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A new method for detection of five alternaria toxins in food matrices based on LC-APCI-MS

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ABSTRACT

A new method for the detection of alternariol (AOH), alternariol monomethyl ether (AME), altenuene (ALT), tentoxin (TEN), and tenuazonic acid (TeA), five alternaria toxins (ATs) was developed by liquid chromatography–triple quadrupole mass spectrometry equipped with atmospheric pressure chemical ionisation (APCI). A single extraction was used to recover the five ATs by apple juices, beers, tomato sauces, olives and dried basil. Different Solid Phase Extractions (SPE) and clean-up were selected to optimize the purification step for each food matrix. Limits of detection and quantification were, respectively, in the range 0.16-12.31 ng g^{-1} and 0.54-41.04 ng g^{-1}.Recovery rates were generally above 70%, except for dried basil and olives. Thirty out of 70 samples analysed (7 apple juices, 14 beers and 9 tomato sauces) resulted positive to at least one alternaria toxin investigated. AOH was the most common AT (14 samples), followed by ALT (10 samples). The highest concentration of ATs was found in commercial apple juices (35.33 ng g^{-1}).

Keywords

Alternaria toxins, apple juice, atmospheric pressure chemical ionization, basil, beer, LC-MS, olives, tomato sauce.

1. INTRODUCTION

Alternaria species are ubiquitous pathogens and saprophytes, indigenous into the soil. Many species are plant pathogens that damage crops in the field and cause postharvest decays. *Alternaria* spp. grows well at low temperatures and it is generally associated with extensive spoilage of fruit and vegetables during storage. Many vegetables become particularly susceptible to *Alternaria* rot as a result of chilling injury (Ostry, 2008). The occurrence of *Alternaria* spp. on several fruit and vegetables makes this pathogen as dangerous as other more extensively studied moulds, such as *Aspergillus* spp., *Penicillium* spp., and *Fusarium* spp. (Stinson, Bills, Osman, Siciliano, Ceponis, & Heisler, 1980). *Alternaria* is able to produce several toxic secondary metabolites, called alternaria mycotoxins (ATs), which include alternariol (AOH), alternariol monomethyl ether (AME), altenuene (ALT), tentoxin (TEN), and tenuazonic acid (TeA) (Scott & Kanhere, 2001). Recent studies reported the presence of these toxins in sorghum, sunflower seeds (Combina, Dalcero, Varsavsky, & Chulze, 1999), cereals (Webley, Jackson, Mullins, Hocking, & Pitt, 1997; Patriarca, Azcarate, Terminiello, & Pinto, 2007), tomatoes (Da Motta & Soares 2000; Asam, Liu, Konitzer, & Rychlik 2011), tobacco (Lucas, Pero, Snow, & Harvan, 1971), wine (Asam, Konitzer, Schieberle, & Rychlik, 2009), carrots (Solfrizzo, De Girolamo, Vitti, & Visconti, 2004), apple juices and beverages (Scott et al., 2001), olives (Visconti, Logrieco, & Bottalico, 1986), and beers (Siegel, Feist, Proske, Koch, & Nehls, 2010). From a toxicological point of view, there is strong evidence that AOH and AME could be mutagenic (An et al., 1989, Brugger et al., 2006). Although the acute toxicity of AOH and AME in mice is low (LD_{50}: 400 mg/kg bw), both compounds show remarkable cytotoxicity in cell culture (Pero, Posner, Blois, Harvan, & Spalding, 1973). Furthermore, it has been suggested that AOH and AME produced by *Alternaria alternata* on grain might be a factor responsible for the increased incidence of human oesophageal cancer in China (Pero et al., 1973; Liu, Qian, Zhang, Dong, Qi, & Guo,

1992). Because ATs can be found in a large number of commercial products, such as juices, tomato derivate, cereals, beers, and carrots, it is necessary to monitor their occurrence in the food production chain with specific analytical methods. Maximum levels admitted for these toxins should be released by the European Food Safety Authority (EFSA) and regulated by the European Union in the next future. Some ATs (ALT, AOH, AME, TEN) are usually extracted from solid and liquid food, through organic solvents or solvent mixtures, such as dichloromethane, methanol, acetonitrile, or ethyl acetate, while in the case of TeA an acidic extraction solvent is suggested (Scott et al., 2001). Purification and concentration procedures usually include solvent partitioning, solid phase extraction (SPE) columns, or solid phase microextraction (SPME) (Scott et al., 2001). For the analytical determination of the main ATs, several methods have been reported in literature; the most important methodologies have been reviewed by Ostry et al., (2008) and Scott et al., (2001). Thin-layer chromatography (TLC), high performance thin-layer chromatography (HPTLC), gas chromatography (GC), liquid chromatography (LC), and ultraviolet (UV) detection were used to detect these mycotoxins. Recently, atmospheric pressure chemical ionisation (APCI) and electrospray ionisation (ESI) LC-mass spectrometry (MS) and LC-MS/MS have been applied for the detection of AOH and AME in apple juices and other fruit beverages (Lau, Scott, Lewis, Kanhere, Cleroux, & Roscoe, 2003).

