

Scientific paper

# Detection of Deoxynivalenol in Wheat by Fourier Transform Infrared Spectroscopy<sup>†</sup>

Biljana Abramović<sup>1</sup>, Igor Jajić<sup>2</sup>, Borislav Abramović<sup>3</sup>,  
Jasenska Čosić<sup>4</sup>, Verica Jurić<sup>2</sup>

<sup>1</sup> Faculty of Sciences, Department of Chemistry, Trg D. Obradovića 3, 21000 Novi Sad, Serbia  
Phone: +38121 4852753, Fax: +38121454065

<sup>2</sup> Faculty of Agriculture, Trg D. Obradovića 8, 21000 Novi Sad, Serbia

<sup>3</sup> High technical School, Školska 1, 21000 Novi Sad, Serbia

<sup>4</sup> Faculty of Agriculture in Osijek, Trg svetog Trojstva 3, Osijek, Croatia

\* Corresponding author: E-mail: abramovic@ih.ns.ac.yu

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## Abstract

The possibility of using two Fourier transform mid-infrared spectroscopic techniques was investigated with the purpose of rapid detection of mycotoxin-producing *Fusarium* fungi on wheat, as an indicator for the presence of the mycotoxin deoxynivalenol (DON). Samples of a single wheat genotype (Monika, blanks and contaminated with *Fusarium graminearum*) were ground and analyzed applying the diffuse reflection (DR) and attenuated total reflection (ATR) modes. The recorded spectra were evaluated with principal component analysis and the blank and contaminated samples were classified by cluster analysis. Besides, the possibility was examined of determining DON on the basis of the ratio of ATR signals at 1709 cm<sup>-1</sup> and 1743 cm<sup>-1</sup>. Reference measurements were performed by high performance liquid chromatography with diode array detection. The concentration range for contaminated samples was 2.51–12.14 mg/kg. Classification efficiency was 100% for ATR spectra, whereas DR spectra did not show so obvious clustering of contaminated and blank samples. The ATR technique appeared advantageous owing to its easier use and interpretation of results, which were better in respect of classification and quantification. Quantification using partial least squares (PLS1) regression, as well as multiple linear regression (MLR) showed good correlation with DON reference data for the mentioned wheat genotype.

**Keywords:** *Fusarium* fungi, attenuated total reflection, mid-infrared spectroscopy, wheat, mycotoxins, chemometrics.

## 1. Introduction

Food safety represents an important issue worldwide. Every year, a large number of crops are affected by fungal invasion, leading to considerable financial losses and impaired health in animals and humans. Toxicity is mainly caused by secondary metabolites of fungi – mycotoxins. Thus, for example, mycotoxin aflatoxin belongs to the group 1 carcinogens, which, according to the International Agency for Research on Cancer (IARC),

means it is carcinogenic to humans, whereas fumonisin B<sub>1</sub> belongs to the group 2B, which means that it is possibly carcinogenic to humans.<sup>1,2</sup> Therefore, mycotoxin prevention and control is a global problem and contaminated commodities impair trade and threaten consumer safety.

Deoxynivalenol (DON or vomitoxin) is a mycotoxin of the chemical structure 12,13-epoxy-3 $\alpha$ ,7 $\alpha$ ,15-trihydroxytrichothec-9-ene-8-one, which belongs to the group of trichothecenes. DON is a secondary fungal metabolite produced by various species of *Fusarium*, especially *Fusarium graminearum* (*Gibberella zeae*) and *Fusarium culmorum*, both of which are important plant pathogens commonly found in cereals and other crops.<sup>3</sup> Although DON is among the least toxic of the trichothecenes, it is the most frequently detected throughout the world, and its

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occurrence is considered to be an indicator of the possible presence of other, more toxic trichothecenes.<sup>4</sup> Consumption of contaminated feed by livestock has been associated with a variety of adverse health effects including feed refusal, reduced weight gain, diarrhea and emesis.<sup>5,6</sup> Maximum permitted levels for DON have not yet been agreed upon, varying thus from country to country and depending on the substrate type. The U.S. Food and Drug Administration permits a maximum of 1.00 mg/kg of DON in finished wheat products ready for human consumption,<sup>4</sup> and limits from 5.00 to 10.0 mg/kg, depending on the type of commodity.<sup>7</sup> Russia<sup>4</sup> permits a maximum level of 1.00 mg/kg of DON in wheat, flour and bran. The same limit is permitted in China<sup>8</sup> and Switzerland<sup>9</sup> in cereals for human consumption. Austria<sup>4</sup>, however, has guidelines of 0.50 mg/kg of DON in wheat and rye and 0.75 mg/kg in durum wheat intended for foods. Health Canada has set the highest guideline of 2.00 mg/kg in uncleaned soft wheat intended for human consumption. In Serbia, the maximum permitted level of DON in feed and groceries has not been set yet, although the presence of DON has been identified.<sup>10</sup> The Commission of the European Communities<sup>11</sup> established the following tolerance values for DON in cereals and cereal-based products: unprocessed cereals other than durum wheat, oats and maize (1.25 mg/kg), unprocessed durum wheat and oats (1.75 mg/kg), unprocessed maize (1.75 mg/kg), cereal flour, including maize flour, maize grits and maize meal (0.75 mg/kg), bread, pastries, biscuits, cereal snacks and breakfast cereals (0.50 mg/kg), pasta (dry, 0.75 mg/kg), and processed cereal-based food for infants and young children and baby food (0.20 mg/kg).

