Assessing sample preparation performance when measuring mycotoxins in grain

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Many mycotoxins – secondary metabolites of fungi that can cause harmful effects when consumed – are not evenly distributed in agricultural commodities and foods. Proficiency tests evaluate the performance of the analytical testing method by providing comminuted material for participants to sub-sample, extract, and analyse as per the participants' analytical method. Therefore the format of these proficiency tests do not assess performance of the crucial steps of sampling, sub-sampling, nor comminution, which are the major contributors to the variability of mycotoxin measurements. A scheme was developed to assess 'performance' of preparing a sample of whole grain wheat for analysis of deoxynivalenol (DON). The scheme involved preparation and analysis of duplicate laboratory samples using a participant's own method for sub-sampling, comminuting, and other handling, up to preparation of the test portion for extraction. Material mimicking infected wheat kernels was prepared and used to fortify portions of blank whole grain wheat to obtain a concentration of approximately 1 mg/kg DON. The fortified whole grain samples were prepared and analysed by collaborators. The variance of test results reflected sub-sampling and comminution processes. Laboratories that sub-sampled the whole grain prior to comminution reported data with variance approximately 10- to 500-fold greater than laboratories that comminuted the entire sample prior to sub-sampling. The ability to distinguish the good and poor sample preparation practises based on variance demonstrates the potential of this scheme as a tool to assess the 'performance' of sample preparation.

Keywords: contaminant, cereal grain, sub-sampling, comminution

INTRODUCTION

Proficiency tests are a common tool used by laboratories to obtain an independent measure of proficiency in performing analytical tests. The comparison of a participant's result against the proficiency test accepted value provides an indication of accuracy. Results from multiple proficiency tests will also provide an indication of precision. Successful participation in proficiency tests are also a criterion for accreditation under ISO17025, the "general requirements for the competence of testing and calibration laboratories".

There are numerous subscription-based proficiency tests available that are based on analysing different commodities for mycotoxins, which are secondary metabolites of fungi that can cause harmful effects when consumed. For mycotoxins in wheat and other cereal grains, the largest proficiency tests provide comminuted whole grain test material to participants. Participants then sub-sample (in some instances) the material to obtain a test portion, or use the entire mass of sample provided in their analytical test method.

However, many mycotoxins are not evenly distributed in agricultural commodities such as grain (Biselli *et al*., 2008; Whitaker *et al*., 2000; Whitaker *et al*., 2003) so the steps of sampling and sample processing such as comminution and sub-sampling have the potential to greatly impact analytical test results if they are not performed in accordance with the Theory of Sampling (TOS). Unfortunately, the format of the proficiency tests that provide comminuted grain as test materials do not assess performance of the crucial steps of sampling, sub-sampling, nor comminution. These steps are the major contributors to the variability of mycotoxin measurements (Tittlemier *et al*., 2019c).

A scheme was developed to assess 'performance' of preparing a sample of whole grain wheat for analysis of deoxynivalenol (DON). DON is produced by species of *Fusarium*, notably *F. graminearum*. Infection of wheat by *F. graminearum* can lead to the disease fusarium head blight, whose symptoms include shrivelled and bleached kernels, and the production of the mycotoxin DON. The concentration of DON observed on kernels is generally related to the degree of fusarium damage (Perkowski *et al.*, 1990; Sinha and Savard, 1997; Tittlemier, 2022). However, severely fusarium-damaged kernels have been shown to contain DON concentrations ranging from less than the limit of quantitation to 320 mg/kg (Tittlemier, 2022).

The scheme involves preparation and analysis of duplicate laboratory samples using a participant's own method for sub-sampling, comminuting, and other handling, up to preparation of the test portion for extraction. A similar scheme involving preparation of test materials was used by Thiex and Ramsey to assess a range of nutritional factors in feed (Thiex and Ramsey, 2023); Ramsey *et al.* also used analysis of moisture content of replicate butter samples to estimate sampling uncertainty (Ramsey *et al*., 2011).

The key to this scheme was having whole grain test material with minimum variability of DON content. Material mimicking infected wheat kernels was prepared using various approaches to minimise the variability of DON content. The most acceptable produced 'kernels' were used to fortify portions of blank whole grain wheat to approximate a relevant scenario of whole grain DON concentration of 1 mg/kg and low fusarium damage. Duplicate laboratory samples of this fortified test material was processed and analysed by a number of participants so that precision, as opposed to accuracy, of the comminution and sub-sampling steps could be assessed. This scheme gave an assessment of "measurement proficiency" (Ramsey *et al*., 2011) as it incorporates the "analytical" stage as well as sample preparation stages.

