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Effect of Industrial Processing on the Distribution of Aflatoxins and Zearalenone in Corn-Milling Fractions

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The aim of this study was to investigate the distribution of aflatoxins and zearalenone levels in various corn-milling fractions. Corn kernels and six derived milling fractions (germ, bran, large and small grits, flour, and animal feed flour) were sampled in an industrial plant; both conventional and organic corns were sampled. To evaluate the effect of cooking, samples of polenta were prepared starting from naturally contaminated flour. Conventional and organic lots showed mycotoxin contamination. For both lots, germ, bran, and animal feed flour showed a marked concentration factor from 239 to 911% accounting for both the low yields of the derived products and the distribution of aflatoxins and zearalenone contamination in the outer parts of the kernels. Conversely, a reduction factor of at least four times from raw material to finished products was observed. Polenta samples were unaffected by the cooking process, with levels of contamination similar to those of starting flour.

KEYWORDS: Aflatoxins; zearalenone; corn; industrial process

INTRODUCTION

Aflatoxins are a group of secondary metabolites produced by the molds *Aspergillus flavus* and *Aspergillus parasiticus* (*1*, *2*). Food products associated with aflatoxin contamination include, among others, cereals such as corn, peanuts, and dairy products. Aflatoxins B_1, B_2, G_1, and G_2 are a principal public health concern and play a pivotal role in the occurrence of primary liver cancer. Since 1993, aflatoxin B_1 has been classified by the International Agency for Research on Cancer as carcinogenic to humans (group 1) (*3*).

Zearalenone is a widely distributed nonsteroidal estrogenic mycotoxin produced by several *Fusarium* species such as *Fusarium graminearum* and *Fusarium culmorum* (*4*). It is found worldwide in a number of cereal crops such as corn, barley, oats, wheat, rice, and sorghum (*5*, *6*). Zearalenone has been implicated in numerous mycotoxicoses in farm animals, especially in pigs. The International Agency for Research on Cancer evaluated the carcinogenic potential of zearalenone. It concluded that there was limited evidence of carcinogenicity of zearalenone in experimental animals and that zearalenone is not classifiable as to its carcinogenicity to humans (group 3) (*3*). Despite its low acute toxicity and carcinogenicity (*7*, *8*), zearalenone exhibits, due to its agonistic effect on the estrogen receptor (*5*, *9*), distinct estrogenic and anabolic properties in several animal species, severely affecting the reproductive system (*7*, *10*). Scientific data about the effects of zearalenone in humans are limited to a few investigations. However, in several cases, zearalenone was suspected to be the causative agent in epidemics of premature thelarche in children (*11*−*13*).

Numerous studies on the natural occurrence of aflatoxins and zearalenone in corn and corn-based foods have been conducted (*14*−*17*). The relevance of the above mycotoxins on human and animal health prompted many countries worldwide to establish appropriate tolerance levels both in foodstuffs directly intended for human consumption and in feeds. In this respect, studies on the distribution of mycotoxins in various parts of the grain and the effects of processing are also fundamental, to minimize the economic impact on the production chain. Only a limited number of papers dealing with the distribution of aflatoxins and zearalenone in corn-milled fractions are available, and no study has so far been performed in Europe. More specifically, a study on aflatoxins distribution has been conducted for rice (*18*), and similar investigations on zearalenone for wheat (*19*−*21*) and corn using a laboratory and commercial scale dry-milling process (*22*) have been reported. Other studies have been conducted concerning the distribution of fumonisins in dry-milled corn fractions (*23*−*25*). Taking into consideration that each study approach was different, the cited studies reported a similar trend in mycotoxin distribution in the various corn-milled fractions.

The fate of aflatoxins and zearalenone during corn processing deserves special consideration with respect to the definition of maximum acceptable level in this cereal. Zearalenone is now regulated in foods in 16 countries with maximum limits varying from 50 to 1000 μg/kg (*26*). At the time of the study, according to EC Regulation 466/01 and because of the lack of information on the fate of aflatoxins during milling, the maximum tolerable

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limit for these toxins in cereals was set at 2 μg/kg for aflatoxin B_1 and 4 μg/kg for total aflatoxins, both in raw and in derived products. In Italy, maximum acceptable levels for zearalenone were put in force both in raw and in derived products at levels of 20 μg/kg for baby foods and 100 μg/kg for cereals and its derived products (*27*). The European legislation on zearalenone was under discussion. In 2002, Regulation EC 257, with the aim of differentiating limits for raw corn grains and corn-milled fractions, put forward a request to Member States of the European Union for the submission of relevant data on this issue. The differentiation of limits could relieve the too stringent legal requirements of raw grain whenever the milling could result in less-contaminated processed fractions.

