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Optimizing sample preparation for homogeneous mycotoxin distribution in official control analyses

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Abstract

Background. The distribution of mycotoxins within cereal batches is highly heterogeneous. Therefore, to obtain representative samples and accurate analytical results, sample homogenization is a critical preparatory step.

Objective. To provide reliable and standardized instructions for preparing an aggregate grain sample within official control framework, four different homogenization procedures were evaluated. The comparison considered variability in mycotoxin distribution, workload, and time constraints.

Methods. The study was conducted on four aggregate samples, each weighing 10 kg, obtained from a maize grain batch according to regulatory provisions of Regulation (EU) 2023/2782. Analytical variability of aflatoxin (B1 and B2) and fumonisin (B1 and B2) was measured.

Results. The contamination levels in each pooled sample and the degree of homogeneity among the procedures were assessed and compared using the relative standard deviation of repeatability (RSD_r) as acceptability criterion.

Conclusions. The procedures involving fine grinding (particle size <0.5 mm) and aqueous slurry preparation yielded the best results in terms of homogeneity.

Key words
- mycotoxin
- official control
- comminution
- slurry
- homogeneity

INTRODUCTION

Mycotoxins are toxic compounds naturally produced by different types of fungi belonging mainly to the genera *Aspergillus*, *Penicillium* and *Fusarium*. In specific environmental conditions of temperature and humidity, these fungi proliferate on vegetable substrates and produce mycotoxins [1, 2]. Thereby, mycotoxins enter the food chain through contaminated crops intended for food and feed production [3, 4].

Mycotoxins are a significant food safety concern, and as such, they have received considerable attention within the framework of European Union (EU) food legislation. Since 2006, mycotoxins have been included in a set of specialized regulations to ensure consumer protection. With this aim, maximum levels of mycotoxins in food and feed are set and control measures of good farming, storage and processing practices are defined to maintain mycotoxins at levels "as low as reasonably achievable" (ALARA). Maximum levels of contaminants in food, including mycotoxins, are established in Regulation (EU) 2023/915 [5] and subsequent amendments. To verify that the legislation is implemented, the EU food safety system has established a set of rules for official control activities for which sampling, analysis, diagnosis and tests shall be included as appropriate Regulation (EU) 2017/625 being the core reference [6]. The provisions related to sampling and analysis methods for the official control of mycotoxin levels in food products are defined by Regulation (EU) 2023/2782 [7].

Sampling plays a crucial role to obtain representative samples of the lot under control, and this is even more critical for the subject matter of mycotoxins, which are recognized to be heterogeneously distributed in a bulk [8-10]. Every step of the analytical control chain for mycotoxin detection shall include the sampling step and the subsample preparation up to obtain the final laboratory sample for analysis. Each of these steps contributes with diverse sources of variability to the uncertainty associated to the final analytical result. While the laboratory work has succeeded in the definition of standard protocols to measure the source of the analytical un-

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certainty, the sampling sources of uncertainty are much higher and difficult to be controlled. It is therefore compulsory to fix criteria which the sampling method should comply with, to have a common approach. The uncertainty that origins because of the implementation of the sampling procedure is out of the scope of this paper. Instead, the aim of the present study is to furnish indications on how to handle the aggregate sample obtained by the implementation of the sampling procedure in official control, to assess the effectiveness of the sample preparation in terms of variability of the mycotoxin content and to guarantee the suitable representativity of the aggregate sample before the arrangement of the official test aliquots, which are used by the laboratory to verify the sample compliance.

According to Regulation (EU) 2023/2782 [7], any food lot shall be sampled following the legislation prescriptions whose guiding criterion is the initial weight of the lot and the weight of the aggregate sample. The incremental samples collected shall be combined to produce an aggregate sample, which, depending on the weight of the lot, can be of a weight from 1 kg to 10 kg. The aggregate sample is considered representative of the lot and the replicate samples (i.e., sample for enforcement, for trade or defense, and for reference or referee), are made available for the lot compliance and for the purposes of the food and feed business operator right of defense, shall be taken from the homogenized aggregate sample. Recent Italian national rule (DL 27/2021) [11] as regards the right of the food business operator (descending from Regulation (EU) 625/2017 [6]) has established the preparation of four (or five in special situations) official aliquots for the accomplishment of the official control. These official aliquots must preserve and guarantee the criterion of representativeness, through an adequate homogenization of the aggregate sample [12, 13].

