

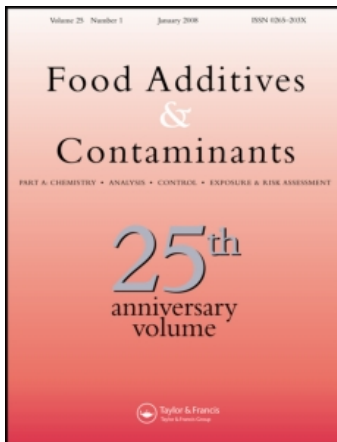
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A. De Girolamo <sup>a</sup>; V. Lippolis <sup>a</sup>; E. Nordkvist <sup>b</sup>; A. Visconti <sup>a</sup>

<sup>a</sup> Institute of Sciences of Food Production (ISPA), National Research Council (CNR), 70126 Bari, Italy <sup>b</sup> National Veterinary Institute, SE-751 89 Uppsala, Sweden

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## Rapid and non-invasive analysis of deoxynivalenol in durum and common wheat by Fourier-Transform Near Infrared (FT-NIR) spectroscopy

A. De Girolamo<sup>a\*</sup>, V. Lippolis<sup>a</sup>, E. Nordkvist<sup>b</sup> and A. Visconti<sup>a</sup>

<sup>a</sup>Institute of Sciences of Food Production (ISPA), National Research Council (CNR), via G. Amendola 122/O, 70126 Bari, Italy; <sup>b</sup>National Veterinary Institute, SE-751 89 Uppsala, Sweden

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Fourier transform near-infrared spectroscopy (FT-NIR) was used for rapid and non-invasive analysis of deoxynivalenol (DON) in durum and common wheat. The relevance of using ground wheat samples with a homogeneous particle size distribution to minimize measurement variations and avoid DON segregation among particles of different sizes was established. Calibration models for durum wheat, common wheat and durum + common wheat samples, with particle size <500 µm, were obtained by using partial least squares (PLS) regression with an external validation technique. Values of root mean square error of prediction (RMSEP, 306–379 µg kg<sup>-1</sup>) were comparable and not too far from values of root mean square error of cross-validation (RMSECV, 470–555 µg kg<sup>-1</sup>). Coefficients of determination ( $r^2$ ) indicated an “approximate to good” level of prediction of the DON content by FT-NIR spectroscopy in the PLS calibration models ( $r^2 = 0.71–0.83$ ), and a “good” discrimination between low and high DON contents in the PLS validation models ( $r^2 = 0.58–0.63$ ). A “limited to good” practical utility of the models was ascertained by range error ratio (RER) values higher than 6. A qualitative model, based on 197 calibration samples, was developed to discriminate between blank and naturally contaminated wheat samples by setting a cut-off at 300 µg kg<sup>-1</sup> DON to separate the two classes. The model correctly classified 69% of the 65 validation samples with most misclassified samples (16 of 20) showing DON contamination levels quite close to the cut-off level. These findings suggest that FT-NIR analysis is suitable for the determination of DON in unprocessed wheat at levels far below the maximum permitted limits set by the European Commission.

**Keywords:** FT-NIR spectroscopy; deoxynivalenol; wheat; PLS regression, discriminant analysis

### Introduction

Deoxynivalenol (DON) is a type B trichothecene mycotoxin produced by fungi of the *Fusarium* genus, in particular *Fusarium graminearum* (*Gibberella zea*) and *Fusarium culmorum*. It is frequently associated with contaminated cereal crops such as wheat, maize, barley, oats, rye and less often with rice, sorghum and triticale (Canady et al. 2001; Schothorst and van Egmond 2004). DON inhibits the synthesis of DNA, RNA and proteins, and has a hemolytic effect on erythrocytes. In animals, acute/subacute oral toxicity of DON is characterized by vomiting, feed refusal, weight loss and diarrhoea (Schlatter 2004).

Based on the temporary tolerable daily intake (TDI) of 1 µg kg<sup>-1</sup> body weight established by the EU Scientific Committee on Food (SCF) (SCF 2002), the European Commission has set maximum permitted levels for DON in a variety of cereal-based foods (European Commission 2007). In particular, 1750 and 1250 µg kg<sup>-1</sup> were set as maximum levels for

unprocessed durum and common wheat, respectively; lower levels were set for wheat flour, bran, germ and pasta (750 µg kg<sup>-1</sup>), for wheat and wheat products for direct human consumption (750 µg kg<sup>-1</sup> and 500 µg kg<sup>-1</sup>), and for wheat-based infants and baby food (200 µg kg<sup>-1</sup>).

Rapid and sensitive methods for the quantification of DON in wheat and wheat-based food products are urgently required by private and public quality control laboratories, as well as in surveys providing data for risk assessments. Several methods based on competitive enzyme-linked immunosorbent assays (ELISA) have been developed for the rapid screening and quantification of DON in cereals. ELISA methods are sensitive, rapid and easy-to-use, but they often show strong cross-reactivity against DON acetyl derivatives, require long incubation times for complete antigen–antibody reaction and commercial kits are still rather expensive. A fluorescence polarization (FP) immunoassay has been recently developed for

\*Corresponding author. Email: annalisa.degirolamo@ispa.cnr.it

the rapid and quantitative analysis of DON in durum wheat, semolina and pasta at levels below the EU maximum permitted levels (Lippolis et al. 2006).

Although these methods can be used for screening purposes, they are destructive and require preparation and extraction steps. Over the last few decades, many researchers have focused on the potential use of infrared spectroscopy in the analysis of food and feed, both in the mid- and near-infrared regions. Infrared spectroscopy (IR) is a rapid analytical technique requiring very little labour, once a calibration is obtained, and no sample extraction and, therefore, does not create chemical waste.

