using an Alliance 2795 HPLC (Waters, Manchester, United Kingdom) coupled with a Quattro Premier T-Wave mass spectrometer. Fifteen microliter samples were injected into a 250 × 4.6 mm i.d., 5 μm, Inertisil octadecyl silane (ODS) analytical column, (Alltech, Deerfield, IL) under a flow rate of 0.95 mL/min of methanol:water 4:1 (isocratic elution). To deliver the samples into the mass spectrometer, a splitter was used at a rate of 2:1 (waste:MS). Samples were ionized using an electrospray (ES) ion source operating in negative mode. The temperatures of block source and probe were set at 100 and 300 °C, respectively. The flow rates of drying gases as well as the three main ionization parameters (capillary, cone, and extractor voltages) were performed using direct infusion into the ES source. The flows of nebulizer and desolvation gases (nitrogen) were 20 and 500 L/h, respectively. The capillary was then set at 3.0 kV for all experiments. For SRM experiments, two transitions were selected for each compound: for **1**, m/z 257 → 215 (monitoring) and m/z 257 → 213 (quantification), using 25 V at the cone and collision energy of 25 eV; for compound **2**, m/z 271 → 256 (quantification) and m/z 271 → 228 (monitoring), using 35 V at the cone and collision energy of 30 eV. The dwell time was varied from 0.1 to 0.8 s (incrementing 0.1 in each analysis), and the interscan delay was varied from 0.005 to 0.05 s (incrementing 0.005 in each analysis). The values for the best sensitivity were achieved to be 0.3 and 0.01 s, respectively; therefore, these values were used during the quantification steps. The collision gas pressure (argon) was set at $5.70.e^{-3}$ mbar for all experiments.

Limits of Detection (LOD) and Quantification (LOQ). The limit of detection (LOD) was defined as the concentration in which the signal-to-noise relationship (S/N) was equal to 3. The limit of quantification (LOQ) was defined as the lowest concentration in which the peak area could be measured with acceptable accuracy and precision (relative standard deviations (RSDs) up to 15%). Measures were also done using the S/N ratio (equal to 10), and they were expressed by relative standard deviation (RSDs). Both limits were obtained by injection/measurement of S/N ratio (*29*).

Specificity, Linearity, and Precision. Specificity was assessed by using specific transitions for each compound in SRM mode. Linearity was achieved by coefficients of determination (r^2) of analytical curves. The precision was evaluated by the analyses of RSDs during both within-day and between-days runs.

Recovery. The accuracy of the method was determined using recoveries. They were calculated by comparing the spiked curves with curves at the same concentration constructed in methanol.

Software. All MS data were acquired and processed using MassLynx NT 4.0 software (Waters, Cheshire, United Kingdom).

RESULTS AND DISCUSSION

Method Development and Specificity. During the establishment of the analytical conditions, different solvents were tested for proceeding with extraction steps. SPE cartridges containing ODS as stationary phase were tested as well. However, the best results were obtained using extraction with acetonitrile 1% acetic acid and cleanup by aminopropyl SPE cartridge.

Concerning the separation and the detection of alternariols, HPLC-MS/MS optimization steps are crucial for developing a suitable method. Therefore, the mobile phase was determined after testing different compositions of solvents and organic modifiers (in reverse mode). The best results were obtained using methanol:water (4:1) without any organic modifier. This

