Effects of grain sampling procedures on fusarium mycotoxin assays in wheat grains

Agnes Champeil, Jean-Francois Fourbet, Thierry Doré

3

4

5   **Effects of Grain Sampling Procedures on *Fusarium* Mycotoxin Assays in**
6   **Wheat Grains**

7

8   AGNES CHAMPEIL*, JEAN-FRANCOIS FOURBET AND THIERRY DORE

9   UMR d'Agronomie INRA/INA P-G, Centre INRA Versailles – Grignon,

10   78850 Thiverval – Grignon, France.

11

12

13

14   Running Head: Grain Sampling for *Fusarium* Mycotoxin Assays

15

16

17

18

19

20

21

22

23

24

25   * Corresponding author. Phone +33130815420, Fax +33130815425,

26   *E mail champeil@grignon.inra.fr, fourbet@grignon.inra.fr, dore@inapg.inra.fr.*

27

29

1

30

31 **Abstract**
32 *Fusarium* mycotoxins are increasingly studied agronomically, chemically and pathologically

33 in the context of food safety, as a means of preventing new major health crises. Reliable

34 mycotoxin techniques and sampling procedures are required for assessment of the effects of

35 different sources of variation on grain mycotoxin content in agronomic experiments. We

36 carried out analyses with the aim of formulating guidelines for grain sampling to increase the

37 reliability of grain mycotoxin measurement in agronomic experiments. We focused on two

38 toxins in wheat samples: deoxynivalenol and nivalenol. With a nested linear mixed model, we

39 estimated that the uncertainty of nivalenol determination was low ($\pm$ 15 µg/kg), whereas that

40 for deoxynivalenol determination was higher ($\pm$ 38 µg/kg). We also found that grinding of the

41 grain decreased the variability of the results. Moreover, despite the heterogeneity in grain

42 mycotoxin content across a given field, we showed that heads can be harvested manually for

43 agronomic experiments provided that sampling is representative (evenly distributed over the

44 entire plot area). Finally, we found that delaying the assay after harvest affected the results

45 obtained and should therefore be avoided.

46

47 **Key words:** deoxynivalenol; nivalenol; sampling; sample preparation; sample conservation

48 effect

49

Introduction

Since the 1990s, several major health crises have shaken the food industry and interest in food safety has increased. One of the key elements of the potential health risk associated with dietary cereals is the accumulation of mycotoxins in grains (*1-3*). Vomiting, reproductive disturbances, leukoencephalomalacia, pulmonary oedema, impairment of the humoral and cellular immune responses, nervous disorders, myocardial hypertrophy and several cancers may result from the ingestion of mycotoxins (*4*). Mycotoxins are fungal secondary metabolism products (*2, 5*) and result from the adaptation of fungal growth to stressful situations (*6*). The pathogenic fungal complex of the genus *Fusarium* is the principal producer of mycotoxins, notably of deoxynivalenol and nivalenol, in grains of growing crops (*7*). *Fusarium* and *Microdochium* also cause a cereal disease, *Fusarium* head blight (*8-10*). *Fusarium* mycotoxins are increasingly being studied in an attempt to prevent new major health crises. Agronomic experiments are carried out to assess the effects of different sources of variation on grain mycotoxin content (*10-15*). Reliable mycotoxin measurement techniques and appropriate sampling procedures are essential for such studies. Mycotoxin contamination is highly heterogeneous in cereal fields (*16*) and grain samples (*17-21*). Almost 90% of the error associated with aflatoxin testing can be attributed to the method used to obtain the original sample (*21*). Moreover, aflatoxin may only be present at high concentrations in less than 0.5% of the peanut crop and concentrations may be as high as 1,000,000 µg/kg in of contaminated peanuts (*17*). We assessed the uncertainty of mycotoxin determination and the effect of mycotoxin sampling procedures on mycotoxin contamination levels for the *Fusarium* mycotoxins, deoxynivalenol and nivalenol. The chemical structures of these toxins are presented in **Figure 1**. We aimed to characterize the errors occurring during each step of the procedure, from the field to the laboratory (**Figure 2**): sampling in the field (mechanical *versus* manual methods); sample preparation (flour or grain); and sample conservation.

76

Materials and methods

Experimental design and mycotoxin analysis

The samples used in this study came from a long-term experiment, the aim of which was to compare different cropping systems and assess the effects of several cropping systems on mycotoxin levels in winter wheat (*12*). Nine agronomic treatments were duplicated in this design and two growing seasons were used (2001/02 and 2002/03). Thirty-six plots were available for this methodological study, of which we used only 16. These plots were chosen according to the variability of head blight attacks. Plots A, B, H and P were cropped under a conventional system, M was cropped under an integrated system, I, N and Q were cropped under an integrated direct drilling system whereas C, D, O, F, J, K, L and R were cropped under an organic system. We extended the range of systems and mycotoxin contents studied by also including two farmers' fields cropped under an organic direct drilling system in 2002/03 (plots E and G): we therefore sampled a total of 18 plots.

90
91 Mycotoxin analyses were performed by the Qualtech laboratory (Vandoeuvre-les-Nancy, France). Levels of the trichothecenes nivalenol and deoxynivalenol were determined. Each sample (flour or grain) received by the laboratory was homogenised at least three times, in a mixer/divider. A small quantity of each sample was taken (20-25g), and in the case of grain samples was ground. Trichothecenes were determined by gas chromatography-mass spectrometry (GC-MS). This method was validated by the French norm NF EN ISO/CEI 17025. The assay laboratory estimates the measurement error for trichothecenes at 20% (differences in extraction rate and errors in sample preparation in the laboratory assay are included).

