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Development and validation of gas chromatography-mass spectrometry method for the detection of zearalenone and its metabolites in cereal-based infant formulas

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ABSTRACT

Food safety is a high priority for human health. Chemical substances contaminated at different stages can cause acute and chronic health problems. Infants are one of the essential risk groups. Studies on endocrine disruptors are particularly important as these substances have many undesirable consequences for future generations, notably their impact on the reproductive system. Zearalenone (ZON), produced by Fusarium fungi, is an endocrine-disrupting mycotoxin with an estrogenic effect. Many species of fungi are localised on agricultural products such as corn, rice, and wheat. Feeding infants in the early stages of life with plant-based formula may lead to harmful effects of mycotoxins. We aimed to investigate whether commercially available cereal-based infant formulas are contaminated with ZON. Gas Chromatography – Mass Spectrometry (GC-MS) was used to determine ZON and metabolites. The method was developed by optimisation studies in sample preparation. The process was accurate, selective, reproducible, and highly sensitive at concentrations below the maximum residue level. ZON was validated using two different working ranges (for 1st working range: 0, 5, 10, 20, 30 ng/g; r^2 are 0.993, 0.995; 1.08 ng/g, LODs are 1.08, 1.01 and 1.2 ng/g; LOQs are 3.25, 3.01 and 3.62 ng/g, and 2n^d working ranges: 0, 1, 2, 3, 4 ng/g; r^2 are 0.996, 0.994, and 0.996; LODs are 0.20, 0.200 ng/g, 0.06 g/ng; LOQs are 0.18, 0.60 ng/g and 0.60 g/ng for ZON, α-ZOL and β-ZOL, respectively). Cereal-based infant formulas sold retail in the market were not contaminated with ZON and its metabolites. Infants consuming these products are not at risk from cereal-based formulas.

Keywords: Cereal-based formulas, Endocrine disrupters, Fusarium mycotoxin, GC-MS, Infant, Zearalenone

Introduction

The safety of food is one of the highest priorities for the health of human beings. Today, producing sufficient quantity and quality foodstuffs for the increasing world population is one of the main issues encountered in the food sector (FAO, 2023). Chemicals used in all stages, from agricultural practices to the end of the production stage, even packaging, can cause many health problems. In addition to chemicals that are intentionally added to foods, such as food additives, chemicals, and various contaminants that are unintentionally contaminated, they cause economic losses and different health problems (Kutluay Şahin & Şahin, 2023; Rather et al., 2017).

Many pathogenic and saprophytic fungi are included in foodstuffs as much as the conditions allow from production to consumption. These fungi cause product losses in terms of quality and quantity and bring significant dangers to human and animal health with the toxins they create. Although there are many toxic fungi in agricultural products and foods, the important mycotoxins frequently observed in public health worldwide are aflatoxins, ochratoxin, fumonisin, deoxynivalenol, zearalenone, trichothecenes, and ergot alkaloids (Awuchi et al., 2021). These mycotoxins, which are much more important for infant and child health because they are in the high-risk group, cause tolerable adverse effects such as emesis and serious adverse effects such as neural tube defects and oesophagal cancer (Bennett & Klich, 2003). Secondary metabolites of filamentous fungi are mycotoxins. They are among the most commonly occurring contaminants. These toxins can enter the food chain at different stages of food production. Zearalenone (ZON) is one of more than 400 detected mycotoxins and an estrogenic mycotoxin mainly produced by Fusarium fungi that infect the plants in the field and cause many diseases (Ropejko & Twarużek, 2021). ZON was first obtained from Fusarium-contaminated corn. ZON is particularly important because it occurs before harvest, and its occurrence cannot be completely prevented by strategies to minimise plant production due to weather conditions. (Thapa et al., 2021).

Exposure to ZON is an important endocrine disrupter for animals and humans due to its estrogenic effect (Song et al., 2021). Infants and children fed with plant-derived formula and similar food products are especially at high risk due to low metabolic rates, body weight, and high physiologic differences (Piacentini et al., 2019).

Due to its adverse effects and toxicities on the endocrine system, extensive research has been conducted on the presence of ZON in food. The European Commission (EC) has established maximum standards for ZON in selected food products. The opinion of the Scientific Panel on Contaminants in the Food Chain of the European Food Safety Authority (EFSA), on a request from the Commission, decided that ZON is accepted as an undesirable substance. To assess the extent of co-occurrence, samples should be analysed simultaneously for deoxynivalenol, ZON, ochratoxin, fumonisin B1+B2 T-2 and HT-2 toxin (Commission Recommendation 2006/576/EC, 2006; Commission Regulation (EC) No. 1881/2006, 2006).

There is strong evidence that cereals and animals worldwide are contaminated with Fusarium mycotoxins, especially ZON. Trade in contaminated materials contributes to the worldwide distribution of mycotoxins. As an example of ZON contamination (average levels) of foodstuffs and animal feeds worldwide, especially in corn and its products, ZON levels of 0.021-1.790 mg/kg for corn products (such as corn crops, silage, and semi-products) were observed in Germany, 0.01-11.8 mg/kg for stored corn in Hungary, 0.004-0.15 mg/kg in Italy, and up to 3.1 mg/kg in the Netherlands (Zinedine et al., 2007). In Europe, maise is the most notable cereal with high levels of ZON contamination compared to the others (oats, wheat, soybean). The FAO/WHO Joint FAO/WHO Expert Committee on Food Additives (JECFA) has set a provisional maximum tolerable daily intake (PMTDI) for ZON at 0.2 μg/kg body weight (European Committee, 2000).

In the present study, we aimed to examine whether ZON and its metabolites were present in commercially available cereal-based infant formulas. Solid Phase Extraction (SPE), a sample preparation technique, was used in optimisation studies to find sensitive, accurate, specific detection and quantification limits. Gas Chromatography-Mass Spectrometry (GC-MS) was used for sample analysis. We aimed to evaluate the residue status of cereal-based infant foods sold at retail in our country regarding ZON and its metabolites and whether infants and children are at risk of potentially contaminated foods. High-performance liquid chromatography (HPLC) and Ultra Performance Liquid Chromatography (UPLC) methods were established to determine ZON in cereal snacks, infant formula and noodles.

Due to the adverse effects of ZON and its metabolites in biological matrices, especially in foods, appropriate analysis methods should check residue levels and take necessary precautions. Some analytical techniques have certain limitations. For example, immunoassays can give potential false positives, weak signals may be obtained by Raman spectroscopy,

and electrochemical analysis has poor selectivity. The most widely used analysis methods for detecting ZON and its metabolites from different matrices are thin layer chromatography, GC-MS, and HPLC (with a fluorescence detector). In our study, we decided to perform our analysis using GC-MS, which does not have the disadvantages of the methods we have given some examples of above.

Materials and Methods

Apparatus

GC- MS was performed on an HP 6890 Agilent Tec. 1200 series instrument (Agilent) Technologies, CA, USA) with a UV detector. GC column- HP5MS (J&W Scien., Milan, Italy) Milli-Q water purification system and mass analyser type — Quadrupole were used. A 140°C oven (CA, USA) was used for thermal degradation.

