# Incidence and levels of the emerging *Alternaria* mycotoxins in spices and herbs produced worldwide and commercialized in Lebanon

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**Short title: Alternaria mycotoxins in spices and herbs**

Abstract

The emerging *Alternaria* mycotoxins tenuazonic acid (TeA), alternariol (AOH), alternariol monomethyl ether (AME), altenuene (ALT) and tentoxin (TTX) are not yet regulated worldwide awaiting for more toxicity and occurrence data. A total of 131 samples of spices (94) and herbs (37) were collected in Beirut (Lebanon) and analysed for TeA, AOH, AME, ALT and TTX by using a UPLC-MS/MS method based on 'extract, dilute and shoot' approach. The limits of detection (LOD) ranged from 1.3 µg/kg (AME) to 6.1 µg/kg (AOH) and limits of quantification (LOQ) ranged from 4.2 µg/kg (AME) to 20.4 µg/kg (AOH). 89% of samples were contaminated by 1-5 mycotoxins whereas 14% of samples were contaminated with a mixture of TeA, AOH, AME and TTX. TeA was the predominant mycotoxin with the highest percentages of positive samples (76%), followed by AME (46%), TTX (37%), AOH (34%) and ALT (5%) with overall mean/max levels of 2453.2/106792.8, 17.1/306, 14.8/179.4, 35.0/636.4 and 2.8/22.1 µg/kg, respectively (middle bound approach). Spices were more contaminated than herbs with higher percentage of positive samples (90 *vs* 81%) and mean levels of total mycotoxins (3391.4 *vs* 305.0 µg/kg). The spices with higher levels of mycotoxin contamination were, in descending order, red chili, paprika, caraway, coriander seeds, onion powder, white pepper and garlic powder whereas within the herbs they were corn silk, violets and marjoram. Based on the mean levels of TeA measured in different spices and herbs, the consumption of >3.3 g red chili, >3.7 g paprika, >16.7 g caraway or >36 g of corn silk will exceed the threshold of toxicological concern (TTC) of TeA (1,500 ng/kg bw per day). For AOH or AME the TTC (2.5 ng/kg bw/day) will be exceeded by consuming >0.5 g of white pepper, >0.9 g of onion powder, > 1.4 g of garlic powder, >1.7 g of black pepper, >1.9 g of sage, >3.3 g of violets or > 4.4 g of thyme leaves. This study highlight the high susceptibility of some spices and herbs to *Alternaria* mycotoxins contamination and emphasizes on the importance of conducting a proper HACCP during the whole production chain of certain spices and herbs in order to identify the critical points, improve the production and manufacturing conditions leading to a consistent reduction of mycotoxins contaminations.

**Keywords*:*** *Alternaria*, herbs, spices, multi-mycotoxin, occurrence, Lebanon, mass spectrometry

## 1. Introduction

Some of the secondary metabolites produced by few *Alternaria* species (*A. alternata*, *A. tenuissima*, *A. arborescens* and relevant species-group, *A. infectoria* sp.-grp and *A. japonica*) are recognised as emerging mycotoxins (Solfrizzo, 2017; Puntscher et al., 2018). These fungi are widespread in humid and semiarid regions and can infect growing plants in the field (Ostry, 2008; Logrieco, Moretti, & Solfrizzo, 2009). Alternariol (AOH), alternariol monomethyl ether (AME), tenuazonic acid (TeA), iso-tenuazonic acid (iso-TeA), altertoxins (ATXs), tentoxin (TTX) and altenuene (ALT) are the main *Alternaria* mycotoxins reported to occur in food and feed, according to the opinion of the EFSA Panel on Contaminants in the Food Chain (EFSA, 2011). These mycotoxins have been recognized potentially harmful and life threatening due to their toxicity and occurrence in different food commodities such as cereals, oilseeds, fruits and vegetables (Arcella, Eskola, & Gómez Ruiz, 2016).

The studies on the toxicity of *Alternaria* mycotoxins have increased rapidly in the last ten years. AOH and AME were found to exhibit mutagenic and genotoxic activities (EFSA, 2011), probably due to DNA damage by indirect mechanisms (Solhaug, Eriksen, & Holme, 2016). It is to be noted that AOH was proven to be rapidly absorbed by the human intestinal lumen (Ostry, 2008), which might expose us to a higher risk. TeA was shown to exhibit toxicity against several animal species such as mice, chicken, dogs including oesophageal cancer in mice (Ostry, 2008; EFSA, 2011). However, the data on the natural occurrence of these mycotoxins in food and feed are not exhaustive and more occurrence data are necessary especially for those matrices not well investigated in the past. Currently, there is no regulation for *Alternaria* mycotoxins worldwide with the exception of Bavarian health and food safety authority who

established a limit of 500 µg/kg for TeA in sorghum/millet-based infant food (Rychlik, Lepper, Weidner, & Asam, 2016).

The first international risk assessment on *Alternaria* mycotoxins (TeA, AOH, AME and TTX) in food and feed was published by the EFSA Panel on Contaminants in the Food Chain in 2011 (EFSA 2011). Due to the very limited data available on the toxicity of these mycotoxins the CONTAM panel used the threshold of toxicity concern (TTC) for risk assessment i.e. 2.5 ng/kg bw per day for the genotoxic AOH and AME and 1,500 ng/kg bw per day for the not genotoxic TeA and TTX. For AOH and AME the estimated mean chronic dietary exposures at the upper bound (UB) and 95$^{th}$ percentile dietary exposures exceeded the TTC value; whereas for TeA and TTX the exposure estimates were below their TTC value, therefore they were judged unlikely to be a human health concern. Later, in 2016, EFSA published the second risk assessment on TeA, AOH, AME and TTX in the European population, based on data of several thousand samples of various foods available from 2010 to 2015 (Arcella et al., 2016). However, most of the results were left-censored data (92%), with only 8% quantified results represented mainly by 'oilseeds' and 'grain milling products' (Arcella et al., 2016). In total, of the 1,290 quantified results the majority were for TeA (n = 822), followed by TTX (n = 196), AME (n = 163) and AOH (n = 109). The data of 115 samples belonging to the food category "herbs, spices and condiments" were also evaluated and the mean levels (middle bound) ranged between 19.9-8801.5 µg/kg for TeA, 4.0-4.5 µg/kg for TTX, 2.8-8.3 µg/kg for AME and 0.8-10.9 µg/kg for AOH. The main recommendation of the EFSA assessment was to collect representative occurrence data on *Alternaria* mycotoxins in food and feed across the European countries to be able to conduct an accurate exposure assessment to these mycotoxins.

The main purpose of this study is to monitor the *Alternaria* mycotoxins in different samples of spices and herbs produced worldwide and commercialized in Lebanon, providing consequently occurrence data useful for the human exposure assessment. The collected samples were analysed for ALT, AOH, TTX, AME and TeA by using an LC-MS/MS method, to provide detailed information on the frequency, levels and multiple occurrence of these mycotoxins.

