

Italian Journal of Food Safety

https://www.pagepressjournals.org/index.php/ijfs/index

eISSN 2239-7132

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Ital J Food Saf 2026 [Online ahead of print]

Please cite this article as:

Camaj Ibrahimi A, Camaj Isa A, Bajra Brahimaj T, et al. **Zearalenone in wheat from Kosovo: baseline surveillance data from 2022-2024**. *Ital J Food Saf* doi:10.4081/ijfs.2026.15745

Submitted: 10-06-2026
Accepted: 19-07-2026

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Zearalenone in wheat from Kosovo: baseline surveillance data from 2022-2024

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Key words: zearalenone, wheat, HPLC–FLD, proximate composition, food safety.

Contributions: Arieta Camaj Ibrahimi: conceptualization, methodology, investigation, data curation, and writing of the original draft. Aferdita Camaj Isa: methodology and investigation. Teuta Bajra Brahimaj: investigation. Indrit Loshi: formal analysis and investigation. Shyhrete Muriqi: data curation and writing of the original draft. All authors read and approved the final version of the manuscript and agreed to be accountable for all aspects of the work.

Conflict of interest: the authors declare that they have no competing interests.

Ethics approval and consent to participate: not applicable. This study did not involve human participants, human data, human tissue, or animals.

Availability of data and materials: the datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Funding: this work was funded by the Ministry of Education, Science, Technology, and Innovation of Kosovo (MASHT). The funder had no role in the study design, sample collection, analysis, interpretation of data, manuscript preparation, or the decision to submit the article for publication.

Acknowledgments: the authors would like to thank the Ministry of Education, Science, Technology, and Innovation of Kosovo (MASHT) for supporting this study.

Declaration of generative artificial intelligence and artificial intelligence-assisted technologies in the writing process: during the preparation of this manuscript, the authors used ChatGPT to improve the English language and readability of the text and to assist with the consultation of relevant scientific literature.

Abstract
Zearalenone (ZEN) is an estrogenic *Fusarium* mycotoxin that may contaminate cereals and cereal-based foods, representing a potential food safety concern in wheat-dependent diets. This study evaluated ZEN occurrence in wheat circulating in the Kosovo food supply during 2022-2024 and assessed proximate composition as complementary information for sample characterization. A total of 80 wheat samples were collected from farms and mills in the Dukagjini Plain and the Kosovo Plain and classified as declared local wheat or declared imported wheat based on information provided at the sampling point. ZEN was determined by high-performance liquid chromatography with fluorescence detection, while proximate composition was assessed using standard analytical methods. ZEN was not detected in any sample above the method limit of detection of 3.17 μg/kg, indicating compliance with the European Union maximum level for ZEN in unprocessed cereal grains during the investigated period. The wheat samples showed a carbohydrate-dominant profile, moderate variability in protein, and relatively stable fat, ash, and phosphorus contents. No statistically significant differences were observed between declared local and declared imported wheat or between the two sampling locations. These findings provide preliminary baseline evidence limited to the analyzed samples and sampling period and should not be interpreted as confirmation of the general or permanent absence of ZEN risk in the Kosovo wheat supply. Continued risk-based surveillance should include additional harvest years, larger sample sets, and broader contaminant coverage, particularly deoxynivalenol, ochratoxin A, T-2 and HT-2 toxins, fumonisins, and, where relevant, pesticide residues.

