Co-occurrence of aflatoxins and ochratoxin A in spices commercialized in Italy

Co-occurrence of aflatoxins and ochratoxin A in spices commercialized in Italy

Ambra Prelle[1], Davide Spadaro[1,2], Angelo Garibaldi[1], Maria Lodovica Gullino[1,2]

[1] Centre of Competence for the Innovation in the Agro-environmental Sector (AGROINNOVA), University of Turin, Grugliasco (TO), Italy.

[2] DISAFA, Dept. Agricultural, Forestry and Food Sciences, University of Turin, Grugliasco (TO), Italy.

Correspondence:

Davide Spadaro, DISAFA, Dept. Agricultural, Forestry and Food Sciences, University of Turin, Via L. da Vinci 44, I-10095 Grugliasco (TO), Italy.

Telephone: +39-011-6708942

Fax: +39-011-6709307

E-mail: davide.spadaro@unito.it

ABSTRACT

A total of 130 spice samples coming from India, China, South America, USA, Northern Africa, Europe and Sub-Saharan Africa were collected in different stores of Northern Italy. They were analyzed for aflatoxins (AFs: AFB_1, AFB_2, AFG_1, AFG_2) and ochratoxin A (OTA) content by liquid chromatography with mass spectroscopy and positive electrospray ionization (LC/ESI-MS/MS), and HPLC with fluorescence detector (FLD), respectively. The analysis showed that 20 (15.4%) and 31 (23,8%) out of 130 samples were contaminated with AFs and OTA, respectively. A low level of total AFs contamination was found in the positive samples, the average concentration was 0.64 ng g^{-1}, far below the maximum threshold admitted by the European legislation (5 ng g^{-1} for AFB_1, and 10 ng g^{-1} for total aflatoxins (AFB_1, AFB_2, AFG_1 and AFG_2). A higher incidence of OTA was found in chili (60.0%) more than in pepper (13.3%), ranging from 2.16 to 16.35 ng g^{-1}, and from 1.61 to 15.85 ng g^{-1}, respectively. Moreover, three spice samples (2.3%) contaminated by OTA trespassed the threshold admitted by the European Regulation (EC, 2010). The co-occurrence of OTA and AFs in spices was detected in 6 out of 130 samples (4.6%), ranging from 1.61 to 15.85 ng g^{-1} and from 0.57 to 3.19 ng g^{-1}, respectively.

Keywords: aflatoxins, ochratoxin A, chili, Italy, pepper.

1. **Introduction**

Spices are widely used all over the world for food preparation, to increase the flavour and aroma, and also in the past as food preservative. Considering the global trade value, the most cultivated spice crops are pepper, capsicum, nutmeg, cumin and cinnamon. Spices are commercially produced in a relatively low number of countries. India is the most important spice producer (74% of the world market), followed by Bangladesh (6%), Turkey (5%) and China (5%) (FAOSTAT, 2010).

The fungal growth and development on spices are favoured by environmental conditions, such as temperature and humidity, and by poor manufacturing conditions in the production region. Moulds decrease quality and quantity of food production, and may also create potential risk for human and animal health, due to the production of secondary metabolites, called mycotoxins. Some mycotoxins, like AFs and OTA, are produced by species of *Aspergillus* and *Penicillium*. These mould species can develop in post-harvest, during drying and storage, particularly when good storage practices are not adopted (Scott, 1984). Aflatoxins (AFs, sum of aflatoxins B_1, B_2, G_1, G_2) and ochratoxin A (OTA) can be found in several types of food commodities, including spices (Wangikar, Dwivedi, Sinha, Sharma, & Telang, 2005).

AFs are difuranocoumarin compounds primarily produced by certain species of *Aspergillus: A. flavus, A. parasiticus, A. nomius,* and *A. tamarri* (Yabe, Nakamura, & Hamasaki, 1999). *A. flavus* and *A. parasiticus* are mainly producers of AFs: aflatoxin B_1 (AFB_1) ,B_2 (AFB_2), G_1 (AFG_1) and G_2 (AFG_2) (Varga, Frisvad, & Samson, 2011). Occurrence of AFs contaminations on several agricultural products, e.g. maize, wheat, rice, spices, dried fruits and hazelnuts, are worldwide reported (Grajewski, Blajet-Kosicka, Twaruzek, & Kosicki, 2012; Jackson, Kudupoje, & Yiannikouris, 2012; Prelle, Spadaro, Garibaldi, & Gullino, 2012). AFs have

been clearly identified as toxic, mutagenic, teratogenic, and carcinogenic compounds. AFB_1 is the most potent carcinogenic compound found in nature (Castells, Marin, Sanchis, & Ramos, 2008). The International Agency for Research of Cancer (IARC, 1993) has classified AFB_1 as a human carcinogen (Group I). OTA is a mycotoxin primarily produced by some strains of *Aspergillus* belonging to the sections *Circumdati* and *Nigri.* In particular, it is produced in warm and tropical climates by *Aspergillus ochraceus*, and in temperate countries by *Penicillium verrucosum.* (Tittlemier, Varga, Scott, & Krska, 2011; Varga, Kevei, Rinyu, Teren, & Kozakiewicz, 1996). OTA is usually found in different foods, such as cereals, spices, coffee, wine and dried fruit (Coronel, Marin, Cano-Sancho, Ramos, & Sanchis, 2012; Spadaro, Lore, Garibaldi, & Gullino, 2010). OTA has been identified as nephrotoxic, hepatotoxic, immunotoxic and teratogenic, and classified in the group 2B by IARC (1993) as a possible carcinogen for humans.