## 2. Materials and methods

### *2.1. Sampling*

The samples of single and mixture of spices (94) and herbs (37) were collected from retail stores located in Beirut (Lebanon). They are listed in Table 1 and classified according to the existence or absence of GMP/HACCP certification from the relevant manufacturing factories. In particular, 58 samples of spices and 23 samples of herbs originated from certified factories, whereas 36 samples of spices and 14 samples of herbs originated from not certified ones. The samples were sent to CNR-ISPA (Italy) where they were subjected to grinding and blending then analysed by LC-MS/MS for ALT, AOH, TTX, AME and TeA.

### 2.2. Chemicals and reagents:

The standard solutions of each mycotoxin were purchased from Romer Labs Diagnostic (Tulln, Austria). In particular, separated standard solutions of ALT (10 µg/ml), AOH (100 µg/ml), TTX (100 µg/ml), AME (100 µg/ml) and TeA (100 µg/ml) in acetonitrile were obtained.

Chromatography-grade methanol (MeOH), acetonitrile, acid formic and ammonium bicarbonate were obtained from sigma-Aldrich (Milan, Italy). Ultrapure water was produced by use of a Milli-Q system (Millipore, Bedford, MA, USA). Regenerated cellulose filters (0.45 µm) were purchased from Sartorius Stedim Biotech (Goettingen, Germany).

### 2.3. Determination of *Alternaria* mycotoxins

#### 2.3.1. Extraction

5 g of grounded spice or herb was weighed and transferred in 250 mL Pyrex screw-capped glass flask and extracted with 20 ml acetonitrile:$H_2O$:formic acid (49:50:1, v/v/v). The sample was shaken manually for 10 s to obtain a homogeneous suspension then the extraction was performed by sonication for 30 min. After centrifugation (4,000×g, 3 min), 200 µl of extract were diluted with 800 µl of ultrapure $H_2O$,

manually shaked and left overnight at 4°C. The extract was then filtered with a regenerated cellulose filters (0.45 μm) syringe and analysed by LC-MS/MS.

A seven spices mixture (allspice, black pepper, cinnamon, ginger, cloves, nutmeg and white pepper) and a mix herbs (thyme, parsley, rosemary, basil, seed of celery) were mixed, grinded and used for recovery experiments and to prepare matrix assisted calibration curves.

Aliquots of commercial solutions of TeA, AOH, ALT, TTX and AME were diluted with acetonitrile and used for spiking purpose according to the spike levels reported in Table 2. Triplicate experiments were performed and recoveries were calculated after subtracting the measured levels of endogenous *Alternaria* mycotoxins measured in the mixture of spices and herbs. Matrix assisted calibration curves were prepared in sample extracts obtained from the mixture of spices and herbs. LOD and LOQ for each mycotoxin were calculated as 3 times and 10 times the noise, respectively.

2.3.2. 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, 50 psi; GS2, air, 30 psi; ionspray voltage -4,500 V.

Quantification of *Alternaria* mycotoxins in 131 samples of spices and herbs was performed by measuring peak areas in the MRM chromatogram, and comparing them with the relevant matrix-matched calibration curves. The five points calibration in matrix ranged between: 0.0125-0.250 ng injected for TeA, ALT, TTX and AME and 0.006 – 0.100 ng injected for AOH.

2.3.3. LC/MS/MS parameters

The separation of ALT, AOH, TTX, AME and TeA was performed using a C18 Gemini analytical column (2 mm × 150 mm, 5 μm particles; Phenomenex). The column oven was set at 40°C. The flow rate of the

mobile phase was 300 µl/min and the injection volume was 10 µl. For the separation of analytes the mobile phase consisted of a binary linear gradient of a mixture of (A), $H_2O$:MeOH (95+5) 1 mM ammonium bicarbonate and (B), MeOH used as follows: 100% A for one min then brought to 100% B in 10 min, then maintained at 100% B for 8 min, then brought to 100% A 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. The mass spectrometer operated in MRM (multiple reaction monitoring) mode. The optimised MS/MS conditions used in this study are reported in (Gambacorta et al., 2018) for the measurement of *Alternaria* mycotoxins. One transition for quantification and three transitions for confirmation were used for all *Alternaria* mycotoxins.The identity of each analyte in the chromatograms of the sample extracts was further confirmed by comparing the retention times and the ion ratios with the calibration standards (Commission Decision 2002/657/EC).

2.3.4. Evaluation of results

Results below LOD or LOQ were given the value half the LOD or LOQ according to the middle bound approach reported by (EFSA, 2010).

2.4. Statistical analysis

Mean, weighted mean, median and standard deviation of the mean were calculated using MS Excel 2013 software (Microsoft Corporation, Redmond, WA, USA). Statistical analyses were performed by using the GraphPad Instat software (Instat, San Diego, CA, USA). Data reported in Fig. 1 and 3 were subjected to the unpaired t-test (one-tail P value). Mean values of the data reported in Tab. 5 were statistically compared with the Tukey-Kramer multiple comparisons test (one way ANOVA). Mean values were judged to be significantly different for P values $< 0.05$.

## 3. Results

### 3.1. Method performance

The results of in-house validation of the LC-MS/MS method for spices and herbs are reported in Table 2. The method was applicable for the analysis of spices and herbs as mean recoveries and mean repeatability ($RSD_r$) of results obtained for spiked samples were all acceptable because ranging 74-118 % and 1-7%, respectively. The use of a modern/powerful LC-MS/MS apparatus allowed us to inject low amount of matrix equivalent (0.5 mg) and to obtain acceptable values of LOD and LOQ for the 5 mycotoxins. In particular, the range values of LOD and LOQ of the five mycotoxins were 1.3 - 6.1 µg/kg and 4.2 - 20.4 µg/kg, respectively (Table 2).

### 3.1. Occurrence of *Alternaria* mycotoxins in different types of spices and herbs

The summary of results on mycotoxin contamination in spices, herbs and relevant mixtures is reported in Table 3 and Figure 1. In general, the mycotoxin levels were higher in spices as compared to herbs. In particular, significantly higher levels of TeA, AOH and ALT were measured in spices whereas no significantly differences were observed for TTX and AME (Figure 1). For spices the incidence of positive samples was higher for TeA (77%) followed by AME (44%), AOH (40%), TTX (40%) and ALT (7%). Only 9 samples (10%) were negative for all tested mycotoxins. Median level of TeA in spices was 41.6 µg/kg whereas median levels of AOH, TTX, ALT and AME were half of their LOD values. The max levels of TeA (106792.8 µg/kg), AOH (636.4 µg/kg), AME (306.0 µg/kg), TTX (179.4 µg/kg) and ALT (22.1 µg/kg) were found in samples of chili powder imported from China, white pepper of unknown origin, onion powder from China, caraway from Egypt and cloves from Madagascar, respectively.

