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PCR-based assay for the detection of *Alternaria* species and correlation with HPLC determination of altenuene, alternariol and alternariol monomethyl ether production in tomato products

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ABSTRACT

Alternaria spp. contamination and subsequent production of mycotoxins is a common problem in vegetable crops. Identification of *Alternaria* species by traditional methods requires specific skills and may not detect toxigenic moulds inactivated by food processing. By using molecular methods such as PCR the detection of *Alternaria* spp. becomes possible directly from the food or feed samples. In this study, a PCR method based on the Internal Transcribed Spacer (ITS) genetic marker has been used for detection of *Alternaria* spp. in raw and processed commercial tomato samples. Occurrence of altenuene, alternariol and alternariol methyl ether in the samples was analysed by high-performance liquid chromatography (HPLC) in order to assess the ability of the PCR assay to identify tomato samples containing *Alternaria* mycotoxins. The PCR assay revealed the presence of *Alternaria* spp. DNA in 41 out of 90 commercial samples (45.6%), while HPLC detected at least one of the *Alternaria* mycotoxins within 31 of the PCR positive samples. Detection of *Alternaria* DNA correlated well with the presence of the analysed *Alternaria* mycotoxins, indicating that the PCR protocol developed in this work for detection of *Alternaria* spp. DNA could be used as an indirect marker of the presence of *Alternaria* mycotoxins in raw and processed tomato products.

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1. Introduction

Alternaria is a ubiquitous fungal genus and includes saprophytic, endophytic and pathogenic species. The cosmopolitan nature of small-spored *Alternaria* species makes them important in a broad range of disciplines. As saprophytic, they can spoil food products and animal feedstuffs by deterioration and by production of biological active compounds. As plant pathogens, they can cause serious problems in agriculture by reducing crop yield in the field and causing considerable economic losses to growers and food processing industry (Bottalico & Logrieco, 1998; Logrieco, Bottalico, Mulé, Moretti, & Perrone, 2003; Ostry, 2008; Pitt & Hocking, 1997). *Alternaria* spp. can produce a wide variety of toxic metabolites belonging to three different structural groups: the dibenzopyrone derivatives, alternariol (AOH), alternariol monomethyl ether (AME), and altenuene (ALT); the perylene derivatives altertoxins (ATX-I and II); and the tetramic acid derivative, tenuazonic acid (TeA), being TeA, dibenzopyrone derivates and ATX-I the main *Alternaria*

mycotoxins that can be found as contaminants of food commodities (Pose, Patriarca, Kyanko, Pardo, & Fernández, 2010).

Exposure to *Alternaria* toxins has been linked to a variety of adverse health effects. AME and AOH are mutagenic, carcinogenic, genotoxic and cytotoxic in microbial and mammalian cell systems (An et al., 1989; Lehmann, Wagner, & Metzler, 2006; Liu et al., 1992; Scott & Stolz, 1980). No comprehensive cancer studies of these *Alternaria* mycotoxins in animals have been carried out; however, precancerous changes were observed in oesophageal mucosa of mice fed 50–100 mg kg^{-1} body weight (b.w.) per day of AME for 10 months (Ostry, 2008; Yekeler, Bitmis, Özcelik, Doymaz, & Calta, 2001). The toxicity of ALT was determined by Panigrahi and Dallin (1994) in brine shrimp larvae. The 50% lethal concentration dose of ALT was 375 g mL^{-1} while the doses for TeA and AOH were 75 and 200 mg mL^{-1}, respectively.

Alternaria mycotoxins have been frequently isolated from raw and processed fruits, vegetables, and oilseeds infected by *Alternaria* rot such as tomato products (Bottalico & Logrieco, 1998; Fente, Jaimez, Vázquez, Franco, & Cepeda, 1998; da Motta & Valente Soares, 2001; Ostry, 2008; Patriarca, Azcarate, Terminiello, & Fernández, 2007; Terminiello, Patriarca, Pose, & Fernández, 2006),

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olives (Visconti, Logrieco, & Bottalico, 1986), citrus fruits (Magnani, De Souza, & Rodrigues-Filho, 2007), cereals (Azcarate, Patriarca, Terminiello, & Fernández, 2008; Mansfield, Archibald, Jones, & Kuldau, 2007; Medina et al., 2006), apples and apple juice (Delgado & Gómez-Cordovés, 1998; Jackson & Al-Taher, 2008), carrots (Solfrizzo, de Girolamo, Vitti, Visconti, & van den Bulk, 2004), pepper, melon (Bottalico & Logrieco, 1998), sunflower (Pozzi et al., 2005) and oilseed rape (Visconti, Sibilia, & Sabia, 1992).

