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

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3 **Rapid and non-invasive analysis of deoxynivalenol in durum and**
4 **common wheat by Fourier-Transform Infrared (FT-NIR) spectroscopy**
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40 **Running title:** FT-NIR analysis of deoxynivalenol in wheat
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Abstract

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 the variation of measurements 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 the external validation technique. Values of root mean square error of prediction (RMSEP, 306-379 $\mu\text{g kg}^{-1}$) were comparable and not too far from values of root mean square error of cross-validation (RMSECV, 470-555 $\mu\text{g kg}^{-1}$). 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 $\mu\text{g kg}^{-1}$ DON to separate the two classes. The model correctly classified 69% of the 65 validation samples with most misclassified samples (16 out 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 DON maximum permitted limits set by the European Commission.

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3 **Keywords:** FT-NIR spectroscopy; deoxynivalenol; wheat; PLS regression, discriminant
4 analysis.
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10 **Introduction**

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14 Deoxynivalenol (DON) is a type B trichothecene mycotoxin produced by fungi of the
15 *Fusarium* genus, in particular *Fusarium graminearum* (Gibberella zea) and *Fusarium*
16 *culmorum*. It is frequently associated with contaminated cereal crops such as wheat,
17 maize, barley, oats, rye and less often with rice, sorghum and triticale (Canady et al.
18 2001; Schothorst and van Egmond 2004). DON inhibits the synthesis of DNA, RNA and
19 proteins, and has a hemolytic effect on erythrocytes. In animals, acute/subacute oral
20 toxicity of DON is characterized by vomiting, feed refusal, weight loss and diarrhea
21 (Schlatter 2004).
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36 Based on the temporary tolerable daily intake (TDI) of 1 µg/kg body weight established
37 by the EU Scientific Committee on Food (SCF) (SCF 2002), the European Commission
38 has set maximum permitted levels for DON in a variety of cereal based foods (European
39 Commission 2007). In particular, 1750 µg kg⁻¹ and 1250 µg kg⁻¹ were set as maximum
40 levels for unprocessed durum and common wheat, respectively; lower levels were set for
41 wheat flour, bran, germ and pasta (750 µg kg⁻¹), for wheat and wheat products for direct
42 human consumption (750 µg kg⁻¹ and 500 µg kg⁻¹), and for wheat-based infants and baby
43 food (200 µg kg⁻¹).
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3 Rapid and sensitive methods for the quantification of DON in wheat and wheat-based
4 food products are strongly demanded by private and public quality control laboratories,
5 as well as in surveys aiming to provide data for risk assessments. Several methods based
6 on competitive enzyme-linked immunosorbent assays (ELISA) have been developed for
7 the rapid screening and quantification of DON in cereals. ELISA methods are sensitive,
8 rapid and easy-to-use, but they often show strong cross-reactivity against DON acetyl
9 derivatives, require long incubation times for complete antigen-antibody reaction and
10 commercial kits are still rather expensive. A fluorescence polarization (FP) immunoassay
11 has been recently developed for the rapid and quantitative analysis of DON in durum
12 wheat, semolina, and pasta at levels below the EU maximum permitted levels (Lippolis et
13 al. 2006).

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

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48 Petterson and Aberg (2003) used near infrared (NIR) transmittance for the determination
49 of DON in whole wheat kernel samples at levels higher than $500 \mu\text{g kg}^{-1}$, whereas NIR
50 reflectance has been used for detection of scab and estimation of DON and ergosterol in
51 single kernels of highly infected wheat (Dowell et al. 1999). A method for the analysis of
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4 *Fusarium* fungi on corn using Fourier transform-mid infrared (FT-MIR) spectroscopy
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6 with attenuated total reflection (ATR) was developed which enabled the segregation of
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8 DON contaminated corn samples from non contaminated (blank) samples (Kos et al.
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10 2003, 2004, 2007; Delwiche and Hareland 2004).
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15 Papers on the application of FT-IR spectroscopy for a determination of DON content in
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17 grain mostly exploit the range below 4000 cm^{-1} . In particular, Mossoba et al. (1996)
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19 reported DON characteristic absorption bands between 1244 and 1750 cm^{-1} . Some
20
21 additional bands at ca. 3500 and close to 3650 cm^{-1} (Young and Games 1994) and not far
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23 from 1700 cm^{-1} (Abramovic et al. 2007) have been reported. The most recent theoretical
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25 calculations of IR bands cover the MIR range below 4000 cm^{-1} (Turker and Gumus
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27 2008), also used by Kos et al. (2007) for corn analysis. Therefore the identification of any
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29 spectral features of DON within the NIR range is highly desirable. FT-IR spectroscopy
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31 allows to perform rapid measurements with an excellent signal-to-noise ratio and high
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33 resolution.
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41 The aim of the present work was to investigate the feasibility of using FT-NIR for the
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43 rapid and non-invasive determination of DON in durum and common wheat.
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46 47 48 **Materials and methods**