Reagents

Water — Purified in-house using the Milli-Q Integral 3 system, Tertiary Butyl Methyl Ether -HPLC grade (Merck; Darmstandt, Germany), Methanol- HPLC grade (Merck; Darmstandt, Germany), Acetone- HPLC grade (Merck; Darmstandt, Germany), Acetonitrile- HPLC grade (Merck; Darmstandt, Germany), Isooktan- HPLC grade (Merck; Darmstadt, Germany), BSTFA: TMCS (99:1) -N, O-Bis(trimethylsilyl)-trifluoroacetamide: Trimethylchlorosilane (99:1) (Sigma-Aldrich (Steinheim, Germany), Sodium chloride -ACS grad (Merck; Darmstadt, Germany), Magnesium sulfate - ACS grad (Sigma-Aldrich (Steinheim, Germany).

Sample Collection and Preparation

The cereal-based infant formulas to be analysed were collected randomly from supermarkets and hypermarkets in different districts of Ankara, Turkey, with 44 samples of other brands and serial numbers. After collecting the samples, they were stored at -18°C until analysis**.** The grain contents of the collected samples are given in Table 1. ZON-producing fungi mostly colonise maise plants, but they also grow on crops such as wheat, barley, sorghum, oats, millet, and rice to a lesser extent. The toxin is also found in grain products such as flour, malt, soy flour, and beer. In recent analyses for the presence of ZON, its metabolites α-zearalenone (α-ZOL) and β-zearalenone (β-ZOL) are present at low levels in corn products, corn silage, and soybean manure in vitro. Therefore, the metabolites should also be considered in studies on the presence of ZON in different matrices in vitro. The samples for which the analysis method was developed, and validation studies were conducted were cereal-based infant formulas. Studies conducted to determine the level of contamination have shown that ZON can be found in the range of 0.001 and 175 mg/kg in cereals for human consumption. It is important to investigate the presence of ZON and its metabolites in commercial cereal-based infant formulas analysed with the developed method since the long-term effects of endocrine disrupting effects, especially with chronic exposure, cannot be predicted in infants, who are in the most sensitive period and open to all kinds of negative effects.

Table 1. Grain contents of the collected samples

Brand	Number of Samples	Grain Contents of the product / Quantity (g)
A	11	Cereal flours (99.5%) (Wheat, oat, rice, millet, barley, corn, rye); calcium carbonate, iron pyrophosphate, potassium iodide, vitamins/200 g
B	11	73% whole grains (52% whole grain wheat flour, 17% whole grain barley flour, 16% whole grain oat flakes, 9% whole grain hulled wheat, 6% whole grain rye flour), 16% corn flour, 10% rice granules, vitamin B1/200 g.
C	11	Cereals (76.5%): wheat flour, hydrolysed wheat flour, rice flour, ground barley, ground rye, corn flour, sucrose, dextrose, calcium carbonate, iron pyrophosphate, potassium iodide vitamins/125 g.
D	11	Cereal flours (45%): rice, whole wheat, oats, millet, barley, corn, rye, wheat fibre, milled sugar, lactose, milk protein, vegetable oil, minerals, vitamins/250 g.

Gas Chromatography-Mass Spectrometry (GC-MS)

Extracts were analysed with a Hewlett-Packard (Agilent Tec., CA, USA) Model 6890 Series GC coupled to a 5973 mass selective detector (MSD). Instrument control and data analysis were performed using Chemstation software.

This study performed an extraction for the GC-MS technique, which requires less matrix load, during the method optimisation stages. This study will provide direct or indirect data to analysts who want to remove unwanted components and perform purification for the target substance or substances in similar studies. The direct use of the optimised method or its re-optimization in similar and different matrices will provide

guidance for more sensitive and lower-limit detection studies. In this study, 4 different commercial cereal-based infant formulas were used.

In GC-MS analysis, the SIM (Selected Ion Monitoring) mode increased sensitivity (Harvey, 2019). To optimise the extraction method, the instrument was also used in full scan (SCAN) mode to identify and characteristically ionise ZON and its metabolites, including non-targeted chemicals in the sample. Agilent HP5MS (J&W Scien., Milan, Italy) 30 m, 0.25 μm film thickness, 0.25 mm diameter column containing diphenyl dimethyl polysiloxane (5%:95%) was used. The column start temperature was 140°C, injector block temperature 230°C, transfer block temperature 250°C, ionisation type EI (70 eV), mass analyser type quadrupole, injection type indivisible, and injection volume 2 μL. Helium (99.99%) was used as carrier gas at constant flow (1.0 mL/min). The GC temperature program is as follows: 140ºC initially, hold for 7.5 min; rate 20ºC/min to 300ºC. Total analysis time 16 min. (GC temperature program: start 140ºC, hold 7.5 min; speed 20ºC/min to 300.)

ZON and its Metabolites Reference Standards

The certified reference powder standard substances used for recovery and calibration plot during the analysis process were ZON (Zearalenone; F-2 toxin), $C_{18}H_{22}O_5$ (Sigma-Aldrich, CAS: 17924-92-4), α-ZOL (α-Zearalenol; (2,4-Dihydroxy-6-(6α,10-dihydroxy-trans-1-undecenyl) benzoic acid μ-lactone), $C_{18}H_{24}O_5$ (Sigma-Aldrich, CAS: 36455-72-8) and β-ZOL (β-Zearalenol; (2,4-Dihydroxy-6-(6α,10-dihydroxy-trans-1-undecenyl) benzoic acid μ-lactone), $C_{18}H_{24}O_5$ (Sigma-Aldrich, CAS: 71030-11-0).

Method Development and Optimisation

Solid-phase extraction (SPE) method was developed to determine ZON and its metabolites in GC-MS. This method determined and adapted the most suitable procedure for target analytes in the matrix using organic solvents to GC-MS. The analysis method includes several steps, and optimisation studies were carried out for each step.

Step 1. Selection of extraction solvent

Water, acetonitrile, methanol, acetone, and tertiary butyl methyl ether, as the most preferred organic solvents for the extraction of analytes, were tested for the extraction of ZON and its metabolites from solid samples, single or mixtures in different ratios of organic solvents (acetonitrile/water, acetonitrile/methanol, methanol/water) were tried. The analysis with tertiary butyl methyl ether as an extract yielded cleaner solutions than the other extract mixtures. Furthermore, its lower boiling point allowed it to volatilise in the sample concentrator in a shorter time, resulting in a significant gain in analysis time. To determine the volume of the extraction solvent, different volumes of tertiary butyl methyl ether were added to other samples, and the volume of the supernatant after centrifugation was determined.

Step 2. Extraction of analytes from the sample

During extraction from the solid phase with an organic solvent, 5 g of the sample is transferred to 50 mL plastic screw-capped propylene centrifuge tubes. 100 μL of 1 μg/mL stock solutions of ZON, α-ZOL, and β-ZOL, 4 g magnesium sulfate (MgSO4), 1 g sodium chloride (NaCl), and 10 mL tertiary butyl methyl ether (TBME) are added to the sample. The tube is tightly capped, and the extractor and sample system are placed in an ultrasonic bath for 1 minute and then vortex-mixed for 1 minute. The optimum TBME volume for the analysis was determined based on optimisation studies. To assess the TBME volume, 5 mL, 6 mL, 8 mL, and 10 mL were tried, respectively, and it was observed that the volumetric recovery in the most stable upper phase was at 10 mL. As a result of the experiments, when 10 mL TBME was added to different samples, 8 mL was recovered. If more than 10 mL of TBME was added to the sample, the experiments were terminated because of intense matrix migration from the sample to the medium.