Within the herbs the incidence of positive samples was higher for TeA (73%) followed by AME (51%), TTX (30%), AOH (19%), whereas all samples were negative for ALT. Seven samples (19%) were negative for all tested mycotoxins. Median levels were 78.8 µg/kg for TeA and 9.3 µg/kg for AME, whereas for AOH and TTX they were half of their LOD values. The max levels of TeA (4868.4 µg/kg),

AME (161.3 µg/kg), TTX (67.0 µg/kg) and AOH (64.2 µg/kg) were found in corn silk of unknown origin, sage of unknown origin, extra thyme from Lebanon and sage from UK, respectively.

In Table 4 are reported the mean levels of each *Alternaria* mycotoxin in the different types of spices and herbs, singles or mixtures. The sums of the means of the 5 mycotoxins for each type of spice/herb are also reported and listed in descending order. The higher contamination of spices with respect to herbs is evident also in this Table. The spices more susceptible to mycotoxin contamination are, in descending order, red chili, paprika, caraway, coriander seeds, onion powder, white pepper and garlic powder whereas within the herbs corn silk, violets and marjoram resulted the more contaminated one's. TeA contributed for 97.4-99.7% of total mycotoxins measured in red chili, paprika, caraway and coriander seeds. Therefore, the sum of ALT, AOH, AME and TTX, when present, contributed for only 0.3-2.6% of total contamination. However, significant mean levels of AOH and AME were found in samples of onion powder (AOH 164.5 µg/kg, AME 137.9 µg/kg) and garlic powder (AOH 57.4 µg/kg, AME 106.3 µg/kg). For these matrices the sum of AOH and AME contributed for 51-68% of total *Alternaria* mycotoxins. High levels of AOH but not AME were found in white pepper (319.7 µg/kg) and black pepper (89.0 µg/kg) for which AOH contributed for 83-86% of total contamination.

Concerning herbs, significant high mean levels of TeA were measured in corn silk (2495.9 µg/kg) and violets (1670.3 µg/kg), whereas significant mean levels of the other mycotoxins were found in sage (AOH 30.5 µg/kg, AME 80.3 µg/kg) and marjoram (TTX 32.3 µg/kg). In corn silk TeA was the only mycotoxin detected whereas in violets TeA was detected together with AOH and AME and contributed for 97% of total contamination.

In Figure 2a and 2b is shown the co-occurrence of multiple mycotoxins in spices and herbs, respectively. Only 10% of spices were negative for the 5 tested mycotoxins, whereas 2%, 16%, 17%, 28% and 27% of samples contained 5, 4, 3, 2 or 1 mycotoxin, respectively. The percentage of herbs negative for the all tested mycotoxins was higher (16%) whereas the percentage of samples containing 4, 3, 2 or 1 mycotoxin

were 8%, 24%, 17% and 35%, respectively. No one sample contained 5 mycotoxins. In Figure 2a and 2b are also shown the different mixtures of mycotoxins occurring in spices and herbs.

In Table 5 are reported the weighted mean levels of each *Alternaria* mycotoxin and the sum of the 5 mycotoxins in spices and herbs classified according to the edible part of the plants. The highest levels of total mycotoxins were measured in fruits (red chili and paprika) followed by flowers (6 different types), seeds (9 different types), bulbs (onion and garlic), leaves (11 different types) and roots (turmeric and ginger). The highest weighted mean level of TeA was found in fruits (red chili and paprika) whereas the highest weighted mean levels of AOH and AME were found in bulbs (garlic and onion).

In Table 6 the samples of spices, herbs and mixtures of spices or herbs were grouped according to their country of origin and listed from the highest to the lowest sum of mean levels of total mycotoxins. Unfortunately, the countries of origin of 49 spices and 16 herbs were unidentified and are reported as unknown. For the samples originated from known countries 56% came from Asia (China, India, Syria, Lebanon, Vietnam and Pakistan), 20% from Africa (Egypt, Madagascar, Sudan), 12% from Europe (Sweden, France, UK) and 12% from America (USA, Mexico, Guatemala). Within the known countries, China and Egypt provided the highest number of spices and herbs, respectively. The Chinese spices contained the highest sum of mean levels of all mycotoxins.

### 3.3. Comparison of spices and herbs with and without HACCP and GMP certification

In Figure 3a and 3b the comparison of results of spices and herbs produced with and without HACCP and GMP certification, respectively is shown. No significant differences were observed for all mycotoxins in herbs (Figure 3b) and for all mycotoxins but ALT in spices (Figure 3a). However, considering the low levels of ALT measured in the few positive samples of spices and the lack of statistical significance for the other mycotoxins, this difference should not be due to the HACCP and GMP certification.

## 4.0 Discussion

The high incidence of positive samples in spices and herbs considered in the present study is in agreement with previous studies reporting a significant incidence of toxigenic *Alternaria* species in these matrices (Gupta, Kumar, Mahajan, Sharma, & Asthana, 2017; González, Romero, Arjona, Larumbe, & Vaamonde, 2017; da Cruz Cabral, Terminiello, Pinto, Nielsen, & Patriarca, 2016; Hashem & Alamri, 2010; Logrieco et al., 2009; McKee, 1995; Hashmi & Ghaffar, 1991). Moreover, the percentages of positive samples registered in our study are comparable with those reported in the first EFSA's report (EFSA 2011) although the levels of *Alternaria* mycotoxins are quite different. In particular, the mean level of TeA found in our spices (3311.1 µg/kg) is about twenty times higher the max level of TeA (163 µg/kg) reported for the food group 'Herbs, spices and condiments' published in the EFSA's scientific opinion (EFSA 2011). A similar situation is observed for mean and max levels of AOH and AME in our spices compared to the EFSA's report, i.e. 45 µg/kg versus 5.0 µg/kg and 16.3 µg/kg versus 1.0 µg/kg, respectively. The high mean levels of *Alternaria* mycotoxins found in red chili and paprika samples in the present study (Table 4) confirm previous results published for paprika powder (Arcella et al., 2016), sweet peppers (Gambacorta et al., 2018) and sweet mouldy peppers (da Crus Cabral et al., 2016). The very high levels of TeA (>1,000 µg/kg) observed in all red chili and paprika samples analysed in this study seems to be related to the particular susceptibility of pepper (*Capsicum spp.*) to fungi producing this mycotoxin rather than their countries of origin that were equally distributed between China, USA, India and Sweden. Very high levels of TeA were also observed in the two samples of caraway (5729.8 and 5030.2 µg/kg) that merit further investigations about the origin of such high contamination in this type of spice. Much lower levels of TeA (93-209 µg/kg) were measured in the 6 samples of cumin seeds, a spice apparently similar but very different to caraway seeds. High levels of TeA, between 1070-5254 µg/kg, were found in 7 samples of mix spices (in descending levels: chicken shawarma, red taouk, tawook, mograbiee, monoghrabieh and kebbe). High levels of AOH, between 71-597 µg/kg, were also found in 6 samples of mix spices (in descending levels: chicken shawarma, francisco, biryani, sausage, kebbe and kefta). Of these, 2 mixtures (chicken shawarma and kebbe) contained high levels of both mycotoxins. It should be

pointed out that all of the spice or herb samples analysed in our study appeared healthy and no one showed any mould symptom.

