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

23

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39

40 **ABSTRACT**

41

42 A total of 130 spice samples coming from India, China, South America, USA, Northern
43 Africa, Europe and Sub-Saharan Africa were collected in different stores of Northern Italy.
44 They were analyzed for aflatoxins (AFs: AFB₁, AFB₂, AFG₁, AFG₂) and ochratoxin A (OTA)
45 content by liquid chromatography with mass spectroscopy and positive electrospray
46 ionization (LC/ESI-MS/MS), and HPLC with fluorescence detector (FLD), respectively. The
47 analysis showed that 20 (15.4%) and 31 (23,8%) out of 130 samples were contaminated with
48 AFs and OTA, respectively. A low level of total AFs contamination was found in the positive
49 samples, the average concentration was 0.64 ng g⁻¹, far below the maximum threshold
50 admitted by the European legislation (5 ng g⁻¹ for AFB₁, and 10 ng g⁻¹ for total aflatoxins
51 (AFB₁, AFB₂, AFG₁ and AFG₂). A higher incidence of OTA was found in chili (60.0%) more
52 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,
53 respectively. Moreover, three spice samples (2.3%) contaminated by OTA trespassed the
54 threshold admitted by the European Regulation (EC, 2010). The co-occurrence of OTA and
55 AFs in spices was detected in 6 out of 130 samples (4.6%), ranging from 1.61 to 15.85 ng g⁻¹
56 and from 0.57 to 3.19 ng g⁻¹, respectively.

57

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

59

60 1. Introduction

61

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

68 The fungal growth and development on spices are favoured by environmental conditions,
69 such as temperature and humidity, and by poor manufacturing conditions in the production
70 region. Moulds decrease quality and quantity of food production, and may also create
71 potential risk for human and animal health, due to the production of secondary metabolites,
72 called mycotoxins. Some mycotoxins, like AFs and OTA, are produced by species of
73 *Aspergillus* and *Penicillium*. These mould species can develop in post-harvest, during drying
74 and storage, particularly when good storage practices are not adopted (Scott, 1984).
75 Aflatoxins (AFs, sum of aflatoxins B₁, B₂, G₁, G₂) and ochratoxin A (OTA) can be found in
76 several types of food commodities, including spices (Wangikar, Dwivedi, Sinha, Sharma, &
77 Telang, 2005).

78 AFs are difuranocoumarin compounds primarily produced by certain species of *Aspergillus*:
79 *A. flavus*, *A. parasiticus*, *A. nomius*, and *A. tamarri* (Yabe, Nakamura, & Hamasaki, 1999). *A.*
80 *flavus* and *A. parasiticus* are mainly producers of AFs: aflatoxin B₁ (AFB₁), B₂ (AFB₂), G₁
81 (AFG₁) and G₂ (AFG₂) (Varga, Frisvad, & Samson, 2011). Occurrence of AFs contaminations
82 on several agricultural products, e.g. maize, wheat, rice, spices, dried fruits and hazelnuts, are
83 worldwide reported (Grajewski, Blajet-Kosicka, Twaruzek, & Kosicki, 2012; Jackson,
84 Kudupoje, & Yiannikouris, 2012; Prella, Spadaro, Garibaldi, & Gullino, 2012). AFs have

85 been clearly identified as toxic, mutagenic, teratogenic, and carcinogenic compounds. AFB₁ is
86 the most potent carcinogenic compound found in nature (Castells, Marin, Sanchis, & Ramos,
87 2008). The International Agency for Research of Cancer (IARC, 1993) has classified AFB₁ as
88 a human carcinogen (Group I). OTA is a mycotoxin primarily produced by some strains of
89 *Aspergillus* belonging to the sections *Circumdati* and *Nigri*. In particular, it is produced in
90 warm and tropical climates by *Aspergillus ochraceus*, and in temperate countries by
91 *Penicillium verrucosum*. (Tittlemier, Varga, Scott, & Krska, 2011; Varga, Kevei, Rinyu,
92 Teren, & Kozakiewicz, 1996). OTA is usually found in different foods, such as cereals,
93 spices, coffee, wine and dried fruit (Coronel, Marin, Cano-Sancho, Ramos, & Sanchis, 2012;
94 Spadaro, Lore, Garibaldi, & Gullino, 2010). OTA has been identified as nephrotoxic,
95 hepatotoxic, immunotoxic and teratogenic, and classified in the group 2B by IARC (1993) as
96 a possible carcinogen for humans.