After vortex mixing, the system was centrifuged at 3500 rpm for 10 minutes. In the study to determine the centrifugation time, 5, 10, and 15 minutes were tried, and it was observed that TBME was separated from the solid phase most clearly and in a stable volume at 10 minutes, while no change was observed at 15 minutes. The 8 mL of TBME collected in the supernatant after centrifugation was taken into a 10 mL glass centrifuge tube with a screw cap using a Pasteur pipette and completely evaporated in a sample concentrator under nitrogen gas at 55°C.

Step 3. Solid Phase Extraction (SPE)-C18 column

To pass the dissolved and dry residues from the sample through the C18 column, 5 mL of 40:60 ratio of methanol: water is placed in a glass centrifuge tube and in an ultrasonic bath for 1 minute. The ultrasonic bath is aimed to quickly dissolve the residues adhered to the wall of the glass tube, which is difficult to dissolve with a vortex. After this process, vortex for 1 minute to dissolve all the free substances in the tube. In the next step, the 40:60 ratio of methanol: water to dissolve the dry residues in the tube and pass through the C18 column was replaced by n-hexane solvent, an oil solvent to remove oil and oil-like matrices. In the process of removing the oils

with n-hexane added to the extracts containing 30:70, 40:60, and 50:50 methanol: water for solvent, it was observed that the n-hexane phase in the upper part was not completely separated from the lower phase containing the solution at 30:70 and 50:50 methanol: water ratios, and a cloudy, suspended image was observed between the phases. 5 mL of 40:60 methanol: water containing homogeneously dissolved matrix and components was filtered through a 0.45 µm nylon filter under vacuum in preparation for the C18 column step. This was done to ensure that the particles from the matrix in the medium did not clog the pores of the C18 cartridge and interfere with the flow under atmospheric pressure, which was preferred to ensure sufficient interaction of the solution with the adsorbent and to avoid loss of analyte. In addition, the loss of sensitivity due to contamination in the GC/MS and, therefore, in the chromatograms was prevented by passing through the pores and being carried to the following stages.

Step 4. SPE amino column (NH2)

It was decided to use an amino-containing column since the sample was rich in carbohydrates; the SPE NH2 column was first conditioned with 5 mL 80: 20 acetone: methanol. The 5 mL of 80:20 acetone: methanol eluate obtained from the C18 column in the previous step was collected by passing through the NH2 column under atmospheric pressure and wholly evaporated in a sample concentrator with nitrogen gas at 55°C. 400 mL of methanol is added to the dry residue at the bottom of the tube, vortex to dissolve and transfer to the derivatisation vial. The solution in the derivatisation vial is evaporated in the sample concentrator at 55°C with nitrogen gas until it becomes a dry residue.

Step 5. Derivatisation

100 µL of N, O-Bis(trimethylsilyl)-trifluoroacetamide: Trimethylchlorosilane (BSTFA: TMCS; 99:1) was added to the dry residue from the elution and vortexed for 1 minute. It was put at 60°C for 60 minutes to ensure that ZON and its metabolites have required thermal stability and volatility for GC. The solution is evaporated again in a sample concentrator under nitrogen gas at 55°C. 25 µL of isooctane is added to the dry residue in the vial and kept in an ultrasonic bath. It is transferred to the insert vial and injected into the GC/MS.

The effect of derivatisation temperature and time on the derivatisation yield was optimised by keeping each experiment at four different conditions, namely, at 60°C for 30 min, 60°C for 60 min, 90°C for 30 min, and 90°C for 60 min in 2 replicates. Among these conditions which are the same temperature and different times), the derivatisation at 90°C for 30 min and 90°C for 60 min caused suppression of analytes, high noise, unidentified peaks, and decreased total ion area in the chromatogram. These conditions are, therefore, excluded from use.

Results and Discussion

To determine the retention times and characteristic ions of ZON, α-ZOL, and β-ZOL, pure reference standards of the analytes were derivatised with BSTFA: TMCS (99:1) and analysed on GC/MS in SCAN mode. Characteristic m/z values were selected for each analyte based on the analysis result. The abundance of diagnostic ions was estimated from the height of the extracted ion chromatograms. In the analyses performed after this stage, the SIM mode created according to the selected m/z values was used to increase the sensitivity. Comparison of the chromatograms of the analytes obtained in both SCAN and SIM modes showed that the characteristic ions overlapped and that these values were in agreement with studies in the literature and with the National Institute of Standards and Technology's (NIST) data from the spectral library on the instrument. When identifying diagnostic ions, only diagnostic ions with a relative intensity greater than 10% in the spectra of pure reference standards were selected, as the mass spectrometric determination is carried out by the recording of full scan spectra in the GC-MS. The signal/noise ratio of all diagnostic ions must be at least 3:1. Level of interest for validation of blank (blind) cereal-based infant formula samples;

i) samples extracted by adding ZON, α-ZOL and β-ZOL at five ng/mg and 10 ng/mg levels,

ii) blank (blind) samples prepared by adding ZON, α-ZOL and β-ZOL at five ng/mg and 10 ng/mg levels (matrix added standards), and

iii) pure standards of analytes prepared at 5 ng/mg and 10 ng/mg working levels were analysed in selected ion monitoring mode (SIM) in GC-MS under the same conditions.

Since the matrix-matched calibration method was used in the validations, matrix-enhanced standards were included in the studies to investigate the performance criteria of the analytical method. After this study, it was observed that the retention times of ZON, α-ZOL, and β-ZOL in the extract corresponded to the calibration standards, matrix-enhanced standards with a tolerance of ± 0.1 min. The acceptance criteria of the ion ratios of the analytes for GC-MS were checked according to Directive 2002/657/EC Implementing Council Directive 96/23/EC on "Performance of Analytical Methods and Interpretation of Results", Procedures for Analytical Quality

Control and Method Validation for the Analysis of Pesticide Residues in Food and Feed (SANTE/11312/2021) and Regulation 2021/808/EU. The ion ratios of ZON, α-ZOL, and β-ZOL obtained by the recovery route agreed with the ion ratios of the matrix-enhanced standards measured under the same conditions. They met the tolerance limits defined for GC-MS in the above-mentioned documents. (Commission Decision, 2002; Guidance *SANTE* 11312/2021, 2021; Commission Implementing Regulation (EU) 2021/808, 2021). Retention times and characteristic ions of ZON, α-ZOL, and β-ZOL are given in Table 2.