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50 *Sample preparation.* A total of 262 wheat (143 durum and 119 common) samples,
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52 belonging to 32 different varieties, were obtained from wheat naturally infected by
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54 *Fusarium graminearum* and *Fusarium culmorum* in experimental fields in Northern Italy
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3 during the period from 2002 to 2006. Wheat samples were ground with a Tecator
4 Cyclotec 1093 (International PBI, Hoganas, Sweden) laboratory mill equipped with a 500
5 μm sieve, and were analyzed by HPLC and by FT-NIR spectroscopy. DON levels, as
6 established by HPLC analysis, ranged from 50 to 2600 $\mu\text{g kg}^{-1}$ in durum wheat and from
7 50 to 3000 $\mu\text{g kg}^{-1}$ in common wheat. Samples containing less than 50 $\mu\text{g kg}^{-1}$ DON (the
8 quantification limit of the HPLC-method) were considered as DON-free. Mean
9 recoveries of DON from durum and common wheat samples ($n = 3$) spiked at levels of
10 200, 800 and 1500 $\mu\text{g kg}^{-1}$ were 75-93% with RSD_r of 1.4-14.2% for durum wheat and
11 79-83% with RSD_r of 1.1-12.2% for common wheat. To investigate the distribution of
12 DON during sieving, five durum wheat samples were ground by a Bühler MLI 204
13 (Bühler S.p.A., Milan, Italy) to pass a 1-mm sieve and analyzed by HPLC for DON. A
14 fraction of these samples was sieved to pass a 300 μm sieve, and the two fractions (below
15 and above 300 μm) were analyzed by HPLC.

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37 *HPLC quantitative analysis.* HPLC quantitative analysis was performed to obtain the
38 reference value of the DON content for all wheat samples tested herein. Wheat samples
39 were analyzed according to the procedure described by Visconti et al. (2004) with minor
40 modifications. Briefly, 25 g of ground wheat was extracted with 100 mL of phosphate
41 buffer solution (PBS, 10 mM sodium phosphate, 0.85% sodium chloride, pH 7.4) by
42 blending at high speed for 2 min with a Sorvall Omnimixer (Sorvall Instruments,
43 Norwalk, Conn.). The extract was filtered through both filter paper (Whatman no. 4) and
44 glass microfiber filter (Whatman GF/A) and 2 mL of the filtered extract were cleaned up
45 by DONTestTM (Vicam, Watertown, MA, USA). After washing the column by passing 5
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3 mL water through it, DON was eluted with 1.5 mL methanol. The eluate was dried under
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5 nitrogen stream at 50°C, re-dissolved in 200 µL of mobile phase (acetonitrile:water,
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7 10:90, v/v) and 50 µL were injected onto an LC system with ultraviolet diode array
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9 detector set to 220 nm.
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15 *FT-NIR analysis.* FT-NIR spectra were recorded using an Antaris II FT-NIR
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17 spectrophotometer (Thermo Electron Corporation, Madison, WI, USA) equipped with an
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19 interferometer (containing a fixed and a moving mirror, a beam-splitter), an integrating
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21 sphere working in diffuse reflection, and an indium and gallium arsenide (InGaAs)
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23 detector. A sample cup spinner allowed the automatic collection of several subsamples
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25 from each sample. These subsamples were averaged to obtain representative spectra of
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27 relatively heterogeneous samples. The integrating sphere's internal reference was also
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29 used to collect the background spectrum. The moving mirror speed was set at 1.63
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31 scans/sec. Approximately 30 g of ground wheat samples were placed into the rotating
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33 sample cup spinner with a quartz cup. Spectroscopic data were recorded as absorbance
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35 between 10000-4000 cm⁻¹ with 8 cm⁻¹ resolution to give a total of 750 points per sample.
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37 Each sample spectrum, consisting of an average of 128 consecutive interferometer scans,
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39 was collected in about 1 min 20 s and stored in a separate data file.
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48 FT-NIR transmission spectra of DON were obtained using the Transmission module
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50 (Thermo Electron Corporation) consisting of a sample holder to allocate glass tubes.
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52 Approximately 1000 µl of solution were transferred into glass culture tubes (6 x 50 mm).
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54 Spectroscopic data were recorded as absorbance between 10000-4000 cm⁻¹ with 8 cm⁻¹
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3 resolution and each sample spectrum consisted of an average of 16 consecutive
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5 interferometer scans.
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10 Commercially available crystalline DON standard (Sigma, Milan, Italy) was dissolved in
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12 acetonitrile to prepare a 1 mg ml^{-1} standard solution. FT-NIR transmission spectrum of
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14 DON was determined after subtraction of the mean spectrum ($n = 10$) of acetonitrile from
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16 the mean spectrum ($n = 10$) of DON standard solution. Furthermore, two sub-samples of
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18 a DON-free durum wheat sample ($< 50 \text{ } \mu\text{g kg}^{-1}$) were spiked with acetonitrile and with
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20 the same volume of DON standard solution (spiking level $10000 \text{ } \mu\text{g kg}^{-1}$), respectively.
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22 The FT-NIR spectrum was obtained after subtraction of the spectrum of acetonitrile
23
24 spiked wheat from that of DON spiked wheat. Similarly, another FT-NIR spectrum was
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26 obtained after subtraction of a DON-free durum wheat spectrum from that of a naturally
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28 contaminated one ($13000 \text{ } \mu\text{g kg}^{-1}$ DON).
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36 *Multivariate data analysis.* Based on DON content (as determined by HPLC analysis)
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38 wheat samples were distributed into classes each covering $100 \text{ } \mu\text{g kg}^{-1}$ DON. Calibration
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40 and validation samples were randomly selected from each class by considering an
41
42 approximate ratio of 2.5:1 between calibration and validation samples. Spectroscopic data
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44 were processed by TQ Analyst[®] professional edition (Thermo Electron Corporation). The
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46 Spectrum Outlier diagnostic (TQ Analyst[®]) was run to seek sample spectra that were the
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48 most different from the other samples. In particular, it was calculated the mean spectrum
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50 for all calibration samples, measured the distance between the mean spectrum and the
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52 spectrum of each calibration sample. The Chauvenet Test estimated whether the
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3 difference between spectra was significant. Once the initial population was free of
4 spectral outliers calibration models were developed and statistically elaborated for
5
6 quantitative analysis using PLS regression with full cross validation (*leave one out*).
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8 DON data obtained by FT-NIR were transformed using Standard Normal Variate (SNV)
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10 (Barnes et al., 1989), first derivative, mean centering and Savitzky-Golay smoothing (2nd-
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12 order polynomial filtering operation performed on 3 points) (Savitzky and Golay, 1964),
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14 and correlated with those obtained by HPLC.
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22 The Partial Least Squares (PLS) models performance were evaluated by calculating the
23 correlation coefficient (*r*) between the reference (HPLC data) and the predicted (FT-NIR
24 data) DON levels, the root mean square error of calibration (RMSEC), the root mean
25 square error of cross-validation (RMSECV) and the number of PLS factors. External data
26 sets (validation sets) were also used to evaluate the performance of the models for
27 prediction, and the root mean square error of prediction (RMSEP) values were calculated.
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29 Validation sets were selected to cover the range of DON-content of interest. RMSEC,
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31 RMSECV and RMSEP were expressed by the same DON level units ($\mu\text{g kg}^{-1}$) in wheat
32 samples. In the development of all calibration models, 10 PLS factors were set up as a
33 maximum number to work with. The optimum number of PLS factors was automatically
34 calculated by the TQ Analyst[®] software corresponding to the lowest number of factors
35 giving the closest to minimum value of the prediction residual error sum of squares
36 (PRESS). As the calculated number of PLS factors gave models that strictly dependent on
37 calibration samples, an appropriate number was chosen giving comparable RMSEC,
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39 RMSECV and RMSEP values.
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3 The accuracy of each model was evaluated based on the coefficient of determination (r^2)
4 for predicted *versus* reference DON levels in calibration and validation sets. It was
5 assumed that with $r^2 = 0.50-0.65$ discrimination between high and low values was
6 possible, $r^2 = 0.66-0.81$ allowed approximate quantitative prediction, $r^2 = 0.82-0.90$
7 allowed good prediction, and excellent calibration model were obtained with $r^2 = 0.91$ or
8 higher (Williams 2003). The prediction accuracy of models was also evaluated based on
9 the residual predictive deviation (RPD), defined as the ratio of the standard deviation of
10 the reference DON values in the validation set to the RMSEP. In general, RPD values of
11 3.1-4.9 indicate NIR calibration models suitable for screening purposes, whereas values
12 of 5.0-6.4 are considered adequate for quality control (Fearn 2002). However, a more
13 qualitative interpretation of the RPD considers values lower than 1.5 insufficient for most
14 applications and values greater than 2 as excellent (Williams 2001; Smyth et al. 2008).

