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HPLC investigation of hidden danger deoxynivalenol (vomitoxin) in baby foods from grain sources in Türkiye

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ABSTRACT

The Food and Agricultural Organization (FAO) declared that at minimum, one quarter of the earth's food supply is infected with mycotoxins. Deoxynivalenol (DON) is the most common *Fusarium* mycotoxin in maize, wheat, rice oats, and barley. The goal of this study is to explore the incidence of DON in cereal-based baby foods in Turkey by using high-performance liquid chromatography (HPLC) with C_{18} column and UV detector at 220 nm. The results were statistically significant ($p< 0.05$) in terms of standard deviation (in parallel analysis). The highest amount of detected DON in baby foods was 0.17 ppm. The samples with DON by HPLC were also confirmed with LC-MS detection (positive and negative mode). Forty-eight commercial cereal-based baby foods were analyzed, and four samples were contaminated (8.3%). DON analysis results show that baby foods containing cereal sources sold in Turkish markets are a significant threat for human health and baby health.

Keywords: deoxynivalenol (DON), LC-MS analysis, baby foods, mycotoxin

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INTRODUCTION

Mycotoxins are easily affected raw materials used in foods, in both the pre-harvest and storage periods. Trichothecenes are produced by several types of fungi such as *Fusarium*, *Myrothecium*, *Trichoderma* and *Cephalosporium*. Deoxynivalenol (DON), commonly referred to vomitoxin, is widely dispersed trichothecene caused especially by *F. graminearium* and *F. culmorum*. The most widespread and frequently recognized mycotoxins that threaten human health are trichothecenes and fumonisins. DON occurs frequently in cereal-based foodstuffs. The possible influence of DON on human health may occur after the consumption of infected foodstuffs. Acute, chronic dietary admission of contaminated food is dangerous. The occurrence of DON in processed cereals and pulses in Turkey were previously researched in Omurtag and Beyoglu[1].

In Turkey, the most recent guideline for DON limits in baby infant supplemental foods is 0.2 ppm[2].

The increase in DON amounts in wheat products also occurs at harvest time since wheat cultivation, Good Agricultural Practices (GAPs) usage, mold strains, temperature, water activity, nutriment accessibility, uses synthesized methods. As is the case for every natural material, mycotoxins may be infected by mold and produce mycotoxins if the environmental conditions (e.g. temperature, moisture) are conducive to it. Human exposure may be through cereals or via animal-based foods (kidneys, liver, milk, eggs). DON's role in the infection is a result of *Tri5* gene disruption leading to a drop in wheat contamination since it cannot generate DON.

Mycotoxins accumulate within the membranes of vegetables, and cereals; then they negatively affect or impede human and animal functions. Studies indicate that temperature, water activity, and development time, have a clear influence on DON exhibition in *F. graminearum, F. culmorum,* as well as *F. meridionale*. The ideal temperature of DON is between 25 °C and 30 °C[3]. Llorens et al.[4], declared that *F. graminearum*, *F. culmorum* growth temperature is between 20 °C and 32 °C, and that fungal growth is reduced at temperatures below at 15 °C. For *F. graminearum,* minimum water activity for growth is 0.90 and the maximum limit is recorded as in excess of 0.99[5]. The highest water activity for DON occurred at 0.97 and 0.99 in wheat[6].

Subjection to trichothecenes is referred to in Southeast Asia as "yellow rain". DON, nivalenol, DAS (diacetoxyscirpenol) and T-2 toxin are parts of this airborne substance and can be detected in tissue, blood, and urine samples of sufferers. The above-mentioned toxins were found in many gastrointestinal

disease epidemics in China and India after the absorption of bread with infected flour, and in rice[7].