In the derivatisation of ZON, its metabolites (α-ZOL and β ZOL) have a potential silylation site containing three hydroxyl groups. ZON has a carbonyl group with a hydrogen atom in the α position and two hydroxyl groups. The presence of molecular ions electron impact (EI) (M+-) leads to the formation of ZON-2TMS, α-ZOL-3TMS, and β ZOL-3TMS derivatisation products, respectively. ZON and its metabolites are converted into trimethylsilyl (3-TMS) ethers by replacing all active hydrogen atoms with the TMS group. As a result of the optimisation studies for derivation, after each condition was repeated four times most accurate and sensitive derivatisation time and temperature (60 min and 60°C) were decided (Figure 1). After this optimisation, the GC/MS data were stable, and the chromatogram results were similar for ZON and its metabolites in the matrix medium.

Application and Validation of the Method to Samples

Method validation studies aim to demonstrate the suitability of an analytical procedure for the desired purpose. Reproducibility studies were performed for the method in two different periods. For this purpose, two separate working ranges (*i*. 0, 5, 10, 20, 30 ng/g and *ii*. 0, 1, 2, 3, 4 ng/g) were validated for ZON, α-ZOL, and β-ZOL, respectively.

Specificity

Specificity is the method's ability to measure only the analyte of interest without interfering with other sample components. The chromatograms of the blank (blind) cereal-based infant formula sample and cereal-based infant formula, in which ZON and its metabolites were compared, were used to assess the specificity of the method. We did not observe any peaks that could be evidence of contamination in the blank cereal-based infant formula samples at the retention times of the analytes, thus demonstrating the specificity of the method.

Linearity

ZON, α-ZOL, and β-ZOL standards were added at four different concentrations (and blank; 5 points) to the analytes-free cereal-based infant formula samples to find the linear working range of the method. The linearity study with cereal-based infant formulas to which blank and standards (ZON, α-ZOL, and β-ZOL) were added was performed using matrix-matched calibration. Two different working ranges (*i.*0, 5, 10, 20, 30 ng/g and *ii.*0, 1, 2, 3, 4 ng/g) were used. Determination coefficients (r^2) for ZON, α-ZOL, and β-ZOL for each two working ranges are 0.993, 0.995, and 0.993 for the first working range (i) and 0.996, 0.994, and 0.996 for the 2nd working range (ii) respectively.

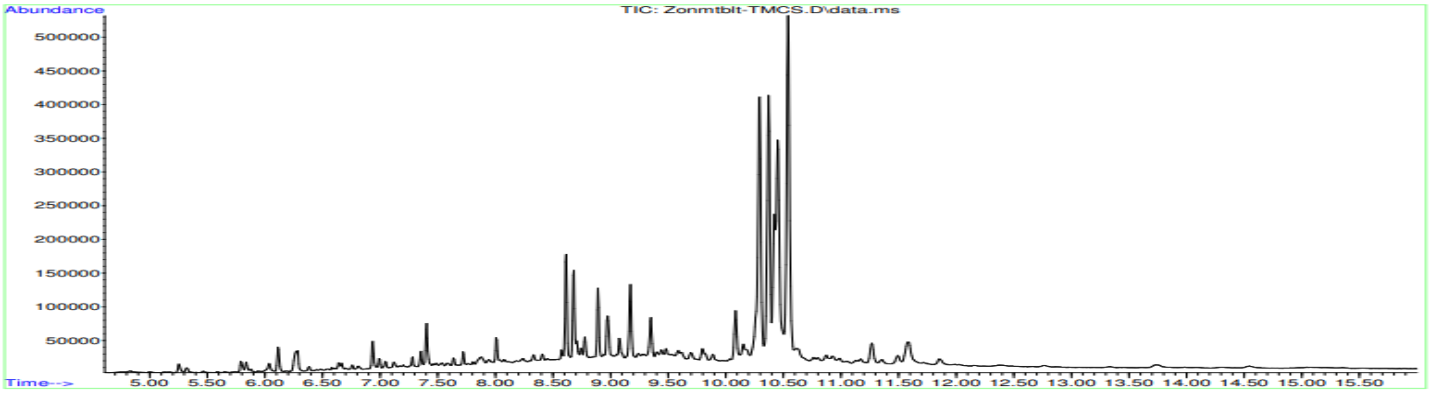

Figure 1. Effect of derivatisation temperature on derivatisation efficiency (Silylation agent BSTFA: TMCS (99:1), 60°C, 60 min.).