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

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46 *Qualitative analysis of different wheat cultivars.* The ability of FT-NIR spectroscopy to
47 discriminate between blank and naturally contaminated samples of different cultivars of
48 both durum and common wheat was evaluated by Discriminant Analysis. Based on the
49 DON content as determined by HPLC analysis, samples were split into two classes, one
50 including samples with less than $300 \mu\text{g kg}^{-1}$ DON (assumed as “blank”) and the other
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3 one including naturally contaminated (from 300 $\mu\text{g kg}^{-1}$ up to about 3000 $\mu\text{g kg}^{-1}$ DON)
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5 wheat samples. Validation samples were randomly selected to cover the range of interest
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7 (up to 2000 $\mu\text{g kg}^{-1}$). Calibration and validation sets contained samples in the
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9 approximate ratio of 3:1. All spectroscopic data were transformed using variance scaling
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11 normalization, Standard Normal Variate (SNV) (Barnes et al., 1989), first derivative,
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13 mean centering and Savitzky-Golay smoothing (2nd-order polynomial filtering operation
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15 performed on 5 points) (Savitzky and Golay, 1964).
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22 Discriminant Analysis was performed to create the distribution model by using the
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24 spectral information from all calibration samples. In particular, during calibration, the
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26 software computed an average spectrum and then generated a distribution model by
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28 estimating the variance at each frequency in the analysis range. When the calibrated
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30 method was used to analyze validation samples, the software performed a Principal
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32 Component Analysis (PCA) on the validation sample spectra and on the variance spectra
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34 to determine score values. The scores were used to produce Mahalanobis distance values,
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36 which in turn were used to rank the classes. Discriminant Analysis assigned the ranking
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38 class based on the level of similarity with the spectrum of the validation sample. The
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40 Mahalanobis distance between the validation sample and each class center was also
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42 recorded.
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51 **Results and discussion**