Pettersson and Aberg (2003) used near infrared (NIR) transmittance for the determination of DON in whole wheat kernel samples at levels higher than  $500 \mu\text{g kg}^{-1}$ , whereas NIR reflectance has been used for detection of scab and estimation of DON and ergosterol in single kernels of highly infected wheat (Dowell et al. 1999). A method for the analysis of *Fusarium* fungi on corn using Fourier transform-mid infrared (FT-MIR) spectroscopy with attenuated total reflection (ATR) was developed which enabled the segregation of DON-contaminated corn samples from non-contaminated (blank) samples (Kos et al. 2003, 2004, 2007; Delwiche and Hareland 2004).

Studies on the application of FT-IR spectroscopy for determination of DON content in grain mostly exploit the range below  $4000 \text{ cm}^{-1}$ . In particular, Mossoba et al. (1996) reported DON characteristic absorption bands between  $1244$  and  $1750 \text{ cm}^{-1}$ . Some additional bands at  $\sim 3500$  and close to  $3650 \text{ cm}^{-1}$  (Young and Games 1994) and not far from  $1700 \text{ cm}^{-1}$  (Abramovic et al. 2007) have been reported. The most recent theoretical calculations of IR bands cover the MIR range below  $4000 \text{ cm}^{-1}$  (Turker and Gumus 2008), also used by Kos et al. (2007) for corn analysis. Therefore, the identification of any spectral features of DON within the NIR range is highly desirable. FT-IR spectroscopy allows the performance of rapid measurements with an excellent signal-to-noise ratio and high resolution.

The aim of the present work was to investigate the feasibility of using FT-NIR for the rapid and non-invasive determination of DON in durum and common wheat.

## Materials and methods

### Sample preparation

A total of 262 wheat (143 durum and 119 common) samples, belonging to 32 different varieties, were obtained from wheat naturally infected by *Fusarium graminearum* and *Fusarium culmorum* in experimental fields in Northern Italy during the period 2002 to 2006. Wheat samples were ground with a Tecator Cyclotec

1093 (International PBI, Hoganas, Sweden) laboratory mill equipped with a  $500 \mu\text{m}$  sieve, and were analyzed by HPLC and FT-NIR spectroscopy. DON levels, as established by HPLC analysis, ranged from 50 to  $2600 \mu\text{g kg}^{-1}$  in durum wheat and from 50 to  $3000 \mu\text{g kg}^{-1}$  in common wheat. Samples containing less than  $50 \mu\text{g kg}^{-1}$  DON (the quantification limit of the HPLC method) were considered as DON-free. Mean recoveries of DON from durum and common wheat samples ( $n=3$ ) spiked at levels of 200, 800 and  $1500 \mu\text{g kg}^{-1}$  were 75–93% with  $\text{RSD}_r$  of 1.4–14.2% for durum wheat and 79–83% with  $\text{RSD}_r$  of 1.1–12.2% for common wheat. To investigate the distribution of DON during sieving, five durum wheat samples were ground by a Bühler MLI 204 (Bühler S.p.A., Milan, Italy) to pass through a 1-mm sieve and analyzed by HPLC for DON. A fraction of these samples was sieved to pass a  $300 \mu\text{m}$  sieve, and the two fractions (below and above  $300 \mu\text{m}$ ) were analyzed by HPLC.

### HPLC quantitative analysis

HPLC quantitative analysis was performed to obtain the reference value of the DON content for all wheat samples tested herein. Wheat samples were analyzed according to the procedure described by Visconti et al. (2004) with minor modifications. Briefly, 25 g of ground wheat was extracted with 100 ml of phosphate buffer solution (PBS, 10 mM sodium phosphate, 0.85% sodium chloride, pH 7.4) by blending at high speed for 2 min with a Sorvall Omnimixer (Sorvall Instruments, Norwalk, CN, USA). The extract was filtered through both filter paper (Whatman no. 4) and glass microfiber filter (Whatman GF/A) and 2 ml of the filtered extract were cleaned up by DONTest<sup>TM</sup> (Vicam, Watertown, MA, USA). After washing the column by passing 5 ml water through it, DON was eluted with 1.5 ml methanol. The eluate was dried under a nitrogen stream at  $50^\circ\text{C}$ , re-dissolved in 200  $\mu\text{l}$  of mobile phase (acetonitrile/water, 10:90, v/v) and 50  $\mu\text{l}$  were injected onto an LC system with ultraviolet diode array detector set to 220 nm.

### FT-NIR analysis

FT-NIR spectra were recorded using an Antaris II FT-NIR spectrophotometer (Thermo Electron Corporation, Madison, WI, USA) equipped with an interferometer (containing a fixed and moving mirror, a beam-splitter), an integrating sphere working in diffuse reflection, and an indium and gallium arsenide (InGaAs) detector. A sample-cup spinner allowed the automatic collection of several subsamples from each sample. These subsamples were averaged to obtain representative spectra of relatively

heterogeneous samples. The integrating sphere's internal reference was also used to collect the background spectrum. The moving mirror speed was set at  $1.63 \text{ scans s}^{-1}$ . Approximately 30 g of ground wheat samples were placed into the rotating sample-cup spinner with a quartz cup. Spectroscopic data were recorded as absorbance between  $10000\text{--}4000 \text{ cm}^{-1}$  with  $8 \text{ cm}^{-1}$  resolution to give a total of 750 points per sample. Each sample spectrum, consisting of an average of 128 consecutive interferometer scans, was collected in approximately 1 min 20 s and stored in a separate data file.

