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Method evaluation of *Fusarium* DNA extraction from mycelia and wheat for down-stream real-time PCR quantification and correlation to mycotoxin levels

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Abstract

Identification of *Fusarium* species by traditional methods requires specific skill and experience and there is an increased interest for new molecular methods for identification and quantification of *Fusarium* from food and feed samples. Real-time PCR with probe technology (Taqman®) can be used for the identification and quantification of several species of *Fusarium* from cereal grain samples. There are several critical steps that need to be considered when establishing a real-time PCR-based method for DNA quantification, including extraction of DNA from the samples. In this study, several DNA extraction methods were evaluated, including the DNeasy® Plant Mini Spin Columns (Qiagen), the Bio robot EZ1 (Qiagen) with the DNeasy® Blood and Tissue Kit (Qiagen), and the Fast-DNA® Spin Kit for Soil (Qbiogene). Parameters such as DNA quality and stability, PCR inhibitors, and PCR efficiency were investigated. Our results showed that all methods gave good PCR efficiency (above 90%) and DNA stability whereas the DNeasy® Plant Mini Spin Columns in combination with sonication gave the best results with respect to *Fusarium* DNA yield. The modified DNeasy® Plant Mini Spin protocol was used to analyse 31 wheat samples for the presence of *F. graminearum* and *F. culmorum*. The DNA level of *F. graminearum* could be correlated to the level of DON ($r^2 = 0.9$) and ZEN ($r^2 = 0.6$) whereas no correlation was found between *F. culmorum* and DON/ZEA. This shows that *F. graminearum* and not *F. culmorum*, was the main producer of DON in Swedish wheat during 2006.
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Keywords: Absolute quantification; Deoxynivalenol; *Gibberella zeae*; TaqMan; qPCR; Zearalenone

1. Introduction

Fusarium species are found in cereals and other cultivated crops in Europe and in other parts of the world (Bahtnager et al., 2002; Miller, 1994; Parry et al., 1995; Placinta et al., 1999; Tanaka et al., 1988). Most of them are able to produce one or more mycotoxins with varying degree of toxicity (Bottalico and Perrone, 2002; Langseth et al., 1999). The trichothecenes constitute the largest group of *Fusarium* toxins found in cereals but also zearalenone (ZEN) and fumonisins are detected (Bahtnager et al., 2002; Hussein and Brasel, 2001; Tanaka et al., 1988). The *Fusarium* toxins have been evaluated by the European Commission (Scientific Committee on Food, 1999, 2000a,b,c, 2001) and by JECFA (WHO, 2001). Deoxynivalenol (DON), T2, and HT-2 were identified as the most critical mycotoxins based on their occurrence in cereals being close to the Tolerable Daily Intake (TDI) (Scientific Committee on Food, 1999, 2001). In 2006, the European Union decided on a uniform legislation to protect the health of the consumers and set limit values for DON, ZEN, Fum B1 and B2 in unprocessed and processed foods including cereals (EC 1881/2006). Limit values will also be determined for T2 and HT-2 (EC 1881/2006). *F. graminearum* (sexual stage = *Gibberella zeae*), *F. culmorum*, *F. avenaceum, F. poae*, and *F. sporotrichioides* are common in grain and are

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causative agents of Fusarium head blight disease (FHB), which causes great yield loss worldwide (Parry et al., 1995). Each *Fusarium* species produce a more or less characteristic set of toxins but strain variations within a species also occurs (O'Donnell et al., 2000). *F. graminearum* and *F. culmorum* are the main producers of DON and ZEN in wheat (WHO, 2001) and consumption of these toxins can cause nausea, diarrhoea, vomiting, skin irritation, and feed refusal (WHO, 2001). *F. graminearum* is common worldwide and dominates in warmer climates whereas *F. culmorum* has been limited to cooler regions (Miller, 1994; Parry et al., 1995). However, there are reports showing that *F. graminearum* is spreading also in the cooler regions of Northern Europe (Bottalico and Perrone, 2002; Waalwijk et al., 2003). The increased cultivation of maize, which is an important host for *F. graminearum*, changes in tillage practice, as well as climatic changes, has been suggested to explain this (Birzele et al., 2002; Dill-Mackay and Jones, 2000).

Quantification and identification of *Fusarium* species have traditionally relied on culture methods and morphological classification that require specific expertise and experience. Culturing methods take time and are dependent on living propagules, which may not be related to toxin levels. In recent years, several PCR-based techniques have been developed to overcome this problem, for review see Edwards et al. (2002). PCR-based identification has several applications such as to study the dynamics of different *Fusarium* species over time and between geographical regions in cereals or other environments or to study disease development in the field. It can also be an important tool in the risk assessment of grain as a screening method to identify samples with potentially high mycotoxin content to reduce costs for chemical analyses.