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53 *Effect of sample preparation on DON analysis by FT-NIR analysis.* Kos et al. (2003;
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55 2007) reported that the repeatability of spectral measurements performed on ground
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3 maize (particle size < 1 mm) was not good enough for attempting classification by MIR-
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5 ATR of DON contaminated maize, and that a sieving procedure (selecting particles with
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7 diameter between 250 and 100 μm) improved the repeatability of spectral measurements
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9 from 20% to 4.4%.
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14 As an initial approach, ground wheat samples were sieved to pass a 300 μm sieve and
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16 analysed by FT-NIR spectroscopy. An alternative sample preparation was used to avoid
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18 the time-consuming sieving step (20-30 min if carried out after grinding). In particular,
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20 wheat samples were milled with a Tecator Cyclotec laboratory mill (Tecator Cyclotec)
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22 equipped with a 500 μm sieve and the obtained fraction was directly analysed by FT-NIR
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24 spectroscopy. Spectra for 50 repetitive measurements of a sieved sample were compared
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26 to those of an unsieved sample of the same wheat variety and with a comparable DON-
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28 content.
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37 In order to statistically evaluate spectra differences between wheat samples with particle
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39 size < 300 μm and < 500 μm , the analysis of variance (ANOVA, one way) was applied to
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41 the two groups of repetitive measurements. No significant difference was found within
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43 each group, while a significant difference ($F = 29.62$, $F_{\text{critic}} = 3.84$) was observed between
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45 the mean spectra of the two groups due to the different distribution of particle sizes and
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47 relevant chemical composition. The PCA score/score plot revealed that the cluster
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49 containing samples with particle size < 300 μm was more scattered than the cluster
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51 containing samples with particle size < 500 μm , showing a more homogeneous
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53 distribution of particle size in the unsieved sample (Figure 1).
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[Insert Figure 1 about here]

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

[Insert Table I about here]

Consequently, the sample preparation approach performed by using a Tecator Cyclotec milling at $< 500 \mu\text{m}$ was preferred in order to avoid both the time-consuming sieving step, and the erroneous DON determination due to the different 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 of 7400-7100 cm^{-1} with a peak at 7095 cm^{-1} , and between 5000 and 5500 cm^{-1} with a peak at 5251 cm^{-1} . The absorption band at 5251 cm^{-1} was much stronger than that at 7095 cm^{-1} . The spectrum obtained after subtraction of the spectrum of acetonitrile spiked wheat from that of DON spiked wheat showed absorption bands at about 7000 and 5250 cm^{-1} (Figure 2, line b) corresponding to those of DON spectrum in acetonitrile. The increased intensity of the

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3 band at 7000 cm^{-1} was due to an overlapping of DON and water absorption bands. The
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5 same absorption bands were observed in the FT-NIR spectrum obtained after subtraction
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7 of a DON-free wheat spectrum from that of a naturally contaminated one (Figure 2, line
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9 c).
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20 DON levels in wheat are generally several orders of magnitude lower than levels of the
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22 main wheat constituents (protein, starch, etc.), and spectra recorded for contaminated and
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24 uncontaminated wheat may differ one from another more because of difference in
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26 chemical composition (due to development of the fungus) than because of DON content.
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28 Thus, subtraction of the spectra can produce spurious features not necessarily related to
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30 DON content due to the relatively low DON levels commonly occurring in wheat.
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32 However, the use of highly contaminated wheat (at levels higher than $10000\text{ }\mu\text{g kg}^{-1}$)
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34 produced subtractive spectra with appreciable FT-NIR spectral bands (particularly at
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36 5251 cm^{-1}) related to DON as shown in Figure 3. The characteristic absorption bands of
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38 DON could not be identified in wheat samples contaminated at lower levels, such as
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40 those used herein (up to $3000\text{ }\mu\text{g kg}^{-1}$) for the three PLS regression models. Nevertheless,
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42 a certain discrimination between low and high DON levels was obtained by considering
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44 the entire spectral range between 10000 and 4000 cm^{-1} (see below).
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53 *DON analysis by FT-NIR spectroscopy.* Three PLS regression models were built by
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55 considering the spectral range between 10000 and 4000 cm^{-1} for durum, common and
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3 durum+common wheat samples, respectively. Table II summarizes sample statistics for
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5 the calibration and validation set of durum, common and durum+common wheat.
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7 Comparing the mean DON levels (ranging from 439 to 887 $\mu\text{g kg}^{-1}$) with median levels
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9 (from 188 to 656 $\mu\text{g kg}^{-1}$) for durum or common wheat samples, a very skewed data
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11 distribution was observed, with many small values and fewer large ones. After
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13 combination of the two wheat species (durum+common wheat) a better distribution of
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15 DON level was obtained and the resulting mean (585-750 $\mu\text{g kg}^{-1}$) and median (395-523
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17 $\mu\text{g kg}^{-1}$) DON levels were closer.
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24 [Insert Table II about here]
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29 Validation sets of common and durum wheat were selected, both consisting of 30
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31 samples. The two PLS models were validated up to about 2000 $\mu\text{g kg}^{-1}$ DON to cover the
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33 range of regulatory levels set by the EU for unprocessed wheat and most wheat based
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35 products. The selection was performed based on DON content (measured by HPLC) in
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37 order to have as many samples as possible of the validation set within the range of DON
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39 content to be considered for the calibration (Table II).
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46 Running the PLS analysis on the calibration sets a positive linear correlation ($r > 0.90$)
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48 was obtained in the measured range for both models, indicating a good fit between HPLC
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50 and FT-NIR data. According to the coefficients of determination (r^2) and slope values,
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52 both > 0.82 , a good prediction of DON content by FT-NIR spectroscopy was achieved in
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54 both PLS calibration models (Figure 3a and 3b).
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[Insert Figure 3 about here]

Furthermore, RMSECV values were 470 and 516 $\mu\text{g kg}^{-1}$ respectively, both corresponding to 4 PLS factors (Table III). 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 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 samples with great accuracy but did not produce the same level of accuracy when applied to validation samples and gave RMSEP and RMSECV values that were too far from RMSEC. Based on these findings, it was decided to run the calibrations with a low number of factors in order to obtain comparable values of RMSEC, RMSEP and RMSECV.