Due to favourable conditions in tropical climates, both mycotoxins can co-contaminate some typologies of spices, not only in the field, but also during drying and storage.

The European Union fixed a maximum admitted level of 5 ng g^{-1} for AFB_1, and 10 ng g^{-1} for total AFs (AFB_1, AFB_2, AFG_1 and AFG_2) intended for chili, chili powder, paprika, white and black pepper, nutmeg, turmeric, ginger and spice mixtures containing one or more of the above-mentioned spices (European Commission, 2010). In the case of OTA, the maximum admitted level for the same spices listed before has been decreased from 30 µg kg^{-1} to 15 µg kg^{-1} since 1/7/2012 (European Commission, 2012).

Despite many papers reported the co-occurrence of AFs and OTA content in spices from different countries, such as Turkey (Ozbey & Kabak, 2012) and Malaysia (Jalili & Jinap, 2012), only one paper reported the AFs content in 28 spice samples marketed in Italy (Romagnoli, Menna, Gruppioni, & Bergamini, 2007).

In Italy, the consumption of spices is low (around 50%), compared to other European countries, such as France, Germany or Spain, but growing quickly, due to the recent immigration from extra-European countries and to the diffusion of ethnical restaurants (Dalpozzo, 2011).

The aim of this study was to analyse the co-occurrence of AFs and OTA in a large number of spice samples collected in different stores of Northern Italy. Two efficient and simple methods of extraction, purification and analysis were validated on several spices: AFs were simultaneous detected by liquid chromatography-tandem mass spectroscopy with electrospray ionization (LC/ESI-MS/MS), and OTA by liquid fluorescence detector (FLD).

2. **Materials and methods**

2.1. Chemicals and reagents

HPLC grade acetonitrile and LC-MS grade methanol, formic acid and acetic acid were purchased from Sigma-Aldrich (St Louis, MO, USA). AFs (AFB_1, AFB_2, AFG_1, AFG_2) and OTA analytical standards were purchased from Sigma-Aldrich, and dissolved in methanol and acetonitrile, respectively, to prepare working standard solutions at the concentration of 10 µg l^{-1}. All standard solutions were stored in the dark at 4°C. NaCl, KCl, Na_2HPO_4, KH_2PO_4, polyethylene glycol (PEG), ammonium formate, $NaHCO_3$ and Tween 20 were purchased from Merck (Merck, Darmstadt, Germany). AflaClean select and OtaClean select immunoaffinity columns were obtained from LCTech (Dorfen, Germany). HPLC eluents were degassed for 5 minutes and filtered through mixed cellulose ester 0.22 µm-filters (Advantec MFS, Pleasanton, CA, USA) before use.

2.2. Samples

A total of 130 spices coming from India, China, South America, USA, Northern Africa, Europe and Sub-Saharan area were purchased randomly in North-Western Italy stores, from September 2011 to February 2012. Due to high different typologies of spices, samples were divided in: 30 samples of pepper, 25 of hot pepper and paprika, 21 of mixed spices (such as curry and food seasoning), and 54 other spices including 15 different types of spices (cinnamon, cloves, ginger, juniper, poppy seeds, coriander, fennel, vanilla, mustard, turmeric, nutmeg, sesame, cardamom, anise, dill).

Two hundred mg of each sample were stored in plastic bags, in the dark, at low relative humidity and 4°C before the analysis. All samples, except powder spices, were pulverized using a food processor, until homogeneous. Spice sampling was done in accordance with sampling provision described on European regulation No 401/2006.

2.3. LC-MS/MS and HPLC apparatus

Liquid chromatography coupled with mass spectrometry was used to detect aflatoxins in spice samples and to confirm OTA absence in spices used for validation method. 1260 Agilent Technologies consisting of binary LC pump and a vacuum degasser; connected with a Varian autosampler Model 410 Prostar (Hansen Way, CA, USA) equipped a 100 µL loop was used as liquid chromatograph and was coupled to a triple quadrupole mass spectrometer Varian 310-MS. The analytical column used for LC separation was a Zorbax Eclipse Plus C18 (100 mm x 4.6 mm, 3.8 µm particle size, Agilent). The chromatographic conditions were: column temperature: 45°C; mobile phase consisting of eluent A (water with 20 mM ammonium formate buffer at pH 3.35) and eluent B (methanol), using a flow rate of 0.3 ml min^{-1}. A gradient elution was applied as follows: 0-5 min, 50% A / 50% B – 30% A / 70% B; 5-10 min, 30% A / 70% B – 10% A / 90% B; 10-11 min, 10% A / 90% B, and 11-15 min, 10% A / 90% B – 50% A / 50% B . Two minutes of post run was applied. The injection volume was 10

159 µl.