Tomato products are widely consumed in Spain, where the consumption of raw and processed tomato were almost 20 kg per capita in 2009 (MARM, 2009). Tomato constitutes the horticultural product with the highest plant processing volume in the country and it is the most extended vegetable crop with approximately 100,000 ha (MARM, 2010). Because of their thin skin, tomatoes are very susceptible to fungal decay, and *Alternaria* is the most common fungus on mouldy tomatoes (Barkai-Golan & Paster, 2008; Terminiello et al., 2006). Direct consumption of mouldy tomatoes by the consumer is not probable, but the presence of mouldy tomatoes being included in processed tomato products is a possibility (Andersen & Frisvad, 2004). Thus, the presence of *Alternaria* spp. and their mycotoxins in tomato products should be evaluated in order to determine a potential risk to consumer health.

The current routine technique for detection and identification of *Alternaria* spp. often requires culture isolation and further morphological and physiological characterization (Simmons, 2007). This process is tedious and time-consuming, requiring days or weeks to obtain a definitive result. Moreover, because of the heat treatments used in food processing, viable microflora counts in the processed foodstuffs are extremely low, and traditional plating methods for the detection of microorganisms cannot be used (Zur, Shimoni, Hallerman, & Kashi, 2002). It should be noted that the mycotoxins produced in raw materials are not destroyed during most food processing operations, resulting in contamination of finished products (Andersen & Frisvad, 2004; Siegel, Feist, Proske, Koch, & Nehls, 2010).

Alternaria mycotoxins have been determined after separation by thin-layer chromatography (TLC), high performance thin-layer chromatography (HPTLC), gas chromatography (GC), and liquid chromatography (LC). However, High-performance liquid chromatography (HPLC) is the most widely used method for the detection of *Alternaria* toxins (Andersen, Smedsgaard, Jorring, Skouboe, & Pedersen, 2006; Magnani et al., 2007; Ostry, 2008; Patriarca et al., 2007; Pose et al., 2010). Even thought these methods are sensitive and specific, they are time-consuming, very laborious, need skilled personnel, and require expensive and sophisticated equipment.

DNA based methods, such as the polymerase chain reaction (PCR), offer alternative tools for detection of viable and non-viable fungal species in food, and could be an indirect marker of the presence of mycotoxins in the foodstuffs (Pasquali et al., 2010).

In this study, a previously developed PCR method based on the ITS genetic marker (Pavón et al., 2011) was applied for detection of *Alternaria* spp. in raw and processed commercial tomato samples. Occurrence of *Alternaria* mycotoxins in the samples was analysed by HPLC in order to assess the ability of the PCR assay to identify tomato samples containing *Alternaria* mycotoxins.

2. Materials and methods

2.1. Sample selection

Twenty samples of raw tomato fruits and 70 samples of tomato products (18 sun-dried tomato samples, 13 canned tomato products, 13 ketchup samples and 26 tomato sauces) were obtained from several local supermarkets and retail shops. Samples were homogenized in a stomacher (IUL Instruments, Barcelona, Spain) and stored at −20 °C in airtight containers until used. Mould counts were determined by plating in Sabouraud-CAF (Liofilchem s.r.l., Roseto degli Abruzzi, Italy).

2.2. DNA extraction and PCR amplification

Total DNA extraction from raw tomato samples and tomato products was performed using the Wizard® DNA Clean-up System kit (Promega Corp., Madison, WI) as previously described (Pavón, González, Pegels, Martín, & García, 2010). DNA concentration was measured with a NanoDrop ND-1000 spectrophotometer (Nano-Drop Technologies Inc., Montchanin, DE).

Specific primer pair Dir1ITSAlt-Inv1ITSAlt was designed in a previous work for amplification of *Alternaria* spp. DNA (Pavón et al., 2011). These primers hybridize on the internal transcribed spacer (ITS1 and ITS2) regions of *Alternaria* spp., and delimit a DNA fragment of approximately 370 bp in all the *Alternaria* spp. analysed.

The primer pair 18Sfweu-18Srveu, designed by Martín et al. (2009), was used as positive amplification control of the assay. These universal primers were expected to amplify a conserved region of 99 bp of the 18S rRNA gene in all raw tomato samples and tomato products analysed.

PCR amplification reactions were done in a total volume of 25 μl. Each reaction mixture contained 100 ng of template DNA, 2 mM MgCl$_2$, 200 μM of each dNTP, 10 pmol of Dir1ITSAlt and Inv1ITSAlt primers, and 1 unit of *Thermus thermophilus* (*Tth*) DNA polymerase (Biotools, Madrid, Spain) in a reaction buffer supplied with the enzyme. PCR amplification was performed in a Progene thermal cycler (Techne, Ltd., Cambridge, UK) under the following conditions: an initial denaturation at 94 °C for 1 min, followed by 50 amplification cycles consisting of 94 °C for 30 s for DNA denaturation, 55 °C for 30 s for primers annealing, and 72 °C for 45 s for DNA extension. A final extension step at 72 °C for 5 min also was included. Positive control amplifications were set using 5 pmol of 18Sfweu-18Srveu primers combined in duplex PCR with Dir1ITSAlt-Inv1ITSAlt primer pair.

