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Bioaccessibility of Deoxynivalenol and its natural co-occurrence with Ochratoxin A and Aflatoxin B_1 in Italian commercial pasta

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ABSTRACT

Cereals products for direct human consumption are rarely contaminated by moulds, unlike raw materials, which are often infected, either in the field or during storage.

In this study, 27 samples of dried pasta characterised by size, packaging and marketing intended for young children consumption were collected and analysed by liquid chromatography (LC) and liquid chromatography–tandem mass spectrometry (LC–MS/MS) for Deoxynivalenol (DON), Ochratoxin A (OTA) and Aflatoxin B_1 (AFB_1) determination. The samples that showed the highest amounts of one of the mycotoxins were cooked for 10 min, digested with an *in vitro* gastrointestinal protocol and bioaccessibility values were calculated. Seven of the 27 samples exceeded from 120% to 225% the legal limit of 200 μg/kg for DON fixed for processed cereal-based baby foods by an European Regulation; all the collected samples were under the OTA legal limit (0.05 μg/kg) fixed by the European Regulation and no sample was contaminated by AFB_1 over the instrumental limit of detection of 0.10 μg/kg. The mean value of gastric bioaccessibility verified for the DON resulted of 23.1%, whereas mean duodenal bioaccessibility was 12.1%.

1. Introduction

Trichothecenes constitute the main group of *Fusarium* toxins commonly detected in grains. Animal studies on trichothecenes toxic effects have been largely reported in literature, whereas human toxicity has been scarcely investigated (Blesa et al., 2010), although dietary exposure to trichothecenes can induce effects on mucosal surface, weight loss, and immunosuppressive and haemorrhaging effects (Kleter and Marvin, 2009).

Deoxynivalenol (DON) is mainly produced by *Fusarium graminearum* and its worldwide surveillance confirms its occurrence in 57% of cereal samples like wheat (Santini et al., 2009). Wheat is considered the most important cereal for human diet and European Union. According to EC 1881/2006 Regulation, the maximum levels of DON in cereal foods intended for direct human consumption is 200 and 750 μg/kg for young children and adults, respectively.

Toxicological studies are not so accurate in order to establish a TDI for each age group, therefore TDI for DON (1 μg/kg bw/day) (SCF, 1999) as well as for all mycotoxins is set for general population.

Aspergillus and *Penicillium* species produce Ochratoxin A (OTA), which was firstly reported in South Africa as a secondary metabolite produced by a strain of *Aspergillus ochraceus* (Van der Merwe et al., 1965). OTA might be involved in the pathogenesis of endemic diseases in the Balkan region, including Balkan Endemic Nephropathy (BEN) (EFSA, 2006). The International Agency for Research on Cancer (IARC, 1993) classified OTA as possible human carcinogen (Group 2B), based on the sufficient evidence for carcinogenicity in animal studies, but inadequate evidence in humans.

The weekly exposure of the general population to OTA ranged from 15 to 60 ng/kg bodyweight (EFSA, 2006). This rate of exposure is below the tolerable weekly intake (TWI) value of 120 ng/kg bw/week reported by EFSA (2006), and 1.5–5.7 ng/kg bw/day reported by an assessment of Canadian authorities (Kuiper-Goodman, 1996). The EU Regulation No. 1881/2006 fixed limits of 0.5 and 3 μg/kg for processed cereal-based foods, respectively intended for young children and adults.

AFs are the most investigated and dangerous mycotoxins produced mainly by *Aspergillus flavus* and *A. parasiticus* (Richard 2008; Iha and Trucksess 2010). AFs are considered the compounds with the highest hepatocarcinogenic activity in several species of animals (Firmin et al., 2010), and it has been demonstrated by epidemiological studies that the Aflatoxin B_1 (AFB_1) is the responsible compound of acute liver intoxications (IARC, 1993). Therefore, EU Regulation No. 1881/2006 fixed limits for the AFB_1 of 0.10 and 2.0 μg/kg in cereals products intended for young children and adults, respectively.

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However, to achieve any effects in a specific tissue or organ, mycotoxins must be available, which refers to the compounds tendency to be extracted from the food matrix, and then be absorbed from the gut via the intestinal cells. In this sense, bioaccessibility has been defined as the fraction of a compound that is released from the food matrix in the gastrointestinal tract and thus becomes available for intestinal absorption (i.e., enters the blood stream) (Benito and Miller, 1998).

In human health risk assessment, ingestion of food is considered a major route of exposure to many contaminants either caused by industrial or environmental contamination or as result of production processes. The total amount of an ingested contaminant (intake) does not always reflect the amount that is available to the body, because only a certain amount of the contaminant will be bioavailable (Carolien et al., 2005). Bioavailability is a term used to describe the proportion of the ingested contaminant in food that reaches the systemic circulation (Fernández-García et al., 2009).

The bioaccessibility and bioavailability of mycotoxins have been evaluated by many scientists. Avantaggiato et al. (2003, 2004) studied the intestinal absorption of zearalenone (ZEA), and DON and nivalenol (NIV), respectively. Carolien et al. (2005) and Kabak et al. (2009) described the applicability of an *in vitro* digestion model to measure the bioaccessibility of AFB_1 and OTA ingested from peanut slurry, buckwheat and infant formulas.

The Mediterranean diet is an independent protective factor for several affections in children, and has been consistently shown to be associated with favourable health outcomes and a better quality of life (Castro-Rodriguez et al., 2008; Sofi, 2009). In Italy, this diet is characterised by higher intake of complex carbohydrates, mostly due to pasta consumption (Trichopoulou et al., 2000). Dry pasta is made from semolina milled from durum wheat, which could be contaminated simultaneously by DON, OTA and AFB_1.