Real-time PCR methods have been described for several *Fusarium* species including *F. graminearum*, *F. culmorum*, *F. avenaceum*, and *F. poae* and correlation between DNA from single or groups of *Fusarium* species and their corresponding mycotoxins has been evaluated (Leisová et al., 2006; Sarlin et al., 2006; Schnerr et al., 2002; Waalwijk et al., 2004; Yli-Mattila et al., in press). In wheat from the Netherlands, correlation between DON and DNA was found for both *F. graminearum* and *F. culmorum* (Waalwijk et al., 2004) whereas in Finnish barley only *F. culmorum* could be correlated to DON production (Sarlin et al., 2006). Schnerr et al. (2002) used the *tri5* as the target gene and showed that DNA from the group of trichothecene-producing species correlated to DON in wheat. However, when using the *tri5* gene as target, no information is obtained on the species contribution to the toxin production. Different PCR-based methods employ different DNA extraction methods as well as different primer and probe systems. The degree of correlation between DNA and mycotoxin in grain has also shown to vary substantially. As a first step to a more standardized approach, this paper presents an evaluation of several DNA extraction methods from fungal mycelia in wheat and an optimized extraction method for the external standard used for absolute quantification of *Fusarium* DNA in grain. We also investigated the correlation between DNA levels of *F. graminearum* and *F. culmorum* and their mycotoxins DON and ZEN in 31 Swedish wheat samples.

2. Materials and methods

2.1. Fungal strains and mycelium production

Fusarium graminearum, strain IBT 1958, and *Fusarium culmorum*, strain IBT 2303, were used to produce DNA standards for the absolute quantification of *Fusarium* in wheat. Both strains are deposited at the DTU culture collection in Lyngby, Denmark. To produce fungal mycelia, the isolates were grown in MEA-broth (LP0039, Oxoid Ltd, Hampshire, UK) in a water bath (100rpm) at 25 ± 0.5°C for 5days. The mycelium was washed twice in sterile tap water, centrifuged for 10min at 4000×*g*, and then freeze-dried (Edwards Modulyo freeze dryer). The freeze-dried mycelia were stored in eppendorf tubes at − 20°C until DNA extraction.

2.2. Wheat samples

Thirty-one wheat samples were selected from field trials from the harvest of 2006 in the middle and south parts of Sweden and collected within the project "Survey of fusarioses and fusariumtoxins in winter wheat" sponsored by Swedish Farmers' Foundation for Agricultural Research.

The wheat samples (100g) were milled to fine powder on a Waring Blender (Torrington, Connecticut, USA) and used for both chemical toxin analysis and DNA extraction. The samples were freeze-dried (see above) and stored at − 20°C until DNA extraction.

2.3. DNA isolations from fungal mycelia

Genomic DNA of *F. graminearum* and *F. culmorum* was extracted from 10mg of freeze-dried mycelia using four different methods (A, B, C, and D). All extractions were performed in duplicates. Method A was based on the commercial kit DNeasy® Plant Mini Spin Columns (Qiagen, Solna, Sweden) but modified in the cell lysis and protein removal steps. Fungal mycelium (10mg), 200µl S3 lysis buffer [66mM Tris, 3.3% Triton-X, 1.65M guanidinium–HCL, 0.825M NaCl, and ddH_2O, pH 7.9; (Mulfinger et al., 2000)], 4µl RNAse A (100mg ml^{-1}, Qiagen), 5µl proteinase K (20mg ml^{-1}, Sigma-Aldrich), and 200µl AP1 lysis buffer (provided by the DNeasy® Plant Mini Spin Column kit) were added to Lysis Matrix A tubes (BIO101 Systems, Qbiogene). The tubes were run in a FastPrep® Cell Disrupter, model FP120 (BIO101 Savant, Qbiogene) for 20s at speed level 5.0. The lysate was incubated in a heating block at 65°C for 1h and thereafter treated according to the protocol supplied with the DNeasy® Mini Spin Column kit (Qiagen). An additional phenol extraction step [once with one volume phenol:chloroform: isoamylalcohol (25:24:1) and twice with one volume chloroform:isoamylalcohol (24:1)] was added after the sample had passed through the QIAshredder column supplied by the DNeasy® Mini Spin Column kit.

Method B used the Bio robot EZ1 (Qiagen) with the DNeasy® Blood and Tissue Kit (Qiagen). Freeze dried mycelia was lysed in 350µl G2 buffer (supplied with the kit) and 250U of lyticase (Sigma-Aldrich, MO, USA) as suggested in the user-

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developed protocol for fungi (supplied by Qiagen). The lysate was incubated in a heating block at 30°C for 30min and then separated from cellular debris by centrifugation at 18000×*g* for 10min. The lysate was transferred to the robot sample tubes and placed in the Bio robot sample rack. DNA was extracted according to the protocol programmed on the EZ1 DNA Bacteria Card and eluted in 200µl elution buffer.

Method C was the commercial Fast-DNA® Spin Kit for Soil (Qbiogene, CA, USA). The mycelium was added to Lysis Matrix A tubes (BIO101 Systems, Q-Biogene) and run in a FastPrep® Cell Disrupter, model FP120 (BIO101 Savant, Qbiogene) for 20s at speed level 5.0 (speed and time were modified from the kit protocol). The extraction was then carried out according to the manufacturer's instructions.

