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Are sample size and sample preparation for mycotoxin quantitation in grain products getting trivialized?

Joseph Kumphanda [a,b], Limbikani Matumba [c,g,*], Maurice Monjerezi [d], Thomas B. Whitaker [e], Sarah De Saeger [f,g,h], Hussaini Anthony Makun [a,g]

[a] Africa Centre of Excellence for Mycotoxin and Food Safety, Federal University of Technology Minna, Niger State, Nigeria
[b] Malawi University of Science and Technology, P.O. Box 5196, Limbe, Blantyre, Malawi
[c] Food Technology and Nutrition Group-NRC, Lilongwe University of Agriculture and Natural Resources (LUANAR), P.O. Box 143, Lilongwe, Malawi
[d] Department of Chemistry, University of Malawi, P.O. Box 280, Zomba, Malawi
[e] Biological and Agricultural Engineering Department, North Carolina State University, Weaver Laboratories, P.O. Box 7625, Raleigh, NC, 27695-7625, USA
[f] Department of Bioanalysis, Centre of Excellence in Mycotoxicology and Public Health, Ghent University, Ghent, Belgium
[g] MYTOX-SOUTH International Thematic Network, Ghent University, Belgium
[h] Department of Biotechnology and Food Technology, Faculty of Science, University of Johannesburg, Doornfontein Campus, Gauteng, South Africa

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ABSTRACT

Sampling and sample preparation (grinding and subsampling) are largest sources of variability that negate precision and accuracy of mycotoxin quantitation, particularly in grains. In general, halving sample or subsample (e.g., ground test portion) size doubles variance. Therefore, this paper reports on trends in sample and test portion masses used for the quantification of mycotoxin in maize between 1991 and 2020 by reviewing articles on mycotoxin quantitation in maize (grain and flour) published during this period. About 50% of the articles did not explicitly state the sample mass that was ground. Sample and test portion masses show a significant ($p < 0.05$) decline over the study period. In addition, over two-thirds of the articles did not specify the type of grinder and sieve sizes used in their analysis. Therefore, our findings suggest that standardized sampling plans with emphasis on laboratory sample size and sample preparation methods for maize are increasingly being overlooked during mycotoxin quantitation and increasing the uncertainty associated with estimating the true mycotoxin concentration in grain lots.

1. Introduction

Mycotoxin contamination in grains, particularly maize, remains a major challenge globally (Logrieco et al., 2021). Mycotoxins are secondary metabolites produced by fungi that can contaminate food and cause serious health problems in both human and animals. To protect consumer health and to reduce economic losses, surveillance, and control of mycotoxins in food and feed has become a major objective for producers, traders, food manufacturers, regulatory authorities and researchers worldwide (Köppen et al., 2010). However, these control activities require capability to quantitatively measure mycotoxins with accuracy and precision.

Typically, the procedure for mycotoxin analysis in grains consists of 3 steps: (1) the sampling step, where a random sample (laboratory sample) of a given size is taken from the lot; (2) the sample preparation step, where an entire laboratory sample is comminuted in a mill or grinder to reduce its particle size and a subsample (called a test portion) is drawn from the comminuted laboratory sample (grinding and subsampling are collectively called the sample preparation step); (3) the analytical step, where mycotoxins in the test portion are extracted and quantitated (Whitaker, 2006). While each of the 3 steps contributes to the total variability (random errors) associated with the mycotoxin testing, studies indicate that the sampling step is generally the largest source of variability followed by sample preparation, and analysis contributes the least (Miraglia et al., 2005; Whitaker, 2006). This is so because only a small percentage of kernels are contaminated and the level of contamination on a single grain can be very large, making it extremely difficult to collect a sample that accurately represents the true

* Corresponding author. Food Technology and Nutrition Group-NRC, Lilongwe University of Agriculture and Natural Resources (LUANAR), P.O. Box 143, Lilongwe, Malawi.
E-mail addresses: jkumphanda@must.ac.mw (J. Kumphanda), alimbikani@gmail.com (L. Matumba).

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mean batch concentration.

