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Quantification of deoxynivalenol and fumonisins in cereals and derived products by high-performance liquid chromatography

Quantifizierung von Deoxynivalenol und Fumonisinen in Getreide und Getreideprodukten mittels Hochleistungsflüssigkeitschromatographie

Hilal Zeynep Sahin, Bülent Kabak

Summary

Mycotoxins are toxic, hazardous chemicals that affect human and animal health. The most common problem that importers are facing when importing cereals from main supplying countries is contamination with mycotoxins. The aim of the present study was to assess the presence of deoxynivalenol (DON) and fumonisins (FUM) in cereals and derived products. A total of 225 samples (60 wheat, 60 maize, 40 rice, 40 pasta and 25 maize chip) collected from Turkey were analysed by high performance liquid chromatographic (HPLC) method. The method performance was satisfactory and allowed to quantify target analytes accurately. DON was determined in 6.7% of wheat samples with a range of 158–653 µg/kg. Only two rice and two pasta samples contain trace amounts of DON, while no DON was detected in maize and maize chip products. Eleven maize samples (18.3%) contained fumonisin B_1 (FB_1), but levels (125–830 µg/kg) were below the European Union maximum level of 4000 µg/kg. FB_1 was not detected in any wheat and rice sample, but it was detected in just one pasta and one maize chip sample. It is recommended to further monitoring both of free and modified *Fusarium* toxins in cereals and derived products throughout Turkey.

Keywords: Deoxynivalenol, fumonisin, food safety, HPLC

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Introduction

Mycotoxins are a group of naturally occurring contaminants produced by toxigenic fungi and have been linked to long-term chronic effects on health, including the induction of cancers and immune deficiency. Among the known mycotoxins, aflatoxins (AFs), ochratoxin A (OTA), deoxynivalenol (DON) and fumonisins (FUM) occur most frequently in food and feed, and pose significant threat to human health (Sweeney and Dobson, 1998).

DON and FUM are synthesized mainly by *Fusarium* species and occur predominantly in cereal grains in the field as well as cereal-based foods. DON is the most prevalent among *Fusarium* toxins and an important indicator of *Fusarium* Head Blight occurrence in wheat. It is synthesized mainly by *Fusarium graminearum* and *Fusarium culmorum* (EFSA, 2013). DON has been associated with vomiting, anorexia and weight gain suppression in animals. However, there is a lack of data about DON toxicity during human long-term exposure. In 2010, the Joint FAO/WHO Expert Committee on Food Additives established a provisional maximum tolerable daily intake of 1 µg/kg body weight per day for DON and its acetylated derivatives (3-acetyl DON and 15-acetyl DON) (EFSA, 2017).

FUM are produced mainly by *Fusarium verticillioides* and *Fusarium proliferatum*. FUM occur worldwide, predominantly in maize and maize-based products and, to a lesser extent, in wheat and other cereals including their derived products. While FUM can be divided into four groups (A-, B-, C-, and P-type), B-type FUM, such as fumonisin B_1 (FB_1), fumonisin B_2 (FB_2), fumonisin B_3 and fumonisin B_4 are the most prominent. FB_1 is the most significant in terms of occurrence and toxicity. It has been reported that FB_1 caused several toxic effects varied from hepatotoxicity and renal toxicity in rats, pulmonary oedema and hydrothorax in pigs, and leukoencephalomalacia in horses. While several observations on the health effects of FUM have been discussed in humans, further studies to investigate the association of FUM exposure with cancer risk, liver cancer, child growth impairment, and neural tube defects in humans are needed. The Panel on Contaminants in the Food Chain established a tolerable daily intake for $FB_{1–4}$ of 1 µg/kg body weight per day (EFSA, 2018).

The International Agency for Research on Cancer (IARC) evaluated the carcinogenicity of some naturally occurring substances in 1993 and classified FB_1 in the Group 2B as a “possible human carcinogen”, while DON was placed in Group 3, not classifiable as to its carcinogenicity to humans (IARC, 1993). In order to protect public health, many countries have reported maximum levels (MLs) for certain contaminants in food products. MLs for six groups of mycotoxins (AFs, OTA, FUM, DON, zearalenone and patulin) are laid down in Commission Regulation EC (No) 1881/2006 (EC, 2006b). This regulation has been amended with Commission Regulation EC (No) 1126/2007 to revise MLs for *Fusarium* toxins in maize and maize products (EC, 2007).