Method D was a CTAB-based method used for GMO-analysis of plant material (Community Reference Laboratory for GM Food and Feed, 2005). DNA was extracted according to the protocol (Community Reference Laboratory for GM Food and Feed, 2005) and dissolved in 200µl AE-buffer (supplied by the DNeasy kit from Qiagen).

To examine RNA contamination, 10µl of each DNA extract was analysed by gel electrophoresis on a 1% agarose gel (Sigma-Aldrich, Type II) in 0.5X TBE-buffer and visualized by ethidium bromide. The DNA was quantified using Nanodrop ND-1000 Spectrophotometer (Saveen Werner, Malmö, Uppsala). To avoid freezing and thawing, the DNA was aliquoted (10–20µl) before storage in − 20°C until analysis. DNA purity was evaluated by comparing the absorbance ratios A260/280 and A260/230. Dilution curves of all extracts were analysed by real-time PCR and the Ct-values were plotted against known (given by the Nanodrop Spectrophotometer) DNA concentrations. The standard curves were compared with respect to PCR efficiency, intercept and r^2-value. The standard DNA extracts were also compared for their ability to quantify *Fusarium* DNA from three grain samples.

2.4. DNA isolation from wheat

Three extraction methods (A, B, and C) were used to isolate total DNA from milled wheat samples (15, 26, and 27). Method D was too laborious and gave too low DNA yield of fungal mycelia to be considered for the grain samples. Methods B and C were performed as previously described for fungal mycelia. Method A was based on the method described by Waalwijk et al. (2004) and similar to method A used for fungal mycelia (using DNeasy® Mini Spin Columns from Qiagen). We modified the method by Waalwijk et al. (2004) by extending the sonication time from 5 to 30s and by increasing the sample size from 10 to 100–200mg. The grain samples were mixed with 400µl S3 buffer (Mulfinger et al., 2000) and sonicated for 30s at maximum amplitude with an ultrasonic processor, model UP 100H (Dr Hielscher GmbH, Stahnsdorf, Germany), using a 5mm sonotrode. After sonication, 8µl RNase A (100mg ml^{-1}, Qiagen), 10µl proteinase K (20mg ml^{-1}, Sigma-Aldrich), and 400µl AP1 buffer (supplied in the DNeasy® Mini Spin Columns) were added and the samples were incubated in a heating block at 65°C for 30min. After incubation, 260µl of AP2 buffer (supplied by the DNeasy® Mini Spin Columns) was added and the samples were loaded on to the QIAshredder spin columns. The DNA extraction was thereafter carried out according to the manufacturer's instructions.

To further increase the yield of total DNA with method B, the Bio robot, the lysis step was modified by sonication of the samples for 30s before addition of the lyticase (method B*) or by using the lysis step from method A (S3 buffer, sonication and incubation at 65°C for 30min) instead of enzymatic lysis (method B**).

All extractions were performed in duplicates and eluted in 200µl elution buffer. The extraction methods for wheat were evaluated with respect to total DNA yield and DNA purity as analysed by the Nanodrop ND-1000 Spectrophotometer (Saveen Werner) and specific yield of *F. graminearum* and *F. culmorum* and the presence of PCR inhibitors as analysed by real-time PCR.

2.5. Primers and probes

The primers (*graminearum* MGB-R, *graminearum* MGB-F for *F. graminearum* and *culmorum* MGB-R, *culmorum* MGB-F for *F. culmorum*) and probes (*graminearum* MGB probe and *culmorum* MGB probe) were designed and evaluated by Waalwijk et al. (2004) for their specificity and sensitivity. The probes to detect the two *Fusarium* species were labelled 5′-terminal with FAM (6-carboxy-fluorescein) and 3′-terminal with MGB/non fluorescent quencher. All primers and probes were purchased from Applied Biosystems, CA, USA. Negative samples were re-analysed with the TaqMan® Exogenous Internal Positive Control (Applied Biosystems) to verify that they were true negative samples and not the result of PCR inhibitors.

2.6. TaqMan analysis

The real-time PCR analyses were performed on an ABI Prism Model 7500 (Applied Biosystems). For each TaqMan® reaction 5µl sample or standard DNA was mixed with 25µl PCR reaction mix containing 1X TaqMan® universal Master mix (no. 4324018, Applied Biosystems), 83nM FAM-labelled probe, and 333nM of each forward and reverse species-specific primer. The thermal cycling conditions included a single step of 2min at 50°C and 10min at 95°C, followed by 40 cycles of 95°C for 15s and 60°C for 1min. All PCR reactions were performed in duplicates.

Standard curves were generated by analysis of 10-fold serial dilutions in the range of 1 to 1×10^4pg μl^{-1} of DNA (duplicate samples) from pure cultures of *F. graminearum* and *F. culmorum*. The quantification limit was 1pg DNA μl^{-1}, which correspond to 1pg DNA mg dry weight $grain^{-1}$. The DNA content is presented as pg DNA mg dry weight $grain^{-1}$.