FT-NIR transmission spectra of DON were obtained using the transmission module (Thermo Electron Corporation) consisting of a sample holder to allocate glass tubes. Approximately  $1000 \mu\text{l}$  of solution were transferred into glass culture tubes ( $6 \times 50 \text{ mm}$ ). Spectroscopic data were recorded as absorbance between  $10000\text{--}4000 \text{ cm}^{-1}$  with  $8 \text{ cm}^{-1}$  resolution and each sample spectrum consisted of an average of 16 consecutive interferometer scans.

Commercially available crystalline DON standard (Sigma, Milan, Italy) was dissolved in acetonitrile to prepare a  $1 \text{ mg ml}^{-1}$  standard solution. FT-NIR transmission spectrum of DON was determined after subtraction of the mean spectrum ( $n = 10$ ) of acetonitrile from the mean spectrum ( $n = 10$ ) of DON standard solution. Furthermore, two subsamples of a DON-free durum wheat sample ( $<50 \mu\text{g kg}^{-1}$ ) were spiked with acetonitrile and with the same volume of DON standard solution (spiking level  $10000 \mu\text{g kg}^{-1}$ ), respectively. The FT-NIR spectrum was obtained after subtraction of the spectrum of acetonitrile-spiked wheat from that of DON-spiked wheat. Similarly, another FT-NIR spectrum was obtained after subtraction of a DON-free durum wheat spectrum from that of a naturally contaminated one ( $13000 \mu\text{g kg}^{-1}$  DON).

### Multivariate data analysis

Based on DON content (as determined by HPLC analysis), wheat samples were distributed into classes each covering  $100 \mu\text{g kg}^{-1}$  DON. Calibration and validation samples were randomly selected from each class by considering an approximate ratio of 2.5:1 between calibration and validation samples. Spectroscopic data were processed by TQ Analyst® professional edition (Thermo Electron Corporation). The Spectrum Outlier diagnostic (TQ Analyst®) was run to seek sample spectra that differed most from the other samples. In particular, the mean spectrum for all calibration samples was calculated, and the distance between the mean spectrum and the spectrum of each calibration sample measured. The Chauvenet test estimated whether the difference between spectra was significant. Once the initial population was free

of spectral outliers, calibration models were developed and statistically elaborated for quantitative analysis using PLS regression with full cross-validation (the leave one out strategy). DON data obtained by FT-NIR were transformed using standard normal variate (SNV) (Barnes et al. 1989), first derivative, mean centring and Savitzky–Golay smoothing (second-order polynomial filtering operation performed on three points) (Savitzky and Golay 1964), and correlated with those obtained by HPLC.

The partial least squares (PLS) models' performance were evaluated by calculating the correlation coefficient ( $r$ ) between the reference (HPLC data) and the predicted (FT-NIR data) DON levels, the root mean square error of calibration (RMSEC), the root mean square error of cross-validation (RMSECV) and the number of PLS factors. External data sets (validation sets) were also used to evaluate the performance of the models for prediction and the root mean square error of prediction (RMSEP) values were calculated. Validation sets were selected to cover the range of DON content of interest. RMSEC, RMSECV and RMSEP were expressed by the same DON level units ( $\mu\text{g kg}^{-1}$ ) in wheat samples. In the development of all calibration models, 10 PLS factors were set as a maximum number to work with. The optimum number of PLS factors was automatically calculated by the TQ Analyst® software, corresponding to the lowest number of factors giving the closest to minimum value of the prediction residual error sum of squares (PRESS). As the calculated number of PLS factors gave models that were strictly dependent on calibration samples, an appropriate number was chosen giving comparable RMSEC, RMSECV and RMSEP values.

The accuracy of each model was evaluated based on the coefficient of determination ( $r^2$ ) for predicted versus reference DON levels in calibration and validation sets. It was assumed that, with  $r^2 = 0.50\text{--}0.65$ , discrimination between high and low values was possible;  $r^2 = 0.66\text{--}0.81$  allowed approximate quantitative prediction;  $r^2 = 0.82\text{--}0.90$  allowed good prediction, and excellent calibration model were obtained with  $r^2 = 0.91$  or higher (Williams 2003). The prediction accuracy of models was also evaluated based on the residual predictive deviation (RPD), defined as the ratio of the standard deviation of the reference DON values in the validation set to the RMSEP. In general, RPD values of 3.1–4.9 indicate NIR calibration models suitable for screening purposes, whereas values of 5.0–6.4 are considered adequate for quality control (Fearn 2002). However, a more qualitative interpretation of the RPD considers values lower than 1.5 insufficient for most applications and values greater than 2 as excellent (Williams 2001; Smyth et al. 2008).

The range error ratio (RER), calculated by dividing the range of the reference DON values in the validation set by the RMSEP, was used as a useful indicator to assess the practical utility of the calibration as a predictive model. Models with RER of less than 3 had little practical utility; between 3 and 10 had limited to good practical utility; above 10 indicated a high practical utility value (Fearn 2002; Williams 2003).

### **Qualitative analysis of different wheat cultivars**

The ability of FT-NIR spectroscopy to discriminate between blank and naturally contaminated samples of different cultivars of both durum and common wheat was evaluated by discriminant analysis. Based on the DON content as determined by HPLC analysis, samples were split into two classes, one included samples with less than  $300 \mu\text{g kg}^{-1}$  DON (assumed as "blank") and the other included naturally contaminated (from  $300 \mu\text{g kg}^{-1}$  up to  $\sim 3000 \mu\text{g kg}^{-1}$  DON) wheat samples. Validation samples were randomly selected to cover the range of interest (up to  $2000 \mu\text{g kg}^{-1}$ ). Calibration and validation sets contained samples in the approximate ratio of 3:1. All spectroscopic data were transformed using variance scaling normalization, standard normal variate (SNV) (Barnes et al. 1989), first derivative, mean centring and Savitzky–Golay smoothing (second-order polynomial filtering operation performed on five points) (Savitzky and Golay 1964).