In addition, according to laboratory assay data, the detection limit (d_l) was 30 µg/kg for trichothecenes, and the quantification limit (q_l) was twice the detection limit (60 µg/kg). For

102   the purpose of this study, mycotoxin contents below d_l or q_l were assigned values equal to

103   half of these limits: 15 and 30 μg/kg, respectively.

104

105   ***Effect of sample preparation and mycotoxin measurement uncertainty***

106   Twenty-four samples from the 18 plots were harvested mechanically (roughly 2 kg in total).

107   They were dried at 80 °C for 48 h. Half of the 24 available samples (nos. 1 to 12) were

108   completely ground and three flour subsamples of roughly 300g each were used for mycotoxin

109   analysis; this procedure for sample preparation before analysis was called "flour-flour" (flour

110   subsamples taken from a sample already ground into flour). For each of the twelve remaining

111   samples, three grain subsamples of roughly 300g each were taken. The subsamples from

112   samples 13 to 15 were completely ground and sent for mycotoxin analysis; we called this

113   procedure "grain-flour" as the original sample was in the form of grain and only converted to

114   flour after subsampling. The subsamples from samples 16 to 24 were not ground and sent

115   directly for analysis (referred to as the "grain-grain" procedure, as both sample and subsample

116   are in grain form). The mycotoxin content data obtained for these 24 samples were also used

117   to evaluate the uncertainty of mycotoxin determination.

118   Mycotoxin analyses are destructive, making it impossible to carry out several measurements

119   on the same sample. It is therefore difficult to characterise the repeatability and

120   reproducibility of the assays rigorously because these two parameters must be determined for

121   a single sample (*22*). However, we estimated the uncertainty of the assays by dividing each of

122   the 24 samples into three subsamples . Measurement uncertainty characterises the dispersion

123   of the values that could reasonably be attributed to the measurand (*22-24*) and may be

124   estimated using a linear mixed model (*25*). We used the following model to describe our data:

125    $$Y_{ijk} = \mu + \alpha_i + (A_i)_j + \varepsilon_{ijk}$$

5

126   where Y_{ijk} is the measured toxin content; μ the general mean toxin content; α_i a variable for

127   the ith sample, which has fixed effect; $(A_i)_j$ a variable for the jth preparation, which has random

128   effect because the toxin content of the jth preparation depended on the toxin content of the

129   sample A_i; and ε_{ijk} the standard error, relating to $_k$, the mycotoxin content of the subsample.

130   The classic *proc glm* with the *random option* program of SAS software was used to calculate

131   the intrapreparation method variance as the difference between the variance of $(A_i)_j$ and the

132   standard error. Measurement uncertainty was then calculated as half the confidence interval

133   (CI) estimated using the following equation:

134    $$\text{Measurement uncertainty} = CI/2 = \left[t_{((1-\alpha/2)(n-1))} \times \sqrt{(variance(A_i)_j - variance\,\varepsilon_{ijk})/n} \right]/2$$

135   where n is the number of subsamples.

136

137   We used all the mycotoxin content data, regardless of the method of sample preparation, to

138   estimate the mean square (population variance) from the variability of a given set of

139   mycotoxin measures . We have therefore estimated mycotoxin measurement uncertainty

140   according to the mixed model described above but with the $(A_i)_j$ term eliminated.Thus, Yij

141   was described by the following relationship: $\mathbf{Y_{ij} = \mu + \alpha_i + \varepsilon_{ij}}$. The preparation methods were

142   not distinguished so $_j$ stands for the $_j^{th}$ subsamples of the $_i^{th}$ sample. In this model, ε_{ij}, which

143   characterises the modelling error, was associated with the maximum value of variability for

144   the assay. The maximum measurement uncertainty can therefore be expressed as:

145    $$\text{Measurement uncertainty} = CI/2 = \left[t_{((1-\alpha/2)(n-1))} \times \sqrt{(variance\,\varepsilon_{ij})/n} \right]/2$$

146   This model is based on three assumptions. The first is equality of the variances for each level

147   of variables. The other assumptions are normality and independence of the variables with

148   random effect: kurtosis and skewness coefficients and the distribution of residues with respect

149   to predicted values were also assessed.

150

151 The effects of preparation procedures on measurement variability were investigated for plots

152 A, B, C and P, Q, R for which two types of sample preparation were carried out. The $Y_{ijk} =$

153 $\mu + \alpha_i + (A_i)_j + \varepsilon_{ijk}$ model was used to estimate (i) interpreparation variance, i.e. the variance

154 of $(A_i)_j$ and (ii) intrapreparation variance, i.e. the ratio between the difference between

155 interpreparation variances (those of $(A_i)_j$) and the standard error (those of ε_{ijk}) and the number

156 of subsample mycotoxin content values ($_k$). We investigated whether there was an

157 interpreparation effect or an intrapreparation effect by means of a Chi^2 test comparing these

158 variances and the population variance. These effects were also estimated by calculating the

159 variation coefficient for mycotoxin content (as the ratio of mean square and mean), and the

160 standard deviation for each preparation.