The present paper describes a new method for the simultaneous detection of five ATs, i.e. ALT, AME, AOH, TEN, and TeA. In addition, different commercial food products and beverages were analysed by LC-MS/MS with APCI ionisation to determine the matrix effect. Seventy samples were analysed to represent the occurrence of ATs on different food products commercialised in Italy.

2. MATERIALS AND METHODS

2.1 Chemicals and reagents

LC-MS grade methanol used as mobile phase, pectinase (from *Aspergillus niger*) and phosphate buffer saline (PBS) solution were purchased from Sigma-Aldrich (St Louis, MO, USA). Methanol, acetonitrile, ethyl acetate (Merck, Darmstadt, Germany) and acetic acid (Sigma-Aldrich) were used as solvents to activate, to condition and to elute solid phase extraction (SPE) columns. Eluents were degassed for 5 minutes and filtered through mixed cellulose ester 0.22 μm filters (Advantec MFS, Inc., Pleasanton, CA, USA) before use. Silica SPE columns with different polarities and polymer-based SPE columns (3 mL with 500 mg stationary phase) were: Discovery® DSC-Si from Supelco (Bellefonte, PE, USA), LiChrolut® Si, LiChrolut® EN from Merck, Strata Florisil, Strata C18-U and Strata X from Phenomenex (Torrance, CA, USA), Bond Elut-Mycotoxin from Agilent Technologies (Santa Clara, CA, USA), C18 Set-Pak and Oasis HLB from Waters (Milford, MA, USA). Standards of ALT, AME, AOH, TeA, and TEN were purchased from Sigma-Aldrich and each one was dissolved separately in methanol to prepare 100 μg mL^{-1} stock solutions stored in refrigerator. Aflatoxin M_1 (AFM_1) was used as internal standard, because it is an hydroxylated metabolite of AFB_1 found in animal derived products and it is not present in plant samples. AFM_1 was purchased from Supelco and diluted in acetonitrile to make a stock solution at the concentration of 0.5 μg ml^{-1}.

A AT stock solution mixture, from these individual stock solutions, was prepared in LC mobile phase and matrix, to obtain calibration curves and to determine ion suppression, recovery, limit of detection (LOD) and limit of quantification (LOQ) for each mycotoxin.

2.2 Samples and sample preparation

Five different matrices (apple juice, tomato sauce, three typologies of beer, green and black table olives and dried basil) were used to optimize and validate the analytical detection and extraction method. The optimization and validation of the method were performed by using the five food matrices, purchased in Italian supermarkets, previously tested for being ATs free. The absence of ATs was confirmed as follows: one aliquot of sample was analysed as such, while other aliquots were spiked with a known concentration of mycotoxin standards. Food samples were prepared, extracted, analysed and compared with calibration curves obtained for each analyte. The maximum matrix effect on instrumental signal, recovery, LOD and LOQ for each analyte were determined. For the validation on apple fruit juices, four commercial apple fruit juices were chosen with fruit content higher than 50%. All samples cloudy were left at 40°C overnight with 1 mL pectinase enzyme solution added to 10 mL sample and then were centrifuged at 4,000 rpm (3,180 g) for 5 minutes to remove solid residues. Five mL clear supernatant were diluted with 15 mL PBS and filtered through a Whatman PVDF 0.45 µm syringe filter (Whatman GmbH, Dassel, Germany). For the validation on tomato, four tomato sauces were blended (VOS power basic, VWR) for homogeneity and 30 mL of sample were centrifuged (tabletop centrifuge, CT6E, VWR) at 6,000 rpm (4,770 g) for 5 minutes. Five mL supernatant were diluted in 15 mL PBS solution and then filtered through a Whatman PVDF 0.45 µm syringe filter. Due to the variable composition of beers, three typologies of beer, such as lager, bitter and brown ale, were used to determine matrix-effect and analytical parameters. Before purification procedure, all beer samples were ultrasonicated (ultrasonic clear, USC600C, VWR) for 5 minutes to eliminate most inner gas. Sample preparation was similar to apple juice preparation. The method of Bircan (2006), used to detect aflatoxins in olives, and the method of Solfrizzo et al., (2004), were investigated to determine the presence of ATs in black and green olives and dried basil. To evaluate the matrix effect and losses in the clean up procedure, an internal standard