2.7. Analysis of ZEN and DON in wheat

Milled grain (10g) was extracted with 40ml acetonitril:water [84:16% (v/v)]. The samples were shaken by hand and put in an ultra sonication bath (Transsonic 1040/H from Elma, Singen, Germany) for 10min. Subsequently, the samples were shaken

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for 2h on a shaker (IKA Werke yellow line OS 5 basic, Staufen, Germany) and centrifuged at 3300 ×*g* for 12min. Six ml of extract was transferred to a Mycosep 224 column (Romer Labs, Tulln, Austria) for clean up and 4ml of the filtrate from the column was transferred to a centrifuge tube. The samples were evaporated to dryness on a VR-Maxi vacuum centrifuge (Heto, Allerod, Denmark) and were redissolved in 1ml methanol:water [25:75 (v/v)] and placed in the ultra sonication bath for a few minutes and mixed on a Whirley mixer. Finally the samples were filtered through a 0.45µm filter and analysed on all LC-MSMS.

The chromatographic separation was performed on a Hewlett-Packard 1100 system with gradient elution. Forty µl was injected on a 250 × 2.1mm BDS Hypersil C 18, 5µm column from Thermo Electron Corporation (Waltham, Massachusetts, US).

MSMS detection was performed with an Applied Biosystems Sciex API 2000 instrument in electrospray negative multiple reaction (MRM) ionisation mode. The detection limits were 10µg kg^{-1} for DON and 2µg kg^{-1} for ZEN. Relative standard deviation on results was 10%.

2.8. Statistical analysis

Statistical analyses were performed using MiniTab 14 Statistical Software and Microsoft Office Excel 2003.

3. Results

3.1. Evaluation of DNA extraction methods from fungal mycelia

The four methods (A, B, C, and D) used to extract DNA from freeze-dried mycelium of *F. graminearum* and *F. culmorum* were evaluated with respect to RNA contamination, DNA yield, and DNA purity (A260/280 and A260/230 ratio). Method A (DNeasy® Plant Mini Spin columns, Qiagen) and C (Fast-DNA® Spin Kit for Soil, Qbiogene) gave the highest total DNA yields, 440 ± 95 and 405 ± 55ng mg^{-1} mycelia, respectively (mean value including both *F. graminearum* and *F. culmorum*, $n = 4$). Method B (extraction robot EZ1 and DNeasy® Blood and Tissue Kit, Qiagen) gave a yield of 210 ± 20ng mg^{-1} mycelia (including both *F. graminearum* and *F. culmorum*, $n = 4$) and the CTAB method (method D) gave a yield of 35 ± 35ng mg^{-1} mycelia (including both *F. graminearum* and *F. culmorum*, $n = 4$). The extraction robot (method B) was the least laborious (approximately 1h for six samples) and the CTAB method was the most laborious (approximately 1day for 10–20 samples).

Table 1
DNA from *F. graminearum* and *F. culmorum* was extracted with methods A, B, C, and D

Method	Species	Efficiency (%), mean (SD)	Intercept (C_T), mean (SD)
A [a]	*F. graminearum*	94(3)	42(2)
	F. culmorum	96(3)	41(1)
B [b]	*F. graminearum*	93(5)	43(1)
	F. culmorum	97(5)	41(2)
C [c]	*F. graminearum*	93(5)	43(1)
	F. culmorum	98(5)	39(1)
D [d]	*F. graminearum*	97(8)	43(4)
	F. culmorum	97(8)	39(3)

Each extract was analysed by real-time PCR in ten-fold serial dilutions and analysed for PCR efficiency and standard curve intercept. The PCR analyses were repeated four times within three months ($n=4$). The R^2-value of the standard curves were always >0.99.

Table 2
Total DNA from three wheat samples (100–120 mg) was extracted with methods A, B, and C

Method	Sample 15 (a/b) (ng DNA mg dry weight $wheat^{-1}$), mean(SD)	Sample 26 (a/b) (ng DNA mg dry weight $wheat^{-1}$), mean(SD)	Sample 27 (a/b) (ng DNA mg dry weight $wheat^{-1}$), mean(SD)
A [a]	105(7)	273(4)	179(19)
B [b]	74(6)	80(6)	74(1)
B* [c]	–	99(10)	–
B** [d]	–	60(6)	–
C [e]	48(3)	106(19)	148(1)

[a]Modified method based on the DNeasy® Plant Mini Spin Column kit by Qiagen.
[b]Bio robot EZ1 from Qiagen with the DNeasy® Blood and Tissue Kit.
[c*]Method B with modifications in the lysis step by sonication of the samples for 30 s before addition of lyticase.
[d**]Method B with modifications in the lysis step by using the lysis step from method A (S3 buffer and sonication).
[e]Fast-DNA® Spin Kit for Soil from Qbiogene.
Each extraction was performed in duplicates and the total amount of DNA extracted with each method is presented as ng DNA mg dry weight $wheat^{-1}$.

