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# Co-occurrence of toxigenic moulds, aflatoxins, ochratoxin A, *Fusarium* and *Alternaria* mycotoxins in fresh sweet peppers (*Capsicum annuum*) and their processed products

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**RESEARCH ARTICLE**

## Abstract

Forty-five samples of a landrace of sweet pepper (*Capsicum annuum*) widely cultivated in Basilicata (Italy) were screened for 17 mycotoxins and potential toxigenic fungal species. Two different LC-MS/MS methods were used for the determination of aflatoxins, ochratoxin A (OTA), *Fusarium* mycotoxins zearalenone (ZEA), fumonisins ($FB_1$ and $FB_2$), nivalenol (NIV), deoxynivalenol (DON), T-2 and HT-2 toxins and *Alternaria* mycotoxins altenuene (ALT), alternariol (AOH), alternariol monomethyl ether (AME), tentoxin (TTX) and tenuazonic acid (TeA). Frequency of potential toxigenic fungal species occurrence was: 87% *Aspergillus* Sect. *Nigri;* 58% *Aspergillus* Sect. *Flavi*; 38% *Aspergillus* Sect. *Circumdati*; 42% *Alternaria* spp.; 33% *Penicillium* spp. and 20% *Fusarium* spp. Frequency of mycotoxin occurrence and mean of positives were: 51% OTA, 29.5 µg/kg, 5 samples above the EU limit of 20 µg/kg; 31% aflatoxins, 12.8 µg/kg, two samples above the EU limit of 5 µg/kg for aflatoxin $B_1$; 91% ZEA, 1.4 µg/kg; 78% $FB_2$, 7.6 µg/kg; 58% $FB_1$, 22.8 µg/kg; 38% NIV, 39.5 µg/kg; 36% DON, 6.9 µg/kg; 20% T-2 toxin, 5.6 µg/kg and 22% HT-2 toxin, 13.8 µg/kg. For the *Alternaria* mycotoxins, 100% of samples contained TeA, 4817.9 µg/kg; 93% TTX, 29.7 µg/kg; 56% AOH, 114.4 µg/kg; 33% AME, 13.0 µg/kg and 9% ALT, 61.7 µg/kg. Co-occurrence of mycotoxins in each sample ranged from 2 to 16 mycotoxins (mean 7). No statistical correlation was found between moulds and their mycotoxins occurrence. Within the four groups of peppers collected herein (fresh, dried, grounded and fried) higher percentages of contamination and mycotoxin levels were measured in grounded peppers, whereas much lower values were observed for fried peppers. The high percentages of positive samples and the high levels of some mycotoxins observed in this study confirm the susceptibility of peppers to mycotoxin contamination and claims for an improvement of the conditions used during production and drying process.

**Keywords:** LC-MS/MS, multi-mycotoxin analysis, processing, drying, toxigenic fungi

## 1. Introduction

Sweet pepper (*Capsicum annuum*) originates from America and is widely cultivated in several regions all over the world having the favourable climatic and soil conditions. A particular landrace of *C. annuum* is extensively cultivated in Basilicata, a region in south Italy. In Basilicata sweet pepper is planted between February and March and harvested in early August when the fruits are coloured a deep red purple. The fruits are mostly sun dried and stored as long necklaces ('serte') prepared by threading the fresh fruits with needle and string. Good manufacturing practices are important during drying of the peppers, they can be of great concern, especially when poor hygienic conditions and uncontrolled water activity ($a_w$) occurs during the drying process and storage of peppers. In this respect, fungi that belong mainly to the genera *Aspergillus* and *Penicillium* could pose a serious problem of spoilage and mycotoxin contamination of these products; they were also reported as the most commonly found moulds on *Capsicum* powder

(Santos *et al.,* 2008). Instead, fungi belonging to *Alternaria* and *Fusarium* species, known as field fungi, could be of phyto-pathological and mycotoxicological risk during the field growth and ripening of the pepper. There have been various reports on the occurrence of mycotoxigenic fungi and mycotoxins in chilly, paprika, and red pepper (Ham *et al.,* 2016; Salari *et al.,* 2012; Santos *et al.,* 2011). Both storage fungi, like *Aspergillus* and *Penicillium* with their main important mycotoxins, aflatoxins and ochratoxin A (OTA), and field fungi, like *Alternaria* and *Fusarium* and their mycotoxins (zearalenone (ZEA), thricothecenes and *Alternaria* mycotoxins) were reported (Da Cruz Cabral *et al.,* 2016; Santos *et al.*, 2008, 2011; Van de Perre *et al.,* 2014). On the other hand, few data are available on mycotoxigenic fungi and multi-mycotoxin occurrence on sweet pepper and no data are available on sweet pepper cultivated in Basilicata region. In this survey, for the first time a broad range of mycotoxins and toxigenic fungi has been investigated on this important typical product of the Basilicata region (Italy).

## 2. Materials and methods

### Reagents and chemicals

Chromatography-grade methanol (MeOH), acetonitrile (ACN), n-hexane, glacial acetic acid, ammonium acetate (for mass spectrometry), dimethyl sulfoxide (DMSO) and phosphate-buffered saline (PBS) tablets were purchased from Sigma-Aldrich (Milan, Italy). Ultrapure water was produced by a Milli-Q system (Millipore, Bedford, MA, USA). Mycotoxin standard mixed aflatoxins, containing aflatoxin $B_1$ ($AFB_1$) (2.0 µg/ml), aflatoxin $B_2$ ($AFB_2$) (0.5 µg /ml), aflatoxin $G_1$ ($AFG_1$) (2.01 µg/ml) and aflatoxin $G_2$ ($AFG_2$) (0.5 µg/ml) in acetonitrile, OTA (10 µg/ml), deoxynivalenol (DON) (100.3 µg/ml), T-2 toxin (T-2) (100.2 µg/ml), HT-2 toxin (HT-2) (100.2 µg/ml), nivalenol (NIV) (100.8 µg/ml), ZEA (100.2 µg/ml), altenuene (ALT) (10 µg/ml), alternariol (AOH) (100 µg/ml), tentoxin (TTX) (100 µg/ml), alternariol methyl ether (AME) (100 µg/ml), tenuazonic acid (TeA) (100 µg/ml) in acetonitrile and the mixed fumonisins standard solution containing fumonisin $B_1$ ($FB_1$) (50 µg/ml), and fumonisin $B_2$ ($FB_2$) (50 µg/ml), in acetonitrile:water (50:50, v/v), were purchased from Romer Labs Diagnostic (Tulln, Austria). Myco6in1$^+$ multiantibody immunoaffinity (IMA) columns were purchased from Vicam L.P. (Watertown, MA, USA). Oasis HLB solid phase extraction (SPE) columns, 60 mg, 3 ml were purchased from Waters (Milford, MA, USA). Polytetrafluoroethylene (PTFE) filters (0.45 µm) and regenerated cellulose filters (0.45 µm) were purchased from Sartorius Stedim Biotech (Goettingen, Germany).

### Samples

45 samples of sweet peppers were collected in the inland areas of the Basilicata region, south Italy. In particular, 26 samples were collected in the province of Potenza (municipalities of Lavello, Palazzo San Gervasio, Banzi, Genzano di Lucania, Chiaromonte, Senise and Noepoli) and 19 samples were collected in the province of Matera (municipalities of Matera, Bernalda, Pisticci, Tursi, Rotondella and Nova Siri). The 45 samples were grouped in four categories: fresh peppers (7 samples of whole fresh peppers), dried peppers (23 samples of whole dried peppers), ground peppers (8 samples of dried and ground peppers), fried peppers (7 samples of dried and fried peppers). Samples of fresh peppers were dried overnight at 50 °C in the oven before mycotoxin analysis. Each sample was finely ground, blended and submitted to microbiological analyses and chemical analysis for the determination of aflatoxins, OTA, ZEA, fumonisins, thrichothecenes and *Alternaria* mycotoxins.

### LC-MS/MS equipment

LC-MS/MS analyses were performed on a triple quadrupole API 5000 system (Applied Biosystems, Foster City, CA, USA), equipped with a ESI interface and an Acquity UPLC system comprising a binary pump and a micro autosampler from Waters (Milford, MA, USA). Interface conditions were: TEM, 450 °C; CUR, nitrogen, 20 psi; GS1, air, 60 psi; GS2, air, 40 psi; ionspray voltage +5,500 V or -4,500 V.

Quantification of mycotoxins in the 45 dried and grounded pepper samples was performed by measuring peak areas in the MRM chromatogram, and comparing them with the relevant matrix-matched calibration curves. The calibration ranges in matrix ranged between: 0.7-185.9 µg/kg for $AFB_1$ and $AFG_1$, 0.2-46.5 µg/kg for $AFB_2$ and $AFG_2$, 0.1-203.4 µg/kg for OTA, 5.2-557.0 µg/kg for DON, 0.9-111.2 µg/kg for T-2 and HT-2, 0.5-557.0 µg/kg for NIV, 0.04-102.0 µg/kg for ZEA, 0.6-555.0 µg/kg for $FB_1$ and $FB_2$, 1.0-100.0 µg/kg for ALT, AOH and AME, 5.0-500.0 µg/kg for TTX and 10.0-1000.0 µg/kg for TeA.

