Near infrared spectroscopy for deoxynivalenol content estimation in intact wheat grain

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ABSTRACT

Non-invasive determination of deoxynivalenol (DON) still presents a challenging problem. Therefore, the present study was aimed at a rapid determination of DON in whole wheat grain by means of FT-NIR spectroscopy, with a wide range of concentrations for potential applications in breeding programs and common systems of quality management using partial least square calibration (PLS) and discriminant analysis technique (DA). Using a set of artificially infected wheat samples with a known content of DON, four PLS models with different concentration range were created. The broadest model predicting DON in the concentration range of 0–90 mg/kg possessed the highest correlation coefficients of calibration and cross validation (0.94 and 0.88); but also possessed the highest prediction errors (SEP = 6.23 mg/kg). Thus the subsequent combination of DA as the wide range predictive model and the low-range PLS model was used. This technique gave more precise results in the samples with lower DON concentrations – below 30 mg/kg (SEP = 2.35 mg/kg), when compared to the most wide-range PLS model (SEP = 5.95 mg/kg). Such technique enables to estimate DON content in collections of artificially infected wheat plants in *Fusarium* resistance breeding experiments.

Keywords: FT-NIR; *Triticum aestivum*; *Fusarium*; DON; discriminant analysis; PLS regression

Mycotoxins are secondary metabolites of microscopic filamentous fungi, which cause several different toxic syndromes. They can occur along the entire food chain; and thus can adversely affect the health and wellbeing of both humans and domesticated animals (Placinta and Mello 1999, Botallico and Perrone 2002). Mycotoxin deoxynivalenol (4-deoxynivalenol, vomitoxine, DON) belongs to a group of substances called *B. trichothecenes*, which are produced by the ascomycotic parasitic fungi of *Fusarium* spp.

They are the most common field pathogens for many crops (e.g. wheat, corn, barley, oat and rye), and they can cause ear rot diseases and grain contamination. The main worldwide DON producers are primarily *F. culmorum*, *F. graminearum* and *F. poae* (Tóth et al. 2005). DON is the most commonly detected trichothecene, with a high persistence during storage, as well as during processing due to its water solubility and heat stability (Pestka 2007).

EU legislation considers DON as a risk for consumers, and maximal residual limits were issued in 2006 (Commission Regulation (EC) No. 1881/2006). This directive established different allowed limits of DON concentrations for the following foodstuffs: e.g. 1.25 mg/kg for unprocessed cereals (other than durum wheat, oats, and maize); 0.5 mg/kg for baking products, pastries, and breakfast cereals; and 0.2 mg/kg for processed cereal-based foods, as well as baby foods. Even guidelines for the concentrations of several trichothecenes in animal feeds were introduced within the EU.

The long-term monitoring of *Fusarium* spp. and DON occurrence in winter wheat samples of Czech origin confirmed 29–78% annually contaminated samples and the legislative limit of DON concentration was exceeded in 2–4% (Štočková and Sýkorová 2008).

In the framework of *Fusarium* resistance research, controlled field trials with a targeted spike infection during flowering time have been carried out for an enhancement of differences in the responses to infection between sensitive and more resistant wheat genotypes. The DON concentration in such samples is ten or even hundred times higher than in samples obtained from fields with natural infections (Mesterházy 1995).

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Several methods are presently applied in DON concentration determination. HPLC quantitative analysis is usually used as the reference method for DON analysis in cereals (Visconti and Pascale 2010). ELISA methods are sensitive, rapid, and easy-to-use; and the new immunomethods, e.g. fluorescent polarization immunoassay, are still being developed as well (De Girolamo and Lippolis 2009). Nevertheless, the breeders and crop markets would like to utilize even quicker and cheaper methods of DON quantification, with regard to large selection activities of breeders and demands of the crop market on a higher frequency of controls.