Introduction
Wheat and wheat-based foods are among the most important staple foods in Kosovo and the wider Western Balkan region, making wheat quality and safety directly relevant to public health, food security, and market stability. Among chemical hazards, mycotoxins are particularly important because contamination can occur throughout the entire "field-to-fork" chain, from field infection and harvest conditions to drying, storage, milling, and market distribution, resulting in variable exposure patterns across seasons and locations (Sarmast *et al*., 2021). Consequently, establishing country-specific baseline data is essential for risk-informed decision-making, targeted mitigation measures, and effective communication with stakeholders involved in production, trade, and food control.
Zearalenone (ZEN) is a *Fusarium*-derived mycotoxin frequently associated with cereal matrices and is of particular concern because of its estrogenic activity and endocrine-disrupting potential. Toxicological evidence indicates that ZEN and its metabolites may adversely affect reproductive and endocrine systems, highlighting the importance of routine monitoring in widely consumed commodities such as wheat (Han *et al*., 2022). Because wheat products contribute substantially to dietary intake, even low-to-moderate contamination may become relevant from an exposure perspective when it occurs frequently or in combination with other mycotoxins (Sarmast *et al*., 2021).
The occurrence of ZEN in wheat is influenced by interacting agroclimatic and post-harvest factors. Field infection by *Fusarium* spp. is affected by temperature and moisture conditions during susceptible growth stages, while post-harvest factors, including drying efficiency and storage humidity, may further influence fungal development and mycotoxin production. Recent evidence suggests that projected climate-related conditions may alter fungal behavior and mycotoxin production in wheat-based matrices, potentially increasing uncertainty and inter-annual variability in contamination patterns (Cervini *et al*., 2024). These considerations strengthen the rationale for periodic, data-driven surveillance rather than isolated measurements.
From a regulatory and trade perspective, analytical results gain practical relevance when interpreted against established legal benchmarks. In the European context, Commission Regulation (EU) 2023/915 establishes maximum levels for ZEN in cereals and cereal-derived products, including unprocessed cereal grains, cereal flour, and related milling products (European Commission, 2023). Although Kosovo is not a Member State of the European Union, interpretation in relation to widely

applied EU regulatory limits supports comparability and facilitates discussion of market compliance and consumer protection.

Beyond food safety, *Fusarium* infection and the associated deterioration of grain quality may affect the technological and nutritional properties of wheat. Studies on Fusarium head blight have reported changes in flour quality and processing performance related to altered enzymatic activity and technological characteristics, which may indirectly reduce the overall value of wheat lots entering the food chain (Peršić *et al*., 2023). In the present study, proximate composition was assessed as supporting information for the characterization of the analyzed wheat samples and for evaluating whether their basic compositional profiles differed according to declared origin or sampling location. These parameters were not considered direct predictors of ZEN occurrence, but rather complementary indicators of sample quality, comparability, and heterogeneity within the investigated wheat supply. Their inclusion provides additional context for interpreting the surveyed sample set and may support future studies integrating compositional characteristics, storage conditions, and mycotoxin occurrence. In parallel, different processing and post-harvest mitigation strategies continue to be investigated for their potential to reduce mycotoxin contamination in wheat and wheat-based products (Romero *et al*., 2021).

To date, published data on ZEN occurrence in wheat circulating in the Kosovo food supply remain scarce, particularly for wheat obtained from different sampling locations and classified as declared local wheat or declared imported wheat based on information provided at the sampling point. This lack of country-specific evidence limits the establishment of reference conditions for future surveillance and comparison with European regulatory benchmarks. Although contemporary mycotoxin research increasingly applies multi-mycotoxin approaches, targeted studies of individual mycotoxins remain relevant when they address a clearly defined national data gap and are based on a structured sampling design and an adequately characterized analytical method.

Therefore, this study aimed to determine the occurrence of ZEN in 80 wheat samples collected from farms and mills in the Dukagjini Plain and the Kosovo Plain using an HPLC-FLD method with immunoaffinity clean-up. The findings were interpreted in relation to the European Union maximum level for ZEN in unprocessed cereal grains. Proximate composition was additionally assessed to characterize the analyzed wheat samples and to examine possible differences according to declared origin and sampling location. By integrating contaminant surveillance, sample characterization, and comparative analysis, the study provides a structured baseline dataset for wheat circulating in the Kosovo food supply and establishes a reference for broader multi-year and multi-mycotoxin monitoring.

Materials and Methods

Study design and sampling

From autumn 2022 to spring 2024, a total of 80 wheat samples intended for human consumption were collected from farms and mills across the two main agricultural areas of Kosovo: the Dukagjini Plain (Rrafshi i Dukagjinit) and the Kosovo Plain (Fusha e Kosovës). Of these, 58 samples were collected in the Dukagjini Plain and 22 in the Kosovo Plain.

