Multimycotoxins occurrence in spices and herbs commercialized in Lebanon

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Short version of title: Multimycotoxins in spices

Abstract

Aflatoxins and ochratoxin A are regulated in Europe for some spices (*Capsicum spp.*, *Piper spp.*, *Myristica fragrans*, *Zingiber officinale*, *Curcuma longa*) and mixtures of spices containing one or more of these spices. No mycotoxin limits are in force for herbs. A total of 132 samples of spices (94) and herbs (38) purchased from Beirut in Lebanon were analysed for 12 mycotoxins (AFB_1, AFB_2, AFG_1, AFG_2, OTA, FB_1, FB_2, HT-2, T-2, ZEA, DON, NIV) by using a UPLC-MS/MS method based on 'dilute and shoot' approach. The limits of detection (LOD) ranged from 0.1 µg/kg (ZEA) to 20.5 µg/kg (DON) and limits of quantification (LOQ) ranged from 0.3 µg/kg (ZEA) to 68.2 µg/kg (DON). 80% of analysed samples were contaminated by 1 to 11 mycotoxins. Total aflatoxins and ochratoxin A were detected in 19 and 30% of spices, 8 and 11% of herbs, respectively. Mean levels of total aflatoxins and ochratoxin A were 168.1 and 7.1 µg/kg in positive spices, 36.1 and 7.0 µg/kg in positive herbs, respectively. 78 and 10% of positive spice samples contained aflatoxin and ochratoxin A at levels higher than the limits, respectively. Total aflatoxin levels higher than the European limits were also measured in some non-regulated spices (allspice, cloves, coriander, fenugreek) and some herbs (rosemary, sage and oregano). Within the non-regulated mycotoxins FB_1 was the most occurring (60% in spices, 55% in herbs) followed by FB_2 (35% in spices, 18% in herbs), ZEA (30% in spices, 3% in herbs), DON (12% in spices, 3% in herbs), T-2 and HT-2 toxins (3-5%), whereas NIV and AFG_2 were never detected. Mean levels of FB_1, FB_2, ZEA and DON in positive samples of spices were 6432.3, 203.2, 30.6, 1751.4 µg/kg, respectively; in positive samples of herbs they were 2826.3, 214.9, 2.8, 589.7 µg/kg, respectively. The whole results demonstrate the higher susceptibility of spices to mycotoxin contamination with respect to herbs. Comparison of results obtained for samples produced with (81) and without (51) HACCP and GMP showed that the implementation of HACCP and GMP practices seems to be effective in reducing the occurrence of regulated mycotoxins but was ineffective for the non-regulated ones. The samples analysed in this study originated from at least 15 Countries and the results obtained gives indications about the occurrence of mycotoxins in relation to the Country of origin of the samples.

The high percentages of positive samples and the high levels of some mycotoxins observed in this study highlight the problem of mycotoxin contamination in spices and herbs consumed in Lebanon. The occurrence of high levels of aflatoxins and OTA in some non-regulated spices and herbs suggests the addition of these matrices in the list of regulated ones. The high number of positive samples and the

high levels of fumonisins observed in this study suggest the inclusions of these mycotoxins in the list of regulated mycotoxins for these matrices.

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

1. Introduction

Mycotoxins are natural food and feed contaminants, mainly produced by moulds of the genera *Aspergillus*, *Penicillium* and *Fusarium*. Mycotoxins, especially aflatoxins (AFs) and ochratoxin A (OTA), can contaminate spices and herbs at various degree of incidence and levels (Zinedine et al., 2006)**.** The exposure of human being to mycotoxins is a life threatening problem, especially in developing Countries where hot and humid climate favours fungal growth and where the food storage conditions are not adequate (Wild & Gong, 2009)**.** According to the annual report of the Rapid Alert System for Food and Feed (RASFF) in 2016, mycotoxins were the first main hazard in border rejection notifications in the European Union, with spices and herbs being the second most affected food category (79 notifications) after the food category including nuts, nuts products and seeds (334 notifications) (European Union, 2016). This contamination could be mainly due to the drying process of the spices, laying down on the ground in the open air where the climatic conditions are ideal for growth of moulds and production of mycotoxins (Martins, Martins, & Bernardo, 2001).

AFs, OTA, some *Fusarium* toxins and Patulin are considered the main mycotoxins and they are regulated in food worldwide (Van Egmond, Schothorst, & Jonker, 2007) . For spices, there are two types of mycotoxins of concern: AFs and OTA (Kabak & Dobson, 2017). However, other

mycotoxins such as zearalenone (ZEA), trichothecenes, fumonisins (FBs), *Alternaria* toxins can also contaminate spices and herbs but their occurrence has been poorly investigated.

AFs are secondary metabolites produced by filamentous fungi *Aspergillus* mainly *A. flavus* and *A. parasiticus* (Reddy, Raghavender, Salleh, Reddy, & Reddy, 2011) . Four naturally occurring AFs were identified B_1, B_2, G_1 and G_2 with AFB_1 being the most common and toxic (Groopman, Kensler, & Wild, 2008) was classified as human carcinogen (group 1) by the International Agency for Research on Cancer (IARC) (1993). European Commission Regulations set maximum limits for AFB_1 and total aflatoxins in some spices (*Caspicum spp., Piper spp., Myristica fragrans, Zingiber officinale, Curcuma longa* and mixtures of spices containing one or more of these spices) of 5 µg/kg and 10 µg/kg, respectively (European Commission, 2015).

OTA, mainly produced by *Penicillium verrucosum*, *A. ochraceus* and *A. carbonarius* can contaminate agricultural products prior to harvest or more commonly during storage (EFSA, 2006). OTA has been classified by the International Agency for Research on Cancer (IARC) as a probable human carcinogen (Group 2B) (IARC, 1993). With regard to OTA, Commission Regulation No. 1137/2015 has set maximum limits of 15-20 µg/kg in the spices and mixtures reported above. No limits are currently in force in spices for other important mycotoxins such as deoxynivalenol (DON), zearalenone and fumonisins that are regulated in other food commodities. No mycotoxin limits are in force for herbs. The European Committee for Standardization (CEN) has published several standard methods for the determination of mycotoxins in food commodities including spices and herbs. However, most of these methods were developed for individual mycotoxin or group of related mycotoxins. Up to our knowledge, no studies have been conducted on the multi-mycotoxin occurrence in spices and herbs consumed in Lebanon.