Considering the foregoing, several researchers have been developing mycotoxin sampling plans aimed at reducing variability of test results since early 1970s and to date regulatory bodies such as the European Commission, USDA, CODEX Alimentarius and other regulations authorities have established standardized sampling plans for various food commodities including maize (Whitaker et al., 1970; van Egmond et al., 2007; CODEX, 2009; European Commission, 2006; GIPSA, 2017). However, it is not clear if these prescribed sampling plans are adhered to, and most importantly, if there are noticeable trends. Therefore, this critical review was set to analyse a 30-year global trend (1990–2020) in sample masses, sample preparation, test portion size, milling equipment and sieve size used in the quantitation of mycotoxins in maize.

2. Methodology

2.1. Inclusion criteria for relevant articles

All articles relative to the subject area were reviewed in three selected journals. These journals were chosen based on their scope and their full coverage of the subject area over a 30-year period from January 1991 to December 2020 and their high impact (ranked as Q1 or Q2 in relevant subject category e.g., Food Science & Technology or Toxicology by Journal Citation Reports™ (JCR). Articles were included if they were original research articles that involved the analysis of mycotoxin in maize (grain or flour).

2.2. Data extraction

To ensure consistency, a standardised excel datasheet was used to capture data. Collected data included mass of ground laboratory sample, mass of test portion used for extraction, type of grinder used for milling, and size of sieve the milled dry sample passed through. Other variables recorded include name of author(s), year of publication, type of mycotoxin(s) analysed and country of publication. In cases where the required information was not explicitly specified in the article, but referred to other documents, detail was obtained from the original documents. The referred published documents that were used included Commission regulation (EU) protocols, ISO protocols, manuals, and other previously published articles. Where the referred articles/documents were not traceable or did not contain the cited detail, it was recorded as a missing value. Where articles indicated a range of sample masses, the minimum value of the range given was recorded. It is worth noting that analysis of lot size and number of incremental samples was out of the scope of this research.

2.3. Statistical analysis

Microsoft Excel (Microsoft Corporation, Redmond, WA, USA) and SPSS version 24 (IBM Corp, Armonk, NY, USA) was used for statistical analysis of sample masses and test portion masses and presentation of data and statistical analysis results. The differences among groups were analysed by one-way ANOVA. Results showing significant differences were subjected to post-hoc Tukey's test with significance for $P < 0.05$.

3. Results

3.1. Overall sampling and sample preparation characteristics

Out of the 192 articles that met the inclusion criteria, 182 involved analysis of maize grain samples while 10 articles had sampled maize flour. Forty seven percent (47%) (86/182) of the articles did not provide information about maize sample mass that was ground (Fig. 1). Five articles indicated that they followed EU guidelines without indicating the actual mass that was ground. Similarly, 4 articles indicated that they followed previously published procedures and did not disclose the exact mass that was ground. The ground mass ranged from 20 to 5000 g and the sample mass means did not significantly ($p > 0.05$) differ across continents although higher sample mass means were recorded in North and South America (Fig. 2). Over two-thirds of the articles did not specify the type of grinder and sieve sizes that were used. (Interestingly, analysis of the capacity of the specified branded grinders revealed that some could not handle a 1 kg sample (data not shown). From the few articles (54) that stated the sieve size used, a significant proportion (31%) did not comply with the sieve aperture upper limit of 1 mm as stipulated in the USDA mycotoxin handbook (GIPSA, 2017). Ten percent (10%) of the articles did not directly indicate test portion mass that was extracted and only referred to previously published articles or documents. One article neither specified the extraction portion mass nor referred to any original document/article. Declared extracted mass ranged from 1 to 50 g and the means did not differ among continents ($p > 0.05$) (Fig. 3).