Therefore, the aim of this work was to investigate the distribution of aflatoxins and zearalenone in corn dry-milling fractions derived from the dry-milling processing in an Italian industrial plant. The obtained results have been utilized by the European Commission, for a more consistent evaluation of the maximum tolerable limits for zearalenone in corn and derived products.

MATERIALS AND METHODS

Dry-Milling Process. The industrial process is based upon dry-milling technology coupled with a wet degermination, incorporated with various stages of preparation of the raw grain and selection and calibration of the finished product. The industrial plant can continuously process 5 tons of maize per hour.

The stored (stage 1) raw commodity was cleaned through a dry stoner, an intensive horizontal scourer, and a vibrating aspirator (stage 2); in this step, the corn was separated from waste (stones, earth, and other foreign matter) and from other types of foliage (cob, broken grains, etc.). The foliage was then sent to the hammermill for the production of animal feed flour.

After the cleaning process, the corn moisture content was increased to 20% (stage 3) by adding water to obtain a softer and swollen grain to facilitate the degermination and peeling processes at the conical degerminator (stage 4). Two channels were available at the exit of the degerminator, the first conveying animal feed flour mixed with small size germ and grits and the second conveying large size germ and grits. The first channel led to a conical turbo aspirator (stage 5) separating the animal feed flour from the other products that were in turn mixed with the dried products conveyed from the second channel and transported through a pneumatic system to the plansifter (stage 6). The products were then separated according to their size into grits and germ of small and large dimensions. These were then sent to two different gravity tables (stages 7a,b) where the germ was separated from the grits. Four different products were obtained as follows: coarse grits, fine grits, large germ, and small germ, which were then stored in three different cells, two for coarse and fine grits and one for the gathered germ. The small grits were processed again using a cyclical process along a horizontal rollermill (stage 8a) and plansifter (stage 8b) to obtain flour with the desired granulometry. Undersized flour was sent, together with the rest of the waste from stages 1 to 5, to the hammer mill for the production of animal feed flour. The reprocessed small grits were transformed into edible flour through additional horizontal rollermills and a plansifter. The corn grits may also be transformed into precooked flour by a steam cooker, a flake rolling press, and a flour dryer. At the time of this study, the production of precooked flour was not in operation. A layout of the industrial plant is given in **Figure 1**.

According to the above-described process, obtained were the following products: corn grains, coarse grits (3.350−6.000 μm), fine grits (2.500−4.000 μm), flour (300−800 μm), bran (fiber content, 14.5%), germ (fat content, 20−22%), and animal feed flour (300−850 μm).

Samples. Samples of raw kernels and derived fractions were drawn from the opening slits of the plant. Samples of each of the above listed products were analyzed for aflatoxins and zearalenone. Because no specific sampling procedures exist for zearalenone, the European Directive CEE 98/53/CE, which defines the aflatoxins sampling procedures, was adopted. In fact, this procedure is consistent with the heterogeneous distribution of zearalenone that is similar to that of aflatoxins within a bulk lot. For a lot of 5 tons and for each of the seven groups of products, 40 incremental samples, 100 g each, were collected in 1 h at regular intervals. All 40 incremental samples were then mixed, ground with a mill, and stored at 4 °C prior to analysis. The entire sampling procedure (1 h per lot) was repeated during production cycles on the same day for two different lots, conventional and organic, respectively.

Processed samples of corn flour were used to prepare polenta. This was prepared utilizing a domestic pressure cooker and by traditional manual methods (mixed using a wooden spoon). A 3:1 ratio of water:flour was used for the pressure cooker procedure and a 6:1 ratio for the manual procedure; only sodium chloride was added for preparing polenta as reported in the recipe.

Aflatoxins Analysis. The method used in this work was derived from the validated study for the determination of aflatoxin B_1 in animal feed (*28*) and adapted to the different corn-milling fractions and polenta samples.