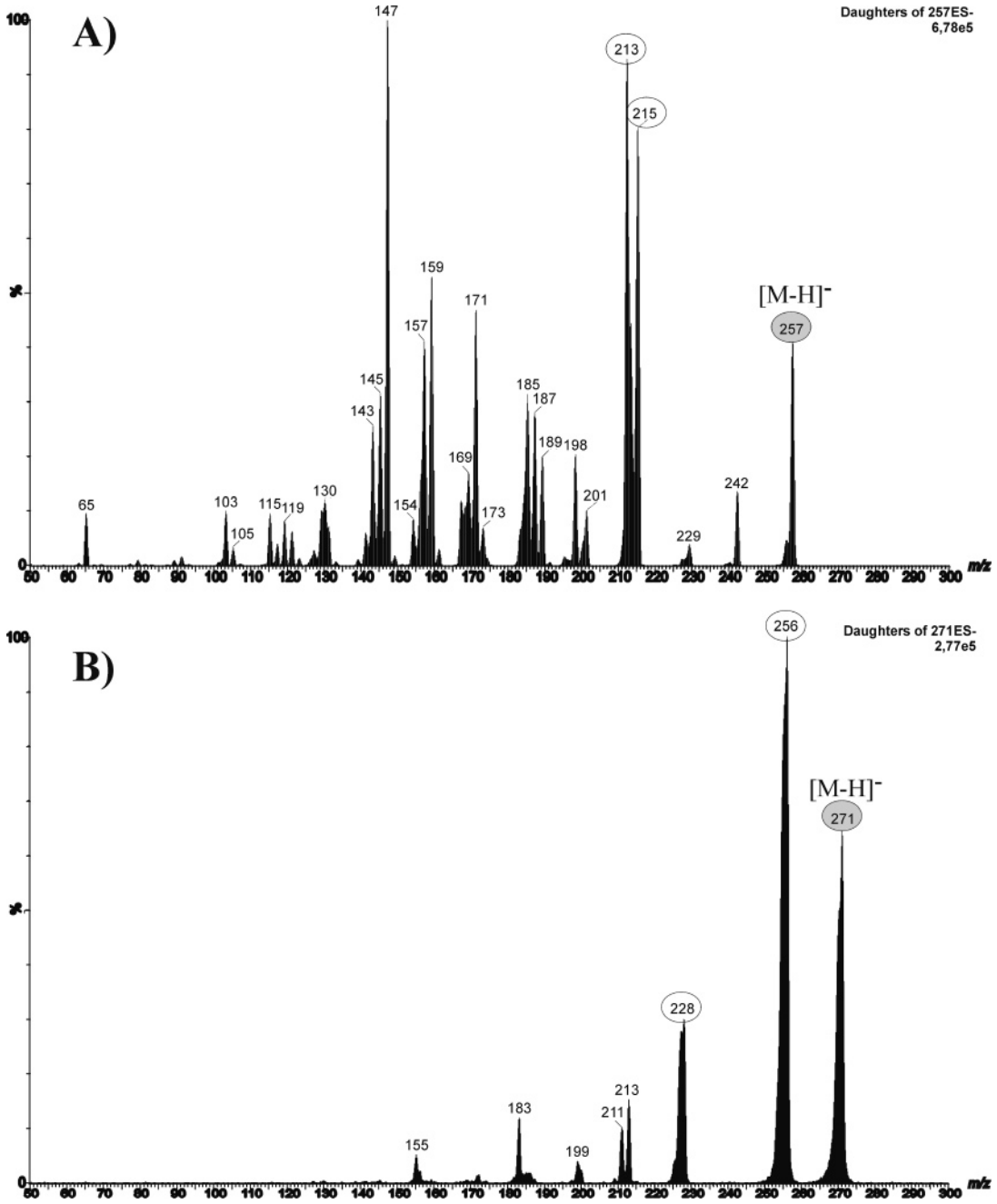

Figure 2. Second-generation ion product spectra obtained under CID–MS/MS: (**A**) alternariol (25 eV); (**B**) alternariol monomethyl ether (35 eV). The marked ions represent the transitions chosen for SRM experiments (●→○).

mobile phase provided both excellent separation between **1** and **2** and good ionization levels under the ES source.

The SRM transitions were chosen on the basis of the stability of product ions obtained under collision-induced dissociation (CID)−MS/MS and also on the basis of interfering compounds from matrixes (flavedo and albedo). The second-generation product ion spectra of compounds **1** and **2** (**Figure 2**) showed deprotonated molecules ([M−H$^-$]) at *m*/*z* 257 and at *m*/*z* 271, respectively. Because of the hydrogen bond between the hydroxyl group at C-3 and the carboxyl group of the lactone, alternariol (**1**) has two likely deprotonation sites, located on hydroxyl groups at C-5 and C-4′, while alternariol monomethyl ether (**2**) has only one on hydroxyl group at C-4′. Thus, the fragmentation pathway for both **1** and **2** was suggested attributing the ionization to the hydroxyl group at C-4′.