### LC-MS/MS parameters for the determination of mycotoxins

#### *Aflatoxins, ochratoxin A, zearalenone, fumonisins and trichothecenes*

The separation of aflatoxins, OTA, ZEA, fumonisins and trichothecenes was performed using an Acquity UPLC BEH phenyl analytical column (2.1×150 mm, 1.7 µm particles; Waters). The column oven was set at 40 °C. The flow rate of the mobile phase was 250 µl/min and the injection volume was 10 µl. For analytes separation a binary linear gradient of acidic MeOH (containing 0.5% acetic acid) in

water (containing 0.5% acetic acid) was developed and used as mobile phase as follows: from 20% to 80% MeOH in 5 min, then maintained at 80% MeOH for 5 min, then brought to 20% MeOH in 0.5 min and left to equilibrate for 4.5 min before the next run. For LC-MS/MS analyses, the ESI interface was used in positive ion mode for $AFB_1$, $AFB_2$, $AFG_1$, $AFG_2$, $FB_1$, $FB_2$, T-2, HT-2 and OTA and in negative ion mode for NIV, DON and ZEA. The mass spectrometer operated in MRM (multiple reaction monitoring) mode. The optimized MS/MS conditions for these mycotoxins are listed in Table 1, in particular, three transitions for confirmation and one transition for quantification for all mycotoxins except for aflatoxins. For $AFB_1$, $AFB_2$, $AFG_1$ and $AFG_2$ two transitions for confirmation and one transition for quantification are given.

*Alternaria mycotoxins*

The separation of ALT, AOH, TTX, AME and TeA was performed using an Acquity UPLC BEH C18 analytical column (2.1×100 mm, 1.7 µm particles; Waters). The column oven was set at 30 °C. The flow rate of the mobile phase was 300 µl/min and the injection volume was 10 µl. For the analytes separation a binary linear gradient of acidic

**Table 1. MS/MS parameters for aflatoxins, ochratoxin A, zearalenone, fumonisins and trichothecenes by the multiple reaction monitoring (MRM) method.[a]**

| Analyte[c] | Precursor ion | Q1 (*m/z*) | Q3 (*m/z*) | DP (V) | EP (V) | CE (V) | CXP (V) |
|---|---|---|---|---|---|---|---|
| $AFB_1$ | $[AFB_1+H]^+$ | 313.4 | 241.2[b] | 160 | 10 | 52 | 13 |
| | | | 213.4 | | | 61 | 14 |
| $AFB_2$ | $[AFB_2+H]^+$ | 315.3 | 287.4 | 150 | 10 | 37 | 15 |
| | | | 259.4[b] | | | 42 | 13 |
| $AFG_1$ | $[AFG_1+H]^+$ | 329.2 | 311.4 | 150 | 10 | 31 | 13 |
| | | | 243.3[b] | | | 38 | 16 |
| $AFG_2$ | $[AFG_2+H]^+$ | 331.1 | 313.3 | 140 | 10 | 35 | 17 |
| | | | 245.3[b] | | | 43 | |
| OTA | $[OTA+H]^+$ | 404.2 | 358.5 | 90 | 10 | 30 | 10 |
| | | | 257.3 | | | 38 | |
| | | | 239.2[b] | | | 47 | |
| NIV | $[NIV+CH_3COO]^-$ | 371.2 | 311.1 | -23 | -6 | -19 | -2 |
| | | | 281.0[b] | | | -19 | -2 |
| | | | 59.1 | | | -38 | -6 |
| DON | $[DON+CH_3COO]^-$ | 355.0 | 295.2 | -50 | -10 | -15 | -20 |
| | | | 265.0 | | -10 | -22 | |
| | | | 59.0[b] | | -4 | -35 | |
| $FB_1$ | $[FB_1+H]^+$ | 722.4 | 370.6 | 50 | 10 | 55 | 10 |
| | | | 334.7[b] | | | 65 | |
| | | | 316.6 | | | 66 | |
| $FB_2$ | $[FB_2+H]^+$ | 706.4 | 354.6 | 70 | 10 | 49 | 15 |
| | | | 336.3[b] | | | 49 | |
| | | | 318.5 | | | 54 | |
| ZEA | $[ZEA-H]^-$ | 317.2 | 273.3 | -100 | -10 | -29 | -10 |
| | | | 175.0 | | | -35 | |
| | | | 131.0[b] | | | -40 | |
| HT-2 | $[HT-2+H]^+$ | 442.4 | 263.5 | 60 | 10 | 19 | 10 |
| | | | 241.2 | 160 | | 52 | 13 |
| | | | 215.2[b] | 60 | | 19 | 10 |
| T-2 | $[T-2+H]^+$ | 484.3 | 245.2 | 45 | 7 | 30 | 3 |
| | | | 215.2[b] | | | | |
| | | | 185.3 | | | | |

[a] CE = collision energy; CXP = collision cell exit potential; DP = declustering potential; EP = entrance potential; Q1 = first quadrupole; Q3 = third quadrupole.
[b] Transitions used for quantification.
[c] $AFB_1$ = aflatoxin $B_1$; $AFB_2$ = aflatoxin $B_2$; $AFG_1$ = aflatoxin $G_1$; $AFG_2$ = aflatoxin $G_2$; DON = deoxynivalenol; $FB_1$ = fumonisin $B_1$; $FB_2$ = fumonisin $B_2$; HT-2 = HT-2 toxin; NIV = nivalenol; OTA = ochratoxin A; T-2 = T-2 toxin; ZEA = zearalenone.

MeOH in water (5 mM ammonium acetate, pH 8.7) was used as mobile phase as follows: 10% MeOH for one min then brought to 100% MeOH in 10 min, then maintained at 100% MeOH for eight min, then brought to 10% MeOH in 0.5 min and left to equilibrate for 4.5 min before the next run. For LC-MS/MS analyses, the ESI interface was used in negative ion mode for *Alternaria* mycotoxins. The mass spectrometer operated in MRM (multiple reaction monitoring) mode. The optimised MS/MS conditions for *Alternaria* mycotoxins are listed in Table 2, in particular, four transitions for confirmation and one transition for quantification for all mycotoxins.

## Determination of mycotoxins

### *Aflatoxins, ochratoxin A, zearalenone, fumonisins and trichothecenes*

The LC-MS/MS method previously described (Lattanzio *et al.,* 2007) was used herein with some modifications depending on the nature of samples analysed. In particular, 10 g of grounded pepper was first extracted with 80 ml phosphate buffered saline (PBS), by shaking for 60 min using an orbital shaker (model 711 VDRL, Asal, Milan, Italy). After centrifugation at 3,000×*g* for 10 min (Allegra X-22R centrifuge, Beckman Coulter, Palo Alto, CA, USA), 35 ml of PBS extract (extract A) was collected and filtered through a glass microfiber filter. Then 105 ml methanol was added to the remaining solid material containing 45 ml PBS, and the sample was extracted again by shaking for 60 min (in this way the extraction solvent was 70% aqueous methanol). After centrifugation (3,000×*g*, 10 min), 10 ml of methanol/PBS extract was diluted with 90 ml PBS and filtered through a glass microfiber filter (extract B). The two extracts (A and B) were separately submitted to clean-up through the same IMA column. In particular, 50 ml of extract B was passed through the IMA column at 1-2 drops per second; the column was then washed with 20 ml PBS to completely remove methanol residues. After passing 5 ml of extract A that was eluted at 1-2 drops per second, the column was washed with 10 ml distilled water. Mycotoxins were eluted from the column with 2×1.5 ml methanol and 2 ml of water. The methanolic eluate was dried under an air stream at 50 °C and reconstituted with 200 µl methanol:water (20:80, v/v), containing 1 mM ammonium acetate and 0.5% acetic acid, filtered with a regenerated cellulose filter and analysed by LC-MS/MS. The following modifications to the method of Lattanzio *et al.* (2007) were used: increased volume of PBS solution for extraction from 50 to 80 ml, the volume of methanol for extraction from 35 to 105 ml; and to completely elute fumonisins from the immunoaffinity column 2 ml water

**Table 2. MS/MS parameters for *Alternaria* mycotoxins by the multiple reaction monitoring (MRM) method.[a]**

| Analyte[c] | Precursor ion | Q1 (*m/z*) | Q3 (*m/z*) | DP (V) | EP (V) | CE (V) | CXP (V) |
|---|---|---|---|---|---|---|---|
| ALT | $[ALT-H]^-$ | 291.4 | 202.9 | -160 | -5 | -45 | -15 |
| | | | 248.0 | | | -35 | |
| | | | 160.9[b] | | | -58 | |
| | | | 188.8 | | | -46 | |
| AOH | $[AOH-H]^-$ | 257.2 | 215.0 | -150 | -3 | -35 | -15 |
| | | | 147.1 | | | -44 | |
| | | | 185.2[b] | | | -38 | |
| | | | 156.9 | | | -40 | |
| TTX | $[TEN-H]^-$ | 413.5 | 141.1 | -150 | -5 | -30 | -15 |
| | | | 213.8[b] | -150 | | -35 | |
| | | | 271.2 | -160 | | -23 | |
| | | | 339.2 | -160 | | -39 | |
| AME | $[AME-H]^-$ | 271.4 | 256.0 | -120 | -10 | -30 | -18 |
| | | | 227.9 | | | -40 | -16 |
| | | | 213.2 | | | -50 | -15 |
| | | | 183.0[b] | | | -55 | -12 |
| TeA | $[TEA-H]^-$ | 196.2 | 111.7 | -100 | -4 | -34 | -10 |
| | | | 139.1 | | | -28 | |
| | | | 69.0[b] | | | -62 | |
| | | | 82.8 | | | -58 | |

[a] Q1 = first quadrupole; Q3 = third quadrupole; DP = declustering potential; EP = entrance potential; CE = collision energy; CXP = collision cell exit potential.
[b] Transitions used for quantification.
[c] ALT = altenuene; AME = allternariol methyl ether; AOH = alternariol; TeA = tenuazonic acid; TTX = tentoxin.

was passed after methanol. To improve the values of the limit of detection (LOD) and limit of quantification (LOQ) a powerful LC-MS/MS apparatus was used.