MATERIALS AND METHODS

Preparation of DON-fortified whole grain wheat

In order to be relevant and realistic, as well as facilitate the assessment of measurement proficiency, the test material was developed to meet a number of criteria. The main criterion was, as much as possible, to limit the constitutional heterogeneity to a bimodal distribution where individual kernels either contained no measureable DON or a consistent amount of DON. While this distribution is not realistic (Tittlemier, 2022), it would minimise the confounding impact of kernel to kernel DON variability on the assessment of DON test result variance due to sub-sampling and commination compared to a continuous distribution of DON in individual kernels. The second criterion was to make the overall concentration of DON in whole grain wheat approximately 1-2 mg/DON. This is a relevant concentration range as it encompasses various established maximum limits for DON in wheat (European Union, 2006; Codex Alimentarius Commission, 2016). The third criterion was to approximate a challenging sampling scenario, where only a few point sources of DON were present in the whole grain sample. Using these criteria, the aim was to prepare 'kernels' with consistent DON concentrations greater than 2000 mg/kg and add a small number of these kernels to clean whole grain wheat.

The first approach attempted to fortify barley kernels with DON from an aqueous solution. Barley sourced from an area with low *Fusarium* presence and Fusarium head blight occurrence was pearled to remove the outer bran layer using an abrasive rice whitener for test milling (Satake Grain Testing Mill

TM, Satake). The barley was pearled to remove any DON that may have been present since this mycotoxin is associated with the outer kernel layer (Schaarschmidt and Fauhl - Hassek, 2018) and also to facilitate absorption of DON from the aqueous solution.

The pearled barley was soaked in an aqueous DON solution (100 mg/kg) prepared from crystalline DON. Over a number of trials, different variables were adjusted to maximise DON absorption including soaking time (24-120 hr), the ratio of kernel mass to DON solution volume (0.012-0.175), and temperature (23-40°C). An aliquot (5 µL) of the DON aqueous solution was also applied directly to pearled and unpearled barley kernels using a syringe (Hamilton 10 µL low-volume syringe).

The second approach involved making pasta dough using an aqueous DON solution, preparing spaghetti, and cutting the strands to obtain kernel-like pieces. An aqueous DON solution (8333 mg/L) was prepared from crystalline DON (purity >98%, TripleBond, Guelph, ON, Canada). Dough was prepared using the aqueous DON solution (5.5 mL) and durum semolina (14.9 g). The dough was mixed at 2500 rpm for 90 s (FlackTek SpeedMixer DAC 300-100 SE) and then manually extruded through a hand press (Titan Owl T-Press 3.5" tool). The die of the hand press was modified using a drill press to make the holes 2.3 mm in diameter. Strands of extruded dough were laid flat and immediately cut to a length of 6.3 mm using a utility knife. The DON-doped pasta kernels were then air dried at room temperature for a minimum of 48 hr (20-21°C).

After characterisation of the DON-doped pasta kernels as described below, 12 of the kernels were added to 1 kg portions of durum wheat. The durum wheat had been visually inspected and no symptoms of fusarium damage were present, suggesting the durum wheat was free of DON. In addition, no DON was detected in a sub-sample analysed using an established liquid chromatographic-mass spectrometric method (Tittlemier *et al.*, 2019b). These 1 kg test materials were provided to collaborating laboratories with the instructions to analyse for DON using their own preparation and analytical equipment and procedures.

Characterisation of DON-fortified kernels

Masses of a sub-set of individual prepared pasta kernels were determined on an analytical balance. Widths and lengths were obtained using a micrometer. The characterised individual pasta kernels were then analysed for DON using a modification of the enzyme-linked immunosorbent assay method (ELISA; Neogen Veratox for DON 5/5 test kit; Neogen, Lansing, MI, USA) described in Tittlemier *et al.*, (2015). Individuall prepared pasta kernels were crushed in between waxed paper using pliers and then extracted with water (3 mL). The mixture was vortexed for 3 min, and then centrifuged for 5 min at 3000 rpm. A 20 µL aliquot of supernatant was diluted with 1980 µL water prior to analysis. Sample extracts that contained DON at concentrations beyond the upper limit of the assay calibration curve were re-prepared at a higher dilution factor and re-analysed.

The stability of the DON concentration in the prepared pasta kernels was also assessed. Pasta kernels (n=5) were analysed individually for DON as described above after 208 days of storage at room temperature (20-21°C).

Assessing sample preparation performance

Prior to providing the 1 kg test materials to collaborating participants, a pair of test material samples were analysed using 'good' and 'bad' processing. For the 'good' processing, the entire 2 x 1 kg was comminuted on a Retsch SR300 rotor beater mill, producing a sample with 99% of mass ≤ 850 µm. Each comminuted 1 kg was then divided on a rotary sample divider (Retsch PT100) to obtain a 50 g test portion. The test portion was extracted and analysed using the ELISA test kit described above, as per described in Tittlemier *et al.*, (2015). Duplicate aliquots from the test portion extract were analysed. For the 'bad' processing, a 50 g portion of whole grain was manually scooped from the 2 x 1 kg test materials. Increments were not taken. The 50 g test portion was then comminuted on a Retsch SR300 rotor beater mill, producing a sample with 99% of mass ≤ 850 µm. The comminuted test portion was extracted and analysed using the same procedure as for the 'good' samples.