The product ions chosen to form the transitions in SRM mode during the development and application of this method provided an excellent specificity to the method, as shown by the SRM chromatograms of blank samples from both flavedo and albedo matrixes (**Figures 3** and **4**). Other transitions were tested as well, but they were not chosen because they were shown to be either nonselective or low-sensitive transitions to be employed in this method. Besides the choice of selective transitions, some parameters such as dwell time and interscan delay were also optimized for an excellent signal-to-noise ratio (S/N). Dwell time is that time spent on each analysis of a determined transition in the SRM mode. Interscan delay is a general parameter for quadrupole mass spectrometry, and it is defined as the time between one scan ending and the next scan starting (*30*). During the optimization of this method, no increment in S/N ratio for both **1** and **2** was observed above the value of 0.3 and 0.01 s for dwell time and interscan delay, respectively.

Sensitivity, Linearity, Precision, and Accuracy. By the optimization of all these parameters, it was possible to obtain

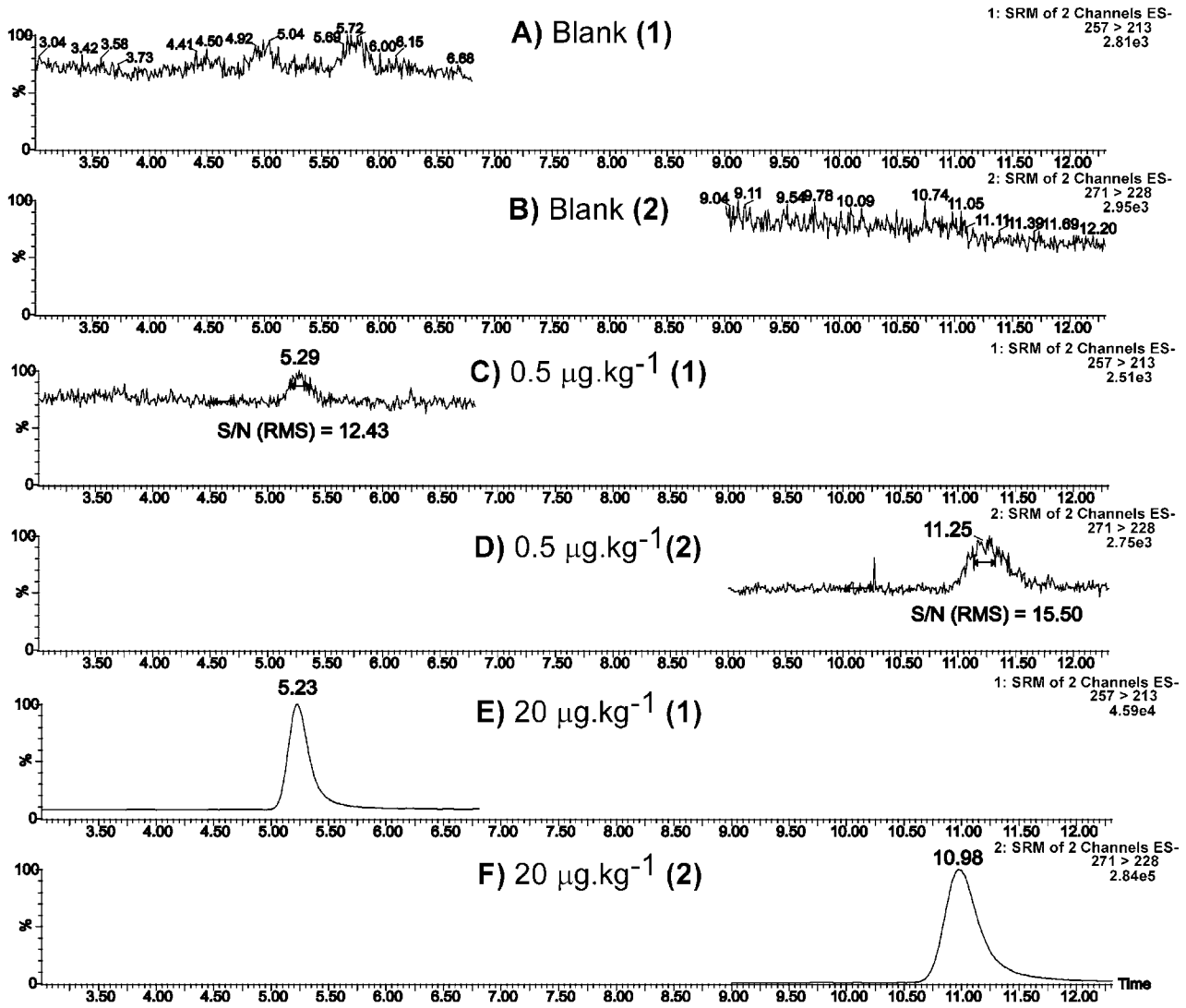

Figure 3. SRM chromatograms obtained during the quantification on flavedo: (**A**, **B**) without mycotoxins (blank); (**C**, **D**) at the concentration near to the limit of quantification; (**E**, **F**) at the highest concentration of analytical curve. The numbers **1** and **2** refer to alternariols.

very low limits. The LOQs were lower than 0.5 μg/kg and the LODs were lower than 0.13 μg/kg for both alternariols (**Figures 3** and **4**). This means that the proposed method has the ability to detect and quantify alternariols in tangerine fruits in ppt levels. Such sensitivity is often necessary to provide a good tool for quality control of these mycotoxins in foodstuffs.

Calibration curves for **1** and **2** were made by spiking these mycotoxins in blank tested samples of both flavedo and albedo. The method showed an excellent linearity over a range of 0.5−20 μg/kg, expressed by the coefficients of determination (r^2), which were greater than 0.997 for all curves.