*Alternaria mycotoxins*

2 g of dried peppers was extracted with 15 ml MeOH:$H_2O$: acetic acid (80:19:1, v/v/v). The sample was shaken manually for 10 s to obtain a homogeneous suspension and then extracted for 45 min at room temperature using and orbital shaker. After centrifugation (4,000×*g*, 10 min), 7.5 ml of extract was diluted with 7.5 ml of 1% (v/v) aqueous acetic acid solution and filtered through a glass microfiber filter. After a preconditioning of the SPE column with 7 ml methanol, followed by 7 ml water and 3 ml 1% (v/v) acetic acid solution, the column was closed and 3 ml 1% (v/v) acetic acid solution was loaded into the SPE column, again. Then, a diluted sample extract was loaded in the SPE column and then opened. The empty test tube that contained the diluted extract was washed with 3 ml 1% (v/v) acetic acid solution and loaded into the SPE column. After elution the column was washed with 7 ml 1% (v/v) aqueous acetic acid solution and with 7 ml *n*-hexane that were discarded, then the column was dried for 10 s with vacuum. The *Alternaria* mycotoxins were eluted from the column into a glass vial containing 100 µl DMSO by passing 6 ml methanol. After the methanol passed, the column was dried for 10 s with vacuum. The methanolic eluate was evaporated to 100 µl at 50 °C using the sample concentrator and a gentle stream of nitrogen. The concentrated sample was vortex-mixed for at least 15 s to re-dissolve the purified sample residues. Afterwards, the DMSO solution was diluted to 1.0 ml with water, filtered with a PTFE syringe filter and analysed by LC-MS/MS.

## Recovery experiments

A mixture of dried and grounded pepper (#1, #14, #16, #30, #31 and #37) was used for recovery experiments of aflatoxins, OTA, fumonisins, ZEA and trichothecenes. For recovery experiments of *Alternaria* mycotoxins, a dried and grounded pepper sample (#17) was used. Triplicate experiments were performed, and recoveries were calculated after subtracting the measured levels of endogenous mycotoxins. The calibration ranges in matrix were between: 0.11-185.9 µg/kg for $AFB_1$ and $AFG_1$, 0.03-46.5 µg/kg for $AFB_2$ and $AFG_2$, 0.1-203.4 µg/kg for OTA, 5.2-557.0 µg/kg for DON, 0.20-111.2 µg/kg for T-2 and HT-2, 0.5-557.0 µg/kg for NIV, 0.1-102.0 µg/kg for ZEA, 0.9-555.0 µg/kg for $FB_1$ and $FB_2$, 0.1-150.0 µg/kg for ALT, 0.1-200 µg/kg for AOH, 0.1-100.0 µg/kg for AME, 0.5-500.0 µg/kg for TTX and 1.0-1000.0 µg/kg for TeA. LOD and LOQ were calculated as 3 times and 10 times the noise, respectively.

## Microbiological analyses

Ten gram of ground samples of typical sweet peppers (*C. annum*) of the Basilicata region were weighted in a sterile plastic container and diluted with 90 ml of sterile distilled water. 100 µl aliquots of appropriate serial decimal dilutions were plated, in triplicate, on Dichloran Rose-Bengal Chloramphenicol Agar (DRBC; Oxoid, Basingstoke, UK) plates. DRBC plates were incubated at 25 °C for 3-5 days. After incubation, all fungal colonies visible on plates were counted and the number of colonies were expressed as cfu/g.

Pure cultures of observed fungal colonies were obtained through monosporic isolation method, preliminary identifications were carried out performing morphological and microscopical analyses. For correct identification at species level the selected pure culture was subjected to sequence analysis by DNA extraction from the pure culture. Wizard® Magnetic DNA Purification System for Food (Promega, Madison, WI, USA) was used to extract total fungal DNA and identifications were confirmed by amplification and sequencing of approximately 500 bp fragments of BenA (coding for the beta-tubulin protein) and 700 bp fragments of CaM (coding for the calcium binding protein calmodulin). Bt2a-Bt2b (Glass and Donaldson, 1995) and CL1-CL2A (O'Donnell *et al.*, 2000) primer pairs were used, respectively.

PCR amplifications were carried out with 5Prime HotMaster Taq DNA Polymerase, nucleotides and buffer (Quanta Bio, Beverly, MA, USA). The PCR reaction mixture contained 1× HotMaster Taq Buffer with $Mg^{2+}$, 200 µM dNTP Mix, 1 U HotMaster Taq DNA Polymerase, 0.1 µM of each primers and 50-100 ng of template DNA. PCR amplification conditions were as follows: for the BenA region an initial denaturation step at 94 °C for 5 min, followed by 35 cycles, 94 °C for 30 s, 58 °C for 50 s, 72 °C for 50 s, and a final elongation step 72 °C for 5 min; for the CaM region an initial denaturation step at 94 °C for 5 min, followed by 40 cycles, 94 °C for 30 s, 55 °C for 30 s, 72 °C for 50 s, and a final elongation step 72 °C for 5 min.

Amplifications were evaluated by electrophoresis of the PCR products in 1.5% agarose gel for 50 min at 80 V in Tris-acetate-EDTA buffer. Sequencing was performed using an AB 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). BigDye Terminator cycle sequencing kit (version 3.1; Applied Biosystems) was used following the manufacturer's manual on both strands by the same primers. Sequence alignment search tool (BLAST) in GenBank (www.ncbi.nlm.nih.gov/blast) was queried after aligning, editing and trimming the sequences by Geneious R10 (http://www.geneious.com, Kearse *et al.*, 2012).

### Statistical analyses

Mean, median and standard error of the mean were calculated using MS Excel 2013 software (Microsoft Corporation, Redmond, WA, USA). SigmaPlot (Systat Software, Inc., San Jose, CA, USA) was used to perform one-way ANOVA followed by Tukey's pairwise multiple-comparison test and a least significant difference (LSD) test at 95% confidence levels ($P$=0.05) to identify significant differences among the four groups: fresh peppers, dried peppers, grounded peppers and fried peppers. For statistical evaluations the negative results (ND) were treated as 1/2 LOD and those <LOQ but >LOD as 1/2 LOQ.

Qualitative correlations between the occurrences of couples of two mycotoxins were performed using the contingency analysis calculating the factor Φ according the formula reported by Hickert *et al.* (2017). Φ factor describes the degree to which the presence of one toxin influences the presence of a second toxin and ranges between -1 (negative correlation) and 1 (positive correlation) and 0 indicates no correlation. Φ ≥0.3 represents a significant relationship between the two analytes. If one of the compared mycotoxins occurs in all samples analysed, no contingency can be calculated (Hickert *et al.*, 2016, 2017).

## 3. Results

The analytical method for the five *Alternaria* mycotoxins used herein was developed within the mandate M/520 (EC, 2013) and is a modified version of the LC-MS/MS method published by Töelgyesi *et al.* (2015). For the determination of aflatoxins, OTA and *Fusarium* mycotoxins we have used a method originally developed for maize (Lattanzio *et al.*, 2007) with minor modifications. In particular, NIV was added in the panel of analysed mycotoxins and the ratio extraction solvent/test portion size was increased, due to the intrinsic characteristics of dried pepper that adsorb more solvent compared to maize flour. The two methods were applicable for the analysis of sweet pepper, as mean recoveries and repeatability of results obtained for spiked samples were all acceptable with the exception of the mean recovery of ALT that was 45%. The results of in-house validation of the two LC-MS/MS methods for sweet pepper are reported in Table 3 and 4. The good sample clean-up and the use of a modern/powerful LC-MS/MS apparatus allowed us to obtain low LOD and LOQ values for the 17 analysed mycotoxins. In particular, for *Alternaria* mycotoxins LODs and LOQs ranged between 0.05 to 0.69 µg/kg and 0.16-2.29 µg/kg, respectively. For the other 12 mycotoxins these values were 0.005-0.24 µg/kg for LODs and 0.017-0.81 µg/kg for LOQs.