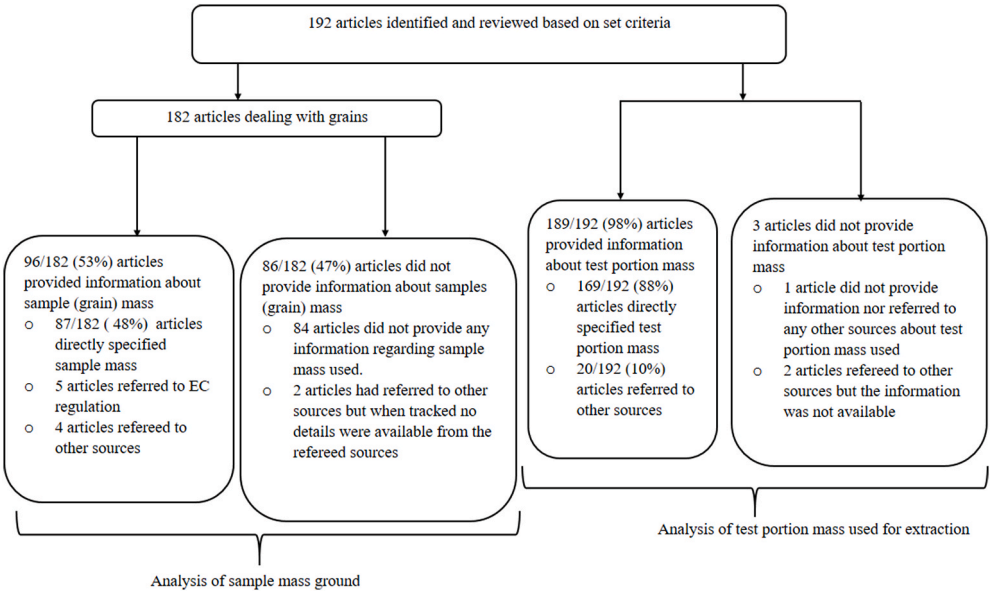

Fig. 1. Schematic diagram showing distribution of articles specifying sample mass ground, and test portion size.

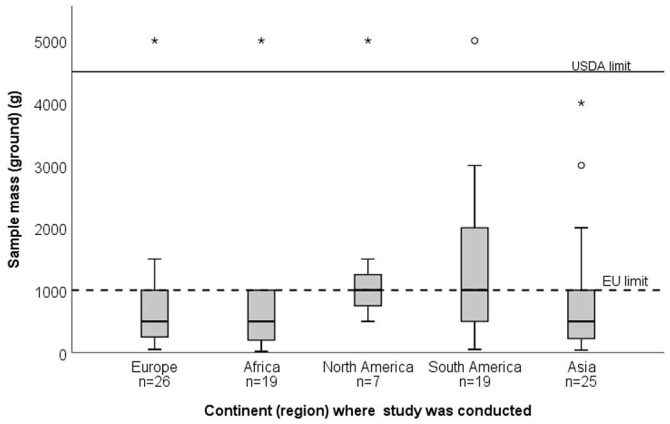

Fig. 2. Distribution of sample mass across the five continents (regions). Circles depict mild outliers (1.5 × IQR), and asterisks depict extreme outliers (3 × IQR).

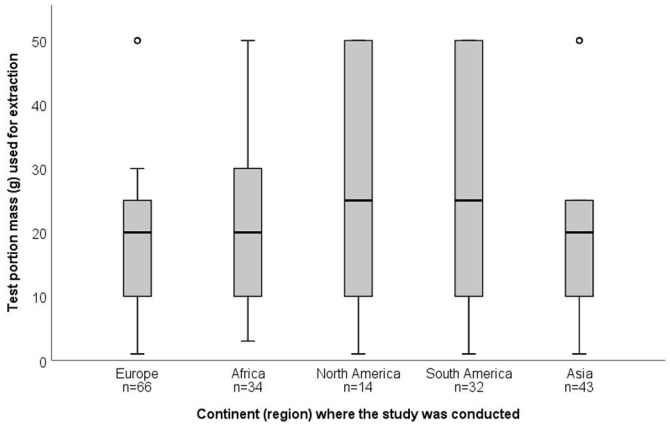

Fig. 3. Distribution of test portion mass across the five continents (regions).

3.2. Trends in mass of ground sample and test portion

The sample mass that was ground for mycotoxin analysis significantly ($p < 0.05$) dropped over the study period (Fig. 4). Notably, during 2011–2020, sample masses ground did not exceed 1 kg [a minimum mass required by Commission Regulation (EC) no 401/2006] (European Commission, 2006).

Similarly, a significant ($p < 0.05$) declining trend in the test portion mass used was observed over the same period with a more pronounced drop from 2001 to 2010 to 2011–2020 (Fig. 5). Notably, during 2011–2020, 50% of the published work involved the use of test portions of not more than 10 g.