Samples of 50 g each were extracted with 250 mL of acetone:water (85:15 v/v) in a blender at high speed for 3 min, and the supernatant was filtered through no. 4 Whatman filter paper. Five milliliters of the clear filtrate was diluted and mixed with 95 mL of phosphate buffer saline (PBS) and then filtered through a microfiber filter paper. For the cleanup of sample extracts, it was essential to utilize the antibody-based immunoaffinity columns (IACs) (*29*), which provide clean extracts and minimize the interference of coextracted compounds. Before the sample was loaded, the column was conditioned with 10 mL of PBS at a flow rate of 2−3 mL/min. Fifty milliliters of the clear filtrate was inserted into a reservoir that fed it into a previously conditioned Aflaprep immunoaffinity column (R-Biopharm Rhône Ltd., Glasgow, United Kingdom) at a flow rate of approximately 3 mL/min. The column was washed with 20 mL of water, applied in two volumes of 10 mL at a flow rate of 3 mL/min, and dried by applying a light vacuum for 5−10 s or passing air through the immunoaffinity column for 10 s by means of a syringe. Aflatoxins were eluted from the column with 1750 μL of methanol; the eluate was collected in a 5 mL calibrated volumetric flask. The flask was topped up with water to give a 5 mL total volume and shaken well. Aflatoxins were quantified by reverse phase HPLC utilizing fluorescence detection, with postcolumn derivatization involving bromination with pyridinium bromide perbromide (PBPB) at a flow rate of 0.4 mL/min to enhance the fluorescence signal produced by aflatoxins B_1 and G_1. The solution of PBPB was prepared by dissolving 25 mg of PBPB in 500 mL of water. One hundred microliters of the diluted eluate was injected into the HPLC system consisting of a PU-980 chromatographic pump and a FP-920 fluorescence detector (Jasco International Co., Ltd., Tokyo, Japan) set at 365 and 435 nm excitation and emission wavelengths, respectively. The analytical column Phenomenex C18, 250 mm × 4.6 mm i.d., 5 μm (Phenomenex, Torrance, CA) was maintained at 40 °C. The mobile phase was water:methanol:acetonitrile (54:29:17, v/v/v) at 1 mL/min flow rate. For each aflatoxin, the limit of detection (LOD) of the method was 0.05 ng/g and the limit of quantification (LOQ) was 0.15 ng/g.

Zearalenone Analysis. The method used in this study was validated in-house adapting the one suggested by the immunoaffinity manufacturer (*30*). The recovery experiments were carried out by adding known amounts of standard solution of zearalenone to blank corn samples.

Twenty-five grams of sample was added to 2.5 g of sodium chloride and extracted with 125 mL of acetonitrile:water (90:10, v/v) in a blender at high speed for 3 min. The supernatant was filtered through no. 4 Whatman filter paper. Twenty milliliters of the clear filtrate was diluted with 80 mL of water, and 25 mL of the diluted filtrate was passed through the Easi-Extract Zearalenone immunoaffinity column (R-Biopharm Rhone Ltd., Glasgow, United Kingdom) at a flow rate of approximately 3 mL/min. The column was washed with 20 mL of water, applied in two volumes of 10 mL at a flow rate of 3 mL/min, and dried by applying a light vacuum for 5−10 s or passing air through the immunoaffinity column for 10 s by means of a syringe. Zearalenone was eluted from the column with 1500 μL of methanol. The eluate was diluted with 1500 μL of water to give 3 mL total volume and then