The main purpose of this study is to provide useful information on multi-mycotoxin incidence and levels in different samples of spices and herbs commercialized in Lebanon. In addition to AFs and OTA, other mycotoxins such as FBs, T-2 toxin (T-2), HT-2 toxin (HT-2), ZEA, DON

and nivalenol (NIV) were analysed by LC-MS/MS, with the aim to provide information on the possible multi-mycotoxin occurrence in herbs and spices consumed in Lebanon.

2. Materials and methods

2.1. Sampling

One hundred and thirty two samples of different kinds of herbs, spices and mixtures were collected from Beirut in Lebanon (Table 1). Of these, 64 samples were of single spices and 30 samples were spices mixtures. Fifty eight samples were purchased from shops selling spices manufactured and packaged according to GMP (Good Manufacturing Practice) and HACCP (Hazard Analysis and Critical Control Point) in certified factories. Thirty six samples were purchased in local shops selling spices in bulk where the manufacturing conditions were unknown (no GMP and HACCP). Furthermore, 38 samples of herbs (single herbs and mixtures) were tested, 23 samples manufactured according to GMP and HACCP rules and 15 samples manufactured without GMP and HACCP rules. All the samples were sent to CNR-ISPA (Italy) with a respect of proper dry storage conditions. At the receiving in Italy, each sample was finely ground, blended and then analysed by LC-MS/MS for Fumonisin B_1 (FB_1), Fumonisin B_2 (FB_2), OTA, T-2, HT-2, Aflatoxin B_1 (AFB_1), Aflatoxin B_2 (AFB_2), Aflatoxin G_1 (AFG_1), Aflatoxin G_2 (AFG_2), Zearalenone (ZEA), DON and NIV.

2.2. Chemicals and reagents:

The standard solutions were purchased from Romer Labs Diagnostic (Tulln, Austria). In particular, separated standard solutions of FB_1 (50 µg/mL) and FB_2 (50 µg/mL) in acetonitrile : water (50:50, v/v) were used. The other standard solutions were in acetonitrile (ACN): OTA (10 µg/mL), HT-2 (100.2 µg/mL), AFB_1 (2.0 µg/mL), AFB_2 (0.5 µg/mL), AFG_1 (2.01 µg/mL), AFG_2 (0.5 µg/mL), T-2 (100.2 µg/mL), ZEA (100.2 µg/mL), DON (100.3 µg/mL), NIV (100.8 µg/mL).

Chromatography-grade methanol (MeOH), ACN, glacial acetic acid and ammonium acetate were obtained from sigma-Aldrich (Milan, Italy). Ultrapure water was produced by use of a Milli-Q system (Millipore, Bedford, MA, USA).

2.3. Determination of mycotoxins

2.3.1. Mycotoxins extraction

The LC-MS/MS method previously described (Gambacorta et al., 2018) for the determination of 12 mycotoxins was used herein with some modifications. In particular, 5 g of grounded spices or herbs were first extracted with 25 mL of ultrapure water, 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), 17.5 mL of water extract was collected (extract A). Then, 17.5 mL of methanol was added to the remaining solid material containing 7.5 mL of water and the sample was extracted again by shaking for 60 min (now the composition of the extraction solution was 70% methanol). After centrifugation at 3,000×g for 10 min the methanol:water (70:30) extract was collected (extract B). One mL of extract A was mixed with 1 mL of extract B, filtered through 0.45 µm regenerated cellulose filter and 10 µL were analysed by LC-MS/MS. For some samples more rich in fibers, it was necessary to use a higher volume of water (up to 50 mL) for the first extraction, due to the high capacity of water absorption of these samples. In these cases, the volume of methanol used for the second extraction was proportionally increased.

A mixture of several spices and herbs was used for recovery experiments and to prepare the extracts for matrix assisted calibration solutions. In particular, a commercially available sample of Seven Spices (ginger, cloves, black pepper, nutmeg, allspice, mahlab, cinnamon) and a sample of Mix Herbs (bay leaves, thyme, parsley, rosemary, basil, celery seeds, summer savory) were mixed, ground and used as 5 g aliquots.

Standard solutions of OTA, FB_1, FB_2, HT-2, T-2, ZEA, DON, NIV, AFB_1, AFB_2, AFG_1 and AFG_2 were used to prepare a mixed spiking solution and five mixed calibration solutions at different concentrations prepared in dried samples extracts of mixture of spices and herbs. Acetonitrile was used to adequately dilute the standard solutions where necessary. The spiking levels of the 12 mycotoxins are reported in Table 2. Recovery experiments were performed in triplicate and the recoveries were calculated after

subtracting the peak area of endogenous mycotoxins eventually present in samples used for these experiments. Matrix assisted calibration curves were prepared in dried sample extracts obtained from the mixtures of spices and herbs (see above). The limits of detection (LOD) and quantification (LOQ) of each mycotoxin were calculated as 3 times and 10 times the noise, respectively. The noise was measured immediately before or after the peak of each analyte.

2.3.2. LC-MS/MS equipment and calibration

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 +5,500 V or -4,500 V.

Quantification of mycotoxins in 132 samples of spices and herbs extracts 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 extracts ranged between: 0.002-0.1 ng injected for AFB_1, AFG_1, OTA, and HT-2, 0.0005-0.025 ng injected for AFB_2 and AFG_2, 0.07-3.5 ng injected for DON, 0.004-0.2 ng injected for T-2, 0.01-0.5 ng injected for FB_1, 0.02-1 ng injected for FB_2, and 0.001-0.05 ng injected for ZEA.