[Insert Table III about here]

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 the 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

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3 model to discriminate between high and low contaminated samples. However, it is
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5 evident that a further improvement of RPD values, and then more accurate PLS models,
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7 could be obtained by increasing the range and the distribution of DON content in the
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9 validation samples. On the other hand, RER values lie within 6 and 7, indicating a good
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11 practical utility of the models for DON prediction in wheat samples (Table III).
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15 Results obtained in the present study were similar to those reported by Pettersson and
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17 Aberg (2003) in terms of correlation coefficient, slope and RMSEP. However, a
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19 remarkable improvement was obtained with the PLS regression models used herein for
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21 prediction of DON in durum and common wheat because calibrations were made on a
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23 larger number of calibration and validation samples. Furthermore DON contamination
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25 levels used herein were close to the DON maximum limits set by the European Union for
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27 unprocessed durum ($1750 \mu\text{g kg}^{-1}$) and common ($1250 \mu\text{g kg}^{-1}$) wheat, whereas
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29 Pettersson and Aberg (2003) calibrated the models at higher and less realistic levels (up
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31 to $10000\text{-}15000 \mu\text{g kg}^{-1}$).
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39 An additional PLS calibration model was built by combining durum and common wheat
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41 samples to evaluate the possibility to include different wheat species belonging to
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43 different varieties in the same model. Also in this case a good fit of the PLS model ($r =$
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45 0.84) was obtained (Figure 3, Table III). The r^2 values indicated that the model
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47 approximated quantitative predictions of DON in the calibration set ($r^2 = 0.71$) and
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49 allowed for discrimination between high and low DON levels in the validation set ($r^2 =$
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51 0.58) (Table III). These results were also confirmed by the RER value (6.3), while the
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53 RPD value (1.0) failed to attain the minimum recommended for quantitative analysis.
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3 Although results obtained in the present study are not fully satisfying for quantitative or
4 semi-quantitative analysis, they provide a good evidence of the feasibility of using FT-
5 NIR spectroscopy as a screening tool for determination of DON in wheat samples.
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8 Furthermore, considering that different wheat species (durum and common) and wheat
9 varieties contain different amount of chemical constituents, such as protein, lipids and
10 moisture with remarkable spectral differences (Cocchi et al., 2006; Morris et al., 2005),
11 our findings indicate that this variability does not affect the applicability of FT-NIR for
12 DON analysis
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24 *FT-NIR qualitative analysis of different wheat cultivars.* The ability of FT-NIR
25 spectroscopy to discriminate between blank and naturally contaminated durum and
26 common wheat samples of different cultivars was evaluated by performing a discriminant
27 analysis on 197 calibration samples. In particular, depending on DON content calculated
28 by HPLC analysis, samples were split into two classes, one including 84 “blank” samples
29 (up to 300 $\mu\text{g kg}^{-1}$ DON) and the other one including 113 “contaminated” samples (> 300
30 $\mu\text{g kg}^{-1}$ DON). The samples were classified as members of each group based on the
31 lowest corresponding Mahalanobis distance, i.e. the multi-dimensional space whose
32 boundaries determine the range of variation.
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[Insert Figure 4 about here]