PCR products (10 μL) were mixed with 2 μL of gel loading solution (Sigma, St.Louis, MO, USA), and loaded in a 2% D1 Low EEO (Hispanlab S.A.) agarose gel containing 1 μg mL^{-1} ethidium bromide in Tris-acetate buffer. Electrophoretic separation was performed at 100 V for 30 min. The resulting DNA fragments were visualized by UV transillumination and analysed using a Chemidoc XRS System.

PCR products obtained from samples that tested negative for the presence of ALT, AOH or AME were gel-purified and sequenced as previously described (Pavón et al., 2010). The sequences obtained were searched for homology to those available at the GenBank-EMBL database using the BLAST program (NCBI software package).

2.3. HPLC analysis

2.3.1. Extraction and cleanup

Alternaria mycotoxins extraction procedure was adapted from Magnani et al. (2007). Briefly, 1 g of tomato samples was extracted with 9 mL of acetonitrile (Panreac, Barcelona, Spain) containing 1% acetic acid (Panreac). This mixture was left in an ultrasonic bath for 5 min and then was filtered through Whatman 40 paper (GE Healthcare, Buckinghamshire, UK). Both filter paper and the flask used during the extraction were washed out three times with 2 mL of the same solvent composition. The solvent was evaporated under vacuum with a Rotavapor® RE 111 (Büchi Labortechnik AG, Flawil, Switzerland), and the residue was reconstituted in 1 mL of methanol (JT Baker, Deventer, Holland). The reconstituted extract was

left in an ultrasonic bath for 1 min and then was diluted with 4 mL of milli-Q water. The cleanup was performed by applying this solution by gravity onto an Oasis HLB 3 cc (60 mg) extraction cartridge (Waters, Milford, MA, USA) previously conditioned with 2 mL of methanol and equilibrated with 2 mL of water. The cartridge was then washed with 2 mL of water and 2 mL of methanol–water (1 + 4). Finally, the toxins were eluted with 5 mL of 1% acetic acid in methanol. This fraction was concentrated under nitrogen flow and was reconstituted in 200 μL of methanol.

2.3.2. Standard preparation

ALT, AOH and AME were purchased from Sigma, in crystallised form. A stock solution of 1000 μg mL^{-1} was prepared in methanol and kept at −20 °C. A working solution (10 μg mL^{-1}) was prepared in methanol. Standards for HPLC calibration and the standards for addition experiments were prepared by diluting working solutions.

2.3.3. HPLC conditions

All analyses were carried out using a HP-1200 high performance liquid chromatograph equipped with a diode array detector (Agilent Technologies, Waldbronn, Germany). Approximately three UV spectra were collected per second from 200 to 600 nm along with chromatographic traces at 210 and 280 nm, all with a 4 nm resolution. The analytical column was a reversed phase Kinetex PFP (100 × 2.1 mm; 2.6 μm, Phenomenex, Torrance, CA, USA) preceded by a 4 × 2 mm PFP guard column (Phenomenex). The injection volume was 1 μl and the column temperature was 40 °C. The mobile phase consisted of a linear gradient acetonitrile-water with a flow rate of 0.3 mL min^{-1} starting at 100% water, reaching 100% acetonitrile after 15 min. 100% acetonitrile was maintained for 3 min. Thereafter the gradient was returned to 100% water in 5 min and allowed to equilibrate for 3 min before the next analysis. Both eluents contained 50 μl trifluoroacetic acid (Panreac) per litre. All solvents were HPLC grade and all chemicals were analytical grade. Working solutions were used for construction of five-point calibration curves (0.5, 1, 2, 5 and 10 μg mL^{-1}), plotting peak areas against concentration (μg mL^{-1}). The detection limit for each mycotoxin was calculated as three times the standard deviation of the blank divided by the slope of the calibration graph. The limit of quantification was calculated as ten times the standard deviation of the blank samples divided by the slope. Confirmation of the identity of ALT, AOH and AME fractions obtained from tomato samples was performed by electro spray ionisation mass spectrometry (ESI-MS) analysis. All the ESI-MS experiments were performed using an ESQUIRE-LC (Bruker Daltonic, Bremen, Germany) ion-trap spectrometer in negative mode. Scanning data were obtained in continuum mode over the mass range *m/z* 100–500. Metabolite standards of ALT, AOH and AME were co-analysed for verification.