160 The triple quadrupole mass spectrometer was operated in the positive electrospray ionization
161 mode (ESI^+). The ionization source conditions were: needle voltage of 2.5 kV, capillary
162 voltage of 60-77 V, source temperature of 50 °C, desolvation temperature of 350°C, cone gas
163 flow rate of 50 psi, desolvation gas flow rate of 50 psi with nitrogen. Multiple reaction
164 monitoring (MRM) mode of operation was used. The $[M+H]^+$ ions of AFs were used as
165 parent ions. The most intense daughter ions, resulting from collision-induced dissociation
166 with argon, were used to detect and quantify AFs content. The argon pressure was set at 1.8
167 psi. The most intense daughter ions detected were: m/z 284.9 at 14 eV of collision energy
168 (CE) for AFB_1, m/z 258.9 at 22 eV CE for AFB_2, m/z 242.9 at 18 eV CE for AFG_1, m/z 245
169 at 24 eV CE for AFG_2, m/z 358 at 18 eV CE and m/z 239 at 30 eV CE for OTA.

170 OTA was detected with a HPLC apparatus 1100 series Agilent equipped with G1311
171 quaternary pump, G1379 degasser, G1313A autosampler, G1316A column thermostat and
172 G1321A FLD – Fluorescence Detector. The mobile phase consisted in an isocratic mixture of
173 acetonitrile:water:acetic acid (49:49:2) for 15 min. Sample (30 µL) was injected into the
174 analytical column Synergi 4u Hydro-RP (250 mm x 4.6 mm, Phenomenex) and detected using
175 333 and 460 nm as wavelengths for excitation and emission, respectively.

176

177

178 *2.4. Aflatoxins extraction and clean up*

179 In the first part of this study, concerning the validation of extraction method, two samples for
180 each matrix, which confirmed to be aflatoxin free, were used as follows: one aliquot of the
181 sample was analysed as such, whilst other aliquots were spiked with a known concentration of
182 mycotoxin standard.

183 The method for AFs extraction from hazelnut, described in a previously published paper

(Prelle *et al*., 2012), was used with slight modification. For 25 g of sample, 5 g of NaCl and 125 ml of extraction solution, methanol: water (80:20) were added, and left for 2 h on a shaker apparatus at 165 rpm. To eliminate the solid phase from the extraction solution, the sample was filtered, first, through a Whatman No. 4 filter paper, and subsequently by using a Whatman CA 0.45 µm syringe filter. A 10 ml aliquot of filtrate was diluted 1:4 in phosphate buffer solution (PBS) and centrifuged at 4,000 rpm. To clean up the samples, immunoaffinity columns (IAC) were used, by loading 10 ml of diluted sample. IAC were washed with 10 ml of PBS solution and then with 10 ml of water and air dried. AFs were eluted with 3 ml of methanol into an amber glass vial. The elute was evaporated at 65°C under air flow and 1 ml of mobile phase was added to the precipitate and vortexed until dissolved. All samples were analysed in triplicate.

2.5. Ochratoxin A extraction and clean up

The absence of OTA, avoiding positive matrix effect of fluorescence signal, was confirmed by analysis through LC-MS/MS. The samples (4g) were put in a 50 mL centrifuge tube with 20 mL of extraction solution, methanol:water (80:20), and left for 5 minutes in ultrasonic apparatus at 25°C. To separate the solid sample from the extraction solution, centrifugation for 15 min at 6,000 rpm was applied. After centrifugation, the solution was diluted 1:4 with PBS solution and filtered by cellulose acetate 0.45 µm syringe filter. Ten ml of diluted and filtered solution were loaded into IAC and, then, 10 ml of washing solution (2.5% NaCl and 0.25% $NaHCO_3$) and 10 ml of ultrapure water were added into column. Before eluting with 3 ml of methanol into an amber glass vial, the column was air dried. The elute was evaporated at 65°C under air flow and 1 ml of eluent was added to the precipitate and vortexed until dissolved. All samples were analyzed in triplicate.