FT-NIR spectroscopy could be another prospective possibility due to its rapidity and cost effectiveness, if a proper calibration is developed. This method is based on the absorption of specific wavelengths in the NIR region by certain functional groups within the tested chemical components, which enables both qualitative and quantitative analyses. The advantages of the Fourier-transformation include improvement in the signal-to-noise ratio, a significant reduction in the scan time, a higher energy throughout, superior spectral resolution and wavelength accuracy (Shiroma and Rodriguez-Saona 2009).

It was proven that DON has its unique absorbance spectrum in the NIR wavelengths, and the content changes of DON influenced the NIR spectra of the whole wheat grain (Peiris et al. 2009). Petterson and Aberg (2002) used near-infrared transmittance for the determination of whole wheat kernel samples in the concentration ranges of 0.4–11.0 mg/kg, using a dilution series of artificially infected wheat. Saito et al. (2009) successfully tested optical sorters with the RGB wavelength and NIR ranges for the removal of *Fusarium*-contaminated grains. De Girolamo and Lippolis (2009) successfully quantified the DON concentration in the range from 0 to 3 mg/kg using FT-NIR spectroscopy in ground durum and common wheat as well.

The aim of our work was to develop a rapid and non-invasive determination of DON in whole wheat grain, with a wide range of concentrations for potential applications in breeding programs and common systems of quality management.

MATERIAL AND METHODS

Experiment and sample characterizations. The 399 winter wheat samples (*T. aestivum* L.), from two different wheat experiments within a defined range of DON concentrations, were analysed using the Fourier transformation near-infrared spectroscopy (FT-NIR).

The first set included ten winter wheat varieties (Sulamit, Simila, Sakura, Petrus, SG-S316-05, Mladka, Radbuza, Bohemia, Rheia, Darwin) cultivated in the breeding station Selgen Stupice for four years (2006–2009) and the mixture of six *F. graminearum* isolates differing in their aggressiveness were used for artificial inoculation according to Chrpová et al. (2008).

The next experiment included advanced breeding lines and registered wheat varieties (46 different materials) in field tests at the Crop Research Institute (CRI) in Prague-Ruzyně, cultivated during 2008. Wheat samples were planted in hill plots. Artificial inoculation of spikes with the highly pathogenic isolate B of *F. culmorum* was performed during mid-flowering according to Chrpová et al (2008). Development of the disease was supported by irrigation of the plots.

The ELISA immuno-assay method (Ridascreen Fast DON commercial kit) was used as a reference method for the calculations of the NIR calibration models. This ELISA kit is widely used at the CRI Prague and its reproducibility was recently verified (compared with a HPLC method), with the values of LOD (limit of detection) and LOQ (limit of quantification) of 0.054 and 0.167 mg/kg, respectively (Chrpová et al. 2008).

FT-NIR measurements. The FT-NIR spectra of intact grains were recorded using an Antaris II FT-NIR spectrophotometer (Thermo Electron Corporation, Madison, USA), equipped with an interferometer, an integrating sphere working in diffuse reflection, plus an indium and gallium arsenide (InGaAs) detector.

Approximately 25 g of intact wheat grains were placed on the rotary sample-cup spinner and 32 interferometer sub-scans in ranges from 10 000 to 4000/cm, with a resolution of 2/cm were applied for the collection of each spectrum sample by means of the software Omnic 7.3 (Nicolet Instruments Co., Madison, USA).

NIR Spectra processing and model calculations. The processing of the collected spectra, development and validation of calibration models for the prediction of DON concentration were carried out with TQ analyst® software. The standard normal variation (SNV) technique was only used with following two statistical methods: partial least squares (PLS) regression, and discriminant analysis (DA). Validation samples (as a part of the tested samples) were independently selected by the TQ analyst software in order to cover spec-

tral variability of the tested set and the range of DON concentration in the approximate ratio of 10:1 (calibration: validation). Each PLS regression was characterized by a correlation coefficient (R), standard error correlation (SEC), correlation coefficient of cross validation (R_{cv}), standard error of cross-validation (SECV), standard error prediction (SEP) and the residual predictive deviation (RPD), defined as the ratio of the standard deviation of the reference DON values in the validation set to the SEP.