### Mycotoxin occurrence

The results of the occurrence of the 17 mycotoxins monitored in this study in fresh, dried, grounded and fried sweet peppers are reported in Table 5. They were grouped according to the appearance and processing methods, i.e. fresh peppers (n=7), dried peppers (n=23), grounded peppers (n=8) and fried peppers (n=7). High percentages of contaminated samples were observed for *Alternaria* mycotoxins. The highest percentage of positive samples for the five *Alternaria* mycotoxins was observed for fresh (43-100%) and grounded peppers (13-100%), followed by dried (0-100%) and fried peppers (0-100%).

TeA was detected in all samples of the four groups reported in Table 5. Very high levels of TeA were observed particularly in fresh samples (mean 27,032 µg/kg) followed by dried samples (mean 914 µg/kg), grounded samples (mean 623 µg/kg) and fried samples (mean 225 µg/kg). TTX was also quite widespread in the four groups (87-100% positives) followed by AOH (14-100%), AME (14-75%) and ALT (0-43%). Comparable levels of AOH, AME, TTX and ALT were measured in positive samples of fresh, dried and grounded peppers, whereas in fried samples the levels were definitely lower and ALT was not detected in all fried samples. TeA levels above the action limit established by the Bavarian Health Protection Authority for millet and sorghum based baby food (500 µg/kg), were measured in 15/45 samples i.e. 4 fresh peppers, 8 dried peppers, 2 grounded peppers and 1 fried peppers.

**Table 3. Results of in-house validation of the LC-MS/MS method for *Alternaria* mycotoxins in sweet pepper.[1]**

| Mycotoxins | Levels (µg/kg) | Recovery (%) | $RSD_r$ (%) | LOD (µg/kg) | LOQ (µg/kg) |
|---|---|---|---|---|---|
| ALT | 23 | 45 | 17 | 0.69 | 2.29 |
| AOH | 10 | 96 | 11 | 0.30 | 0.99 |
| AME | 1.6 | 97 | 4 | 0.047 | 0.16 |
| TTX | 21 | 82 | 3 | 0.63 | 2.1 |
| TeA | 12.5 | 106 | 19 | 0.37 | 1.24 |

[1] ALT = altenuene; AME = allternariol methyl ether; AOH = alternariol; TeA = tenuazonic acid; TTX = tentoxin; $RSD_r$ = relative standard deviation of repeatability; LOD = limit of detection; LOQ = limit of quantification.

**Table 4. Results of in-house validation of the LC-MS/MS method for aflatoxins, ochratoxin A, fumonisins, zearalenone and trichothecenes in sweet pepper.[1]**

| Mycotoxins | Levels (µg/kg) | Recovery (%) | $RSD_r$ (%) | LOD (µg/kg) | LOQ (µg/kg) |
|---|---|---|---|---|---|
| NIV | 2.02 | 118 | 9 | 0.06 | 0.20 |
| DON | 6.12 | 119 | 8 | 0.18 | 0.61 |
| $FB_1$ | 8.01 | 65 | 6 | 0.24 | 0.81 |
| $FB_2$ | 3.5 | 100 | 10 | 0.11 | 0.36 |
| ZEA | 0.4 | 82 | 19 | 0.012 | 0.04 |
| HT-2 | 0.7 | 105 | 5 | 0.022 | 0.072 |
| T-2 | 2.01 | 96 | 1 | 0.060 | 0.20 |
| $AFB_1$ | 0.32 | 79 | 3 | 0.010 | 0.033 |
| $AFB_2$ | 0.08 | 90 | 8 | 0.005 | 0.017 |
| $AFG_1$ | 0.32 | 79 | 4 | 0.011 | 0.035 |
| $AFG_2$ | 0.08 | 98 | 8 | 0.030 | 0.11 |
| OTA | 2.2 | 73 | 4 | 0.070 | 0.22 |

[1] $AFB_1$ = aflatoxin $B_1$; $AFB_2$ = aflatoxin $B_2$; $AFG_1$ = aflatoxin $G_1$; $AFG_2$ = aflatoxin $G_2$; DON = deoxynivalenol; $FB_1$ = fumonisin $B_1$; $FB_2$ = fumonisin $B_2$; HT-2 = HT-2 toxin; NIV = nivalenol; OTA = ochratoxin A; T-2 = T-2 toxin; ZEA = zearalenone; $RSD_r$ = relative standard deviation of repeatability; LOD = limit of detection; LOQ = limit of quantification.

In Figure 1, the mean levels of each *Alternaria* mycotoxin measured in the four groups of peppers are compared and statistically evaluated. The mean levels of TeA and TTX followed the trend fresh > dried > grounded > fried peppers. A similar trend was observed for AOH and AME but the highest levels were measured in grounded peppers. Fried peppers showed the lowest mycotoxins mean levels and in most cases they were statistically different from the levels measured in the other three groups of peppers (Figure 1).

Aflatoxins were detected in 88% of grounded peppers, 22% of dried peppers, and 14% of fresh and fried peppers (Table 5). The highest mean level of total aflatoxins in positive samples was measured in dried peppers (105.9 µg/kg), followed by fresh peppers (13.9 µg/kg), grounded peppers (1.7 µg/kg) and fried peppers (0.056 µg/kg). One sample of dried peppers contained very high levels of aflatoxins ($AFB_1$ 155.7 µg/kg, $AFB_2$ 9.9 µg/kg, $AFG_1$ 318.1 µg/kg, $AFG_2$ 45.4 µg/kg, total aflatoxins 529.1 µg/kg) which were largely above the EU limits of $AFB_1$ (5 µg/kg) and total aflatoxins (10 µg/kg). Another sample of fresh pepper contained $AFB_1$ (13.5 µg/kg) above the EU limit.

All samples of grounded peppers were contaminated with OTA, followed by fresh peppers (57%), dried peppers (39%) and fried peppers (29%). The highest mean level of OTA was measured in dried peppers (53.9 µg/kg) followed by grounded peppers (23.6 µg/kg), fried peppers (0.71 µg/kg) and fresh peppers (0.59 µg/kg). An OTA level above the EU limit (20 µg/kg) was recorded in three samples of dried peppers (142.5, 160.7 and 177.4 µg/kg) and two samples of grounded peppers (42.3 and 101.1 µg/kg). Very low levels of OTA and aflatoxins were measured in the three positive samples of fried peppers (two samples contained OTA 0.69 and 0.74 µg/kg, and one sample 0.04 µg/kg total aflatoxins).

A high frequency of positive samples was also observed for *Fusarium* mycotoxins. ZEA was quite widespread in the four groups of peppers since the percentages of positive samples were 100% for fresh and fried peppers, 88% for grounded peppers and 87% for dried peppers. However, mean levels were all ≤2.8 µg/kg, while the highest level was 53.6 µg/kg. When comparing the four groups, the highest percentage of contamination was observed for grounded peppers, since NIV, $FB_1$ and $FB_2$ were detected in 100% of samples and 50-88% of samples also contained ZEA, DON, T-2 and HT-2 (Table 5).

In total, the measured levels of each *Fusarium* mycotoxin in the 45 samples were always <250 µg/kg and the highest level was recorded for NIV and $FB_1$ (243.9 µg/kg). Co-occurrence of $FB_1$ and $FB_2$ was observed in 65% of samples containing $FB_1$ and/or $FB_2$ (24/37). $FB_2$ alone was recorded in 30% (11/37) and $FB_1$ alone in 5% (2/37) of positive samples. Within the 25 samples containing DON and/or NIV, DON+NIV co-occurred in 32% of samples (8/25), DON and NIV occurred alone in 32% (8/25) and 36% (9/25) of positive samples, respectively. For the 41 samples positive to ZEA, 56% (23/41) contained DON and/or NIV, whereas the remaining samples representing 44% (18/41) were negative for DON and NIV. T-2 and HT-2 co-occurred in almost all samples positive for T-2 or HT-2.