2.3.4. Standard addition experiments

Uncontaminated raw tomato samples, previously analysed with negative results for presence of *Alternaria* mycotoxins, were spiked with toxin working solutions (10 μg mL^{-1}) to reach 0.5, 1 and 2.5 μg g^{-1} of ALT, AOH or AME. Spiked tomato samples were analysed using the same procedure described for the tomato samples (metabolite extraction, cleanup and HPLC analysis). Recovery tests were based on triplicate spiking and triplicate analysis.

3. Results and discussion

3.1. PCR assay development

The *Alternaria* specific primer pair Dir1ITSAlt-Inv1ITSAlt amplified a DNA fragment of approximately 370 bp in all the *Alternaria* spp (Pavón et al., 2011).

Commercial tomato products like tomato sauce, ketchup and canned tomato are heat and pressure treated products, where some degree of DNA degradation is possible, reducing the PCR signal. For this reason, the PCR amplification conditions were modified in this work, including fifty cycles of amplification, in order to guarantee a trustworthy *Alternaria* DNA detection, without compromising the sensitivity of PCR assay. No cross-species amplification was obtained from other fungal, bacterial, animal, or plant species analysed, despite the use of 50 amplification cycles (results not shown).

3.2. HPLC method performance

The developed HPLC method with diode-array detection was able to resolve the major toxins of *Alternaria* (ALT, AOH and AME) in less than 16 min, with retention times of 10.8, 12.8 and 15.6 min, respectively. Calibration curves for ALT, AOH and AME were drawn by linear regression of the least-squares method using peak area of the standard as response vs. concentration. The correlation coefficients were >0.999 for ALT and AME, and 0.98 for AOH. The limit of detection for ALT, AOH and AME were 1.7, 19.7 and 0.37 μg L^{-1}, corresponding to 0.34, 3.94 and 0.074 μg kg^{-1}, respectively. The detection limit was calculated as three times the standard deviation of the blank divided by the slope of the calibration graph. The results of the level and precision of recoveries for the *Alternaria* mycotoxins tested are shown in Table 1. The average recoveries for three levels of addition (*n* = 9) of pure standards to tomato paste were 98.4%, 111% and 97.1% for ALT, AOH and AME. Recoveries obtained in this work were equivalent to recoveries reported in other surveys of *Alternaria* toxins (Azcarate et al., 2008; Fente et al., 1998; Lau et al., 2003; Magnani et al., 2007).

3.3. Analysis of commercial tomato products

The detection of *Alternaria* DNA and the occurrence of ALT, AOH and AME in commercial tomato products are shown in Table 2. The PCR assay revealed the presence of *Alternaria* spp. DNA in 41 out of 90 samples (45.6%), while HPLC detected at least one of the *Alternaria* mycotoxins within 31 out of the 90 samples (34.4%). Twenty tomato fruit samples were analysed, 14 of which were mouldy fruits and 6 unspoiled tomatoes. Mould counts were negative in unspoiled peeled tomato samples, while counts in mouldy tomatoes were in the range of 10^2–10^3 CFU/g. All the unspoiled tomatoes tested negative in the *Alternaria* PCR assay, and no *Alternaria* toxins were detected, whereas all the mouldy tomatoes showed amplification of *Alternaria* DNA, and the presence of toxins was confirmed in 11 of the 14 mouldy tomatoes (78.6%), with maximum concentrations of 11,780; 73,490 and 140 μg kg^{-1}, for ALT, AOH and AME, respectively. These concentrations of ALT and AOH are higher than those reported by Stinson, Osman, Heisler, Siciliano, and Bills (1981) in whole tomatoes, with a maximum level of 1100 μg kg^{-1} for ALT and 5300 μg kg^{-1} for AOH.

Regarding processed foodstuffs, in this study 27 out of 70 tomato products (38.6%) were positive in the analysis of *Alternaria* spp. DNA. Sun-dried tomato samples not only showed higher incidence of *Alternaria* DNA than canned tomato products, ketchup

Table 1
Recoveries of ALT, AOH and AME added to tomato pulp, determined by HPLC.

Concentration (μg g^{-1})	Recovery (%) ± SD			Replicates
	ALT	AOH	AME	
0.5	98.2 ± 4.8	114.4 ± 2.5	101.3 ± 0.9	3
1	102.2 ± 3.8	112.8 ± 1.9	93.6 ± 1.9	3
2.5	94.8 ± 1.4	105.9 ± 4.3	96.5 ± 3.0	3

Table 2
Results obtained in the PCR and HPLC analysis (mean and standard deviation) of commercial tomato products.