Each collaborating participant was provided with 2 x 1 kg test materials and instructed to analyse for DON using their own equipment and preparation and analytical procedures.

RESULTS AND DISCUSSION

Preparation of DON-fortified whole grain wheat

The initial attempts to fortify barley kernels with DON did not produce valuable material. The kernel DON concentrations obtained ranged from 11.8 ± 0.5 up to 64 ± 3 mg/kg, orders of magnitude lower than the minimum target of 2000 mg/kg. Variation of the different parameters (heat, soaking time, mass of kernels/DON solution volume) did not appreciably increase the final DON content of the kernels. In addition, direct application of DON solution to the individual barley kernels using a micro-syringe was unfeasible. The added solution remained on the surface of the kernels, risking physical loss and compromising the amount of DON remaining on the kernel.

The second approach of incorporated DON into pasta kernels during the preparation of dough was successful. Table 1 provides data on the physical characteristics of the prepared pasta kernels compared to sound wheat kernels, and Table 2 summarises the DON content and compares to concentrations of DON in Fusarium-damaged wheat kernels.

Table I. Physical characteristics of pasta kernels and sound wheat kernels[1]

	Mass (mg)		Width (mm)		Length (mm)	
	Prepared	**Wheat**	**Prepared**	**Wheat**	**Prepared**	**Wheat**
Mean[2]	35	36	2.66	2.90	6.03	5.90
% RSD[3]	13	23	4		8	
Minimum	25	18	2.48		5.13	
Maximum	42	56	2.82		7.14	

[1]unpublished data
[2]n=30
[3]relative standard deviation

The physical characteristics of the prepared pasta kernels were similar to sound (ie. good quality wheat). The masses were very similar, and as desired, the variability and range of masses of the prepared pasta kernels (25-42 mg, 13% relative standard deviation (RSD) was not as wide as for the wheat kernels (18-56 mg, 23% RSD). The prepared pasta kernels were thinner and longer than the kernels, but still realistic as Fusarium-damaged kernels can be shrivelled and lighter than sound grain (Sinha and Savard, 1997).

Table II. DON content (mg/kg) of pasta kernels and Fusarium-damaged wheat kernels[1]

	Prepared pasta kernels	**Fusarium damaged kernels**
Mean	2640[2]	52[3]
% RSD[4]	13	143
Minimum	2185	<0.34
Maximum	3455	263

[1](Tittlemier 2022)
[2]n=30
[3]n=100
[4]relative standard deviation

The concentration of DON in the prepared pasta kernels averaged 2640 mg/kg, exceeded the minimum target of 2000 mg/kg. As with mass, the variability and range of DON concentrations in the prepared pasta kernels (2185-3455 mg/kg, 13% RSD) was not as wide as for the wheat kernels (less than test limit of quantitation-263 mg/kg, 143% RSD).

The prepared pasta kernels maintained integrity over time. The concentration of DON in the kernels was not significantly different after 208 days of storage. This is consistent with two years stability of DON observed in wheat (Tangni *et al.*, 2017).

The high DON concentrations in the prepared pasta kernels facilitated preparation of whole grain test material with a very low proportion of 'contaminated kernels'. This provided a challenging heterogeneity scenario with which to test the sample preparation assessment scheme. Only 12 prepared pasta kernels were added to 1 kg of sound grain (approximately 28,600 kernels) to obtain an overall concentration of approximately 1.5 mg/kg DON. The 0.04% mass ratio of 'contaminated kernels' in the test material is well below the tolerances for the mass percentage of Fusarium-damaged kernels in in higher quality grades range from 0.3-0.8% (Canadian Grain Commission, 2023a).

Assessing sample preparation performance

The difference in sample preparation performance was readily apparent in the DON test results from the sample pairs initially prepared using 'good' and 'bad' processing. The 'good' process of comminution of the entire 1 kg laboratory sample prior to sub-sampling using a rotary sample divider to obtain the 50 g test portion is shown in Figure 1 as participant 8. The 'bad' process of manually scooping out 50 g in one increment, and then comminuting the 50 g test portion, is shown as participant 9. The 'bad' process shows a large variance between results from the two 1 kg laboratory samples (4.8 mg^2/kg^2) whereas the 'good' process shows a variance approximately 530-fold lower (0.00080 mg^2/kg^2).