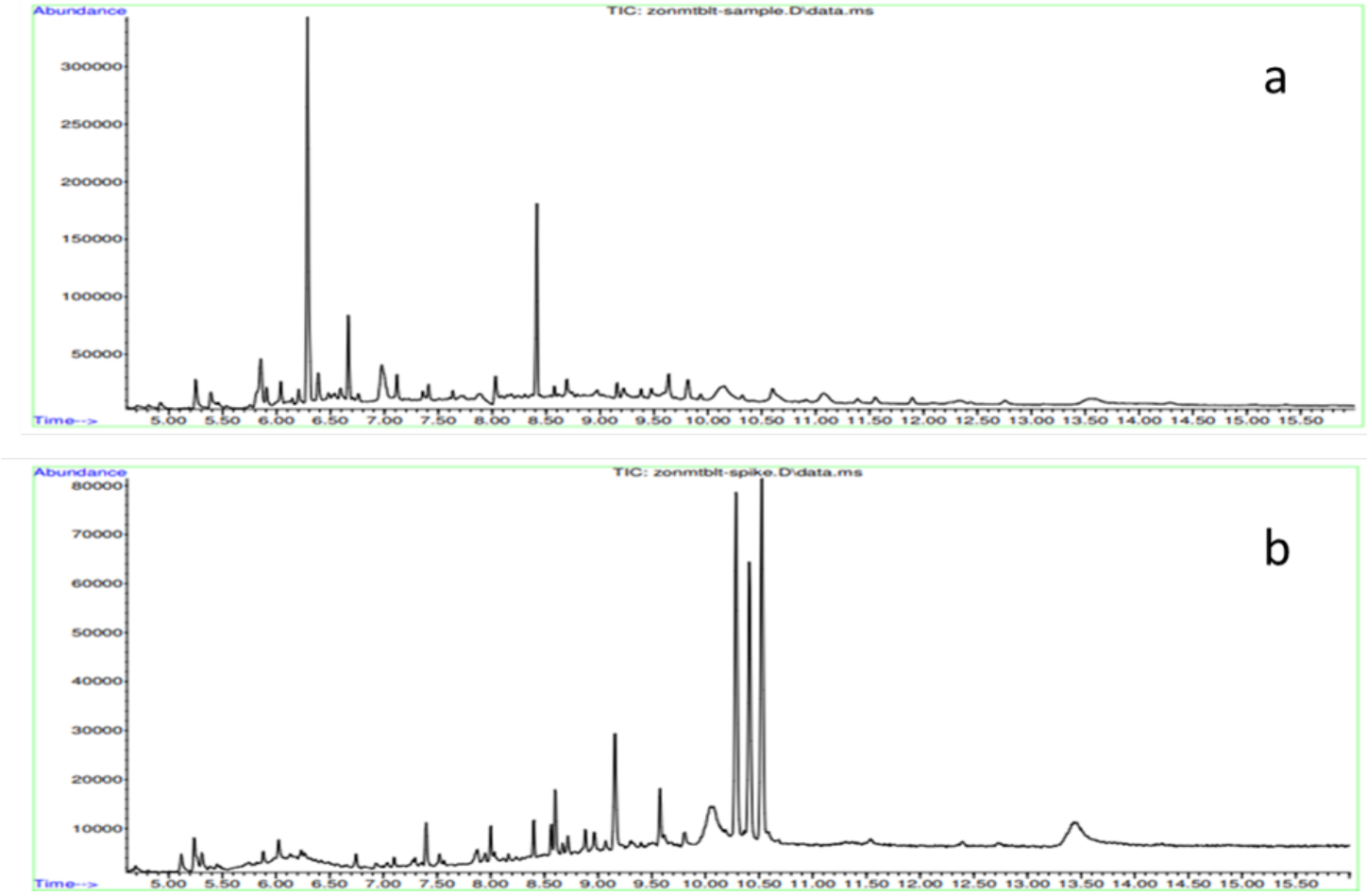

Figure 2. a. Chromatogram of blank cereal-based infant formula sample. **b.** Chromatogram of the cereal-based infant formula sample to which ZON, α-ZOL, and β-ZOL standards were added (spiked sample).

Accuracy, Recovery, and Precision

ZON, α-ZOL, and β-ZOL at three different concentrations (5, 10, 20 ng/g) were added to blank cereal-based infant formula samples. Six parallel studies were performed in 2 different periods. Mean recovery, average % recoveries, and precision values were calculated from repeatability studies. The same studies and calculations were performed for ZON, α-ZOL, and β-ZOL concentrations of 1, 2, and 3 ng/g (Table 3).

Limit of Detection (LOD) and Limit of Quantification (LOQ)

Validation was performed under the same conditions as in the method optimisation studies, i.e., for the linear working range of 0, 5, 10, 20, and 30 ng/g, including the maximum residue limit (MRL) with the existing column and linear. After the successful results were obtained, the method was validated within the linear working range of 0, 1, 2, 3, and 4 ng/g by using a new (never used) column and liner, which are the factors affecting the separation in GC-MS. The Limit of Observability (LOD; Low observed detection, limit of detection) and Lower limit of detection (LOQ) for the studies were determined according to the calculation method using the parameters of the analytical curve, i.e. linear regression method (ICH, 2005; Ribani et al., 2007; Topagi et al., 2010). In both validation studies, 12 replicate analyses were performed separately for each working level in the linear working ranges, and a linear calibration curve was drawn with recovery values against all points in the linear working range.

LOD and LOQ values were determined from the following equations.

$LOD = 3{,}3\ \sigma/S$ $LOQ=10\ \sigma/S$

σ= The standard deviation of the response
(Standard deviation of the y-intercept of the regression line.)
S = The slope of the calibration curve

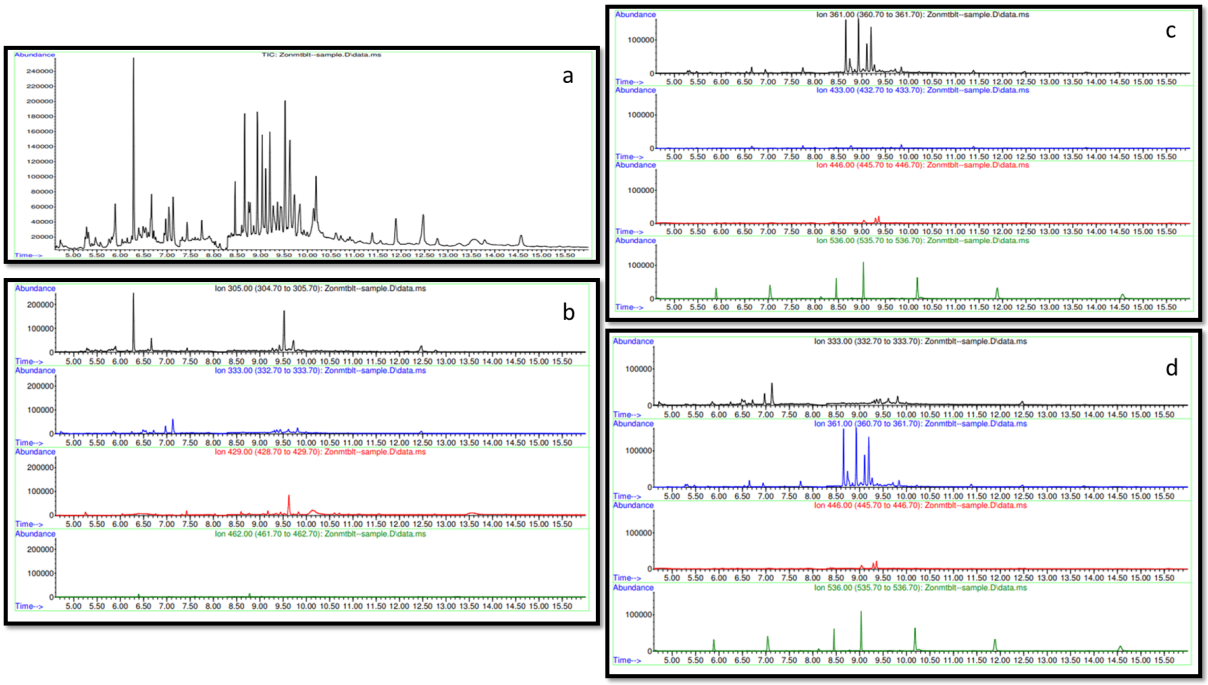

Figure 3. a. Chromatogram of a cereal-based infant formula sample (SIM mode); **b.** Chromatogram of ZON in the cereal-based infant formula sample (SIM mode); **c.** Chromatogram of α-ZOL in the cereal-based infant formula sample (SIM mode); **d.** Chromatogram of β-ZOL in the cereal-based infant formula sample (SIM mode).