Type of product (No samples)	Alternaria spp. PCR system[*]	ALT (μg kg^{-1})	AOH (μg kg^{-1})	AME (μg kg^{-1})
Unspoiled tomatoes (6)[a]	−	nd[**]	nd	nd
Mouldy tomatoes (3)[b]	+	nd	nd	nd
Mouldy tomato TA091	+	11,780 ± 190	73,490 ± 810	0
Mouldy tomato TA113	+	nd	26,960 ± 740	140 ± 2
Mouldy tomato TA114	+	nd	30,400 ± 170	64 ± 3
Mouldy tomato TA115	+	nd	29,270 ± 800	nd
Mouldy tomato TA116	+	5450 ± 100	29,530 ± 1060	57 ± 6
Mouldy tomato TA117	+	nd	24,670 ± 270	45 ± 6
Mouldy tomato TA118	+	nd	28,090 ± 320	nd
Mouldy tomato TA119	+	nd	31,610 ± 1450	nd
Mouldy tomato TA120	+	nd	28,830 ± 510	nd
Mouldy tomato TA121	+	nd	28,040 ± 410	57 ± 2
Mouldy tomato TA122	+	nd	27,510 ± 400	nd
Canned tomatoes (11)[c]	−	nd	nd	nd
Canned tomato TA035	+	870 ± 70	nd	nd
Canned tomato TA062	+	740 ± 10	nd	nd
Ketchup (7)[d]	−	nd	nd	nd
Ketchup (2)[e]	+	nd	nd	nd
Ketchup TA068	+	21,320 ± 490	nd	nd
Ketchup TA074	+	nd	680 ± 40	nd
Ketchup TA075	+	nd	460 ± 10	nd
Ketchup TA077	+	910 ± 30	nd	nd
Sun-dried tomatoes (6)[f]	−	nd	nd	nd
Sun-dried tomato TA030	+	nd	350 ± 20	112 ± 2
Sun-dried tomato TA031	+	nd	60 ± 1	118 ± 10
Sun-dried tomato TA072	+	380 ± 30	170 ± 10	72 ± 4
Sun-dried tomato TA073	+	nd	420 ± 20	54 ± 4
Sun-dried tomato TA096	+	nd	340 ± 30	38 ± 4
Sun-dried tomato TA097	+	nd	350 ± 10	39 ± 2
Sun-dried tomato TA098	+	nd	980 ± 40	nd
Sun-dried tomato TA099	+	280 ± 30	620 ± 40	nd
Sun-dried tomato TA100	+	nd	300 ± 40	nd
Sun-dried tomato TA101	+	nd	620 ± 20	nd
Sun-dried tomato TA102	+	1010 ± 10	430 ± 30	nd
Sun-dried tomato TA103	+	nd	300 ± 40	nd
Tomato sauce (19)[g]	−	nd	nd	nd
Tomato sauce (5)[h]	+	nd	nd	nd
Tomato sauce TA044	+	19,220 ± 300	nd	nd
Tomato sauce TA060	+	22,820 ± 70	nd	nd

[*] (+) Amplification of the 370 bp amplicon from Alternaria spp./(−) No amplification of the PCR product.
[**] Not detected. <0.34 μg kg^{-1} for ALT, <3.94 μg kg^{-1} for AOH, and <0.074 μg kg^{-1} for AME.
[a] Unspoiled tomatoes: TA028, TA029, TA094, TA095, TA111 and TA112.
[b] Mouldy tomatoes: TA090, TA092 and TA093.
[c] Canned tomatoes: TA032, TA033, TA034, TA052, TA053, TA063, TA065, TA066, TA086, TA087 and TA108.
[d] Ketchup: TA036, TA037, TA038, TA039, TA040, TA069 and TA076.
[e] Ketchup: TA078 and TA079.
[f] Sun-dried tomatoes: TA071, TA104, TA105, TA056, TA057 and TA058.
[g] Tomato sauce: TA042, TA043, TA045, TA048, TA049, TA051, TA059, TA061, TA067, TA080, TA081, TA082, TA083, TA084, TA085, TA088, TA089, TA106 and TA107.
[h] Tomato sauce: TA046, TA047, TA050, TA109 and TA110.

or tomato sauces, but also higher incidence of mycotoxins. As shown in Table 2, 12 out of 18 sun-dried tomato samples (66.7%) contained Alternaria DNA, and they were also contaminated with Alternaria toxins, with a maximum concentration of ALT, AOH and AME of 1010; 980 and 118 μg kg^{-1}, respectively. On the contrary, only 2 out of 13 (20.5%) canned tomato products were positive in the analysis of Alternaria DNA and only ALT was detected, with a maximum concentration of 870 μg kg^{-1}. Moreover, 6 out of 13 (46.2%) ketchup samples tested positive for the amplification of Alternaria spp. DNA, four of which were also contaminated with ALT or AOH, with a maximum level of 21,320 and 680 μg kg^{-1}, respectively. Finally, 7 out of 26 (26.9%) tomato sauces were positive by PCR, ALT was the only mycotoxin detected, and it was present in two of the tomato sauces analysed, with a maximum concentration of 22,820 μg kg^{-1}. The high incidence of Alternaria mycotoxins in sun-dried tomato samples could be due to the drying process, particularly during the early stages, when the

environmental conditions are appropriate for Alternaria growth and mycotoxin production. It should be noted that the mould counts in the sun-dried tomato samples that tested positive by PCR were in the range of 10–100 CFU/g, but plating on Sabouraud-CAF did not show the presence of viable moulds in the rest of tomato products analysed.

