

Occurrence of Zearalenone and Enniatin B in Swiss Wheat Grains and Wheat Flours

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Featured Application: The current investigation was part of a collaborative research project aiming at finding innovative decontamination strategies to prevent food waste and reintroduce safe whole wheat grain into the food value chain.

Abstract: Wheat is one of the world's key staple foods, but it is often contaminated with mycotoxin-producing microorganisms, resulting in a large amount of food waste every year. The contamination of wheat grains harvested in 2020 and 2021 in Switzerland, as well as of wheat flours bought in local stores with the two mycotoxins zearalenone (ZEA) and enniatin B (ENB) was investigated. The quantification was performed using LC-MS/MS. ZEA, the level in different cereals and food products of which is regulated by law, was detected in half of the grain samples at levels below 100 µg/kg, except for one sample contaminated with 147 µg/kg. No ZEA was detected in the commercial wheat flours. The emerging mycotoxin ENB was detected in all samples of wheat grains and flours, at levels between 3 and 938 µg/kg. The harvest year was shown to affect the ENB content (p value < 0.01), and in particular the humid weather conditions encountered in 2021 during the month of harvest. The refining grade of the flours showed no influence on the contamination by ENB, indicating that the contamination with ENB can occur not only on the surface layers but also on the inner layers on the wheat grain. As chronic exposure to ENB can therefore not be excluded, decontamination solutions are needed to prevent food waste and further improve the food safety of wheat-based products.

Keywords: enniatin B; mycotoxins; wheat; wheat flour; zearalenone



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1. Introduction

Wheat is one of the first crops that was domesticated by humans more than 10,000 years ago and is still one of our key staple foods today, playing a major role in the daily nutrition of humankind worldwide. As a source of energy, carbohydrates, proteins, fibers, vitamins B and E, and other micronutrients, wheat is the basis for hundreds of recipes and offers high nutritional value, especially when consumed in the form of whole grain. Some evidence suggests that the regular consumption of whole-grain cereals may have a role in the prevention of some chronic diseases [1].

However, wheat is often contaminated by molds, which can counterbalance its beneficial properties. According to the Food and Agriculture Organization (FAO), 25% of the global wheat production is contaminated by molds either before the harvest or after harvest during storage and processing [2]. Therefore, food safety has become a challenge in recent years. Indeed, filamentous fungi can produce toxic secondary metabolites called mycotoxins. Even if milling and separation into pure white flour and bran fractions have been shown to result in a significant reduction of microbial contamination [3], mycotoxins are relatively stable compounds chemically and are not totally removed during processing steps and can be therefore found later in the feed and food chains [4,5]. The ingestion of

food contaminated by mycotoxins is of concern for both human and animal health, causing both acute intoxications and long-term effects [6].

Among the microorganisms producing mycotoxins, *Fusarium* species often infect wheat grains. Most *Fusarium* species can produce one or more mycotoxins. Among the most studied are zearalenone (ZEA), deoxynivalenol (DON), and the emerging mycotoxins of the Enniatin family. While the quantity of ZEA and DON in cereals is controlled and regulated by the European Food Safety Authority (EFSA), enniatins have not yet been considered in the legislation. Despite the statement of the EFSA that an acute exposure to enniatins does not indicate a concern for human health [7], scientific evidence has proven the cytotoxic effects of enniatin B (ENB) and has shown concern about chronic exposure [7–9].

The contamination of wheat with zearalenone and enniatin B has been widely studied worldwide [5,10–13]. In Switzerland, a broad study conducted between 2007 and 2014 established an average contamination of wheat with zearalenone of 39 µg/kg; 7% of the wheat samples in this study exceeded the EFSA limit of 100 µg/kg [11]. However, no other recent publications on zearalenone contamination of wheat in Switzerland were available at the time of writing. Concerning the contamination of wheat grown in Switzerland by the emerging mycotoxin enniatin B, no data have been published to date. In France, a study conducted between 2012 and 2014 reported that enniatin B is the prevalent enniatin in small grain cereals, accounting for 68% of the total enniatin content [10].