161

162 ***Effect of the harvest procedure***

163 Three in-field sampling procedures were investigated (one mechanical and two manual

164 harvest methods) and compared on three plots (P, Q and R). Grain mycotoxin content was

165 measured just after harvest. For mechanical harvesting, grain sampling was based on the

166 98/53/CE directive (*26*), which was subsequently modified by the 2002/27/CE directive (*27*),

167 a document that lays down the sampling procedure for official controls of aflatoxin level. This

168 directive was used because there is no equivalent text dealing with *Fusarium* toxins.

169 According to this directive, for plots with yields below 1 tonne, 10 samples of 100 g each

170 must be collected and pooled to give a total sample of 1 kg. The samples (in our case roughly

171 2 kg) were then dried at 80 °C for 48 h. For the first method of manual harvest (the "hundred

172 method"), we collected ten randomly selected samples of 100 heads each from each

173 experimental plot (at least 1 kg). For the second method (the "quadrat method") we collected

174 the heads from nine quadrats made up of 1m x 2 adjacent rows from each experimental plot

175 (900g-1 kg). The harvested heads were dried at 80 °C for 48h and the glumes and rachis were

176   separated from the grains. Grains from all the hand-harvested heads were pooled to give a

177   total sample per plot for each method. The three samples from each plot were completely

178   ground and subsamples of flour (each weighing approximately 300g) were sent for mycotoxin

179   analysis.

180

181

182   *Effect of the grain storage procedure*

183   The effect of the grain storage procedure was investigated on several plots. In each case,

184   subsamples of approximately 300g were collected for mycotoxin analysis. Five kinds of

185   storage process were tested: storage at room temperature for eight months (plots A, B and C);

186   at 4 °C for two months (plots H, I and J); at –20°C for two months (plots H, I and J); at room

187   temperature for two months (plots H, I and J and M, N and O); and no storage at all (assay

188   performed immediately after harvest) for all nine plots.

189

190   We evaluated the effect of the different harvest and storage methods used by comparing the

191   variances associated with these methods with the estimated population variance for each

192   toxin, by means of a Chi^2 test. If a significant effect was observed, Bonferroni correction was

193   applied.

194

195   **Results**

196   *Mycotoxin measurement uncertainty*

197   For each sample, the various mycotoxin measurements obtained are presented in **Table 1**. We

198   checked that ε_{ij} for deoxynivalenol and nivalenol analyses were randomly distributed (results

199   not shown) and followed a Gaussian distribution: the coefficients of kurtosis and skewness for

200   deoxynivalenol were 0.65 and 0.25 respectively, and those for nivalenol were 2.328 and –

201 0.23, respectively. This variable with a random effect was therefore normally distributed and

202 independent. No significant differences were observed in the variance of ε_{ij} (according to

203 Bartlett's test with $\alpha \leq 35\%$) for nivalenol. For deoxynivalenol content, eight samples

204 (numbers 1, 9, 14, 16, 17, 18, 20, 23) presented ε_{ij} variances significantly higher than those

205 for the other 16 samples. To take into account the three assumptions on which the model was

206 based, the uncertainty of nivalenol determinations was calculated using all the samples

207 whereas that for deoxynivalenol was estimated using the 16 samples for which no significant

208 inequality was observed in the variances of ε_{ij} (according to Bartlett's test with $\alpha = 10\%$).

209

210 For nivalenol, measurement uncertainty was 15 µg/kg (mean square: 956.4), whereas for

211 deoxynivalenol, measurement uncertainty was at least 38 µg/kg (mean square: 153.6). It

212 should be noted that (i) if the samples excluded due to heterogeneity in variance were

213 included, then measurement uncertainty was even higher for deoxynivalenol (81 µg/kg) and

214 (ii) six of the eight samples excluded from the calculation of deoxynivalenol measurement

215 uncertainty corresponded to grain-grain preparations rather than grain-flour or flour-flour

216 preparations.

217

218 For plots A, B and C, the standard deviations of deoxynivalenol and nivalenol measurements

219 were lower after the flour-flour procedure than after the grain-grain procedure, with values of

220 62 and 5 versus 98 and 9, respectively. With the exception of the deoxynivalenol

221 measurements for plot A, the variability of measurements (estimated by the coefficient of

222 variation on each plot) was lower for analyses on flour samples than for those on grain

223 samples (**Figure 3a**). For grain samples taken for plots P, Q and R, variability was also lower

224 if subsamples were ground (grain-flour procedure) than if they were not ("grain-grain"

225 procedure), except for the deoxynivalenol measurements for plot P (**Figure 3b**). The standard

226 deviation was 54 for deoxynivalenol and 3 for nivalenol for samples sent for analysis in the

227 form of flour, versus 76 and 16, respectively, for samples sent for analysis in the form of

228 grain. The results from plots A, B, C, P, Q and R therefore suggest that the variability of the

229 mycotoxin measurements may be reduced by early grinding of the samples. The results

230 obtained with the $Y_{ijk} = \mu + \alpha_i + (A_i)_j + \varepsilon_{ijk}$ model provided no evidence of an intrapreparation

231 effect: according to the population variance analysis, the variability of intrapreparation

232 mycotoxin levels (grain or flour) was similar. However, this model revealed an

233 interpreparation effect on deoxynivalenol contamination ($\alpha = 0.05$) for plots A, B, C and P,

234 Q, R. There was also an interpreparation effect on nivalenol contamination ($\alpha = 0.01$) for

235 plots P, Q and R: mycotoxin contamination levels were higher for grain samples than for flour

236 samples, except for nivalenol contamination in plots P, Q and R, for which the opposite result

237 was obtained.