The precision was evaluated with five determinations per concentration during the intraday assay for repeatability, and with four during the interday assays for intermediate precision. In the latter, quadruplicates of each point were analyzed in three nonconsecutive days, with an interval of one week between each day. The intra and interbatch RSDs for both **1** and **2** were lower than 7% on flavedo and lower than 14.5% on albedo, respectively. The relative errors were all lower than 11% and 14.5% on flavedo and albedo, respectively. Concerning the quantification of mycotoxins in foodstuffs, values of both RSDs and relative errors up to 20% are usually acceptable (*31*). Therefore, these outcomes indicate that the proposed method is very precise and reproducible.

The accuracy was achieved by measuring absolute recoveries, and these were almost the same for both mycotoxins in these two tangerine matrixes. However, the nominal values of recoveries also suggest that the extraction procedure or even the cleanup process was not completely sufficient, since the overall recoveries are around 40%. However, these values are in accordance with those reported by Lau et al. (*25*), who obtained recoveries of 36% for **1** and 20% for **2** in orange juices using a similar approach for extraction and purification of these mycotoxins. These values strongly suggest the occurrence of considerable matrix effects during the extraction, cleanup, or even during the ionization under ES source (ion suppression). Apparently, this occurs with both citrus juices and citrus fruits. However, this behavior is not observed during the extraction of **1** and **2** from other juices or fruits, like apples, in which recoveries around 90% were achieved using similar conditions (*25*, *32*).

To detect alternariol (**1**) and alternariol monomethyl ether (**2**) in samples of tangerines, this method was applied to flavedo and albedo tissues of fruits with and without symptoms of Alternaria brown spot disease. In general, the amounts of **1** were 5 times greater than the amounts of **2** in flavedo obtained from fruits with symptoms (**Table 1**). In addition, both toxins were detected in all flavedo samples obtained from fruits with