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

99 The European Union fixed a maximum admitted level of 5 ng g⁻¹ for AFB₁, and 10 ng g⁻¹ for
100 total AFs (AFB₁, AFB₂, AFG₁ and AFG₂) intended for chili, chili powder, paprika, white and
101 black pepper, nutmeg, turmeric, ginger and spice mixtures containing one or more of the
102 above-mentioned spices (European Commission, 2010). In the case of OTA, the maximum
103 admitted level for the same spices listed before has been decreased from 30 µg kg⁻¹ to 15 µg
104 kg⁻¹ since 1/7/2012 (European Commission, 2012).

105 Despite many papers reported the co-occurrence of AFs and OTA content in spices from
106 different countries, such as Turkey (Ozbey & Kabak, 2012) and Malaysia (Jalili & Jinap,
107 2012), only one paper reported the AFs content in 28 spice samples marketed in Italy
108 (Romagnoli, Menna, Gruppioni, & Bergamini, 2007).

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

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

118

119 2. Materials and methods

120

121 2.1. Chemicals and reagents

122 HPLC grade acetonitrile and LC-MS grade methanol, formic acid and acetic acid were
123 purchased from Sigma-Aldrich (St Louis, MO, USA). AFs (AFB₁, AFB₂, AFG₁, AFG₂) and
124 OTA analytical standards were purchased from Sigma-Aldrich, and dissolved in methanol and
125 acetonitrile, respectively, to prepare working standard solutions at the concentration of 10 µg
126 l⁻¹. All standard solutions were stored in the dark at 4°C. NaCl, KCl, Na₂HPO₄, KH₂PO₄,
127 polyethylene glycol (PEG), ammonium formate, NaHCO₃ and Tween 20 were purchased
128 from Merck (Merck, Darmstadt, Germany). AflaClean select and OtaClean select
129 immunoaffinity columns were obtained from LCTech (Dorfen, Germany). HPLC eluents
130 were degassed for 5 minutes and filtered through mixed cellulose ester 0.22 µm-filters
131 (Advantec MFS, Pleasanton, CA, USA) before use.

132

133 2.2. Samples

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

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

145

146 *2.3. LC-MS/MS and HPLC apparatus*

147 Liquid chromatography coupled with mass spectrometry was used to detect aflatoxins in spice
148 samples and to confirm OTA absence in spices used for validation method. 1260 Agilent
149 Technologies consisting of binary LC pump and a vacuum degasser; connected with a Varian
150 autosampler Model 410 Prostar (Hansen Way, CA, USA) equipped a 100 µL loop was used
151 as liquid chromatograph and was coupled to a triple quadrupole mass spectrometer Varian
152 310-MS. The analytical column used for LC separation was a Zorbax Eclipse Plus C18 (100
153 mm x 4.6 mm, 3.8 µm particle size, Agilent). The chromatographic conditions were: column
154 temperature: 45°C; mobile phase consisting of eluent A (water with 20 mM ammonium
155 formate buffer at pH 3.35) and eluent B (methanol), using a flow rate of 0.3 ml min⁻¹. A
156 gradient elution was applied as follows: 0-5 min, 50% A / 50% B – 30% A / 70% B; 5-10
157 min, 30% A / 70% B – 10% A / 90% B; 10-11 min, 10% A / 90% B, and 11-15 min, 10% A /
158 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 AFB₁, m/z 258.9 at 22 eV CE for AFB₂, m/z 242.9 at 18 eV CE for AFG₁, m/z 245
169 at 24 eV CE for AFG₂, 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

184 (Prelle *et al.*, 2012), was used with slight modification. For 25 g of sample, 5 g of NaCl and
185 125 ml of extraction solution, methanol: water (80:20) were added, and left for 2 h on a
186 shaker apparatus at 165 rpm. To eliminate the solid phase from the extraction solution, the
187 sample was filtered, first, through a Whatman No. 4 filter paper, and subsequently by using a
188 Whatman CA 0.45 μ m syringe filter. A 10 ml aliquot of filtrate was diluted 1:4 in phosphate
189 buffer solution (PBS) and centrifuged at 4,000 rpm. To clean up the samples, immunoaffinity
190 columns (IAC) were used, by loading 10 ml of diluted sample. IAC were washed with 10 ml
191 of PBS solution and then with 10 ml of water and air dried. AFs were eluted with 3 ml of
192 methanol into an amber glass vial. The elute was evaporated at 65°C under air flow and 1 ml
193 of mobile phase was added to the precipitate and vortexed until dissolved. All samples were
194 analysed in triplicate.