Discriminant analysis was performed to create the distribution model using the spectral information from all calibration samples. In particular, during calibration, the software computed an average spectrum and then generated a distribution model by estimating the variance at each frequency in the analysis range. When the calibrated method was used to analyze validation samples, the software performed a principal component analysis (PCA) on the validation sample spectra and on the variance spectra to determine score values. The scores were used to produce Mahalanobis distance values, which in turn were used to rank the classes. Discriminant analysis assigned a ranking class based on the level of similarity with the spectrum of the validation sample. The Mahalanobis distance between the validation sample and each class centre was also recorded.

## **Results and discussion**

### **Effect of sample preparation on DON analysis by FT-NIR analysis**

Kos et al. (2003, 2007) reported that the repeatability of spectral measurements performed on ground maize (particle size  $<1 \text{ mm}$ ) was not good

enough to attempt classification by MIR-ATR of DON-contaminated maize, and that a sieving procedure (selecting particles with diameter between 250 and  $100 \mu\text{m}$ ) improved the repeatability of spectral measurements from 20 to 4.4%.

As an initial approach, ground wheat samples were sieved to pass through a  $300 \mu\text{m}$  sieve and analysed by FT-NIR spectroscopy. An alternative sample preparation was used to avoid the time-consuming sieving step (20–30 min if carried out after grinding). In particular, wheat samples were milled with a Tecator Cyclotec laboratory mill (Tecator Cyclotec) equipped with a  $500 \mu\text{m}$  sieve and the obtained fraction was directly analysed by FT-NIR spectroscopy. Spectra for 50 repetitive measurements of a sieved sample were compared to those of an unsieved sample of the same wheat variety and with a comparable DON content.

To statistically evaluate spectra differences between wheat samples with particle size  $<300$  and  $<500 \mu\text{m}$ , the analysis of variance (ANOVA, one-way) was applied to the two groups of repetitive measurements. No significant difference was found within each group, while a significant difference ( $F = 29.62$ ,  $F_{\text{critic}} = 3.84$ ) was observed between the mean spectra of the two groups due to the different distribution of particle sizes and relevant chemical composition. The PCA score/score plot revealed that the cluster containing samples with particle size  $<300 \mu\text{m}$  was more scattered than the cluster containing samples with particle size  $<500 \mu\text{m}$ , showing a more homogeneous distribution of particle size in the unsieved sample (Figure 1).

Furthermore, HPLC analysis of the different fractions obtained after sieving at  $300 \mu\text{m}$  of five wheat samples showed a clear segregation of DON between fractions with respect to the original ground wheat (Table 1). In particular, mean DON levels decreased by 84% in wheat fractions with a particle size  $>300 \mu\text{m}$ , but increased by 115% in fractions with a particle size  $<300 \mu\text{m}$ .

Consequently, the sample preparation approach performed using Tecator Cyclotec milling at  $<500 \mu\text{m}$  was selected to avoid both the time-consuming sieving step and erroneous DON determination due to the different size distribution in the sieved fractions.

### **DON absorption in the NIR region**

The major FT-NIR absorption bands of DON dissolved in acetonitrile (Figure 2, line a) were identified in the range  $7400\text{--}7100 \text{ cm}^{-1}$  with a peak at  $7095 \text{ cm}^{-1}$  and  $5000\text{--}5500 \text{ cm}^{-1}$  with a peak at  $5251 \text{ cm}^{-1}$ . The absorption band at  $5251 \text{ cm}^{-1}$  was much stronger than that at  $7095 \text{ cm}^{-1}$ . The spectrum

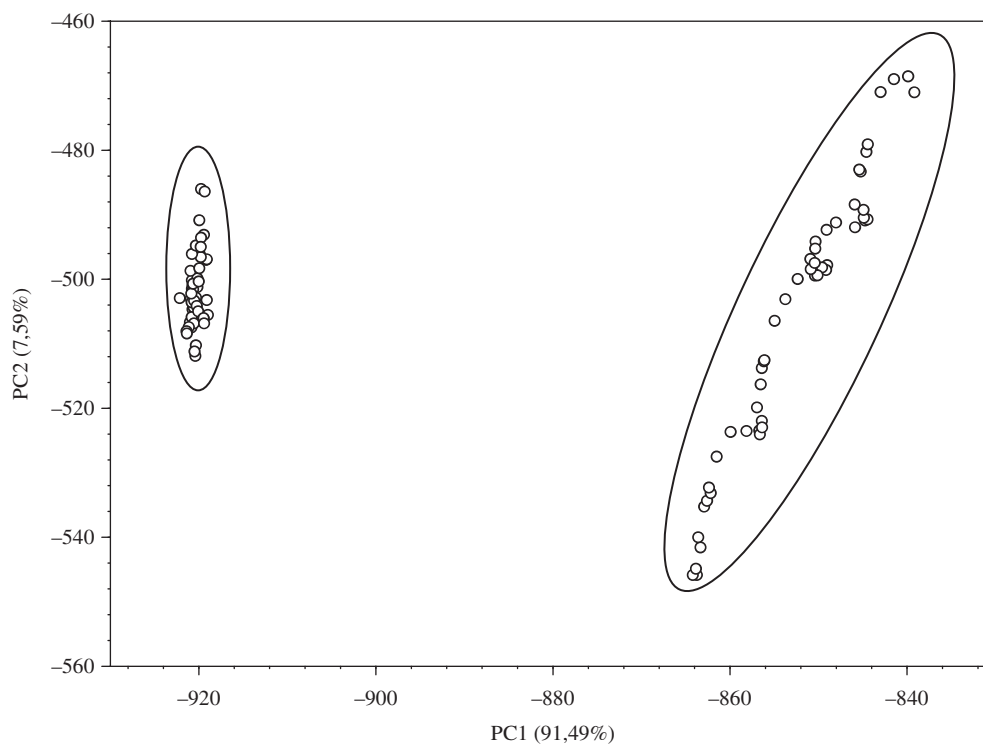


Figure 1. Score/score plot of spectral data (PC1 versus PC2) from 50 repetitive measurements of two wheat samples with different particle sizes. The two clusters represent wheat samples with a particle size  $< 500 \mu\text{m}$  (left, unsieved) and  $< 300 \mu\text{m}$  (right, sieved).