Two characteristics were used as a qualitative measure of the relevant DA. Performance index (PI), as the average Mahalanobis distance ratio in the range of values 0–100% was automatically calculated by the TQ analyst. The percentage of eliminated and misclassified samples needed for obtaining of robust models (PI ≥ 90) was another qualitative measure of the relevant DA as well.

The basic statistical characterization of DON concentration in a set of calibration samples (mean, median, standard deviation, skewness, kurtosis, minimum, and maximum), as well as individual PLS regression figures were defined using the Statistica 7.0 CZ statistical software (StatSoft, Inc., Tulsa, USA).

RESULTS AND DISCUSSION

DON distribution. The basic statistical characterization of DON concentration in the wheat samples is presented in Figure 1. The reference values of the determined DON concentrations (measured using ELISA) ranged from 0 to 178.48 mg/kg. Nevertheless, extremely high DON concentrations (above 100 mg/kg) very rarely occurred and 50% of the wheat samples did not exceed 1.50 mg/kg, and 44% of the tested samples showed DON concentration values below the declared hygienic limit of 1.25 mg/kg. The Shapiro-Wilks W test proved, as being significant, that the obtained data did not have a normal distribution. These results confirmed that the evaluation of breeding experiments, based on artificial inoculation, requires a wide-ranging analytical method which is able to estimate high levels of DON concentrations, as well as (on the other hand) to be able to determine low concentrations of DON with a reasonable accuracy.

Spectral characteristics of whole grains. During the development of the PLS models' calibration, two spectral regions (1390–1770 nm, and 1880–2070 nm) with the highest correlation coefficients to the DON concentration were identified (Figure 2). These two wavelength regions provided the best prediction parameters of calculated PLS models and currently covered the typical wavelength absorption bands of DON (1408 nm, 1904 nm, and 1919 nm) mentioned by Peiris et al. (2009). In accordance with De Girolamo and Lippolis (2009), these selected regions also reflected differences of chemical composition in particular wheat samples, that could be caused by primary variabilities of the varieties, as well as by various abiotic and biotic factors including *Fusarium* infection. Thus, the successive DON prediction seems to be based on the ability to differentiate the spectral variability caused by changes in the principal wheat constituents (protein, starch, etc.) from changes in the DON content.

PLS model calculations. The initial widest-ranged PLS model (Table 1, Figure 3) did not cover all ranges of DON concentrations in the measured grains, since the wheat samples with concentrations

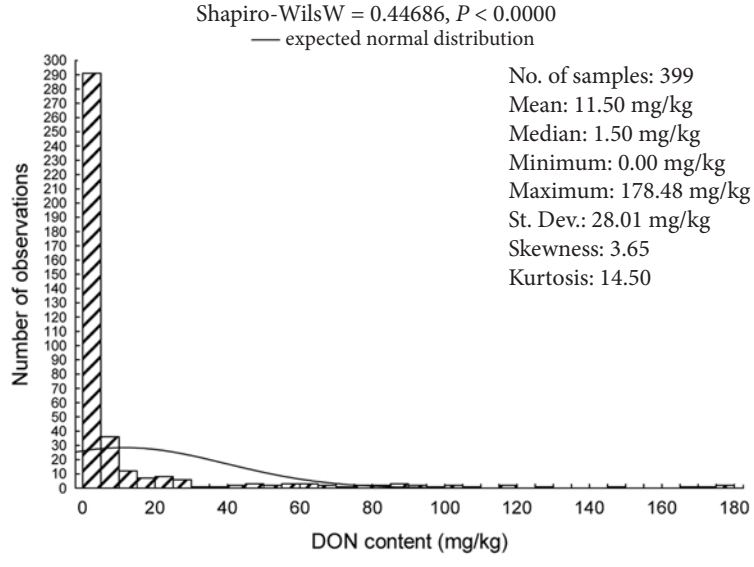

Figure 1. Distribution of deoxynivalenol (DON) concentration in a calibration samm ple set assessed by ELISA (2006–2009)