195

196 2.5. *Ochratoxin A extraction and clean up*

197 The absence of OTA, avoiding positive matrix effect of fluorescence signal, was confirmed
198 by analysis through LC-MS/MS. The samples (4g) were put in a 50 mL centrifuge tube with
199 20 mL of extraction solution, methanol:water (80:20), and left for 5 minutes in ultrasonic
200 apparatus at 25°C. To separate the solid sample from the extraction solution, centrifugation
201 for 15 min at 6,000 rpm was applied. After centrifugation, the solution was diluted 1:4 with
202 PBS solution and filtered by cellulose acetate 0.45 μ m syringe filter. Ten ml of diluted and
203 filtered solution were loaded into IAC and, then, 10 ml of washing solution (2.5% NaCl and
204 0.25% NaHCO₃) and 10 ml of ultrapure water were added into column. Before eluting with 3
205 ml of methanol into an amber glass vial, the column was air dried. The elute was evaporated
206 at 65°C under air flow and 1 ml of eluent was added to the precipitate and vortexed until
207 dissolved. All samples were analyzed in triplicate.

208

209 *2.6. Method validation*

210 Validation of AFs and OTA methods was optimized studying apparent recovery rate (R%),
211 limits of detection (LOD) and quantification (LOQ), based on the IUPAC definition
212 (Thompson, Ellison, & Wood, 2002), and, to validate the extraction method and
213 chromatographic performances, capacity factor (k'), repeatability of recovery (RSD) and
214 capacity factor ($RSDk'$) were calculated. Following the guidelines of Commission Decision
215 2002/657/EC, these parameters were validated. The recovery (R%), RSD, LOD and LOQ
216 were determined on pepper, chili and four spices chosen by “others” (cinnamon, clove,
217 nutmeg and sesame) and two spice by “mixed” group (curry and food seasoning). Samples
218 were spiked with four concentrations of standard solutions of AFB₁ (0.5, 5, 10, 25 ng ml⁻¹)
219 for pepper, cinnamon and seasoning food and (1, 5, 10, 25 ng ml⁻¹) for chili, clove, nutmeg,
220 sesame and curry. Validation levels for total AFs were obtained spiking at 3, 5, 10, 25 ng ml⁻¹
221 ¹), and for OTA at 5, 10, 25, 50 ng ml⁻¹ with standard solutions. $RSDk'$ and k' were
222 calculated, by analysing spiked cinnamon, clove, food seasoning and pepper samples at 10 ng
223 ml⁻¹ of AFB₁, total AFs, and OTA. Each test was performed three times.

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

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

233 repeatability of chromatographic analysis and were calculated for each mycotoxin (IUPAC,
234 1997).

235

236 *2.7. Statistical analysis*

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

248

249 **3. Results and discussion**

250

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

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

258 agreement with a FAO report (FAOSTAT, 2012) about the main spice producers, most of the
259 analysed samples were coming from India (74), followed by North Africa (33), and China
260 (11).

261

262 *3.1. Analytical performance*

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

283 70% for all spices tested, despite the matrix complexity of some kind of spice, such as curry
284 and food seasoning, composed by different quantity of other spices. In the other hand the k'
285 and RSDk' values obtained reported in Table 3, demonstrated the high repeatability and
286 hardiness (RSDk'% < 0.1) of optimized chromatographic method, despite different spices
287 analysed.

288

289 3.2. Mycotoxin occurrence

290 AFs were found in 20 (15.4%) out of 130 samples analysed with levels ranging from 0.59 to
291 5.38 ng g⁻¹. The average concentration was 0.96 ng g⁻¹. Five samples of each typology of
292 spice (pepper, chili, mixed and others) resulted positive to one or more AFs. The AFs
293 contamination range was between 0.59 and 3.68 ng g⁻¹, with 11 (8.5%) samples contaminated
294 by AFB₁, with a mean on contaminated samples of 0.31 ng g⁻¹, 14 (4.6%) by AFB₂
295 (contamination mean: 1.07 ng g⁻¹), 3 (2.3%) by AFG₁ (0.42 ng g⁻¹), and 7 (5.4%) by AFG₂
296 (2.26 ng g⁻¹) (Table 4).