A number of analytical techniques such as direct competitive enzyme-linked immunosorbent assay, thin-layer chromatography, gas chromatography, liquid chromatography, and fluorimetry, have been developed for the determination of DON in cereals and cereal-based foods and feeds.<sup>3–5,12</sup> The most frequently used techniques today are gas and liquid chromatography. However, the application of these methods is time consuming, tedious, and requires a high level of experience and expertise. Several sample preparation steps are needed, which results in a low sample throughput, since extensive extraction and clean-up steps must be performed before separation and detection either by gas or liquid chromatography. However, in view of large amounts of cereals that are processed in the food and feed industries each year and high infection rates, frequent checks are needed, which requires a large number of samples.

For this reason the possibility was examined of applying visual inspection of wheat kernels.<sup>13</sup> However, the method is labor-intensive and often the fungus resides inside the kernel,<sup>14</sup> making visual inspection impossible. Besides, it is not possible to evaluate samples after their grinding, which is a common step to overcome the inhomogeneity problem and obtain a representative sample.<sup>15</sup>

Infrared spectroscopy has found widespread use in the analysis of food and feed in recent years.<sup>16</sup> It provides

quick and reliable information about the sample. Some infrared spectroscopic techniques have been also developed as a means of identifying samples infected by fungi and estimating DON content.<sup>17–21</sup> Thus, for example, near-infrared reflectance (NIR) spectroscopy was used for detection of scab and estimation of DON and ergosterol in single kernels of highly infected wheat,<sup>17</sup> whereas Ruan et al.<sup>18</sup> developed a neural network based procedure for determining DON levels in barley, also by NIR spectroscopy. Pettersson and Åberg<sup>19</sup> used near-infrared transmittance (NIT) spectroscopy for the determination of DON in wheat kernel samples at levels just above the proposed EU maximal limits in wheat flour.

Besides, the possibility has been examined of a rapid detection of DON and ergosterol on corn by two mid-infrared spectroscopic techniques – diffuse reflection (DR)<sup>20</sup> and attenuated total reflection (ATR).<sup>20,21</sup> The ground sample was sieved and the particle size fraction between >250 and 100  $\mu\text{m}$  was used for mid-infrared ATR measurements.<sup>21</sup> In DR measurements, additional sample preparation included a dilution step with potassium bromide (1% w/w).<sup>20</sup> In both cases the calculation was based on the major part of the fingerprint area (the range between 1800 and 800  $\text{cm}^{-1}$ ). The measured spectra were evaluated with principal component analysis (PCA) and the blank and contaminated samples were classified by cluster analysis. Under the given conditions, the classification efficiency was 100% for ATR spectra, but DR spectra did not show as obvious a clustering of contaminated and blank samples. The concentration range for DON determination in contaminated samples was 0.30–2.60 mg/kg.

The aim of this work was to investigate the possibility of identification and quantification of DON in wheat (Monika genotype) on the basis of spectra obtained by Fourier transform mid-infrared spectroscopy (FTIR) with diffuse and attenuated total reflection. The measured spectra were evaluated using PCA, whereby blanks and contaminated samples were classified by cluster analysis. Also, the objective was to examine whether there is a characteristic change in the mid-infrared spectra of infected wheat which would enable establishing a univariant approach to the determination of DON concentration.

## 2. Experimental

### 2.1. Sample Preparation

All wheat samples were taken from one single genotype (Monika, Agricultural Institute Osijek) in order to eliminate any interference in mid-infrared spectra caused by genotype variations. Artificial infection (inoculation) of wheat was performed when 50% of ears were in the flowering stage, which is considered to be optimal for this purpose. The isolated *F. graminearum* originated from wheat grains and the isolate cultivation was carried out on

a PDA medium. Each of fourteen batch samples, consisting of 160 ears, was sprayed with a suspension of mycelia and spores (10,000 spores/mL suspension), using 20 mL of the suspension. In order to ensure sufficient humidity for infection and initial development of the disease the ears were kept covered with transparent PVC bags for 24 hours. At full ripeness, the ears were collected and threshed manually, and the grains were stored in a refrigerator at  $-20\text{ }^{\circ}\text{C}$ . Three control samples were sprayed with distilled water, the rest of the procedure being identical. Prior to recording FTIR spectra, each sample was prepared by grinding in a laboratory mill in such a way that 93% passed through a sieve with pore diameter of 0.8 mm.