Assuming the sampling procedure yields a representative aggregate sample of the lot under control, the mycotoxin concentration in the lot is inferred from the analytical result obtained from a small laboratory test portion (typically around 5-50 grams) taken from the official aliquot. Even when sampling provisions are strictly followed according to regulatory procedures to obtain the aggregate sample, special care must be taken to ensure the official aliquots are homogeneous and truly representative. Handling of aggregate sample requires particular attention, especially regarding homogenization. In fact, in the context of official control, decisions on accepting or rejecting a lot are based on the contamination level measured in the laboratory test portion. According to Member Sates rules, the replicate samples for enforcement, defense and reference are envisaged to be taken from the homogenized aggregate sample for the purposes the rights of the food business operator [7]. If the replicate samples are not accurately formed from homogenized aggregate sample, there is a significant risk of misclassification, which can lead to trade disputes as well as economic and health repercussions.

In the case of cereals and nuts, the goal of the homogenization of the aggregate sample, is achievable by two different approaches: the dry milling and the water-slurry mixing. The dry milling involves a milling process on the whole aggregate sample using commercial mills capable of grinding the cereal with appropriate particle size. The slurry mixing involves the simultaneous grinding and blending of the sample with a controlled amount of water through a homogenizer. The preparation of the homogenized aggregate sample shall be carried out following a procedure which guarantees harmonized and standardized operations for the control of particle size distribution and time employed. All the handling of the aggregate sample for the sake of the homogeneity shall be done in an appropriate premise, where technical personnel is available for the implementation of all steps and all the instrumental tools are accessible as well. In case the laboratory takes the burden of the implementation of the homogenization procedure, the whole aggregate sample received shall be submitted to the homogenization procedure.

Several studies investigated the effects of different approaches for preparing the sample and the effects, in terms of accuracy and precision, of different particle size of the testing material on the determination of mycotoxins in foodstuffs. Velasco and Morris [14] evaluated the possibility of extracting aflatoxins from water-slurried products. Copra, maize, cottonseed, cottonseed meal, peanuts, peanut butter and peanut meal were successfully analyzed. The precision of slurry analysis for these products, expressed as coefficient of variation (CV, percent), ranged from 2.6 to 7.8 compared to 5.7 to 20.8 for analysis by standard procedures. The use of water-slurry could successfully reduce the variability associated with aflatoxin analysis because i) the distribution of particles is more uniformly achieved with a slurry than with a dry ground product, and ii) high oil content seeds are readily reduced to a fine particle size whereas, because of clogging, only a coarse grind is possible with conventional mills.

Spanjer *et al.* [15] made a comparison between dry milling and water-slurry mixing as comminuting step preceding analysis of aflatoxin B1 in almonds, pistachios and mixed spices and ochratoxin A in cocoa and green coffee beans. The homogenization process was evaluated in terms of CV, which resulted higher for dry milling than for water-slurry mixing. The authors concluded that sample comminution is best performed by water-slurry mixing which, by producing smaller particles, leads to the lowest possible CVs and reveals the best estimate of the mycotoxin content of a lot, reducing to a minimum subsampling error as well as chances of false-positive or -negative values.

To determine differences in mean aflatoxins contamination and subsample variance from dry and slurry homogenizations, Bircan [16] tested triplicates of 10 kg dried fig samples (naturally contaminated) of six different lots, collected from exporting companies in accordance with the EU legislation. Taking into consideration the waste disposal and storage difficulties associated with slurry homogenization, he considered both dry and slurry homogenization as suitable methods to obtain a homogeneous subsample for such a difficult matrix. Dry mixing had slightly higher CV values

compared with slurry mixing, however he highlighted that to obtain a homogeneous fig dough it is imperative to blend the dried figs sufficiently to break up any large particles, which contribute to the variability.

Lippolis *et al*. [17] compared water-slurry mixing and dry milling procedures investigating the distribution of ochratoxin A (OTA) and deoxynivalenol (DON) in naturally contaminated wheat. Although a normal distribution and a good repeatability of DON measurements was observed for both water-slurry mixing (CV% 4.6) and dry milling (CV% 6.4) procedures, for OTA determination reliable results could be obtained only by slurry mixing (CV% 4.0), whereas dry milling comminution resulted in an inhomogeneous distribution with a high variability (CV% 75.2). This study supports the advantage, in terms of variability of results, derived from the use of the slurry mixing approach to achieve a complete homogenization of the laboratory sample.