The main aims of this study are to carry out surveillance for DON, OTA and AFB_1 on wheat-based products and to ascertain the bioaccessibility of DON in a model system that simulates the digestion steps. This mycotoxin was chosen for bioaccessibility evaluation because its level in several pasta samples exceeded the legislative limits fixed for babies and infants.

2. Materials and methods

2.1. Materials

Potassium chloride (KCl), potassium thiocyanate (KSCN), monosodium phosphate (NaH_2PO_4), sodium sulphate ($NaSO_4$), sodium chloride (NaCl), sodium bicarbonate ($NaHCO_3$), urea, α-amilase, hydrochloric acid (HCl), pepsin, pancreatin and bile salts were obtained from Sigma–Aldrich (Madrid, Spain). Acetonitrile, methanol and ethyl acetate were purchased from Fisher Scientific (Madrid, Spain). Deionised water (<18 MΩ cm resistivity) was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Chromatographic solvents and water were degassed for 20 min using a Branson 5200 (Branson Ultrasonic Corp., CT, USA) ultrasonic bath.

DON, OTA and AFB_1 standards were purchased from Sigma–Aldrich (Milan, Italy) and stored at 4 °C in the dark. They were dissolved in methanol at 1 mg/mL concentration (stock solution). Working standard solution of DON was prepared by diluting standard stock solution with a suitable solvent mixture ($CH_3OH:H_2O$, 70:30, v/v), whilst working standard solutions of OTA and AFB1 were obtained by diluting stock solutions with methanol.

2.2. Sampling

Dry pasta samples of different brands ($n = 27$), characterised by little size, marketing and packaging referring to young children, were collected from several supermarkets located in the city of Naples (Italy). The selected samples included pastas of several forms such as penne, starlets, cut noodles, short pasta mixed, macaroni, bow pasta, spiral shaped pasta. Samples were milled with a knife mill (Grindomix GM 200, Retsch, Haan, Germany) and then divided with a subsample divider (PT 100 Retsch, Haan, Germany). For every sample, a 200 g-subsample was collected in a plastic bag and kept at −20 °C until mycotoxin analysis.

2.3. DON extraction

The extraction of DON was carried out according to the method of Santini et al. (2009). Briefly, 10 g of finely grounded pasta were mixed with 50 ml acetonitrile:water (84:16; v/v), and extracted by agitation on a mechanical shaker (Intercontinental equipment, Hidalgo, TX) during 1 h. The mixture was filtered using a paper filter (LABOR, Microglass Heim SRC, Naples, IT) and 5 mL of the filtered extract was completely evaporated using a centrifugal evaporator (Speed Vacuum Thermo Electron Corporation Milford, MA, USA). The extract was then dissolved in 1 mL of methanol and finally filtered through a 0.22 μm cellulose filter (Chemtek Analytica, Bologna, IT) before liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis. Each sample was analysed in triplicate.

2.4. OTA extraction

OTA extraction was performed according to Winnie et al. (2009) with slight modifications. Twenty five grams of finely grounded pasta together with 100 mL of acetonitrile:water (60:40, v/v) mixture were homogenised utilising an Ultra-Turrax (T 25 basic, IKA-Werke, Germany) at 2000 rpm during 2 min. The samples were centrifuged (Thermo-scientific Centrifuge IEC CL30R, UK) at 4000 rpm during 5 min, 10 mL of the supernatant were collected and diluted with 40 mL of PBS solution.

The sample was then purified by an immunoaffinity column (ICs) (Ochratest, VICAM, Watertown, MA, USA). In details, the ICs were first washed with 10 mL of water, the sample extract was charged on the column and eluted at 1–2 drops/s, then the column was washed with 20 mL of water and eluted with 4 mL of methanol.

The methanolic extract was dried with a centrifugal evaporator, resuspended in 1 mL of methanol, filtered with a 0.22 μm filter (Phenomenex, Torrance, CA) and injected in the LC apparatus. Each sample was analysed in triplicate.

2.5. AFB_1 extraction

For the extraction of AFB_1 the method reported by Herrera et al. (2009) was applied with slight modifications. In details, 25 g of grounded sample were mixed with 2.5 g of NaCl and 50 mL of a mixture methanol:water (80:20, v/v). The sample was homogenised using an Ultra-Turrax (T 25 basic, IKA-Werke, Germany) during 2 min at 2000 rpm and centrifuged (Thermo-scientific Centrifuge IEC CL30R, UK) during 5 min at 4000 rpm. 10 mL of the supernatant was collected and diluted with 40 mL of PBS solution before the purification step.

Sample was purified by ICs Aflatest (VICAM, Watertown, MA, USA). ICs were washed with 10 mL of water, the sample extract was charged on the column and eluted at 1–2 drops/s. After washing with 20 mL of water, the column was eluted with 4 mL of methanol. The methanolic extract was dried with a centrifugal evaporator, resuspended in 1 mL of methanol, filtered with a 0.22 μm filter (Phenomenex, Torrance, CA) and injected in the LC apparatus. For every sample, a triplicate analysis was performed.

2.6. LC–MS/MS analysis of DON

LC analysis for DON was carried out using a system consisting of two micropumps (Series 200, PerkinElmer, Waltham, MA, USA). A Gemini column (C_{18} 5 μm particle size, 150 × 4.60 mm, pore size 110 Å, Phenomenex, USA) heated at 50 °C was used; the flow rate was set to 0.8 mL/min, and the injection volume was 20 μL. Mobile phase A was a $H_2O:CH_3OH$ mixture (90:10, v/v) containing 5 mmol/L ammonium acetate, whereas mobile phase B consisted of $CH_3OH:H_2O$ (90:10, v/v) mixture also containing 5 mmol/L ammonium acetate. The following binary gradient was applied: initial condition 10% B; 0–7 min, 35% B; 7–9 min, 80% B; 9–13 min constant at 80% B; 13–15 min 100% B, finally returning to the initial conditions in 3 min.