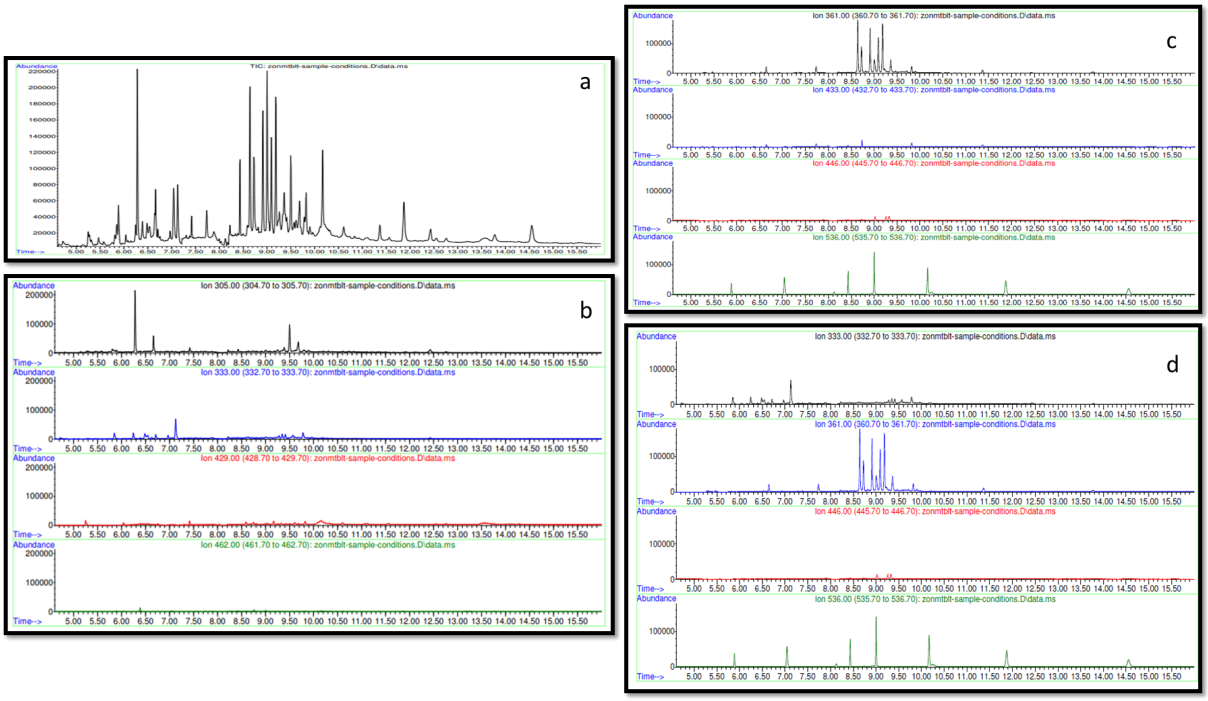

Figure 4. a. Chromatogram of the cereal-based infant formula sample stored under unstable conditions (temperature, humidity, etc.) (SIM mode); **b.** The chromatogram of ZON in the cereal-based infant formula sample was stored under unstable conditions (temperature, humidity, etc.) (SIM mode); **c.** Chromatogram of α-ZOL in the cereal-based infant formula sample stored under unstable conditions (temperature, humidity, etc.) (SIM mode); **d.** Chromatogram of β-ZOL in the cereal-based infant formula sample stored under unstable conditions (temperature, humidity, etc.) (SIM mode).

Table 2. Retention times and ions of ZON, α-ZOL, and β-ZOL (m/z)

Analyte	Retention Time (Mean ± SD)	Molecular weight	Characteristic ions (m/z)	Quantitation ion(s) (m/z)
ZON-2TMS	10.31±0.01	318	305, 333, 429, 462	333
α-ZOL-3TMS	10.44±0.01	320	361, 433, 446, 536	446
β-ZOL-3TMS	10.57±0.01	320	333, 361, 446, 536	333

TMS: Trimethylsilyl derivatives

Table 3. Average recovery, average % recoveries, the limit of repeatability (r), and relative repeatability standard deviation (% RSDr) data for cereal-based infant formulas (ZON, α-ZOL, and β-ZOL added).

Added Concentration (ng/mg)	Average Recovery (ng/mg)		Average % Recoveries		Precision	
	First Repeatability	Second Repeatability	First Repeatability	Second Repeatability	Limit of Repeatability (r)	Relative repeatability standard deviation RSDr (%)
ZON						
5 (n=6)	4.32	3.92	86.48	78.38	0.71	5.87
10 (n=6)	9.36	9.62	93.61	96.21	0.47	1.79
20 (n=6)	20.71	21.09	103,55	105.45	0.26	0.45
1 (n=6)	0.97	1.01	97.00	101.00	0.02	0.74
2 (n=6)	1.86	1.94	93.00	97.00	0.03	0.58
3 (n=6)	2.89	2.85	96.30	95.00	0.02	0.25
α-ZOL						
5 (n=6)	4.68	4.82	93,61	96,40	0.37	2.82
10 (n=6)	9.62	9.65	96,20	96,50	0.40	1.49
20 (n=6)	20.02	19.80	100.11	99.00	0,12	0.21
1 (n=6)	0.96	0.99	95.57	99.23	0.03	1.12
2 (n=6)	2.03	1.87	101.57	93.63	0.06	1.06
3 (n=6)	3.04	3.12	101.28	103.93	0.02	0.23
β-ZOL						
5 (n=6)	4.42	4.56	88.40	91.20	0.35	2.83
10 (n=6)	9.54	9.48	95.40	94.80	0.38	1.42
20 (n=6)	19.52	19.70	97.60	98.50	0.18	0.33
1 (n=6)	0.99	0.95	99.00	94.48	0.01	0.36
2 (n=6)	2.09	1.86	104.70	92.87	0.03	0.51
3 (n=6)	2.83	2.66	94.23	88.77	0.02	0.25

n: Number of repetitions

The accuracy of the LOD and LOQ values determined according to the calculation method using the parameters of the analytical curve was checked by performing recovery studies. For the working range of 0, 5, 10, 20, and 30 ng/g, the detection limit (LOD) of ZON was found to be 1.08 ng/g, 1.01 ng/g for α-ZOL and 1.2 ng/g for β-ZOL. For the working range 0, 1, 2, 3, 4 ng/g, the ZON detection limit was 0.20 ng/g, α-ZOL 0.20 ng/g, β-ZOL 0.06 ng/g. As a result of calculations using the parameters of the analytical curve, for the working range of 0, 5, 10, 20, and 30 ng/g, the limit of detection of ZON was found to be 3.25 ng/g, α-ZOL 3.01 ng/g, β-ZOL 3.62 ng/g. For the working range 0, 1, 2, 3, 4 ng/g, the ZON's determination limit was 0.60 ng/g, α-ZOL 0.60 ng/g, β-ZOL 0.18 ng/g.

Evaluating the Results of Cereal-Based Infant Formulas

Validation studies of the developed method were performed for 0, 5, 10, 20, and 30 ng/g and 0, 1, 2, 3, and 4 ng/g working ranges. Parameters of specificity, linearity, accuracy, precision, recovery, LOD, and LOQ were examined. Since there is no guideline for validating the analytes whose contamination levels were investigated in cereal-based infant formulas, the method in the internationally accepted guideline ICH Q2(R1)2 was followed (ICH, 2005). In addition, in the recovery study carried out by enrichment of the samples, the calculation was made according to the "ISO 5725" standard for the accuracy and precision of the measurement methods and results (ISO 5725-1:2023 (en), 2023). The chromatogram of each cereal-based infant formula sample was measured against two different calibration levels at both working intervals (1st and 2nd working intervals). As a result, ZON and metabolites were not detected in any of the cereal-based infant formula samples at both calibration intervals at the LOD. In order to check whether the instrument was receiving a signal, but not quantitatively at the LOD, all fragment ions of each sample ZON, α-ZOL, and β-ZOL were input into the instrument software and checked for possible contamination by SIM scanning. No fragment ions of ZON and its metabolites were detected in any cereal-based infant formula samples; therefore, no signal was detected. Figure 4-7 shows the chromatogram of a cereal-based infant formula sample and ZON, α-ZOL, and β-ZOL in the cereal-based infant formula samples (SIM mode).