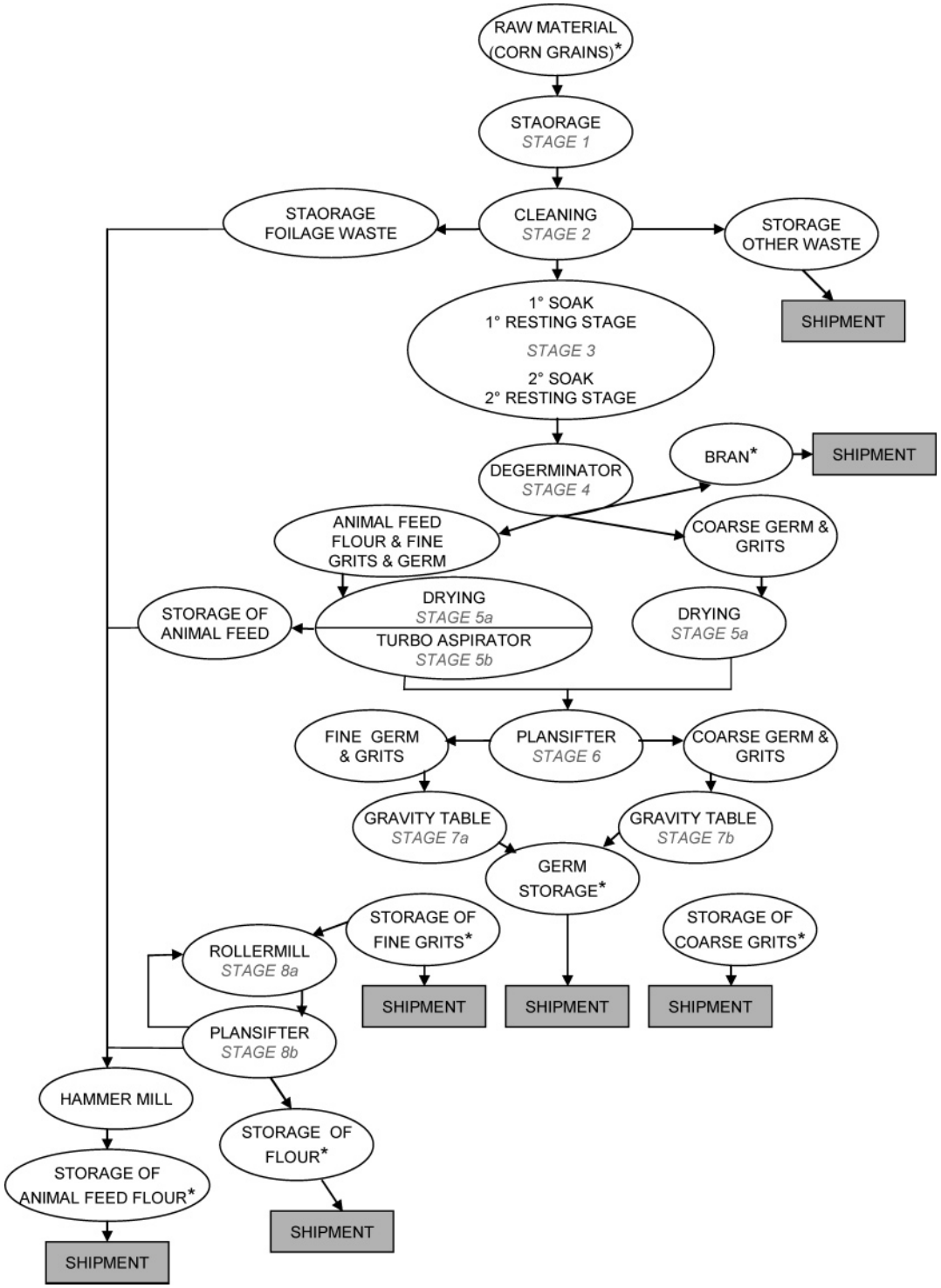

Figure 1. Layout of the industrial plant showing sampling points (marked with an asterisk).

well shaken. One hundred microliters of diluted eluate was injected into the same HPLC system used for aflatoxins, under the following conditions: 150 mm × 4.6 mm i.d., 5 μm, C18 reverse phase column (Phenomenex, Torrence, CA); oven temperature of 40 °C; excitation wavelength 274 and 440 nm emission; and mobile phase acetonitrile: water:methanol (46:46:8, v/v/v) at a flow rate of 1 mL/min. The LOD of the method was 3.0 ng/g, and the LOQ was 9.0 ng/g.

For all of the investigated mycotoxins, the precision and accuracy were determined by analyzing five replicates of naturally contaminated corn kernels at 1 μg/kg of aflatoxins and 100 μg/kg of zearalenone and spiked samples at five different levels of contamination. In both cases, the obtained results were in accordance with the Comité Européen de Normalisation (CEN) Standard 13505 guideline that deals with the performance characteristics for mycotoxins analysis (*31*).