MS/MS data were acquired using an API 3000 triple-quadrupole mass spectrometer (Applied Biosystems, Ontario, Canada). All analyses were performed with an atmospheric pressure chemical ionisation (APCI) interface with the following settings: probe temperature 450 °C, corona current (NC) ±2 μA (depending on use in positive or negative mode). The declustering potential (DP) and collision energy (CE) were optimised for each compound by direct infusion of standard solution (10 μg/mL) into the mass spectrometer at a flow rate of 8 μL/min, using a Model 11 syringe pump (Harvard Apparatus, Holliston, MA, USA) and a solution of 5 mmol/l ammonium acetate in $CH_3OH:H_2O$ (90:10, v/v) mixture as liquid carrier at a flow rate of 200 μL/min. The acquisition was carried out by selected reaction monitoring (SRM) both in the negative and positive ion mode (Blesa et al., 2010).

2.7. LC-FLD analysis of OTA and AFB_1

OTA and AFB_1 analysis were performed using an LC (Shimadzu-Japan) equipped with an autosampler SIL-20A, two pumps LC-20AD and a fluorimetric detection RF-20AXL (OTA: λ_{ex}: 360 nm; λ_{em}: 460 nm; AFB_1: λ_{ex}: 360 nm, λ_{em}: 440 nm). For AFB_1 analysis, a Gemini column was used, whereas for OTA analysis, an Onyx Monolithic column (3 μm particle size C_{18}, 100 × 3.0 mm) (Phenomenex, USA) was employed. Mobile phase for OTA analysis was used in isocratic conditions: 65% A (1% CH_3COOH

in H_2O), 35% B (1% CH_3COOH in CH_3CN) at the flow rate of 1 mL/min (Winnie et al., 2009). In the case of AFB_1, the mobile phase was used in isocratic conditions: 95% A (H_2O), 5% B (CH_3CN:CH_3OH, 50:50, v/v) and the flow rate setted was of 1 mL/min (Giray et al., 2007).

2.8. In vitro digestion model

The procedure used in this study was adapted from the method outlined by Gil-Izquierdo, et al. (2002), slightly modified. The method consists of an initial saliva/pepsin/HCl digestion for 2 h at 37 °C, to simulate the mouth and the gastric conditions, followed by a digestion with pancreatic juice for 2 h at 37 °C to simulate duodenal digestion.

For the saliva/pepsin/HCl digestion, the sample (3 g) of cooked pasta (10 g of dry pasta were cooked in 500 mL of hot water during 10 min) were mixed with 6 mL of artificial saliva composed by: KCl (89.6 g/L), KSCN (20 g/L), NaH_2PO_4 (88.8 g/L), $NaSO_4$ (57 g/L), NaCl (175.3 g/L), $NaHCO_3$ (84.7 g/L), urea (25 g/L) and 290 mg of α-amilase. The pH of the solution was adjusted at 6.8 with HCl 0.1 N. The mixture was introduced in a plastic bag, containing 40 mL of water and was homogenised in a Stomacher IUL Instruments (Barcelona, Spain) during 3 min.

Immediately, 0.5 g of pepsin (14,800 U) dissolved in HCl 0.1 N was added, the pH was adjusted at a value of 2 with HCl 6 N, and then incubated at 37 °C in an orbital shaker (250 rpm) (Infors AG CH-4103, Bottmingen, Switzerland) for 2 h.

After the gastric digestion, the pancreatic digestion was simulated as follows: The pH was increased to 6.5 with $NaHCO_3$ 0.5 N and then 5 mL of a mixture pancreatin (8 mg/mL) and bile salts (50 mg/mL) (1:1; v/v), dissolved in 20 mL of water, were added and incubated at 37 °C in an orbital shaker (250 rpm) for 2 h.

After each step of digestion, 30 mL of the obtained extract were centrifuged at 4000 rpm and 4 °C during 1 h. To determine the DON concentration, 10 mL of the supernantant of the gastric and the intestinal phases (saliva/pepsin/HCl and pancreatin–bile digestions) were extracted with an acetonitrile–water (84:16; v/v) mixture, and then analysed by LC–MS/MS according to the method described in Section 2.3.

The simulated digestion gastrointestinal model, applied in order to reproduce the physiological condition of the child, was basically the same of the one employed for adults with slight modifications. In particular, the pH of the stomach was fixed at 3.0, the quantity of pepsin used for the gastric digestion was reduced to 0.02 g, and in the intestinal digestion the amount of pancreatin and bile salts were reduced to 0.0005 and 0.03 g, respectively (Jovaní et al., 2004). The schematic representation of the *in vitro* digestion model is represented in the Fig. 1.

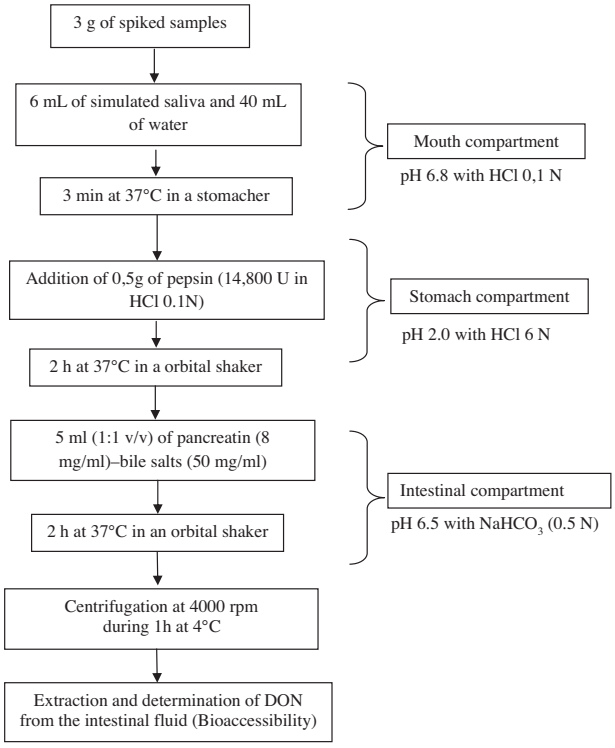

Fig. 1. Schematic representation of the *in vitro* digestion model.