Table 1. Deoxynivalenol content in ground durum wheat prior and after sieving ( $300 \mu\text{m}$  sieve).

Wheat sample (#)	Unsieved ( $\mu\text{g kg}^{-1}$ )	Sieved, $< 300 \mu\text{m}$ ( $\mu\text{g kg}^{-1}$ )	Recovery (%)*	Sieved, $> 300 \mu\text{m}$ ( $\mu\text{g kg}^{-1}$ )	Recovery (%)*
#1	813	995	122	577	71
#2	1466	1619	110	1226	84
#3	819	886	108	696	85
#4	776	846	109	751	97
#5	1277	1588	124	1085	85
Mean $\pm$ SD			$115 \pm 8$		$84 \pm 9$

\*Percentage of deoxynivalenol in the sieved fraction relative to the original (unsieved) wheat sample.

obtained after subtraction of the spectrum of acetonitrile-spiked wheat from that of DON-spiked wheat showed absorption bands at  $\sim 7000$  and  $5250 \text{ cm}^{-1}$  (Figure 2, line b), corresponding to those of the DON spectrum in acetonitrile. The increased intensity of the band at  $7000 \text{ cm}^{-1}$  was due to an overlapping of DON and water absorption bands. The same absorption bands were observed in the FT-NIR spectrum obtained after subtraction of a DON-free wheat spectrum from that of a naturally contaminated one (Figure 2, line c).

DON levels in wheat are generally several orders of magnitude lower than levels of the main wheat

constituents (protein, starch, etc.), and spectra recorded for contaminated and uncontaminated wheat may differ due to differences in chemical composition (owing to development of the fungus) rather than DON content. Thus, subtraction of the spectra can produce spurious features, not necessarily related to DON content, due to the relatively low DON levels commonly occurring in wheat. However, the use of highly contaminated wheat (at levels higher than  $10000 \mu\text{g kg}^{-1}$ ) produced subtractive spectra with appreciable FT-NIR spectral bands (particularly at  $5251 \text{ cm}^{-1}$ ) related to DON, as shown in Figure 3. The characteristic absorption bands of DON could

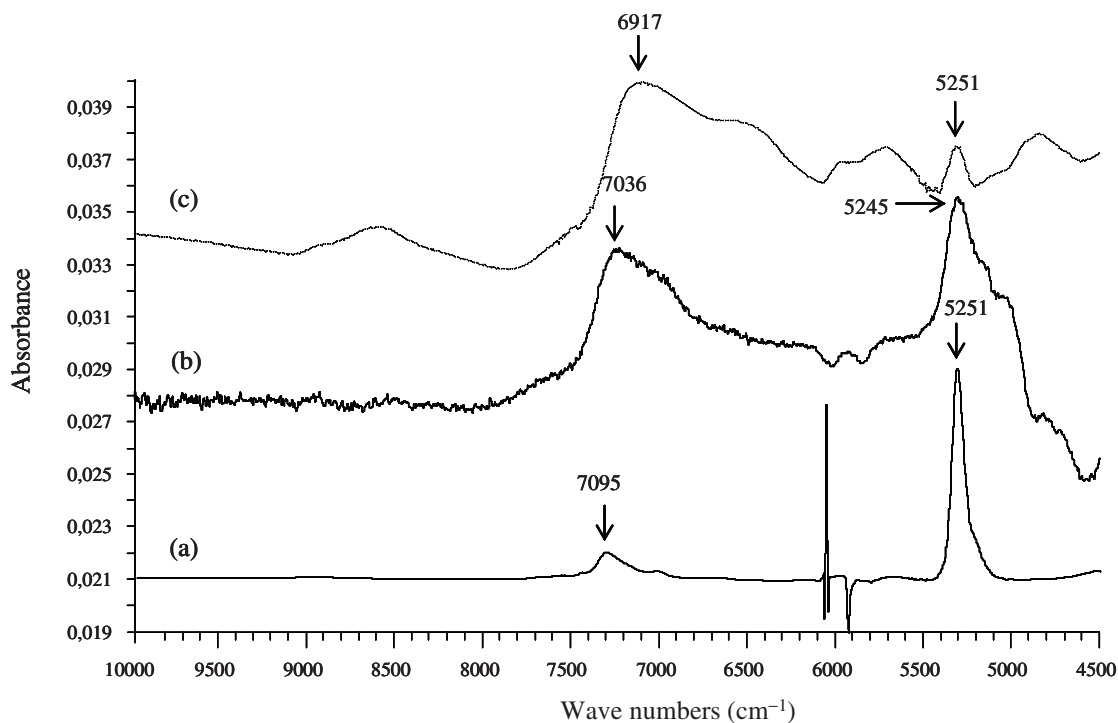


Figure 2. FT-NIR subtractive spectra of deoxynivalenol in the acetonitrile spectrum from that of acetonitrile (line a), the acetonitrile-spiked wheat spectrum from that of DON-spiked ( $10000 \mu\text{g kg}^{-1}$ ) wheat (line b) and the deoxynivalenol-free wheat spectrum from that of naturally contaminated ( $13000 \mu\text{g kg}^{-1}$ ) wheat.

not be identified in wheat samples contaminated at lower levels, such as those used herein (up to  $3000 \mu\text{g kg}^{-1}$ ) for the three PLS regression models. Nevertheless, a specific discrimination between low and high DON levels was obtained by considering the entire spectral range between 10000 and  $4000 \text{ cm}^{-1}$  (see below).