Damiani *et al*. [18] evaluated the effect of particle size of comminuted maize on fumonisins recovery during the extraction step. Maize samples were ground, and the resulted flours were separated in different fractions by their particle size (1,000-250 µm). For all samples, the efficiency of the extraction from each fraction increased as particle size decreased. Concentrations in fractions with smaller particle size were 1.3 to 4 times higher than the concentration measured in unfractionated samples. The authors attributed this behavior to an increase of the area available for solvent extraction, considering that the smaller the particle size the higher the surface available for the solvent. This work demonstrated that comminuting the sample into smaller particle size could not only reduce the variance of test results but also affect the accuracy.

All the studies agree on the need of homogenization, but no one has given instruction on how to demonstrate that the protocol adopted ensures complete homogenization. The dry milling or slurry process should obtain particles with appropriate particle size, and the preparation of the homogenized aggregate sample shall be carried out following a procedure which guarantees harmonized and standardized operations for the control of particle size distribution and time employed. A recent audit conducted by the Directorate-General for Health and Food Safety of the European Commission (IT GFA 2023-766) emphasized the importance to provide robust evidence of sample homogeneity for the mycotoxin subject matter. Consequently, the Competent Authority (CA) should ask local CA and official laboratories for comprehensive instructions to demonstrate that achieved homogeneity of the aggregate sample is sufficient. Accordingly, a detailed report on homogeneity testing must be submitted including the number of samples taken and analyzed, the statistical evaluation of the data obtained and, importantly, the acceptance criteria applied for homogeneity assessment.

Slurry preparation is widely recognized as an effective approach for ensuring homogeneity and the European guidance document for competent authorities for the control of compliance with EU legislation on aflatoxins explicitly acknowledges it as an appropriate procedure [19]. However, in the context of official control, not all laboratories may have the capacity or equipment to perform slurry preparation, especially when dealing with large bulk sample quantities. In such cases, grinding the sample is an effective alternative, provided that its effectiveness is thoroughly demonstrated.

In the present study, four different homogenization procedures were evaluated to provide reliable guidance for standardizing the preparation of cereal aggregate samples obtained according to the sampling prescriptions of Regulation (EU) 2023/2782 [7]. The assessment focused on workload, time requirements, and variability in mycotoxin distribution. Homogeneity among the procedures was assessed by measuring the analytical variability of aflatoxins (B1 and B2) and fumonisins (B1 and B2) contamination within each aggregate sample, using the relative standard deviation of repeatability (RSD_r) as acceptability criterion. Additionally, the particle size distribution analysis presented was introduced and considered as a further tool for verifying homogeneity.

MATERIALS AND METHODS

Samples

A 400 kg batch of maize grain was made available by an Italian milling company. After applying the sampling provisions of Regulation (EU) 2023/2782 four times, four 10 kg sacks of maize grain were obtained as aggregate samples representative of the lot. Each aggregate sample was processed using a different procedure: i) hand-mixing of the raw grains (referred to as "grain"); ii) coarse milling, performed using a RAS mill – Romer Analytical Sampling mill (Coring-System Diagnostix GmbH, Gernsheim, Germany) (referred to as "coarse"); iii) fine milling, performed using a Retsch ZM 200 (Retsch GmbH, Haan, Germany) (referred to as "fine"); iv) water-slurry mixing, obtained by the addition of water to the sample in a 1:1 ratio and carried out using a Silverson EX-50 batch mixer (Silverson Machines Ltd, Waterside, Chesham, UK) (referred to as "slurry").

From each homogenized sample, 10 testing aliquots of 25 g maize each were collected. For the dry samples (grain, coarse, and fine), the material was spread evenly on a laboratory bench to form a square with a thickness of approximately two centimeters. This square was then divided into 100 small squares, each containing about 100 g of material. Following a Z-shaped sampling pattern, 10 small squares were selected, from which the material was taken to prepare the 10 testing aliquots. For the slurry samples, aliquots were taken directly from the homogenized slurry.

The test sample were found to be contaminated with aflatoxins B1 and B2, and with fumonisins B1 and B2, therefore the study is focused on these mycotoxins.

Analytical determination

Aflatoxins were analyzed in the samples according to method EN 14123:2007 [20], a high-performance liquid chromatographic method with post-column derivatization and immunoaffinity column cleanup. The application of this method required a matrix extension in order to include maize. For this purpose, the perfor-

mances of the analytical method were verified for the analysis of maize. The values obtained were found to be in compliance with the performance criteria reported in the CEN standard. The analysis of fumonisins were conducted according to method EN 16187:2015 [21]. This analytical procedure employs an immunoaffinity column purification step followed by a high-performance liquid chromatographic detection with a pre-column derivatization using ortho-phthalaldehyde (OPA) and 2-mercaptoethanol. In compliance with quality control procedures, recovery tests were conducted, and QC samples were analyzed to verify the performance of the method during the analytical sessions.