238

239 *Effect of harvest and sample storage methods*

240 All the results obtained were very similar (**Table 2**), but deoxynivalenol and nivalenol levels

241 tended to be higher in cases of manual harvest by the quadrat method than in cases of

242 "hundred harvest" or mechanical harvest. This trend was confirmed by the Chi^2 test ($\alpha = $

243 0.10). Bonferroni's test graded ($\alpha = 0.05$) the deoxynivalenol levels obtained by the quadrat

244 method were higher than those obtained by the other methods, and nivalenol levels obtained

245 by the quadrat method were higher than those obtained by the mechanical method.

246

247 Deoxynivalenol levels seemed to be lower when measured two and eight months after harvest

248 than when they were measured at harvest (**Table 3**). This result was confirmed by the results

249 of a Chi^2 test with $\alpha = 0.10$ and a Bonferroni's test ($\alpha = 0.05$) performed on whole plots. A

250 similar trend was observed for nivalenol contamination but was found to be non-significant

251 (Chi^2 test with $\alpha = 0.10$) for plots with the four types of storage tested. On plots on which only

252 two types of storage were tested, the type of storage was found to have a significant effect

253 (Chi^2 test with $\alpha = 0.05$) on nivalenol contamination. The results of the Bonferroni's test ($\alpha =$

254 0.05) showed that nivalenol contamination after eight months of storage was lower than that

255 with no storage, but no difference was observed between two months of storage and no

256 storage.

257

258 **Discussion**

259 The results of any assay are biased by measurement uncertainty resulting from the variability

260 of the sample (dependent on the method used to select samples, sample size, sample quality)

261 and variability of the measurements (dependent on the measurement method, operator, kind of

262 analytical method and number of analytical measurements) (*25, 28*). Sampling constitutes the

263 greatest source of error, followed by subsampling and analysis (*29*).

264

265 The laboratory that performed the analysis in this study estimated the variability of its assays

266 at 20%. We found that the measurement uncertainty for a sample, estimated by means of

267 mycotoxin analysis on subsamples, was low: **nivalenol determinations were accurate to**

268 **within 20 µg/kg** up to a minimum nivalenol concentration of 60 µg/kg (the quantification

269 limit) and the measurement uncertainty was less than 26% of the concentration of nivalenol

270 measured. Thus, **the subsampling procedure adopted did not increase the variability of**

271 **mycotoxin concentrations measured**. However, it should be pointed out that these

272 encouraging results were obtained with only a small number of plots. It would also be useful

273 to analyse more highly contaminated samples.

274

275 **The accuracy of deoxynivalenol measurement was lower, with a measurement**

276 **uncertainty of up to 40 μg/kg**. However, this result corresponds to 22% of the measurement

277 mean, similar to the variability of other analyses. Our results also show that **grinding grain**

278 **as soon as possible may minimise errors**. Similar results have been obtained for aflatoxin in

279 shelled peanuts (*28*) and for deoxynivalenol in wheat (*25, 30*). Indeed, the trend towards

280 lower variability when samples or subsamples were ground probably reflected the grinding of

281 a larger number of grains than would be the case for a grain sample ground in the laboratory

282 just before testing. This may increase the uniformity of the sample, resulting in lower

283 variability. These findings require confirmation and should be taken into account in future

284 agronomic studies.

285

286 The mixed model used made it possible to estimate the mean and the mean square of

287 deoxynivalenol and nivalenol contamination levels of a "field population". We considered the

288 population to be variable, with a random effect. This made it possible to take into account

289 correlations between several measurements carried out on several subsamples originating

290 from a given sample, although we assumed that assays were independent. In fact, subsample

291 content determinations are independent, but measurement results are not themselves

292 independent because analyses were carried out on subsamples taken from the same given

293 initial sample.

294

295 Part of the reason for the choice of this model lies in the fact that a model lacking an

296 interaction term between the mycotoxin levels of sample and subsample, $Y_{ijk} = \mu + \alpha_i + \beta_j +$

297 ε_{ijk}, may be biased by this interaction, should such an interaction exist. Moreover, a classic

298 model including an interaction between subsample and sample, $Y_{ijk} = \mu + \alpha_i + \beta_j + \gamma_{ij} + \varepsilon_{ijk}$

299 $_{ijk}$, may be biased by the independence of subsample assays: in our case, the subsamples are

300   taken from the same sample plot and are therefore not true repetitions, which must be taken

301   from different plots cropped in a similar fashion.

302

303   Despite the heterogeneity of mycotoxin content within a field (*16*), **mycotoxin**

304   **contamination may be analysed following harvesting by manual means if the**

305   **heterogeneity of contamination is taken into account by representative sampling, evenly**

306   **distributed over the entire area of the plot**. Taking ten samples of one hundred heads

307   seemed to give better results than analyses of the heads in nine quadrats made up of 2 rows x

308   1 m, probably simply because the size of the sample considered was greater.