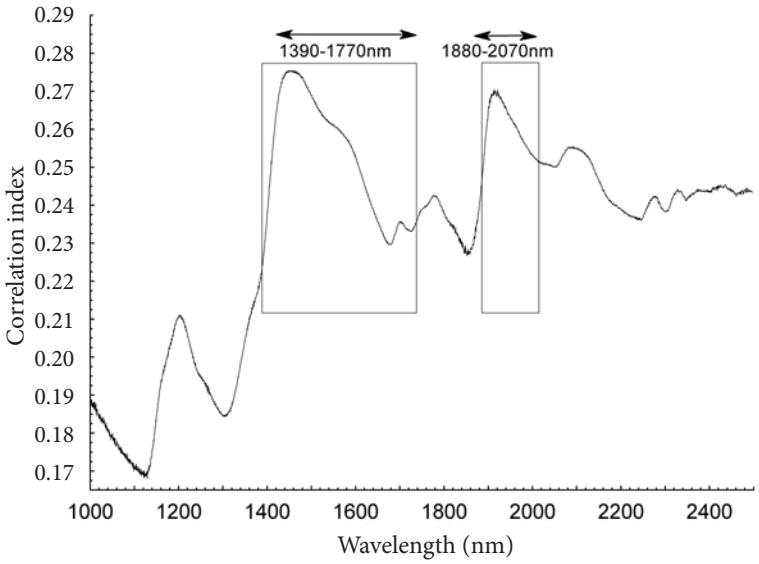

Figure 2. Correlation indexes of individual wavelengths in relation to deoxynivalenol (DON) concentration

above 92 mg/kg were eliminated from this calibration model. These eliminations partially included spectral outliers (as defined by the Chauvenet test); and also partially included samples with a high prediction error in the cross-validation diagnostic. Nevertheless, the total percentage of sample eliminations was in the acceptable 4–6% range in all of the PLS models developed.

The predictive parameters of PLS model 1 showed the similar values of R (0.93) and R_{cv} (0.88) and a higher value of RPD (3.02) presented by De Girolamo and Lippolis (2009). Regarding a thirty fold wider range of DON concentration in our PLS model, the prediction errors (RMSEC, RMSECV, and RMSEP) were significantly higher. According to Williams (2003), the obtained coefficient of determination (R^2_{cv} = 0.77) reached in PLS model 1 would allow approximate quantitative predictions. The obtained value of RPD would also indicate our NIR calibration models suitable only for screening purposes (Williams 2003, Smyth et al. 2008). The uncertain prediction of lower DON concentrations is well documented in Figures 4. Although the absolute prediction errors were decreasing as the DON concentrations increased, the relative predictive errors (RPE) dramatically increased along with the decrease of DON concentration below 10 ppm.

Consequently, three other PLS calibration models with closer ranges of DON concentrations were derived (Table 1; models 2–4). The PLS models 2 and 3 showed comparable parameters (R = 0.89–0.92; R_{cv} = 0.83–0.85) with the PLS model 1. Furthermore, their values of RMSEC, RMSECV, and RMSEP were 3–7 times lower. Nevertheless, the RPD values oscillated around 2.6 which does not offer a higher robustness of both PLS models in their actual ranges compared to the PLS model 1. Other essential limiting factor in using a low-range PLS model was that the models were not able to reliably exclude the samples with DON content above the upper limit of the calibration range.