2.3.3. LC-MS/MS parameters for the determination of mycotoxins

The MS/MS parameters for the mycotoxins considered in this study are reported in (Gambacorta et al., 2018). In particular, each sample extract was analysed twice, in positive ion mode (AFB_1, AFB_2, AFG_1, AFG_2, OTA, FB_1, FB_2, T-2, HT-2), and in negative ion mode (NIV, DON and ZEA).

Three transitions for confirmation and one transition for quantification were used 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 were used.

The separation of the mycotoxins considered in this study was performed according to (Gambacorta et al., 2018) with some modifications by 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 positive ion mode, the separation was performed with the following binary linear gradient of acidic MeOH (containing 0.5% acetic acid, 1 mM ammonium acetate) in water (containing 0.5% acetic acid, 1 mM ammonium acetate): from 20% to 80% acidic MeOH in 20 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 negative ion mode, the separation was performed with the following binary linear gradient of acidic MeOH (containing 0.5% acetic acid, 1 mM ammonium acetate) in water (containing 0.5% acetic acid, 1 mM ammonium acetate): 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.

2.4. Statistical analysis

Mean, median and standard deviation of results 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 were subjected to the unpaired t-test (one-tail P value). Values were judged to be significantly different if P values were < 0.05.

3. Results and discussion

3.1. Method performance

The method performance characteristics obtained from triplicate analyses of a mixture of seven spices and a mixture of seven herbs spiked with the 12 mycotoxins and quantified by use of matrix-matched

calibration curves are reported in Table 2. Acceptable mean recoveries, between 72% (ZEA) and 105% (FB_2), were obtained for most of the analysed mycotoxins whereas the mean recovery of AFB_1 and AFB_2 were 60% and 61%, respectively. The repeatability of results was acceptable for also mycotoxins and ranged between 1% (FB_1 and HT-2) and 24% (NIV). For the calculation of the limits of detection (LOD) and quantification (LOQ) the signal-to-noise ratio of 3 and 10, respectively were used. LOD values ranged from 0.1 (ZEA) to 20.5 µg/kg (DON) whereas the LOQs values ranged from 0.3 (ZEA) to 68.2 µg/kg (DON) (Table 2). The criteria established in the EU Regulation 519/2014 for aflatoxins require 70-110% recovery for levels of 1-10 µg/kg and 80-110% for levels >10 µg/kg, whereas the repeatability (RSDr) values, at the spiking levels tested herein (15 µg/kg AFB_1 and 4 µg/kg AFB_2, Table 2), should be lower than 9.8% for AFB_1 and 12.1% for AFB_2 as calculated by multiplying per 0.66 the result obtained from Horwitz equation; in our case RSDr is 6% for both AFB_1 and AFB_2 (Table 2). Although our recovery results for AFB_1 and AFB_2 are lower than the established values the repeatability of recovery results are acceptable. Moreover, the values of LOD and LOQ of our method for AFB_1 and AFB_2 are sufficiently low (Table 2) and comparable or better of those obtained for the other mycotoxins reported in Table 2 that gave acceptable recoveries. The method performances obtained in this study for the analysed mycotoxins are comparable to those reported in other studies using a similar approach for sample extraction and partial cleanup before LC-MS/MS determinations (Gambacorta et al., 2018; Motloung et al., 2018)).

3.2. Multi-mycotoxin occurrence in spices and herbs commercialized in Lebanon

Table 3 represents the summary of results on multi-mycotoxin occurrence in 94 samples of spices and 38 samples of herbs collected from the Lebanese market. While assessing the regulated mycotoxins (AFB_1, total aflatoxins and OTA), the percentage of positive samples ranged from 16 to 30% and from 8 to 11% for spices and herbs, respectively (Table 3). For aflatoxins the percentage of positive spice samples were about double as compared to herbs whereas for OTA the percentage of positive spice

235 samples were three times higher compared to herbs (Table 3). The average level of AFB_1 in the 15
236 positive spice samples was 193.4 (µg/kg), which is about forty times higher than the European limit of
237 AFB_1 in some spices (5µg/kg) laid down in the Commission Regulation (EC) No. 165/2010. AFB_2 was
238 detected at low levels in 3 spice samples (red chili, red pepper, curry powder, 0.2-4.4 µg/kg), AFG_1 in
239 4 samples (cloves, anise, nutmeg seeds, mixture of spices at 0.2-85.7 µg/kg) whereas AFG_2 was never
240 detected. For total aflatoxins the average level (168.1 µg/kg) in the 18 positive spice samples was about
241 17 times higher than the European limit (10 µg/kg).
242 For the herbs the situation was less worrying because only 3 samples (rosemary, sage and oregano)
243 were contaminated with only AFB_1 but at levels higher than 5 µg/kg with a mean level (36.1 µg/kg) 7
244 times higher than the European limit. No herb samples were found contaminated by AFB_2, AFG_1 and
245 AFG_2.
246 OTA was detected in 28 samples of spices in the range of 0.7-33.9 µg/kg and the mean level of positive
247 samples (7.1 µg/kg) was below the European limits (15-20 µg/kg) Commission Regulation No.
248 1137/2015. The mean level of OTA (7.0 µg/kg) in the 4 positive samples of herbs (0.7-9.8 µg/kg) was
249 similar to those observed in spices but the percentage of positive samples was 1/3 of spices (Table 3).
250 While assessing the non-regulated mycotoxins, FB_1 was highly present in spices (64% positives) and
251 of herbs (55% positives). Mean FB_1 levels in positive spices (6432.3 µg/kg) and herbs (2826.4 µg/kg)
252 represent the highest mean values among the twelve mycotoxins analysed in this study. For FB_2 lower
253 percentages of positive samples (35% for spices, 18% for herbs) and mean levels (230.2 µg/kg for
254 spices and 75.2 µg/kg for herbs) were observed, and again spices were more contaminated compared
255 to herbs. A significant percentage of positive samples was also observed for ZEA (30%) and to a lesser
256 extend DON (12%) in spices (Table 3). On the other hand the mean level of DON (1751.4 µg/kg) in
257 positives samples of spices was 57 times higher than that of ZEA (30.6 µg/kg). Only one sample of
258 herbs (zaatar halabi) contained ZEA (2.8 µg/kg) and another sample of herb (safflower) contained DON
259 (589.7 µg/kg). The occurrence of T-2 and HT-2 toxins in spices was quite limited, as only 3-4% of