A common problem of PCR-based methods for food analysis is failure of DNA amplification due to the presence of inhibitory substances in the samples or to DNA shearing. Thus, it is essential to include positive controls in order to avoid false-negative results. The absence of the control product would indicate a technical problem in the process and would avoid false-negative findings (Zur et al., 2002). For this reason, a positive amplification control was performed in each PCR experiment by using 18Sfweu and 18Srveu primers. These primers successfully amplified a conserved 99 bp fragment on the 18S rRNA gene of all the food samples analysed. These results confirm that the food samples appearing
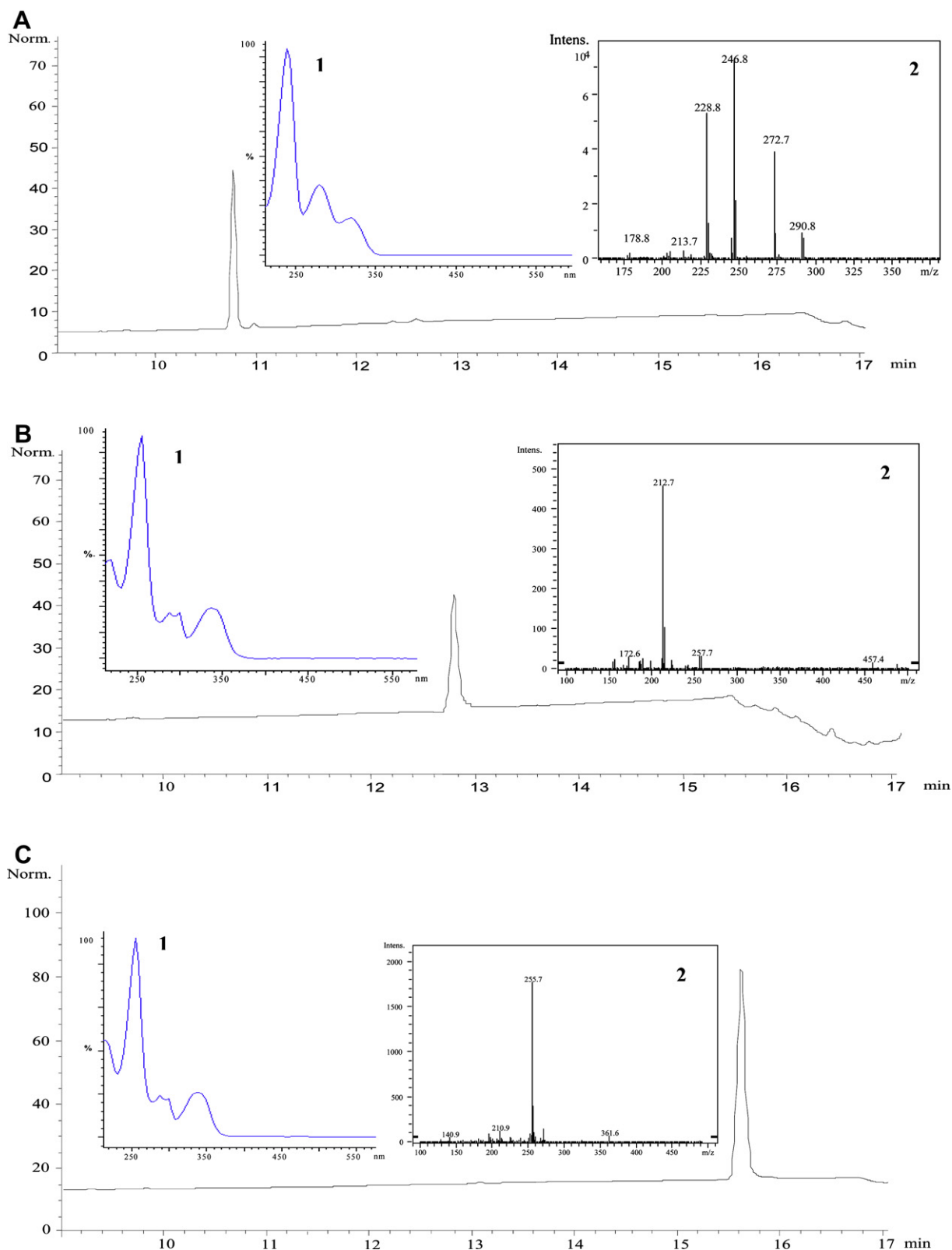
Fig. 1. HPLC-DAD and MS analysis of *Alternaria* mycotoxin standards. UV−VIS chromatogram (210 nm) from ALT (**A**), AOH (**B**), and AME (**C**). The numbers **1** and **2** refer to UV−VIS (200−600 nm) and ESI-MS spectra of the target compound, respectively.

negative in the *Alternaria*-specific PCR assay did not correspond to false-negative results.