155 method was used for the most complex matrix, i.e. olives, by adding 100 ng g^{-1} AFM1, after
156 homogenisation,. For both methods, 250 g olives were pitted and chopped, by using a food
157 processor until homogeneity (Polymix System PX-MFC 90D, VWR), while the dried basil
158 samples were used as such. The method of Bircan (2006) was slightly modified as follow: 25
159 g sample were added to 100 mL methanol for olives, and to 200 mL methanol for dried basil.
160 The mixture, left on an orbital shaker (Advanced 3500, VWR; 130 rpm) for 3 min until
161 homogeneity, was then filtered through a filter paper (Whatman # 4) and centrifuged for 5
162 min at 4,000 rpm (3,180 g). Five mL supernatant were then withdrawn and diluted with 15
163 mL PBS solution. The same method of Solfrizzo et al., (2004), applied to extract AOH and
164 AME toxin from carrots, was also used for olives and basil: 25 g samples were added to 1 g
165 NaCl and 100 or 200 mL extraction solvent solution (acetonitrile:methanol:distilled water -
166 45:10:45, v/v/v), respectively for olives or dried basil. The mixture was shaken at 130 rpm for
167 30 minutes with an orbital shaker, and then centrifuged at 4000 rpm (3,180 g) for 5 min. Five
168 mL supernatant were withdrawn and diluted with 15 mL PBS solution. Ten apple juices, 8
169 tomato sauces and 2 tomato juices, 30 beers (10 for each type), 7 black and 3 green olives and
170 10 basil dried were analysed to confirm the validity and reliability of the optimized methods.
171 All samples were stored in the dark at 4°C before analysis.
172
173 **2.3 Solid phase extraction**
174 Solid phase extraction (SPE) was carried out with a 24-position SPE vacuum manifold from
175 Supelco. The SPE procedure used was similar for each kind of cartridge. The cartridges were
176 activated with 5 mL methanol, by following the product instruction, and conditioned with 5
177 mL PBS solution. Five mL diluted samples were loaded into the cartridges and, before
178 elution, sorbents were washed with 5 mL ultrapure water and air dried. To evaluate the
179 recovery, different solvents were used: methanol, methanol with 1% acetic acid, and ethyl

acetate. Samples were evaporated under gentle air flow at 65°C, reconstituted in 500 µL LC mobile phase (water:methanol, 50:50), and transferred to polypropylene filter vials Mini-UniPrep^{TM} (Whatman).

2.4 LC/APCI-MS/MS analysis

A 1260 Agilent Technologies system consisting of binary LC pump and a vacuum degasser; coupled with a Varian autosampler Model 410 Prostar (Hansen Way, CA, USA) equipped with a 100 µL loop, was used as liquid chromatograph. The chromatographic column used for LC separation was a Pursuit XRs Ultra C18 (100 mm x 2.0 mm, 2.8 µm particle size, Varian). Atmosphere pressure chemical ionization (APCI) was carried out on Varian MS-310 triple-quadrupole instrument. LC-MS system, data acquisition and processing were managed by MS Workstation (6.9.3 version, Varian). Chromatographic conditions were as follows: column temperature at 30°C; mobile phase consisting of eluent A (water) and eluent B (methanol) and a flow rate of 0.2 mL min^{-1}. A gradient elution was applied as follows: 0-10 min (50% A/ 50% B – 20% A / 80% B); 10-16.50 min, isocratic step 20% A / 80% B. Five minutes at initial conditions were set as post run to re-condition the chromatographic column for a new analysis. The injection volume was 10 µL. Mass calibration and resolution adjustment on the resolving quadrupoles were performed with electrospray source (ESI), by using a 10^{-5}mol L^{-1} standard solution polypropylene glycol introduced by a Harvard 11 plus infusion pump (Harvard Apparatus Inc., Holliston, MA, USA). The APCI conditions were: corona discharge voltage 2.0 kV, shield voltage 400 V, temperature and gas flow-rate for vaporization were respectively 500°C and 20 psi. Temperature and gas flow-rate for drying were 300°C and 25 psi. The target ATs were prepared in methanol: water (1:1, v/v; 1 ng mL^{-1} each AT) and infused at 10 µL min^{-1} flow rate..