Each sample represented a separate wheat lot, generally weighing up to approximately 1 tonne. For each lot, incremental portions of approximately 100 g were collected from ten spatially distributed sampling points and combined to form an aggregate sample weighing approximately 1-2 kg. The aggregate samples were thoroughly mixed to improve representativeness before transport to the laboratory.

Information on the harvest year was available for most, but not all, wheat lots. Most samples were associated with the 2022 and 2023 harvests, while some samples declared as imported wheat may have originated from the 2021 harvest. Because harvest-year information was based on declarations provided at the sampling point and was not independently verified for every lot, the period 2022–2024 refers to the sampling and surveillance period rather than exclusively to the harvest years represented in the study.

The sampling location refers to the place where the sample was collected and does not necessarily represent the geographical origin of wheat production. Samples obtained from mills could include both wheat declared as local and wheat declared as imported, based on information provided at the sampling point. Several samples were declared as imported wheat, mainly from Serbia and Croatia.
The wheat samples were dry at the time of collection and were transported to the laboratory in clean paper bags. Upon arrival, they were registered and stored in a dry, dark room at ambient temperature to minimize moisture uptake and further fungal development. Sample preparation and laboratory analyses were performed within several weeks of collection.

Ethical considerations

This study involved market/farm wheat samples and did not include human or animal subjects. Laboratory work followed standard food safety and analytical quality procedures to ensure data integrity and traceability.

Zearalenone determination by HPLC-FLD

Instrumentation and chromatographic conditions

ZEN was determined using high-performance liquid chromatography with fluorescence detection (HPLC-FLD). The system consisted of a binary pump, manual injector, and fluorescence detector set at excitation 274 nm and emission 440 nm. Separation was achieved on a reversed-phase C18 column (250 × 4.6 mm, 5 µm) at ambient temperature. The mobile phase was acetonitrile:water (65:35, v/v) at a flow rate of 1.2 mL/min. Injection volume was 50 µL and total run time was 10 min per injection.

Standards and calibration

A ZEN stock solution (50 µg/mL) was prepared in acetonitrile. Working calibration standards (10–2000 ng/mL) were prepared by serial dilution. External calibration was performed by plotting peak area versus concentration; linearity was accepted at $R^2 > 0.999$. Standard solutions were stored at 4°C and brought to room temperature prior to use.

Sample extraction and clean-up

Ground wheat (25 g) was extracted with 100 mL of 80% methanol (v/v) by shaking for 30 min at room temperature. The extract was filtered through Whatman No. 1 filter paper. An aliquot of filtrate was diluted with phosphate-buffered saline (PBS, pH 7.4) prior to immunoaffinity clean-up.

Immunoaffinity purification

A 10 mL portion of the diluted extract was passed through a multi-toxin immunoaffinity column (IAC) at approximately 1 drop/s. The column was washed with 10 mL PBS followed by 10 mL deionized water. ZEN was eluted with 3 mL methanol followed by 2 mL deionized water. The eluate was evaporated to dryness under nitrogen at 50°C. The residue was reconstituted in 250 µL mobile phase, filtered, and transferred to HPLC vials for analysis.

Method validation

The HPLC-FLD method was evaluated for linearity, accuracy, precision, selectivity, and analytical sensitivity in accordance with Commission Regulation (EC) No 401/2006 and the applicable European Commission performance requirements for mycotoxin analysis (European Commission, 2006). External calibration was performed using seven ZEN working standards over the range of 10-2000 ng/mL, corresponding to an equivalent matrix concentration range of 2.5–500 µg/kg under the applied sample-preparation conditions. Acceptable linearity was defined as $R^2 > 0.999$.

Method accuracy was assessed by fortifying blank wheat samples at 100, 500, and 1000 µg/kg. Recoveries ranged from 85% to 95%. Intra-day precision, expressed as RSDr, and within-laboratory inter-day precision, expressed as RSDwR, were both below 5%.

The limit of detection was calculated as 3.3 × SD/slope, and the limit of quantification as 10 × SD/slope, where SD represented the standard deviation of the background response from blank

samples and the slope was obtained from the calibration curve. The resulting LOD and LOQ were 3.17 and 10 μg/kg, respectively.
Selectivity was evaluated using blank and fortified wheat extracts. No interfering chromatographic peak was observed at the ZEN retention time, which was approximately 2.54 min. Immunoaffinity clean-up provided selective purification of the wheat extracts and supported the use of external calibration. Detailed validation characteristics are presented in Table 1.