Reagents: chemicals and solvents used for the extraction and clean-up solutions were ACS grade or equivalent (Sigma-Aldrich, St. Louis, USA). Water used was deionized and, for HPLC (high-performance liquid chromatography), purified through a Mill-Q treatment system (Millipore, London, UK). For HPLC analysis, methanol and acetonitrile were HPLC grade (Sigma-Aldrich, St. Louis, USA). Phosphate buffer solution (PBS) was prepared from potassium chloride (0.2 g) (Sigma-Aldrich, St. Louis, USA), potassium dihydrogen phosphate (0.2 g) (Carlo Erba, Milan, Italy), anhydrous disodium hydrogen phosphate (1.2 g) (Panreac Química S.L.U, Castellar del Vallès, Spain), and sodium chloride (8 g) (Sigma-Aldrich, St. Louis, USA) added to distilled water (900 mL). After dissolving, the pH was adjusted to 7.4 and the solution was made to 1 L. Sodium dihydrogen phosphate was from Carlo Erba (Milan, Italy), PBPB (pyridinium hydrobromide perbromide) was from Merck KGaA (Darmstadt, Germany) and OPA was from Sigma-Aldrich (Steineim, Germany). Disodium tetraborate and 2-mercaptoethanol were from Sigma-Aldrich (St. Louis, USA).

Standard solutions: aflatoxins certified standard solution was purchased from Biopure (Tulln, Austria) as a mix in acetonitrile. Fumonisins stock solution was obtained from Biopure (Tulln, Austria) as mix of fumonisins B1 and B2 in acetonitrile:water (50:50, v/v).

Flour sieving

Flour sieving was manually carried out on the coarse and fine flours, by passing them through ISO-certified sieves (Giuliani Tecnologie, Turin, Italy) with meshes of 2.8 mm, 2.0 mm, 1.4 mm, 0.5 mm and 0.212 mm. The percentage of particles passing each sieve was measured by weighing each single fraction and comparing it with the initial amount of material. No sample loss was observed during the sieving.

Laboratory milling equipment

Different milling machines are available on the market with different load capacity of the hopper. In this study the following laboratory milling equipment have been used.

The RAS mill (Romer Analytical Sampling mill) with a 7 kg grain capacity hopper can work with a 2 kg/minutes speed to obtain a coarse milled sample. A Retsch ZM 200, assembled with a 0.5 mm ring sieve and a 3 kg cassette collecting cyclone, able to work with 0.5 kg/minutes speed was employed to obtain a fine milled sample. Finally, a Silverson EX-50 batch mixer was used to prepare the water-slurry maize sample, starting from the maize kernels.

RESULTS AND DISCUSSION

Four 10 kg aggregate maize grain samples were processed to obtain grain, coarse, fine, and slurry testing aliquots. With the aim to verify the grade of homogenization in the four practices, the mycotoxin distribution and variability, and the granulometry distribution were scrutinized.

Aflatoxin and fumonisin contamination distribution

Each of the four 10 kg aggregate samples (grain, coarse, fine and slurry) was sub-sampled to obtain 10 replicates of testing aliquots to be analyzed on the same day. During the analytical sessions, recovery tests were carried out, and quality control (QC) samples were tested to verify method performances. To handle the contamination values, all analytical test values resulted as lower than the limit of quantification were treated with the substitution approach and were replaced by 0 [22].

Mean concentration values (corrected for the recovery and expressed in µg/kg), standard deviations (SD), and RSD_r obtained for grain, coarse, fine and slurry samples for each mycotoxin are reported in *Table 1*.

Table 2 shows data for the sum of aflatoxins, AFs (AFB1+AFB2), and sum of fumonisins, FBs (FB1+FB2). *Figure 1* shows the box plots of single data for AFs and FBs values obtained; to note that box plots for fine and slurry show the lower variability either for AFs or FBs. To note that a 25 g test portion of maize in grain contained approximately 80 maize kernels, whereas 50 g of slurry (water:maize in a 1:1 ratio) contained 25 g of an emulsified, homogenized 10 kg maize kernel sample, without visible intact maize kernels.