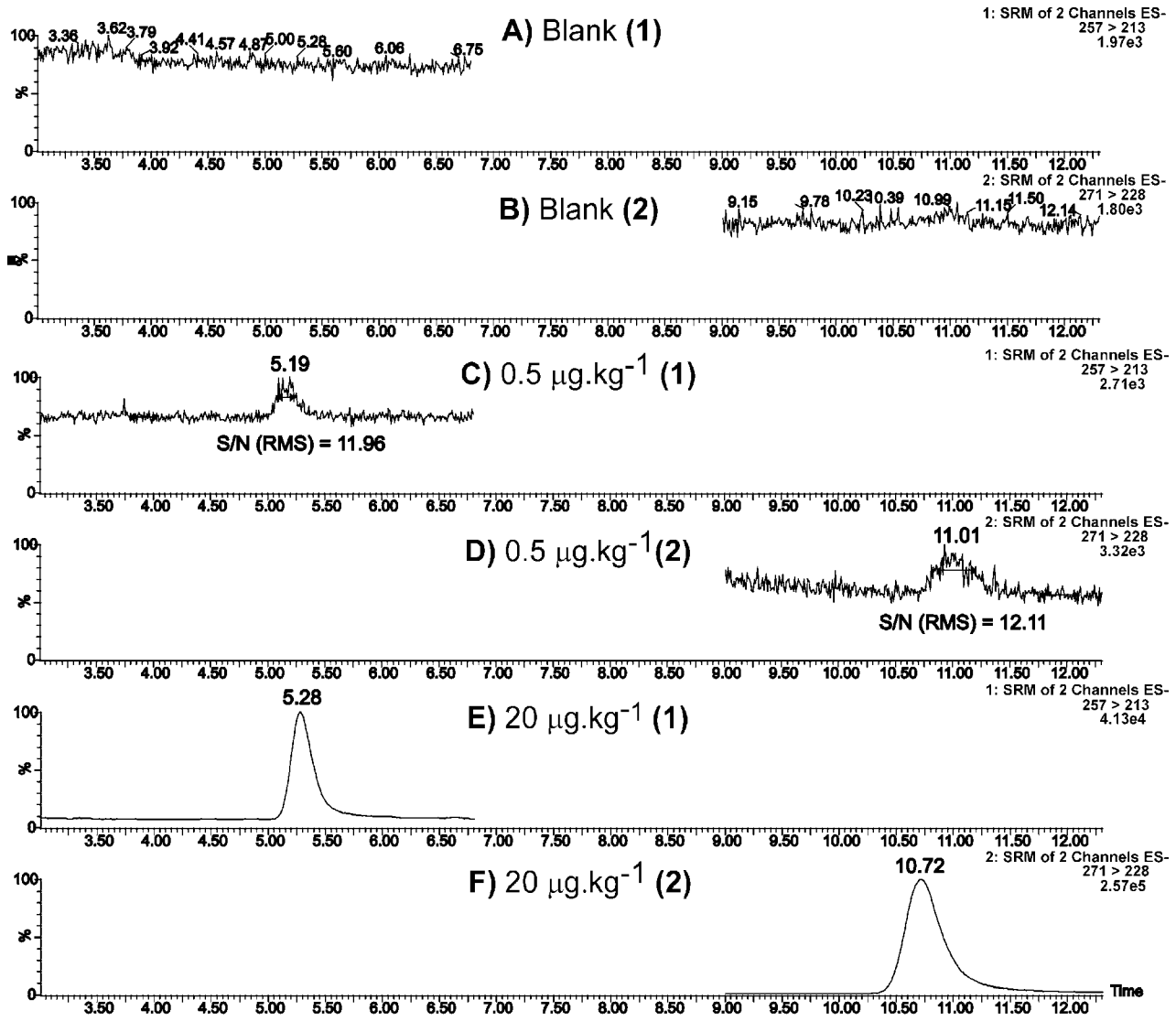

Figure 4. SRM chromatograms obtained during the quantification on albedo: (**A**, **B**) without mycotoxins (blank); (**C**, **D**) at the concentration near to the limit of quantification; (**E**, **F**) at the highest concentration of analytical curve. The numbers **1** and **2** refer to alternariols.

Table 1. Analysis of Tangerines with and without Symptoms of Alternaria Brown Spot Disease