Alternaria mycotoxins were not detected in 7 of the 27 processed tomato samples (2 ketchup samples and 5 tomato sauces, 25.9%)

and 3 of the 14 mouldy tomatoes (21.4%) that tested positive by PCR. The absence of ALT, AOH and AME in a positive PCR sample can be explained by several reasons. First, it could be due to contamination by *Alternaria* species that do no produce the mycotoxins
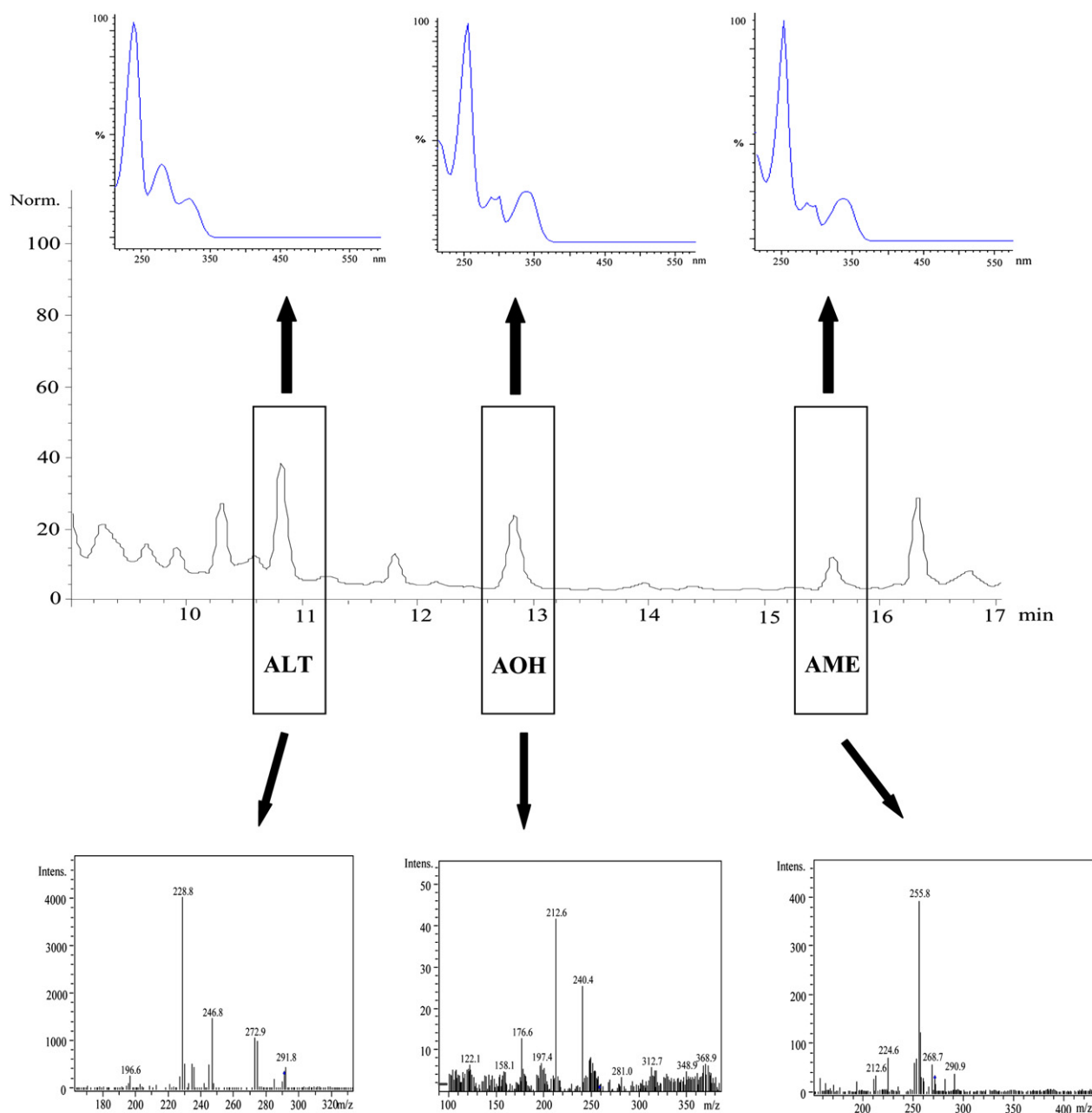
Fig. 2. HPLC-DAD and MS analysis of sun-dried tomato A extract where ALT, AOH and AME were tentatively identified. UV–VIS chromatogram (210 nm) from sample extract. UV–VIS spectra (200–600 nm) of the target compounds are shown above the chromatogram, and the corresponding ESI-MS spectra at the bottom of the figure.