While nuts products are the major source of human exposure to AFs, cereals seem to be the main source of *Fusarium* toxins (DON, FUM etc.) (EFSA, 2013; EFSA, 2014) and OTA (EFSA, 2006). Wheat is Turkey’s main food staple, followed by maize (TUIK, 2018). However, few data about DON and FUM contamination of cereals and cereal-based products from Turkey. The main objective of the present study was therefore to investigate DON and FUM (FB_1 and FB_2) occurrence in wheat, maize, rice, pasta and maize chip samples collected from Turkey.

Materials and methods

Samples

A total of 225 samples of wheat (n= 60), maize (n= 60), rice (n= 40), pasta (n= 40) and maize chip (n= 25) intended for human consumption were collected from retail stores, supermarkets, wholesale places and grain-milling factories in Turkey. All grain samples originated from the main production areas of Turkey (Central Anatolia, Thrace, Mediterranean and Black Sea region). Concerning pasta, 90% were durum wheat-based, and 10% were maize-based. During the period of December 2017–November 2019, an aggregate sample of at least 500 g of product (except for maize chip) was taken. Due to the possible heterogeneous distribution of target analytes in products, the aggregate samples were ground with milling machines (C.W. Brabender Instruments, Inc., Germany and IKA®, Germany) and homogenised. All milled samples were stored in plastic bags at –18 °C to prevent any further mould development and mycotoxin production until analysis.

Chemicals, reagents and standards

Acetonitrile and methanol were purchased from Sigma–Aldrich (St. Louis, MO, USA) and were of high-performance liquid chromatography (HPLC) grade. Anhydrous monobasic potassium phosphate, sodium phosphate dibasic, o-phthaldialdehyde (OPA, ≥99%) and 2-mercaptoethanol (≥99%) were also procured from Sigma–Aldrich (St. Louis, MO, USA). Potassium chloride was from VWR International (Leuven, Belgium). Ortho-phosphoric acid (85%), di-sodium tetraborate decahydrate, sodium hydroxide and sodium chloride were supplied from Merck (Darmstadt, Germany). Ultrapure water was produced using a Milli Q water purification system (Millipore, Molsheim, France).

The DONtest™ (product code: G1005) and FumoniTest™ (product code: G1008) immunoaffinity columns (IACs) were procured from Vicam® (Watertown, MA, USA). Mycotoxin standards (DON, FB_1 and FB_2) were purchased from Sigma-Aldrich (St. Louis, MO, USA). DON stock standard solution (200 µg/mL) was prepared in methanol. FB_1 and FB_2 stock standard solutions were combined to prepare a mixed working standard solution in acetonitrile-water (50:50, v/v) (50 µg/mL for each).

Sample extraction and IAC clean-up

DON was extracted from cereals and cereal-based samples according to the method developed by Cahill et al. (1999). A representative sample of 25 g was extracted with 100 mL of water. A volume of 2 mL of filtered extract was diluted with 18 mL of water and filtered with a glass microfiber filter. The whole filtrate was then passed through Vicam® DONtest™. After washing step, DON was eluted from the column with 1 mL of methanol (2 x 0.5 mL), and then the eluate was diluted with 1 mL of purified water.

FUM extraction from grain samples was slightly different, it was performed following the AOAC Official method 2001.04 (Visconti et al. 2001), based on Shephard et al. (1990), with slight modifications. FUM were extracted from grain samples with acetonitrile-methanol-water

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(25:25:50, v/v/v) and 2.5 g of NaCl. An aliquot (10 mL) of each filtered extract was diluted with 40 mL of phosphate-buffered saline solution and cleaned up by FumoniTest™. FUM were eluted from the IACs with 1 mL of methanol. The eluate was evaporated just to dryness, and the residue was dissolved in 1 mL of acetonitrile-water (50:50, v/v). A volume of 200 µL of the reconstituted grain extract was then transferred into an HPLC vial, and 200 µL OPA based reagent was added to form fluorescent FUM derivatives.

Chromatographic analysis

The equipment used for the determination of DON and FUM was reversed-phase HPLC (Shimadzu, Tokyo, Japan) coupled with photodiode array (HPLC-PDA) and fluorescence detectors (HPLC-FLD), respectively. An inertsil® ODS-3 analytical column (150x4.6 mm, 5 µm, GL Sciences Inc., Japan) was used. The isocratic eluents of water-methanol (85:15, v/v) and methanol–0.1M NaH_2PO_4 (77:23, v/v) for DON and FUM analyses, respectively, were eluted at 1 ml min^{-1}. The injection volumes were 100 µL. PDA detector was set at 219 nm.