3. Results and discussion

3.1. Analytical performance

During the optimisation of the extraction procedures, a representative sample of pasta was used. For every different extraction, blank and fortified pasta (30, 100 and 250 μg/kg of DON and 0.1, 10 and 50 μg/kg of OTA and AFB_1) were used. Biological fluids obtained from digestion of uncontaminated pasta were also spiked with DON at the same concentrations above reported to develop recovery tests.

According to Santini et al. (2009) and Gonzalez-Osnaya et al. (2010) the mixture acetronitrile:water (84:16; v/v) was suitable and gave the highest recoveries.

For the recovery analysis, five replicates of the samples (10 g for pasta and 10 mL for biological fluid) spiked with three investigated mycotoxins were prepared with blank pasta, and processed as previously described.

Calibration curves were obtained by injecting in duplicate standard solutions at different concentrations for each mycotoxin; the areas obtained from integration of the peaks were correlated linearly with the concentrations. For the determination of DON, standard solutions of 1, 10, 20, 50, 100, 250 and 500 ng/mL were prepared by diluting the stock solution (1000 μg/mL) with a CH_3OH:H_2O mixture (70:30, v/v). For the determination of OTA, standard solutions of 0.05, 0.1, 0.5, 1, 10 and 50 ng/mL were prepared with CH_3OH, whereas for the determination of AFB_1, standard solutions of 0.1, 0.5, 1, 5, 10 and 50 ng/mL were prepared with CH_3OH.

Mycotoxin identification was performed by comparing the retention times of the peaks present in the samples with pure standards. Quantification of mycotoxins was carried out by comparing peak areas of investigated samples with the calibration curve of standards. The mean DON recovery, was independent of spiking levels assayed, was of 89.5% with a relative standard deviation (RSD) of 3.5%. For AFB_1 and OTA, the mean recoveries obtained were 76.01% (RSD = 4.24%) and 77.50% (RSD = 9.19%), respectively (Table 1).

The detection limits (LODs) obtained were 1.0 μg/kg for DON, 0.05 μg/kg for OTA and 0.1 μg/kg for AFB_1, whereas the limits of quantification (LOQs) were 2.0 μg/kg for DON, 0.15 μg/kg for OTA (Fig. 4) and 0.3 μg/kg for AFB_1. Fluorescence chromatograms of AFB_1 and OTA can be seen in Figs. 2 and 3, respectively. In Fig. 4 is reported the total ion chromatogram (TIC) of DON.

3.2. Occurrence of DON, OTA and AFB_1 in the analysed samples

The occurrence of the three mycotoxins analysed in this study is reported in Tables 2 and 3. In particular, DON was detected in 81.4% of the 27 samples finding the highest level of contamination (450.0 μg/kg) in sample 22, whereas the lowest was evidenced in sample 13 where the DON concentration was 35.1 μg/kg.

Seven samples (25%) exceeded the maximum permitted level of DON (200 μg/kg) by the EU for processed cereal-based foods destined to babies and infants. Eleven samples (40.7%) resulted contaminated with values below 100 μg/kg, whilst four of the analysed samples (14.81%) evidenced a contamination range variable from 100 to 200 μg/kg. Five of the samples (18.52%) were under instrumental LOD for DON. The mean DON content was

Table 1
Mean recoveries, LOD and LOQ for the investigated mycotoxins in dry pasta.

Mycotoxin	Mean recovery ± RSD (%)	LOD (μg/kg)	LOQ (μg/kg)
OTA	77.50 ± 9.19	0.05	0.15
AFB_1	76.01 ± 4.24	0.10	0.30
DON	89.50 ± 3.54	1.00	2.00

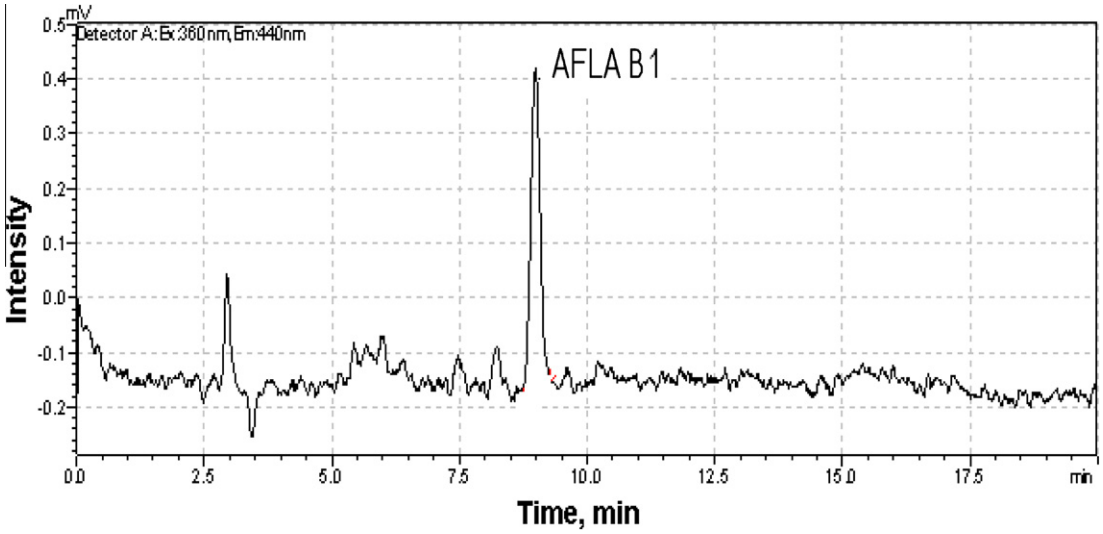

Fig. 2. Fluorescence chromatogram of a AFB_1 standard at 1 μg/kg.