## 2. 2. FTIR Spectroscopy

Spectra were recorded using a Thermo Nicolet Nexus 670 spectrometer with deuterated triglycine sulfate (DTGS) as detector which requires no maintenance. The interferometer was operated at scan rate of  $0.63\text{ cm s}^{-1}$ . All spectra were acquired at  $4\text{ cm}^{-1}$  resolution between  $650$  and  $4000\text{ cm}^{-1}$ . Air was used as a background spectrum, and 100 scans were coadded for each spectrum. Prior to taking every spectrum the baseline was recorded under the same conditions, to eliminate the effect of possible changes in the quality of the air (content of  $\text{CO}_2$  and humidity). Fourier transformation was performed with a Mertz phase correction and Happ-Genzel apodization function.

*Diffuse reflection spectroscopy.* DR spectra were recorded using a single-cup holder. In the front position it holds the platen from the Si-Carb sampling tool. The cup holds approximately 0.25 g of sample. A gold disk mounted in the back position is used for the background measurements. No special preparation of the air contained in the sample chamber was performed. Baseline was taken with respect to gold.

*Attenuated total reflection spectroscopy.* Use was made of an ATR accessory with ZnSe-crystal, which allowed 12 internal reflections and pathlength of  $12\text{ }\mu\text{m}$ . Angle of incidence was  $45^{\circ}$ . A pressure applicator with a torque knob pressed the sample against the crystal and ensured the application of a reproducible pressure during the measurement.

## 2. 3. Reference Method

Data from conventional method for DON in each sample were obtained after extraction and clean-up. Separation and detection were performed using HPLC-DAD method.<sup>12</sup>

## 2. 4. Data Analysis

Unbiased raw data, without smoothing, baseline corrections or other manipulations, were collected. The spectra were acquired using software provided with the

spectrometer. All spectra (used for statistical treatment) were averaged from five repeat ATR/DR measurements, each recorded from a new sub-sample. These averaged spectra were normalized.

After recording and data treatment, the spectra were converted from the internal Omnic Spectral File to the Microsoft Office Excel Comma Separated Values File for further data analysis. Several spectral windows were chosen for data analysis. Further data analysis was performed with Statistica, version 7.1 (StatSoft, Inc. (2006). STATISTICA (data analysis software system), version 7.1. www.statsoft.com.), and Unscrambler 9.7 (Camo, Oslo, Norway). The number of data points varied from 50 to 580 for each spectrum before PCA calculations. PCA was based on covariance matrix of active variable. Results were displayed as score/score plots. Cluster analysis of relevant principal components for classification purposes was performed using Euclidean distance and Ward's method for linkage rule. The K-mean-clustering method was employed to obtain graphs of cluster means.

Partial least squares (PLS1) regression was used for attempting quantification on the basis of the range DR, as well as ATR spectra. In the case of the determination based on one or two points use was made of multiple linear regression (MLR). Data were correlated with DON results from reference measurements.

## 3. Results and Discussion

Kos et al.,<sup>21</sup> in their analysis of corn, found that for obtaining satisfactory reproducibility of ATR measurements it was necessary to separate a sample fraction with particle diameter between  $100\text{--}250\text{ }\mu\text{m}$ , which required additional 30 minutes for sieving after grinding. In view of this we examined first the effect of particle size on the reproducibility of measurements under our experimental conditions. In Figure 1 are shown normalized spectra of the ground non-contaminated wheat (Monika genotype)

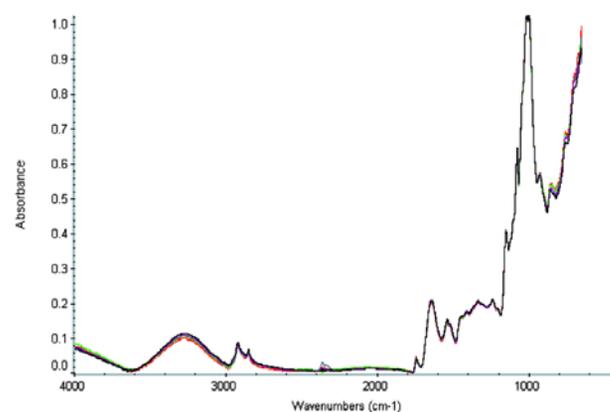


Figure 1. Repeatability of 5 normalized spectra measurements of the ground non-contaminated wheat

obtained by the ATR technique. As can be seen, the repeatability of spectral measurements was good enough for attempting classification when the grain size was  $90\% \leq 0.8$  mm. Such finding is probably a consequence of a larger surface area of the crystal ( $\sim 70$  mm<sup>2</sup>) in contrast to the one used by Kos et al.<sup>21</sup> ( $\sim 3$  mm in diameter), as well as of a larger number of internal reflections (12 vs. 3).