For the determination of FB_1 and FB_2, the analyses were performed on HPLC-FLD after pre-column derivatization with OPA based reagent. Due to the FUM are not fluorescent, OPA derivatives of FUM were prepared and injected onto the HPLC-FLD system within 30 seconds. Fluorescence detector programme was as follows: 335 nm for excitation and 440 nm for emission. The retention times were about 6.5, 14.5, and 23 min for FB_1, FB_2 and DON, respectively.

Method validation

The determination of DON and FUM in cereals and cereal-derived products was in-house validated with respect to the linearity, limit of detection (LOD), limit of quantification (LOQ), recovery and repeatability. Linearity was constructed over a concentration range of 100–2000 µg/L for DON, and 25–5000 µg/L for both FB_1 and FB_2.

The sensitivity (method LOD and LOQ), recovery and repeatability were assessed by spiked experiments using blank samples of wheat, maize, rice and pasta. Method LOD and LOQ values were assessed by analysing eight independent blank wheat, maize, rice and pasta matrices spiked with 100 µg/kg for DON, 50 µg/kg for FB_1 and 50 µg/kg for FB_2. LOD and LOQ values were calculated as 3 and 10 times the standard deviation of replicate analyses (n= 8). The recovery of DON and FUM were determined at a level of 250 µg/kg in wheat, maize, rice and pasta matrices in five replicates. The repeatability of the method was calculated by performing recovery experiments (n= 5) and was expressed as the relative standard deviation (RSD, %).

Results and discussion

Validation data

The calibration curves were linear over the concentration ranging from 100 to 2000 µg/L for DON, and from 25 to 5000 µg/L for both FB_1 and FB_2. For target analytes, the coefficient of determination (R^2) calculated by linear regression was higher than 0.99.

Table 1 summarizes validation data. LODs of DON, FB_1 and FB_2 in different matrices were in the range of 15.1–16.7, 14.3–14.8 and 12.7–13.6 µg/kg, respectively, while LOQs were in the range of 50.3–55.8, 47.6–49.2 and 42.2–45.4 µg/kg for DON, FB_1 and FB_2, respectively. As LOQ values were below the EU MLs, the methodology could be considered adequate for these mycotoxins measurement in cereals and products thereof. Good recoveries (85.4–93.1% for DON, 91.7–93.9% for FB_1 and 91.1–93.4% for FB_2) and intra-day repeatabilities (RSDr) (4.75–7.34% for DON, 5.04–7.12% for FB_1 and 4.32–6.04% for FB_2) were obtained, which were in the range of performance criteria set in the Commission Regulation No 401/2006: Recoveries of 60–110% and 60–120%, and RSD values of ≤20% and ≤30% for DON and FUM, respectively, for the mass fraction of 250 µg/kg (EC, 2006a).

Occurrence of DON and FUM in cereals and derived products

Table 2 shows DON and FUM incidence and levels in wheat, maize, rice, pasta and maize chip. Simultaneous occurrence of DON and FUM was not observed in analysed samples. DON was detected above the LOD in only two rice samples (72.4 and 106. 3 µg/kg), but no positive findings were recorded for maize and maize chip. Contamination of pasta with DON was only found in two (durum wheat-based) out of 40 analysed samples at levels of 52.2 and 61 µg/kg. In wheat, DON was detected in 4 out of 60 samples, but at levels lower than the EU ML of 1250 µg/kg. DON levels in wheat ranged from 158 to 653 µg/kg, with lower bound and upper bound averages of 20.7 and 35.9 µg/kg, respectively.

These values are much lower than a previous observation by Şahindokuyucu et al. (2010) who detected DON in 38.3% of 60 maize silage samples in the range of 24.2–100.3 µg/kg. In another report, DON occurred in 7 out of 65 wheat and wheat-based products (11%) and 6 maize and maize-based products (9%) commercialized in Turkey ranging from 132 to 9589 µg/kg (Bakırcı, 2014).

It has been observed that DON is most frequently found and at higher concentrations in wheat when compared to other cereals. In wheat samples, the incidence of DON was 100% (203–4130 µg/kg) in Austria (Berthiller et al. 2009), 74.4% (14.5–41157 µg/kg) in China (Ji et al. 2014), 65% (115–278 µg/kg) in Croatia (Pleadin et al. 2012), 3.5% (>54–4590 µg/kg) in Czech Republic (Polišenská et al.

TABLE 1: The validation data.