Model 4, with the closest predictive range of DON concentration 1–5 mg/kg showed the lowest RMSEC, RMSECV, and RMSEP. However, its prediction confidence was also the lowest of all the models (R = 0.70; R_{cv} = 0.67; RPD = 1.43). It is evident that the attempts at predictions of DON concentrations with FT-NIR spectroscopy in intact grain samples and in concentration ranges close to the legislative limit are limited. It is also not possible to expect a similar sensitivity as in

Table 1. PLS model characterizations for prediction of deoxynivalenol (DON) concentration by FT-NIR

Model	Prediction range of model (DON mg/kg)	No. of samples	R_{cal}	RMSE (mg/kg)	R_{cv}	RMSECV (mg/kg)	RMSEP (mg/kg)	No. of factors	RPD
1	0–92	399	0.94	5.45	0.88	7.10	6.23	9	3.02
2	0–30	367	0.92	1.91	0.85	2.50	2.43	11	2.60
3	0–13	339	0.89	1.08	0.83	1.30	1.22	10	2.63
4	0–5	298	0.70	0.88	0.67	0.92	0.95	5	1.43

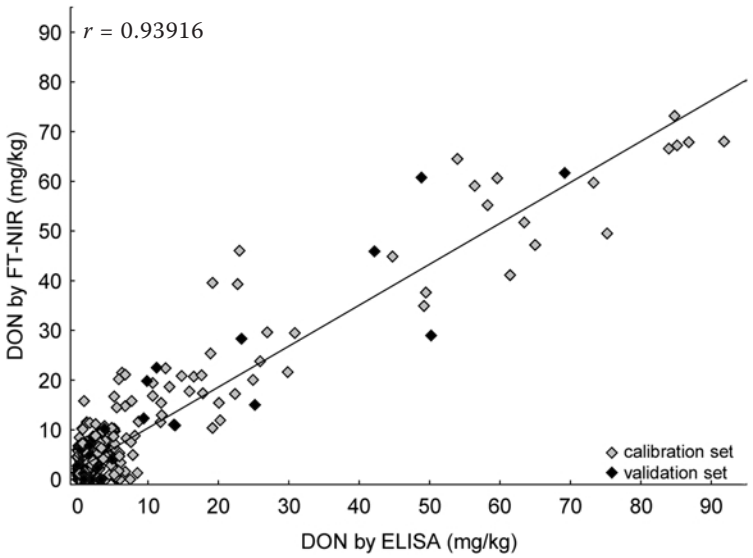

Figure 3. Partial least square (PLS) regression model 1 of deoxynivalenol (DON) concentration determined by the FT-NIR and the reference method (ELISA)

a model based on prediction of DON concentration in acetonitrile (Peiris et al. 2009). Pettersson and Aberg (2002) obtained a higher reliability of DON prediction in intact grains. However, their calibration was based on dilution series of small number of contaminated wheat samples. That ensures better calibration parameters but likely limits the applicability of this calibration in real samples.

Despite the fact that DON concentration does not have to be in any close relationship to the intensity of the *Fusarium* infection (Brennan et al. 2007), the more reliable prediction of DON