In the context of a broader research project aiming at proposing strategies to decontaminate wheat grains with active agents such as functional microorganisms and enzymes in order to reintroduce them into the food value-chain [14], wheat grains cultivated in Switzerland in 2020 and 2021, as well as wheat flours purchased in local stores in Switzerland were analyzed. The goal was to assess the contamination of wheat grains and flours by the regulated ZEA and to assess the contamination of the before-mentioned products with the not yet regulated mycotoxin ENB.

2. Materials and Methods

2.1. Chemicals and Samples

Pure standards of zearalenone and enniatin B were obtained commercially from Sigma-Aldrich (product numbers 32939 and E5411, respectively; Merck AG, Zug, Switzerland). All solvents and mobile phase modifiers were of LC-MS grade. Acetonitrile, methanol, ammonium formate, and acetic acid were supplied by Sigma-Aldrich (Merck AG, Zug, Switzerland). Water was supplied by Carl Roth AG (Arlesheim, Switzerland). Formic acid was supplied by VWR International GmbH (Dietikon, Switzerland).

Seven samples of wheat grains harvested during the summer 2020 and 2021 in Canton Vaud (Switzerland, latitude 46.7283) were provided by GMSA Mills (Groupe Minoteries SA, Granges-près-Marnand, Switzerland). The sampling was done with an automatic sampler according to the Commission Regulation (EC) No. 401/2006 [15] on moving grains during the filling of the cell. The grains were cleaned with a laboratory cleaner and separator (10 mm round mesh and 3 mm triangular mesh) to remove impurities. Then 100 to 200 g of the global sample was used for the analysis of the mycotoxins.

Wheat flours with different milling grades (whole grain, type 405, type 1050) were bought in a local store in Zürich (Switzerland). Details about the samples are given in Table 1. The term ‘conventional’ refers to conventional agriculture and the term ‘organic’ to organic agriculture. The different labels are also provided, namely, IP-Suisse (label of the association IP-Suisse for food products from integrated production) [16], Demeter (label of the Demeter federation for products from biodynamic agriculture) [17], and Bioland (official label of the Federal Republic of Germany for organically produced foodstuffs) [18], as well as the quality classes of the wheat grains harvested (Top, class 1 and class 2) [16].

Table 1. List of samples analyzed during the study.

Samples	Form	Land of Origin	Type	Year of Harvest	Label
G1	Grains	Switzerland	Whole grain	2021	Conventional (Quality: Class 2)
G2	Grains	Switzerland	Whole grain	2021	Conventional (Quality: Class 2)
G3	Grains	Switzerland	Whole grain	2021	Conventional (Quality: Class 1)
G4	Grains	Switzerland	Whole grain	2021	IP-Suisse (Quality: Class 2)
G5	Grains	Switzerland	Whole grain	2021	Conventional (Quality: Class Top)
G6	Grains	Switzerland	Whole grain	2020	IP-Suisse (Quality: Class 2)
G7	Grains	Switzerland	Whole grain	2020	Conventional (Quality: Class 1)
F1	Flour	Germany	Whole grain	NC *	Organic (label Bioland)
F2	Flour	Germany, Austria, Czech Republic	405	NC *	Organic (Demeter label)
F3	Flour	Hungary, Austria	1050	NC *	Organic (Demeter label)

* NC: not communicated.

2.2. Extraction Method

Fifty grams of wheat grains was ground in closed disposable milling tubes using an Ika Tube-Mill 100 device (Ika-Werke GmbH, Staufen, Switzerland) under a fume hood for 1 min at 25,000 rpm. The samples were then extracted according to Scarpino et al. (2019), with some modifications [19]; 5 g of wheat flour were weighed out in triplicate in 50 mL centrifuge tubes and extracted with 20 mL of a 79:20:1 acetonitrile:water:acetic acid mixture for 90 min in an overhead shaker at room temperature. The tubes were then centrifuged for 10 min at 4400 rpm.