53 Figure 4 shows graphically the Mahalanobis distance between each sample and the
54 “blank” and “contaminated” classes that were selected for the X- and Y- axis of the plot,
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3 respectively. The boundaries, displayed as perpendicular lines in the plot, show the 95%
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5 confidence intervals for the two classes. All calibration and validation samples relevant to
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7 the “blank” class are expected to be clustered in the upper left corner of the plot, while
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9 samples falling in the lower right corner define the “contaminated” class. As observed in
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11 Figure 4, a few calibration samples of the two classes fall in the uncertainty area (lower
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13 left corner, 40 out of 197). Most of them were “blank” (16%) or contained DON levels
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15 between 300 and 500 $\mu\text{g kg}^{-1}$ (30%), indicating that the model had a limited degree of
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17 specificity for wheat samples contaminated with DON levels close to the cut-off limit.
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19 When the obtained model was applied to a set of 65 validation samples, it correctly
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21 classified 69% of samples as “blank” or “contaminated” wheat. The remaining 20
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23 samples were incorrectly classified as false positive or negative sample. Also in this case,
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25 most of misclassified samples were “blank” (55%) or contained DON levels ranging from
26
27 300 to 400 $\mu\text{g kg}^{-1}$ (25%), confirming that the goodness of the discrimination model was
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29 greatly influenced by the low DON cut-off limit fixed to distinguish the two classes.
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31 Lower cut-off levels, i.e. 50 $\mu\text{g kg}^{-1}$ (the quantification limit of the HPLC method), 100
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33 and 200 $\mu\text{g kg}^{-1}$, led to lesser precise models, while higher cut-off levels gave poorly
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35 reliable models due to problems associated with the relatively limited number of samples
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37 with high DON levels. However, these results still recommend the use of FT-NIR as a
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39 sorting tool for screening purpose, since the legal limit at present is well over 300 $\mu\text{g kg}^{-1}$
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41 for most commodities (with the important exception of baby food where the limit is 200
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43 $\mu\text{g kg}^{-1}$). An improvement to this qualitative model could be obtained by increasing the
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45 number of samples, and in particular, by increasing the number of high DON
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47 contaminated samples.
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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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2 **Figure 1.** Score/score plot of spectral data (PC1 vs PC2) from 50 repetitive measurements of
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4 two wheat samples with different particle sizes. The two clusters represent wheat samples
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6 with particle size < 500 μm (left, unsieved) and < 300 μm (right, sieved).
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9 **Figure 2.** FT-NIR subtractive spectra of deoxynivalenol in acetonitrile spectrum from that of
10 acetonitrile (line a), of acetonitrile spiked wheat spectrum from that of DON spiked (10000
11 $\mu\text{g kg}^{-1}$) wheat (line b) and of deoxynivalenol-free wheat spectrum from that of naturally
12 contaminated (13000 $\mu\text{g kg}^{-1}$) wheat.
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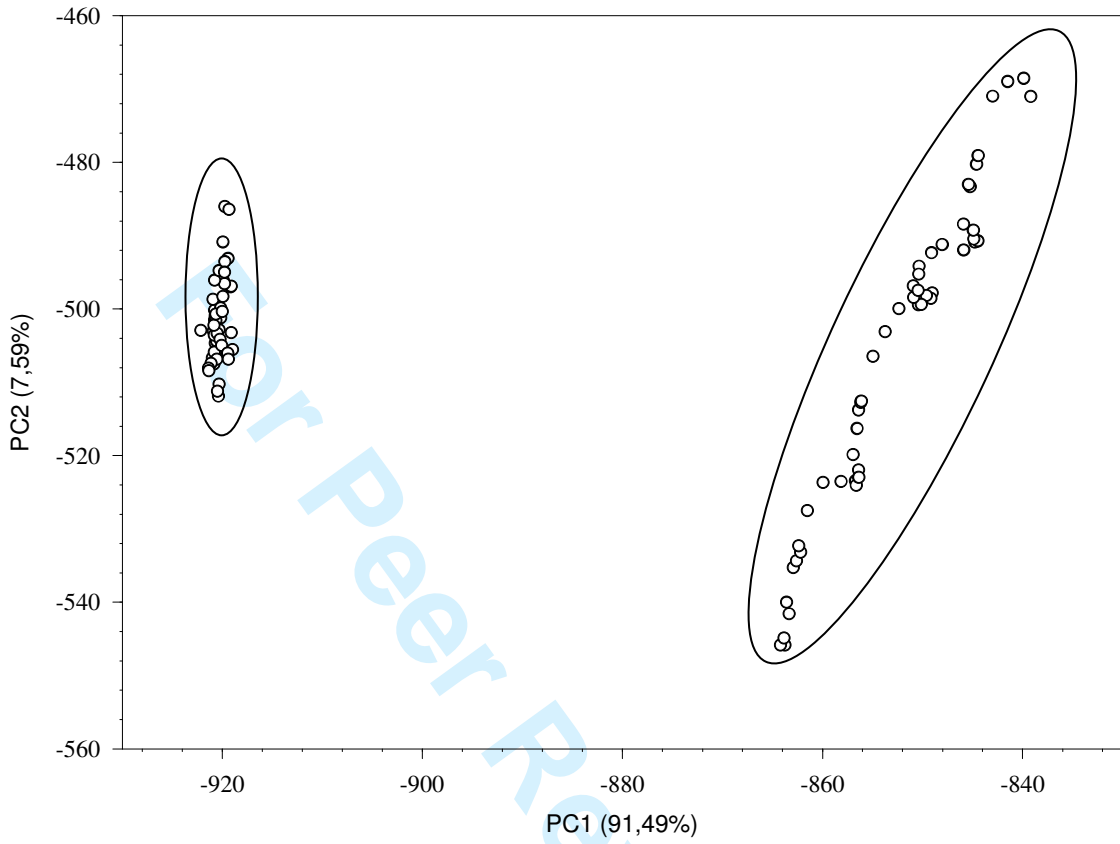
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18 **Figure 3.** PLS regression models of deoxynivalenol content determined by FT-NIR and the
19 reference method (HPLC) of durum (3 a), common (3 b) and durum+common (3 c) wheat
20 samples.
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26 **Figure 4.** Pairwise distance plot reporting the Mahalanobis distance between each sample and
27 the "Blank" and "Contaminated" classes.
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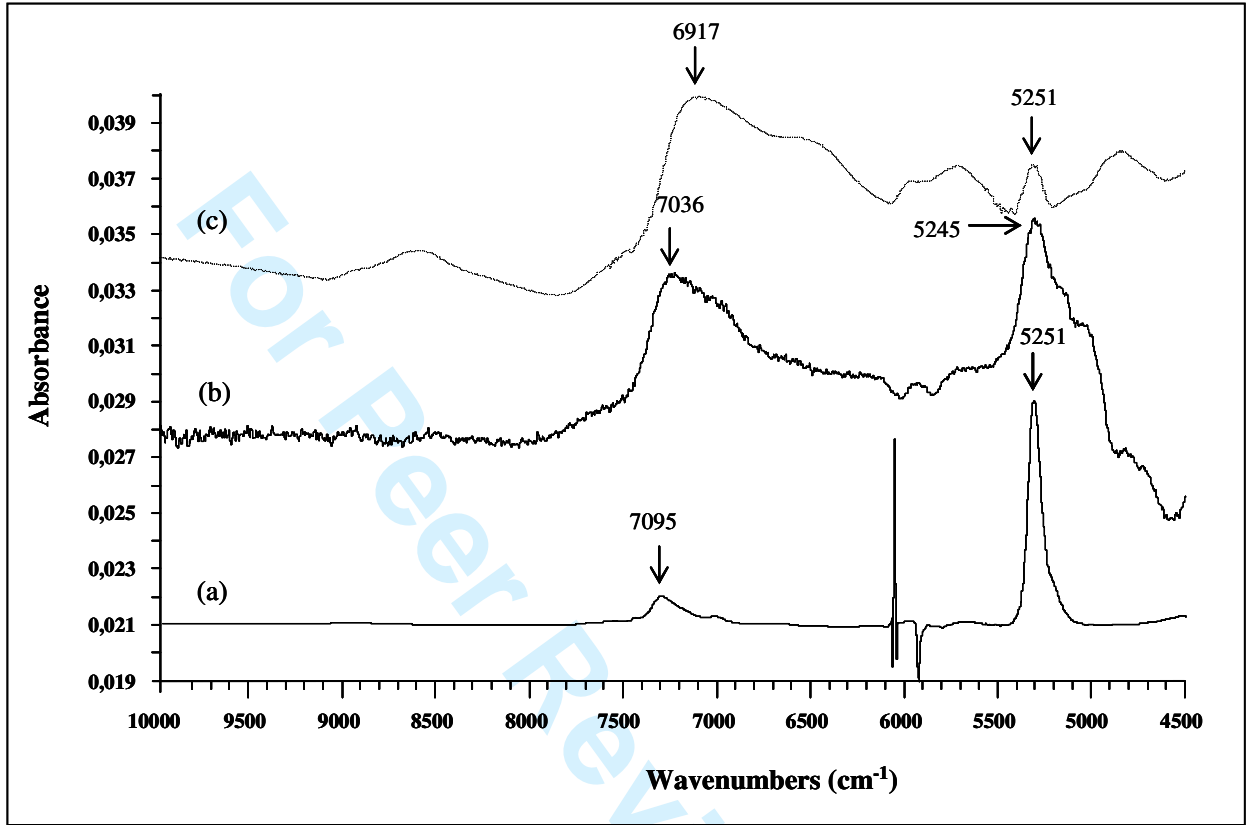
1 **Figure 1.**