There was no visible contamination of RNA in any of the DNA extracts as seen by gel electrophoresis (data not shown). The A260/280 ratios were close to 1.8 for DNA extracted with methods A and C (1.81–1.91 for method A and 1.79–1.96 for method C) but the A260/230 ratios were lower with C (0.1–0.2) than with A (1.8–2.34). This may indicate high concentration of salt or organic solvents in the DNA extracted with method C. The A260/280 and A260/230 ratios for DNA extracted with method B were 1.67–2.00 and 0.30–1.81, respectively. Because of the low yield from the CTAB method (method D), close to the limit of quantification of the Nanodrop Spectrophotometer, the absorbance ratios could not be accurately calculated. Method A was the best DNA extraction method for fungal mycelia with respect to DNA yield and purity.

All DNA extracts were analysed by real-time PCR in ten-fold dilutions in the range of 10ng $µl^{-1}$ to 1pg $µl^{-1}$ of DNA. The genome sequence of *F. graminearum* predicts a genome size of 36Mb, which equals 0.04pg. This value was used to calculate the number of genome equivalents in the DNA standard solutions of both *F. graminearum* and *F. culmorum*. Standard curves were generated with the 7500 System Software (Applied Biosystems) and the slope and intercept were compared between the DNA extracts of the different extraction methods (Table 1). The slope was used to determine the efficiency ($E = 10^{-1/\text{slope}} - 1$) of the PCR reaction.

There were no significant differences in the PCR efficiency nor the standard curve intercept of the four extraction methods

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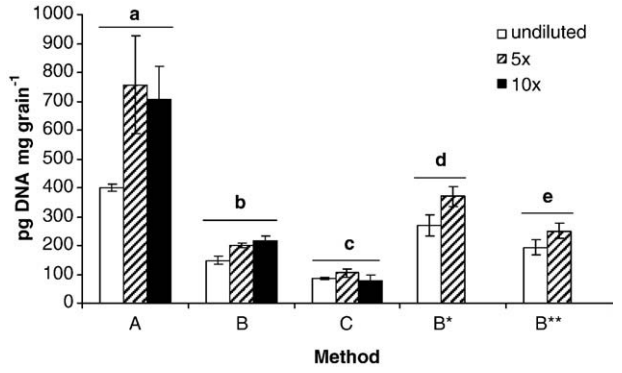

Fig. 1. Total DNA was extracted from wheat using extraction method A (modified method based on the DNeasy® Plant Mini Spin Column kit by Qiagen), method B (Bio robot EZ1 from Qiagen with the DNeasy® Blood and Tissue Kit), method B* (Bio robot EZ1with modifications in the lysis step by sonication of the samples before enzymatic lysis), method B** (Bio robot EZ1 with modifications in the lysis step by using S3 buffer and sonication instead of enzymatic lysis), and method C (Fast-DNA® Spin Kit for Soil from Qbiogene). The DNA extracts were diluted 5- and 10-fold and the undiluted and diluted sub samples were analysed by real-time PCR for the presence of PCR inhibitors. Each extraction was made in duplicates and each sub sample was analysed in duplicates by real-time PCR analysis. Error bars represent ± standard error. The different letters (a–e) indicate significant different levels of DNA ($P<0.05$, ANOVA).

(Table 1). All methods gave high PCR efficiency (> 90%). The intercept was generally lower for *F. culmorum* than for *F. graminearum*. The standard curve was linear for five (methods A, B, C) or four (method D) orders of magnitude.

To evaluate the DNA stability at − 20°C, real-time PCR analysis was repeated four times within a three-month period. Both PCR efficiency and intercept of the standard curve varied between analyses but no time-dependent tendencies could be identified (data not shown) and the results from the four analyses were therefore considered as repetitions (Table 1). To study DNA stability during storage, DNA was quantified with the Nanodrop spectrophotometer prior to each PCR analysis. A decrease in DNA concentration was seen after storage in − 20°C (data not shown) but this decrease was eliminated by heat-treatment in 90°C for 1min before quantification.

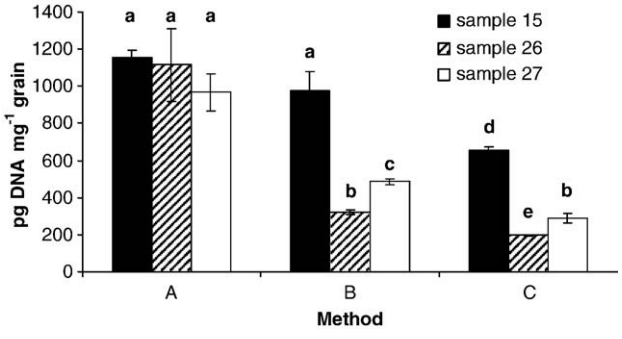

Fig. 2. Total DNA was extracted from three wheat samples (15, 26, and 27) using extraction method A (modified method based on the DNeasy® Plant Mini Spin Column kit by Qiagen), method B (Bio robot EZ1 from Qiagen with the DNeasy® Blood and Tissue Kit) and method C (Fast-DNA® Spin Kit for Soil from Qbiogene). The standard DNA from fungal mycelia of *F. graminearum*, strain IBT 1958, used for absolute DNA quantification, was extracted with method A. Each DNA extraction was performed in duplicates and each extract was analysed in duplicates by real-time PCR. All samples were diluted 5-fold prior to PCR analysis. Error bars represent±standard error. The different letters (a–f) indicate significant different levels of DNA ($P<0.05$, ANOVA).