205 **2.5 Method validation**

206 Validation of the ATs method optimized in this study concerned sensitivity, linearity, possible
207 matrix effects (ME), apparent recovery rate (R%), repeatability (RSDr), and limits of
208 detection (LOD) and quantification (LOQ). These parameters were validated by following the
209 guidelines of Commission Decision 2002/657/EC,. To evaluate the R% and RSDr of the five
210 ATs, three blank samples for each matrix were spiked with analyte standards to achieve four
211 different contamination concentrations, prior to extraction, and after extraction for the ME.
212 The spiking levels for each matrix were 50, 100, 200, and 500 ng g^{-1} (except for TeA on
213 olives, due to the higher LOD and LOQ: 70, 100, 200, and 500 ng g^{-1}), and prepared in six
214 replicates. In addition six samples of olives were spiked with 100 ng g^{-1} and internal standard.
215 The spiked samples were left at room temperature for an hour until total solvent evaporation.
216 Thereafter, the samples were extracted, cleaned up, and analysed by LC-MS. Linear
217 regression analyses were obtained for each matrix. Four-point calibration curves for all
218 mycotoxins were plotted at different concentrations. Linear regression was used to plot the
219 peak area ratio (y) of each mycotoxin to its concentration. Each point was repeated in
220 triplicate. The limits of detection (LOD) and quantification (LOQ) of each method for the five
221 mycotoxins were determined. LOD was calculated based on the concentration of the analyte,
222 whose peak area was three times the area of the noise of a blank sample ($S/N \geq 3$). LOQ was
223 calculated by taking three replicates of the lowest calibration standard when $S/N \geq 10$. The
224 matrix effect (ME) and the recovery (R%) were calculated, using a protocol presented by
225 Matuszewski, Constanzer, & Chavez-Eng (2003) with the following formulas:

226 $ME(\%) = B/A \times 100$

227 $R(\%) = C/B \times 100$

228 where A is the average peak area in the standard solution, B is the average peak area in the
229 spike after extraction, and C is the average peak area in the sample extract spiked before

extraction. In the calibration with the internal standard, ratios of the peak areas of AT standards and AFM1 concentrations (y) were plotted versus AT concentrations.

2.6. Statistical analysis

The calibration curves used for quantification were calculated by least-squares method. Samples with a mycotoxin concentration higher than the LOD were considered positives, whereas samples with concentrations lower than the LOD were considered negatives. Mean AT concentrations were calculated only for the positive samples higher than the LOQ. Experimental results are reported as mean ± standard deviation.

3. RESULTS AND DISCUSSION

The aim of this study was to develop a method to analyse and quantify simultaneously 5 ATs in different food matrices, by using single clean-up procedure and analytical run. The originality of this method is based on the detection of mycotoxins with different chemical and physical properties on different food matrices, which can interfere with chromatographic separation and detection, such as decrement of analyte ionization.

3.1. LC-MS/MS analysis

Most of the published methods for determination of ATs relied on an electrospray ionisation (ESI) source (Lau et al., 2003; Siegel et al., 2010). In the first experiments, we focused on the choice of the best LC ionization method, by comparing atmospheric pressure chemical ionization (APCI) and ESI. Ionisation efficiencies of ESI and APCI were evaluated on ATs standard solutions at 200 µg L^{-1}. Both techniques offered high sensitivity and specificity, by providing similar intensity signal for ALT, AOH, AME and TEN. Only the signal of TeA obtained with APCI was three times higher compared to the signal obtained with ESI (Figure

1). For each AT, mass spectrometric parameters were obtained with positive and negative APCI ionization mode, by using direct injection into the spectrometer. Chromatographic and mass spectrometer data are summarized in Table S1: precursor and product ions, cone (V) and collision (eV) voltages are indicated for each compound. The mobile phase was chosen based on the ionization and separation efficiencies. Due to good separation performance, methanol was employed as strong elution mobile phase, whereas water was chosen as weak elution mobile phase. The separation of ATs was obtained by gradient conditions for 16.50 minutes (Figure 2) and retention times were 2.35 min for TeA, 4.30 min for ALT, 6.15 min for AOH, 6.9 min for TEN, and 10.28 min for AME.