2.6. Method validation

Validation of AFs and OTA methods was optimized studying apparent recovery rate (R%), limits of detection (LOD) and quantification (LOQ), based on the IUPAC definition (Thompson, Ellison, & Wood, 2002), and, to validate the extraction method and chromatographic performances, capacity factor (k'), repeatability of recovery (RSD) and capacity factor (RSDk') were calculated. Following the guidelines of Commission Decision 2002/657/EC, these parameters were validated. The recovery (R%), RSD, LOD and LOQ were determined on pepper, chili and four spices chosen by "others" (cinnamon, clove, nutmeg and sesame) and two spice by "mixed" group (curry and food seasoning). Samples were spiked with four concentrations of standard solutions of AFB_1 (0.5, 5, 10, 25 ng ml^{-1}) for pepper, cinnamon and seasoning food and (1, 5, 10, 25 ng ml^{-1}) for chili, clove, nutmeg, sesame and curry. Validation levels for total AFs were obtained spiking at 3, 5, 10, 25 ng ml^{-1}), and for OTA at 5, 10, 25, 50 ng ml^{-1} with standard solutions. RSDk' and k' were calculated, by analysing spiked cinnamon, clove, food seasoning and pepper samples at 10 ng ml^{-1} of AFB_1, total AFs, and OTA. Each test was performed three times.

Limits of detection (LOD) and quantification (LOQ) of each method for all mycotoxins were assessed. LOD was defined as three times the electronic baseline noise and LOQ as ten times the level of the baseline noise. The baseline noise was obtained with a blank sample for each matrix processed following the tested procedures. LOD and LOQ were calculated, analysing six blank samples and for total AFs were obtained by meaning the values of each mycotoxin. The recovery was calculated, using a protocol presented by (Matuszewski, Constanzer, & Chavez-Eng, 2003).

Capacity factors (k'), intended as a measure of the time the sample component resides in the stationary phase relative to the time in the mobile phase, were used to assure reliability and

repeatability of chromatographic analysis and were calculated for each mycotoxin (IUPAC, 1997).

2.7. Statistical analysis

Normal distribution of toxin contents, means, standard errors and validation data were analysed with SPSS software (SPSS Institute, Inc, 2000, Version 18.0). The calibration curves used for quantification were calculated by least-squares method. Samples with a concentration of AFs and OTA higher that LOD were considered positives, whilst samples with concentrations lower than LOD were considered negatives. Mean AFs and OTA concentrations were calculated only on the positive samples higher than the LOQ. The Kruskal-Wallis test was used to compare the mean AF and OTA levels among the different typologies (pepper, chili, mixed and others), and geographical origins of spice samples, while the Mann-Whitney test was used to compare the mean OTA and AFs levels in pepper/chili samples and grain/powder of pepper and chili samples, using the null hypothesis that the levels were not different.

3. **Results and discussion**

This study represents the first report about monitoring of AFs and OTA co-occurrence in a large number of spice samples marketed in Italy. Two extraction and detection methods for AFs and OTA were validated on chili, pepper, cinnamon, nutmeg, food seasoning and curry matrices by LC/ESI-MS/MS and by FLD, respectively.

Due to the limited consumption, and consequently limited availability of spices in Italy, with the exception of pepper and chili, we grouped the spices in four categories: pepper, chili, others and mixed. The geographical origins of the spices analysed are shown in table 1. In

agreement with a FAO report (FAOSTAT, 2012) about the main spice producers, most of the analysed samples were coming from India (74), followed by North Africa (33), and China (11).

3.1. Analytical performance

The analytical methods were validated considering: linearity, apparent recovery rate (R%), capacity factor (k'), repeatability of recovery (RSD) and capacity factor (RSDk), limits of detection (LOD) and quantification (LOQ) for AFs and OTA methods. Data of performed analytical methods are summarized in Table 2. Due to complexity of chili and pepper matrix, LOD and LOQ obtained for OTA detection, 0.86 ng g^{-1} and 2.61 ng g^{-1} for pepper, and 0.70 ng g^{-1} and 2.13 ng g^{-1} for chili, respectively, were higher than other spices tested, These results could be explained by presence of matrix compounds which interfere with analytical signal increasing baseline noise (Prelle, Spadaro, Denca, Garibaldi & Gullino, 2013). OTA recovery rate ranged from 70.8 % at 25 ng g^{-1} of sesame to 102.1 % at 10 ng g^{-1} of nutmeg. According to Castagnaro et al. (2006), we could explain these recovery results obtained as interaction between several interferences on specific identification antigen-antibody present into IAC. In particular, the specific antibodies identification could be reduced by the formation of open-ring OTA at alkaline pH and by cross-reaction due to the presence of nonchlorinated analogue OTB (Castegnaro, Tozlovanu, Wild, Molinie, Sylla, & Pfohl-Leszkowicz, 2006). Repeatability of OTA measurements, ranging from 0.3% for curry to 18.2% for food seasoning, resulting in accordance with requirement established by EU regulation. On the contrary the values of LOD and LOQ for chili and pepper for aflatoxin detection, ranging from 0.08 ng g^{-1} of AFB1 to 0.41 of AFs, respectively, for pepper and from 0.17 ng g^{-1} of AFB1 to 0.71 of AFs, respectively, for chili resulted less influenced by matrix interferences on analytical signal than OTA detection. Recovery values for AFs and AFB1 were above of

70% for all spices tested, despite the matrix complexity of some kind of spice, such as curry and food seasoning, composed by different quantity of other spices. In the other hand the k' and RSDk'values obtained reported in Table 3, demonstrated the high repeatability and hardiness (RSDk'% < 0.1) of optimized chromatographic method, despite different spices analysed.