3.2. Evaluation of DNA extraction methods from wheat samples

Methods A, B and C were evaluated with respect to total DNA yield (grain DNA + fungal DNA), DNA purity, presence of PCR inhibitors, and yield of target DNA (*F. graminearum* and *F. culmorum*). Total DNA yield varied between samples and between methods. DNA extraction was most efficient, i.e. gave the highest yield of total DNA, with method A (Table 2). The extraction robot (B) generally gave lower total DNA yield than methods A and C but was more consistent between the three samples. With the addition of sonication before enzymatic lysis (method B*) it was possible to increase total DNA yield from the Bio Robot but it was still lower than for methods A and C

Table 3
DNA from 31 wheat sample were extracted with method A (modified method based on the DNeasy® Plant Mini Spin Column kit by Qiagen) in duplicates (2 × 200 mg)

Wheat samples	*F. graminearum* [a] (pg DNA mg dry weight grain $^{-1}$), mean(SD)	*F. culmorum* [a] (pg DNA mg dry weight grain^{-1}), mean(SD)	DON [b] (ppb)	ZEN [b] (ppb)
1	310(75)	<1	84	26
2	155(1)	1(1)	151	6
3	45(5)	80(4)	54	6
4	80(30)	1(1)	<10	3
5	280(40)	<1	143	2
6	55(15)	<1	33	<2
7	420(275)	30(1)	283	120
8	10(5)	<1	<10	<2
9	3(1)	2(1)	<10	<2
10	20(3)	70(4)	188	10
11	2(2)	<1	<10	<2
12	300(35)	<1	390	<2
13	210(50)	70(5)	335	10
14	6(3)	<1	<10	<2
15	1990(395)	125(5)	1530	843
16	1290(335)	2(1)	1533	466
17	880(110)	1(1)	756	251
18	1550(60)	<1	851	254
19	1530(95)	100(5)	1203	126
20	1490(275)	185(5)	1219	170
21	1170(190)	120(0)	955	216
22	150(10)	130(2)0	324	60
23	450(30)	<1	524	2
24	130(10)	8(1)	148	45
25	370(75)	10(0)	213	49
26	1285(340)	2(0)	1193	<2
27	995(85)	1(0)	1015	56
28	40(25)	0(0)	12	<2
29	215(20)	7(2)	290	<2
30	20(9)	3(1)	75	<2
31	105(40)	1(0)	139	32

Each DNA extract was analysed for the concentration of *F. graminearum* and *F. culmorum* ($n=2$) by real-time PCR analysis. The concentration of deoxynivalenol (DON) and zearalenone (ZEN) were analysed as single samples by LC-MSMS.

[a] The detection limit for *Fusarium* DNA was 1 pg DNA mg dry weight grain^{-1}.

[b] The detection limit for DON was 10 ppb and for ZEN 2 ppb.

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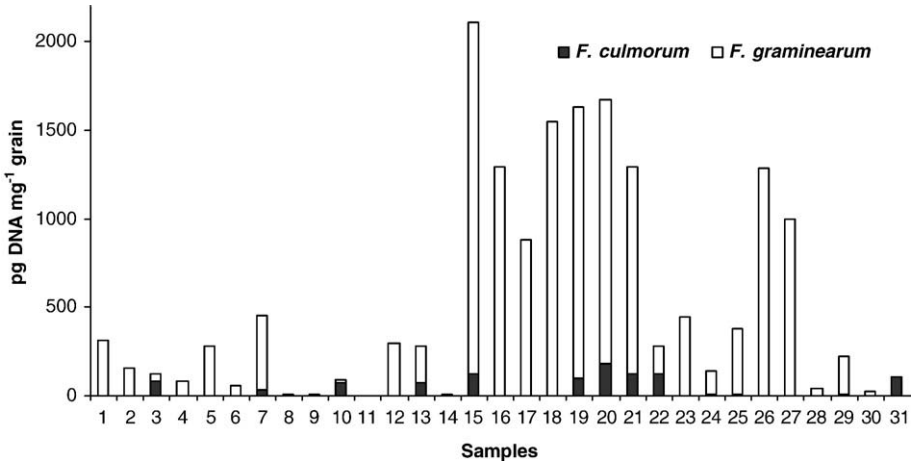

Fig. 3. Distribution of DNA from *F. graminearum* and *F. culmorum* in 31 Swedish wheat samples from the 2006 harvest. Each wheat sample was extracted in duplicates and each extract was analysed in duplicates in the real-time PCR.