Quality control
Each analytical batch included reagent blanks, spiked samples, and calibration check standards. Calibration standards were injected at the beginning, mid-sequence, and end of runs to monitor instrument stability. Detector sensitivity and retention time consistency were monitored throughout.

Proximate composition analysis
Sample preparation
Wheat samples were cleaned to remove foreign materials (stones, dust). Samples were dried at 105°C to constant mass, milled to pass a 1 mm sieve, and stored in sealed containers at room temperature until analysis.

Protein
Total protein was determined by the Kjeldahl method according to ISO 5983-1:2005 (ISO, 2005). Nitrogen content was quantified by titration and converted to protein using a factor of 6.25.

Fat
Total fat was determined by Soxhlet extraction according to ISO 6492:1999 (ISO, 1999). Approximately 5 g of dried, ground sample was extracted with petroleum ether for 6 h using a Soxhlet apparatus. Fat content was expressed as a percentage of dry weight.

Ash
Crude ash was determined according to ISO 5984:2022 by incineration to constant mass, and expressed as % of dry weight (ISO, 2022).

Phosphorus
Phosphorus was determined according to ISO 6491:1998 (colorimetric method) (ISO, 1998). Digestion released orthophosphate, which reacted with molybdate reagent to form a blue complex measured at 820 nm. Results were calculated from a calibration curve and expressed as percentage of dry weight (%).

Carbohydrates
Total carbohydrates were calculated by difference:
Carbohydrates (%) = 100 − (Moisture + Protein + Fat + Ash)
Moisture was determined gravimetrically by drying at 105°C to constant weight.

Quality control
All nutritional analyses were performed in triplicate. Certified reference materials (wheat flour with certified composition) were included to verify performance. Method blanks were included to detect contamination or procedural errors.

Data processing and statistical analysis
Chromatographic data were acquired and processed using Chromeleon software. ZEN concentrations were quantified by external calibration. Statistical analyses were performed in IBM SPSS Statistics

(Version 25) (IBM Corp, 2017). Descriptive statistics were computed, and independent-samples t-tests were used where applicable. Statistical significance was set at $p < 0.05$.

Results

Zearalenone results

ZEN was determined in 80 wheat samples using the HPLC–FLD method. ZEN was not detected in any sample above the method limit of detection (LOD = 3.17 µg/kg). Accordingly, all samples were compliant with the EU maximum level for ZEN in unprocessed cereal grains (100 µg/kg, except unprocessed maize grains intended for wet milling and except rice), as established in Commission Regulation (EU) 2023/915 (European Commission, 2023).

Proximate composition of wheat samples

The proximate composition of the 80 wheat samples is summarized in Table 2. Carbohydrates were the predominant component, with a mean content of 76.62% and a range of 73.14-79.47%. Protein content averaged 11.04% and showed the greatest variability among the measured parameters, ranging from 9.54% to 13.13%. Fat and ash contents were comparatively stable across the sample set, with mean values of 1.64% for both parameters. Phosphorus content averaged 0.29%. Overall, the results provide a concise compositional characterization of the analyzed wheat samples and support subsequent comparisons according to declared origin and sampling location.

Comparison between declared local and declared imported wheat

Table 3 compares the proximate composition of declared local wheat (DLW, n = 23) and declared imported wheat (DIW, n = 57), based on the origin information provided at the sampling point. No statistically significant differences were observed between the two groups for carbohydrates, protein, fat, ash, or phosphorus ($p > 0.05$). Mean values were similar across all measured parameters, indicating broadly comparable proximate composition between declared local and declared imported wheat during the study period. Levene's test indicated unequal variances for fat and phosphorus; therefore, Welch's t-test was used for these parameters. The statistical conclusions remained unchanged.

Comparison by sampling location

Table 4 compares the proximate composition of wheat samples according to sampling location: the Dukagjini Plain (n = 58) and the Kosovo Plain (n = 22). No statistically significant differences were observed between the two sampling locations for carbohydrates, protein, fat, ash, or phosphorus ($p > 0.05$). Overall, the measured compositional parameters were broadly comparable between samples collected from the Dukagjini Plain and those collected from the Kosovo Plain.