Matrix	DON				FB_1				FB_2			
	LOD (µg/kg)	LOQ (µg/kg)	Recovery (%)	RSD_r (%, n=5)	LOD (µg/kg)	LOQ (µg/kg)	Recovery (%)	RSD_r (%, n=5)	LOD (µg/kg)	LOQ (µg/kg)	Recovery (%)	RSD_r (%, n=5)
Wheat	16.3	54.1	86.0	7.34	14.8	49.2	93.9	5.04	13.6	45.4	91.6	6.04
Maize	15.1	50.3	93.1	4.75	14.3	47.6	94.2	7.12	12.7	42.2	92.5	5.81
Rice	16.7	55.8	85.4	5.99	14.5	48.5	91.7	5.78	13.1	43.7	91.1	4.48
Pasta	15.9	52.8	89.5	5.13	14.5	48.4	93.1	6.45	13.0	43.2	93.4	4.32

RSD_r: Intra-day repeatability

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2008), 96.7% in (>4–5510) µg/kg) in Finland (Nathanail et al. 2015), 72% (230–1880 µg/kg) in Hungary (Tima et al. 2016), 59.5% (13–1230 µg/kg) in Italy (Alkadri et al. 2014), 34.5% (57–1840 µg/kg) in Serbia (Jajić et al. 2008) and 62% (>300–6178) in Spain (Vidal et al. 2013).

While no measurable amounts of DON were detected, during the present study, in any maize and maize chip sample, different studies conducted in various countries showed DON occurrence in maize samples with different incidence levels: 100% (42–3680 µg/kg) in Austria (Berthiller et al. 2009), 72.5% (18–273 µg/kg) in Cameroon (Njobeh et al. 2010), 84% (170–2630 µg/kg) in Canada (Tran et al. 2012), 71% (215–2942 µg/kg) in Croatia (Pleadin et al. 2012), 86% (225–2963 µg/kg) in Hungary (Tima et al. 2016) and 32% (27–2460 µg/kg) in Serbia (Jajić et al. 2008). These differences could be due to climate difference, agricultural practices, seasonal variances, pesticide use, storage conditions, etc. However, this study is in compliance with the result obtained by Hanvi et al. (2019), who did not detect DON in any of the 55 maize samples from Togo.

Among the cereals, rice is the third most important crop in Turkey daily intake preceded by wheat and maize (TUIK 2018). This is the first data dealing with DON contamination of rice cultivated in Turkey. However, Turkey climatic conditions are more favourable to ochratoxigenic mould growth in rice. During a previous study that we performed, OTA was found in 3 out of 58 rice samples (5.2%) up to a level of 0.98 µg/kg (Golge and Kabak, 2016).

Regarding DON incidence in pasta, similar results were recorded in a study conducted in Thailand by Poapolathep et al. (2008). However, DON levels (170 and 350 µg/kg) were higher than those found during the present survey. In 2003 DON was found in 44% of pasta samples (8/17) with a range of concentration of 9–77 µg/kg and with an average concentration of 19 µg/kg (Cirillo et al. 2003). In Spain, DON was determined in 62.7% of pasta samples (47/75) with a range of 10.9–623 µg/kg (González-Osnaya et al. 2011). In the Netherland, four of the 26 pasta samples contained DON with levels of 91–295 µg/kg (De Nijs et al. 2016). In our recent study, we found that DON occurred only in one out of 10 pasta samples at a trace amount (49.3 µg/kg) (Golge and Kabak, 2020).

FB_1 and FB_2 were absent in wheat and rice samples, while only one maize-based dry pasta and one maize chip sample contained FB_1 at a trace amount. No FB_2 was detected in all maize samples, while FB_1 was found in 11 out of 60 maize samples (18.3%), but at levels lower than EU ML of 4000 µg/kg. FB_1 concentrations in maize samples ranged between 125 and 830 µg/kg, with an average middle bound level of 97.5 µg/kg.

These values are well below than FUM concentrations of maize and maize based products reported in the literature. Bakırcı (2014) observed that 74% of maize and maize-based products (42 out of 57 samples) commercialised in Turkey contained FB_1 up to a level of 9589 µg/kg. In another study conducted in Turkey, Ocak and Bostan (2010) found FB_1 in 56% of maize flour samples at levels ranging from 257 to 5580 µg/kg. They also reported FB_1 exceeding the EU ML in 24% of maize flour samples (6 samples). Among cereals, maize is known to have a high risk not only for FUM but also for AFs contamination. The climates of Turkey are more favourable for AFs-producing fungi and AFs are the main mycotoxin group in agricultural products. In our previous study, aflatoxin B1 was detected in 66.7% of maize flour samples up to a level of 1.12 µg/kg (Kara et al. 2015).