DON was proven to be the reason for vomiting (hence the term "vomitoxin"), under-nourishment, diminished weight gain, anorexia, and an impaired immune system. The symptoms described for DON in humans consist of abdominal pain, nausea, vomiting, dizziness, headache, throat irritation, diarrhea, and blood in the stool[7]. Effects on the immune system, especially on IgA were demonstrated with experimental animal studies. Humoral and cellular immunity, as well as a tendency for infectious diseases, were demonstrated in studies on experimental mice[8]. Reports of vomiting caused by the consumption of moldy cereal grains, even when baked into bread in the form of flour, have been reported for animals and humans since 1916 by numerous investigators in various parts of the world. The causative agents that had previously eluded detection were identified in a new trichothecene vomitoxin from corn contaminated in the field with *F. graminearum* by Vesonder et al.[9].

DNA damage in human hepatoma cells (Hep G2) via DON-influenced oxidative stress was shown by Stepanova et al.[10] who demonstrated that the temporary intrauterine subjection of DON results in its durable availability in piglets' plasma, causing the alteration of the body's immune system. DON impairs protein synthesis and is toxic for the body's immune system in a variety of animals, resulting in gastrointestinal diseases, lower growth, and a growing threat of contagious illness. The Codex Alimentarius Commission manages food sourced mycotoxins that are risky for people, have set the maximum tolerable levels (MTLs) of DON as 0.2 ppm of cereal-based foods for children. 1 ppm of flour, meal, semolina, or flakes sourced from wheat, maize or barley, 2 ppm of wheat, maize and barley for additional treatment can diminish levels of DON before usage within foods. The Food and Drug Administration (FDA) put the limit for DON in processed human food as 1 ppm. Health Canada set DON levels for wheat as 2 ppm for raw foods and 1 ppm for children's foodstuffs. The European Union (EU)'s DON standards are in line with the American and Canadian regulations. The Codex Alimentarius Commission (CAC) set the allowed limit as 2 ppm of DON in raw wheat, barley, and maize. EU countries are applying a 0.75 ppm DON limit in flour intended for raw materials for several years[5]. The European Union set the highest levels for DON as 0.2 ppm in infant and baby foodstuffs. The lowest limit of quantification (LOQ) is 0.2 ppm for DON for baby food in EU[11].

The NOAEL in the study with mice is 1 ppm feed, equal to 0.1 ppm b.w. / day[12]. In another trial, the results of DON on humoral and cellular defense systems in

mice was investigated; a 'no effect' level for immune system related outcomes in mice was demonstrated between 0.25 ppm and 0.50 ppm b.w. / day[13]. For single mycotoxins, the approximate daily subjection compared with the toxicological thresholds of mycotoxins without carcinogenic potency, i.e. tolerable daily intake (TDI) or highest tolerable daily intake (PMTDI) was: DON (TDI 1000 ng kg^{-1}.$bw.day^{-1}$)[14]. For DON, a tolerable daily intake (TDI) was shown as 1 μg DON / kg.bw/ day by the European Food and Safety Authority (EFSA) for people[11]. *Fusarium* toxin exposure in the diets of children and adults were assessed worldwide, but no data on baby foods and baby biscuits is available for DON in Turkey. This study will address the lack of data for the DON limitation in the healthy growth of those populations. The HPLC and clean-up procedures were adapted by Omurtag and Beyoglu[1].

LC-MS / MS development and validation were used for the coincident detection of *Fusarium* mycotoxins in single chromatographic runs in cereals and cereal-derived foods[15].

Pereira et al.[16] evaluated DON in processed cereal-based baby food by GC-MS. Scott et al.[17] first evaluated DON in wheat and grains by GLC-EC. DON in concentrations of 0.01-4.3 mg / g revised for recovery were reaffirmed in 15 positive samples by GLC-MS (SIM). Omurtag and Beyoglu[1] evaluated the incidence of DON in 83 processed cereals and pulses in Turkey and concluded that 6 out of 68 cereal contain significant amounts of DON, with the highest level being 2.67 ppm.

The objective of this research was to investigate the possible health risks of DON in baby foods while raising a healthy generation.

METHODOLOGY

Materials

All the baby food samples were purchased from stores and pharmacies from Istanbul, Turkey. Only 4 of 48 total samples were baby foods and the remaining of them were in the food supplement category with grain and fruit additives (Table 1). Each sample (1 kg) was blended by an Erwaka blender. A 50-g aliquot of 250 g subsample was taken for analysis and stored at -20°C until analysis.