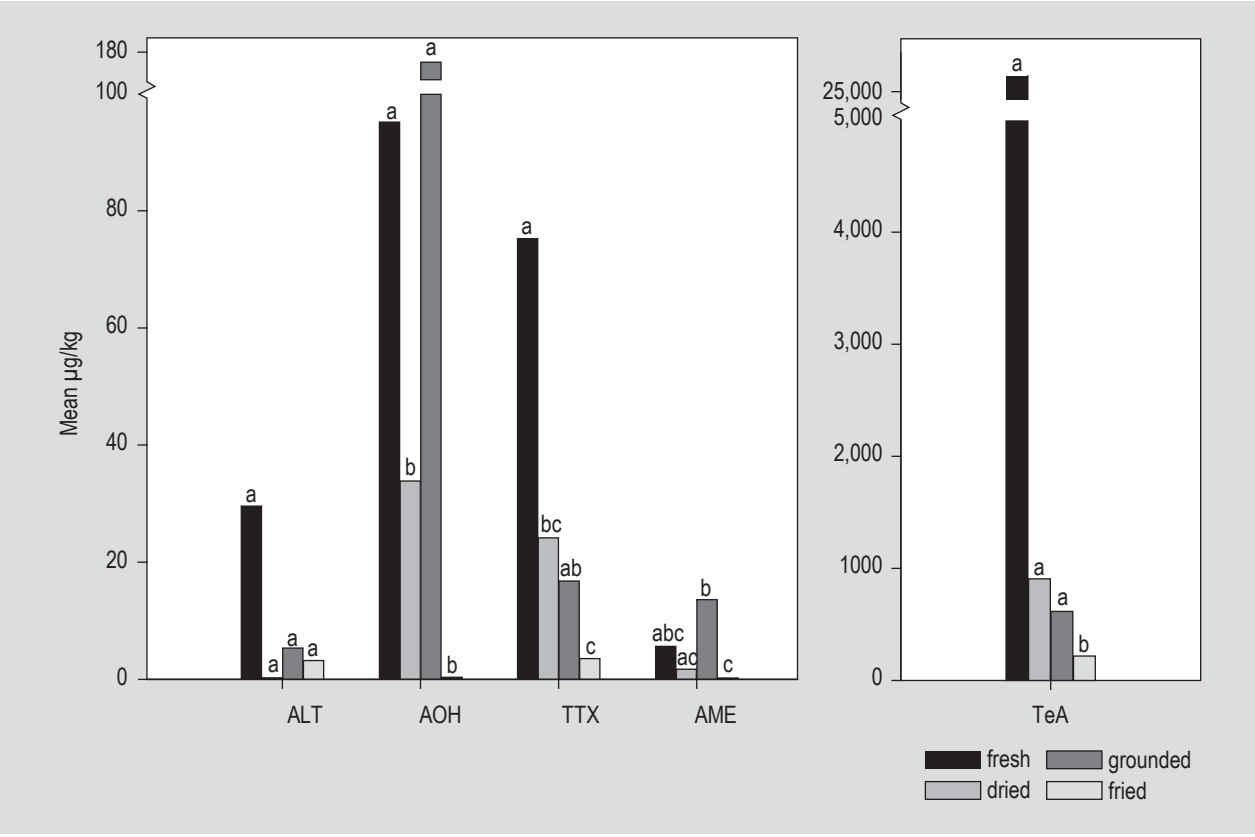


**Figure 1. Mean levels (µg/kg) of *Alternaria* mycotoxins in fresh, dried, grounded and fried peppers. ALT = altenuene; AME = allternariol methyl ether; AOH = alternariol; TeA = tenuazonic acid; TTX = tentoxin. Different letters indicate significant differences between groups (*P*<0.05).**

In Figure 2, the mean levels of aflatoxins, OTA and *Fusarium* mycotoxins measured in the four groups of peppers are compared and statistically evaluated. The mean levels of $FB_1$ followed the same trend as TeA and TTX, i.e. fresh > dried > grounded > fried peppers. In contrast the trend of $FB_2$ was different; its mean levels in dried and grounded peppers were higher than in fresh peppers. The mean levels of the other monitored mycotoxins varied between the four groups of peppers (Figure 2). Again, as observed for *Alternaria* mycotoxins, fried samples showed the lowest mean levels of aflatoxins, OTA and *Fusarium* mycotoxins.

The results of the statistical contingency analysis are reported in Table 6. Obviously, the best positive correlation (Φ=1.0) was found between $AFB_1$ and total aflatoxins. A strong and significant positive correlation was found between HT-2 and DON (Φ=0.72) followed by $FB_1$ and AOH (Φ=0.59), DON and T-2 (Φ=0.56) and NIV and HT-2 (Φ=0.56). As expected, significant positive correlations were observed between HT-2 and T-2 (Φ=0.53). Other significant positive correlations (Φ≥0.3) were observed for the couples T-2/$AFB_2$, NIV/$AFB_1$, AME/ALT, OTA/$FB_1$, NIV/AOH, $AFB_2$/AOH, $AFB_2$/AME, $FB_1$/$FB_2$, AME/$FB_1$ with Φ ranging from 0.49 to 0.40. ZEA did not significantly correlate with any of the other mycotoxins (Φ≤0.23). The Φ value for TeA could not be calculated because the mycotoxin was present in all samples.

### Occurrence of toxigenic fungi

The results of mycological analyses show that *Aspergillus* section *Nigri* and *Flavi* moulds were the most occurring toxigenic species in the 45 samples of peppers (Table 7). However, also the other three potential toxigenic genera considered herein were widely found with an incidence ranging from 20 to 40%. Only sample (#9) was found uncontaminated by any toxigenic fungal genus. All the others were contaminated by one (8 samples), two (10 samples), three (11 samples), four (11 samples) or five (4 samples) different toxigenic genera.

The highest level of contamination by moulds found in pepper reached $10^7$ cfu/g for the *Aspergillus* genus, $10^6$ cfu/g for the *Penicillium* and *Fusarium* genera, and $10^4$ cfu/g for the *Alternaria* genus. *Aspergillus* appears to be widespread in almost all samples and the most frequently isolated species belong to *Aspergillus niger*, *Aspergillus tubingensis*, *Aspergillus welwitschiae*, *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus ochraceus*. All these species were firstly characterised by micro and macro-

**Table 5. Results of mycotoxins determined in fresh, dried, grounded and fried sweet peppers.[1]**

| Mycotoxins | Fresh (n=7) | | | Dried (n=23) | | | Grounded (n=8) | | | Fried (n=7) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Mean µg/kg (range) | Median µg/kg | % positives | Mean µg/kg (range) | Median µg/kg | % positives | Mean µg/kg (range) | Median µg/kg | % positives | Mean µg/kg (range) | Median µg/kg | % positives |
| $AFB_1$ | 13.5 (<LOQ-13.55) | 13.55 | 14 | 31.2 (<LOQ-155.7) | 0.02 | 22 | 1.3 (<LOQ-4.6) | 0.84 | 88 | 0.017 (<LOQ-0.016) | 0.016 | 14 |
| $AFB_2$ | 0.34 (<LOQ-0.34) | 0.34 | 14 | 9.9 (<LOQ-9.9) | 9.9 | 4 | 0.20 (<LOQ-0.78) | 0.075 | 75 | ND | ND | – |
| $AFG_1$ | ND | ND | – | 159.1 (<LOQ-318.1) | 159.1 | 9 | 0.47 (0.15-0.93) | 0.34 | 38 | 0.040 (<LOQ-0.040) | 0.040 | 14 |
| $AFG_2$ | ND | ND | – | 45.4 (<LOQ-45.4) | 45.5 | 4 | 0.12 (<LOQ-0.12) | 0.12 | 13 | ND | ND | – |
| Total aflatoxins | 13.9 (<LOQ-13.9) | 13.9 | 14 | 105.9 (<LOQ-529.1) | 0.1 | 22 | 1.7 (<LOQ-6.4) | 0.93 | 88 | 0.056 (<LOQ-0.056) | 0.056 | 14 |
| OTA | 0.59 (<LOQ-0.95) | 0.64 | 57 | 53.9 (<LOQ-177.4) | 0.89 | 39 | 23.6 (0.33-101.1) | 11.2 | 100 | 0.71 (<LOQ-0.74) | 0.72 | 29 |
| NIV | ND | ND | – | 34.9 (<LOQ-206.0) | 2.7 | 30 | 53.4 (<LOQ-243.9) | 19.3 | 100 | <LOQ | <LOQ | 29 |
| DON | ND | ND | – | 4.8 (<LOQ-12.4) | 2.8 | 22 | 10.6 (<LOQ-59.8) | 1.3 | 88 | 3.05 (<LOQ-3.8) | 3.1 | 57 |
| $FB_1$ | 34.8 (9.4-139.6) | 9.5 | 100 | 26.1 (<LOQ-243.9) | 1.3 | 48 | 7.6 (<LOQ-17.5) | 5.9 | 100 | ND | ND | – |
| $FB_2$ | 4.2 (<LOQ-22.3) | 0.44 | 100 | 10.8 (<LOQ-176.9) | <LOQ | 74 | 6.7 (<LOQ-28.2) | 1.7 | 100 | <LOQ | <LOQ | 43 |
| ZEA | 0.23 (0.15-0.53) | 0.18 | 100 | 2.8 (<LOQ-53.6) | 0.1 | 87 | 0.18 (<LOQ-0.32) | 0.18 | 88 | 0.12 (0.050-0.26) | 0.10 | 100 |
| HT-2 | ND | ND | – | <LOQ | <LOQ | 9 | 34.4 (<LOQ-75.9) | 30.4 | 50 | 0.089 (<LOQ-0.14) | 0.10 | 71 |
| T-2 | ND | ND | – | ND | ND | – | 8.4 (<LOQ-27.1) | 3.8 | 75 | <LOQ | <LOQ | 43 |
| ALT | 68.8 (<LOQ-139.9) | 57.9 | 43 | ND | ND | – | 40.3 (<LOQ-40.3) | 40.3 | 13 | ND | ND | – |
| AOH | 111.1 (<LOQ-270.7) | 73.7 | 86 | 78.0 (<LOQ-428.4) | 9.8 | 43 | 176.4 (1.6-1,110.8) | 34.6 | 100 | 1.6 (<LOQ-1.6) | 1.6 | 14 |
| TTX | 75.4 (<LOQ-217.5) | 21.6 | 100 | 27.9 (<LOQ-345.4) | 7.9 | 87 | 16.9 (4.6-49.1) | 13.7 | 100 | 3.6 (2.3-5.3) | 3.6 | 100 |
| AME | 10.0 (<LOQ-17.8) | 10.1 | 57 | 10.7 (<LOQ-20.2) | 10.1 | 17 | 18.3 (<LOQ-36.7) | 19.3 | 75 | 2.0 (<LOQ-2.0) | 2.0 | 14 |
| TeA | 27,032.4 (<LOQ-67,828.1) | 21,299.2 | 100 | 914.0 (4.4-8,248.5) | 306.1 | 100 | 622.8 (208.2-1,989.2) | 409.0 | 100 | 224.9 (4.3-1,319.9) | 4.5 | 100 |

[1] $AFB_1$ = aflatoxin $B_1$; $AFB_2$ = aflatoxin $B_2$; $AFG_1$ = aflatoxin $G_1$; $AFG_2$ = aflatoxin $G_2$; ALT = altenuene; AME = allternariol methyl ether; AOH = alternariol; DON = deoxynivalenol; $FB_1$ = fumonisin $B_1$; $FB_2$ = fumonisin $B_2$; HT-2 = HT-2 toxin; LOD = limit of detection; LOQ = limit of quantification; ND = not detected; NIV = nivalenol; OTA = ochratoxin A; T-2 = T-2 toxin; TeA = tenuazonic acid; TTX = tentoxin; ZEA = zearalenone.