Discussion

The absence of detectable ZEN in all analyzed wheat samples indicates that, during the investigated period, wheat circulating in the assessed Kosovo supply chain did not contain ZEN above the analytical detection limit of 3.17 µg/kg. This finding indicates compliance with the EU maximum level for ZEN in unprocessed cereal grains (European Commission, 2023). However, the result should be interpreted as baseline evidence for the sampled period rather than as proof of permanent absence of ZEN risk. *Fusarium* mycotoxin occurrence can vary substantially across harvest years, weather conditions, storage practices, and supply-chain routes. Therefore, non-detect results remain relevant for food safety monitoring because they provide reference data for future surveillance of wheat in Kosovo.

The relevance of this baseline evidence is supported by the structure of the Kosovo wheat supply, which includes both domestic production and imported wheat. Samples collected from mills may represent wheat lots exposed to different production, transport, and storage conditions, and the origin of some samples was based on information declared at the sampling point. For this reason, routine

monitoring of ZEN and other cereal-associated contaminants remains important for both declared local and declared imported wheat circulating through the food supply chain (Kosovo Agency of Statistics, 2022, 2024).

The present findings are consistent with the known variability of ZEN occurrence in cereal matrices. Previous studies have shown that ZEN may be detected in wheat grains or cereal-based products, but occurrence depends strongly on matrix, season, region, processing level, and post-harvest conditions (André *et al*., 2022; Ji *et al*., 2023). In addition, ZEN may co-occur with other *Fusarium* toxins, particularly deoxynivalenol (DON), which supports the need for broader monitoring strategies even when one toxin is not detected in a specific dataset (Golge and Kabak, 2020; dos Santos *et al*., 2021; Thapa *et al*., 2021). The broader public health and economic relevance of *Fusarium* mycotoxins in wheat supply chains has also been emphasized in European contexts, reinforcing the value of structured food safety monitoring frameworks (Johns *et al*., 2022).

Proximate nutritional parameters were assessed as supporting indicators of wheat quality and sample comparability. The carbohydrate-dominant profile and moderate protein levels observed in this study are consistent with typical wheat compositional ranges reported for wheat-based products (Oyeyinka and Bassey, 2023). Protein showed greater variability than fat, ash, and phosphorus, which may reflect the influence of genotype, environmental conditions, and agronomic management (Wolters *et al*., 2022; Szuba-Trznadel *et al*., 2024; Xu *et al*., 2024). The absence of statistically significant differences between declared local and declared imported wheat, as well as between the two sampling locations, suggests broadly comparable proximate composition within the assessed sample set. These results should be interpreted as complementary quality information rather than as direct predictors of ZEN occurrence. Declared origin alone should also not be considered a strong predictor of wheat composition, particularly in market- and mill-based samples where lots may be selected, blended, or distributed according to processing needs.

This study has some limitations that should be considered when interpreting the findings. First, only ZEN was analyzed, while other relevant cereal-associated mycotoxins, including DON, ochratoxin A, T-2/HT-2 toxins, and fumonisins, were not included. Second, the classification of samples as declared local wheat or declared imported wheat was based on information provided at the sampling point and was not independently verified through documentary traceability. Third, the sample size and sampling period provide baseline evidence but do not allow conclusions for all harvest years or all supply-chain conditions. Future monitoring should therefore include larger sample sets, multiple harvest seasons, and multi-mycotoxin approaches, together with pesticide residue screening where relevant.

Conclusions

This study provides baseline evidence on ZEN occurrence in wheat circulating in the Kosovo food supply during 2022-2024, with proximate composition included as complementary information for sample characterization. ZEN was not detected above the method limit of detection (3.17 μg/kg) in any of the 80 analyzed samples, indicating compliance with the European Union maximum level for ZEN in unprocessed cereal grains during the investigated period. The proximate composition data showed broadly comparable compositional profiles, with no statistically significant differences according to declared origin or sampling location. These findings are limited to the analyzed sample set and sampling period and should not be interpreted as evidence of the general or permanent absence of ZEN risk in the Kosovo wheat supply. Continued risk-based monitoring should include additional harvest years, larger and more systematically distributed sample sets, and broader contaminant coverage, particularly deoxynivalenol, ochratoxin A, T-2 and HT-2 toxins, fumonisins, and, where relevant, pesticide residues.