3.2. Mycotoxin occurrence

AFs were found in 20 (15.4%) out of 130 samples analysed with levels ranging from 0.59 to 5.38 ng g^{-1}. The average concentration was 0.96 ng g^{-1}. Five samples of each typology of spice (pepper, chili, mixed and others) resulted positive to one or more AFs. The AFs contamination range was between 0.59 and 3.68 ng g^{-1}, with 11 (8.5%) samples contaminated by AFB_1, with a mean on contaminated samples of 0.31 ng g^{-1}, 14 (4.6%) by AFB_2 (contamination mean: 1.07 ng g^{-1}), 3 (2.3%) by AFG_1 (0.42 ng g^{-1}), and 7 (5.4%) by AFG_2 (2.26 ng g^{-1}) (Table 4).

No sample was contaminated by AFs above the maximum admitted threshold established by the European legislation (European Commission, 2010). In fact, the highest AFB_1 level detected was 1.95 ng g^{-1}, 2.5 times lower than the maximum threshold specific for AFB_1. In contrast with the results obtained by Romagnoli *et al*. (2007) on monitoring of AFs on spices purchased in Italy, we detected a lower incidence of contamination and a lower level of AFs. Although pepper and chili are usually more susceptible substrates to mycotoxins contamination (Reddy, Mayi, Reddy, Thirumala-Devi, & Reddy, 2001), our results showed no differences among the spice typologies analysed. The low amount of AF content detected in our study agrees with the results reported by similar studies ((Jalili & Jinap, 2012; Ozbey & Kabak, 2012). The incidence of positive samples was slightly higher in the mixed and chili samples, 23.8% and 20.0%, respectively, compared to pepper (16.6%) and other (9.26%)

spices. The Kruskal-Wallis test showed that the level of contamination of AFs in different countries were not statistically different (p=0.586). Average AF content in pepper and chili samples were calculated with Mann-Whitney test, and resulted not statistically different (p=0.248).

Considering the OTA contamination in the spice samples, the incidence resulted higher than AFs: 23.8% spices were positive to OTA, while only 15.4% to AFs. The values of OTA contamination ranged from LOD to 19.06 ng g^{-1}. The contamination OTA mean on positive samples was 6.18 ng g^{-1}. Fifteen out of 25 (60%) chili samples were contaminated at levels ranging from 2.16 to 16.35 ng g^{-1}, 7 out of 21 (33.3%) mixed samples at levels ranging from 1.84 to 19.06 ng g^{-1}. Pepper (13.3%) and other samples (9.3%) resulted positive ranging from 1.61 to 15.85 and from 2.21 to 11.08, respectively (Table 4).

Similarly to AFs, results of Kruskal-Wallis test showed no significant differences among the geographical origins of the samples. The Mann-Whitney test, instead, reported significant differences on OTA contamination levels between chili and pepper (p<0.05). Significant differences were also noticed for grain and powder chili (p<0.05). Chili powder showed higher OTA content (78.9%) than chili grains, probably, due to the higher contact surface presented to the microbial population, which can favor the mycotoxin release.

The current monitoring showed a higher incidence of OTA than AFs in spice samples marketed in Italy; moreover, concentration levels of OTA detected in three (2.3%) samples out of 130 trespassed the threshold admitted by the European Regulation (EC, 2010).

The co-occurrence of OTA and AFs in spices was detected in 6 out of 130 samples(4.6%), in particular in chili, pepper and curry samples. OTA and AFs concentration ranged from 1.61 to 15.85 ng $g^{-1}$and 0.57 to 3.19 ng g-1, respectively. Several studies were performed over the last decade on the co-occurrence of AFs and OTA in spices. Most of the studies highlighted that the reason for a high mycotoxin contamination is the susceptibility of spices to fungal

contamination, and consequently to mycotoxin production, connected to environmental conditions, such as high humidity and temperature (Patharajan et al., 2011), of the producer countries located in tropical and sub-tropical areas. Regarding the co-occurrence of AFs and OTA in same substrate, Sedmikova, Reisnerova, Dufkova, Barta, & Jilek (2001) demonstrated that OTA could increase the mutagenic activity of AFB_1. In agreement with our results, most of previous work reported high incidence of AFs and OTA in pepper and chili. In particular, during the last decade, AFs and OTA were monitored in over 1,500 spice samples, resulting in a contamination incidence ranging from 30 to 70%. Despite the wide range of spices present, attention mainly focused on pepper and chili, due to their high demand in the market worldwide and to their susceptibility to fungal contamination, especially during the drying processes (Shundo, de Almeida, Alaburda, Lamardo, Navas, Ruvieri, et al., 2009). According to the available literature and to our results, chili and pepper are more frequently contaminated by AFs and OTA than other spices, such as cumin or cinnamon. This is the first study on AFs and OTA co-occurrence in a large number of spice samples marketed in Italy. Future studies will also monitor the fungal population present on the spices, which could be related to the AFs and OTA contamination, with microbiological an molecular tools (Spadaro, Patharajan, Karthikeyan, Lorè, Garibaldi, & Gullino, 2011).