Several spectral regions were investigated and the one between 1800 and 650 cm<sup>-1</sup> was selected for the calculations in both techniques, to minimize the influence of noise and irrelevant information (Figures 2 and 3). The remaining parts of the DR and ATR spectra were excluded from the calculations because the intensity of the OH-

stretching vibration at  $\sim 3300$  cm<sup>-1</sup> had a rather large variation, mainly because of the drying process, which was not very reproducible and whose only purpose was to stop the fungal growth. The C–H stretching bands of CH<sub>2</sub> group at 2925 and 2855 cm<sup>-1</sup> are ubiquitous and therefore not specific enough for the purpose of the study. The range between 2250 and 2400 cm<sup>-1</sup> was removed because this region is sensitive to the changes of CO<sub>2</sub> in the atmosphere.

Because of the small differences between spectra of contaminated sample and blank (see Figures 2 and 3) a visual classification was not possible, so that multivariate methods for the extraction of relevant information seemed

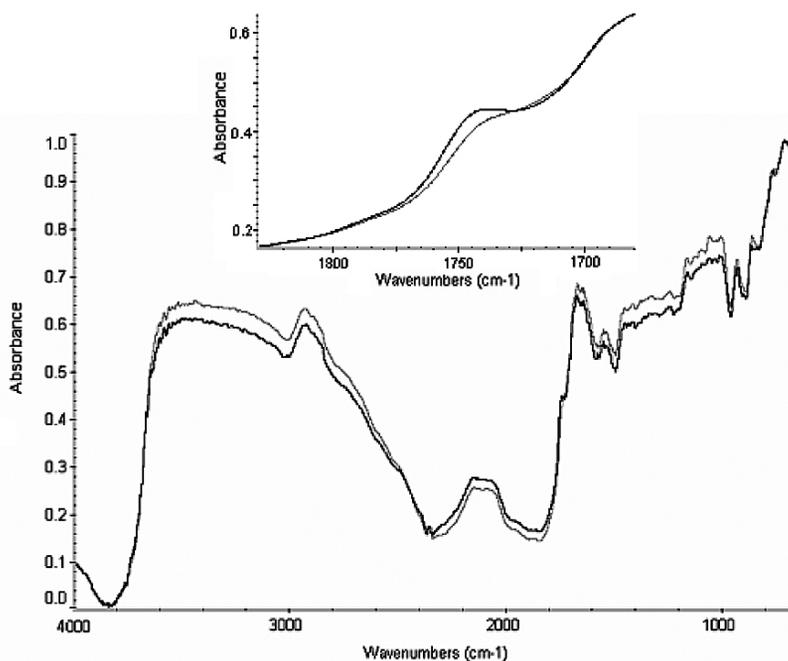


Figure 2. DR spectrum of contaminated (light trace) and non-contaminated (dark trace) wheat

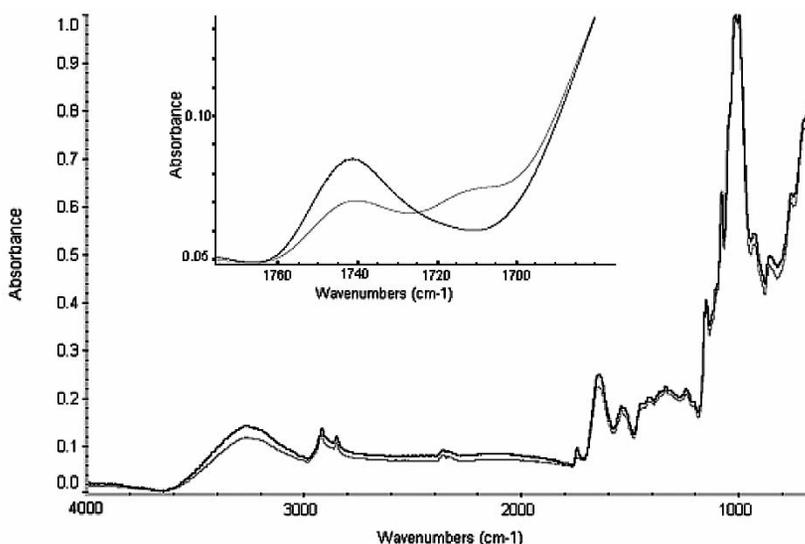
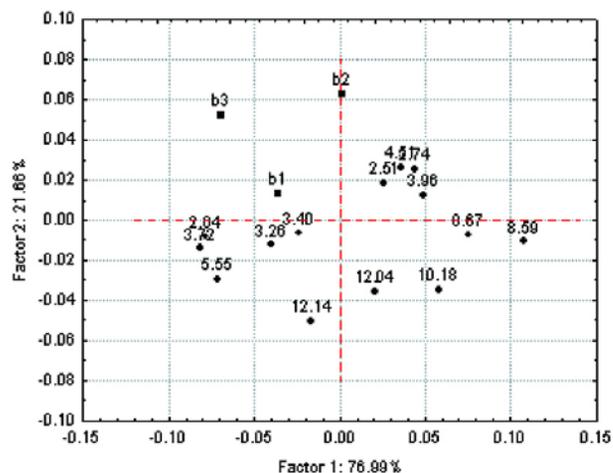


Figure 3. ATR spectrum of contaminated (light trace) and non-contaminated (dark trace) wheat

to be necessary. Sample numbers in all plots give DON concentrations in mg/kg obtained from reference measurements, whereas the blanks are marked with “b”.