Table 1. Deoxynivalenol in baby foods processed with cereal sources determined by HPLC

Samples From Turkey	Processed baby foods with cereals sources		
	DON by HPLC (positive / total)	DON level by HPLC (LOD-LOQ) (ppm)	Mean of DON (ppm)
Baby Food (A)	0/2[p]	-	-
Baby Food with Milk, Rice (B_1)	0/2[p]	-	-
Baby Food with Organic Grain Rice ©	½[m]	0.05-0.17	0.13*
Baby Food with Milk, Rice (B_2)	0/2[m]	-	-
Baby Food with Milk, Rice (B_3)	0/2[p]	-	-
Baby Food with Cereal (D_1)	½[m]	0.05-0.17	0.12*
Baby Food (D_2)	0/2[m]	-	-
Baby Food with Milk ©	0/2[m]	-	-
Baby Food with Milk, Biscuit (F)	0/2[p]	-	-
Baby Food with Milk, Honey, Semolina (G1)	½[p]	0.05-0.17	0.07*
Baby Food with Organic Grain, Oat (H)	0/2[m]	-	-
Baby Food with Grain, Fruit, Yogurt (J)	½[m]	0.05-0.17	0.17**
Baby Food with Organic Grain, Apple, Milk (K)	0/2[m]	-	-
Baby Food with Whole Grain with Milk (L)	0/2[p]	-	-
Baby Food with Milk, Five Fruits (M)	0/2[p]	-	-
Baby Food with Apple, Seven Grain (N)	0/2[p]	-	-
Baby Food with Milk, Banana, Apricot (O)	0/2[p]	-	-
Baby Food with Organic Grains (P)	0/2[m]	-	-
Baby Food with Milk, Fennel, Grains ©	0/2[m]	-	-
Baby Food with, Milky, Apple, Grains (S)	0/2[m]	-	-
Baby Food with Milk, Banana, Rice (T)	0/2[m]	-	-
Baby Food with Milk, Honey, Semolina (G_2)	0/2[m]	-	-
Baby Food with Milk, Pear (U)	0/2[p]	-	-
Baby Food with Milk, Honey, Semolina (G_3)	0/2[p]	-	-
A, C, E, F, H, J, K, L, M, N, O, P, R, S, T, U: Different brands' baby food samples' codes.			

B_1, B_2, B_3, D_1, D_2, G_1, G_2, G_3: Different brands with similar ingredients' codes

DON, deoxynivalenol; [m] received from retailer; [p] received from pharmacy, *: > LOD, **: =LOQ, >LOD.

Chemicals

All solvents were of high purity and were purchased from Carlo Erba (methanol) and Merck (acetonitrile), while the DON standard was purchased from Sigma-Aldrich. Ultra-pure water purified by an Elgaflex-3-Ultra-Purified Water Machine was used in all the solution preparation steps and HPLC separation.

Preparation of stock solutions

A DON stock standard solution was prepared as a 1 mg / mL concentration in acetonitrile and from this stock solution, the 40 ng / mL mother liquor, which served as a source for dilutions, was diluted to 5 different concentrations with a methanol: water (20:80 v/v) mixture, and standard solutions of the calibration chart for HPLC analysis were created with them.

Sample preparation

After baby food samples (50 g) were blended with acetonitrile: aqua (21:4 v/v), a suspension mixture was decanted by a filtering process with Whatman no:4. The filtrate was evaporated with a vacuum dryer under inert atmosphere at 60°C. After sample extracts were dissolved acetonitrile: aqua (21:4 v/v), they were loaded to Alumina–Celite–Charcoal clean-up disposable column for stabilizing the DON composition. After collecting the elations, they were evaporated with inert vacuum atmosphere at 60°C (Extract A)[18].

