Szent István University

Detection of defects and characterization of quality parameters on crops using optical methods

PhD THESIS

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The applicant met the requirement of the PhD regulations of the Szent István University of Budapest and the thesis is accepted for the defence process.

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BACKGROUND OF THE WORK, OBJECTIVES

Although the technology of hyperspectral imaging appeared about thirty years ago, its spread and the study of its application possibilities are an actual issue today. The application of the technique in the food industry has several advantages, as it allows non-contact, real-time measurement, it can enable the prediction of the value of constituents and its distribution, and it also allows the detection of certain quality defects.

The method was used to study a number of raw materials and foods of plant and animal origin in the literature, by estimating various quality parameters and detecting contamination and defects. A relatively new direction of research is the emergence of microbiological infections, including the investigation of fungal infections with a hyperspectral visual system, which is still a new, largely unexplored area.

The appearance of various fungal infections on foods and raw materials can pose a direct food safety threat, such as the secondary metabolites, mycotoxins produced by fungi of the Fusarium genus. Another group of fungal infections causes significant economic losses by causing rapid deterioration of products, therefore their detection is also a key issue.

My aim is to study the applicability of optical methods, including hyperspectral imaging, in order to determine the quality and food safety characteristics of food raw materials and crops, and to detect their defects. Within this, I conducted my research in three areas:

I studied the detectability of the characteristic pathogen of cobweb disease and green mold (*Cladobotryum dendroides, Trichoderma aggressivum*) on champion caps. I investigated the detectability of various synthetic and biological antifungal agents used against mold infections (natamycin, prochlorase-Mn, *Bacillus subtilis*) and the separability of the samples treated with them. By artificially infecting the mushroom samples with the characteristic pathogen of cobweb disease and green mold, I followed the process of the development of the infection with measurements.

In the case of watermelon, I estimated the distribution of quality parameters (pH, soluble solid content: SSC) on the cutting surface of the fruit based on the measured hyperspectral data using regression models.

I examined the classification of susceptible and resistant maize hybrids, different levels of toxins and different Fusarium strains, as well as the estimated concentration of DON, fumonisin B1 and B2 toxins present by measuring ground maize samples artificially infected with different Fusarium strains.

MATERIAL AND METHOD

Measurements on mushroom and watermelon were performed with Headwall XEVA-1648 XC134 hyperspectral imaging system at the Faculty of Food Sciences, Szent István University, Department of Physics and Control. The recording of hyperspectral images for mycotoxin-contaminated maize samples was performed at the Universität für Bodenkultur (VIRIS lab for Analytical Ecogeochmeistry) in Vienna with a push broom system, produced by Zeutec GmbH. Argus software, developed at the Department of Physics and Control (FIRTHA, 2011) controlled the hyperspectral image processing system, the sensors and the stepper motor for both measuring systems.

During the measurement, various noises may occur due to the instrument or external factors, which affect the measured values. The effect of these factors can be reduced by different pre-processing methods. The obtained hyperspectral data were subjected to noise filtering (median filter), pixel correction, and averaging, as well as various spectral pretreatments such as normalization, moving average, Savitzky-Golay smoothing, and SNV method.

During the analysis of the data, I used box diagram and performed the analysis of hyperspectral data using different statistical methods. I used the PCA method as an exploratory and variable selection method (for mold-infected mushroom samples). The classification of the groups in the case of mushroom samples was performed by LDA, FDA and SVM methods. In the case of mycotoxin-contaminated ground maize samples, the different classes were separated by PLS-DA method. Continuous variables (watermelon pH and SSC, mycotoxin contamination of maize samples) were estimated by PLS regression. I used cross-validation and MCCV method to validate the models.

Research of white button mushroom

Segmentation of hyperspectral images was performed with CuBrowser (FIRTHA and ÉDER, 2012) MATLAB software, normalization and Savitzky-Golay smoothing were applied to the spectra. Model building and validation was performed with RStudio (R Project) software.