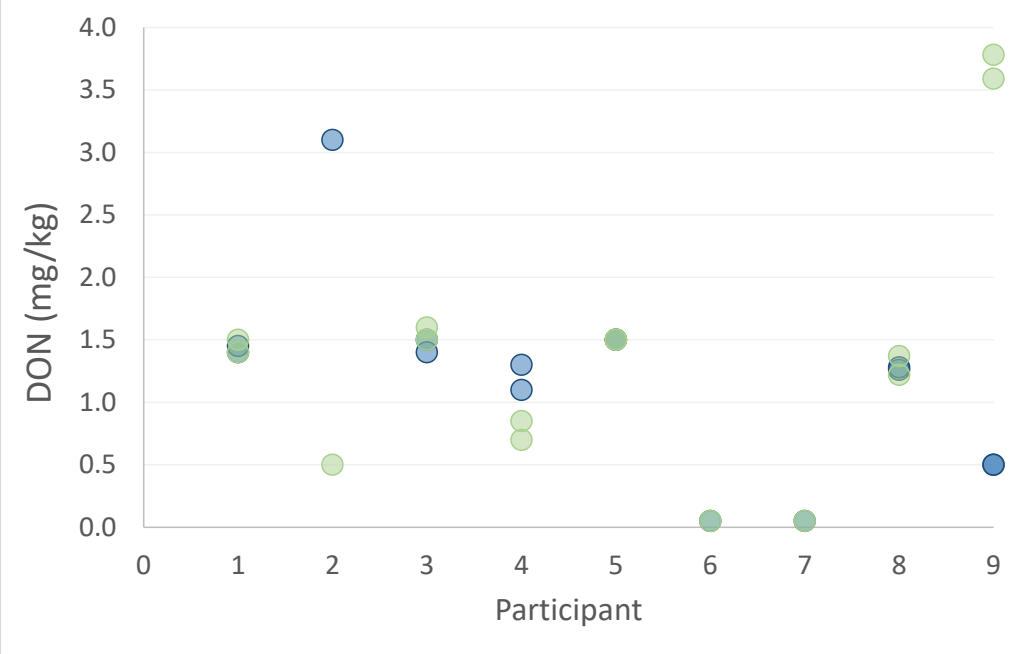

Figure 1. DON testing results from analysis of 2 x 1 kg whole grain test materials. Blue and green circles delineate the two 1 kg samples provided to each participant. Two aliquots from each test portion extract of the 1 kg samples were analysed, except for participants 2, 6, and 7.

A variety of results scenarios were observed when considering results from all participants (Figure 1). Results from participants 1, 3, and 5 resembled the 'good' process (ie. participant 8). Results from participant 2 resembled the 'bad' process as shown by participant 9. Participants 6 and 7 reported results as less than the limit of detection of their analytical test (0.05 mg/kg). Finally, results from participant 4 appear to show an intermediate scenario between 'good' and 'bad' processing.

Methodological information provided by participants easily explained the results observed. The sample processing performed by participants 1, 3, and 5 involved comminuting the entire 1 kg samples using a rotor beater mill that produced a sample with 99% of mass ≤ 850 µm and then sub-sampling using a

rotary sample divider to obtain a 50 g test portion. This process of comminution followed by sub-sampling that takes a number of increments is indicated by the TOS reduce the fundamental error.

Using a less-desired process according to the TOS, participants 2, 6, and 7 sub-sampled whole grain and then performed comminution. Results obtained from these participants either indicated an inaccurate absence of DON above 0.05 mg/kg, or relatively high variance (3.4 mg^2/kg^2).

The preparation used by participant 4 involved a burr-type mill to comminute the entire 1 kg samples. This particular mill also incorporates a sub-sampling step in the equipment after comminution (Romer Mill Series II). This equipment has been shown to produce comminuted wheat with approximately 80% of mass ≤ 850 µm (Tittlemier *et al*., 2017). Even though the entire 1 kg samples were comminuted and then sub-sampled in concordance with the TOS, this equipment may not be fit for purpose. The variance in results between the two samples from participant 4 was 0.18 mg^2/kg^2, which was approximately 20-25 times lower than observed for the 'bad' sampling processes. The applicability of this equipment would ultimately depend on the level of variance deemed acceptable by those using the test results.

CONCLUSIONS

A method was developed to produce samples of whole grain wheat containing a relatively consistent concentration of DON using 'kernels' prepared from fortified pasta. A scheme involving preparation and analysis of duplicate 1 kg laboratory samples using a participant's own methods and equipment to prepare a test portion for extraction and analyse for DON was demonstrated to provide information that could distinguish between processes that reduced fundamental error according to the TOS and those that did not. This scheme could be a valuable tool for laboratories (and laboratory clients) to assess and quantify the performance of their sample preparation to complement assessments of analytical test performance currently provided by existing mycotoxin proficiency testing schemes.

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