HPLC procedure

Extract A was solved with a methanol: aqua (20:80 v/v) solution mixture (1 mL). After it was filtered to vials with IsoLab Syringe filters (cellulose acetate 0.22 μm) PVDF cartridge, vial samples (50 μL) were auto injected to the HPLC system from a 100-vial tray into an autosampler unit. For the calibration graph, diluted DON standards of five different concentrations (3-40 ng / mL) were qualitatively and quantitatively analysed for 15 min in triplicate with HPLC. DON was detected at retention time of 6.2 min at UV 220 nm. Detected DON contents were verified with further LC-MS analysis on negative and positive mass fragment mode (Figure 1). All of the peak areas from standards and samples were recorded for quantitative analysis. Concentrations of baby food samples were calculated with the linear equation obtained from the calibration graph ($y = 2.402x + 3.416$ (r= 0.9770))[18]. The detection limits of DON contents at baby foods were found to be 3 times and 10 times of mean standard deviation of noise. The method linearity was measured as of 0.05-3 ppm in recognized DON compounds. Chemwindow detection limit analysis data were found to be highly significant.

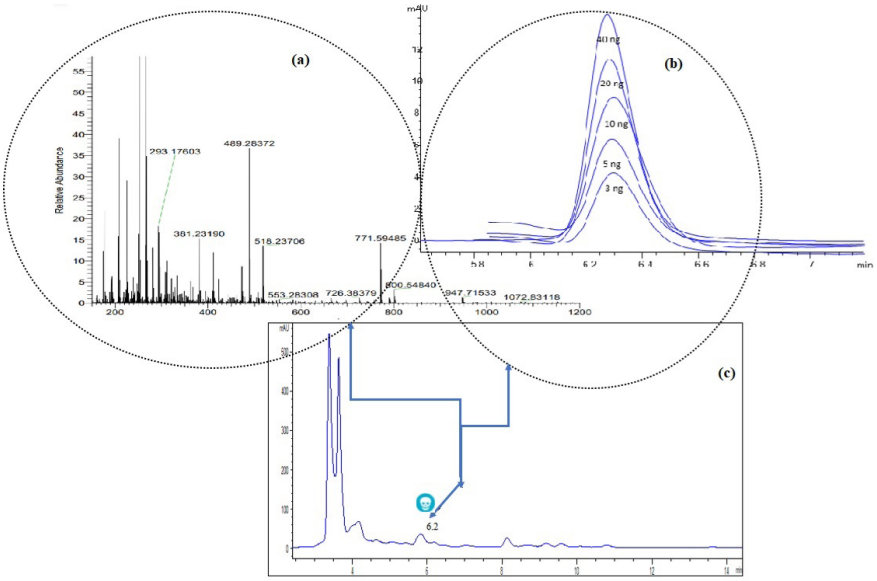

Figure 1. (a): DON chromatogram with MS, (b): Overlapped peaks for DON standards (3-40 ng), (c): DON detected in baby food samples' UV chromatogram (0.17 ppm)

HPLC apparatus

The Shimadzu LC-2010C-HT HPLC compact system (Japan) utilized in the analysis consisted of a degasser, a thermostable column unit, a gradient manager pump, an autosampler unit, and a UV detector. UV wavelength was set at 220 nm. The experiments were performed using an encapped purospher star reverse phase- C_{18} column with guard column (4.6 × 250 mm, 5 µm) (Merck, Germany). Data procurement (peak area, retention time) was carried out with the use of the Shimadzu Chemwindow Program. An auto-injection receptacle with 50 µL loop volume ensured the correct concentration of the baby food samples.

Operating conditions

The isocratic mobile phase was methanol: aqua (70:30 v/v), and flow rate was 0.7 $ml.min^{-1}$. The column temperature was set at 25-30°C with a column oven. The mobile phase was purchased ultra purely and the isocratic solvent flow was secured with the degasser system and filter cartridge system[18].