samples were positive at levels ranging between 3.8-16.7 µg/kg. Low incidence of positive samples (3-5%) and low levels of T-2 and HT-2 (0.9-36.6 µg/kg) were also observed for herbs. NIV was never detected in all samples of spices and herbs. As observed for aflatoxins and OTA, also for the non-regulated mycotoxins the percentage of positive samples and relevant mean levels were higher in spices than herbs, therefore herbs should be considered less susceptible to mycotoxin contamination.

Table 4 represents the number of samples contaminated with aflatoxins and/or OTA at levels higher than the European limits established for some spices. In particular, 10 single spice (2 allspice, 2 red chilli, 1 red pepper, 1 coriander, 1 paprika, 1 nutmeg, 1 fenugreek and 1 cloves) and 3 mixtures of spices (1 Moghrabieh Spices, 1 Red Taouk Spices, and 1 Hot Curry Spices) were contaminated with AFB_1 at levels higher than the European limit (5 µg/kg). Four of these spices (allspice, coriander, fenugreek and cloves) are not included in the list of regulated spices reported in EC Regulation N. 165/2010.

Within the herb samples, 3 single herbs (1 rosemary, 1 sage and 1 oregano) were contaminated with AFB_1 at level higher than 5 µg/kg. Moreover, the rosemary and sage samples contained total aflatoxins higher than 10 µg/kg.

Only three spice samples (1 paprika, 1 nutmeg and 1 mixture of spices) contained OTA at level higher than the EU limit (15 µg/kg). The OTA levels in the four positive herb samples were below 15 µg/kg. Although the percentages of positive samples for aflatoxins and OTA are lower than those reported in other studies (Kabak & Dobson, 2017) the mean levels observed in our study are much higher (Table 3).

3.3. Comparison of multi-mycotoxin (regulated and non-regulated) occurrence in different type of single spices, herbs and mixtures.

Table 5 represents the mean levels of regulated mycotoxins (total aflatoxins and OTA) and the mean levels of the sum of non-regulated mycotoxins in 21 different spices, 30 spices mixtures, 18 different

herbs and 3 herbs mixtures listed in this Table. These results helps to identify the spice or herb more susceptible to mycotoxin contamination. In particular, 7 spices were found heavily contaminated with aflatoxins at values higher than the European limit. Out of these 7 spices, three (red chilli, paprika and nutmeg seeds) are regulated under European Commission No. 165/2010, whereas the other four spices (allspice, cloves, coriander seeds and fenugreek grain) are not regulated. Among the 3 regulated spices, the mean total aflatoxin levels were 7 to 19 times higher than the European limit (10 µg/kg). Among the 4 non-regulated spices, the mean total aflatoxin levels were 7 to 112 times higher than 10 µg/kg. One sample of Anise also contained AFG_1 (5.3 µg/kg) and one sample of Cardamom contained only AFB_1 (3.9 µg/kg).

Within the herbs 2 samples (rosemary and sage) contained aflatoxins at levels 4 to 6 times higher than 10 µg/kg whereas one sample of oregano contained 8.7 µg/kg AFB_1.

While assessing the OTA occurrence, 8 spices (black pepper, red chili, paprika, garlic powder, turmeric, cumin, nutmeg seeds and anise) were found contaminated but only one sample (nutmeg seeds) at level higher the European limit of 15 µg/kg.

Within the 18 herbs listed in Table 5 only one sample of chamomille and one sample of sage contained measurable levels of OTA but below 15 µg/kg.

While assessing the non-regulated mycotoxins, 90% of spices and 89% of herbs were contaminated. Among the spices, only caraway and cloves samples were negative for all non-regulated mycotoxins analysed in this study, although cloves contained high level of aflatoxins (Table 5).

Within the spices, garlic and onions powders presented the highest levels of fumonisins, up to 115231.9 µg/kg of FB_1+FB_2. Low levels of fumonisins (10 to 135 µg/kg) were previously reported in garlic and onions powders by (Boonzaaijer, van Osenbruggen, Kleinnijenhuis, & van Dongen, 2008), (Waśkiewicz, Beszterda, Bocianowski, & Goliński, 2013) and Tonti et al., 2017). The very high fumonisin levels found in this study emphasize the importance of exploring the origin of fumonisin contamination of garlic and onion powders. Other spices containing high levels (>2000 µg/kg) of non-

310 regulated mycotoxins were, in descending order: cardamom seeds, paprika, cumin, red chilli, sesame,
311 anise, black pepper and cinnamon.
312 Among the herbs, only primrose and thyme grinded were negative for non-regulated mycotoxins. One
313 of the two corn silk samples presented very high levels of fumonisins (11683 µg/kg of FB_1 and 215
314 µg/kg of FB_2). Low levels of fumonisins (0.03 to 29.4 µg/kg) were previously reported in corn silk by
315 (Abbas et al., 2017). Corn silk (*Stigma Maydis*) is an important herb used as such or as extract in
316 healthcare and tea in many part of the world (Hasanudin, Hashim, & Mustafa, 2012). More samples of
317 corn silk and their extracts should be collected and analysed in future studies to better assess the
318 fumonisin contamination in this herb.
319 Taken together, the results of Tables 3, 4 and 5 clearly show a higher incidence of positives and higher
320 mycotoxin levels in spices as compared to herbs. However, for some samples of herbs (rosemary, sage,
321 corn silk and oregano) high levels of aflatoxins or fumonisins were observed which prompt further
322 studies on these herbs for a possible future regulation. These studies should also include those spices
323 currently not listed in EC regulation but found highly contaminated by aflatoxin in the present study
324 (allspice, cloves, fenugreek and coriander seeds).
325