3.2 Extraction and clean-up

In previous works several solvents and solutions were investigated to elute standard solutions of the five mycotoxins with SPE cartridges. Scott et al. (2001) and Delgado & Gomez-Cordoves (1998) suggested acetonitrile-acetic acid and acetonitrile–formic acid solutions to elute AME and AOH by C-18 and aminopropyl SPE, while chloroform and ethyl acetate were used as solvents to extract AOH, TeA and AME by Da Motta & Soares (2010). Due to different polarities of the analytes, also methanol was tested as elution solvent. Preliminary results demonstrated a higher elution capability of methanol compared to acetonitrile, providing over 60% recovery for AOH, ALT, AME and TEN, instead of 35%. Ethyl acetate was chosen to elute TeA instead of chloroform in order to avoid the use of chlorinated solvents, providing over 70% recovery. Samples were subjected to a further clean-up step by using different types of SPE cartridges to obtain a satisfactory recovery rate of the five ATs. A preliminary screening was carried out on nine SPE cartridges with different polarities (Discovery® DSC-Si, LiChrolut® Si, LiChrolut® EN, Strata Florisil, Strata C18-U and Strata X from Phenomenex, Bond Elut-Mycotoxin, C18 Set-Pak and Oasis HLB), to assess the

recovery performance by passing ATs standard solution through the columns. Four out of nine cartridges (LiChrolut® EN, Oasis HLB, Strata C18-U and Strata X) showed higher recoveries ranging between 65% to 115%. Subsequently, the selected SPE columns were tested for purification efficiency for each food matrix.

3.2.1 Apple juices

To determine the best SPE cartridge to detect ATs on apple juice, a toxin-free sample was spiked with 200 ng mL^{-1} standard solution, using the extraction procedure already described. Among the SPE columns tested, LiChrolut® EN showed the best recovery rate for the five ATs (figure 3). TA was the only mycotoxin retained and completely eluted from the four cartridges tested. After the purification of the matrix with LiChrolut® EN, the eluted solution appeared clear and transparent. This SPE column method was used for the validation tests. Our extractive SPE method was different from that described in previous works (Delgado et al., 1998; Lau et al., 2003; Scott et al., 2001), where two cartridges – one C-18 not end-capped type and one NH_2 type – were used to determine only AOH and AME on fruit juices and beverages. By using only one cartridge, we obtained similar recovery results for the same mycotoxins. Calibration range, validation level, recovery, RSDr, RSDi, LOD, LOQ and matrix effect are reported in table 1. Linear calibration curves covering a concentration range from 1 to 1000 ng mL^{-1} were obtained for the five mycotoxins with r^2 range from 0.993 to 0.998. Acceptability values were obtained by determining accuracy and repeatability with recovery and RSDr. The LOQ value ranged from 0.93 ng mL^{-1} for TEN to 23.73 ng mL^{-1} for TeA. Furthermore, we did not observed a substantial variation of signal suppression due to the apple juice matrix.

3.2.2 Beers

Lager, bitter and brown ale beers have highly different compositions, due to low molecular compounds with different polarities that can determine chemical noise background and matrix suppression of the analyte signal (Araùjo et al., 2005). For this reason, specific SPE extractions for each beer typology were performed. Our results showed that different extraction techniques and purification methods are required for different kinds of beers (Figure 3). As showed in table 1, lager and bitter beer required C-18 SPE column to obtain respectively from 87.5% to 80.6% and from 67.1% to 94.5% recovery rate, while LiChrolut® EN – the cartridge applied for apple juices – was preferable for stout beers with a recovery rate >75%. For the stout beer, a dark coloured precipitate was found after elution with the other cartridges. Throughout the validation experiments, a good reliability of the generated data was reported for the three matrices tested (table 1): the analyte recovery tested at different levels ranged from 57.5% to 111.9%, RSDr did not exceed 37.3% and all analytes showed linearity in the range 0.993-0.998 (r^2). The matrix effects was similar between lager and bitter beers. Only for stout beer, a variation of signal suppression was observed (Zachariasova, Cajka, Godula, Malachova, Veprikova, & Hajslova, 2010). To our knowledge, this is the first report about the development of a method based on a rapid extraction procedure of five ATs in beer. Only two previous works were published on analysis and quantification of ATs in beer without SPE extraction: Siegel et al. (2010) developed a TeA method with ethyl acetate extraction and quantification after derivatization on different kinds of beers, while AOH and AME were detected by Zachariasova et al. (2010) with a multi-mycotoxins method based on different extraction steps.

3.2.3. Tomato products

Several techniques for extraction and clean-up of ATs from tomato products were described, by using large amounts of solvents, and several purification steps with possible loss of