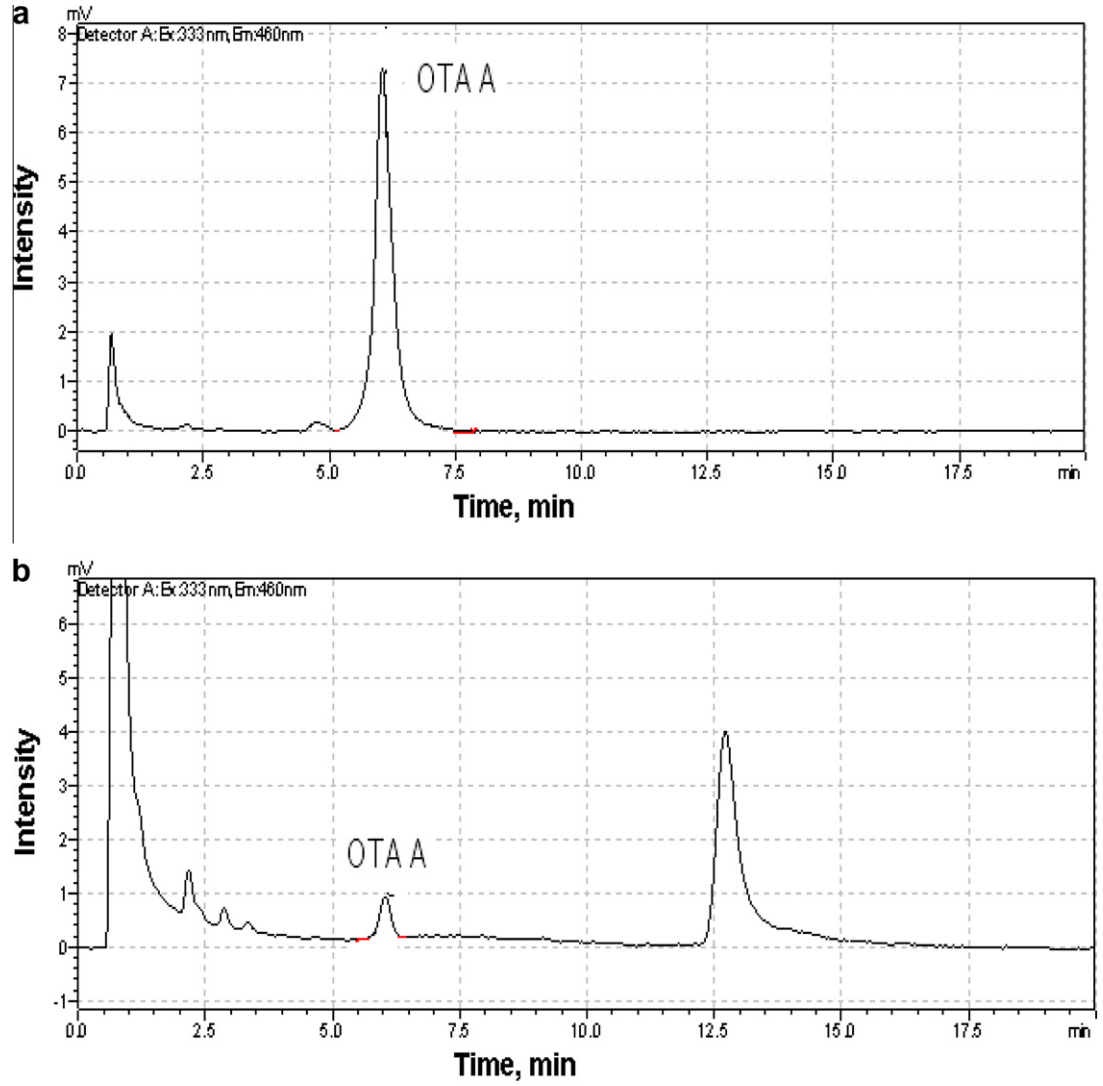

Fig. 3. Fluorescence chromatogram of (a) OTA standard at 5 μg/kg (b) Sample naturally contaminated with OTA.