297 No sample was contaminated by AFs above the maximum admitted threshold established by
298 the European legislation (European Commission, 2010). In fact, the highest AFB₁ level
299 detected was 1.95 ng g⁻¹, 2.5 times lower than the maximum threshold specific for AFB₁. In
300 contrast with the results obtained by Romagnoli *et al.* (2007) on monitoring of AFs on spices
301 purchased in Italy, we detected a lower incidence of contamination and a lower level of AFs.
302 Although pepper and chili are usually more susceptible substrates to mycotoxins
303 contamination (Reddy, Mayi, Reddy, Thirumala-Devi, & Reddy, 2001), our results showed
304 no differences among the spice typologies analysed. The low amount of AF content detected
305 in our study agrees with the results reported by similar studies ((Jalili & Jinap, 2012; Ozbey &
306 Kabak, 2012). The incidence of positive samples was slightly higher in the mixed and chili
307 samples, 23.8% and 20.0%, respectively, compared to pepper (16.6%) and other (9.26%)

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

312 Considering the OTA contamination in the spice samples, the incidence resulted higher than
313 AFs: 23.8% spices were positive to OTA, while only 15.4% to AFs. The values of OTA
314 contamination ranged from LOD to 19.06 ng g⁻¹. The contamination OTA mean on positive
315 samples was 6.18 ng g⁻¹. Fifteen out of 25 (60%) chili samples were contaminated at levels
316 ranging from 2.16 to 16.35 ng g⁻¹, 7 out of 21 (33.3%) mixed samples at levels ranging from
317 1.84 to 19.06 ng g⁻¹. Pepper (13.3%) and other samples (9.3%) resulted positive ranging from
318 1.61 to 15.85 and from 2.21 to 11.08, respectively (Table 4).

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

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

328 The co-occurrence of OTA and AFs in spices was detected in 6 out of 130 samples(4.6%), in
329 particular in chili, pepper and curry samples. OTA and AFs concentration ranged from 1.61 to
330 15.85 ng g⁻¹ and 0.57 to 3.19 ng g⁻¹, respectively. Several studies were performed over the last
331 decade on the co-occurrence of AFs and OTA in spices. Most of the studies highlighted that
332 the reason for a high mycotoxin contamination is the susceptibility of spices to fungal

333 contamination, and consequently to mycotoxin production, connected to environmental
334 conditions, such as high humidity and temperature (Patharajan et al., 2011), of the producer
335 countries located in tropical and sub-tropical areas. Regarding the co-occurrence of AFs and
336 OTA in same substrate, Sedmikova, Reisnerova, Dufkova, Barta, & Jilek (2001)
337 demonstrated that OTA could increase the mutagenic activity of AFB₁. In agreement with our
338 results, most of previous work reported high incidence of AFs and OTA in pepper and chili.
339 In particular, during the last decade, AFs and OTA were monitored in over 1,500 spice
340 samples, resulting in a contamination incidence ranging from 30 to 70%. Despite the wide
341 range of spices present, attention mainly focused on pepper and chili, due to their high
342 demand in the market worldwide and to their susceptibility to fungal contamination,
343 especially during the drying processes (Shundo, de Almeida, Alaburda, Lamardo, Navas,
344 Ruvieri, et al., 2009). According to the available literature and to our results, chili and pepper
345 are more frequently contaminated by AFs and OTA than other spices, such as cumin or
346 cinnamon. This is the first study on AFs and OTA co-occurrence in a large number of spice
347 samples marketed in Italy. Future studies will also monitor the fungal population present on
348 the spices, which could be related to the AFs and OTA contamination, with microbiological
349 and molecular tools (Spadaro, Patharajan, Karthikeyan, Lorè, Garibaldi, & Gullino, 2011).

350

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355

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456 **Tables**

457

458 **Table 1.** Geographical origin of spice samples.

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Origin	Spices			
	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

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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

G₂

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

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

Mycotoxins	Positive/total (%)	Mean of contamination \pm SD (ng g ⁻¹)	Number of positive samples (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±0.025	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					

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