(Table 2). The A260/280 and A260/230 ratios were 1.87 ± 0.01 and 1.73 ± 0.12 for method A, 1.78 ± 0.02 and 2.04 ± 0.13 for method B, and 2.05 ± 0.28 and 0.49 ± 0.37 for method C.

Undiluted and diluted (5- and 10-fold) sub samples of the wheat DNA extracts were analysed by real-time PCR. No significant differences could be identified between the undiluted and diluted samples, partly due to large standard error for method A (dilution 5 and 10×; Fig. 1). However, the estimated levels of target DNA were higher in the diluted sub samples than in the undiluted samples for all methods, indicating the presence of PCR inhibitors. For further quantification of *Fusarium* DNA, all samples were diluted five times to avoid PCR inhibitors. The estimated target DNA concentrations in all samples (diluted or undiluted) were significantly lower in samples extracted with methods B and C than with method A ($P < 0.001$ for undiluted and $P < 0.05$ for diluted samples, ANOVA; Fig. 1). Target yield could be increased with the BioRobot by modifying the cell lysis steps (methods B* and B**).

Target DNA (*F. graminearum*) was quantified by real-time PCR in DNA extracts from three wheat samples each extracted with three methods (methods A, B, and C; Fig. 2). When extracted with method A, no significant difference (ANOVA) in estimated DNA content was seen between the three samples (Fig. 2). However, when extracted with methods B and C, the DNA content differed significantly between the samples (ANOVA, $P = 0.01$ and $P = 0.001$ for B and C, respectively; Fig. 2). Method C extracted significantly lower DNA levels than method B (ANOVA, $P < 0.05$) for all samples.

External standard DNA extracted with methods A, B, and C were compared for the absolute quantification of target DNA in the three wheat samples (15, 26, and 27). ANOVA analysis showed that the estimated concentrations of *Fusarium* DNA differed significantly when using the three standards ($P < 0.001$). The estimated target DNA levels when using methods B and C for extraction of standard DNA were 68–73% and 36–41%, respectively, compared to when using method A. Tukey's test showed that standard B gave significantly lower target DNA estimations than standard A and that standard C gave significantly lower target DNA levels than both standard A and B ($P < 0.05$).

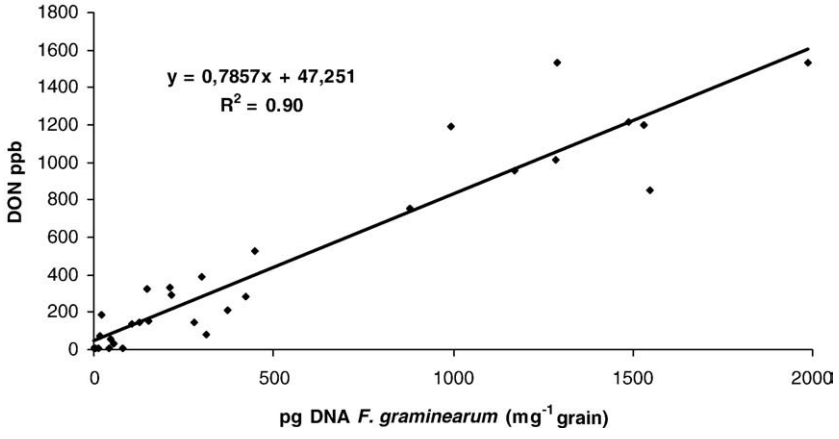

Fig. 4. The concentration of *F. graminearum* DNA (pg DNA mg wheat^{-1}) analysed by real-time PCR (mean value, see Table 3) correlated to the concentration of DON analysed by LC-MSMS in 31 Swedish wheat samples from the 2006 harvest.

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For further quantification of *F. graminearum* and *F. culmorum* in wheat, method A was chosen for extraction of both external DNA from fungal mycelia and sample DNA from wheat.

3.3. Detection of *F. graminearum* and *F. culmorum* DNA in 31 Swedish wheat samples

Method A was used to extract total DNA from 31 Swedish wheat samples from the harvest of 2006 and *F. graminearum* and *F. culmorum* DNA was quantified by real-time PCR (Table 3, Fig. 3). *F. graminearum* was detected in 100% of the samples of which 77% of the samples contained over 10pg DNA mg^{-1} dry weight grain. *F. culmorum* was detected in 70% of the samples but only 30% of the samples had levels over 10pg DNA mg^{-1} dry weight grain. Overall, *F. graminearum* was the most dominating of the two DON producing species in the samples (Table 3, Fig. 3).