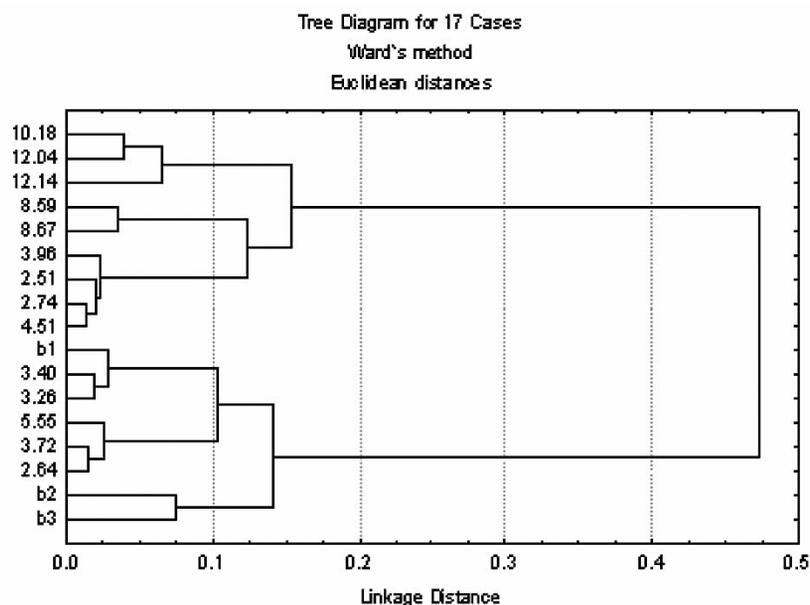
However, the classification results obtained by PCA and cluster analyses were not satisfactory when either of the two techniques was applied in the mentioned spectral region. Because of that we attempted to narrow the spectral range to determine an optimal one which would yield satisfactory classification. It appeared that an optimal spectral range for DR was between 1830 and 1710  $\text{cm}^{-1}$  (Figure 2, insert), and for ATR from 1765  $\text{cm}^{-1}$  to 1690  $\text{cm}^{-1}$  (Figure 3, insert). Namely, for the majority of spectra of infected wheat the carbonyl peak at 1739  $\text{cm}^{-1}$  (DR), and 1743  $\text{cm}^{-1}$  (ATR) decreased compared to that of uninfected wheat (Monika genotype). It was also discovered serendipitously that the height of the shoulder at 1709  $\text{cm}^{-1}$  in the ATR technique was related to fungal infection in wheat. Similar results were also obtained by Gordon et al.<sup>22</sup> in studying the applicability of neural network pattern recognition of photoacoustic FTIR for the detection of corn kernels infected with mycotoxigenic fungi. Elevation of the shoulder is an empirical feature for which no biochemical rationale has been proposed.<sup>22</sup>

Figure 4 shows a score/score plot for several wheat samples calculated from DR data. All data were averaged from five consecutive measurements and then normalized and baseline corrected at 1830  $\text{cm}^{-1}$ . The PCA score/score plot shows a good separation between blank and contaminated samples, but blank 1 (b1) clusters in the contaminated area and therefore cannot be identified as a blank sample. When cluster analysis was applied to the principal components, this effect was enhanced and a clear pattern



**Figure 4.** PCA of averaged, normalized and baseline corrected DR spectra (1830–1710  $\text{cm}^{-1}$ ) of wheat with varying of DON content. Sample numbers in the plot give DON concentrations in mg/kg. Blanks are designated with b1 – b3

of two clusters made up of contaminated and blank samples could not be identified, as shown in Figure 5. Blank 1 is not classified as non-contaminated, which clusters among the contaminated samples. Besides, linking of sub-clusters of contaminated samples was not adequate. Namely, in one of the cases the cluster of wheat with DON content from 2.64 to 5.55 mg/kg is linked with the blank, and in the other case the cluster of wheat with DON content from 2.51 to 4.51 mg/kg links to the clusters containing samples that are significantly contaminated with DON. Such inappropriate classification is understandable