326 **3.4. Comparison of mycotoxins occurrence in samples of spices and herbs according to the**
327 **manufacture production**

328 Table 6 reports the mean levels of mycotoxin measured in positive samples of spices and herbs
329 collected from shops selling products manufactured and packaged with or without the HACCP and
330 GMP procedures. While assessing the regulated mycotoxins, a significant difference was noted for
331 AFB_1 and total aflatoxins. In particular, without HACCP and GMP procedures the mean levels of AFB_1
332 and total AFs were about 10 times higher. Similar result was observed for OTA but the difference was
333 not significant.
334

335 The higher levels of aflatoxins and OTA measured in samples manufactured without HACCP and
336 GMP could be due to a low or no control and inadequate storage and manufacturing conditions used
337 by these food manufacturers, as a consequence of the lack of HACCP and GMP implementation. In
338 fact 68% of the 19 samples contaminated with levels of AFB_1, total AFs or OTA higher than the limits
339 established in the EC No. 165/2010, originated from producers not using HACCP and GMP. Note that
340 the samples manufactured in factories implementing HACCP and GMP the presence and levels of
341 aflatoxins and OTA are checked to assess the compliance with the European limits. Our findings are in
342 agreement with Ramesh *et al.*, 2013 who demonstrated the importance to maintain a high level of
343 control of these foodstuffs, by implementing HACCP "from farm to fork" to provide high quality and
344 safe food for enhancing food safety and global food security (Ramesh, Sarathchandra, & Sureshkumar,
345 2013). A direct relationship between moisture and aflatoxin content was found for red chilli and it was
346 demonstrated that inadequate storage conditions initiated the aflatoxin development (Sahar et al.,
347 2015).
348 While assessing the non-regulated mycotoxins, in general the mean mycotoxin levels were higher in
349 samples with HACCP and GMP as compared to those not using these procedures, with the exception
350 of ZEA and T-2 toxin. (Table 6). These results could be partially explained by the lack of regulations
351 for these mycotoxins in spices and herbs, therefore the producers implementing HACCP and GMP
352 procedures do not control presence and levels of these mycotoxins in their products probably because
353 they are not regulated. Moreover, considering that these mycotoxins are produced by *Fusarium* species
354 mainly in the field and much less during the storage, the post-harvest implementation of HACCP and
355 GMP have limited efficacy in controlling the accumulation of these mycotoxins. Therefore, the annual
356 and seasonal climatic conditions in the Countries of origin should be the main factors contributing in
357 the accumulation of these mycotoxins. In Figure 1a are shown the mean levels of total aflatoxins and
358 OTA and the percentages of positives of spices and herbs in relation to their Country of origin. In
359 particular, 67 samples originated from 15 Countries whereas the origin of 66 samples was unknown. In

Figure 1b are represented the results obtained for non-regulated mycotoxins, i.e. sum of FB_1+FB_2 and ZEA+DON, HT-2+T-2.

The contamination of aflatoxins was concentrated in samples originating from 7 out of the 15 Countries considered in this study and the mean levels of positive samples were particularly high (>50 µg/kg) in samples coming from Egypt, Lebanon and Madagascar (Figure 1a).

All samples originated from China, USA, Guatemala, Sweden, UK, Vietnam, Mexico and India were positive for fumonisins whereas ZEA and/or other trichothecenes were detected in a low percentage of samples originated in China, USA, Sweden, Syria, France and Sudan (Figure 1b).

Although the limited number of samples for which the Country of origin was known the results shown in Figures 1a and 1b give some indications on the relationship between Country of origin and mycotoxin contamination.

4. Conclusions

This study gives a clear picture of the multi-mycotoxin occurrence in a consistent number of herbs (38) and spices (94) commercialised in Lebanon and originated from at least 15 Countries. Spices resulted more contaminated as compared to herbs. In addition, significant contaminations of aflatoxins and OTA were demonstrated in some non-regulated spices as well as some herbs, which suggests to add these matrices in the list of regulated spices. The implementation of HACCP and GMP practices seems to be useful in reducing the contamination of regulated mycotoxins but seems to be ineffective for non-regulated ones. Human exposure to mycotoxins in Lebanon through the consumption of herbs and spices should be high since 80% of analysed samples were contaminated and 44% contained more than one mycotoxin. Further studies should be conducted to assess the consumption of herbs and spices in Lebanon in order to quantify the mycotoxin risk for Lebanese deriving from consumption of this food category. The results of this study suggest that some herbs and spices, previously not considered, should be included in the EC Regulation. Moreover, in addition to aflatoxins and OTA, also fumonisins should

be regulated in those herbs and spices that showed the highest levels and incidence of positive samples in this study.

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Conflicts of Interest

The authors declare no conflict of interest.