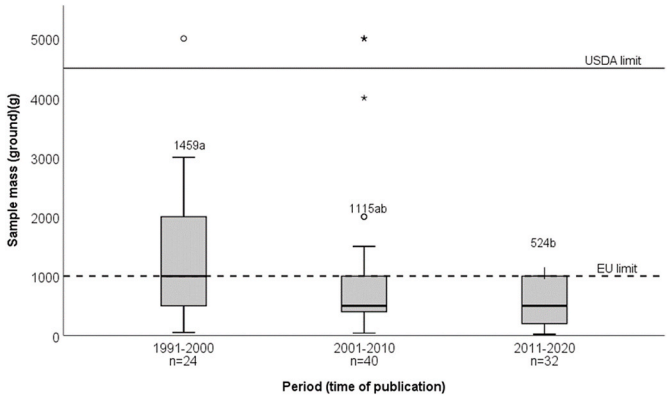

Fig. 4. Distribution of sample mass over 30 years categorised in 10-year blocks. Five articles that indicated to have followed EU recommendation were assumed to have used 1 kg. Circles depict mild outliers (1.5 × IQR), and asterisks depict extreme outliers (3 × IQR). The value above each boxplot is a mean, and means followed by the same letter are not significantly different *($p \leq 0.05$)* according to Tukey's HSD test.

4. Discussion

This meta-analysis has unearthed two critical issues: First, both the mass of maize that is ground and the mass of test portion used for extraction during mycotoxin quantitation has been decreasing over time, a trend likely to have compromised reliability of test results. Second, a significant proportion of published work related to mycotoxin quantitation in maize omitted details of the sample preparation step such as mass of maize ground, particle size of comminuted sample and test portions.

The reduction in maize sample size for mycotoxin quantitation is worrisome considering that sample mass relates inversely to sampling variance. This is so because contaminated grains in a lot are unevenly distributed and only a small proportion of the grains are contaminated (Johansson, Whitaker, Giesbrecht, et al., 2000). Therefore, they can easily be missed giving an impression of mycotoxin low risk leading to acceptance of bad lots. Conversely, if highly contaminated grains are included in a small sample, they can skew the results towards high overall mycotoxin concentration of a sample portion thus exaggerating the risk and rejecting good lots (Cucullu et al., 1977; Johansson, Whitaker, Giesbrecht, et al., 2000; Shotwell et al., 1974; Whitaker & Wiser, 1969). As exemplified by Whitaker (2003), a single grain contaminated with 400,000 μg/kg aflatoxin in a 4.5 kg maize sample can result in an overall sample concentration of 26 μg/kg aflatoxin while the concentration could even go up to 234 μg/kg if the same grain is in a 500 g sample. Decreasing sample size of shelled maize at 20 μg/kg aflatoxin from 4.54 to 0.91 kg (factor of 5) increases sampling variance by the same factor of five from 268.1 to 53.5 (Whitaker, 2006).

This phenomenon cannot be changed by advanced technology and yet, in the studies considered here, the analysts have been opting to reduce the mass. This observation may be attributed to challenges associated with handling of large samples, i.e., the homogenizing of large quantities of ground material, disinfection of contaminated mills and waste disposal. Unfortunately, this has been normalised hence the use of grinders with capacity of less 1 kg.

Like the sample size, the decreasing trend in test portion could be contributing to variance in mycotoxin results and misrepresentation of the physical contamination. Just like with sample size, reduction in test

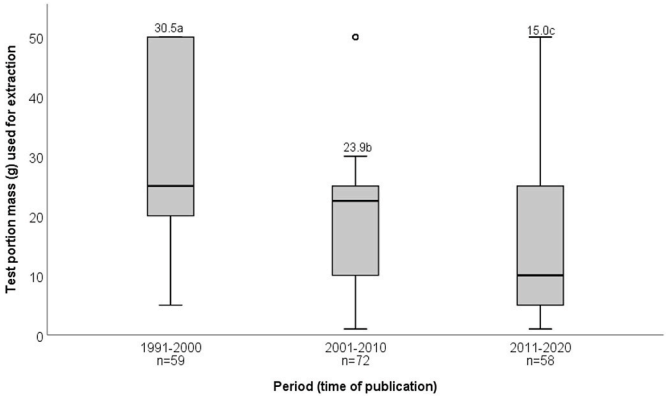

Fig. 5. Distribution of test portion mass used for extraction by publication year over 30 years categorised in 10 year periods. Circles depict mild outliers (1.5 × IQR). The value above each boxplot is a mean, and means followed by the same letter are not significantly different ($p \leq 0.05$) according to Tukey's HSD test.