The level of variability of aflatoxin and fumonisin content in the aggregate samples was measured by means of RSD_r obtained by 10 independent analyses. Among

Table 1
Mean values, standard deviation (SD) and relative standard deviation of repeatability (RSD_r) for each mycotoxin (replicates n=10)

	Mean±SD (µg/kg)				RSD_r (%)			
	AFB1	AFB2	FB1	FB2	AFB1	AFB2	FB1	FB2
Grain	7.80±18.88	0.99±2.84	12,380±4,654	1,266±1,000	242	285	38	79
Coarse	101.74±140.49	3.74±4.08	14,690±1,195	1,549±493	138	109	8.1	32
Fine	57.92±1.21	2.26±0.31	16,607±919	1,685±366	2.1	14	5.5	22
Slurry	52.78±0.88	2.55±0.08	17,199±865	2,367±402	1.7	3.1	5.0	17

AFB1: aflatoxin B1; AFB2: aflatoxin B2; FB1: fumonisin B1; FB2: fumonisin B2.

Table 2
Mean values, standard deviation (SD) and relative standard deviation of repeatability (RSD_r) for the sum of AFs (AFB1+AFB2) and FBs (FB1+FB2) (replicates n=10)

	Mean (µg/kg)		SD (µg/kg)		RSD_r (%)	
	AFs	FBs	AFs	FBs	AFs	FBs
Grain	8.8	13,647	22	5,119	247	38
Coarse	105	16,239	144	1,374	137	8.5
Fine	60	18,292	1.3	1,198	2.1	6.6
Slurry	55	19,567	0.9	1,123	1.7	5.7

AFs: sum of aflatoxins AFB1 and AFB2; FBs: sum of fumonisins FB1 and FB2.

the four aggregate samples, the RSD_r varied from 1.7% and 3.1% (in slurry) to 242% and 285% (in grain) for AFB1 and AFB2, respectively, and from 5.0% and 17% (in slurry) to 38% and 79% (in grain) for FB1 and FB2, respectively.

RSD_r showed similar trend for each mycotoxin class in the four different procedures, with RSD_r in grain >RSD_r in coarse >RSD_r in fine ≈RSD_r in slurry. In the grain procedure, aflatoxins showed much higher variability than fumonisins (242.2% for AFB1 *vs* 38% for FB1), while in the fine procedure (smaller particle size) variability for aflatoxins was lower than the one for fumonisins (1.7% for AFB1 *vs* 5.7% for FB1).

As regards aflatoxins, when comparing contamination levels across the different procedures, from grain up to water-slurry, the data clearly reveal that aflatoxin contamination tends to be distributed in spots, with highly contaminated individual grains interspersed with uncontaminated ones. In the grain procedure, each testing aliquot represents only few kernels, resulting in a high variability among the 10 analyses. The contamination is detected randomly; therefore, the average contamination is lower compared to other procedures. From grain up to water-slurry, as the degree of comminution increases, the variability decreases, and the average value stabilizes as it is reflected in reduced SD and RSD_r.

As regards fumonisins, the impact of comminution degree on contamination variability is less pronounced. *Fusarium,* a plant pathogen affecting harvested maize, tends to cause widespread contamination throughout the crop. As a result, from grain to water-slurry, a noticeable reduction in variability can be observed (*Table 1* and *2*), along with the stabilization of average contamination levels, reflected by a decreased RSD_r that becomes apparent after the first comminution process (coarse).