The supernatant was passed through a clean-up Oasis[®] Prime HLC cartridge (Waters AG, Baden, Switzerland) for the removal of fatty acids and phospholipids. No cartridge conditioning was performed. First a 0.4 mL aliquot of the supernatant was passed through the column (3cc, 150 mg) and discarded. A 1 mL aliquot of the supernatant was then passed through the column and collected in an amber HPLC vial. All samples were extracted and analyzed in triplicate. Then 10 µL of the cleaned extracts was injected into the LC–MS system.

2.3. HPLC–MS/MS Analysis

LC–MS/MS analyses were conducted on a system consisting of an Agilent 1290 Infinity II chromatographic system coupled to an Agilent 6530 Q-TOF mass spectrometer. Separation of analytes was performed using an Agilent Poroshell 120 EC-C18 (2.1 × 100 mm, 2.7 µm) column protected by a guard column (Agilent EC-18, 2.1 × 5 mm, 2.7 µm).

For ZEA analysis, the flow rate was set to 0.28 mL/min, and the column temperature was set at 35 °C. The two elution mobile phases were made up of water + 0.1% acetic acid (mobile phase A) and methanol + 0.1% acetic acid (mobile phase B). Gradient elution was as follows: 0–0.5 min, 10% B; 6–15 min, 98% B; 15.10–17 min, 10% B. Re-equilibration time was 6 min. Injection volume was 10 µL.

For ENB analysis, the flow rate was set to 0.25 mL/min, and the column temperature was set at 35 °C. The two elution mobile phases were made up of water + 0.1% formic acid + 5 mmol ammonium formate (mobile phase A) and methanol + 0.1% formic acid + 5 mmol ammonium formate (mobile phase B). Gradient elution was as follows: 0–0.5 min, 10% B; 6–15 min, 98% B; 15.10–17 min, 10% B. Re-equilibration time was 5 min. Injection volume was 10 µL.

Both LC methods were used the same MS parameters. The MS analyses were performed using an Agilent 6530 Q-TOF instrument in negative ionization mode (ESI[−]) in the spectral range of 100–1000 Da. Nitrogen served as the nebulizer and collision gas. The MS parameters were as follows: gas temperature, 350 °C; drying gas, 10 L/min; nebulizer, 40 psi; sheath gas temperature, 350 °C; sheath gas flow, 11 L/min; capillary voltage, 3500 V (for ZEA)/3000 V (for ENB); fragmentor voltage, 100 V.

The mycotoxins were identified in wheat extracts using Mass Hunter Qualitative Analysis Software (v 10.0, Agilent) by comparing their retention time and m/z to those of the reference standards.

For the quantification, a matrix-matched calibration was prepared by spiking 5 g of cleaned wheat grains (Zwicky, Müllheim-Wigoltingen, Switzerland) with different concentrations of ZEA and ENB standards. The grains spiked with the mycotoxins were mixed using a vortex and allowed to stand overnight under the fume hood. On the next day, the grains were ground and extracted using the same extraction protocol as used for the samples, in triplicate. The following linear regressions were used for quantification:

ZEA: linear range: 200 $\mu\text{g}/\text{kg}$ –15 $\mu\text{g}/\text{kg}$. Equation: $y = 1 \times 10^8 x - 1473.3$; $R^2 = 0.9999$. LOD = 7.5 $\mu\text{g}/\text{kg}$; LOQ = 15 $\mu\text{g}/\text{kg}$.

ENB: linear range: 2000 $\mu\text{g}/\text{kg}$ –3 $\mu\text{g}/\text{kg}$. Equation: $y = 3 \times 10^8 x + 518,566$; $R^2 = 1$. LOD = 1.5 $\mu\text{g}/\text{kg}$; LOQ = 3 $\mu\text{g}/\text{kg}$.