Acknowledgements

Work carried out within the framework of the project “ITACA – Technological Innovation, Automation and new Analytical Controls to improve the quality and safety of the Piedmontese food products” funded by the Piedmont Region (Italy).

References

Castegnaro, M., Tozlovanu, M., Wild, C., Molinie, A., Sylla, A., & Pfohl-Leszkowicz, A. (2006). Advantages and drawbacks of immunoaffinity columns in analysis of mycotoxins in food. *Molecular Nutrition & Food Research, 50*(6), 480-487.

Castells, M., Marin, S., Sanchis, V., & Ramos, A. J. (2008). Distribution of fumonisins and aflatoxins in corn fractions during industrial cornflake processing. *International Journal of Food Microbiology, 123*(1-2), 81-87.

Coronel, M. B., Marin, S., Cano-Sancho, G., Ramos, A. J., & Sanchis, V. (2012). Exposure assessment to ochratoxin A in Catalonia (Spain) based on the consumption of cereals, nuts, coffee, wine, and beer. *Food Additives and Contaminants Part a-Chemistry Analysis Control Exposure & Risk Assessment, 29*(6), 979-993.

Dalpozzo D. (2011). Le spezie continuano a crescere e arrivano anche i sali esotici. *Mark Up, 2011* (07/08), 109.

European Commission (2012). Commission Regulation (EU) No 594/2012 of 5 July 2012 amending Regulation (EC) 1881/2006 as regards the maximum levels of the contaminants ochratoxin A, non dioxin-like PCBs and melamine in foods. *Official Journal of the European Union*, L176/43-L176/45.

European Commission (2010). Commission Regulation (EU) No 165/2010 of 26 February 2006 amending Regulation (EC) No 1881/2006 setting maximum level for certain contaminants in foodstuffs as regards aflatoxins. *Official Journal of the European Union*, L50/8-L50/12.

European Commission (2006). Commission Regulation (EU) No 401/2006 of 23 February 2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. *Official Journal of the European Union*, L70/12-L70/34.

FAOSTAT. (2012). Website: http://faostat.fao.org

Grajewski, J., Blajet-Kosicka, A., Twaruzek, M., & Kosicki, R. (2012). Occurrence of mycotoxins in Polish animal feed in years 2006-2009. *Journal of animal physiology and animal nutrition, 96*(5), 870-877.

IARC, (1993). Some naturally occuring substances: food items and constituents, heterocyclic aromatic amines and mycotoxins, in: IARC Monographs on the Evaluation of Carcinogenic Risk to Humans. *IARC Scientific Publication*, 56, 489-521.

IUPAC (1997). Compendium of Chemical Terminology, 2nd ed. (the "Gold Book"). Compiled by A. D. McNaught and A. Wilkinson. Blackwell Scientific Publications, Oxford.

Jackson, L. C., Kudupoje, M. B., & Yiannikouris, A. (2012). Simultaneous multiple mycotoxin quantification in feed samples using three isotopically labeled internal standards applied for isotopic dilution and data normalization through ultra-performance liquid chromatography/electrospray ionization tandem mass spectrometry. *Rapid Communications in Mass Spectrometry, 26*(23), 2697-2713.

Jalili, M., & Jinap, S. (2012). Natural occurrence of aflatoxins and ochratoxin A in commercial dried chili. *Food Control, 24*(1-2), 160-164.

Matuszewski, B. K., Constanzer, M. L., & Chavez-Eng, C. M. (2003). Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS. *Analytical Chemistry, 75*(13), 3019-3030.

Ozbey, F., & Kabak, B. (2012). Natural co-occurrence of aflatoxins and ochratoxin A in spices. *Food Control, 28*(2), 354-361.

Patharajan, S., Reddy, K.R.N., Karthikeyan, V., Spadaro, D., Lorè, A., Gullino, M.L. & Garibaldi, A. (2011). Potential of yeasts antagonists on *in vitro* biodegradation of ochratoxin A. *Food Control, 22,* 290-296.

Prelle, A., Spadaro, D., Garibaldi, A., & Gullino, M. L. (2012). Aflatoxin monitoring in Italian hazelnut products by LC-MS. *Food Additives & Contaminants Part B-Surveillance, 5*(4), 279-285.

Prelle, A., Spadaro, D., Denca, A., Garibaldi, A. & Gullino, M.L. (2013). Comparison of clean up method for ochratoxin A in wine, beer, roasted coffee and chili commercialized in Italy. *Toxins, 5,* 1827-1844.