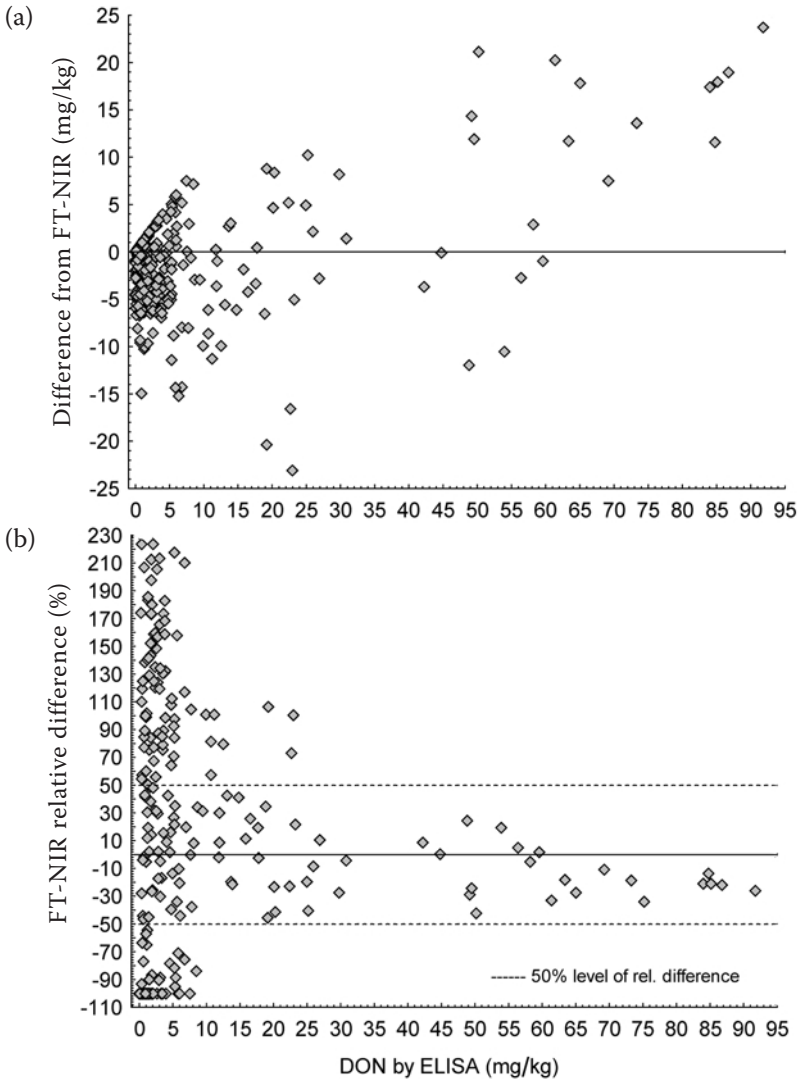

Figure 4. (a) Specific absolute and (b) specific relative differences of deoxynivalenol (DON) content predicted by partial least square (PLS) model 1