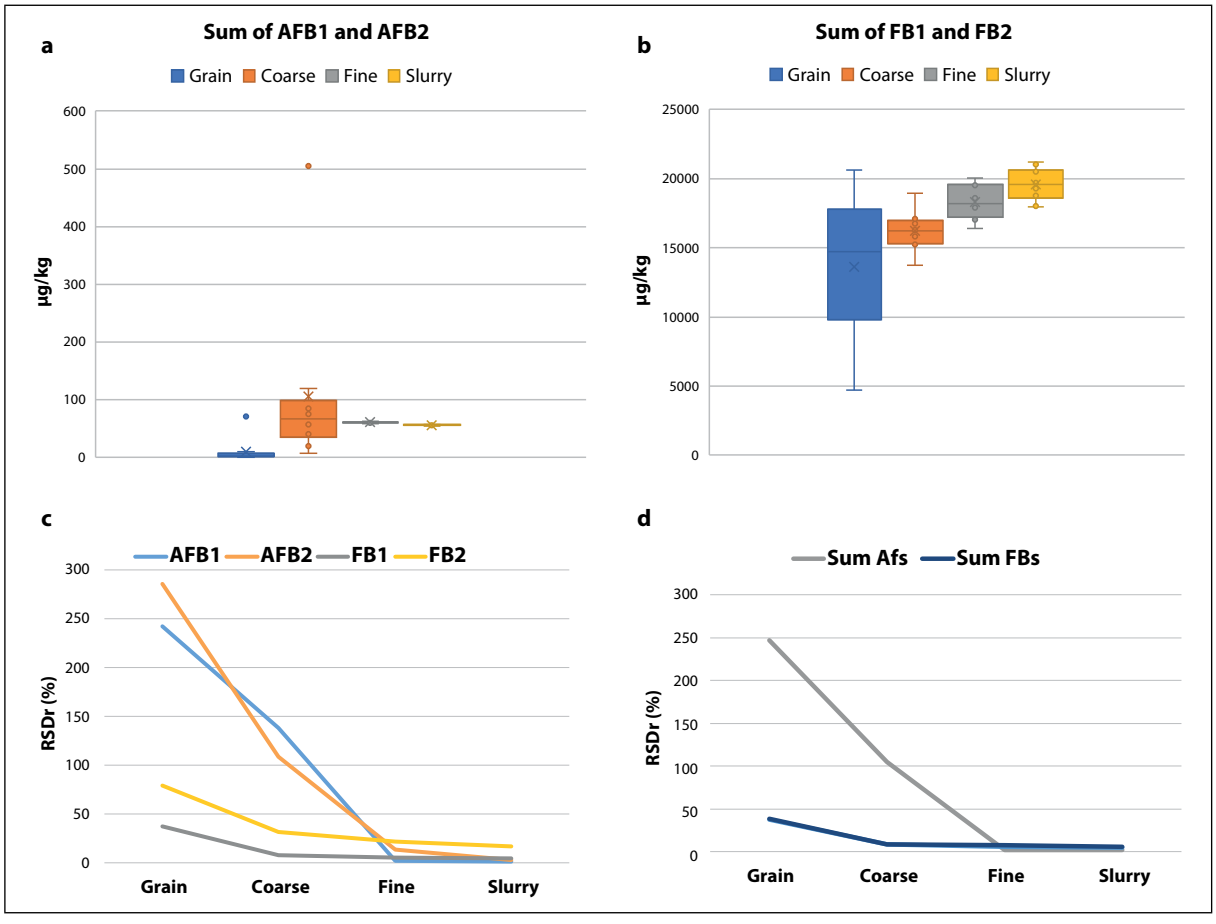

Figure 1
Box plot describing the distribution of the contamination values for the sum of aflatoxins (a) and sum of fumonisins (b), respectively; (c) and (d), trend of RSD_r (relative standard deviation of repeatability) along the four processed samples (grain, coarse, fine and slurry) for the four mycotoxins and for the sum of aflatoxins and fumonisins (d).

Regulation (EU) 2023/2782 [7] (and its amendments) reports prescriptions for analytical requirements of method precision, either in terms of repeatability (the maximum tolerable variability in repeated analyses within one laboratory), and of reproducibility (the maximum tolerable variability among laboratories), and regardless of the mycotoxin analyzed, the RSD_r acceptance criterion is set at 20%. For assessing the homogeneity of samples, the RSD_r can be assumed as a key parameter indicating the effectiveness of the sample preparation.

Taking these RSD_r value as the acceptance criteria of homogeneity, the performances obtained for the sum of AFB1 and AFB2 in grain (247%) and in coarse (137%) are considered unsatisfactory, while the values obtained in fine (2.1%) and in slurry (1.7%) are considered highly satisfactory. On the other hand, for the sum of FBs, while the values obtained for grain (38%) is considered unsatisfactory, the values for coarse (8%), fine (7%) and slurry (6%) are considered all satisfactory. All three procedures are thus considered suitable to homogenize this contaminant and it is confirmed that fumonisins can reach homogeneity with a lower comminution grade. Considered as single, FB1 performed well in coarse, fine and slurry procedure reaching satisfactory and comparable RSD_r values, while FB2 showed insufficient precision for coarse (31.8%), almost sufficient for fine (21.7%) and satisfactory for slurry (17.0%) procedure. The higher variability observed for FB2 highlights its less stable chromatographic signal, which is attributable to limitations in the analytical method. In fact, while FB1 contains a primary amine group that reacts efficiently with OPA forming a stable and highly fluorescent derivative, FB2, lacking this primary amine, produces a weaker derivatization and gives a less stable chromatographic signal.