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Figures

a

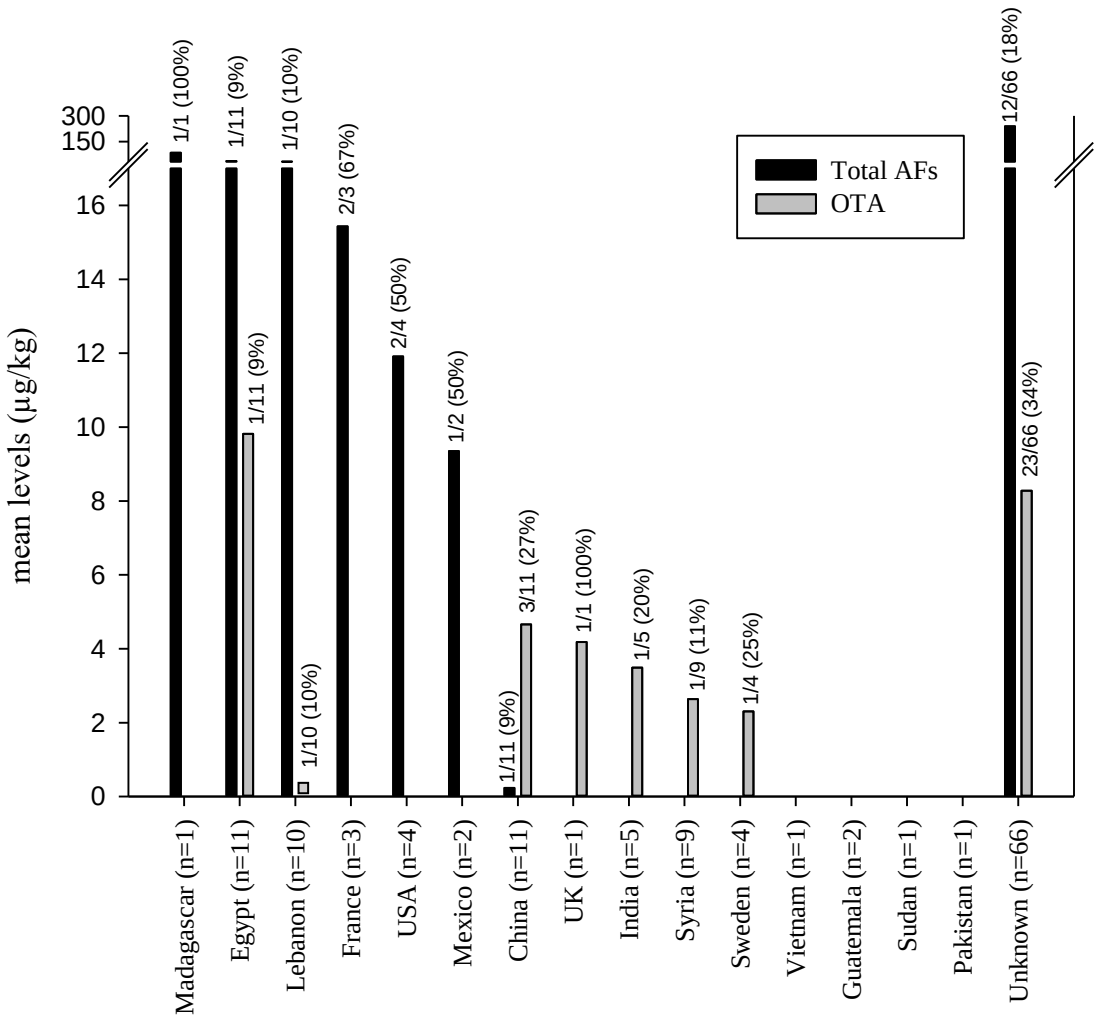

Figure ~~2a~~1a

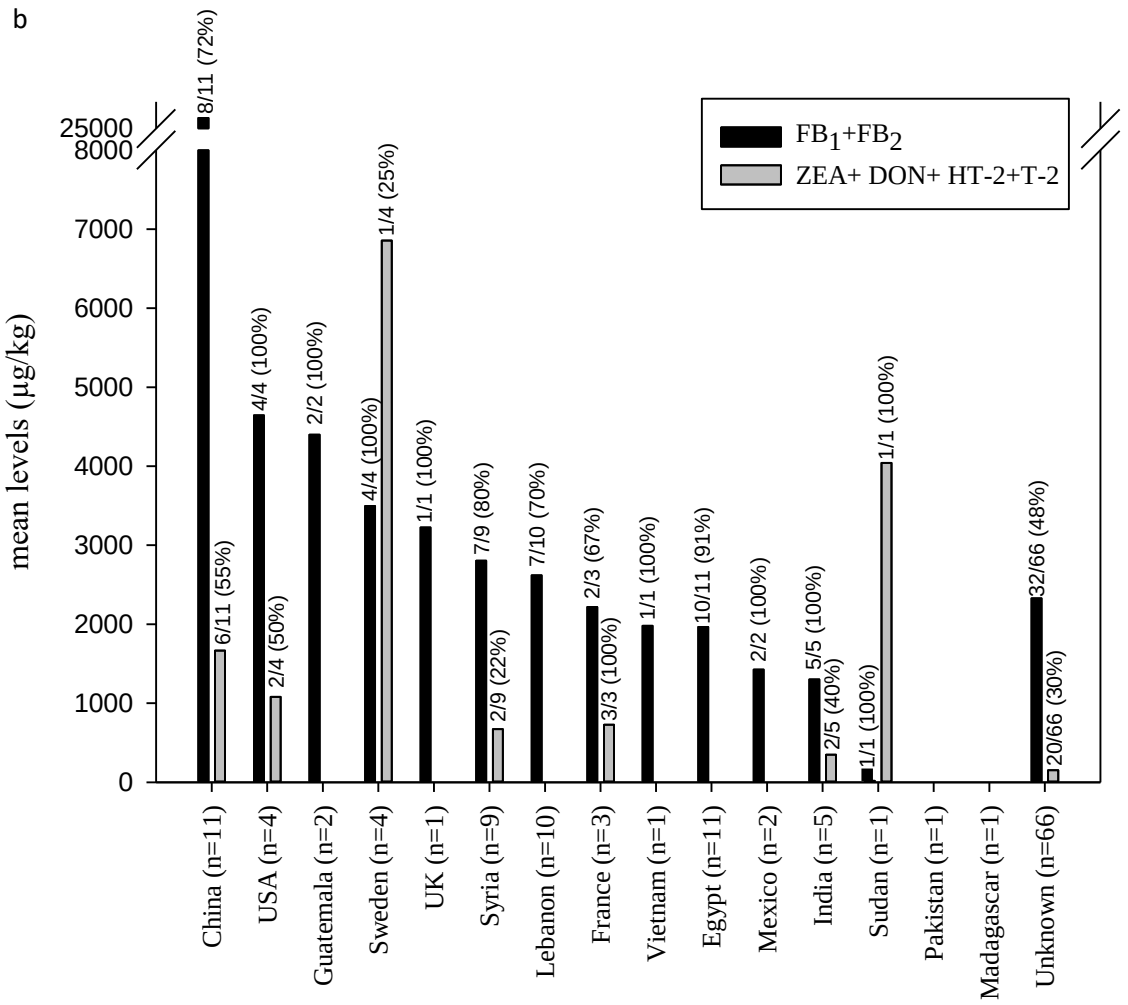
b
mean levels (µg/kg)
25000
8000
7000
6000
5000
4000
3000
2000
1000
0
FB1+FB2
ZEA+ DON+ HT-2+T-2
8/11 (72%)
6/11 (55%)
4/4 (100%)
2/4 (50%)
2/2 (100%)
4/4 (100%)
1/4 (25%)
1/1 (100%)
7/9 (80%)
2/9 (22%)
7/10 (70%)
2/3 (67%)
3/3 (100%)
1/1 (100%)
10/11 (91%)
2/2 (100%)
5/5 (100%)
2/5 (40%)
1/1 (100%)
1/1 (100%)
32/66 (48%)
20/66 (30%)
China (n=11)
USA (n=4)
Guatemala (n=2)
Sweden (n=4)
UK (n=1)
Syria (n=9)
Lebanon (n=10)
France (n=3)
Vietnam (n=1)
Egypt (n=11)
Mexico (n=2)
India (n=5)
Sudan (n=1)
Pakistan (n=1)
Madagascar (n=1)
Unknown (n=66)