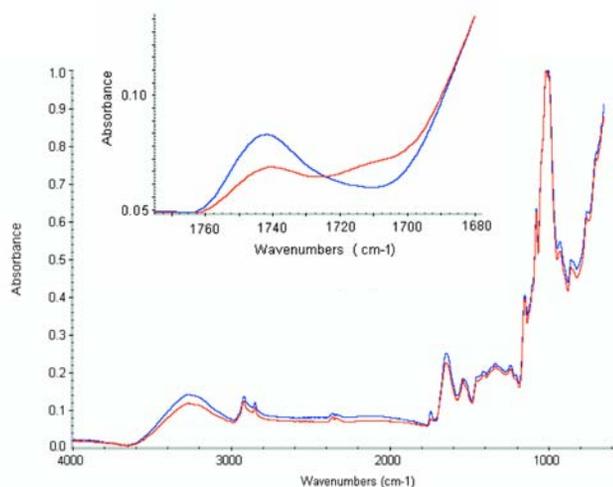


**Figure 5.** Tree of cluster analysis of averaged, normalized and baseline corrected DR spectra (1830–1710  $\text{cm}^{-1}$ ) of wheat with varying of DON content. Sample numbers in the plot give DON concentration in mg/kg. Blanks are designated with b1 – b3

having in mind that the changes in the DR spectra, on the basis of which the classification is made, are very small (Figure 2, insert).

Classification results for ATR data show a different picture. Figure 6 shows a score/score plot of principal components PC1 and PC2 calculated from averaged ATR spectra. Data treatment was kept to a minimum by averaging and normalizing spectra and baseline corrected at 1765  $\text{cm}^{-1}$ . Two clusters made up of blank and contaminated samples are clearly distinguishable. Again, these two clusters are also reflected in the resulting dendrogram after performing cluster analysis with the first two PCs enabling a correct classification for all samples and blanks (Figure 7). These results show a clear advantage of the ATR model over the one that was created using data from DR measurements, so that further studies were carried out using exclusively ATR technique.

Quantification was also attempted by applying a PLS1 model to the data. The same spectral window was



**Figure 6.** PCA of averaged, normalized and baseline corrected ATR spectra (1765–1690  $\text{cm}^{-1}$ ) of wheat with varying of DON content. Sample numbers in the plot give DON concentrations in mg/kg. Blanks are designated with b1 – b3

used for the calculations and the data treatment (averaging and normalizing) was identical. Table 1 gives numerical details for investigated PLS1 regression. The calibration line for DR data leads to an underestimation of predicted values, whereas the slope of the regression line modeled from ATR data is close to 1, which indicates good quality of the regression. Full cross-validation was performed on the datasets. As the difference between the coefficients of correlation for calibration and validation for ATR data is small, the model can be trusted.

It is also evident from Figure 7 that the cluster of infected wheat is clearly divided into two subclusters. Because of that the graphs of means clusters using K-mean clustering method for ATR spectra were obtained by setting the number of clusters to be 3. The obtained graphs of means clusters are shown in Figure 8. As observed in Figure 3, there is the mentioned characteristic decrease of the absorbance at 1743  $\text{cm}^{-1}$  and its increase at 1709  $\text{cm}^{-1}$ . Having this in mind we examined whether the classification of wheat samples could be achieved using PCA and cluster analysis only on the basis of the absorbance at 1709  $\text{cm}^{-1}$  and ratio of absorbances (signals) at 1709 and 1743  $\text{cm}^{-1}$  ( $A(1709)/A(1743)$ ). As can be seen from Figures 9 and 10, the classification results are similar to those obtained using the spectral range from 1765  $\text{cm}^{-1}$  to 1690  $\text{cm}^{-1}$  (Figures 6 and 7). Similar results were also obtained on the basis of the absorbance at 1743  $\text{cm}^{-1}$  and mentioned ratio of absorbances (signals).

Quantification was also attempted by applying a MLR model to the data (Table 1). In this case too, the slopes of the regression lines are close to 1, which indicates the good quality of the regression. Full cross-validation indicates that the model can be trusted, and the agreement of the correlation of calibration and validation is somewhat better on the basis of the absorbance at 1743  $\text{cm}^{-1}$  and ratio  $A(1709)/A(1743)$ .

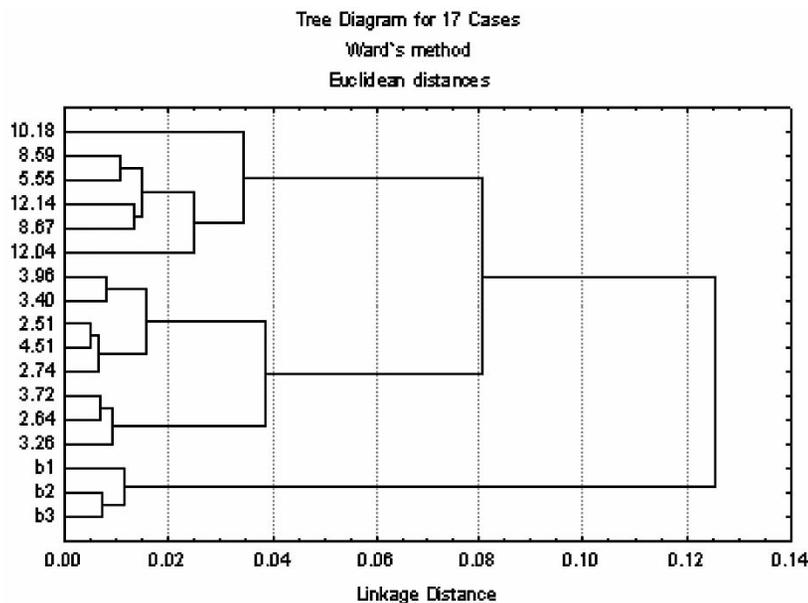
Encouraged by the obtained results we examined the possibility of determining DON content on the basis of the univariant approach. To this purpose we examined the