The intake of spices from routinely prepared dishes have been recently assessed in southern India and represent 0.8-2.2% of the total dry matter content of a diet (Siruguri & Bhat, 2015). Based on the mean levels of TeA reported in Table 4, the consumption of >3.3 g red chili, >3.7 g paprika, >16.7 g caraway or >36 g of corn silk will exceed the TTC value of TeA which is 1,500 ng/kg bw per day. For AOH or AME the TTC (2.5 ng/kg bw/day) will be exceeded by consuming >0.5 g of white pepper, >0.9 g of onion powder, > 1.4 g of garlic powder, >1.7 g of black pepper, >1.9 g of sage, >3.3 g of violets or > 4.4 g of thyme leaves. Most of these amounts are within the range of mean spice intakes (0.21 – 3.0 g) reported by Siriguri and Bath 2015. The dietary exposure assessment to *Alternaria* mycotoxins in the European population, published in 2016, demonstrated that for the genotoxic AOH and AME the estimated mean chronic dietary exposures at the upper bound and 95$^{th}$ percentile, dietary exposures exceeded the TTC value of 2.5 ng/kg bw per day. On the other hand, the exposure estimates of the non-genotoxic TeA and TTX were much lower than the TTC value of 1,500 ng/kg bw per day therefore they were unlikely to be a human health concern (Arcella et al., 2016). The results reported herein demonstrate, for the first time, that the normal consumption of some spices or herbs exceed the TTC values of TeA, AOH or AME.

The lack of statistical significance between samples with and without HACCP and GMP certification may be explained with more than one hypothesis. For example, the worldwide absence of regulation for *Alternaria* mycotoxins or the inappropriateness of the HACCP and GMP practices currently used by producers. Therefore, the emanation of appropriate regulation worldwide is desirable together with the development of proper HACCP and GMP specific for *Alternaria* mycotoxins. HACCP should be conducted taking into account that the contamination is affected by the variability of the environmental conditions, agronomic practices, ripeness as well as the soil conditions that vary in different producing countries (Ssepuuya et al., 2018). In addition, the storage, processing, packaging and distribution

297 conditions should be carefully analysed to identify critical points that promote further grow of toxigenic
298 *Alternaria* species that are conducive of mycotoxins accumulation in spices and herbs.
299 Many spices are grown and harvested in poor sanitary conditions in areas with high environmental
300 temperature and humidity that increase microbiological contamination. Numerous studies have indicated
301 high microbial loads in spices and herbs that suggest a need for better control in all aspects of the
302 production, processing and usage of these products to prevent food spoilage and food-borne illnesses due
303 to contaminated spices and herbs (McKee 1995). However, it has also been reported that samples of sumac
304 contained very rare fungal colony counts suggesting antifungal properties of this spice. Negligible
305 mycotoxin contamination was observed in the sumac samples analysed in the present study and previously
306 (El Darra et al., 2019) which may confirm the antifungal property of this spice. The anti-microbial activity
307 of some spices may alter the gut microbiota and influence various metabolic diseases (Gupta et al., 2017).
308

309 **4. Conclusions**

310 The results of this study suggest that spices and herbs produced worldwide can often be contaminated
311 with high levels of *Alternaria* mycotoxins. The consumption of some spices or herbs can results in high
312 exposure to these mycotoxins that exceed their TTC values. This is the first study on the incidence and
313 levels of *Alternaria* mycotoxins in spices and herbs produced worldwide and commercialised in Lebanon.
314 As observed for other type of mycotoxins, spices showed a higher incidence and levels of *Alternaria*
315 mycotoxins as compared to herbs. TeA was the major contaminant in spices and herbs. Red chili, Paprika,
316 caraway and corn silk presented the highest contaminations in *Alternaria* mycotoxins. No significant
317 difference was noted between the samples collected from HACCP and GMP certified and non-certified
318 producers. This study highlight the high susceptibility of some spices and herbs to *Alternaria* mycotoxins
319 contamination and emphasizes on the importance of conducting a proper HACCP during the whole
320 production chain of certain spices and herbs in order to identify the critical points, improve the production
321 and manufacturing conditions leading to a consistent reduction of mycotoxins contaminations. These

activities are urgently needed in the spices and herbs producing countries before setting suitable worldwide limits for these mycotoxins.

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**Table 1. Collected samples of spices (94) and herbs (37) produced with and without HACCP and GMP rules**

| | | With HACCP/GMP (58) | Without HACCP/GMP (36) |
|---|---|---|---|
| **Single Spices (64)** | Allspice (3) | 1 | 2 |
| | Black pepper (4) | 3 | 1 |
| | White pepper (2) | 1 | 1 |
| | Red chili (7) | 6 | 1 |
| | Paprika (3) | 2 | 1 |
| | Garlic powder (2) | 1 | 1 |
| | Onion powder (4) | 3 | 1 |
| | Turmeric (2) | 1 | 1 |
| | Cinnamon (3) | 2 | 1 |
| | Ginger (3) | 1 | 2 |
| | Fennel (2) | 1 | 1 |
| | Caraway (2) | 1 | 1 |
| | Cloves (2) | 1 | 1 |
| | Coriander seeds (2) | 1 | 1 |
| | Cumin (5) | 4 | 1 |
| | Fenugreek grain (4) | 3 | 1 |
| | Nutmeg seeds (2) | 1 | 1 |
| | Anise (3) | 2 | 1 |
| | Sumac (2) | 1 | 1 |
| | Cardamon seeds (4) | 3 | 1 |
| | Sesame (3) | 2 | 1 |
| **Mixture Spices (30)** | Four spices (1) | 1 | 0 |
| | Seven spices (2) | 1 | 1 |
| | Steak black pepper (1) | 1 | 0 |
| | Ten spices (1) | 1 | 0 |
| | Falafel spices (2) | 1 | 1 |
| | Kibby spices (2) | 1 | 1 |
| | Moghrabieh spices (2) | 1 | 1 |
| | Biryani spices (2) | 1 | 1 |
| | Chicken shawarma spices (1) | 1 | 0 |
| | Rice spices (2) | 1 | 1 |
| | Kefta spices (2) | 1 | 1 |
| | Kabseh spices (2) | 1 | 1 |
| | Sausage spices (1) | 1 | 0 |
| | Francisco spices (3) | 1 | 2 |
| | Red taouk spices (2) | 1 | 1 |
| | Hamburger spices (1) | 1 | 0 |
| | Chicken spices (1) | 0 | 1 |
| | Curry powder (2) | 1 | 1 |