Confirmation with LC-MS

A Thermo Orbitrap Q-Exactive system of LC-MS was utilized for the confirmation of DON. The detector was applied in electron spray ionization (ESI) mode, with an electron spray voltage equivalent to 3.8 kV. Operating conditions were as follows: the column was a Fortis C_{18} – 150 x 3.0 mm of 3 μm film thickness; gradient pump flow rate and carrier gas flow rate were 0.35 mL / min and 10 mL / min, respectively. Initially (for the first 3 min.), dual gradient flow consisting of 50% mobile phase A (1% Formic acid – H_2O), 50% mobile phase B (1% Formic acid – MeOH) was completed with 100% mobile phase B while DON detection was performed. The temperatures of the capillary and heater ion source gas were adjusted to 320°C. The quantification was performed using a mass fragment scan range of 150-1200 *m/z* from the component library for DON, and its retention time was 6.2 min. The temperature and relative humidity under which the analysis was applied, were respectively[19]: (22.0 ± 5.0) °C, (50 ± 15) rh%.

RESULTS and DISCUSSION

DON occurrence in cereal grains and processed products was found to be an important food safety and global health concern, especially in developing countries. The intake risk evaluation of DON for baby foods was not known.

DON was the most abundant toxin in processed cereal-based foodstuffs for children and toddlers, with an incidence of 21.3%. The maximum DON found in the EU was as 0.2 ppm in only one sample[20]. DON was analyzed in 35 samples of commercial baby foods. Herrera et al.[19], noticed that DON was found in higher amounts in baby foods with organic sources. DON was detected in 20% of all samples (one of them was exceeded the EU limit of 0.2 ppm). In a survey where cereal based baby and infant foods were evaluated, DON was found in 71% of all samples. DON was also tested in commercial baby foods from Qatar. 27% of samples were found to contain DON, and this mycotoxin was the only one exceeding EU limits[21]. The occurrence of DON in beer in Turkey was investigated by HPLC[22]. DON was detected from urine after exposure. Sarkanj et al.[23], firstly, analyzed DON with LC–ESI–MS / MS in samples collected from 40 pregnant Croatian women, and it was found that 97.5% of the urine samples exceeded the limit of detection. Huybrechts et al.[24] detected DON and its derivative with an LC–MS / MS method.

DON levels exceeding legal limits were found in several cereal-based baby foods[25]. In our study, the highest amount of detected DON in baby foods was 0.17 ppm. Recovery (%) was 101.4 (SD: 2.17, n=5). Detected baby food samples

were those made with grain, semolina, rice and cereals. The rice sample was of organic origin. Cuce[26] investigated DON with ELISA and could detect it in tarhana, lentils, wheat and wheat flour in Turkey; it was concluded that temperature and humidity have a direct relationship with DON growth. LOD and LOQ validation criteria for DON were set in line with the International Conference on Harmonization guidelines[18].

Baby foods and baby biscuits are largely consumed as matter of convenience and saving time. Many countries consume high amounts of baby foods and baby biscuits; it is important to test DON presence in those products too.

As a result of the pandemic, securing immune system support with healthy foods gained importance. The Food and Agriculture Organization of the United Nations (FAO) declared 2021 the "International Year of Fruits and Vegetables". Uncontaminated dietary products from the source are in focus worldwide. Growing with clean food is a fundamental for a healthy generation. For sustainable secure food access, the results of this study on baby food contamination levels by DON will lead to set criteria on this era.

The previous sentence is beyond repair. Also, dietary DON intake in chicken affected the body defense response to virus vaccines and influenced the serum clinical parameters[27]. Thus, during baby and children's vaccinations, the effect of chronic exposure to DON should be considered for its effect on vaccine responses as well as immune system responses.

STATEMENT OF ETHICS

This study does not require any ethical permission.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflict of interests that could have appeared to influence the work reported in this paper.

AUTHOR CONTRIBUTIONS

O.E.E. and D.Y. conceived and wrote the original draft. G.Z.O provided advice and supervision. O.E.E., G.S.E. and M.E.T. collected the data that shown in the Figure 1 and Table 1. D.Y. and O.E.E. summarized the data of published articles. O.E.E., G.Z.O., and D.Y. finally revised the manuscript.

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