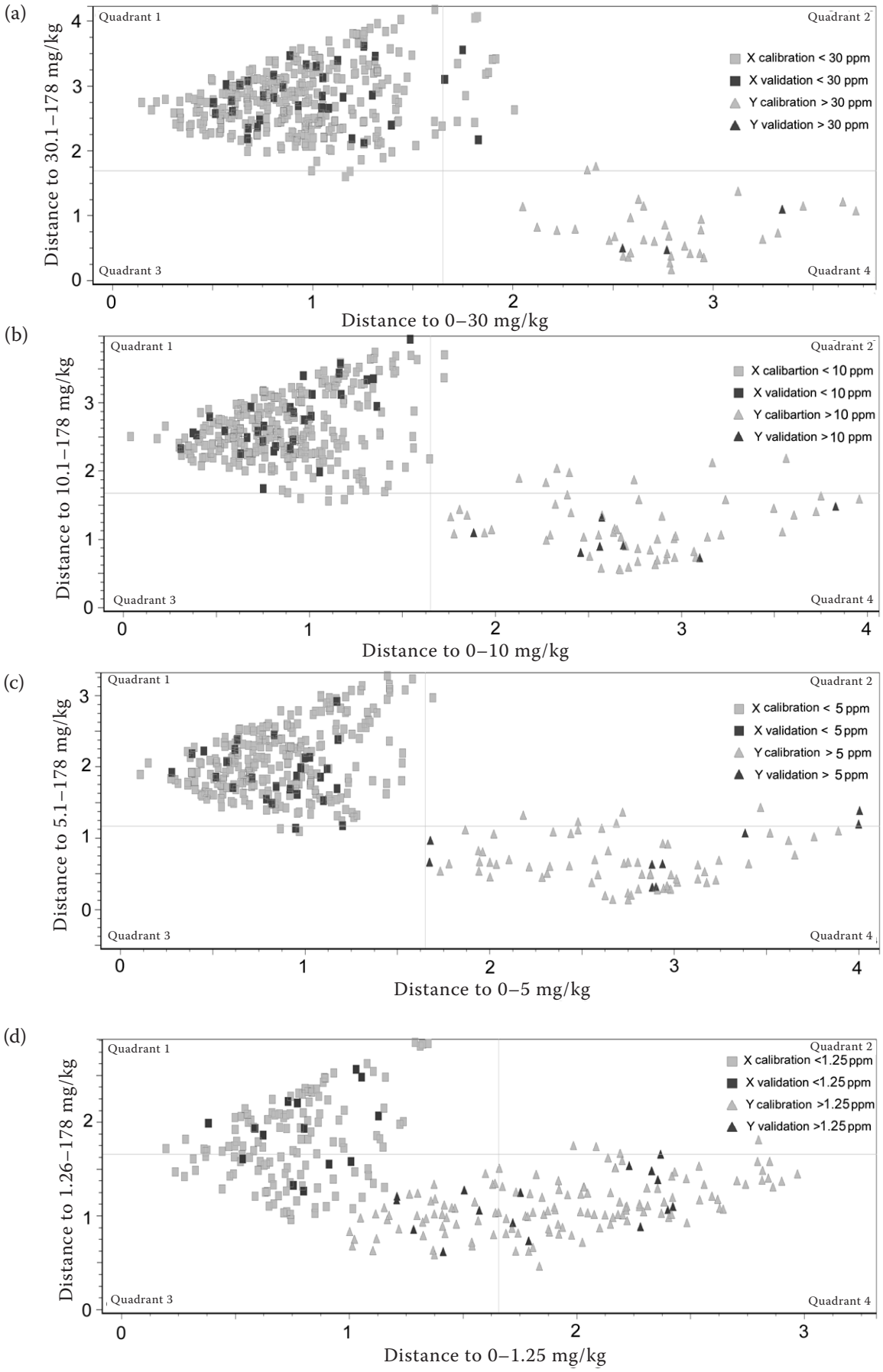

Figure 5. Distance plot l of discriminant analysis technique (DA) for the classification limit of deoxynivalenol (DON) content of (a) 30 mg/kg; (b) 10 mg/kg; (c) 5 mg/kg and (d) 1.25 mg/kg

Table 2. Model parameters of calculated discriminant analyses for different classification limits of deoxyniva-lenol (DON) concentration

DA model No.	Classification categories (mg/kg)	No. of samples	Σ Excluded + misclassified (%)	Performance index
1	0.00–30.00	360	6.39	93.3
	30.10–178.00	39	7.69	
2	0.00–10.00	327	6.42	93.4
	10.10–178.00	72	12.50	
3	0.00–5.00	291	6.53	92.1
	5.10–178.00	108	28.70	
4	0.00–1.25	179	19.55	89.1
	1.26–178.00	220	27.73	

DA – discriminant analysis technique

concentrations of samples markedly overstepping hygienic limit could be explained by a higher activity of *Fusarium* infections which negatively affect the wheat grain properties. These changes can be readily recorded in NIR spectra and statistically evaluated (Antes et al. 2001, Prange et al. 2005).

Improvement of DON prediction by FT-NIR spectroscopy. Further increases in the accuracy of the PLS predictive models of DON concentrations in intact grains could be achieved by the precise control of growing conditions and a selection of calibrated samples reflecting the genetic background of the tested materials. Additional possibilities might be found in a suitable combination of statistical methods. The combination of two methods: discriminant analysis (DA) and subsequent PLS quantitative analysis, with an appropriate calibration range, seemed to us perspective. This procedure should eliminate the reduced ability of low-range PLS models to recognize a sample with a DON concentration above the calibration range, and to take advantage of the lower prediction errors of the lower-range PLS models. The three suggested DON concentration limits for calculations of DA, corresponded with the upper concentration limit of each smaller-ranged PLS model 2–4. It should enable a collective combination of both models (DA and PLS) and a reduction of predictive errors in materials with lower DON concentrations (< 30, < 10, and < 5 mg/kg), compared to the direct application of PLS model 1. The fourth concentration limit presented the legislative value of DON concentrations for unprocessed wheat (1.25 mg/kg). Consequently, four calibration models of DA were obtained (Figures 5a–d, Table 2).