| | | With HACCP/GMP (23) | Without HACCP/GMP (14) |
|---|---|---|---|
| **Single Herbs (34)** | Chamomille flowers (2) | 1 | 1 |
| | Safflower (2) | 1 | 1 |
| | Basil (2) | 1 | 1 |
| | Bay leaves (2) | 1 | 1 |
| | Corn silk (2) | 1 | 1 |
| | Rosemary (2) | 1 | 1 |
| | Sage (3) | 2 | 1 |
| | Oregano (3) | 2 | 1 |
| | Hibiscus (2) | 1 | 1 |
| | Primrose (2) | 1 | 1 |
| | Marjoram (2) | 1 | 1 |
| | Molokia (2) | 1 | 1 |
| | Mint dried (3) | 2 | 1 |
| | Parsley dried (1) | 1 | 0 |
| | Violets (1) | 1 | 0 |
| | Thyme grinded (1) | 1 | 0 |
| | Thyme flower (2) | 1 | 1 |
| **Mixture Herbs (3)** | Mix herbs (1) | 1 | 0 |
| | Extra Thyme (1) | 1 | 0 |
| | Zaatar Halabi (1) | 1 | 0 |

**Table 2. Results of in-house validation of the LC-MS/MS method for the Alternaria toxins considered in this study in a mixture of spices and herbs.**

| Alternaria toxins | Spike level (µg/kg) | Recovery (n=3) (%) | $RSD_r$ (%) | LOD (µg/kg) | LOQ (µg/kg) |
|---|---|---|---|---|---|
| TeA | 50 | 98 | 6 | 1.4 | 4.6 |
| | 100 | 110 | 4 | | |
| | 500 | 115 | 1 | | |
| AOH | 25 | 105 | 7 | 6.1 | 20.4 |
| | 50 | 70 | 3 | | |
| | 200 | 78 | 6 | | |
| ALT | 25 | 122 | 6 | 2.6 | 8.5 |
| | 50 | 119 | 3 | | |
| | 200 | 112 | 1 | | |
| TTX | 25 | 125 | 5 | 2.9 | 9.7 |
| | 50 | 95 | 4 | | |
| | 200 | 98 | 4 | | |
| AME | 25 | 95 | 1 | 1.3 | 4.2 |
| | 50 | 65 | 1 | | |
| | 200 | 62 | 5 | | |

$RSD_r$ = relative standard deviation of repeatability; LOD=limit of detection; LOQ=limit of quantification.

**Or**

| *Alternaria* toxins | Spiking levels (µg/kg) | Mean recovery (n=9) (%) | $RSD_r$ range (%) | LOD (µg/kg) | LOQ (µg/kg) |
|---|---|---|---|---|---|
| TeA | 50, 100, 500 | 108 | 1 - 6 | 1.4 | 4.6 |
| AOH | 25, 50, 200 | 85 | 3- 7 | 6.1 | 20.4 |
| ALT | 25, 50, 200 | 118 | 1 - 6 | 2.6 | 8.5 |
| TTX | 25, 50, 200 | 106 | 4 - 5 | 2.9 | 9.7 |
| AME | 25, 50, 200 | 74 | 1 - 5 | 1.3 | 4.2 |

$RSD_r$ = relative standard deviation of repeatability; LOD=limit of detection; LOQ=limit of quantification.

**Versione tabella 3 con le miscele di spezie ed erbe raggruppate per tipo di miscela**

**Table 3. Whole results of spices and herbs. Mean levels of Alternaria toxins in different single and mixture spices a) and herbs b) samples.**

**a)**

| Spices | Alternaria toxins | | | | | Total Alternaria toxins µg/kg |
|---|---|---|---|---|---|---|
| | TeA µg/kg | AOH µg/kg | ALT µg/kg | TTX µg/kg | AME µg/kg | |
| Red chili (7) | 27255.5 | 12.2 | 3.6 | 32.5 | 19.2 | 27323.0 |
| Paprika (3) | 24331.3 | 28.7 | 2.3 | 71.8 | 29.2 | 24463.3 |
| Caraway (2) | 5380.0 | 3.1 | 1.3 | 139.3 | 0.7 | 5524.4 |
| White pepper (2) | 20.3 | 319.7 | 1.3 | 1.5 | 24.0 | 366.8 |
| Coriander seeds (2) | 650.5 | 3.1 | 1.3 | 1.5 | 0.7 | 657.1 |
| Onion powder (4) | 90.5 | 164.5 | 1.3 | 47.9 | 137.9 | 442.1 |
| Black pepper (4) | 10.5 | 89.0 | 1.3 | 1.5 | 0.7 | 103.0 |
| Garlic powder (2) | 138.0 | 57.4 | 1.3 | 17.9 | 106.3 | 320.9 |
| Turmeric (2) | 263.5 | 3.1 | 2.8 | 1.5 | 25.2 | 296.1 |
| Fennel (2) | 160.9 | 3.1 | 1.3 | 8.4 | 8.0 | 181.7 |
| Cumin (5) | 145.8 | 3.1 | 1.3 | 25.1 | 1.2 | 176.5 |
| Ginger (3) | 44.3 | 5.4 | 5.2 | 1.5 | 5.7 | 62.1 |
| Cinnamon (3) | 7.7 | 26.1 | 6.7 | 1.5 | 15.8 | 57.8 |
| Nutmeg seeds (2) | 22.0 | 12.7 | 1.3 | 1.5 | 8.3 | 45.8 |
| Cloves (2) | 14.9 | 3.1 | 11.7 | 1.5 | 11.7 | 42.9 |
| Sumac (2) | 13.5 | 6.6 | 1.3 | 7.6 | 10.5 | 39.5 |
| Allspice (3) | 0.7 | 8.0 | 1.3 | 1.5 | 14.3 | 25.8 |
| Cardamon seeds (4) | 13.1 | 3.1 | 1.3 | 1.5 | 0.7 | 19.7 |
| Fenugreek grain (4) | 12.5 | 3.1 | 1.3 | 2.2 | 5.9 | 25.0 |
| Sesame (3) | 0.7 | 3.1 | 1.3 | 1.5 | 2.9 | 9.5 |
| Anise (3) | 0.7 | 3.1 | 1.3 | 1.5 | 0.7 | 7.3 |
| **Mean of means** | **2789.4** | **36.3** | **2.5** | **17.7** | **20.5** | **2866.2** |
| **Median of means** | **22.0** | **5.4** | **1.3** | **1.5** | **8.3** | **103.0** |
| **Mixture Spices** | **TeA µg/kg** | **AOH µg/kg** | **ALT µg/kg** | **TTX µg/kg** | **AME µg/kg** | **Alternaria toxins µg/kg** |
| Chicken shawarma spices (1) | 12071.0 | 610.7 | 1.3 | 21.3 | 14.7 | 12719.0 |
| Red taouk spices (2) | 4731.4 | 24.0 | 1.3 | 21.6 | 7.3 | 4785.6 |
| Moghrabieh spices (2) | 1405.3 | 29.2 | 1.3 | 32.7 | 7.8 | 1476.3 |
| Francisco spices (3) | 1105.8 | 216.4 | 1.3 | 8.5 | 5.9 | 1337.9 |
| Kibby spices (1) | 744.5 | 57.8 | 1.3 | 55.7 | 7.6 | 866.9 |
| Curry powder (2) | 729.7 | 6.6 | 1.3 | 38.2 | 11.2 | 787.0 |
| Kabseh spices (2) | 331.9 | 9.6 | 1.3 | 4.8 | 0.7 | 348.3 |
| Steak black pepper (1) | 312.9 | 3.1 | 1.3 | 7.6 | 0.7 | 325.6 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Falafel spices (2) | 195.3 | 3.1 | 1.3 | 12.2 | 9.4 | 221.3 |
| Kefta spices (2) | 131.2 | 36.9 | 1.3 | 6.5 | 13.8 | 189.7 |
| Biryani spices (2) | 103.5 | 65.4 | 1.3 | 1.5 | 0.7 | 172.3 |
| Ten spices (1) | 150.0 | 3.1 | 1.3 | 7.8 | 0.7 | 162.9 |
| Chicken spices (1) | 49.3 | 53.2 | 1.3 | 1.5 | 14.6 | 119.9 |
| Sausage spices (1) | 0.7 | 100.2 | 1.3 | 1.5 | 0.7 | 104.4 |
| Rice spices (2) | 15.3 | 49.0 | 1.3 | 1.5 | 15.5 | 82.6 |
| Four spices (1) | 27.9 | 22.2 | 1.3 | 1.5 | 0.7 | 53.6 |
| Hamburger spices (1) | 6.6 | 17.3 | 1.3 | 1.5 | 9.5 | 36.2 |
| Seven spices (2) | 15.1 | 10.5 | 1.3 | 1.5 | 4.9 | 33.3 |
| **Mean of means** | **1229.3** | **73.2** | **1.3** | **12.6** | **7.0** | **1323.5** |
| **Median of means** | **172.7** | **26.6** | **1.3** | **7.1** | **7.5** | **205.5** |