309

310   *Fusarium* mycotoxins are known to be stable to heat and chemical treatments (*31, 32*), so the

311   lower levels of mycotoxin contamination recorded when toxin levels were not assessed

312   immediately after harvest probably does not correspond to a real decrease, resulting instead

313   from high measurement uncertainty or from changes in the sample during storage. Our

314   calculations suggest that high measurement uncertainty is not responsible for the observed

315   decrease. The second possibility, that changes occur in the sample during storage, therefore

316   appears more likely. Without more data on the question, it is possible for example that mould

317   could have either modified the grain samples and thus the toxin extraction rate, or have

318   degraded the toxin with an enzyme such as acetyltransferase Ayt1p (33), This enzyme was

319   found to be responsible for a decrease in the amount of deoxynivalenol six weeks after

320   inoculation in a previous study (34). A third explanation is a modification of the ratio of

321   acetonitrile / water during grain storage: this ratio strongly influences the extraction rate of

322   deoxynivalenol and nivalenol, and may also explain our results. **It would therefore seem**

323   **advisable to sort and grind samples immediately after harvest and, if this is not possible,**

324   **to minimise the time interval between harvest and analysis.**

325

326   These results, which are of potential value for agronomic research, are also likely to be useful

327   for the harmonisation of mycotoxin-sampling plans (*28*). They may also contribute to the

328   standardisation of maximum limits, which currently differ between countries (*35*), and

329   thereby facilitate international trade (*28, 36*).

330

336

References

1. Le Boulc'h, V.; Bedouret, S.; Poyet, V.; Barchietto, T.; Hazouard, D.; De Paepe, I.; Seng, J.M. Un test innovant pour contrôler la qualité sanitaire des céréales : détection des champignons potentiellement producteurs d'une famille de mycotoxines, les trichothécènes. *AFPP - 6ème conférence internationale sur les maladies des plantes, Tours, France*; 2000, Vol. 1, pp. 451-458.

2. Galtier, P.; Comera, C.; Oswald, I.; Puel, O. Mycotoxines : origines et toxicités. *AFPP - 6ème conférence internationale sur les maladies des plantes, Tours, France*; 2000, Vol. 1, pp. 77-86.

3. Gilbert, J.; Tekauz, A. Review: Recent developments in research on Fusarium head blight of wheat in Canada. *Can. J. of Plant Pathol.* **2000**, *22*, 1-8.

4. Placinta, C.M.; D'Mello, J.P.F.; Macdonald, A.M.C. A review of worldwide contamination of cereal grains and animal feed with *Fusarium* mycotoxins. *Anim. Feed Sci. Technol.* **1999**, *78*, 21-37.

5. Chelkowski, J. Fungal pathogens influencing cereal seed quality at harvest. In *Cereal grain: mycotoxins, fungi and quality in drying and storage.* (J. Chelkowski, ed.); Developments in Food Science, 26, Elsevier; Amsterdam, 1991, pp 53-56.

6. Thouvenot, D. Moisissure et mycotoxine. In *Mycotoxines : un prochain enjeu de sécurité alimentaire ?*; (CERAAF, ed.); Les cahiers de recherche, N°2, 2002, pp 14-17.

7. Maurin, N.; Chenet, I. Espèces de *Fusarium* présentes dans les cultures de céréales en France. *Phytoma.* **1993**, *453*, 20-22.

8. Cassini, R. Facteurs favorables ou défavorables au développement des fusarioses et septorioses du blé. *Proc. Meeting of sections cereals and physiology, Dijon*; 1970, pp. 271-279.

361   9. Parry, D.W.; Jenkinson, P.; McLeod, L. *Fusarium* ear blight (scab) in small grain cereals -

362   a review. *Plant Pathol.* **1995**, *44*, 207-238.

363   10. Champeil, A.; Doré, T.; Fourbet, J.F. Review - *Fusarium* head blight: Epidemiological

364   origin of the effects of cultural practices on head blight attacks and the production of

365   mycotoxins by *Fusarium* in wheat grains. *Plant Sci.* **2004**, *166*, 1389-1415.

366   11. Dill-Macky, R.; Jones, R.K. The effect of previous crop residues and tillage on *Fusarium*

367   head blight of wheat. *Plant Dis.* **2000**, *84*, 71-76.

368   12. Champeil, A.; Fourbet, J.F.; Doré, T. Influence of cropping system on *Fusarium* head

369   blight and mycotoxin levels in winter wheat. *Crop Protect.* **2004**, *23*, 531-537.

370   13. Teich, A.H. Epidemiology of wheat (*Triticum aestivum L.*) scab caused by *Fusarium* spp.

371   In *Fusarium: mycotoxins, taxonomy and pathogenicity*; (J. Chelkowski, ed.); Elsevier;

372   Amsterdam, 1989, pp 269-282.

373   14. Lemmens, M.; Haim, K.; Lew, H.; Ruckenbauer, P. The effect of nitrogen fertilization on

374   *Fusarium* head blight development and deoxynivalenol contamination in wheat. *J.*

375   *Phytopathol.* **2004**, *152*, 1-8.

376   15. Cromey, M.G.; Shorter, S.C.; Lauren, D.R.; Sinclair, K.I. Cultivar and crop management

377   influences on fusarium head blight and mycotoxins in spring wheat (*Triticum*

378   *aestivum*) in New Zealand. *New Zealand J. Crop Horticult. Sci.* **2002**, *30*, 235-247.