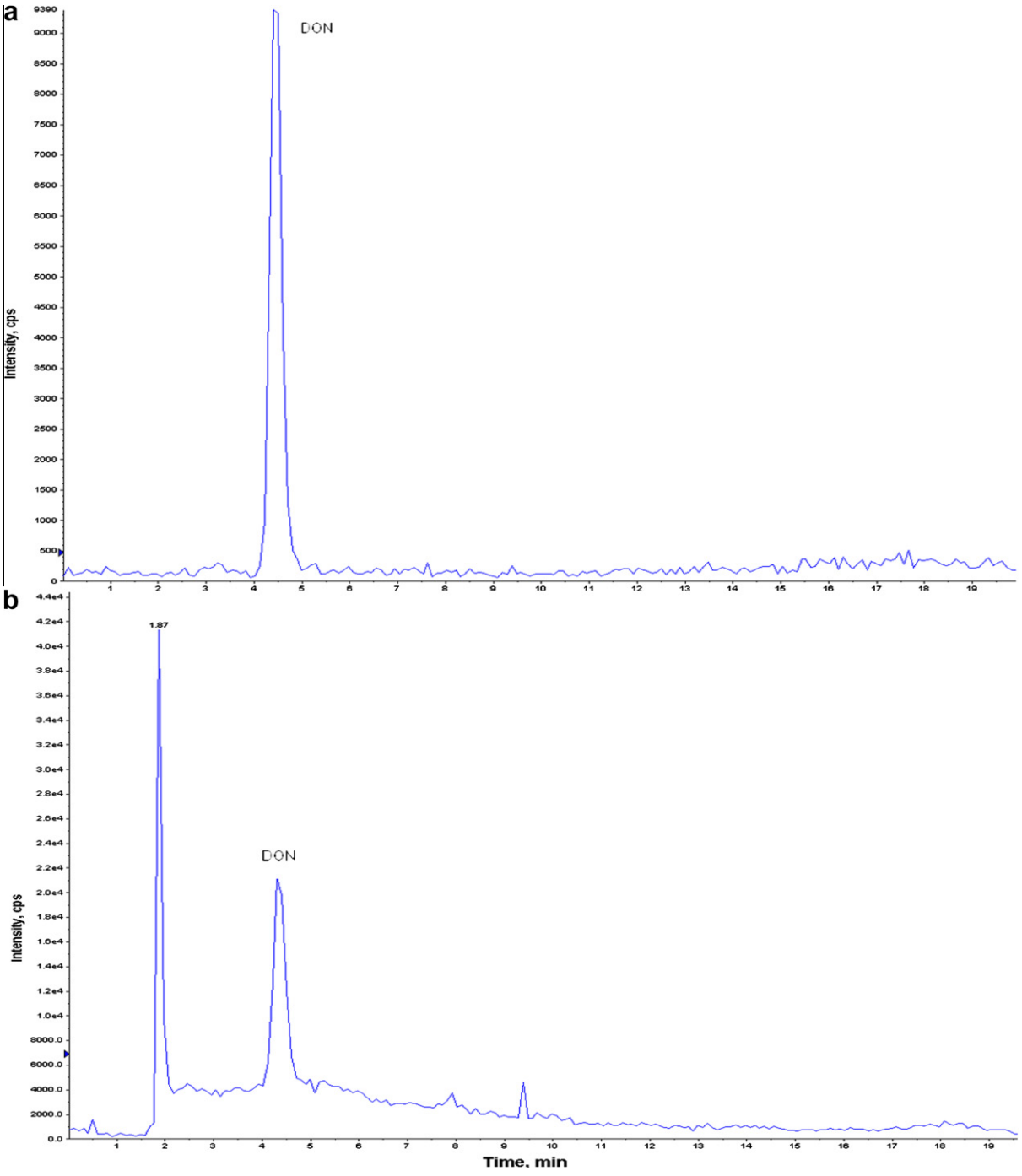

Fig. 4. Total ion chromatogram (TIC) (a) DON standard at 100 μg/kg (b) Sample of pasta sample naturally contaminated with DON.

162.14 μg/kg. All the samples show DON levels below the maximum permitted in pasta established by the EU for adults (750 μg/kg).

Schollenberger et al. (2005) calculated the intake for trichothecene toxins for adults, children and babies, in an area of southwest Germany, in 1998 and 1999. The mean DON content in pasta reported was 149 μg/kg in 1998 and 395 μg/kg in 1999. No exceeding of the tolerable daily intake (TDI) of DON, NIV and the sum of HT-2 and T-2, as stated by the EU, was found for adults (70 kg body weight (BW) and babies (10 kg BW), independently of the year and level of consumption. For children (20 kg BW) the intake of DON exceeded the TDI in both years.

Cano-Sancho et al. (2010) determined HT-2 toxin (HT2) and T-2 toxin (T2) from type A and DON from type B in 479 cereal-based food products (breakfast cereals, snacks and pasta) from Spain. Their results showed that DON was the main trichothecene present with percentages of positive samples ranging from 1.4% to 100.0%. However, despite the high incidence of DON, only five samples were above EU limits.

González-Osnaya et al. (2010) studied the occurrence of DON and T-2 toxin in pasta commercialised in Spain. An incidence of these mycotoxins, varying from 9.3% to 62.7% was reported by these authors. The mean value of DON was 137.1 μg/kg; regarding to the OTA analysis, twenty-six samples (96.3%) resulted

Table 2
Occurrence of DON and OTA in samples of analysed pasta.

Pastas	Level DON[a] (μg/kg)	Level OTA (μg/kg)
Sample 1	350.00 ± 2.00	0.12 ± 0.04
Sample 2	387.00 ± 1.55	0.11 ± 0.03
Sample 3	371.00 ± 2.54	0.20 ± 0.03
Sample 4	41.30 ± 1.70	0.25 ± 0.02
Sample 5	134.00 ± 3.75	0.52 ± 0.01
Sample 6	270.00 ± 4.20	0.09 ± 0.01
Sample 7	79.90 ± 1.50	0.32 ± 0.04
Sample 8	121.00 ± 2.74	0.12 ± 0.09
Sample 9	61.20 ± 3.84	−0.17 ± 0.04
Sample 10	62.00 ± 1.57	0.32 ± 0.05
Sample 11	51.70 ± 4.21	0.24 ± 0.01
Sample 12	73.80 ± 2.80	0.35 ± 0.02
Sample 13	35.10 ± 1.64	0.14 ± 0.08
Sample 14	58.50 ± 1.52	0.14 ± 0.06
Sample 15	61.80 ± 2.13	0.21 ± 0.01
Sample 16	299.00 ± 4.89	0.25 ± 0.09
Sample 17	39.80 ± 2.92	0.10 ± 0.01
Sample 18	106.00 ± 4.93	0.18 ± 0.04
Sample 19	176.00 ± 2.12	0.23 ± 0.09
Sample 20	98.00 ± 1.97	0.37 ± 0.07
Sample 21	240.00 ± 2.29	0.14 ± 0.02
Sample 22	450.00 ± 3.20	<0.05
Sample 23	<1	0.15 ± 0.02
Sample 24	<1	0.13 ± 0.05
Sample 25	<1	0.12 ± 0.01
Sample 26	<1	0.21 ± 0.06
Sample 27	<1	0.18 ± 0.03

[a] Average values ± standard deviation from the means of three replications are presented.

contaminated by OTA over its LOD (0.05 μg/kg). The highest level of contamination reported was 0.52 μg/kg for the sample 5 whilst the average content of OTA was 0.3 μg/kg. Only one of the samples exceeded (3.7%) the maximum level permitted (0.50 μg/kg) in baby food established by the EU for these products. Fourteen samples (51.85%) contained OTA levels below 0.2 μg/kg, whereas eleven samples (40.74%) evidenced a contamination range variable from 0.2 to 0.5 μg/kg.