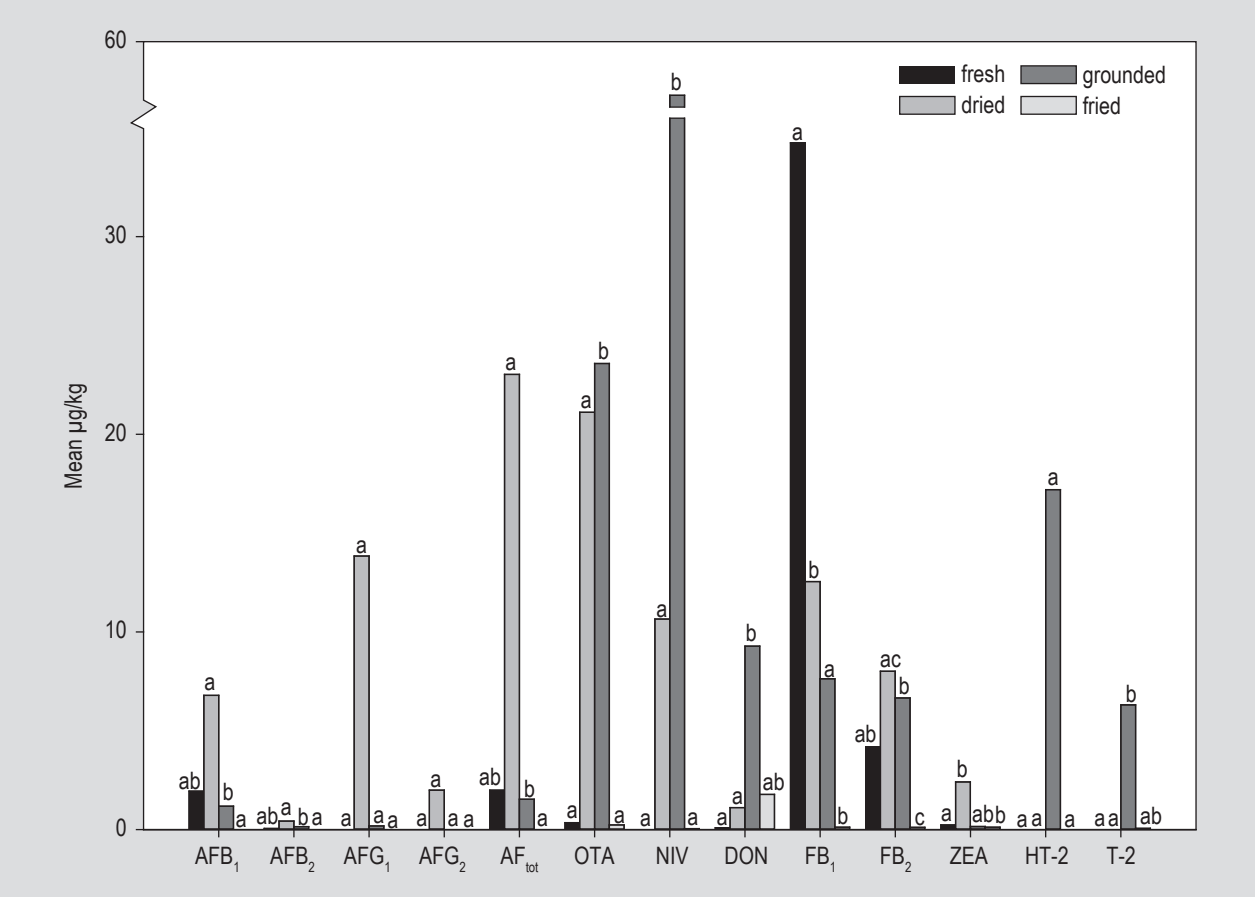


**Figure 2. Mean levels (µg/kg) of aflatoxins $B_1$, $B_2$, $G_1$, $G_2$ and total aflatoxins ($AFB_1$, $AFB_2$, $AFG_1$, $AFG_2$, $AF_{tot}$), ochratoxin A (OTA), nivalenol (NIV), deoxynivalenol (DON), fumonisin $B_1$ and $B_2$ ($FB_1$ and $FB_2$), zearalenone (ZEA), T-2 and HT-2 toxins (T-2, HT-2) in fresh, dried, grounded and fried peppers. Different letters indicate significant differences between groups ($P$<0.05).**

morphological characters, and then confirmed by sequences of two housekeeping genes with a 99-100% homology with the sequences of the respective type strains. Although most of them are responsible of aflatoxin and OTA production, no correlation was found between cfu load and mycotoxin content, as per the other genera and their relative mycotoxins. However, a small correlation was found for aflatoxins content and cfu of *Aspergillus* section *Flavi* ($r^2$=0.66). In the 14 samples contaminated by aflatoxins the presence of *Aspergillus* section *Flavi* was always assessed, but in the other 12 samples contaminated by the *Flavi* group no aflatoxins were detected.

## 4. Discussion

The particular landrace of sweet pepper (*C. annuum*) considered in this study is exclusively and widely cultivated in Basilicata region and represents 71% of the total *Capsicum* cultivated in this region (Montesano *et al.,* 2012). This particular sweet pepper is well known all over Europe for its unique characteristics, such as the thin epicarp, high content of mineral salts and vitamin C, low content of water, and the peduncle does not detach itself after drying. The thin epicarp facilitates the drying process, whereas the capacity of peduncle to remain tightly attached to the fruit allows the preparation of necklaces (serte) of fresh fruits that are usually sun dried and much less frequently dried in oven (Loperfido, 2012; Montesano *et al.,* 2012). Dried fruits are consumed all over the year, both whole and finely ground, and used as condiment in typical dishes or as ingredient in the preparation of salami to provide taste and colour. They can also be fried in olive oil and eaten as garnish or starters.

This is the first study on the occurrence of 17 mycotoxins and their potential toxigenic fungal species in this landrace of *C. annuum* that has adapted very well in Basilicata region during the last 400-500 years (Loperfido, 2012). In Europe, aflatoxins and OTA are regulated in dried fruits of *Capsicum* spp. and most of the published papers dealing with this commodity have mainly investigated aflatoxins and to a lesser extent OTA (Berthiller *et al.*, 2016; European Commission, 2015; Iqbal *et al.*, 2017; Jalili, 2016; Kabak and Dobson, 2017; Pesavento *et al.*, 2016; Prelle *et al.*, 2014; Santos *et al.*, 2008; Singh and Cotty, 2017; Tosun and Ozden, 2016). Since LC-MS/MS has become popular