#### ***DON analysis by FT-NIR spectroscopy***

Three PLS regression models were built by considering the spectral range between 10000 and  $4000 \text{ cm}^{-1}$  for durum, common and durum+common wheat samples, respectively. Table 2 summarizes sample statistics for the calibration and validation set of durum, common and durum+common wheat. Comparing the mean DON levels (ranging from 439 to  $887 \mu\text{g kg}^{-1}$ ) with median levels (from 188 to  $656 \mu\text{g kg}^{-1}$ ) for durum or common wheat samples, a very skewed data distribution was observed, with many small and fewer large values. After combination of the two wheat species (durum+common wheat), a better distribution of DON level was obtained and the resulting mean ( $585\text{--}750 \mu\text{g kg}^{-1}$ ) and median ( $395\text{--}523 \mu\text{g kg}^{-1}$ ) DON levels were closer.

Validation sets of common and durum wheat were selected, both consisting of 30 samples. The two PLS models were validated up to  $\sim 2000 \mu\text{g kg}^{-1}$  DON to

cover the range of regulatory levels set by the EU for unprocessed wheat and most wheat-based products. The selection was performed based on DON content (measured by HPLC) to have as many samples as possible of the validation set within the range of DON content to be considered for the calibration (Table 2).

Running the PLS analysis on the calibration sets, a positive linear correlation ( $r > 0.90$ ) was obtained in the measured range for both models, indicating a good fit between HPLC and FT-NIR data. According to the coefficients of determination ( $r^2$ ) and slope values, both  $> 0.82$ , a good prediction of DON content by FT-NIR spectroscopy was achieved in both PLS calibration models (Figure 3a and b).

Furthermore, RMSECV values were 470 and  $516 \mu\text{g kg}^{-1}$ , respectively, both corresponding to four PLS factors (Table 3). The performance of these models for DON prediction on the validation sets gave RMSEP values of  $306 \mu\text{g kg}^{-1}$  for durum wheat and  $348 \mu\text{g kg}^{-1}$  for common wheat. These values were not too far from the RMSECV values obtained in cross-validation. The  $r^2$  values of the validation sets (0.62–0.63) indicated that the PLS models were able to discriminate between low and high DON levels in durum and common wheat. An improvement of RMSECV and  $r^2$  values was obtained by using the recommended number of PLS factors (5–6); however, the obtained PLS models quantified the calibration