| | | Alternaria toxins | | | | | |
|---|---|---|---|---|---|---|---|
| **b)** | **Herbs** | **TeA µg/kg** | **AOH µg/kg** | **ALT µg/kg** | **TTX µg/kg** | **AME µg/kg** | **Total Alternaria toxins µg/kg** |
| Corn silk (2) | | 2495.9 | 3.1 | 1.3 | 1.5 | 0.7 | 2502.4 |
| Violets (1) | | 1670.3 | 45.7 | 1.3 | 1.5 | 9.3 | 1728.1 |
| Marjoram (2) | | 406.5 | 3.1 | 1.3 | 32.3 | 19.9 | 463.1 |
| Oregano (3) | | 75.0 | 13.5 | 1.3 | 21.0 | 29.0 | 139.8 |
| Sage (3) | | 137.6 | 30.5 | 1.3 | 3.6 | 80.3 | 253.4 |
| Molokia (2) | | 159.2 | 3.1 | 1.3 | 1.5 | 0.7 | 165.6 |
| Thyme grinded (1) | | 77.3 | 3.1 | 1.3 | 28.8 | 33.9 | 144.4 |
| Mint dried (3) | | 80.2 | 11.8 | 1.3 | 6.2 | 17.5 | 116.9 |
| Thyme flower (2) | | 67.2 | 3.1 | 1.3 | 1.5 | 32.1 | 105.1 |
| Safflower (2) | | 80.1 | 3.1 | 1.3 | 1.5 | 0.7 | 86.6 |
| Chamomille flowers (2) | | 75.7 | 3.1 | 1.3 | 1.5 | 0.7 | 82.2 |
| Rosemary (2) | | 50.4 | 3.1 | 1.3 | 1.5 | 17.6 | 73.8 |
| Bay leaves (2) | | 48.2 | 3.1 | 1.3 | 1.5 | 7.8 | 61.8 |
| Primrose (2) | | 0.7 | 20.3 | 1.3 | 1.5 | 15.7 | 39.4 |
| Hibiscus (2) | | 0.7 | 3.1 | 1.3 | 1.5 | 0.7 | 7.3 |
| Basil (2) | | 46.6 | 3.1 | 1.3 | 1.5 | 0.7 | 53.1 |
| Parsley dried (1) | | 0.7 | 3.1 | 1.3 | 1.5 | 0.7 | 7.2 |
| **Mean of means** | | 319.2 | 9.4 | 9.1 | 6.5 | 15.8 | 359.9 |
| **Median of means** | | 75.7 | 3.1 | 1.3 | 1.5 | 9.3 | 105.1 |

| **Mixture Herbs** | **TeA µg/kg** | **AOH µg/kg** | **ALT µg/kg** | **TTX µg/kg** | **AME µg/kg** | **Alternaria toxins µg/kg** |
|---|---|---|---|---|---|---|
| Mix herbs (1) | 212.0 | 32.5 | 1.3 | 16.2 | 35.7 | 297.7 |
| Extra Thyme (1) | 186.3 | 3.1 | 1.3 | 67.0 | 19.9 | 277.6 |
| Zaatar Halabi (1) | 141.0 | 3.1 | 1.3 | 24.1 | 16.6 | 186.1 |
| **Mean of means** | | | | | | |
| **Median of means** | | | | | | |

**Oppure questa versione più snella con la media delle miscele di spezie e di erbe**

**Table 3. Whole results of spices and herbs. Mean levels of Alternaria toxins in different single and mixture spices a) and herbs b) samples.**