analytes. One of the first methods for TeA extraction from tomato was described by Scott et al. (2001) with organic solvents, such as methanol, dichloromethane, sulphuric acid, and hexane as defatting solvent. Other authors (Da Motta et al., 2000; Asam et al., 2010) used the same solvent to extract and clean-up also AOH and AME with some modifications. Our goal was to use a simple method capable of obtaining satisfactory results in terms of recovery rate, without using large amounts of solvent. Since most tomato samples are liquid (e.g. tomato pulp, juice, purée), the apple juice extraction method, previously described, was tested. Preliminary results showed an excellent recovery rate with a clear extract and no solid precipitate. SPE cartridge screening permitted to choose Strata X as the best extraction cartridge, with recovery rates ranging from 50.54 for AME to 102.23 for AOH. Validation values are listed in table 1: matrix effect is consistent, as already highlighted for stout beer, especially for TEN and TeA. Linear calibration curves covering a concentration range from 5 to 1,000 ng mL^{-1} were obtained for all mycotoxins with r^2 range from 0.990 to 0.997. Acceptable values were obtained studying accuracy and repeatability by recovery and RSDr. The LOQ value ranged from 0.70 ng mL^{-1} for AME to 4.06 ng mL^{-1} for TeA.

3.2.4. Olives and dried basil

The diversity and the complexity of olives and dried basil did not facilitate the extraction and analysis of toxins. In addition, matrix interferences must be carefully considered: olives and basil are rich in polyphenols and fatty acids which may interfere with extraction and analysis (Reboredo-Rodriguez, Gonzales-Barreiro, Cancho-Grande, & Simal-Gandara, 2012). The recovery rate, obtained with the two extraction procedures described above on olives and basil spiked with standard solutions of ATs, demonstrated that the method of Solfrizzo et al. (2004), which used solvents with different polarity, was the only one capable of extracting the five toxins by both matrices. On the other side, only AME and AOH were detected with the

355 method of Bircan (2006). Higher recovery rates for the five ATs were obtained with C-18 U
356 cartridge for both matrices. Anyway, a low recovery was not considered an obstacle for a
357 reliable determination, because the other performance data, such as precision and linearity,
358 were good. Due to the matrix complexity of olives, an internal standard was used on such
359 samples, to test the validation parameters in presence or absence of AFM1, as internal
360 standard. The results obtained did not show significant differences in terms of R (%), RSD
361 (%) and ME (%) on the same samples, with or without internal standard (supplementary table
362 S2), so AFM1 was not anymore used as internal standard procedure. Calibration curves
363 obtained in the matrix solutions revealed a good linearity for all analytes, with correlation
364 coefficients not lower than 0.93, LOD and LOQ, recovery rates and matrix effect for olives
365 and basil were summarized in table 2. Due to the complexity of olives and basil, also
366 highlighted by the significant matrix effect values obtained, recovery results were lower than
367 for the other matrices. Only TEN showed recovery rate >70% for both matrices. Since this
368 was the first method developed to extract and detect five ATs on olives and basil, the
369 recovery rates obtained could be considered positive. Similar behaviours were found, despite
370 the specificity of each method validated and the diversity of matrices: AME and TEN had a
371 very low value for LOD and LOQ, and TeA signal was subjected to negative matrix effect.
372 The recovery rates demonstrated that the method performance was good for the tested
373 mycotoxins/matrices, such as apple juice, lager and bitter beer.
374
375 **3.3. Mycotoxins analysis in commercial food samples**
376 The optimized and validated methods were used to measure the presence of ATs in different
377 food products commercialised in Italy. In total 70 samples (10 apples juices, 30 beers, 10
378 tomato products, 10 olives and 10 dried basils) were analysed (Table 3). In 30 out of 70
379 samples analysed, at least one AT was found (7 apple juices, 14 beers and 9 tomato products).

In 7 positive samples the contamination level was lower than the LOQ. Two samples out of 10 apple juices analysed were contaminated by TeA and ALT. TeA concentrations found were 24.3 and 45.3 ng g^{-1}, respectively, while ALT occurred only in one sample at 45.6 ng g^{-1}. Among the positive beer samples detected, only lager and bitter beers, respectively 7/10 and 2/10 samples were contaminated by AOH, ALT and TEN. The stout beers analysed were free from alternaria toxin. AOH was the most widespread toxin found in 8 samples of beer with the highest concentration (23.2 ng g^{-1}). In two samples of lager beer, already contaminated with AOH, a low level of ALT and TEN contamination was detected. Tomato products showed the highest incidence of contamination, with 9 out of 10 samples positive. The results obtained showed a major contamination by AOH and ALT: 4 samples were contaminated by ALT, 4 by ALT and AOH, and 1 by AOH and TEN. Despite the high incidence of ATs in tomato products, low contamination levels were detected, ranging from 1.8 to 4.7 ng g^{-1}. In olive and dried basil samples ATs were not detected. In general, AOH was the most common AT found in 17 samples, followed by ALT (in 6 samples), TEN and TeA (both present in 2 samples). The highest concentration level of ATs was found in the commercial apple juice samples.