The accuracy of the ZEA method was evaluated using certified reference material from Romer Labs (contaminated wheat flour, Romer Labs, Tulln, Austria). The recovery percentage was 93%, which is in the appropriate range of the Commission Regulation EC No. 401/2006 criteria for ZEA [15]. As no certified reference material is yet commercially available for ENB, the percentage of recovery for ENB could not be evaluated.

For evaluation of the repeatability (RSD_R), extraction was carried out on the same sample on different days and analyzed. The RSD_R for ZEA was between 1.0 and 13.3%, and the RSD_R for ENB was between 5.8 and 16.3%.

2.4. Statistical Analysis

Statistical analysis was done using the XLSTAT statistical and data analysis solution for Excel (Premium Edition 2022.2.1). Student's t -tests were conducted with an alpha value of 1%.

3. Results

The results of both ZEA and ENB contamination in the wheat grains and wheat flours are shown in Figure 1.

ZEA was detected and quantified in 5 out of 10 samples. When found, the amount of ZEA was below the regulated value of 100 $\mu\text{g}/\text{kg}$ in unprocessed cereals other than maize [20] for four samples and exceeded the regulation in sample G3 in which the quantity of ZEA reached 147.7 $\mu\text{g}/\text{kg}$ (average of three replicates). ZEA was not detected in the commercial wheat flours.

On the other hand, all investigated samples were found to contain ENB at several micrograms per kilogram, ranging from 3.3 to 937.8 $\mu\text{g}/\text{kg}$. The relatively high standard deviation for ENB in samples G3 and G4 could be explained by a non-homogeneous contamination of the grains, giving for each extraction replicate a different value. The results obtained here are in line with the quantities of ENB found in wheat grains previously [10]. Interestingly, wheat flours bought in the supermarket were also contaminated with ENB; the amounts found were between 97.6 and 200.9 $\mu\text{g}/\text{kg}$. The whole grain and pastry (type 405) flours had similar ENB concentrations, and type 1050 (first clear flour) showed a significantly higher contamination ($p < 0.01$, t -test), showing that the refining grade of the flours does not prevent the emerging mycotoxin ENB from being found in the final products.

No correlations could be found between the agricultural system, labelling, or grain quality classification and the amount of mycotoxin contamination. However, when classifying the results according to the harvest year, the grains harvested in 2021 (samples G1 to G5) were significantly more contaminated with ENB than the samples harvested in 2020 ($p < 0.01$, t -test). For ZEA, no contamination could be detected in wheat grains harvested in 2020, where ZEA was detected in all wheat grains harvested in 2021.

Climatic variables such as temperature, precipitations, and humidity are known to play a determining role in fungal growth and therefore on the potential production of

mycotoxins [11]. Those three parameters are summarized in Figure 2 for the months of May, June, and July (pre-harvest and harvest period in Switzerland [11]) for the years 2020 and 2021.