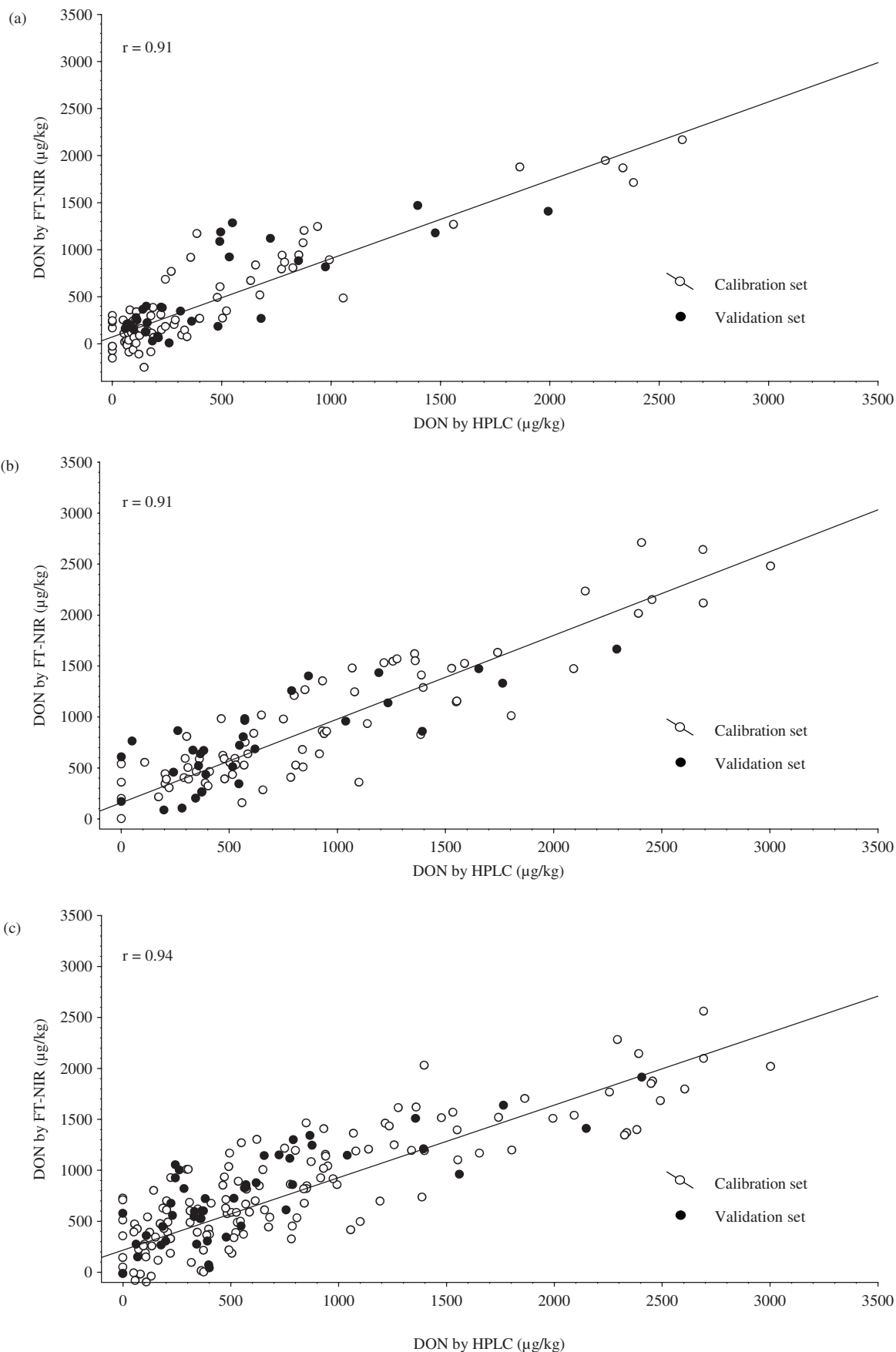


Figure 3. PLS regression models of deoxynivalenol content determined by FT-NIR and the reference method (HPLC) for durum (a), common (b) and durum + common (c) wheat samples.



Table 2. Statistical summary of deoxynivalenol content by HPLC in the calibration and validation sets of durum, common and durum + common wheat with a particle size &lt;500 µm.

	Calibration set			Validation set		
	Durum	Common	Durum + Common	Durum	Common	Durum + Common
No. of samples	76	77	149	30	30	48
Range (µg kg <sup>-1</sup> )	0–2600	0–3000	0–3000	60–1990	0–2290	0–2400
Median (µg kg <sup>-1</sup> )	188	656	523	245	530	395
Mean (µg kg <sup>-1</sup> )	439	887	750	459	658	585
SD (µg kg <sup>-1</sup> )	590	720	722	470	681	533

SD = standard deviation.

Table 3. Calibration and validation results for deoxynivalenol detection by FT-NIR in durum, common and durum + common wheat with a particle size &lt;500 µm.

Parameters	Durum	Common	Durum + Common
Slope <sub>calibration</sub>	0.83	0.82	0.71
$r_{calibration}$	0.91	0.91	0.84
$r^2_{calibration}$	0.83	0.82	0.71
Slope <sub>validation</sub>	0.79	0.62	0.71
$r_{validation}$	0.80	0.79	0.76
$r^2_{validation}$	0.62	0.63	0.58
RMSEP (µg kg <sup>-1</sup> )	306	348	379
RMSECV (µg kg <sup>-1</sup> )	470	516	555
PLS factors	4	4	5
RER	6.3	6.6	6.3
RPD	1.6	2.0	1.0

$r$ , correlation coefficient;  $r^2$ , determination coefficient; RMSECV, root mean square error of cross-validation; RMSEP, root mean square error of prediction set; PLS, partial least squares; RER, ratio error range; RPD, residual predictive deviation.

samples with great accuracy but did not produce the same level of accuracy when applied to validation samples, giving RMSEP and RMSECV values that were too far from the RMSEC. Based on these findings, it was decided to run the calibrations with a low number of factors to obtain comparable values of RMSEC, RMSEP and RMSECV.

In addition to the RMSECV,  $r^2$  and RMSEP, the PLS models were evaluated using the RER and RPD values. Both the RER and RPD standardize the RMSEP value of the model against the range and standard deviation of the reference data in the validation set. RPD values obtained for durum and common wheat (1.6–2.0) show the ability of the model to discriminate between high and low contaminated samples. However, it is evident that a further improvement in RPD values, and thus more accurate PLS models, could be obtained by increasing the range and the distribution of DON content in the validation samples. On the other hand, RER values lie within

6 and 7, indicating a good practical utility of the models for DON prediction in wheat samples (Table 3).

Results obtained in the present study were similar to those reported by Pettersson and Aberg (2003) in terms of correlation coefficient, slope and RMSEP. However, because calibrations were made on a larger number of calibration and validation samples, a remarkable improvement was obtained with the PLS regression models used herein for prediction of DON in durum and common wheat. Furthermore, DON contamination levels used herein were close to the DON maximum limits set by the European Union for unprocessed durum (1750 µg kg<sup>-1</sup>) and common (1250 µg kg<sup>-1</sup>) wheat, whereas Pettersson and Aberg (2003) calibrated the models at higher and less realistic levels (up to 10000–15000 µg kg<sup>-1</sup>).

An additional PLS calibration model was built by combining durum and common wheat samples to evaluate the possibility to include wheat species belonging to different varieties within the same model. In this case, a good fit of the PLS model ( $r=0.84$ ) was also obtained (Figure 3; Table 3). The  $r^2$  values indicated that the model approximated quantitative predictions of DON in the calibration set ( $r^2=0.71$ ) and allowed for discrimination between high and low DON levels in the validation set ( $r^2=0.58$ ) (Table 3). These results were also confirmed by the RER value (6.3), while the RPD value (1.0) failed to attain the minimum recommended for quantitative analysis. Although results obtained in the present study are not totally satisfactory for quantitative or semi-quantitative analysis, they provide good evidence of the feasibility of using FT-NIR spectroscopy as a screening tool for determination of DON in wheat samples. Furthermore, considering that different wheat species (durum and common) and wheat varieties contain different amount of chemical constituents, such as protein, lipids and moisture with remarkable spectral differences (Morris et al. 2005; Cocchi et al. 2006), our findings indicate that this