| a) Spices | Alternaria toxins TeA µg/kg | AOH µg/kg | ALT µg/kg | TTX µg/kg | AME µg/kg | Total Alternaria toxins µg/kg |
|---|---|---|---|---|---|---|
| Allspice (3) | 0.7 | 8 | 1.3 | 1.5 | 14.3 | 25.8 |
| Black pepper (4) | 10.5 | 356 | 5.2 | 5.8 | 2.6 | 380.1 |
| White pepper (2) | 20.3 | 639.5 | 2.6 | 2.9 | 48.1 | 713.4 |
| Red chili (7) | 27255.5 | 12.2 | 3.6 | 32.5 | 19.2 | 27323 |
| Paprika (3) | 24331.3 | 28.7 | 2.3 | 71.8 | 29.2 | 24463.3 |
| Garlic powder (2) | 138 | 57.4 | 1.3 | 18 | 106.3 | 321 |
| Onion powder (4) | 90.5 | 164.5 | 1.3 | 48 | 138 | 442.3 |
| Turmeric (2) | 263.5 | 3.1 | 2.8 | 1.5 | 25.2 | 296.1 |
| Cinnamon (3) | 7.7 | 26.1 | 6.7 | 1.5 | 15.8 | 57.8 |
| Ginger (3) | 66.1 | 3.1 | 7.2 | 1.5 | 0.7 | 78.6 |
| Fennel (2) | 161 | 3.1 | 1.3 | 8.4 | 8 | 181.8 |
| Caraway (2) | 5380 | 3.1 | 1.3 | 139.3 | 0.7 | 5524.4 |
| Cloves (2) | 14.9 | 3.1 | 22.1 | 1.5 | 0.7 | 42.3 |
| Coriander seeds (2) | 650.5 | 3.1 | 1.3 | 1.5 | 0.7 | 657.1 |
| Cumin (5) | 145.8 | 3.1 | 1.3 | 25.1 | 1.2 | 176.5 |
| Fenugreek grain (4) | 12.5 | 3.1 | 1.3 | 2.2 | 5.9 | 25 |
| Nutmeg seeds (2) | 22 | 12.7 | 1.3 | 1.5 | 8.3 | 45.8 |
| Anise (3) | 0.7 | 3.1 | 1.3 | 1.5 | 0.7 | 7.3 |
| Sumac (2) | 13.5 | 6.6 | 1.3 | 7.6 | 10.5 | 39.5 |
| Cardamon seeds (4) | 13.1 | 3.1 | 1.3 | 1.5 | 0.7 | 19.7 |
| Sesame (3) | 0.7 | 3.1 | 1.3 | 1.5 | 2.9 | 9.5 |
| Mixture Spices (30) | 1168.1 | 72.1 | 1.3 | 14.9 | 7.7 | 1264.0 |

| c) Herbs | Alternaria toxins TeA µg/kg | AOH µg/kg | ALT µg/kg | TTX µg/kg | AME µg/kg | Total Alternaria toxins µg/kg |
|---|---|---|---|---|---|---|
| Chamomille flowers (2) | 75.7 | 3.1 | 1.3 | 1.5 | 0.7 | 82.2 |
| Safflower (2) | 80.1 | 3.1 | 1.3 | 1.5 | 0.7 | 86.6 |
| Basil (2) | 0.7 | 3.1 | 1.3 | 1.5 | 0.7 | 7.2 |
| Bay leaves (2) | 48.2 | 3.1 | 1.3 | 1.5 | 7.8 | 61.8 |
| Corn silk (2) | 2495.9 | 3.1 | 1.3 | 1.5 | 0.7 | 2502.4 |
| Rosemary (2) | 50.4 | 3.1 | 1.3 | 1.5 | 17.6 | 73.8 |
| Sage (3) | 137.6 | 30.5 | 1.3 | 3.6 | 80.3 | 253.4 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Oregano (3) | 75 | 13.5 | 134.2 | 21 | 29 | 272.7 |
| Hibiscus (2) | 0.7 | 3.1 | 1.3 | 2.2 | 0.7 | 7.9 |
| Primrose (2) | 0.7 | 20.3 | 1.3 | 1.5 | 15.7 | 39.4 |
| Marjoram (2) | 406.5 | 3.1 | 1.3 | 32.3 | 19.9 | 463.1 |
| Molokia (2) | 159.2 | 3.1 | 1.3 | 1.5 | 0.7 | 165.6 |
| Mint dried (3) | 80.2 | 11.8 | 1.3 | 6.2 | 17.5 | 116.9 |
| Parsley dried (1) | 0.7 | 3.1 | 1.3 | 1.5 | 0.7 | 7.2 |
| Violets (1) | 1670.3 | 45.7 | 1.3 | 1.5 | 9.3 | 1728.1 |
| Thyme grinded (1) | 77.3 | 3.1 | 1.3 | 28.8 | 33.9 | 144.4 |
| Thyme flower (2) | 67.2 | 3.1 | 1.3 | 1.5 | 32.1 | 105.1 |
| Mixture Herbs (3) | 179.8 | 12.9 | 1.3 | 35.7 | 24.1 | 253.8 |

**Table 4: Summary of the results obtained for the occurrence of Alternaria toxins in the 94 spice samples (a) and 37 herbs samples (b) commercialized in Lebanon. Five Alternaria toxins were determined: Tenuazonic acid (TeA), Alternariol (AOH), Altenuene (ALT), Tentoxin (TTX), and Alternariol methyl ether (AME).**

**a)**

| **Spices= 94** | **TeA** | **AOH** | **ALT** | **TTX** | **AME** |
|---|---|---|---|---|---|
| min (µg/kg) | 0.7 | 3.1 | 1.3 | 1.5 | 0.7 |
| max (µg/kg) | 106792.8 | 636.4 | 22.1 | 179.4 | 306.0 |
| mean (µg/kg) | 3311.1 | 45.0 | 2.1 | 16.8 | 16.3 |
| median (µg/kg) | 41.6 | 3.1 | 1.3 | 1.4 | 0.6 |
| **n positive** | **72** | **38** | **7** | **38** | **41** |
| **% positive** | **77** | **40** | **7** | **40** | **44** |

| **Herbs (n=37)** | **TeA** | **AOH** | **ALT** | **TTX** | **AME** |
|---|---|---|---|---|---|
| min (µg/kg) | 0.7 | 3.1 | 1.3 | 1.5 | 0.7 |
| max (µg/kg) | 4868.4 | 64.2 | 400.0 | 67.0 | 161.3 |
| mean (µg/kg) | 273.4 | 9.7 | 12.1 | 9.7 | 19.1 |
| median (µg/kg) | 78.8 | 3.0 | 1.3 | 1.4 | 9.3 |
| **n positive** | **27** | **7** | **1** | **11** | **19** |
| **% positive** | **73** | **19** | **3** | **30** | **51** |

Figure 1 Comparison of *Alternaria* mycotoxins mean levels in spices (n=94) and herbs (n=37). Different letters between pairs of means represent significant differences ($P$<0.05).