Fine milling and water-slurry mixing showed comparable variability and average values without statistical difference (t test, 2 tails, same variance, $p>0.05$, accept null hypothesis).

Results obtained in this study are comparable to those reported by other authors [14-18] that investigated and compared procedures of dry milling and water-slurry mixing as successful comminuting step preceding analysis of other matrices and/or mycotoxins. Velasco and Morris [14], for slurried, maize, cottonseed, peanuts, and copra showed aflatoxins CVs below 6%, while for dry milled samples, CVs were in the range between 5% and 21%. Lippolis [17] for slurried wheat reported OTA and DON CVs below 5% and ranges between 6% and 75% for dry milled procedures. This confirms that slurry is the ideal approach for ensuring adequate sample homogeneity and that dry milling procedures needs to be adjusted and standardized to reach comparable performances. It is noteworthy that the variability in dry milling products can be significant among laboratories due to differences in procedures and equipment, making it necessary to establish clear acceptability criteria for the homogeneity assessment.

Granulometry

Comminution is a process that enables reduction of the average particle size of a solid material through crushing, cutting, blending, milling or pulverizing, thereby decreasing its heterogeneity [23]. To evaluate comminution efficiency, the distribution of the particle size of both coarse and fine fractions was studied. A portion of maize flour coarsely milled by using a RAS mill (Romer Analytical Sampling mill), was subject to sieve-shaking fractionation resulting in five particle size fractions (*Figure 2, a-e*): F6, the coarsest fraction (granulometry >2.8 mm) accounted for 3.5%; F5, particles between 2.0 mm and 2.8 mm, made up 26.7%; F4, particles between 1.4 mm and 2.0 mm, were 40.0%; F3, particles ranging from 0.5 mm to 1.4 mm, comprised 20.1%; and F2, the finest fraction (granulometry <0.5 mm), represented 9.7%. Similarly, a portion of maize milled flour underwent to sieve-shaking fractionation, producing three different fractions (*Figure 2, f-h*): F3, the coarsest particles (granulometry >0.5 mm,) with 3.6%, F2, particles sized 0.5-0.212 mm, with 68.7%, and F1, finest particles (granulometry <0.212 mm,), with 27.7%.

Table 3 shows the percentage of particles in each fraction obtained after sieve-shaking fractionation for coarse and fine maize flour.

Taking a threshold fraction of 0.5 mm, the percentage of the particles with granulometry ≤0.5 mm in the coarse milling was 9.7%, while the percentage of par-

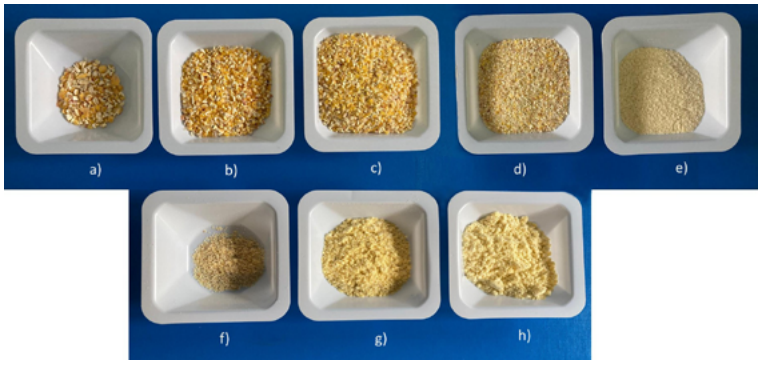

Figure 2
Different fractions obtained after sieve-shaking fractionation of the coarsely milled flour. Particles with granulometry >2.8 mm (a), 2.0-2.8 mm (b), 1.4-2.0 mm (c), 0.5-1.4 mm (d) and <0.5 mm (e). Different fractions obtained after sieve-shaking fractionation of the finely milled flour. Coarsest fraction particles with granulometry >0.5 mm (f), 0.5-0.212 mm (g) and <0.212 mm (h).