Figure ~~2b~~1b

Captions

Figure ~~2a~~1a

Mean levels (µg/kg) and incidence of positive samples for total aflatoxins and ochratoxin A in relation to the origin of spices and herbs samples.

Figure ~~2b~~1b.

Mean levels (µg/kg) and incidence of positive samples for the non-regulated mycotoxins (FB_1+FB_2) and (ZEA+DON+T-2+HT-2) in relation to the origin of spices and herbs samples.

Tables

Table 1. List of 94 spices (64 singles and 30 mixtures) and 38 herbs (35 singles and 3 mixtures) analysed

Spices		Herbs	
Single spices (64)	Mixture of spices (30)	Single herbs (35)	Mixture of herbs (3)
Allspice (3)	Four spices (1)	Chamomille flowers (2)	Mix herbs (1)
Black pepper (4)	Seven spices (2)	Safflower (2)	Extra thyme (1)
White pepper (2)	Steak black pepper (1)	Saffron (1)	Zaatar halabi (1)
Red chilli (7)	Ten spices (1)	Basil (2)	
Paprika (3)	Falafel spices (2)	Bay leaves (2)	
Garlic powder (2)	Kibby spices (2)	Corn silk (2)	
Onion powder (4)	Moghrabieh spices (2)	Rosemary (2)	
Turmeric (2)	Biryani spices (2)	Sage (3)	
Cinnamon (3)	Chicken shawarma spices (1)	Oregano (3)	
Ginger (3)	Rice spices (2)	Hibiscus (2)	
Fennel (2)	Kefta spices (2)	Primrose (2)	
Caraway (2)	Kabseh spices (2)	Marjoram (2)	
Cloves (2)	Sausage spices (1)	Molokia (2)	
Coriander seeds (2)	Francisco spices (3)	Mint dried (3)	
Cumin (5)	Red taouk spices (2)	Parsley dried (1)	
Fenugreek grain (4)	Hamburger spices (1)	Violets (1)	
Nutmeg seeds (2)	Chicken spices (1)	Thyme grinded (1)	
Anise (3)	Curry powder (2)	Thyme flower (2)	
Sumac (2)			
Cardamon seeds (4)			
Sesame (3)			

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

Mycotoxins	Spike level (µg/kg)	Recovery (%)	RSDr (%)	LOD (µg/kg)	LOQ (µg/kg)
AFB_1	15	60	6	0.5	1.6
AFB_2	4	61	6	1.3	3.9
AFG_1	15	73	4	0.9	2.9
AFG_2	4	95	6	0.2	0.8
OTA	10	93	5	0.5	1.5
FB_1	50	98	1	2.1	7.2
FB_2	50	105	4	4.4	14.5
HT-2	40	103	1	0.6	1.9
T-2	20	98	5	0.3	0.8
ZEA	15	72	23	0.1	0.3
DON	500	98	20	20.5	68.2
NIV	200	95	24	4.3	14.5

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

Table 3. Summary of multi-mycotoxin results obtained for spices (94) and herbs (38) commercialized in Lebanon. Percentage of positive samples, mean and median concentrations (µg/kg) of each mycotoxin for positive samples are reported.

	Regulated mycotoxins			Non-regulated mycotoxins						
a) Spices (n=94)	AFB_1	total AFs	OTA	FB_1	FB_2	HT-2	T-2	ZEA	DON	NIV
min (µg/kg)	2.2	2.2	2.0	18.2	15.1	6.4	3.8	0.4	76.5	na
max (µg/kg)	1118.3	1118.3	34.0	113474.5	1757.4	16.7	11.9	305.4	6850.6	na
mean (µg/kg)	193.4	168.1	7.1	6432.3	230.2	10.0	7.3	30.6	1751.4	na
median (µg/kg)	124.3	102.8	4.2	1568.0	53.4	8.3	6.0	4.9	890.6	na
n. positives	15	18	28	60	33	4	3	28	11	0
% positive	16	19	30	64	35	4	3	30	12	0
b) Herbs* (n=38)										
min (µg/kg)	8.7	8.7	4.2	16.1	19.8	0.9	4.4	2.8	589.7	na
max (µg/kg)	62.7	62.7	9.8	12410.3	214.9	36.6	4.4	2.8	589.7	na
mean (µg/kg)	36.1	36.1	7.0	2826.4	75.2	18.7	4.4	na	na	na
median (µg/kg)	36.8	36.8	7.0	1663.0	45.3	18.7	4.4	na	na	na
n. positives	3	3	4	21	7	2	1	1	1	0
% positive	8	8	11	55	18	5	3	3	3	0

na: not applicable

* in Europe mycotoxins are not regulated in herbs

Table 4. Incidence of spices and herbs contaminated with AFB_1, total aflatoxins and OTA higher than the EC limits for some spices.