Reddy, S. V., Mayi, D. K., Reddy, M. U., Thirumala-Devi, K., & Reddy, D. V. R. (2001). Aflatoxins B-1 in different grades of chillies (Capsicum annum L.) in India as determined by indirect competitive-ELISA. *Food Additives and Contaminants, 18*(6), 553-558.

Romagnoli, B., Menna, V., Gruppioni, N., & Bergamini, C. (2007). Aflatoxins in spices, aromatic herbs, herb-teas and medicinal plants marketed in Italy. *Food Control, 18*(6), 697-701.

Sedmikova, M., Reisnerova, H., Dufkova, Z., Barta, I., & Jilek, F. (2001). Potential hazard of simultaneous occurrence of aflatoxin B-1 and ochratoxin A. *Veterinarni Medicina, 46*(6), 169-174.

Shundo, L., de Almeida, A. P., Alaburda, J., Lamardo, L. C. A., Navas, S. A., Ruvieri, V., & Sabino, M. (2009). Aflatoxins and ochratoxin A in Brazilian paprika. *Food Control, 20*(12), 1099-1102.

Spadaro, D., Lore, A., Garibaldi, A., & Gullino, M. L. (2010). Occurrence of ochratoxin A before bottling in DOC and DOCG wines produced in Piedmont (Northern Italy). *Food Control*, *21*(9), 1294-1297.

Thompson, M., Ellison, S. L. R., & Wood, R. (2002). Harmonized guidelines for single-laboratory validation of methods of analysis - (IUPAC technical report). *Pure and Applied Chemistry, 74*(5), 835-855.

Tittlemier, S. A., Varga, E., Scott, P. M., & Krska, R. (2011). Sampling of cereals and cereal-based foods for the determination of ochratoxin A: an overview. *Food Additives and Contaminants Part a-Chemistry Analysis Control Exposure & Risk Assessment, 28*(6), 775-785.

Varga, J., Frisvad, J. C., & Samson, R. A. (2011). Two new aflatoxin producing species, and an overview of Aspergillus section Flavi. *Studies in Mycology,* (69), 57-80.

Varga, J., Kevei, E., Rinyu, E., Teren, J., & Kozakiewicz, Z. (1996). Ochratoxin production by Aspergillus species. *Applied and Environmental Microbiology, 62*(12), 4461-4464.

Wangikar, P. B., Dwivedi, P., Sinha, N., Sharma, A. K., & Telang, A. G. (2005). Teratogenic effects in rabbits of simultaneous exposure to ochratoxin A and aflatoxin B-1 with special reference to microscopic effects. *Toxicology, 215*(1-2), 37-47.

Yabe, K., Nakamura, M., & Hamasaki, T. (1999). Enzymatic formation of G-group aflatoxins and biosynthetic relationship between G- and B-group aflatoxins. *Applied and Environmental Microbiology, 65*(9), 3867-3872.

Tables

Table 1. Geographical origin of spice samples.

	Spices			
Origin	**Pepper (n)**	**Chili (n)**	**Mixed (n)**	**Others (n)**
South America	2	2	0	0
India	25	15	8	26
North Africa	2	2	13	16
Europe	0	0	0	1
China	1	5	0	5
Sub-Saharan Africa	0	1	0	5
USA	0	0	0	1
Total samples (n)	**30**	**25**	**21**	**54**

461 **Table 2.** Recovery of AFs and OTA spiked into pepper and cinnamon samples.