379   16. Raimbault, J.M.; Orlando, D.; Grosjean, F.; Leuillet, M. Méthodes d'échantillonnage et de

380   quantification des mycotoxines - Réussir à trouver des aiguilles dans une meule de

381   foin. *Perspect. Agri.* **2002**, *278*, 32-35.

382   17. Whitaker, T.B.; Wiser, E.H. Theoretical investigations into the accuracy of sampling

383   shelled peanuts for aflatoxin. *J. Am. Oil Chem. Soc.* **1969**, *46*, 377-379.

384   18. Armitage, D. Grain sampling methods to achieve consumer confidence and food safety. In

385   *HGCA Research Review*; Home Grown Cereals Authority; London UK, 2003.

386 19. Hart, P.; Schabenberger, O. How to sample wheat to accurately determine vomitoxin

387   levels. http://www.msue.msu.edu/imp/modab/26309701.htm, 1999, date on last

388   accessed 18th February, 2004.

389 20. Codex Alimentarius Commission. Report of the 33rd session of the codex alimentarius

390   committee on food additives and contaminants. ALINORM 01/12A. FAO/WHO;

391   2001, pp. 300.

392 21. GIPSA. Grain fungal diseases and mycotoxin reference. GIPSA, Technical Services

393   Division, Kansas City, 1999, pp. 54.

394 22. Feinberg, M. *La validation des méthodes d'analyse - Une approche chimiométrique de*

395   *l'assurance qualité au laboratoire*; Masson: Paris, 1996, pp. 397.

396 23. ISO. Guide to the Expression of Uncertainty in Measurement. *2001 ALINORM 01/23*.

397   1993.

398 24. Codex Alimentarius Commission. Report of the 23rd session of the codex committee on

399   methods of analysis and sampling - ALINORM 01/23. FAO/WHO; 2001, pp. 51.

400 25. Hart, L.P. Variability of vomitoxin in truckloads of wheat in a wheat scab epidemic year.

401   *Plant Dis.* **1998**, *82*, 625-630.

402 26. Commission Directive. Commission directive 98/53/EC of the 16 July 1998 on laying

403   down the sampling methods and the methods of analysis for the official control of the

404   levels for certain contaminants in foodstuffs. *Official Journal* **1998**, *201*, 93-101.

405 27. Commission Directive. Commission directive 2002/27/EC of the 13 March 2002

406   amending Directive 98/83/EC on laying down the sampling methods and the methods

407   of analysis for the official control of the levels for certain contaminants in foodstuffs.

408   *Official Journal* **2002**, *75*, 44-45.

409 28. Whitaker, T.B. Standardisation of mycotoxin sampling procedures: An urgent necessity.

410   *Food Control* **2003**, *14*, 233-237.

411 29. Whitaker, T.B.; Dickens, J.W. Variability of aflatoxin test results. *J. Am. Oil Chem. Soc.*
412   **1974**, *51*, 214-218.

413 30. Whitaker, T.B.; Hagler, W.M., Jr.; Giesbrecht, F.G.; Johansson, A.S. Sampling, sample
414   preparation, and analytical variability associated with testing wheat for
415   deoxynivalenol. *J. AOAC Int.* **2000**, *83*, 1285-1292.

416 31. Vidal, D.R. Propriétés immunosuppressives des mycotoxines du groupe des
417   trichothécènes. *Bulletin de l'institut Pasteur.* **1990**, *88*, 159-192.

418 32. Quillien, J.F. Les mycotoxines. Institut National de la Recherche Agronomique (INRA) -
419   Centre de Réseaux pour l'Innovation en Agriculture et Agroalimentaire (CRIAA);
420   2002, pp. 24.

421 33. Mitterbauer, R.; Adam, G. *Saccharomyces cerevisae* and *Arabidopsis thaliana*: Useful
422   model systems for the identification of molecular mechanisms involved in resistance
423   of plants to toxins. *Eur. J. of Pl. Pathol,* **2002**, *108*, 699-703.

424 34. Miller, J.D.; Young, J.C. Deoxynivalenol in an experimental *Fusarium graminearum*
425   infection of wheat. *Can. J. of Pl. Pathol.* **1985**, *7*, 132-134.

426 35. Food and Agriculture Organization. Worldwide regulations for mycotoxins 1995- A
427   compensium. FAO Food and Nutrition; 1997, pp. 64.

428 36. FAO/WHO/UNEP. Report of the Third Joint FAO/WHO/UNEP International Conference
429   on Mycotoxins - MYC-CONF/99/REPe. FAO/WHO/UNEP International; 1999, pp.
430   22.

431

432

433 **Table 1**: Mycotoxin levels were determined for three subsamples per sample, with each
434 sample corresponding to a plot. No result indicates that no analysis was carried out; <d_l
435 mycotoxin not detected (< 30 µg.kg^{-1}); <q_l mycotoxin level lower than the quantification limit
436 (< 60 µg.kg^{-1}).
437