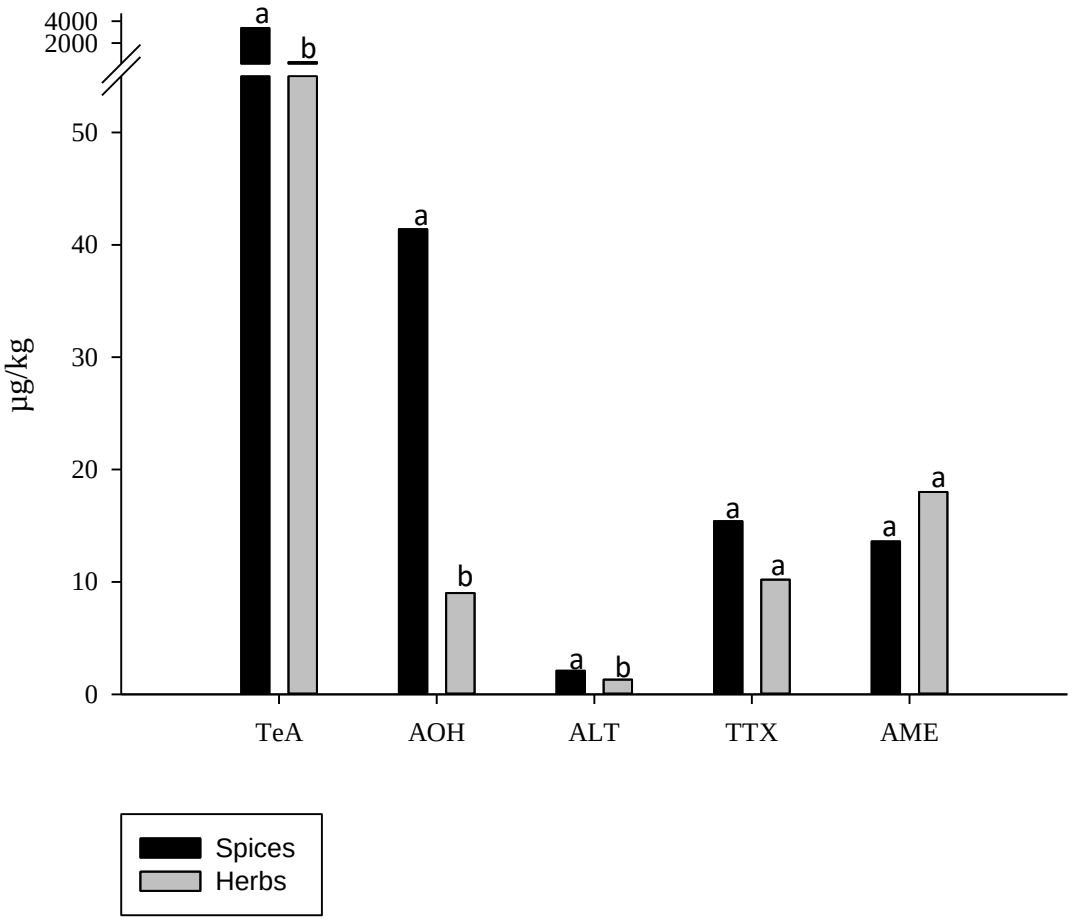
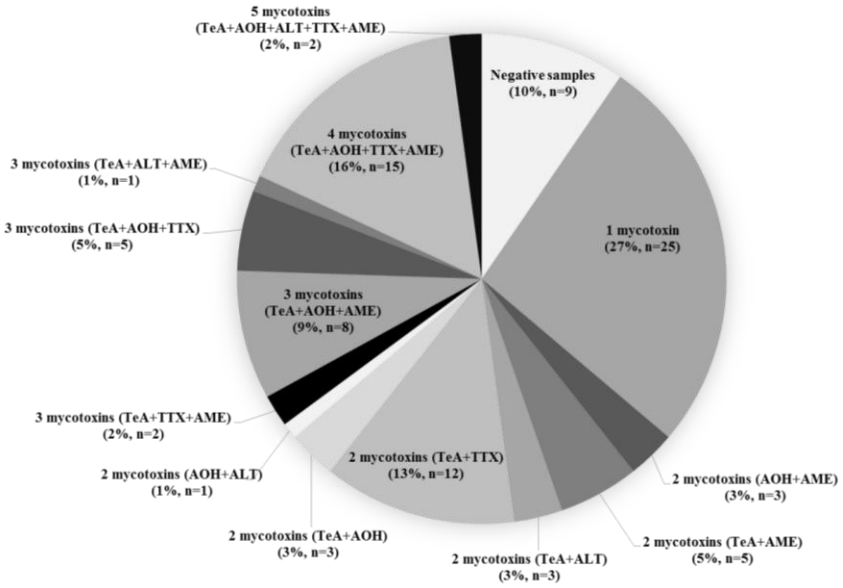


Figure 2. Mycotoxin combinations in spices **a)** and herbs **b)**

**a)**




**b)**

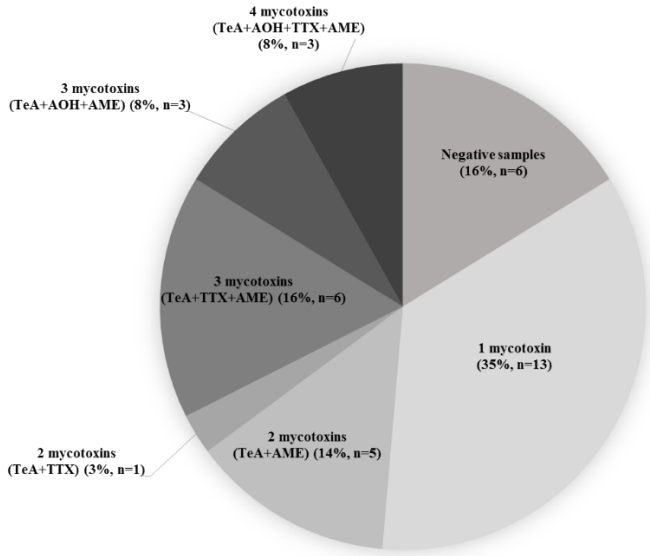
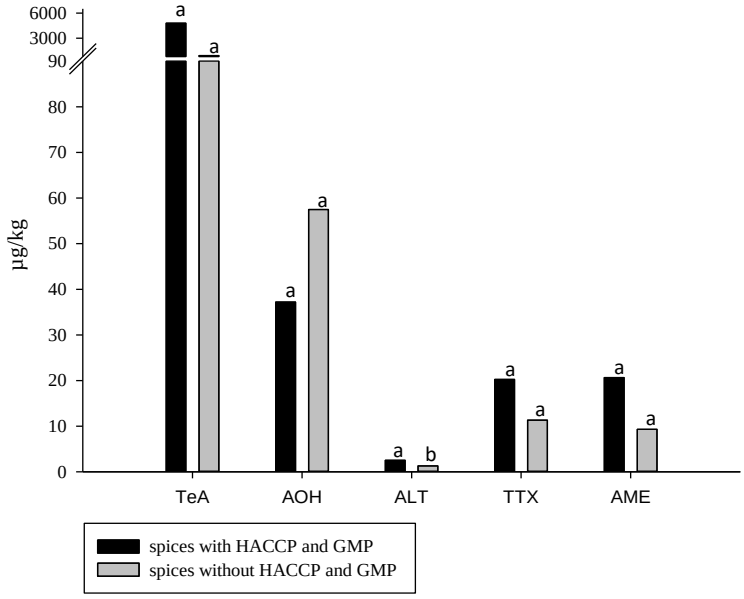


Figure 3. Comparison of *Alternaria* mycotoxins (TeA, AOH, ALT, TTX, AME) in spices (n=94) produced with a) or without b) HACCP and GMP certification. Different letters between pairs of means represent significant differences ($P$<0.05).

**a)**




**b)**