		amounts of mycotoxins (mean ± SD) in (μg/kg)			
		flavedo		albedo	
samples ($n = 3$)	source (place, city/state)	alternariol **(1)**	alternariol monomethyl ether **(2)**	alternariol **(1)**	alternariol monomethyl ether **(2)**
healthy 1	farm, Aguaí/SP	2.5 ± 0.3	≤LOD	n.d.	n.d.
healthy 2	farm, Aguaí/SP	3.7 ± 0.4	0.9 ± 0.1	n.d.	n.d.
healthy 3[a]	local market, Recife/PE	n.d.	n.d.	n.d.	n.d.
healthy 4[a]	local market, Recife/PE	n.d.	n.d.	n.d.	n.d.
with symptoms 1	farm, Aguaí/SP	17.4 ± 1.0	3.5 ± 0.2	n.d.	n.d.
with symptoms 2	farm, Aguaí/SP	13.3 ± 0.9	2.5 ± 0.2	n.d.	n.d.
with symptoms 3	farm, Aguaí/SP	14.1 ± 0.9	2.9 ± 0.2	n.d.	n.d.
with symptoms 4	farm, Aguaí/SP	13.1 ± 0.9	2.5 ± 0.3	n.d.	n.d.

[a] Acquired in Recife city (northeast of Brazil), a region in which this disease has not been reported yet; n.d.: not detected.

symptoms of Alternaria brown spot. However, neither alternariol (**1**) nor alternariol monomethyl ether (**2**) was detected in albedo samples, even on those obtained from fruits with symptoms of Alternaria brown spot (**Table 1**). This indicates that these toxins are not accumulated inside tangerine fruits, suggesting that flavedo tissues might act as a barrier for fungal penetration. This contrasts with the observation of Agrios (*33*), who observed that a small lesion promoted by Alternaria brown spot at the surface of citrus fruits may indicate an extensive spread of the infection inside the fruit. These present results suggest that the infection is not always closely related with the production of such mycotoxins inside of the fruits. Some authors have attributed antimicrobial properties to albedo tissue because of the high concentration of flavonoids and other phenolic acids in it (*34*, *35*). Although some of these compounds can also be found in flavedo tissues, no antimicrobial activity has been

attributed to this tissue as yet (*34*). Therefore, the presence of such compounds in albedo tissue might inhibit the fungal spread, preventing the production of **1** and **2** in the tangerine fruits.

The levels of contamination obtained in this method (**Table 1**) were lower than those previously reported by Logrieco et al. (*36*), who have analyzed mandarins with symptoms of Alternaria black rots, a nonspecific disease of citrus plants. These authors reported the detection of 0.92 mg/kg of **1** and 0.17 mg/kg of **2**, suggesting the higher production of these toxins *in situ* when compared with strains of *Alternaria* responsible for causing Alternaria brown spot, including that used in this present work.

Low amounts of alternariols were also detected in healthy samples but only in those originating from São Paulo state, the main producer in Brazil, and once again only in flavedo (**Table 1**). In samples that originated from Recife, a city in the northeast region of Brazil and in which this disease has not been reported yet, neither of the alternariols were detected. In addition, four different tangerine juices, which are sold throughout Brazil, were also analyzed, and no alternariol was detected. These results confirm that Alternaria brown spot disease is not currently affecting cultivars of tangerines in the northeast region of Brazil.

Currently, the most effective approach to control mycotoxin levels in consuming food products is the use of validated analytical methods to analyze target compounds present in these products. Such analyses must be conducted in all stages of production to ensure the quality control of the whole process. The results presented here suggest a very sensitive and reproducible method for quantifying alternariol and alternariol monomethyl ether in fruits of tangerines. We also have checked the presence of such mycotoxins on different parts of tangerines, that is, on flavedo and on albedo tissues. Our results indicate that alternariols are not accumulated on albedo tissues of tangerine fruits with symptoms of Alternaria brown spot. They were only detected on flavedo samples, indicating the production of these toxins during the infection. Therefore, a good way for consuming tangerines as well as other citrus fruits or even juices safely is removing the flavedo tissue before the consumption, since it apparently acts as a barrier for these toxins.

SAFETY

Both alternariol and alternariol monomethyl ether are toxic by inhalation, if swallowed or even if in contact with skin. Alternariol monomethyl ether may also cause harm to the unborn child (*37*).

ABBREVIATIONS USED

ODS, octadecyl silane; ES, electrospray; SRM, selected reaction monitoring; CID, collision-induced dissociation; S/N, signal-to-noise; LOQ, limit of quantification; LOD, limit of detection; RSD, relative standard deviation; FAO, Food and Agriculture Organization; ppt, parts per trillion.

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