	Number of samples	AFB_1 n. samples > 5 µg/kg	total AFs n. samples > 10 µg/kg	OTA n. samples > 15 µg/kg
Spices	94	13 (14%)	14 (15%)	3 (3%)
Herbs*	38	3 (8%)	2 (5%)	0

Table 5. Occurrence of regulated and non-regulated mycotoxins in spices and herbs and relevant mean levels of positive samples

a)		Total aflatoxins		Ochratoxin A		Non-regulated mycotoxins*	
	Spices	Positive/total	µg/kg	Positive/total	µg/kg	Positive/total	µg/kg
	Allspice	2/3	66.8	0/3	nd	2/3	1426.2
	Black pepper	0/4	nd	1/4	2.3	4/4	2089.6
	White pepper	0/2	nd	0/2	nd	1/2	1.4
	Red chili	3/7	187.9	2/7	7.7	7/7	2585.9
	Paprika	1/3	190.1	2/3	11.4	3/3	5791.2
	Garlic powder	0/2	nd	1/2	5.1	1/2	23832.0
	Onion powder	0/4	nd	0/4	nd	4/4	56843.5
	Turmeric	0/2	nd	1/2	2.4	2/2	568.2
	Cinnamon	0/3	nd	0/3	nd	3/3	2083.6
	Ginger	0/3	nd	0/3	nd	3/3	1572.1
	Fennel	0/2	nd	0/2	nd	1/2	160.5
	Caraway	0/2	nd	0/2	nd	0/2	nd
	Cloves	2/2	127.9	0/2	nd	0/2	nd
	Coriander seeds	1/2	75.3	0/2	nd	1/2	80.5
	Cumin	0/5	nd	1/5	3.5	3/5	2829.0
	Fenugreek grain	1/4	1118.2	0/4	nd	3/4	558.0
	Nutmeg seeds	1/2	138.2	1/2	33.9	2/2	898.8
	Anise	1/3	5.3	1/3	2.6	2/3	2154.8
	Sumac	0/2	nd	0/2	nd	2/2	375.8
	Cardamon	1/4	3.9	0/4	nd	3/4	16712.1
	Sesame	0/3	nd	0/3	nd	2/3	2384.9
	Mix spices	5/30	18.1	18/30	6.0	20/30	1419.7
		18/94	143.0**	29/94	7.0**	69/94	5782.3**

b)		Total aflatoxins		Ochratoxin A		Non-regulated mycotoxins*	
	Herbs	Positive/total	µg/kg	Positive/total	µg/kg	Positive/total	µg/kg
	Chamomille	0/2	nd	1/2	9.8	1/2	3473.4
	Safflower	0/2	nd	0/2	nd	1/2	1091.2
	Saffron	0/1	nd	0/1	nd	1/1	1264.0
	Basil	0/2	nd	0/2	nd	1/2	1663.0
	Bay leaves	0/2	nd	0/2	nd	1/2	745.8
	Corn silk	0/2	nd	0/2	nd	1/2	11897.9
	Rosemary	1/2	36.8	1/2	< LOQ	1/2	373.0
	Sage	1/3	62.7	1/3	4.2	2/3	1619.3
	Oregano	1/3	8.7	0/3	nd	2/3	8030.1
	Hibiscus	0/2	nd	0/2	nd	1/2	224.2
	Primrose	0/2	nd	0/2	nd	0/2	nd
	Marjoram	0/2	nd	1/2	< LOQ	1/2	2392.6
	Molokia	0/2	nd	0/2	nd	1/2	103.2
	Mint dried	0/3	nd	0/3	nd	2/3	4286.9
	Parsley dried	0/1	nd	0/1	nd	1/1	3170.6
	Violets	0/1	nd	0/1	nd	1/1	1268.6
	Thyme grinded	0/1	nd	0/1	nd	0/1	nd
	Thyme flower	0/2	nd	0/2	nd	1/2	3296.9
	Mix herbs	0/3	nd	0/3	nd	3/3	509.1
		3/38	36.1**	4/38	3.9**	22/38	2743.9**

*Mean of the sum of FB_1, FB_2, ZEA, DON, T-2, HT-2

** weighted mean of positive samples

nd: not detected

Table 6. Mean levels (µg/kg) ± SE of FB_1, FB_2, DON, AFB_1, total aflatoxins, ZEA, HT-2, T-2, OTA, NIV in positive samples of spices and herbs produced with and without HACCP and GMP rules.

Mycotoxins	with HACCP and GMP		without HACCP and GMP		P-value
	µg/kg ± SE	n. of samples	µg/kg ± SE	n. of samples	
FB_1	5996.3 ± 2371.9	65	3470.7 ± 2464.2	16	0.0210
FB_2	197.0 ± 94.9	25	129.8 ± 75.2	15	0.1932
DON	1654.6 ± 594.1	12	nd	0	na
AFB_1	22.4 ± 9.4	6	239.6 ± 89.5	12	0.0067
Total AFs	23.3 ± 8.5	11	240.0 ± 89.5	12	0.0006
ZEA	12.0 ± 5.6	12	40.3 ± 19.6	17	0.2355
HT-2	15.4 ± 10.8	3	10.3 ± 3.2	3	0.5000
T-2	2.4 ± 2.0	2	7.2 ± 2.4	3	0.2000
OTA	4.3 ± 1.0	16	7.5 ± 2.1	16	0.0856
NIV	nd	0	nd	0	na

nd: not detected

na: not applicable