3.4. *F. graminearum* and *F. culmorum* content correlated to deoxynivalenol (DON) and zearalenone (ZEN) levels

The concentration of DON and ZEN was analysed in the wheat samples (Table 3). To evaluate whether the extraction method based on the modified DNeasy® Plant Mini Spin Column protocol in combination with real-time PCR can be used for the estimation of mycotoxin content in wheat, the concentration of *Fusarium* DNA was compared to the concentration of DON and ZEN in the wheat samples. In linear regression analysis, we found that *F. graminearum* correlated well with DON ($R^2 = 0.9$; Fig. 4) and ZEN ($R^2 = 0.6$; data not shown) and that *F. culmorum* did not correlate with ZEN ($R^2 = -0.1$) and only weakly with DON ($R^2 = 0.2$). The correlation between DON and the sum *F. graminearum* and *F. culmorum* DNA was lower than for *F. graminearum* DNA alone ($R^2 = 0.7$).

4. Discussion

In recent years, several methods for identification and quantification of *Fusarium* spp. from cereal grain using real-time PCR have been described (Dyer et al., 2006; Edwards et al., 2002; Leisová et al., 2006; Reischer et al., 2004; Sarlin et al., 2006; Schnerr et al., 2001, 2002; Waalwijk et al., 2004; Yli-Mattila et al., in press). In these studies, DNA extraction methods, primers, probes, and PCR technology differ making quantification results difficult to compare.

Currently, different DNA extraction methods are used for both grain samples and DNA standards and it is not clear how these have been evaluated. According to the guidance document on minimum performance requirements for analytical methods of GMO testing of food and feed, real-time PCR methods should have a slope of the standard curve in the range of − 3.1 and − 3.6, corresponding to a PCR efficiency of 80 to 110% and the R^2 coefficient of the standard curve should be ≥ 0.98 (European Network of GMO Laboratories, 2005). These guidelines should also be valid for the analysis of fungal DNA in food and feed. In this study, we evaluated four different DNA extraction methods of which all gave PCR efficiencies and standard curve R^2 coefficients within these intervals (Table 1). Yet, significant differences were still found between the methods when they were used to estimate *Fusarium* DNA in wheat DNA extracts. The standard curves were not superimposable, possibly due to impurities in the DNA extracts, which may have influenced the estimation of standard DNA concentration. Correct measurement of DNA in the standard is crucial for correct DNA quantification in the samples.

Extraction efficiency and presence of PCR inhibitors are important factors to consider when choosing extraction method for DNA quantification from natural samples. The three methods evaluated in this study differed in both extraction efficiency and presence of PCR inhibitors. The DNeasy-based method (method A) had the highest DNA extraction efficiency for both total and target DNA (Table 2, Figs. 1 and 2) but required a five-fold sample dilution to eliminate PCR inhibitors (Fig. 1).

The DNeasy-based method (method A) was chosen to quantify *F. graminearum* and *F. culmorum* DNA from 31 wheat samples. The results showed that *F. graminearum* was the dominating DON-producer in all wheat samples but three (Fig. 3, Table 3) and this confirms previous findings that *F. graminearum* can dominate also in cooler temperate geographical regions (Bottalico and Perrone, 2002; Waalwijk et al., 2003). Mycotoxin analysis showed that 84% of the samples contained > 10ppb of DON and 52% of the samples contained > 10ppb of ZEN. Two wheat samples contained DON above the EC limit value (1250ppb), whereas eight of the samples contained ZEN above the EC limit value (100ppb). To investigate whether the *Fusarium* DNA levels could be correlated to the levels of DON and ZEN in the samples, linear regression analysis was performed on the results. This analysis showed that the concentration of both DON ($r^2 = 0.9$) and ZEN ($r^2 = 0.6$) correlated well with the DNA levels of *F. graminearum* (Fig. 4) but not with *F. culmorum* ($r^2 = 0.2$ and − 0.1, respectively) indicating that *F. graminearum* was the most important contributor to DON and ZEN production in these samples. Regression analysis of the sum of *F. graminearum* and *F. culmorum* DNA did not improve the correlation between DNA and DON in the samples ($r^2 = 0.7$). The results showed that 1250ppb DON, which is the EC limit value for DON in unprocessed cereals, corresponded to approximately 1400pg DNA of *F. graminearum* mg^{-1} dry weight wheat. No samples with DON levels under the limit value of 1250ppb contained more than 1400pg DNA mg^{-1} dry weight wheat. Sarlin et al. (2006) showed that DON correlated well to the group of trichothecene-producing *Fusarium* in North American barley and that approximately 1000pg DNA mg $barley^{-1}$ of target DNA corresponded to the limit value 1250ppb. They suggested that barley samples from North America containing more than 500pg DNA mg^{-1} barley indicated a high risk of DON over the EC limit value. We have shown that the limit of 500pg DNA mg^{-1} dry weight grain is also relevant when using the real-time PCR method described in this study in the risk assessment of Swedish wheat. However, more data are necessary in order to set reliable DNA limits to estimate the mycotoxin content in Swedish grain. This study showed that *F. graminearum* was an important producer of DON in Swedish wheat during 2006 and that the method based on the DNeasy Plant Mini kit (Qiagen) in combination with real-time PCR can be used to quantify *Fusarium* DNA and to predict high DON levels in wheat.

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