RESULTS AND DISCUSSION

To estimate the effect of the technological process on the distribution of aflatoxins and zearalenone throughout the milled fractions, two different corn lots (one conventional and one organic) processed in the described industrial plant were taken into consideration. For both lots, the analysis of aflatoxins and zearalenone on the raw corn kernels and on the derived milling fractions was carried out and the obtained results are illustrated in **Tables 1** and **2**. The average recovery factors ranged from 120.0 to 76.0%, from 108.7 to 75.3%, and from 104.8 to 87.7% for aflatoxins B_1, B_2, and G_1, respectively; as for zearalenone, the recovery factors ranged from 103.1 to 80.3%.

Table 1. Aflatoxins B_1 and B_2 and Zearalenone Average Levels and Distribution Factors in Dry-Milling Corn Industrial Processing (Conventional Lot)

	aflatoxin B_1		aflatoxin B_2	zearalenone	
material	μg/kg ± SD	distribution factor (%)	μg/kg ± SD	μg/kg ± SD	distribution factor (%)
corn grain	0.93 ± 0.02	100	0.15 ± 0.01	89.6 ± 0.1	100
germ	3.15 ± 0.26	339	0.28 ± 0.04	214.4 ± 1.3	239
bran	5.06 ± 0.14	544	0.67 ± 0.01	271.5 ± 6.5	303
coarse grit	0.51 ± 0.03	55	<LOQ	13.3 ± 0.8	15
fine grit	0.21 ± 0.01	23	<LOQ	13.4 ± 0.1	15
flour	0.20 ± 0.01	22	<LOQ	10.4 ± 0.1	12
corn meal for feeds	3.28 ± 0.08	353	0.35 ± 0.01	314.5 ± 1.3	351
polenta (paiolo)	<LOQ[a]		<LOQ[b]	<LOQ[c]	
polenta (pressure cooker)	<LOQ		<LOQ	<LOQ	

[a] LOQ = 0.15 μg/kg. [b] LOQ = 0.15 μg/kg. [c] LOQ = 9.0 μg/kg.

Table 2. Aflatoxins B_1 and B_2 and Zearalenone Average Levels and Distribution Factors in Dry-Milling Corn Industrial Processing (Organic Lot)

	aflatoxin B_1		aflatoxin B_2	aflatoxin G_1	zearalenone	
material	mean ± SD (μg/kg)	distribution factor (%)	mean ± SD (μg/kg)	mean ± SD (μg/kg)	mean ± SD (μg/kg)	distribution factor (%)
corn grain	2.83 ± 0.01	100	<LOQ[b]	0.38 ± 0.01	9.8 ± 0.7	100
germ	10.58 ± 0.18	374	0.67 ± 0.01	0.36 ± 0.01	30.8 ± 1.5	314
bran	25.78 ± 3.11	911	1.96 ± 0.01	1.85 ± 0.15	39.5 ± 1.8	403
coarse grit	1.42 ± 0.01	50	<LOQ	<LOQ[c]	<LOQ[d]	
fine grit	0.81 ± 0.01	29	<LOQ	<LOQ	<LOQ	
flour	0.69 ± 0.01	24	<LOQ	<LOQ	<LOQ	
corn meal for feeds	21.21 ± 1.84	750	0.87 ± 0.01	0.91 ± 0.01	41.6 ± 0.6	424
polenta (paiolo)	<LOQ[a]		<LOQ	<LOQ	<LOQ	
polenta (pressure cooker)	0.16 ± 0.01	6	<LOQ	<LOQ	<LOQ	

[a] LOQ = 0.15 μg/kg. [b] LOQ = 0.15 μg/kg. [c] LOQ = 0.15 μg/kg. [d] LOQ = 9.0 μg/kg.

Aflatoxins B_1 and B_2 and zearalenone were detected both in the conventional and in the organic lot; aflatoxin G_1 was detected only in the organic lot. For all mycotoxins, the levels of contamination of the raw kernels were lower than the maximum tolerable limits currently proposed by the European Commission for aflatoxins and by Italian law in regards to zearalenone. Aflatoxins and zearalenone levels in the derived products showed a rather similar pattern of distribution, with some slight differences between conventional and organic lots. With respect to the raw grain contamination, germ, bran, and animal feed flour showed a marked concentration factor, while a reduction factor was observed in large and small corn grits and in the samples of flour intended for the preparation of polenta.