Preparation sample when sampling	sent for analyse	Sample	Plot	Deoxynivalenol, µg/kg			Nivalenol, µg/kg		
				sub sample 1	sub sample 2	sub sample 3	sub sample 1	sub sample 2	sub sample 3
Flour	Flour	1	A	150	160	320	<d_l	<d_l	<d_l
		2	B	240	290	240	<d_l	<d_l	<d_l
		3	C	330	310	390	<q_l	<q_l	<d_l
		4	D	60	100	120	<q_l	<q_l	<d_l
		5	E	370	380		<q_l	<q_l	
		6	F	110	90	<d_l	<d_l	<d_l	<d_l
		7	G	200	190		<q_l	<q_l	
		8	H	240	250	250	<q_l	<q_l	<q_l
		9	I	800	650	600	120	150	150
		10	J	110	100	130	60	60	80
		11	K	110	100		60	<d_l	
		12	L	100	<d_l		70	<d_l	
Grain	Flour	13	P	190	160	100	<q_l	<q_l	<q_l
		14	Q	550	400	430	80	80	70
		15	R	110	90	120	<q_l	<q_l	<q_l
Grain	Grain	16	A	210	330	170	<d_l	<d_l	<q_l
		17	B	360	120	210	<d_l	<q_l	<d_l
		18	C	350	500	360	<q_l	<d_l	<d_l
		19	M	<q_l	<d_l	<d_l	<d_l	<d_l	<d_l
		20	N	500	700	600	<q_l	<q_l	<q_l
		21	O	350	340	340	<d_l	60	<q_l
		22	P	200	240	200	<q_l	<q_l	<q_l
		23	Q	550	340	550	60	<d_l	60
		24	R	<d_l	60	110	<d_l	<d_l	<q_l

438

439 **Table 2**: Mycotoxin contamination determination according to harvest method. $<q_l$ mycotoxin
440 level lower than quantification limit (< 60 µg.kg^{-1}).
441

		Plot P	Plot Q	Plot R	Bonferroni
Deoxynivalenol, µg/kg	Quadrat harvest	350	600	240	a
	Hundred harvest	180	550	160	b
	Mechanical harvest	190	550	110	b
Nivalenol, µg/kg	Quadrat harvest	70	60	90	a
	Hundred harvest	$<q_l$	70	60	ab
	Mechanical harvest	$<q_l$	80	$<q_l$	b

442
443

444 **Table 3**: Mycotoxin contamination according to type of storage. No result indicates that no
445 analysis was carried out; $<d_l$ mycotoxin not detected (< 30 µg.kg^{-1}); $<q_l$ mycotoxin level
446 lower than the quantification limit (< 60 µg.kg^{-1}).
447

		0 months	2 months			8 months
	Plots	no conservation	ambient temperature	4 °C	-20 °C	ambient temperature
Deoxynivalenol, µg/kg	H	230 ⎫	240 ⎫	230 ⎫	230 ⎫	
	I	800 ⎬ a	500 ⎬ b	630 ⎬ b	600 ⎬ b	
	J	140 ⎭	140 ⎭	120 ⎭	110 ⎭	
	A	150 ⎫				220 ⎫
	B	310 ⎬ a				240 ⎬ b
	C	600 ⎭				370 ⎭
	M	$<d_l$ ⎫	$<q_l$ ⎫			
	N	1100 ⎬ a	600 ⎬ b			
	O	600 ⎭	340 ⎭			
Nivalenol, µg/kg	H	$<q_l$ ⎫	$<q_l$ ⎫	$<q_l$ ⎫	$<q_l$ ⎫	
	I	110 ⎬ a	70 ⎬ b	90 ⎬ b	90 ⎬ b	
	J	70 ⎭	50 ⎭	$<q_l$ ⎭	50 ⎭	
	A	60 ⎫				$<q_l$ ⎫
	B	440 ⎬ a				$<q_l$ ⎬ b
	C	$<d_l$ ⎭				$<q_l$ ⎭
	M	$<d_l$ ⎫	$<d_l$ ⎫			
	N	200 ⎬ a	$<q_l$ ⎬ a			
	O	$<q_l$ ⎭	60 ⎭			