In spite of comparable high PI values (89–93) obtained from final DA models, the ratio of ex-

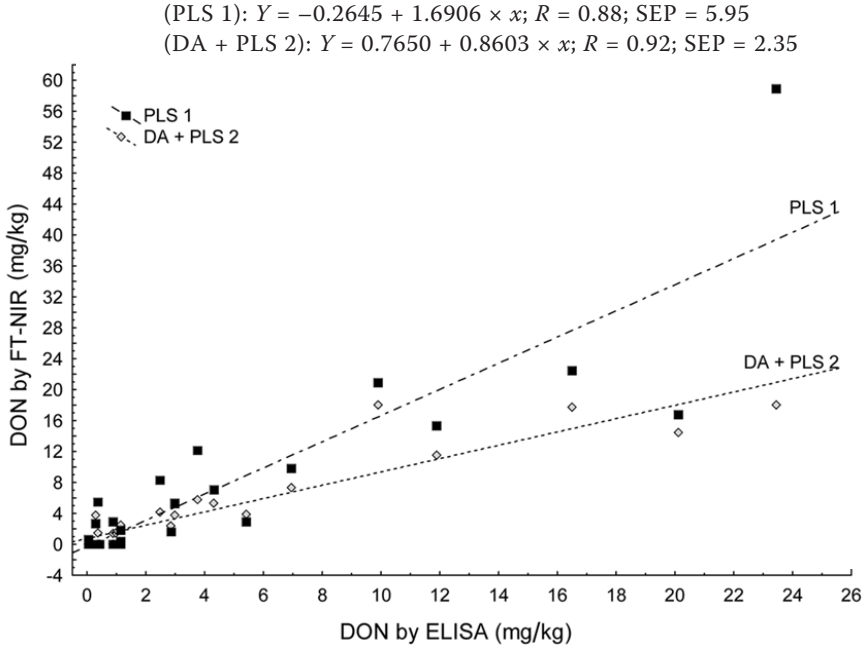

Figure 6. The accuracy of deg oxynivalenol (DON) prediction with direct application of partial least square (PLS) regression model 1 and application of dise criminant analysis technique (DA) and PLS regression model 2 (Linear regression)

cluded and misclassified samples (quadrant 2 and 3) increased with a decreasing limit of DON concentration. Especially, DA models 3 and 4 have already indicated a high ratio of misclassified samples (20–30%). On the other hand, De Girolamo and Lippolis (2009) reported a successive classification between blank and contaminated sample, despite the fact that 20% of calibration samples fell in the uncertainty area. They also confirmed that the goodness of the discrimination model was greatly influenced by the selection of proper DON cut-off limit between both classes.

The benefit of the combination of DA with the highest classification limit of 30 mg/kg and PLS model 2 with a similar range of DON concentrations compared to the application of PLS predictive model 1 is displayed in Figure 6. It is evident that the regression model computed from the combination of DA with PLS regression 2 for 27 common validation samples showed a higher correlation coefficient (r = 0.92), and a lower SEP (2.35 mg/kg) to the reference values, compared to the PLS regression 1 (r = 0.88; SEP = 5.95 mg/kg). Thus, although this statistical combination did not enable to reliably distinguish wheat samples with DON concentrations below hygienic limit, the detection of more resistant and sensitive wheat genotypes in the framework of resistance breeding experiments will be possible.

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