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Table 1. Validation characteristics of the HPLC-FLD method for the determination of zearalenone in wheat.

Validation parameter	Procedure or result
Analyte	Zearalenone (ZEN)
Matrix	Wheat
Analytical technique	HPLC-FLD with multi-toxin immunoaffinity column clean-up
Calibration approach	External calibration with ZEN standards prepared in acetonitrile
Number of calibration levels	Seven
Calibration range	10–2000 ng/mL
Equivalent concentration range in wheat	2.5–500 µg/kg
Linearity acceptance criterion	$R^2 > 0.999$
Fortification levels	100, 500 and 1000 µg/kg
Recovery	85–95%
Intra-day precision, RSDr	<5%
Inter-day precision, RSDwR	<5%
Limit of detection	3.17 µg/kg
Limit of quantification	10 µg/kg
LOD calculation	3.3 × SD/slope
LOQ calculation	10 × SD/slope
SD used for LOD and LOQ	Standard deviation of the background response from blank samples
Selectivity	No interfering chromatographic peak was observed at the ZEN retention time
Matrix-effect assessment	No relevant chromatographic interference was observed at the ZEN retention time after immunoaffinity clean-up
Justification for external calibration	The immunoaffinity clean-up produced sufficiently selective extracts, while method performance was verified through recovery experiments using fortified wheat samples
Approximate ZEN retention time	2.54 min
Regulatory basis	Commission Regulation (EC) No 401/2006 and the applicable European Commission performance requirements for mycotoxin analysis

Table 2. Proximate composition of wheat samples (n=80), expressed on a dry-weight basis.

Nutritional parameter (%)	Mean ± SD	Median	Min-Max
Carbohydrates	76.62±1.15	76.62	73.14-79.47
Protein	11.04±0.73	11.04	9.54-13.13
Fat	1.64±0.11	1.64	1.32-1.92
Ash	1.64±0.06	1.64	1.54-1.83
Phosphorus	0.29±0.02	0.29	0.27-0.37

Table 3. Comparison of proximate composition between declared local wheat (DLW, n=23) and declared imported wheat (DIW, n=57).

Nutritional parameter (%)	DLW, Mean ± SD	DIW, Mean ± SD	Mean difference (DLW–DIW)	*t*-value	p-value
Carbohydrates	76.68±1.04	76.60±1.21	0.08	0.27	0.79
Protein	11.18±0.76	10.99±0.71	0.19	1.07	0.29
Fat	1.66±0.07	1.63±0.13	0.02	1.03	0.31
Ash	1.64±0.05	1.65±0.06	−0.01	−0.58	0.56
Phosphorus	0.30±0.01	0.30±0.02	0.004	1.05	0.30

Values are expressed on a dry-weight basis. Independent-samples t-tests were used to compare declared local and declared imported wheat. Welch's t-test was applied for fat and phosphorus because Levene's test indicated unequal variances ($p < 0.05$). Statistical significance was set at $p < 0.05$.

Table 4. Comparison of proximate composition by sampling location: Dukagjini Plain (n=58) and Kosovo Plain (n=22).

Nutritional parameter (%)	Dukagjini Plain, Mean ± SD	Kosovo Plain, Mean ± SD	Mean difference	*t*-value	*p*-value
Carbohydrates	76.73±1.26	76.35±0.78	0.38	1.30	0.197
Protein	11.02±0.72	11.11±0.76	−0.09	−0.49	0.628
Fat	1.65±0.11	1.61±0.12	0.04	1.52	0.133
Ash	1.65±0.06	1.64±0.05	0.01	0.99	0.326
Phosphorus	0.299±0.018	0.300±0.018	−0.001	−0.12	0.908

Values are expressed on a dry-weight basis. Independent-samples t-tests were used to compare samples according to sampling location. Statistical significance was set at $p < 0.05$.