448

449   FIGURES LEGENDS
450
451   **Figure 1**: Chemical structures of type B trichotecenes.
452
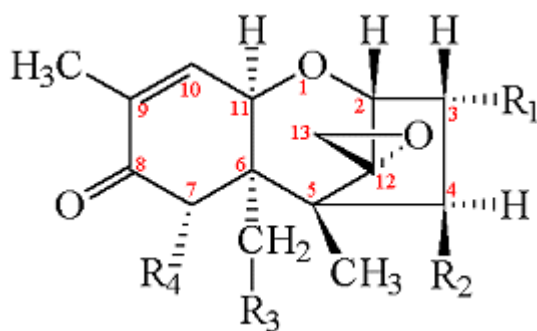453   **Figure 2**: Steps of the grains sampling procedures
454
455   **Figure 3**: Effect of sample preparation on the variability of mycotoxin contamination
456   measurement for plots A, B and C (a) and for plots P, Q and R (b).
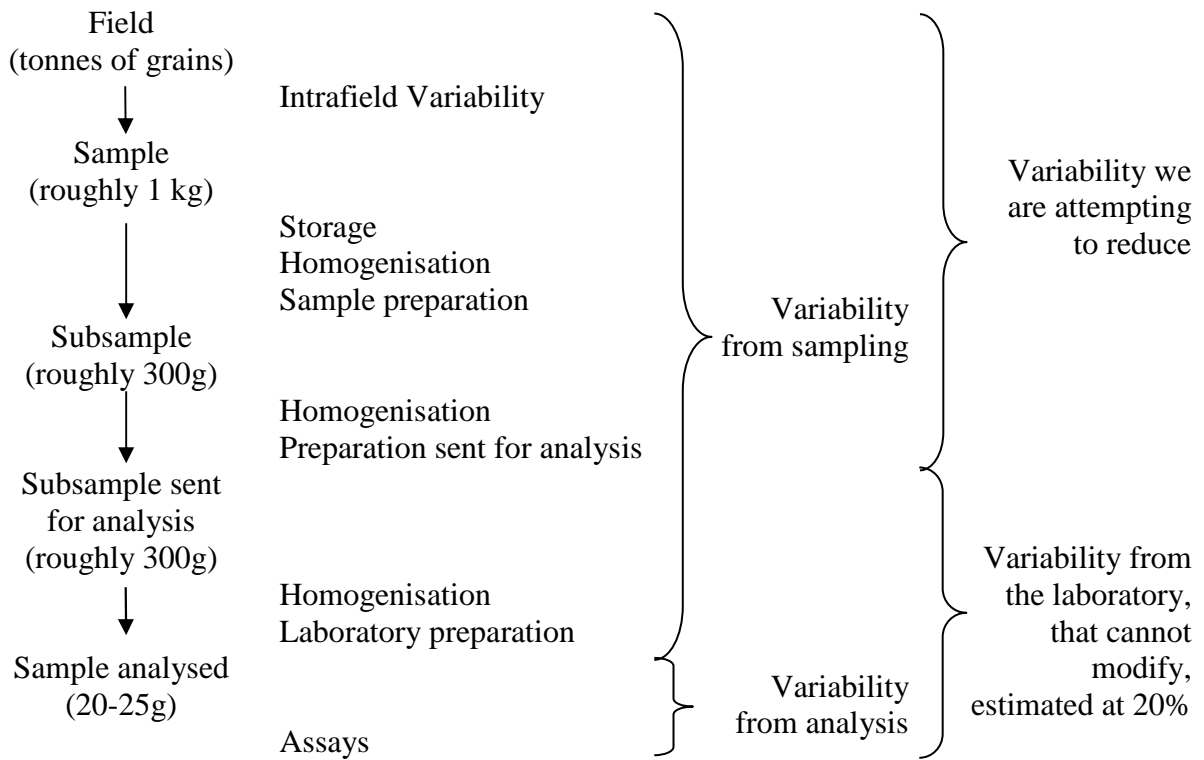457
458

Figure 1:

Type B trichothecenes: Deoxynivalenol (R1 = OH, R2 = H, R3 = OH, R4 = OH)
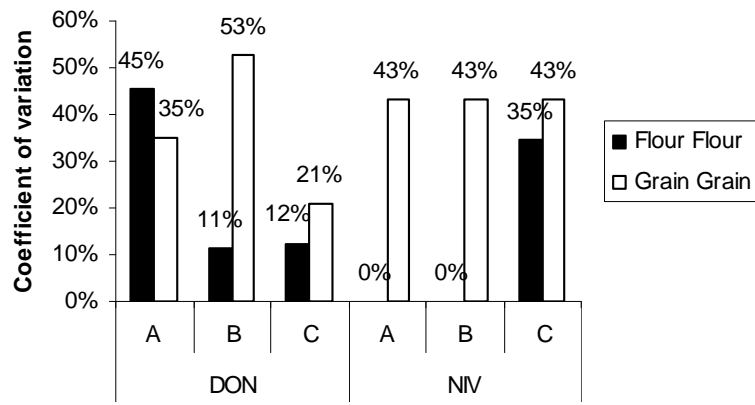   Nivalenol (R1 = OH, R2 = OH, R3 = OH, R4 = OH)

461
462

463   Figure 2:
464
465

Field
(tonnes of grains)
↓   Intrafield Variability

Sample
(roughly 1 kg)

Storage
Homogenisation
Sample preparation

Subsample
(roughly 300g)

Homogenisation
Preparation sent for analysis

Subsample sent
for analysis
(roughly 300g)

Homogenisation
Laboratory preparation

Sample analysed
(20-25g)

Assays

Variability
from sampling

Variability
from analysis

Variability we
are attempting
to reduce

Variability from
the laboratory,
that cannot
modify,
estimated at 20%
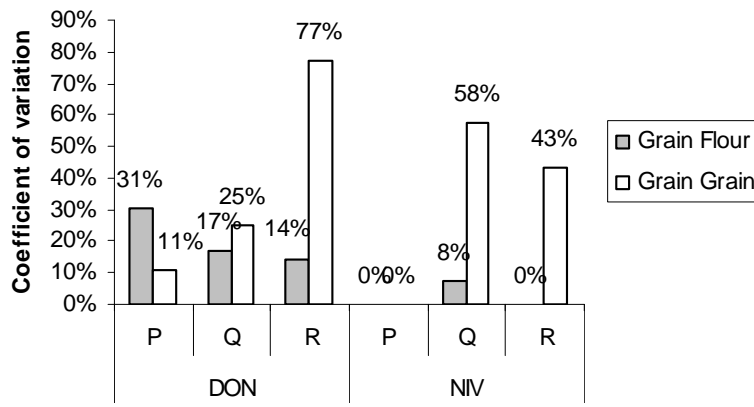
466

467   Figure 3a:

468

469

470

471

472

473

474

475

476

477

478

479

480

481
482　Figure 3b:
483
484
485
486
487
488
489
490
491
492