There are many reports worldwide based on the presence of FUM in various cereals and cereal-based products. In more than half of maize samples from different origins including Cameroon (55%, 37–24225 µg/kg) (Njobeh et al. 2010), China (92.5%, 200–37000 µg/kg) (Sun et al. 2011), Croatia (90%, 37–4438 µg/kg) (Pleadin et al. 2012), Serbia (100%, 880–2950 µg/kg) (Krnjaja et al. 2013) and Togo (88%, 101–1838 µg/kg) (Hanvi et al. 2019) FB_1 was detected.

With regard to maize and maize-based products, a total of 228 RASFF notifications were received between the years 2002 and 2018. AFs and FUM in maize and maize products are the most recurrent issues with 121 and 82 notifications, respectively. Of these 82 notifications on FUM, more than half (46 notifications) were originated from Italy. There were also five notifications concerned with

TABLE 2: The incidence and concentration of DON and FUM in cereals and derived products.

Food product	Parameter	DON	FB_1	FB_2	FUM (FB_1+FB_2)
Wheat (n = 60)	No. positive samples[a] (%)	4 (6.7)	–[b]	–	–
	Range (min–max, µg/kg)	158–653	–	–	–
	Mean of positive samples (µg/kg)	310	–	–	–
	MB (LB–UB, µg/kg)[c]	28.3 (20.7–35.9)	7.4 (0–14.8)	6.8 (0–13.6)	14.2 (0–28.4)
Maize (n = 60)	No. positive samples[a] (%)	–	11 (18.3)	–	11 (18.3)
	Range (min–max, µg/kg)	–	125–830		125–830
	Mean of positive samples (µg/kg)	–	500		500
	MB (LB–UB, µg/kg)[c]	7.6 (0–15.1)	97.5 (91.7–103.3)	6.3 (0–12.7)	102.7 (91.7–113.3)
Rice (n = 40)	No. positive samples[a] (%)	2 (5)	–	–	–
	Range (min–max, µg/kg)	72.4–106.3	–	–	–
	Mean of positive samples (µg/kg)	89.4	–	–	–
	MB (LB–UB, µg/kg)[c]	12.4 (4.5–20.3)	7.3 (0–14.5)	6.6 (0–13.1)	13.8 (0–27.6)
Pasta (n = 40)	No. positive samples[a] (%)	2 (5)	1 (2.5)	–	1 (2.5)
	Range (min–max, µg/kg)	52.2[d]–61.0	47.6[d]	–	47.6
	Mean of positive samples (µg/kg)	56.6	47.6	–	47.6
	MB (LB–UB, µg/kg)[c]	10.4 (2.8–17.9)	8.3 (1.2–15.3)	6.5 (0–13.0)	14.6 (1.2–28.0)
Maize chip (n = 25)	No. positive samples[a] (%)	–	1 (4)	–	1 (4)
	Range (min–max, µg/kg)	–	107	–	107
	Mean of positive samples (µg/kg)	–	107	–	107
	MB (LB–UB, µg/kg)[c]	7.6 (0–15.1)	11.1 (4.3–18.0)	6.3 (0–12.7)	17.2 (4.3–30.2)

[a] Positive samples: Mycotoxin level ≥ LOD; [b] –, not detected, i. e., below LOD; [c] MB (LB–UB): middle bound (lower bound–upper bound). LB: results below the LOD were replaced with 0, MB: results below the LOD were replaced with LOD/2, UB: results below the LOD were replaced with the value of LOD; [d] < LOQ

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simultaneous occurrence of high levels of AFs and FUM in maize.

Limited studies have shown no discernable FUM risk in pasta. In a previous study by Cirillo et al. (2003), no FB_1 was found in pasta samples commercialised in Italy. Similarly, Tolosa et al. (2017) could not detect FB_1 and FB_2 in 58 durum wheat pasta collected from local markets of Italy. During the period of 2002–2018, there were only 7 RASFF notifications on mycotoxins in pasta, 4 of which concerning DON, 2 of which concerning FUM and one of which concerning OTA. However, this is the first data about FUM contamination in dry pasta commercialised in Turkey. It is not surprised that FB_1-positive pasta sample was maize-based, due to maize is more susceptible to FUM contamination than wheat.

Conclusions

This study was designed to measure DON and FUMs contamination in cereals and cereal-derived products commercialised in Turkey using the HPLC method. The sensitivity of the analytical method is suitable to meet the limit of target mycotoxins established in the EU legislation, and the validation parameters meet the requirements of performance criteria set in the Commission Regulation No 401/2006. The present study observed that there is no reason to be concerned about DON and FUM incidence in cereals and products thereof from Turkey. However, the contamination level varies from year to year depending on the climatic region and climatic season, and thus contamination range may be wider. Further studies are needed to determine not only free *Fusarium* toxins but also their modified or bound forms in cereals and cereal-based foods.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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