portion mass by a given factor causes increase in variance with the same factor (Johansson, Whitaker, Giesbrecht, et al., 2000; Whitaker et al., 1994). It is with this understanding that standard protocols, such as USDA, recommend using test portion size of at least 50 g (GIPSA, 2015). To the contrary, the present analysis has revealed that test portion mass as small as 1 g was used (Fig. 3). While we acknowledge the advancement in technology that has led to discovery of grinders capable of producing very fine particles and more homogenous meriting the use of reduced test portion (Tittlemier et al., 2017), it is unlikely that the variance associated with testing 1 g would be equal or less to that associated with testing 50 g sample of standard particle size. Unfortunately, most of the authors who used such small test masses did not disclose the particle size or grinder sieve size involved.

Fig. 6 (a) is built from modelled variances for sampling and sample preparation derived from Johansson et al.' equations (1) and (2) (Johansson, Whitaker, Hagler, et al., 2000) to further illustrate the effect of reducing sample size and test portion on increasing variance. From the equations, combined variances (sampling plus sample preparation) associated with testing a lot of maize when using a Romer mill and two extreme sampling plans of '50 g sample and 1 g test portion' and '5.0 kg sample and 50 g test portion' would be 3619 and 48 respectively.

$$Sampling\ variance = \left(\frac{1.13}{ns}\right) \times 11.361C^{0.98} \quad (1)$$

where ns is sample size in kg

$$\text{Sample preparation} = \left(\frac{50}{nss}\right) \times 1.254C^{1.27} \quad (2)$$

where *nss* is subsample size in g.

A graphical comparison of risks of misclassification of maize lots when using two extreme sampling plans of '50 g sample and 1 g test portion' versus '5.0 kg sample and 50 g test portion' for using regulatory limit of 10 μg/kg for total aflatoxin is displayed in Fig. 6(b). There is about 95% and 50% probability of accepting a lot above 10 μg/kg and 100 μg/kg respectively, when using the former sampling plan.

The present analysis did not investigate the motivation for the reduction in the sample size. Perhaps the small test position is preferred due to 1) economic reason as it requires correspondingly less organic solvent volume, 2) ease in extraction as 1–5 g test samples can fit in a 50 mL tube that could easily be hand-shaken or fit onto small shakers and, (3) avoidance of handling large volume of wet laboratory wastes. Whatever the reason, certainly none of the above can out weight cost of misclassifying a maize lot. Otherwise, there are other ways of overcoming the hypothesized challenges. For instance, it is feasible to improve precision of mycotoxin quantitation in maize with reduced organic solvent volume through slurry procedure (Kumphanda et al., 2019).

Lastly, ethos of scientific writing require that researchers concisely and clearly outline procedures, equipment and materials used with sufficient detail for reproducibility purposes. The omission of details on mass of maize ground and test portions, particle size of comminuted sample and test portions, not only negates reproducibility but also has a potential of misleading other analysts. For instance, the current trend of extracting mycotoxin from a test portion of 1–5 g and leaving out details of grinder/sieves used, grinding and sieving procedure and the resulting particle size, is likely to mislead other analysts into extracting mycotoxin from such small test portions with courser particles. More so, full disclosure of methodology and materials would allow readers to ascertain reliability of the results as they would assess the procedures, materials and equipment used.