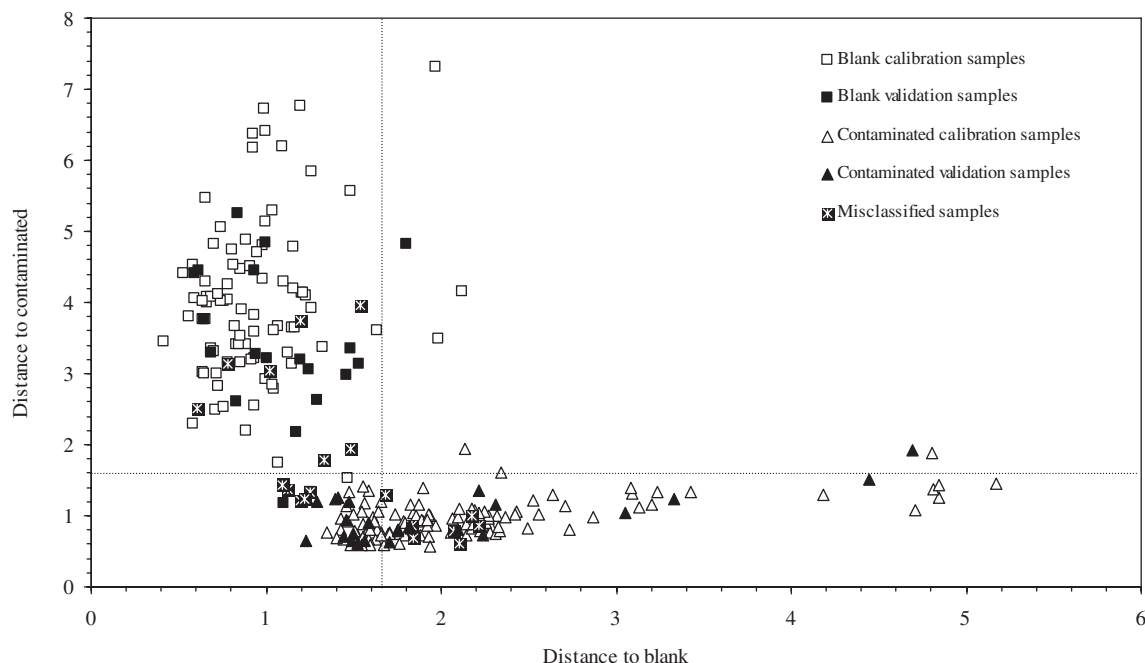


Figure 4. Pairwise distance plot reporting the Mahalanobis distance between each sample and the “Blank” and “Contaminated” classes.

variability does not affect the applicability of FT-NIR for DON analysis

#### *FT-NIR qualitative analysis of different wheat cultivars*

The ability of FT-NIR spectroscopy to discriminate between blank and naturally contaminated durum and common wheat samples of different cultivars was evaluated by performing a discriminant analysis on 197 calibration samples. In particular, depending on DON content calculated by HPLC analysis, samples were split into two classes, one included 84 “blank” samples (up to  $300 \mu\text{g kg}^{-1}$  DON) and the other included 113 “contaminated” samples ( $>300 \mu\text{g kg}^{-1}$  DON). The samples were classified as members of each group based on the lowest corresponding Mahalanobis distance, i.e. the multi-dimensional space whose boundaries determine the range of variation.

Figure 4 shows graphically the Mahalanobis distance between each sample and the “blank” and “contaminated” classes that were selected for the  $x$ - and  $y$ -axis of the plot, respectively. The boundaries, displayed as perpendicular lines in the plot, show the 95% confidence intervals for the two classes. All calibration and validation samples relevant to the “blank” class are expected to be clustered in the upper left corner of the plot, while samples falling in the lower right corner define the “contaminated” class. As

observed in Figure 4, a few calibration samples of the two classes fall in the uncertainty area (lower left corner, 40 of 197). Most of them were “blank” (16%) or contained DON levels between  $300$  and  $500 \mu\text{g kg}^{-1}$  (30%), indicating that the model had a limited degree of specificity for wheat samples contaminated with DON levels close to the cut-off limit. When the obtained model was applied to a set of 65 validation samples, it correctly classified 69% of samples as “blank” or “contaminated” wheat. The remaining 20 samples were incorrectly classified as false positives or negatives. In this case, most of misclassified samples were also “blank” (55%) or contained DON levels ranging from  $300$  to  $400 \mu\text{g kg}^{-1}$  (25%), confirming that the goodness of the discrimination model was greatly influenced by the low DON cut-off limit fixed to distinguish the two classes. Lower cut-off levels, i.e.  $50 \mu\text{g kg}^{-1}$  (the quantification limit of the HPLC method),  $100$  or  $200 \mu\text{g kg}^{-1}$ , led to lesser precise models, while higher cut-off levels gave poor reliability due to problems associated with the relatively limited number of samples with high DON levels. However, these results still endorse the use of FT-NIR as a sorting tool for screening purpose, since the legal limit at present is well over  $300 \mu\text{g kg}^{-1}$  for most commodities (with the important exception of baby food where the limit is  $200 \mu\text{g kg}^{-1}$ ). An improvement to this qualitative model could be obtained by increasing the number of samples and, in particular, by increasing the number of high DON-contaminated samples.

## Conclusions

Performances of discriminant analysis for qualitative models and of PLS regression models for semi-quantitative prediction for DON contamination of wheat makes FT-NIR analysis a useful tool to discriminate between high and low DON-contaminated samples. FT-NIR analysis is suitable for the determination of DON in a specific wheat species (durum or common) or in both at levels far below the DON maximum permitted limits set by the European Commission for unprocessed wheat. FT-NIR analysis might be used for rapid, non-invasive, inexpensive and user-friendly screening of large numbers of wheat samples.

The FT-NIR models developed herein merit further implementation in a larger study involving more calibration and validation samples with a uniform distribution of DON levels

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