In conclusion, in this paper we developed and described new sensitive methods to detect and monitor the occurrence of five ATs in different food matrices, such as apple juices, beers, tomato products, olives and dried basil. We also gathered a preliminary information about the level of contamination of food products commercialised in Italy. The presence of alternaria toxins in some food samples analysed, suggests the need of a wider monitoring of the food products present on the market, and indicates the importance of setting up maximum thresholds for AFs in Europe and internationally to ensure a high level of food safety.

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Tables

Table 1.

Validation results in tomato products, apple juices, lager, bitter and stout beers.

			tomato products			apple juices			lager beers			bitter beers			stout beers		
AT	Calibration range (µg mL^{-1})	Validation levels (µg mL^{-1})	R (%)	RSDr (%)	LOD (µg mL^{-1}), LOQ (µg mL-1), ME (%)	R (%)	RSDr (%)	LOD (µg mL^{-1}), LOQ (µg mL-1), ME (%)	R (%)	RSDr (%)	LOD (µg mL^{-1}), LOQ (µg mL-1), ME (%)	R (%)	RSDr (%)	LOD (µg mL^{-1}), LOQ (µg mL-1), ME (%)	R (%)	RSDr (%)	LOD (µg mL^{-1}), LOQ (µg mL-1), ME (%)
ALT	50-1000	50	66,6	1,4	9.71, 32.35, -8	96,6	13,28	5.82, 19.41, 5	74,9	29,5	4, 13.34, 18	85,9	9,27	2.22, 7.4, 12	64,8	32,63	12.31, 41.04, -12
		100	79,7	5,1		90,5	7,09		90,9	9,36		90,5	7,09		76,4	32,68	
		200	72	12,2		87,5	3,66		87,7	7,91		94,5	13,45		73,4	29,59	
		500	87,5	3,7		87,5	2,56		87,5	3,67		57,5	19,94		57,5	33,9	
AOH	10-1000	50	102,2	3,1	1.01, 3.35, -0.06	101	0,59	3.19, 10.63, 14	63,1	8,25	0.62, 2.08, 15	82,6	11,72	1.8, 5.99, 20	111,9	6,82	8.08, 29.92, -7
		100	98,1	1,2		102,6	8,4		101,9	3,86		89	4,84		86	3,02	
		200	102,2	1,8		89,8	4,38		87,5	8,82		70	10,28		97,5	3,1	
		500	81,3	1,2		76,7	7,98		81,3	26,69		60,8	23,05		89,2	12,99	
AME	5-1000	50	82	2,5	0.21, 0.7, -11	101,5	28,15	0.45, 1.5, 5	83,8	15,25	0.46, 1.53, 3	86,2	15,77	0.26, 0.87, 18	97,5	18,18	0.16, 0.54, -12
		100	66,9	38,2		93,4	15,1		81,1	6,47		75	24,24		82,8	3,34	
		200	50,8	4,6		91,9	10,56		75,7	18,21		95,6	29,54		74,2	45	
		500	51	1,1		72,1	10,48		65,5	6,01		79,9	3,78		84,9	3,01	
TEN	10-1000	50	100,4	5,6	0.5, 1.67, -13	99,2	6,41	0.28, 0.93, 12	89,2	18,75	0.39, 1.29, 15	82,7	20,21	0.92, 3.06, 7	108,9	17,68	0.2, 0.68, 6
		100	83,9	13,9		66,7	13,09		88,9	3,8		59,4	4,95		91,7	37,28	
		200	108,5	0,8		101	2,36		95,4	18,33		67,1	19,95		98	12,93	
		500	78,8	7,3		81,3	3,48		72,6	12,9		62,3	1,91		69,8	11,26	
TeA	20-1000	50	51,1	13,5	1.22, 4.06, -15	65,1	5,75	7.12, 23.73, -7	71,1	19,64	3.12, 10.39, -8	66,5	15,62	3.12, 10.39, -14	68,1	12,58	6.1, 20.33, -18
		100	68,7	6,2		73,9	11,31		74,8	15,33		71,8	8,67		72,1	18,66	
		200	70,1	10,2		75,3	7,06		80,6	9,88		76,3	11,23		77,7	13,57	
		500	67	5,8		70,7	3,97		71,3	7,41		61	10,6		63,1	21,65	

484

485

Table 2.