During the optimisation of the analysis steps and method validation studies, approximately 16 samples from 4 companies were kept under unstable conditions in room conditions. Some were analysed for control purposes after their expiry dates had passed. No residues of ZON and its metabolites were found in the analysis of the samples kept under unstable conditions (temperature, humidity, etc.), under the influence of environmental stress, in spring, summer, and autumn climatic conditions. This result suggests that when the sample packages were opened, they were not contaminated with ZON and its metabolites, and even if they were, the analytes degraded over time under environmental conditions or the environmental conditions did not contain parameters that promote fungal growth. Figure 8-11 shows the chromatogram of a cereal-based infant formula sample and ZON, α-ZOL, and β-ZOL in the cereal-based infant formula sample stored under unstable conditions (temperature, humidity, etc.) (SIM mode).

People need basic nutrients to maintain their lives. In addition to the benefits of these basic nutrients that ensure the continuity of life, they can also contain many chemicals or toxic substances that adversely affect health. Basic food sources such as grains and grain feed are food or raw materials that are candidates for various adverse events at each stage, from planting in the field to storage on the shelves. Fungi, formed due to the natural flora in the field or the subsequent conditions that allow them to reproduce, are the main factors in forming undesirable contaminants in such plants. After the fungi settle on the plant as phytoparasites, they release many different mycotoxins into the environment during their metabolic activities. One of these mycotoxins produced by Fusarium fungi is ZON. ZON mimics or replaces estrogen in the organism after exposure to plant sources, causing many adverse effects on the hormonal system and, thus, on target organs and tissues (Ekwomadu et al., 2021).

Contamination of cereal-based infant formulas with ZON and its derivatives may cause many hormonal/toxic effects on infant and child health (Mostrom, 2011). Detection of these substances, which can be found as undesired contaminants in foods with high sensitivity, is essential in predicting adverse effects and minimising the risk as much as possible. ZON has low acute toxicity and carcinogenicity. However, most animal clinical symptoms are associated with ZON's estrogenic and anabolic properties (Döll & Dänicke, 2022).

The pig organism is physiologically similar to the human organism. Therefore, the potential adverse effects of ZON exposure in pigs are helpful for pathologic and physiologic comparison with humans (Fischer & Schnieke, 2022).

Tiemann and Danicke (2007) investigated the effects of ZON and deoxynivalenol, a ZON metabolite, on various non-reproductive and reproductive organs in female pigs. 22 mg ZON kg(-1) in the diet causes uterine-like changes in the reproductive system of pigs and affects follicular and embryo development. ZON and its metabolites have been shown to

have competitive binding to estrogen receptors *in vitro*. Feeding pigs a 9 mg DON kg(-1)-contaminated diet may impact protein synthesis and humoral and cellular immune responses and affect liver and spleen cell structures. It has been observed to cause reproductive changes in pigs. In *vivo* and *in vitro* exposure to DON reduced oocyte and embryo development. In pigs fed wheat contaminated with high concentrations of Fusarium toxin, there is evidence of spleen and liver dysfunction without clinical signs (Tiemann & Dänicke, 2007). Since ZON has an estrogen-like structure, it binds to various estrogen receptors (ERs). Reproductive toxicity, which leads to reproductive disorders in animals, is one of the major toxic effects observed with ZON; several acute toxicity studies have given oral LD50s of ZEA above 2000, 4000, and 5000 mg/kg bw in mice, rats, and Guinea pigs, respectively. Reproductive toxicity, hepatotoxicity, genotoxicity, carcinogenicity, and immunotoxicity were observed with ZON exposure (Han et al., 2022).

Humidity above 20% is favourable for ZEN production by fungi, and temperature is between 20 and 25°C for three weeks. Countries with hot and rainy climates can produce high levels of ZEN in feedstuffs (Dogan and Dal, 2022). Contaminated food and feed remediation strategies using chemical, physical, and biological methods to reduce or eliminate the toxic effects of ZON are essential to improve food safety and prevent economic losses and recalls of contaminated foods (Wu et al., 2021).

The SPE method is widely used in different matrices for biological compounds and other components. In recent years, liquid-liquid extraction, etc., has also been used. However, the SPE technique has many advantages, such as being more selective in complex biological matrices, allowing the detection of much lower levels of analytes, separating different compounds or chemicals in the same matrix environment, and obtaining much cleaner extracts (Apfell et al., 2021). Using liquid-liquid extraction instead of SPE to analyse samples with complex matrices, such as cereal-based infant formula samples, will result in instrument working costs (full columns, autosampler injector, ion source, etc.) as the dense matrix will be injected directly into the instrument. In addition, simultaneous analyses will cause deviations in the evaluation and comparison of samples injected into the instrument with similar methods and in the drawing of calibration curves. The SPE technique has always been preferred by analysts for sustainable accuracy and reproducibility, especially in instruments such as GC and GC-MS, where sensitivity is adversely affected by various impurities.

In the present study, different steps of the existing SPE methods were optimised with modifications at specific critical points, and the most suitable technique was adapted to GC-MS. The GC-MS method evolved due to optimisation studies, and the limit of determination was based on two different calibration curves. In the selection of broad-range calibration levels, the calibration curve moved away from the origin, discrepancies were observed between the lower and upper calibration points, and two different calibration ranges (one for quantities less than five ng/g and the second for five>ng/g) were decided in order for the results to be close to the actual values and the calibration curve to approach the origin. The advantage of the optimised method is that the limits of detection and determination are much lower than in many other studies. The results of our analysis and similar studies in previously published studies (extraction methods, techniques used, LOD, and LOQ) are presented in Table 4.

In our study, LOD was obtained by the MRL demanded by many countries, especially the EU, and especially the MRL permitted by the Turkish Food Codex (Communiqué on Maximum Residue Limits of Contaminants in Foodstuffs (Communiqué No: 2008/26), even at lower levels. As a result of the different infant formula samples analysed, no level that could be evidence of residue was detected, even at the diagnostic limit.

The fact that the analysed cereal-based infant formula samples were not contaminated with ZON and its metabolites does not mean that all similar samples are the same. For this reason, our study is tabulated for comparison with other studies involving similar products, similar methods, optimisation studies, survey studies, and market studies (Table 4).

The reasons for not detecting contamination in our samples and some other studies (Table 4)include the selection of quality raw materials, keeping the product in an environment with minimal superficial deformations that will allow the growth of fungi, washing the product in order to remove contaminants that may cause uniformity in terms of taste and possible health problems, separation of the shell part, many different processes to ensure the uniformity of the packages, physical and chemical (inert sorbents) processes to remove contaminants. In addition, as is well known, fungi are aerobic organisms and require oxygen to continue their vitality. If commercial products are packaged in an airtight manner, the maintenance of viability will not be ensured even if contaminated with the fungus that causes mycotoxin growth. Moreover, consideration should be taken into account, especially for maise and its products, which are the most likely to be contaminated with ZON and its metabolites and do not contain

corn alone. Still, they were included in a homogeneous mixture with other cereals and grain feeds. For this reason, the unit amount analysed reduces the possibility of the sample being contaminated or makes it difficult to determine the possible contamination level by remaining below the LOD and LOQ.