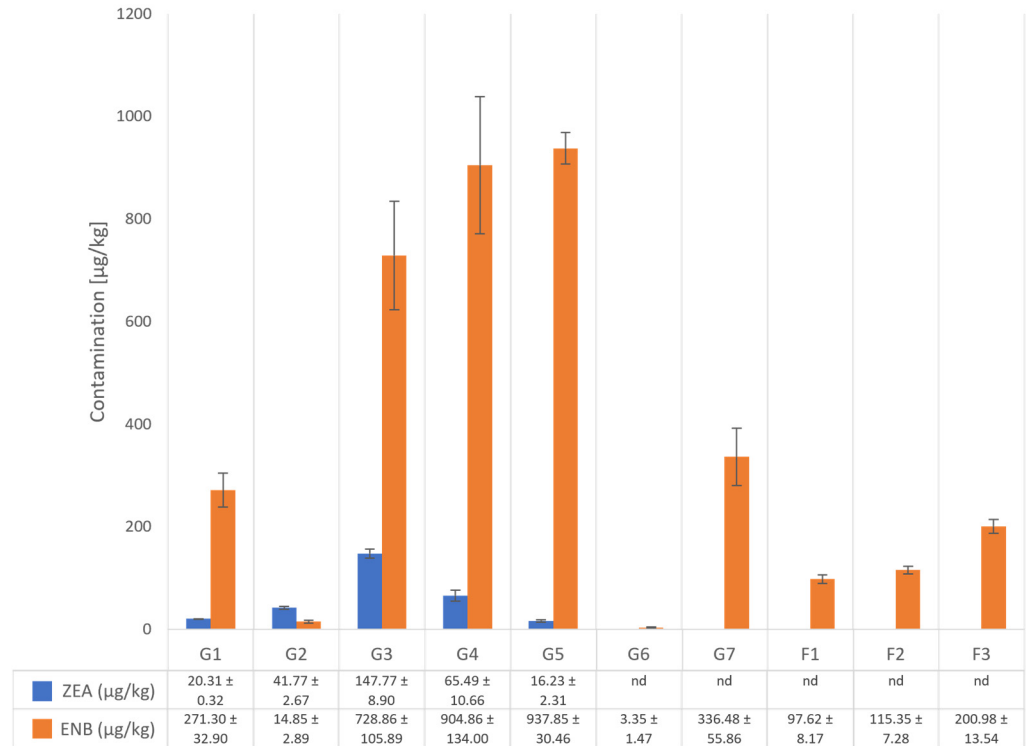


Figure 1. Amount of zearalenone (ZEA) and enniatin B (ENB) in wheat grains (G samples) and wheat flours (F samples) expressed as µg/kg; nd: not detected.

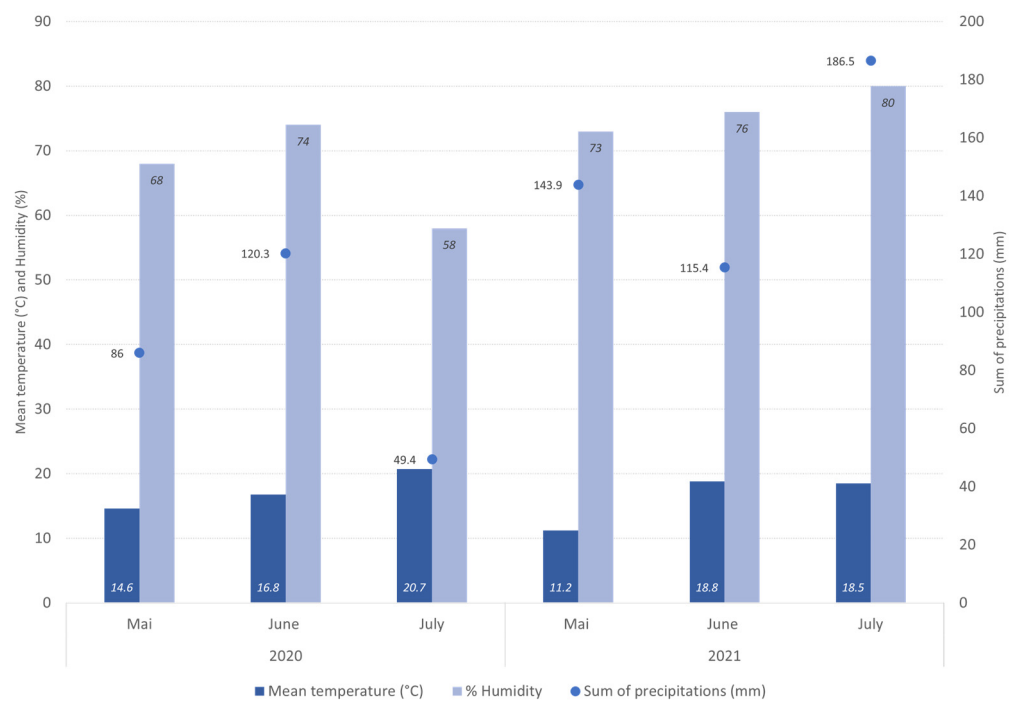


Figure 2. Average temperature, percentage of humidity, and total precipitation in Canton Vaud (Switzerland) for the months of May, June, and July of the years 2020 and 2021 (data from Agrome-teo.ch, station Arnex-sur-Orbe).