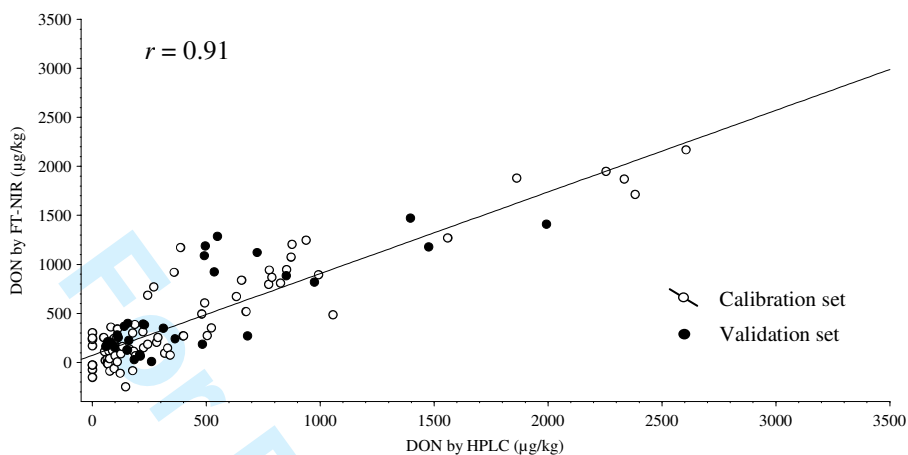
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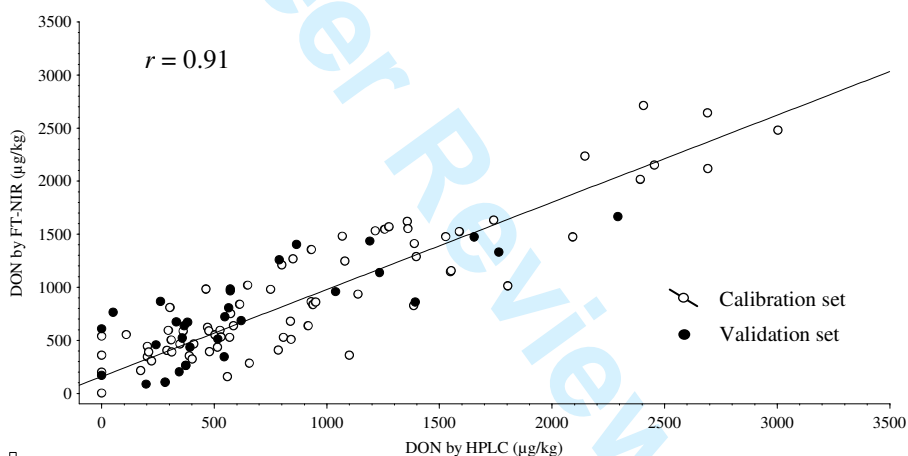
1 **Figure 2.**