Validation results in olives and dried basil.

	olives					dried basil				
AT	Calibration range (µg mL^{-1})	Validation levels (µg mL^{-1})	R (%)	RSDr (%)	LOD (µg mL^{-1}), LOQ (µg mL-1), ME (%)	Calibration range (µg mL^{-1})	Validation levels (µg mL^{-1})	R (%)	RSDr (%)	LOD (µg mL^{-1}), LOQ (µg mL-1), ME (%)
ALT	70-1000	70	55,2	17	18.91, 63.04, -6	50-1000	50	51,1	15,3	11.93, 39.76, -8
		100	82,9	7,6			100	59,7	12,3	
		200	59,5	29,4			200	51,9	12,8	
		500	66,4	36,6			500	57,5	5,2	
AOH	20-1000	50	55,7	6,1	5.03, 16.8, -32	10-1000	50	69,3	5,7	1.52, 5.07, -2
		100	62	13			100	68,9	14,3	
		200	71,8	15,6			200	70,2	13	
		500	53,2	15,8			500	56,7	11,3	
AME	5-1000	50	58,2	39	0.71, 2.36, -13	10-1000	50	52	18,7	1.38, 4.6, -11
		100	51,1	10,4			100	52,7	9,1	
		200	60	0,5			200	53,4	9,5	
		500	62,7	6			500	56,2	6,2	
TEN	1-1000	50	56,2	29,2	0.27, 0.88, 12	20-1000	50	88	16,3	4.14, 13.7, -13
		100	104,6	1,2			100	65	13,7	
		200	108,9	16,9			200	72	13,5	
		500	95,8	4,1			500	63,2	14,6	
TeA	50-1000	50	51,2	13,5	6.13, 20.4, -9	30-1000	50	50,3	17,2	6.13, 20.23, -15
		100	57,9	9,9			100	62,3	13,9	
		200	60,1	6,6			200	63,8	10,3	
		500	60,9	11,8			500	62,2	9,4	

Table 3.

Alternaria toxin occurrence in food products commercialised in Italy.

Type of samples		ALT	AME	AOH	TEN	TeA
apple juice	N° samples analysed	10	10	10	10	10
	N° positive samples	1	0	0	0	2
	range (ng g^{-1})	45.6	-	-	-	24.3 – 45.3
	RSDr range (%)	5.1	-	-	-	5.8 - 7.7
beers	N° samples analysed	30	30	30	30	30
	N° positive samples	1	0	9	1	0
	range (ng g^{-1})	14.5	-	6.04 - 23.2	10.9	-
	RSDr range (%)	4.3	-	0.99 - 14.95	11.3	-
tomato products	N° samples analysed	10	10	10	10	10
	N° positive samples	8	0	5	1	0
	range (ng g^{-1})	3.8 - 4.82	-	4.0 - 6.8	4.7	-
	RSDr range (%)	3.36 - 15.99	-	0.06 - 11.61	2.51	-
olives	N° samples analysed	10	10	10	10	10
	N° positive samples	0	0	0	0	0
dried basil	N° samples analysed	10	10	10	10	10
	N° positive samples	0	0	0	0	0

Supplementary data

Table S1.

MS/MS parameters for the analysis of ATs by MRM APCI-positive and negative ionization mode.

Alternaria toxins	**Precursor ion *(m/z)***	**Cone voltage (V)**	**Product Ions *(m/z)***	**Collision energy (eV)**
ALT	293 (+)	70	256.9[a]	14
			258	30
AME	271 (-)	40	255.7	18
			227.7[a]	22
AOH	256(-)	50	256.7	12
			112[a]	18
TEN	413(-)	50	270.8[a]	14
			140.8	18
TeA	198(+)	25	153	12
			124.8[a]	16

[a] Most abundant product ion

Table S2.

Comparison between extraction and analysis with and without internal standard.

	Olives					
	without AFM1 (100 ng g^{-1})			with AFM1 (100 ng g^{-1})		
AT	R(%)	RSD (%)	ME (%)	R(%)	RSD (%)	ME (%)
ALT	82.9	7.6	-6	80.4	2.6	-6
AOH	62.0	13.0	-32	60.3	9.5	-33
AME	51.1	10.4	-13	54.1	9.4	-12
TEN	104.6	1.2	-12	101	1.5	-12
TeA	57.9	9.9	-9	53.6	8.3	-10

506 **Figure legends**

507

508 **Fig 1.** Comparison between TeA signals obtained with ESI and APCI ionisation techniques.

509

510 **Fig. 2.** LC/APCI-MS/MS chromatogram in Multiple Reaction Monitoring of ATs at 500 ng

511 mL^{-1}.

512

513 **Fig. 3.** Recovery by using four SPE columns for five alternaria toxins (ALT, AOH, AME,

514 TEN and TeA) spiked at 200 ng mL^{-1} each on: A) apple juices; B) lager beers; C) bitter beer;

515 D) stout beer with error bars (RSDr%).