As can be seen in Figure 2, even though the mean temperatures were quite similar in 2020 and 2021, the total precipitation in May 2021 was higher than in May 2020 (143.9 mm in 2021 vs. 86 mm in 2020). During the harvest month in July, 2021 also showed higher precipitation (186.5 mm vs. 20.7 mm in 2020) and higher humidity (80% vs. 58% in 2020). Therefore, it is thought that the particularly humid conditions during the summer of 2021 led to a higher contamination of wheat grains by the mycotoxins ZEA and ENB in our study, as seen in Figure 1.

4. Discussion

The results of this study show that except for one sample, the ZEA content of the analyzed wheat grains and flours did not exceed the legal concentration of 100 µg/kg for unprocessed cereals other than maize [20], except for one sample of wheat grains (G3). However, with up to 938 µg ENB per kilogram found in wheat grains and up to 200 µg/kg found in wheat flours, the chronic exposure to the mycotoxin ENB via the consumption of wheat flour in our daily life cannot be excluded.

As underlined by Bertero et al. [21], missing regulations for emerging mycotoxins drive gaps in routine analysis; the emerging mycotoxins such as ENB are neither routinely determined nor monitored and thus can go undetected. In comparison, ZEA, the levels of which are regulated, was only found in half of the samples, in levels below the current regulation for unprocessed cereals, except for one sample. Therefore, our study intends to fill the knowledge gap on emerging mycotoxins, providing information on ENB contamination of wheat grains and commercial flours found in Switzerland.

Temperature and precipitation play a critical role in determining the mycotoxin concentration levels during the growth phase according to Vogelgsang et al. (2017) in their review [11]. Global warming is bringing major changes in the weather, such as increases in temperatures and CO₂ levels, which are predicted to increase the risk of mycotoxin contamination of all crops [22,23]. Combined with the global political and economic context of 2022, where cereals shortages in Europe became a reality [24], the results of this study also show a need for decontamination solutions for mycotoxins, especially when 5–10% of the world's food is wasted because of fungal spoilage [25].

It has been shown that the contamination by microorganisms of wheat grains is located close to the surface, and that removing the outer layers of the grains (pericarp) could lead to a substantial reduction of the microbial contamination [3]. However, during this process, a large amount of nutritionally valuable components is lost, for example dietary fiber, ferulic acid, arabinoxylans, vitamin B, and phenolic compounds contained in the outer layers of the wheat grains. Moreover, other studies show that the reduction of mycotoxin contamination by applying milling technologies is toxin-dependent [26]. As shown in our study, the emerging mycotoxin ENB can also be found in several micrograms per kilogram in commercial wheat flours, the whole grain flour not being significantly more contaminated than the type 405 white wheat flour, indicating that although the *Fusarium* contamination stays at the surface layer, the ENB contamination migrates toward the inner layers of the wheat grain.

For all those reasons, mycotoxin decontamination strategies are needed in order to improve both food safety and food quality and are currently under investigation. The objective would be to reintroduce safe whole grains that are rich in nutritionally valuable compounds into the food value chain.

5. Conclusions

Using HPLC–MS/MS to detect and quantify two mycotoxins in wheat grains and flours, this study provides insights into the level of contamination of wheat samples with the regulated mycotoxin zearalenone and the emerging mycotoxin enniatin B in Switzerland for the purpose of finding adequate decontamination solutions.

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