**Table 6. Results of statistical contingency analysis.[1,2]**

| | $AFB_1$ | $AFB_2$ | $AFG_1$ | $AFG_2$ | $AF_{tot}$ | OTA | NIV | DON | $FB_1$ | $FB_2$ | ZEA | HT-2 | T-2 | ALT | AOH | TTX | AME | TeA |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| $AFB_1$ | – | 0.69 | 0.54 | 0.32 | 1.00 | 0.27 | 0.47 | 0.30 | 0.19 | 0.13 | -0.13 | 0.10 | 0.38 | -0.04 | 0.31 | -0.01 | 0.34 | na |
| $AFB_2$ | 0.69 | – | 0.50 | 0.46 | 0.69 | 0.34 | 0.36 | 0.38 | 0.40 | 0.11 | -0.06 | 0.31 | 0.49 | 0.06 | 0.42 | 0.12 | 0.41 | na |
| $AFG_1$ | 0.54 | 0.50 | – | 0.55 | 0.58 | 0.38 | 0.37 | 0.12 | 0.07 | 0.05 | 0.12 | 0.26 | 0.29 | -0.12 | 0.22 | 0.10 | 0.28 | na |
| $AFG_2$ | 0.32 | 0.46 | 0.55 | – | 0.32 | 0.21 | 0.05 | 0.07 | 0.18 | -0.14 | 0.07 | 0.14 | 0.16 | -0.07 | 0.19 | 0.06 | 0.30 | na |
| $AF_{tot}$ | 1.00 | 0.69 | 0.58 | 0.32 | – | 0.27 | 0.47 | 0.30 | 0.19 | 0.13 | -0.13 | 0.10 | 0.38 | -0.04 | 0.31 | -0.01 | 0.34 | na |
| OTA | 0.27 | 0.34 | 0.38 | 0.21 | 0.27 | – | 0.30 | 0.17 | 0.42 | 0.12 | -0.15 | -0.01 | 0.27 | 0.15 | 0.38 | 0.10 | 0.04 | na |
| NIV | 0.47 | 0.36 | 0.37 | 0.05 | 0.47 | 0.30 | – | 0.19 | 0.02 | 0.31 | 0.08 | 0.02 | 0.56 | -0.08 | 0.42 | 0.21 | 0.23 | na |
| DON | 0.30 | 0.38 | 0.12 | 0.07 | 0.30 | 0.17 | 0.19 | – | 0.07 | -0.16 | -0.09 | 0.72 | 0.56 | -0.23 | -0.08 | 0.01 | 0.07 | na |
| $FB_1$ | 0.19 | 0.40 | 0.07 | 0.18 | 0.19 | 0.42 | 0.02 | 0.07 | – | 0.41 | -0.11 | -0.08 | 0.09 | 0.27 | 0.59 | 0.13 | 0.41 | na |
| $FB_2$ | 0.13 | 0.11 | 0.05 | -0.14 | 0.13 | 0.12 | 0.31 | -0.16 | 0.41 | – | 0.21 | -0.23 | 0.00 | 0.17 | 0.38 | 0.07 | 0.26 | na |
| ZEA | -0.13 | -0.06 | 0.12 | 0.07 | -0.13 | -0.15 | 0.08 | -0.09 | -0.11 | 0.21 | – | 0.17 | -0.04 | 0.10 | 0.03 | 0.23 | 0.06 | na |
| HT-2 | 0.10 | 0.31 | 0.26 | 0.14 | 0.10 | -0.01 | 0.02 | 0.72 | -0.08 | -0.23 | 0.17 | – | 0.53 | 0.10 | -0.17 | -0.07 | 0.08 | na |
| T-2 | 0.38 | 0.49 | 0.29 | 0.16 | 0.38 | 0.27 | 0.56 | 0.56 | 0.09 | 0.00 | -0.04 | 0.53 | – | -0.16 | 0.11 | 0.13 | 0.35 | na |
| ALT | -0.04 | 0.06 | -0.12 | -0.07 | -0.04 | 0.15 | -0.08 | -0.23 | 0.27 | 0.17 | 0.10 | 0.10 | -0.16 | – | 0.28 | 0.08 | 0.44 | na |
| AOH | 0.31 | 0.42 | 0.22 | 0.19 | 0.31 | 0.38 | 0.42 | -0.08 | 0.59 | 0.38 | 0.03 | -0.17 | 0.11 | 0.28 | – | 0.30 | 0.54 | na |
| TTX | -0.01 | 0.12 | 0.10 | 0.06 | -0.01 | 0.10 | 0.21 | 0.01 | 0.13 | 0.07 | 0.23 | -0.07 | 0.13 | 0.08 | 0.30 | – | 0.19 | na |
| AME | 0.34 | 0.41 | 0.28 | 0.30 | 0.34 | 0.04 | 0.23 | 0.07 | 0.41 | 0.26 | 0.06 | 0.08 | 0.35 | 0.44 | 0.54 | 0.19 | – | na |
| TeA | na | na | na | na | na | na | na | na | na | na | na | na | na | na | na | na | na | – |

[1] $AFB_1$ = aflatoxin $B_1$; $AFB_2$ = aflatoxin $B_2$; $AFG_1$ = aflatoxin $G_1$; $AFG_2$ = aflatoxin $G_2$; $AF_{tot}$ = total aflatoxins; ALT = altenuene; AME = allternariol methyl ether; AOH = alternariol; DON = deoxynivalenol; $FB_1$ = fumonisin $B_1$; $FB_2$ = fumonisin $B_2$; HT-2 = HT-2 toxin; NIV = nivalenol; OTA = ochratoxin A; T-2 = T-2 toxin; TeA = tenuazonic acid; TTX = tentoxin; ZEA = zearalenone.
[2] na = not applicable for this mycotoxin.

**Table 7. Mycological analysis of pepper samples evidenced the huge varieties of Fungal genus found on this spice.**

| Descriptive statistics (n=45) | *Aspergillus* section *Nigri* | *Aspergillus* section *Flavi* | *Aspergillus* section *Circumdati* | *Alternaria* spp. | *Penicillium* spp. | *Fusarium* spp. |
|---|---|---|---|---|---|---|
| Average (cfu/g) | $1.7\times10^6$ | $3.8\times10^3$ | $3.9\times10^5$ | $2.8\times10^3$ | $9.9\times10^4$ | $1.4\times10^5$ |
| Maximum | $33.9\times10^6$ | $8.2\times10^4$ | $1.3\times10^7$ | $6.5\times10^4$ | $3\times10^6$ | $3\times10^6$ |
| Median | $1.5\times10^3$ | $1\times10^2$ | ND[1] | ND | ND | ND |
| Standard deviation | $7.8\times10^6$ | $14.7\times10^3$ | $1.9\times10^6$ | $1\times10^4$ | $4.8\times10^5$ | $6.2\times10^5$ |
| No. of contaminated samples | 39 | 26 | 17 | 19 | 14 | 9 |
| Frequency (%) | 86.7 | 57.7 | 37.8 | 42.2 | 31.1 | 20 |

[1] ND = not detected.

in analytical laboratories, multi-mycotoxin occurrences have been investigated in *Capsicum* spp. (Santos *et al.*, 2011; Yogendrarajah *et al.*, 2014).

The occurrence of aflatoxins and/or OTA in *Capsicum* commercialised in various countries have been recently reviewed (Kabak and Dobson, 2017). The high incidence of OTA (51%) and to a lesser extent of aflatoxins (31%) found in our study is not surprising, and even higher incidences of positive samples for OTA (87-100%) have been reported in Turkish red pepper flakes (Tosun and Ozden, 2016). A high incidence of positive samples for aflatoxins and OTA has been reported for chili pepper samples from Pakistan, and paprika and chili samples commercialised in Spain (Iqbal *et al.*, 2017; Santos *et al.,* 2010). In a recent survey on chilli pepper imported in Italy, aflatoxins were detected in 41% of samples and 5% of samples exceeded the EU limit for $AFB_1$ (Pesavento *et al.,* 2016). In a previous survey on spices, including chili peppers imported and commercialised in Northern Italy, aflatoxins and OTA were detected in 20 and

60%, respectively, of samples at levels below the EU limits (Prelle *et al.,* 2014). The widespread occurrence of OTA in peppers was also confirmed in a survey conducted in samples imported in Iran (Jalili, 2016). Low levels of OTA (1-2 µg/kg) were detected in 10% of Korean red pepper samples, whereas aflatoxins were absent (Ham *et al.,* 2016). Also in Brazil and China only OTA was detected in 4% of paprika samples, whereas aflatoxin was absent (Reinholds *et al.,* 2016). The levels of aflatoxins and OTA measured in the 6 irregular samples identified in our study are comparable to the contamination levels found in samples of *Capsicum* subjected to RASFF notification in 2011 and originating from tropical and subtropical areas (Kabak and Dobson, 2017). The high levels of OTA and aflatoxins measured in our study are of particular concern, since they are up to 9 times the limit of OTA and up to 31 times the limit of $AFB_1$ and 53 times the limit of total aflatoxins.

The high incidence of samples positive for ZEA, thichothecenes and fumonisins is partially due to the low values of LODs of the method used in this study (Table 3 and 4). Fumonisins, DON, ZEA and HT-2 were detected in few samples of chili paste, but no results were reported on co-occurrence of these mycotoxins in the same sample (Patel *et al.,* 1996). The occurrence of $FB_1$ in paprika (11% positives) imported from Brazil and China has been reported (Reinholds *et al.,* 2016), as well as ZEA in paprika (39% positives) and chili (46% positives) commercialised in Spain (Santos *et al.*, 2010).