Table 3
Percentage of particles of coarse and fine flours in each fraction after sieve-shaking fractionation

Fraction	F1	F2	F3	F4	F5	F6
Sieve (mm)	Ø≤0.212	0.212<Ø≤0.5	0.5<Ø≤1.4	1.4<Ø≤2.0	2.0<Ø≤2.8	Ø>2.8
Particles in coarse flour[1] (%)	-	9.7	20.1	40.0	26.7	3.5
Particles in fine flour[2] (%)	27.7	68.7	3.6	-	-	-

[1]RAS mill-Romer Analytical; [2]Retsch ZM 200-Retsch GmbH.

ticles below the same granulometry in fine flour was 96.4% (68.7+27.7). Following the definition of homogeneity provided in ISO/FDIS 6498:2012 [24], that is, the degree to which a property or a constituent is uniformly distributed throughout a material, a higher proportion of smaller particles indicates greater homogeneity due to increased comminution. The granulometry level and its distribution offer only a rough indication of the potential homogeneity; nonetheless, finer particle size generally corresponds to improved uniformity in the material. Consequently, fine flour exhibits potential homogeneity of the test portions.

Thus, the dry milled sample was considered homogeneous for aflatoxin analysis when finely ground, as demonstrated by an acceptable RSD_r (2.1% for AFs), and when at least 90% of particles exhibited a granulometry below 0.5 mm. For fumonisin analysis, homogeneity was achieved with coarse grinding, where the RSD_r was also acceptable (6.1% for FBs), and at least 60% of the particles with a granulometry below 2 mm.

Therefore, a finely milled dry sample with an RSD_r below 20% and at least 90% of particles smaller than 0.5 mm can be considered suitably homogeneous for aflatoxin analysis. Conversely, for fumonisin analysis, a coarsely milled dry sample with a RSD_r below 20% is considered adequately homogeneous when at least 60% of the particles are smaller than 2 mm.

Working time to carry out the procedures

To handle 10 kg of aggregate maize sample with the RAS mill, 5 minutes were taken, loading the hopper with 2 maize portions, to obtain a coarsely ground sample. Five more minutes were used to disassemble and clean the helical feed worm device and the hopper with a special brush. A grand total of around 20 minutes was taken to obtain the final sample in the coarse procedure.

Using the Retsch ZM 200, to handle 10 kg of maize aggregate sample, 30 minutes were taken. Five more minutes were used to disassemble and properly clean the cassette, the sieve and the rotor. A grand total of around 40 minutes was taken to obtain the final sample in the fine procedure.

Finally, the Silverson EX-50 batch mixer was used to prepare the water-slurry maize sample. For the purpose, 10 kg of maize kernels were placed in a 20 L capacity vessel, and 10 L of water were gradually added to produce a homogeneous paste. Around 15 minutes were taken to obtain a properly slurried and well emulsified sample without visible intact maize kernels. 10 more minutes were taken to clean the rotor by removing the vessel containing the sample and using a 10 L clean vessel to rinse the rotor with water. A grand total of around 30 minutes was taken to obtain the final sample in the slurry procedure.

CONCLUSIONS

The effect caused by the heterogeneous nature of mycotoxin contamination distribution in agricultural products creates major challenges for generating representative samples to be tested during official controls. Sampling regulations specifically addressed to mycotoxins official control establish procedures to be strictly followed to guarantee representativity of the aggregate sample. Due to the high variance in the mycotoxin distribution, the sample preparation to obtain the aggregate sample represents a critical step to be carefully carried out especially in the context of official controls.

In this study, the fine flour procedure (which produces the 96.4% of particles size <0.5 mm) and water-slurry procedure, were the sample preparation options that achieved the highest homogeneity (RSD_r <7%), in 30 and 40 minutes workload respectively.

It was verified that the degree of homogeneity of the 10 kg cereals aggregate sample is closely related to the homogeneization procedure employed. Both the RSD_r and the percentage of particles with size <0.5 mm serve as reliable critera for assessing homogeneity. Furthermore, if a defined percentage of particles (e.g., ≥90%) meet the specific size threshold (e.g., ≤0.5 mm), this particle size criterion can complement the RSD_r and act as a robust proxy for verifying homogeneity, consistent with the reliability of slurry method. Consequently, when a specified percentage of particles falls below the defined size threshold (e.g., from the output of this study, ≥90% <0.5 mm for aflatoxins and 60% <2.0 mm for fumonisins), sample homogeneity can be considered assured (RSD_r <20%). This approach enables a robust and objective assessment of the effectiveness of sample preparation procedures adopted by official laboratories to achieve complete homogenisation of the material.

Authors' contributions

FD, EG, BDS conceived and designed the study; FD, MR, BDS wrote the manuscript; EP, EG, MR did formal analyses; EP, MEG, MR revised and edited the manuscript. All Authors revised the manuscript for important intellectual content and agreed with this article's contents.

Conflict of interest statement

The Authors declare no competing interests.

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