Winnie et al. (2009) determined the occurrence of OTA in 274 samples of dry pasta of Canadian origin between 2004 and 2006. Incidence of contamination above 0.5 μg of OTA per kg was 21%, 18% and 66% in the years 2004, 2005 and 2006, respectively. Mean levels of OTA in these 3 years were, respectively, 0.3, 0.2 and 0.7 μg/kg, and maximum levels were, respectively, 1.8, 1.4 and 3.3 μg/kg.

A mean OTA incidence of 16.7% in infant cereals of Turkish origin was reported by Kabak, 2009, with a maximum level of 0.3 μg/kg.

Zinedine et al. (2007) determined the occurrence of aflatoxins in cereals from Rabat (Morocco), finding contamination levels between 0.03 and 0.15 μg of AFB_1/kg in wheat. Moreover, Zinedine et al. (2010) studied the occurrence of OTA in 68 samples of breakfast and infants cereals from Morocco and showed that all analysed infant cereals were OTA free; however, four samples of breakfast cereals destined to general population were contaminated with OTA at levels ranged from 5.1 to 224.6 μg/kg.

Villa and Markaki (2009) evaluated the occurrence and the risk assessment of AFB_1 and OTA in breakfast cereals from Athens market. The presence of AFB_1 in 56.3% of the samples (mean 1.42 μg/kg AFB_1) was detected. Seven samples were found contaminated at levels higher than the EU limit, whilst OTA was detected in 60% of the samples (mean 0.18 μg/kg).

The results of our study were comparable with the data reported by Herrera et al. (2009), who compared DON, OTA and AFB_1 levels in conventional and organic durum semolina. The AFB_1 was no detected in any of durum semolina samples. The percentage of durum semolina samples positive for the presence of OTA was 8.3% in conventional and 20% in organic samples. The occurrence of samples that resulted positive for DON was of 16.7% in conventional products and 20% in organic products, whereas DON mean levels resulted lowest in conventional samples (77 μg/kg) respect to the organic samples (89 μg/kg).

In Algerian wheat and derived products, Riba et al. (2010) investigated the presence of AFs. AFB_1 was detected in 56.6% of the wheat samples and derived products (flour, semolina and bran), with contamination levels ranging from 0.1 to 37.4 μg/kg.

In year 2009, Soubra et al. assessed the occurrence of aflatoxins, OTA and DON in some foodstuffs available in the Lebanese market and evaluated the potential risk to the health of children and teenagers in Beirut from dietary exposure to these mycotoxins. The calculated intake for aflatoxins exceeded its respective PTDI in all groups by a factor ranging from 3 to 7. The intakes of OTA and DON were found to be below the threshold of toxicological concern established for these mycotoxins by international expert groups, although the intake of DON in children at the highest percentile (P95) was close to its PTDI.

The Italian population consumes 76.7 g/day of pasta (28 kg/persona/year) (ISMEA-AC NIELSEN, 2003), which would correspond to 12.4 μg/day of DON, taking into account the mean occurrence evaluated in this study.

The EFSA estimates 1.4 μg/kg bw/day the DON intake for European consumers, in particular for an adult (70 kg body weight) the Tolerable Daily Intake of DON (TDI) is 70 μg/kg/day and the pasta intake corresponding to 17.7% of TDI in adult and 62.1% for a children between 3 and 5 years of age, with 20 kg body weight (Cole et al., 2000).

The value for DON intake assessed in this study is less than the TDI values for adults, but the subgroup of children can be considered a critical group because the cereal intake can be markedly higher than in adults and in many cases children can have a diet not so varied as the adults.

3.3. DON contents in the gastric and duodenal fluids (Bioaccessibility)

Mean recoveries were performed on the fortified intestinal fluid (free from the contamination of DON) (n = 5) at levels of DON (0.1–500 μg/g). The DON recoveries obtained in this study varied from 80.2 ± 2.3% to the 92.5 ± 3.1%. The values obtained for recoveries and relative standard deviations of the method used are in agreement with those of the Commission Regulation (EC) No 401/2006 for methods of analysis of mycotoxins in foodstuffs (European Commission, 2006). Intra-day (n = 5) and inter-day (five different days) variation values ranged between 1.5 and 2.1, respectively

Table 3
Incidence and levels of DON and OTA in pasta samples.

Mycotoxin	Positive sample/frequency (%)	Mean (μg/kg)	Max. level (μg/kg)	<LOD	Frequency distribution n (%)		
DON	22 (81.48%)	162.14	450.00	5 (18.52%)	<100 μg/kg 11 (40.74%)	100–200 μg/kg 4 (14.81%)	>200 μg/kg 7 (25.93%)
OTA	26 (96.30%)	0.21	0.52	1 (3.70%)	<0.2 μg/kg 14 (51.85%)	0.2–0.5 μg/kg 11 (40.74%)	>0.5 μg/kg 1 (3.70%)

* The statistical data showed have been obtained no considering the no-detected samples the data were evaluate with SPSS program (SPSS Inc., Chicago, IL, 326 release 15 for Windows).

Table 4
Bioaccessibility of DON for pasta samples after gastric and duodenal digestion.