The high incidence of OTA (51%) and $FB_2$ (78%) in our 45 samples can be explained by the widely presence of black aspergilli (*Aspergillus* section *Nigri*) on this product (87%), as it is well known that two of the most diffused species of this group, *A. niger* and *A. welwitschiae*, are able to produce both toxins (Hong *et al.*, 2013). Similar results by Santos *et al.* (2011) showed evidence of high occurrence of *Aspergillus* spp., mainly among section *Nigri*, with respect to the other mycotoxigenic fungi identified in capsicum powder. However, the percentage of toxigenic strains within these two species is low and variable (Susca *et al.,* 2016), which can explain why no correlation among presence of toxins and cfu/g of these species was found. In this respect, we found 4/5 samples with a high cfu load of *Aspergillus* section *Nigri,* but no ochratoxin or fumonisins inside. While in other food samples, like raisins and grapes, a good correlation is found between OTA contamination and occurrence of *A. carbonarius*, as in this species a high percentage of strains are OTA producers (Perrone *et al.*, 2013). Similar results we found for aflatoxins and *Aspergillus* section *Flavi*; but with a higher correlation between toxin amount and fungal presence. This could be explained by the fact that within *Aspergillus* section *Flavi* the percentage of toxigenic strains is higher with respect to the *Aspergillus s*ection *Nigri*. In general, the potential toxigenic fungal species found on this peculiar sweet pepper are numerous and cover most of the main important toxigenic genera (*Alternaria*, *Aspergillus*, *Fusarium* and *Penicillium*), and this is in accordance with the wide diversity of mycotoxins found on this product. Particularly, the genus *Alternaria* and *Fusarium* occur in relatively low percentages with respect to their toxins. This could be partially explained by the fact that these genera are usually considered as phytopatogenic and occur mainly on fresh fruits or live plants, while our survey is mainly from dried peppers. This means that fungal contamination for these two genera occurs in the field with the subsequent mycotoxin accumulation; then, in the final product (dried pepper) only the mycotoxins were detected and not the relevant toxigenic fungal species. Finally, our findings support the hypothesis of previous reports that mycotoxin contamination of *Capsicum* products may occur both in the field and/or during storage (Santos *et al.,* 2008, 2011).

The occurrence of *Alternaria* mycotoxins in sweet peppers, including chili and paprika, has been reported in few papers (Asam *et al.,* 2012; Da Cruz *et al.*, 2016; Lohrey *et al.,* 2013; Van de Perre *et al.,* 2014; Yogendrarajah *et al.,* 2013, 2014). Lohrey *et al.* (2013) and Asam *et al.* (2012) measured only TeA in pepper sauce, paprika and other spices; Yogendrarajah *et al.* (2013, 2014) measured AME and other mycotoxins in chilli; Van de Perre *et al.* (2014) AOH, AME and other mycotoxins in bell peppers; Da Cruz *et al.* (2016) AOH, AME and TeA in spoiled pepper fruits. This is the first report on the occurrence of five *Alternaria* mycotoxins (TeA, AOH, AME, TTX, ALT) in sweet peppers.

It should be underlined that none of the 45 pepper samples analysed in this study was negative for all the 17 searched mycotoxins. Only six samples contained 2-4 mycotoxins, whereas the remaining 39 samples contained a number of mycotoxins ranging from 5 to 16 with a mean of 7 mycotoxins per sample. As shown in Figure 3, the ground pepper samples contained the highest numbers of mycotoxins co-occurring in each sample. The low values of the LODs obtained with the LC-MS/MS methods used this study could only partially explain the high percentages of positive samples and the high number of mycotoxins detected in each sample.

Figure 3 also shows the mean level of total mycotoxins measured in each sample, as well as the individual mycotoxin order from the highest level to the lowest level. TeA was the mycotoxin occurring at highest level in 43/45 samples, whereas in one sample of fresh pepper (#6) AOH occurred at highest level and in another of dried pepper (#4) OTA occurred at highest level. When comparing the four groups of peppers for the mean of the sum of occurring mycotoxins, the lowest mean level was obtained for fried peppers (231 µg/kg) followed by grounded peppers (961 µg/kg), dried peppers (1,054 µg/kg) and fresh peppers (27,280 µg/kg).

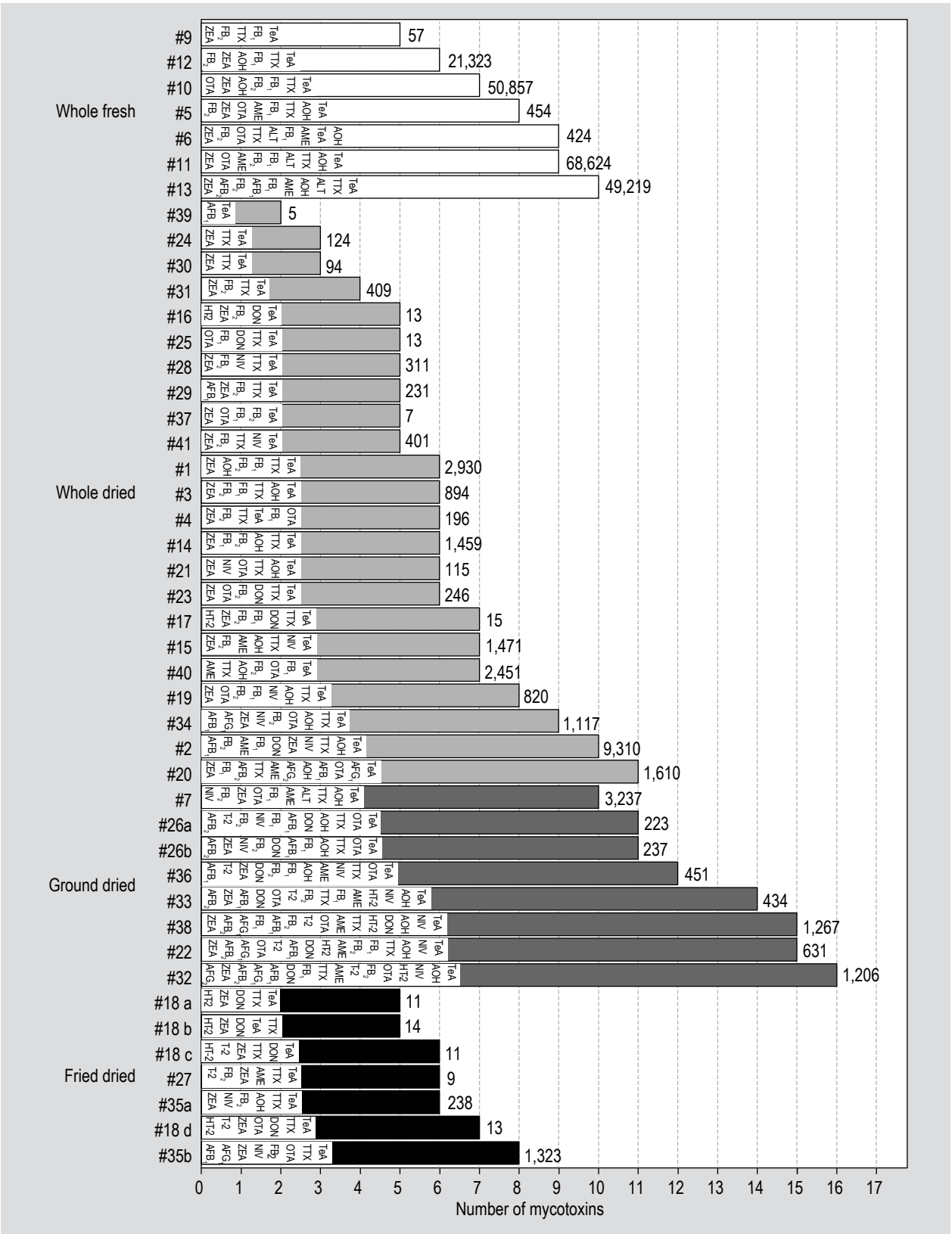


**Figure 3. Co-occurrence of mycotoxins in the 45 pepper samples. Bars represent the number of mycotoxins occurring in each sample. Within the bars, the mycotoxins are shown in descending order based on their levels. On top of each bar the sum of total mycotoxin levels (µg/kg) is shown. $AFB_1$ = aflatoxin $B_1$; $AFB_2$ = aflatoxin $B_2$; $AFG_1$ = aflatoxin $G_1$; $AFG_2$ = aflatoxin $G_2$; ALT = altenuene; AME = allternariol methyl ether; AOH = alternariol; DON = deoxynivalenol; $FB_1$ = fumonisin $B_1$; $FB_2$ = fumonisin $B_2$; HT-2 = HT-2 toxin; NIV = nivalenol; OTA = ochratoxin A; T-2 = T-2 toxin; TeA = tenuazonic acid; TTX = tentoxin; ZEA = zearalenone.**

The significantly lower levels of contamination measured in fried peppers compared to fresh, grounded and dried peppers (Figures 1, 2, 3) does not mean that those samples were originally less contaminated. The frying process could have degraded or extracted (into the oil) the mycotoxins from the peppers thus reducing their levels. This hypothesis should be confirmed with appropriate experiments before the frying process can be defined a good cooking method to reduce mycotoxin levels in peppers and other food commodities susceptible to mycotoxin contamination.

The high co-occurrence of mycotoxins demonstrated for sweet pepper in this study is particularly worrying for consumers, especially because each toxin has a different toxicity that can be additive, synergistic or antagonistic. It is therefore necessary to test the toxicity of numerous and different mycotoxin mixtures that should be selected according to their natural co-occurrence. In parallel, human biomonitoring studies should be encouraged and focused on multi-mycotoxins in order to identify and quantify human exposure to mycotoxin mixtures particularly dangerous to human health.

The results of this study have a broad significance and are not limited to Basilicata region, because *Capsicum* spp. are widely cultivated around the world. Therefore, high levels of mycotoxin co-occurrence are also expected in other countries, if the processing and storage conditions are not adequate. The high percentages of positive samples and the high levels of some mycotoxins observed in this study claim for an improvement of the conditions used during the production and drying process of sweet peppers in the Basilicata region (Italy). These improved conditions can also be used worldwide to control and reduce fungal and mycotoxin contamination in *Capsicum* spp.

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