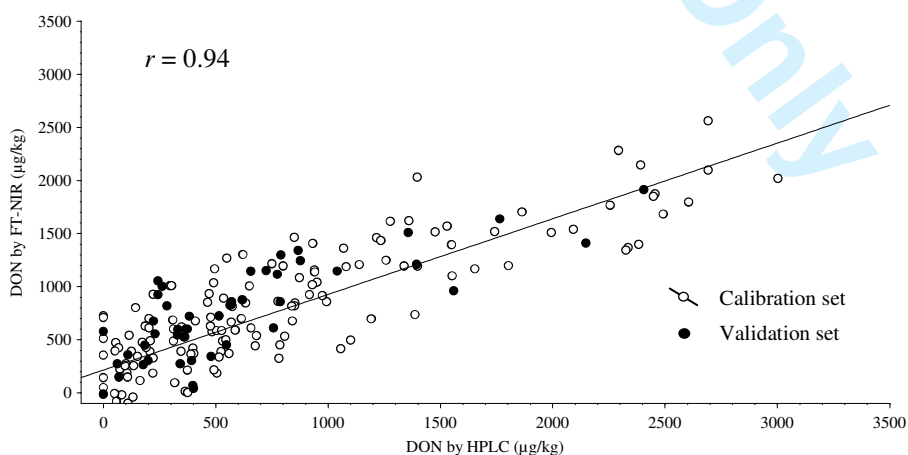
1 **Figure 3 (a)**



23 **Figure 3 (b)**



43 **Figure 3 (c)**



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1 **Figure 4.**

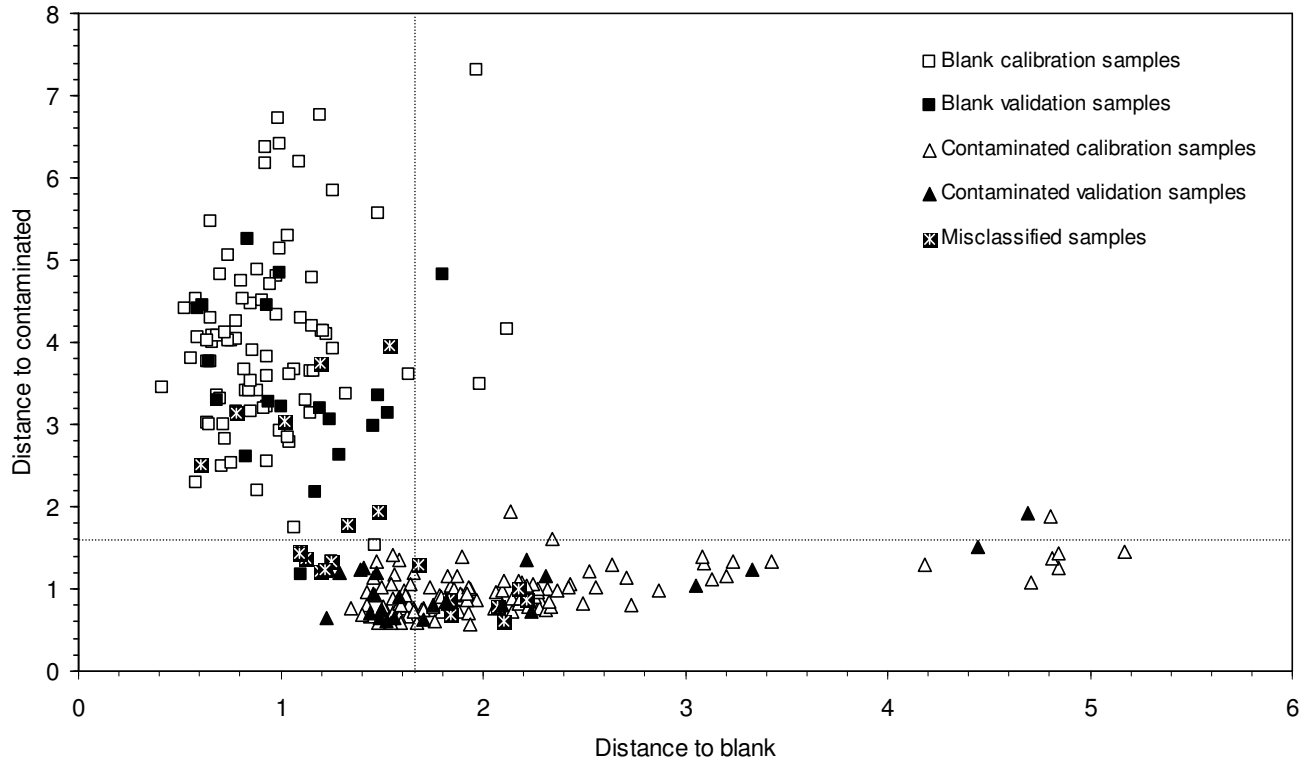


Table I. Deoxynivalenol content in ground durum wheat prior and after sieving (300 μm sieve).

Wheat sample (#)	Unsieved ($\mu\text{g}/\text{kg}$)	Sieved, < 300 μm ($\mu\text{g}/\text{kg}$)	Recovery (%)*	Sieved, > 300 μm ($\mu\text{g}/\text{kg}$)	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.

Table II. Statistical summary of deoxynivalenol content by HPLC in the calibration and validation sets of durum, common and durum+common wheat with particle size < 500 μm .

	Durum	Common	Durum+common	Durum	Common	Durum+common
	Calibration set			Validation set		
N. of samples	76	77	149	30	30	48
Range ($\mu\text{g}/\text{kg}$)	0-2600	0-3000	0-3000	60-1990	0-2290	0-2400
Median ($\mu\text{g}/\text{kg}$)	188	656	523	245	530	395
Mean ($\mu\text{g}/\text{kg}$)	439	887	750	459	658	585
SD* ($\mu\text{g}/\text{kg}$)	590	720	722	470	681	533

*SD = standard deviation

Table III. Calibration and validation results for deoxynivalenol detection by FT-NIR in durum, common and durum+common wheat with particle size < 500 μm .

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

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Deleted: RMSEC, root mean square error of calibration set;

* 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.

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RMSEC ($\mu\text{g}/\text{kg}$)	240	303	386

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