Pasta	DON μg/kg	DON μg/kg cooked pasta	Gastric digestion Gastric bioaccessibility (%)	Duodenal digestion Duodenal bioaccessibility (%)
Sample 1I[a]	350.00 ± 2.00	25.32 ± 1.1	19.72 ± 1.73	8.40 ± 1.12
Sample 2I	387.00 ± 1.55	42.10 ± 3.1	2.12 ± 0.11	1.11 ± 0.01
Sample 3I	371.00 ± 2.54	40.21 ± 1.9	16.76 ± 2.11	10.71 ± 0.25
Sample 5I	134.00 ± 3.75	15.22 ± 0.5	20.40 ± 1.80	10.42 ± 0.14
Sample 12A[b]	73.80 ± 2.80	10.27 ± 0.3	41.49 ± 2.42	24.13 ± 1.71
Sample 12I	73.80 ± 2.80	11.36 ± 0.1	38.41 ± 2.95	17.91 ± 0.80

Average values ± standard deviation from the means of three replications are presented.
[a] I: infant.
[b] A: adult.

for DON. These values are below 15% which is the maximum variation for certification exercises for several mycotoxins.

In the risk assessment related to the mycotoxins exposure, it needs to be remembered that all the bioactive compounds must be bioaccessible to act on the different organs or tissues of the human body. The bioaccessibility is defined as the fraction of a compound present in a food that pass unmodified the complex of the enzymatic reactions of the gastrointestinal digestion and that is potentially accessible to the absorption by the cells of the intestinal epithelium (Benito and Miller, 1998).

To evaluate the real exposure to DON in adults and children, its bioaccessibility was evaluated with a simulated gastrointestinal digestion optimised for both groups of population. Six pasta samples were selected amongst 27 analysed samples to be used in the study.

Firstly, to verify if the pasta cooking, necessary for the bioaccessibility study, had any effect on the DON concentration present in the samples, each pasta sample was analysed for the DON content using the method of Santini et al. (2009) before the application of the simulated gastrointestinal digestion model. As it can be shown in the Table 4, the lost of DON contained in the analysed samples, due to the boiling, ranged from 10.2 μg/kg of the sample 12A to 42.1 μg/kg of the sample 2I evidencing a mean percentage of DON equal to 12% compared with the content in raw pasta.

Visconti et al. (2004) investigated the reduction of DON during durum wheat processing and spaghetti cooking. With respect to the uncleaned wheat, the average level of DON was 20% in cooked spaghetti.

In the Table 4 there are reported the results regarding the gastric and duodenal bioaccessibility expressed as DON concentration and as % respect to the initial DON concentration in pasta. The sample 12 has been digested with adult model system, whereas the rest have been treated with the alternative digestive process for children. The DON percentages in the gastric fluid ranged from 2.12% to 41.5% with a mean value for this gastrointestinal model of 23.1%.

High values of gastric bioaccessibility were also evidenced in the sample 12I (child digestion) and 5I (child digestion) with 38.4% and 20.4%, respectively. DON was only detected after adult gastric digestion in the sample 12.

The mean DON bioaccessibility value after duodenal process was of 12.1%, with values ranging from 1.1% to 24.1%. The highest DON duodenal bioaccessibility value was reported by the sample 12 (adult digestion) where the data evidenced was of 24.1 ± 1.7%, whereas the lowest was detected in the sample 2 (child digestion), with 1.1 ± 0.01%.

Considering only the samples treated with the child digestion the mean DON duodenal bioaccessibility data was of 9.7%. This data is very interesting by the toxicological point of view, because proved that a 9.7% of the DON present in pasta samples, did not interact with the enzymes of the digestion system and arrived unmodified to the intestinal lumen, available for the absorption by the cells of the intestinal epithelium. Therefore, the DON amounts present in the pasta samples (7.3–38 μg/kg) could interact with the intestinal epithelium cells, and these concentrations are cytotoxic on several cell lines as demonstrated by Diessing et al. (2001) and Daeniche et al. (2011). Moreover, considering the small dimension of the child intestinal epithelium, the DON concentrations present in the intestinal fluid could probably produce more damage to the intestinal enterocytes respect to that in an adult.

In literature, there are few studies on the mycotoxins bioaccessibility, which are very interesting because try to correlate the bioactive compounds presence in food with the real exposure risks.

In particular, Avantaggiato et al. (2003), studied the intestinal absorption of ZEA by using a laboratory model that mimics the metabolic processes of the gastrointestinal tract of healthy pigs. Approximately 32% of ZEA intake (247 mg) was released from the food matrix to the bioaccessible fraction during 6 h of digestion and was rapidly absorbed at intestinal level.

Carolien et al. (2005) described the applicability of an *in vitro* digestion model allowing the measurement of the bioaccessibility of ingested mycotoxins from food as an indicator of oral bioavailability Bioaccessibility of AFB_1 from peanut slurry and OTA from buckwheat was 94% and 100%, respectively.

Recently, Kabak et al. (2009) determined the bioaccessibility of the AFB_1 (90%), and OTA (30%) in pistachio nuts, buckwheat and infant foods using an *in vitro* model under fed condition.

It has been also demonstrated that mycotoxin bioaccessibility performances depends on several factors, such as food product, contamination level, compound and type of contamination (spiked versus naturally contaminated) (Kabak et al., 2009). Our *in vitro* study has demonstrated differences in levels of DON bioaccessibility during the child digestion processes, attributable to different typologies of pasta and initial contamination level.

The combination of surveillance data and bioaccessibility provided in this study suggested that DON exposure risk should be reconsidered by JEFCA Commission and that more studies must be done in this topic.

Considering that the legal limit for baby food protects until 2 years old, whilst in Italy and most of Mediterranean countries pasta consumption starts at age of 2–3, a new more restrictive limit for child than for adult (750 μg/kg) should be auspicable by the European Commission. Alternatively, the general limit could be decreased to reduce the exposure of the young population, and consequently the adult population.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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