analysed, like those from the *Alternaria infectoria* species-group, which produce metabolites like infectopyrones and novae-zelandins, but not ALT, AOH or AME (Andersen, Sorensen, Nielsen, van den Ende, & de Hoog, 2009; Christensen et al., 2005). More-over, some species from the *Alternaria alternata* species-group, like *Alternaria longipes*, do not always produce these mycotoxins (Andersen, Kroger, & Roberts, 2001). Furthermore, not only does the production depend on the fungal strain, but also on the substrate on which it grows and on the environmental growth conditions.

Sequence analysis of the PCR products obtained from the samples that tested positive for *Alternaria* DNA and negative for the presence of mycotoxins was performed to identify the *Alternaria* species-groups present in those samples. The sequences obtained have been registered in the EMBL database with the following accession numbers: tomato sauce TA046 (FR863589), tomato sauce TA047 (FR863590), tomato sauce TA050 (FR863591), tomato sauce TA109 (FR863597), tomato sauce TA110 (FR863598), mouldy tomato TA090 (FR863592), mouldy tomato TA092 (FR863593), mouldy tomato TA093 (FR863594), ketchup TA078 (FR863595) and ketchup TA079 (FR863596). DNA from *A. infectoria* species-group was detected in three tomato sauce samples (TA050, TA109 and TA110) and in the three mouldy tomatoes analysed (TA090, TA092 and TA093), explaining why ALT, AOH and AME were absent in these samples. Moreover, DNA from *A. alternata* species-group was present in two tomato sauce samples (TA046 and TA047) and two ketchup samples (TA078 and TA079). As stated above, some species from this group may not produce the mycotoxins analysed, but sequencing of the ITS PCR products did not allow identification up to the species level in the *A. alternata* species-group (Pavón et al., 2011).

In order to confirm the identity of the *Alternaria* mycotoxins detected in tomato products by HPLC-diode array, the positive samples were subjected to ESI-MS analysis. ALT, AOH and AME fragment ions obtained from the positive samples analysed resemble those obtained from the standards (Figs. 1 and 2).

Since *Alternaria* species are ubiquitous in nature and occur commonly as post-harvest pathogens of tomatoes, it is not surprising that *Alternaria* mycotoxins are found in tomato products (Pose et al., 2010). The concentration of AOH detected in processed tomato products was lower than that reported by Terminiello et al. (2006) in tomato puree samples (187–8756 µg kg^{-1}). Moreover, higher concentrations of AME have been reported by Stinson et al. (1981) and Terminiello et al. (2006), with maximum concentrations of 800 and 1734 µg kg^{-1}; respectively. In Brazil, da Motta and Valente Soares (2001) reported that no AOH or AME was detected in 80 samples of tomato products.

In this work, a good correlation was found between PCR detection of DNA from *Alternaria* and the presence of ALT, AOH or AME in tomato samples. The results revealed that 31 out of 41 (75.6%) PCR positive samples were contaminated with at least one of the *Alternaria* mycotoxins analysed. A positive correlation between the PCR detection of fungal DNA and mycotoxins has been reported for *Fusarium culmorum* and the presence of nivalenol in cereal samples (Pasquali et al., 2010). Also, Sarlin et al. (2006) reported a correlation between *Fusarium graminearum* DNA concentration and the deoxynivalenol content in North American barley and malt samples.

Although *Alternaria* spp. are the most frequent fungal species invading tomatoes (Barkai-Golan & Paster, 2008; Logrieco, Moretti, & Solfrizzo, 2009), so far there are no specific regulations for any of the *Alternaria* toxins in foods. However, considering their potential effects on human health and the frequency of their presence in tomato products, systematic testing for *Alternaria* mycotoxins in these commodities is desirable to evaluate the consumer health risk (Pose et al., 2010; Terminiello et al., 2006).

In conclusion, a positive correlation has been demonstrated between the PCR detection of *Alternaria* DNA and the presence of ALT, AOH or AME in the raw and processed tomato products. Accordingly, this PCR-based assay could be used as a quality and biosecurity marker of raw materials or processed food where *Alternaria* spp. and their toxins can be present.

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