When evaluating the positive results observed in similar studies, the most critical parameter enabling mycotoxin development is the climate zone of the country where the products are grown or supplied. The absence of a positive result in the samples may have been because the raw materials were developed in places with a continental climate, the post-harvest humidity of the product was not in an environment that promotes fungal growth, or the regional flora from which the raw material was sourced did not contain ZON-producing Fusarium fungi.

In this study, the LOD for ZON was 0.20 ng/g, and the LOQ was 0.6 ng/g. The LOD for ZON is slightly above the TDI (0.25 μg/kg body weight day) (EFSA, 2014) and a provisional maximum tolerable daily intake (PMTDI; 0.5 μg/kg body weight day). In the analytical evaluation, all four fragment ions of ZON should be detected at the LOQ. In the analyzed samples, no ions belonging to ZON were detected in the fragment ion controls made by the software. In addition, the LOD for β-ZOL was determined as 0.06 ng/g below TDI and PMTDI (1-2 ng/ g) (JECFA, 2000). Since ZON and its other metabolite α-ZOL can be expected to be present in the environment where β-ZOL is present, it constituted a supporting basis.

In light of this information, it is concluded that the analysed samples are predicted to be free of contaminants at the TDI value and that no estrogenic effect due to ZON would be observed if consumed.

There are several studies in which ZON in flour, feed and cereals were measured by GC-MS and HPLC methods (Liao et al., 2009; Luo et al., 2022; Pascari et al., 2023). Advantages and disadvantages of the detection method developed in this paper compared to previous detection methods: The limits of detection (LOD) of ZON, α-ZOL, and β-ZOL were found to be 0.2 ng/g, 0.2 ng/g, 0.06 ng/g, respectively, and when compared with different previous studies (Table 4), these LOD values were lower. Therefore, the method we developed is more sensitive for analysing related substances. Although the MRL for ZON in infant formula is set at 20 ng/g, the long-term consequences of lower levels of low toxic/endocrine-disrupting effects with chronic exposure are not fully known. In addition, given that ZON has been identified as an endocrine disruptor with its estrogenic effect and that infants, especially in the high-risk group, will also be exposed to estrogenic effects from various other sources (xenoestrogens), it is very important to avoid risks by providing awareness of the danger related to the long-term negative effects of the cumulative, plus effect that may occur in the organism. For this reason, developing a highly sensitive method to analyse ZON, α-ZOL, and β-ZOL at levels lower than the MRL value is necessary and useful for studies to be carried out in this direction. In the GC-MS method developed by method optimisation, the derivatisation process required to analyse these substances and the extra steps and procedures to eliminate the matrix effect and remove unwanted components and substances (clean-up) are considered a disadvantage. Still, the developed method is superior to other methods because it provides a diagnosis at lower limits. GC-MS, one of the mass spectrophotometric measurement techniques, provides qualitative and quantitative verification of substances. However, only qualitative and quantitative screening of substances can be performed with HPLC and UPLC techniques. Mass spectrometric devices are needed to confirm suspected positive and positive results obtained from HPLC and UPLC systems. Therefore, our method, performed with GC-MS, can be used as a direct qualitative and quantitative confirmation method. Each of the commercial forms analysed contained different ingredients with different contents and ratios, and the developed and optimised extraction method enabled the analysis of all these different infant formulas with a single method. The derivatisation process used in the GC-MS technique has also made the substances quantitatively analysable at lower limits, increasing the possibility of more sensitive detection of substances compared to HPLC techniques. The study carried out can be a guide for toxicology science, especially in low-toxic effect research.

Table 4. The results of our study and similar studies in previous published studies

Analyte	Matrice	Extraction	Clean up	Instrument	LOD (ng/g)	LOQ (ng/g)	Ref.
ZON	Maize, millets	ACN.H_2O	MycoSep	LC-APCI-MS	0.3–3.8	-	(33)
ZON	Wheat flour	ACN.H_2O	MycoSep	LC-ESI-MS	10	-	(34)
ZON	Grain	ACN.H_2O MeOH. H_2O	Oasis HLB, IAC, MycoSep	HPLC	0.5		(35)
ZON	Cereal-based infant formulas	MeOH:ACN: H2O	SPE, C_{18}, IAC	LC-fluorescence detector UV spectrometry	2.5	3	(36)
ZON	Baby food, breakfast cereals, snack corn, bread	ACN.H_2O	IAC	HPLC	0.65	1.95	(37)
ZON	Baby food, pasta for soup, corn-based snack	ACN.H_2O	IAC	HPLC	4	2.5	(38)
				UPLC	8.1-10	8.0–9.2	
ZON	white flour, mixed flour, corn flour, baby food	ACN.H_2O MeOH. H_2O	IAC	LC-FD	3.75	12.5	(39)
ZON	Unpopped popcorn	ACN	SPE, C_{18}	GC-MS	16	48	(40)
ZON	Cereals and swine feed	MeOH: ACN	IAC	LC-FLD	2-6	-	(41)
α-ZOL					3-6		
ZON	Semolina	ACN	SPE, C_{18}	GC–QqQ-MS/MS	-	<10	(42)
ZON	Wheat, rice, corn-based infant formula	ACN.H_2O	SPE, C_{18}	GC-MS	-	10	(43)
ZON	Cereal-based infant formula	ACN: MeOH	LLE	LC-MS/MS	1.5	5	(44)
α-ZOL					1.5	5	
β-ZOL					2	2.5	
ZON	Milk-based cereal infant formula	ACN:H2O	IAC	LC-MS/MS	1.33	4	(45)
ZON	Cereal-based infant formula	TBME; MeOH:H2O	SPE, C_{18}	GC-MS	0.20 and 1.08	0.6 and 3.25	The present study (2 working conditions)
α-ZOL					0.20 and 1.01	0.6 and 3.03	
β -ZOL					0.06 and 1.2	0.12 and 3.06	

Immunoaffinity column, IAC; Acenonitrile: ACN, MeOH, Methylalcohol, TBME; Methyl tert-butyl ether; LC-APCI-MS; Liquid Chromatography- Atmospheric Pressure Chemical Ionization-Mass Spectrometry, LC-ESI-MS; Liquid Chromatography-Electrospray-Ionization-Mass Spectrometry HPLC; High-Performance Liquid Tandem Mass Spectrometry, UPLC; Ultra Performance Liquid Chromatography, LC-FD; Liquid Chromatography-Fluorescence Detection, GC-MS; Gas Chromatography-Tandem Mass Spectrometry.

Conclusion

In conclusion, by optimising the method for the analysis of ZON and its metabolites α-ZOL and β-ZOL in cereal-based infant formula, it was shown that the method developed was precise, accurate, selective, reproducible, and highly sensitive at concentrations lower than the maximum residue level. According to the results obtained from the analysed samples, it was determined that cereal-based infant formulas sold retail in the market are not contaminated with Zearalenone and its metabolites. It has been shown that infants and children consuming these products are not at risk from cereal-based formula.

Compliance with Ethical Standards

Conflict of interest: The author(s) declares that they have no actual, potential, or perceived conflict of interest for this article.

Ethics committee approval: The authors declare that this study does not include experiments with human or animal subjects, so ethics committee approval is not required.

Data availability: Data will be made available on request.

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