The observed variation in the levels of aflatoxins and zearalenone in the milled fractions can be associated with both the yield of the milling process and the distribution of mycotoxins in the various parts of the grain as a consequence of the fungal attack. The average yields obtained in the milling plant were 65, 10, 7, 5, and 13% for grits, flour, germ, bran, and animal feed flour, respectively. Therefore, the highest levels of contamination in bran and germ were associated with their lower industrial yields. In fact, because of the low number of contaminated grains with respect to the total number of grains in the lot, the ratio of the contaminated fraction/total mass in bran or germ is higher than the equivalent ratio for grits. Therefore, it is understandable for the above that the actual levels of mycotoxins in bran and germ are higher than in the other fractions, such as grits and flour.

In addition, the levels of aflatoxins and zearalenone found in germ and bran were higher than in grits and polenta. This can also be attributed to the actual contamination in the external part of the grains due to the fungal attack and to the poor transfer of the mycotoxin to the inner parts. The high mycotoxin concentration in germ is also consistent with the composition of this fraction, since the high level of fats is favorable for mold attack (*32*). It should, however, be noted that the presence of mycotoxin in the derived corn oil and margarine is likely to be negligible, due to the potential degradation of the mycotoxin following the usually adopted alkaline treatment in the processing of germ (*33*).

Aflatoxins and zearalenone concentration in animal feed flour can be considered as a result of the two above-mentioned factors. Notably, bran can represent a risk both for consumers with a high intake of dietary fibers and for animals as it is usually included in feed formulations.

The average distribution pattern of aflatoxin B_1 and zearalenone for conventional and organic lots is shown in **Tables 1** and **2**, where the percentage of increase/decrease mycotoxin distribution factor is also reported. For both toxins, it can be observed that the pattern is similar with an increase of the average percentages in germ, bran, and feed flour up to 911% for aflatoxin B_1 in organic bran and 424% for zearalenone in organic feed flour. In contrast, a decrease of percentages showing a reduction of contamination in grits and flour was observed with neglectable contamination levels in polenta.

Concentration/reduction factors are substantially consistent when organic and conventional lots are compared for zearalenone and for aflatoxin B1 with the exception of bran and corn meal for feed. Both of these fractions are intended for animal consumption and presumably need less requirements with respect to those intended for human consumption. The waste produced during the cleaning step is not eliminated but integrated to the corn meal for feed; as a consequence, this fraction shows a variability in composition that can explain the doubled concentration factor reported in tables. Possible considerations could also be related to the starting status of the lot. The cleaning step is a coarse step and is always conducted in the same way, but if a lot is more infested, it is possible to find

a higher concentration in bran and in corn meal for feed, resulting in a higher concentration factor. Moreover, a sampling error should always be taken into account, even if the sampling procedure has been performed according to the European Directive CEE 98/53/CE.

In conclusion, a quantitative estimate of the fate of the two toxins from the raw grain to the end products leads to a reduction factor for aflatoxins of roughly four times and of roughly 10 times for zearalenone in conventional and organic lots. Conversely, for the byproducts such as germ, bran, and animal flour, where a concentration factor occurred, an increase of three times for aflatoxins and zearalenone was obtained as a minimum in the conventional lot; roughly eight times for aflatoxin B_1 and four times for zearalenone in the organic lot. The results of this study showed that the distribution of aflatoxins and zearalenone during corn processing deserves special consideration with respect to the definition of maximum acceptable levels in cereal and in its derived products. In fact, two opposite situations cooccur during the milling, the first concerning fractions intended for human consumption in which a reduction factor has been observed and the second concerning fractions for animal feeding where a concentration factor was noted. Therefore, the maximum tolerable limits should balance these opposing features, relieving the raw grain of too stringent legal requirements in consideration of obtaining less contaminated milled/processed fractions but at the same time considering that a higher value for the raw grain would lead to a quite high concentration level in the products intended for animals.

Furthermore, it should be noted that no comparison was made between different contamination levels of mycotoxin for conventional and organic lots, since the collected samples were not representative for this kind of consideration. The results obtained in this study have been adopted by the EC Regulation 2174/2003 (*34*) that was recently issued setting differentiated limits for aflatoxing in corn intended for further processing from all other cereals for which more stringent maximum limits were put into force.

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