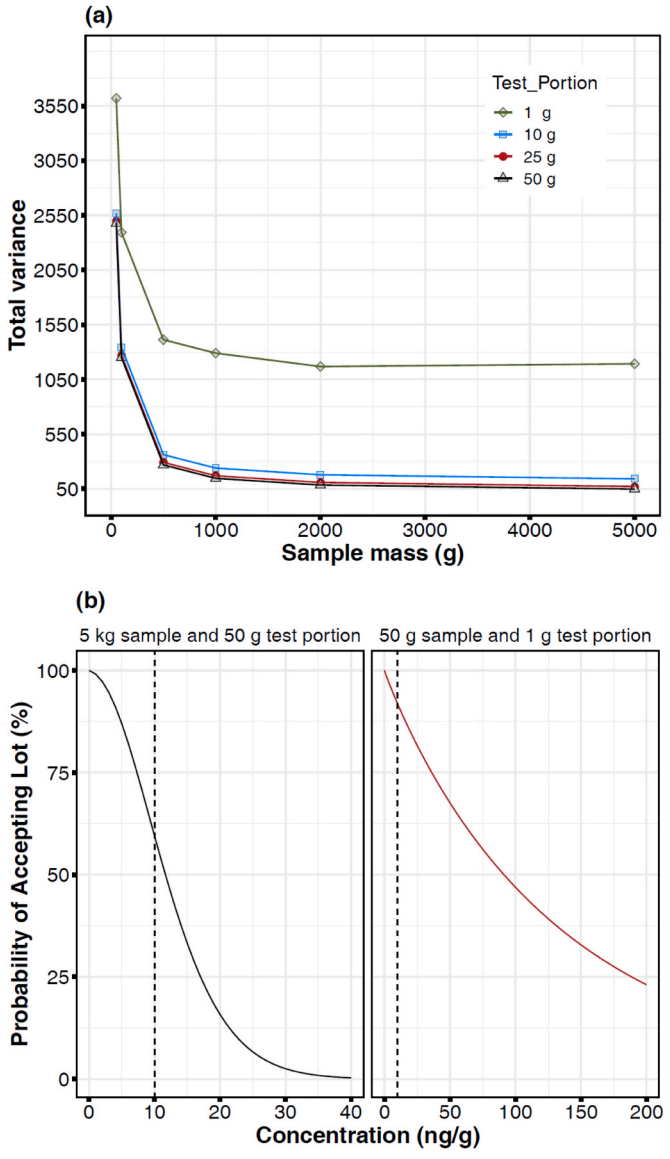

Fig. 6. Effect of size of ground sample and test portion on mycotoxin analysis. (a) A plot of combined variances for various sampling plan. (b) Operating characteristic (OC) curves for testing aflatoxin in shelled maize using (i) a single 50 g sample and a 1 g test portion and (ii) a single 5.0 kg sample and a 50 g test portion. Both calculations assume using a Romer mill, 1 aliquot, HPLC, and an accept/reject limit of 10 μg/kg for total aflatoxin. All samples must test 10 μg/kg for a lot to be accepted. The curves were generated using FAO's calculator for OC curves, accessed at http://tools.fstools.org/mycotoxins.

5. Conclusion

The overall goal of sampling and sample preparation in mycotoxin quantitation is to provide a representative sample for analysis so that analytical results statistically reflect mycotoxin contamination of a given lot. In this study, we demonstrate that, over the past 3 decades, there has been a significant decline in the masses of the ground sample and test portion used in the analysis of mycotoxins in maize (grains and meal) reported in high impact journals dedicated to mycotoxin research. Based on evidence generated by previous research carried out by Whitaker and many others, the reduction in sample size and test portion mass is significantly contributing to the total variance associated with the mycotoxin testing thus compromising the reliability of test results. In addition, there is laxity and inconsistency in the way materials and methods are reported, putting the requirement of reproducibility in question. There are omissions of details of mass of maize ground (laboratory sample) and test portions, particle size of comminuted sample and test portions. By ignoring official sampling and sample preparation procedures/guidelines analysts are likely to be overstating or

understating the status of mycotoxin contamination in maize lots. In case of trade of maize, this would lead to rejecting good lots (seller's risk) and accepting bad lots (buyer's risk). These results highlight the need to revive research on sampling and sample preparation research to further uncover and inform uncertainties associated with the current ills. Moreover, as official sampling procedures of huge grain lots might be tedious and time-consuming, research towards alternative sampling strategies such as dust sampling needs further efforts. More importantly, relevant food safety bodies and journal editors should ensure that minimum sampling and sample preparation conditions are applied and adequately reported.

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