**Table 1.** Correlations of DR and ATR spectral data with reference data from DON measurements

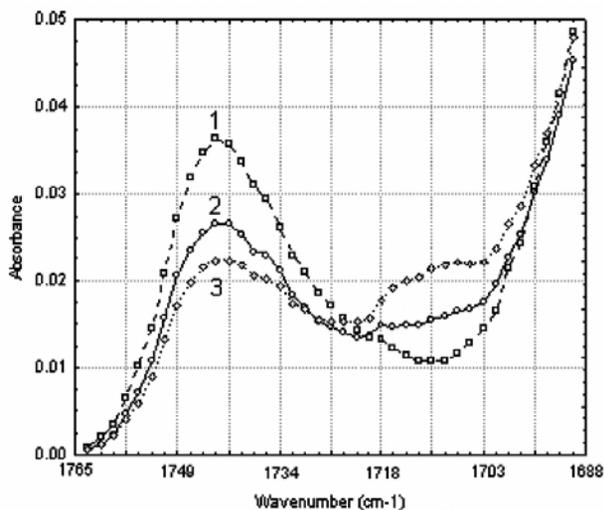
Reference parameter	DR				ATR					
	1830–1710 $\text{cm}^{-1}$ *		1765–1690 $\text{cm}^{-1}$ *		A1 and A3*		A2 and A3*		A3*	
	Calibration	Validation	Calibration	Validation	Calibration	Validation	Calibration	Validation	Calibration	Validation
Slope	0.7641	0.5427	0.8795	0.8373	0.8896	0.8764	0.8863	0.8000	0.8772	0.8693
Offset	1.164	2.104	0.5948	0.7064	0.5451	0.6308	0.5609	0.7787	0.6059	0.6369
RMSEC/RMSEP	1.879	2.706	1.343	1.639	1.286	1.604	1.304	1.899	1.355	1.483
SEC/SEP	1.936	2.784	1.384	1.686	1.325	1.653	1.344	1.946	1.397	1.523
Correlation	0.8741	0.7528	0.9378	0.9171	0.9432	0.9113	0.9415	0.8739	0.9366	0.9237
Bias	-2.66e-7	-0.1534	-1.54e-7	-0.0965	4.07e-7	0.0208	3.23e-7	-0.210	-1.54e-7	8.41e-3

Data given for calibration set and after full cross validation. A1 –  $A(1743 \text{ cm}^{-1})$ ; A2 –  $A(1709 \text{ cm}^{-1})$ ; A3 –  $A(1709/1743 \text{ cm}^{-1})$ ;

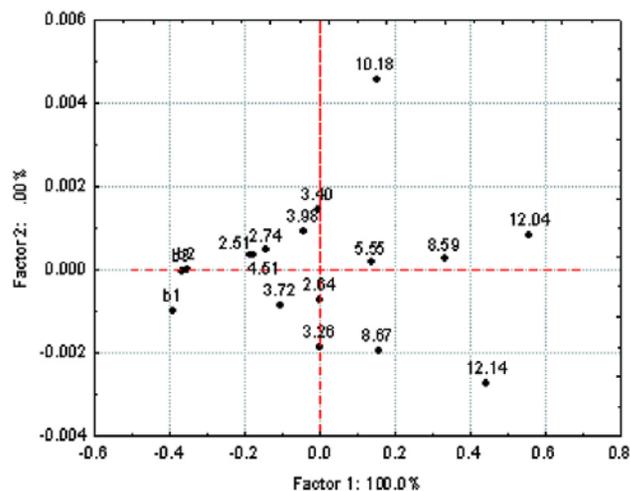
\* – PLS1 regression; \* – MLR; RMSEC – root mean square error of calibration; RMSEP – root mean square error of prediction; SEC – standard error of calibration; SEP – standard error of prediction.