Analytes	Matrix	Fortification (ng ml^{-1})	Recovery (n=3) (%)	RSD (%) (n=3)	LOD (ng g-1) (n=6)	LOQ (ng g-1) (n=6)
Aflatoxin B_1	pepper	0.5, 5, 10, 25	91.8, 97.8, 99.6, 99.7	3.8, 0.6, 2.8, 3.5	0.08	0.28
Aflatoxins B_1, B_2, G_1, G_2	pepper	3, 5, 10, 25	79.1, 82.8, 82.5, 83.3	25.6, 23.5, 25.0, 25.4	0.12	0.41
Aflatoxin B_1	chili	1, 5, 10, 25	100.6, 97.3, 93.4, 90.5	1.8, 2.0, 2.9, 2.7	0.17	0.55
Aflatoxins B_1, B_2, G_1, G_2	chili	3, 5, 10, 25	78.04, 83.4, 85.5, 88.0	1.9, 1.1, 2.5, 1.2	0.21	0.71
Aflatoxin B_1	cinnamon	0.5, 5, 10, 25	99.8, 99.8, 99.6, 99.9	0.1, 2.7, 3.1, 3.0	0.12	0.41
Aflatoxins B_1, B_2, G_1, G_2	cinnamon	3, 5, 10, 25	80.5, 81.5, 81.9, 83.1	24.8, 25.5, 25.8, 23.7	0.18	0.61
Aflatoxin B_1	nutmeg	1, 5, 10, 25	79.0, 77.4, 77.6, 82.1	12.3, 14.3, 8.8, 8.9	0.2	0.66
Aflatoxins B_1, B_2, G_1, G_2	nutmeg	3, 5, 10, 25	76.9, 80.3, 89.7, 87.4	9.1, 5.9, 12.7, 7.3	0.65	2.2
Aflatoxin B_1	clove	1, 5, 10, 25	81.6, 92.7, 90.3, 85.1	13.3, 16.9, 12.4, 10.5	0.17	0.58
Aflatoxins B_1, B_2, G_1, G_2	clove	3, 5, 10, 25	83.9, 96.6, 87.3, 81.2	15.6, 13.0, 9.4, 11.4	0.57	1.94
Aflatoxin B_1	sesame	1, 5, 10, 25	73.2, 73.0, 77.8, 83.6	5.9, 3.2, 7.3, 5.6	0.19	0.62
Aflatoxins B_1, B_2, G_1, G_2	sesame	3, 5, 10, 25	82.7, 81.1, 77.3, 81.9	20.3, 13.2, 10.4, 12.6	0.33	1.12
Aflatoxin B_1	food seasoning	0.5, 5, 10, 25	97.2, 97.6, 87.8, 81.8	4.7, 1.1, 2.9, 1.8	0.13	0.45
Aflatoxins B_1, B_2, G_1, G_2	food seasoning	3, 5, 10, 25	92.8, 93.1, 94.3, 83.8	18.6, 6.3, 4.7, 5.1	0.26	0.88
Aflatoxin B_1	curry	1, 5, 10, 25	71.6, 74.7, 79.8, 80.3	19.6, 17.7, 15.9, 15.3	0.25	0.83
Aflatoxins B_1, B_2, G_1,	curry	3, 5, 10, 25	73.0, 71.6, 78.6, 79.4	17.3, 17.1, 18.0, 17.9	0.77	2.62

G_2						
OTA	pepper	5, 10, 25, 50	91.1, 94.8, 97.8, 98.7	2.1, 2.9, 4.8, 2.1	0.86	2.61
OTA	chili	5, 10, 25, 50	81.1, 79.3, 88.4, 87.8	4.7, 8.6, 4.5, 6.7	0.70	2.13
OTA	cinnamon	5, 10, 25, 50	97.4, 96.3, 99.2, 95.6	3.4, 4.5, 4.5, 3.6	0.03	0.09
OTA	nutmeg	5, 10, 25, 50	80.2, 102,1, 84.6, 71.0	17.8, 7.8, 0.8, 0.4	0.03	0.10
OTA	clove	5, 10, 25, 50	78.4, 80.4, 75.3, 76.0	2.3, 17.1, 13.0, 0.68	0.02	0.07
OTA	sesame	5, 10, 25, 50	73.5, 74.5, 70.8, 75.4	6.0, 6.1, 6.9, 14.5	0.05	0.14
OTA	food seasoning	5, 10, 25, 50	78.1, 78.2, 82.7, 77.3	1.8, 14.5, 18.2, 2.5	0.05	0.15
OTA	curry	5, 10, 25, 50	79.6, 72.9, 78.3, 71.2	0.3, 8.2, 9.4, 0.8	0.04	0.12

462 **Table 3.** Analytical chromatographic performance.

Analytes	Matrix	Capacity factor (k')	RSDk' (%)
AFs	pepper	38.1, 36.6, 34.1, 31.8	0.06, 0.07, 0.09, 0.1
OTA	pepper	5.2	0.04
AFs	cinnamon	38.4, 36.7, 34.3, 31.9	0.05, 0.07, 0.07, 0.09
OTA	cinnamon	5.2	0.05
AFs	food seasoning	38.0, 36.6, 34.3, 31.7	0.03, 0.04, 0.03, 0.06
OTA	food seasoning	5.2	0.03
AFs	clove	37.9, 36.2, 34.1, 31.0	0.09, 0.1, 0.1, 0.12
OTA	clove	5.3	0.07

463

464 **Table 4.** Occurrence and levels of AFs and OTA in the spices analysed.

			Number of positive samples (ng g^{-1})				
Mycotoxins	**Positive/total (%)**	**Mean of contamination ± SD (ng g^{-1})**	**< LOD**	**LOD-5.0**	**5.0-10.0**	**10.0-15.0**	**>15.0**
AFB_1	11/130 (8.5%)	0.31±0.003	8	3	-	-	-
AFB_2	6/130 (4.6%)	1.07±0.086	4	2	-	-	-
AFG_1	3/130 (2.3%)	0.42±0025	3	-	-	-	-
AFG_2	7/130 (5.4%)	2.26±0.191	3	4	-	-	-
OTA	31/130 (23.8%)	6.18±0.639	-	15	11	2	3
OTA+AFs	6/130 (4.6%)	0.85±0.030					

465