**Figure 7.** Tree of cluster analysis of averaged, normalized and baseline corrected ATR spectra (1765–1690  $\text{cm}^{-1}$ ) of wheat with varying of DON content. Sample numbers in the plot give DON concentration in mg/kg. Blanks are designated with b1 – b3



**Figure 8.** Graphs of means clusters obtained applying K-mean clustering method for ATR spectra. Clusters: 1 – blanks; 2 – 2.51 – 4.51 mg/kg of DON; 3 – 5.55 – 12.14 mg/kg of DON



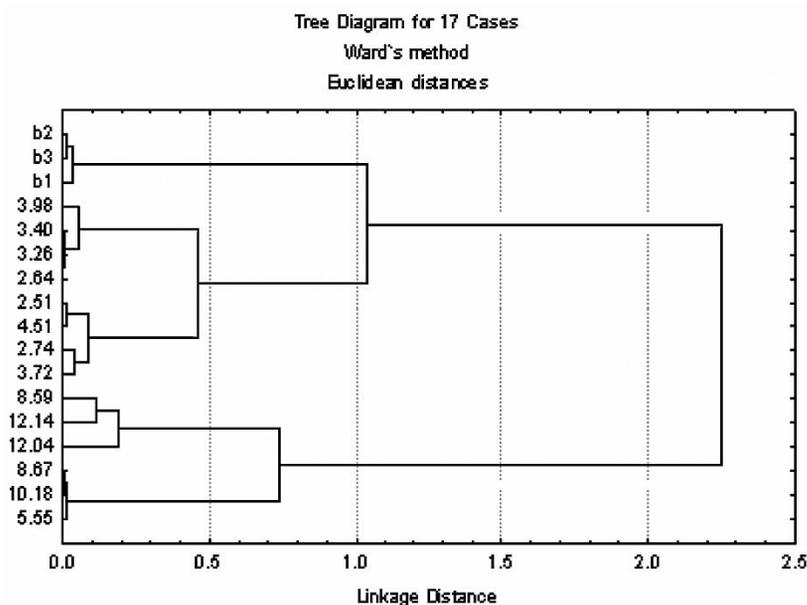
**Figure 9.** PCA of averaged, normalized and baseline corrected ATR spectra of wheat with varying of DON content based on  $A(1709)$  and  $A(1709)/A(1743)$ . Sample numbers in the plot give DON concentrations in mg/kg. Blanks are designated with b1 – b3

correlation of DON concentration with the ratio  $A(1709)/A(1743)$ . A satisfactory correlation was found, the corresponding coefficient being 0.937 (Table 1).

## 4. Conclusion

This study illustrates the performance and the major advantages and disadvantages of the DR and ATR spectroscopic techniques for the multivariate classification and estimation of fungal infection wheat by *Fusarium graminearum*.

All 17 samples from a single genotype (Monika) were classified correctly with data obtained using the ATR technique, in contrast to the DR technique. These results show a clear advantage of the ATR model over the one that was created using data from DR measurements. The application of a larger ATR crystal, as well as the larger number of internal reflections, made it possible to omit separate sample fraction with particle diameter between 100–250  $\mu\text{m}$ , which shortened significantly the analysis time. At the same time, the analyzed sample was larger, so that we could have a representative sample onto the cry-



**Figure 10.** Tree of cluster analysis of averaged, normalized and baseline corrected ATR spectra of wheat with varying of DON content based on  $A(1709)$  and  $A(1709)/A(1743)$ . Sample numbers in the plot give DON concentration in mg/kg. Blanks are designated with b1 – b3

stal. The sample preparation for ATR measurements was straightforward and no treatment except for grinding was necessary. The instrumentation required no cryogenics and/or purging, making the system suitable for in-situ measurements, especially in combination with commercially available portable devices. Besides, it was found that the measurement of absorbances at only two wavelengths ( $1709$  and  $1743\text{ cm}^{-1}$ ) allows a rapid assessment of DON content in wheat, i.e. without application of multivariate methods. In the future study it would be interesting to analyze some other wheat genotypes, although we think that one should not expect different results.

## 5. Acknowledgment

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## Povzetek

Proučevali smo možnost uporabe dveh spektroskopskih tehnik FTIR za hitro detekcijo glive *Fusarium graminearum* na žitu, kot indikatorja prisotnosti mikotoksina deoksinivalenola (DON). Neokužene in okužene vzorce žita smo zmleli in analizirali s tehniko difuznega odboja (DR) in atenuiranega popolnega odboja (ATR). Posnete spektre smo ovrednostili z metodo glavnih osi ter vzorce klasificirali s klustersko analizo. Poleg tega smo preverili možnost določevanja DON na osnovi razmerja signalov ATR pri  $1709\text{ cm}^{-1}$  in  $1743\text{ cm}^{-1}$ . Primerjalne meritve smo opravili z metodo tekočinske kromatografije visoke ločljivosti z detekcijo DAD. Koncentrationsko območje okuženih vzorcev je bilo 2,51 do 12,14 mg/kg. Uspešnost klasificiranja vzorcev je bila 100 % za spektre ATR, tehnika DR pa ni dala očitnih klastrov okuženih in neokuženih vzorcev. Dosegli smo zadovoljiv korelacijski koeficient (0.968) med omenjenim razmerjem signalov ATR in koncentracijo DON. Tehnika ATR zagotavlja enostavnejšo uporabo in interpretacijo rezultatov, ki omogočajo tako klasifikacijo okuženih in neokuženih vzorcev, kot tudi kvantifikacijo DON.