<u>Detection of cobweb disease and the classification of white button mushroom treated with fungicides</u>

The samples were divided into four groups, three groups were treated with fungicides: 1. natamycin, 2. Proclorase-Mn, 3. *Bacillus subtilis*, and one group was left untreated. The four groups were divided into two parts and each part was directly infected with a suspension of cobweb (*Cladobotrynum dendroides* suspension). The groups were classified by FDA and SVM methods, the models were validated by MCCV method.

<u>Study of the development of cobweb disease and green mold on champignon caps</u>

Samples were divided into 3 groups: control, artificially infected with *Cladobotryum dendroides*, and artificially infected with *Trichoderma aggressivum*. I performed PCA on the data, I selected the 5 most important wavelength values for the classification according to the obtained loading values: 1014 nm, 1459 nm, 994 nm, 1397 nm and 980 nm. The samples were then classified on the basis of the data measured at the selected wavelength values, using SVM and FDA methods, and cross-validation was performed on the built classification models.

Prediction of quality parameters on watermelon

Slices were cut from the watermelon samples at the diameter, then radially divided into 3 parts according to the structure of the fruit, sampled at 3 different distances at designated points, and pH and SSC values were measured at the selected points.

During the pre-processing of the spectra, I used a 3-point moving average (to remove the effect of noises appearing as peaks on the spectra) and SNV transformation, and the statistical analysis was performed with MATLAB software (MATLAB, 2016). The quality parameters (SSC, pH) and the value of the radius measured from the centre were estimated by PLS regression and cross-validation was used for the model validation.

Study of of maize samples contaminated with mycotoxins

In the next experiment, I examined two types of maize hybrids obtained from different sources, one was more sensitive and the other was more resistant to Fusarium infection. Three groups of samples were artificially infected with different Fusarium species: *Fusarium culmorum, Fusarium graminearum,* and *Fusarium verticilloides*. The fourth group was not treated. The toxin content of the samples - deoxynivalenol. fumonisin B1 and B2, - was determined by an LC-MS / MS based method (Malachová et al., 2014).

RESULTS

Detection of cobweb disease on white button mushroom

Figure 1 shows the normalized spectra taken from healthy tissue sections of control samples and infected areas, with clear spectral differences between them. Known water absorption peaks around 1200 nm and 1450 nm can be observed.

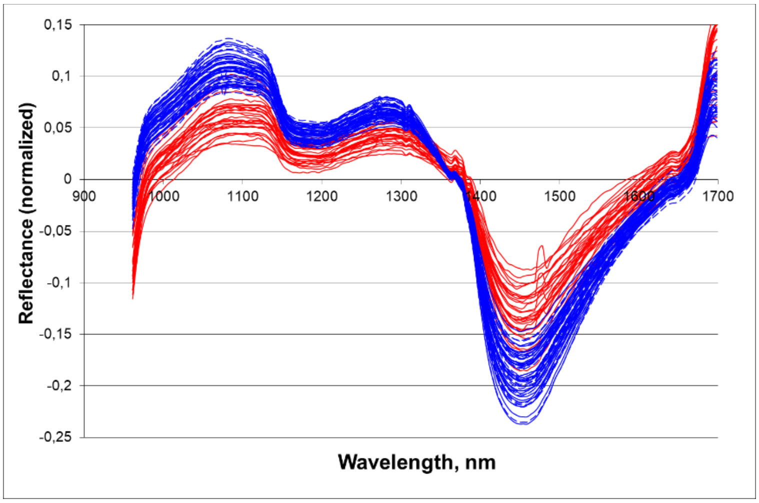

Figure 1: Normalized spectra measured on the first day of the experiment, red: spot caused by *Cladobotryum*, blue: control

With the method, the condition of the tissues can be visualized and the cause of the tissue damage can be determined (Figure 2). By normalizing the spectra measured by the HSI system at 1454 and 1200 nm, the appearance of infection can be detected at an early stage.

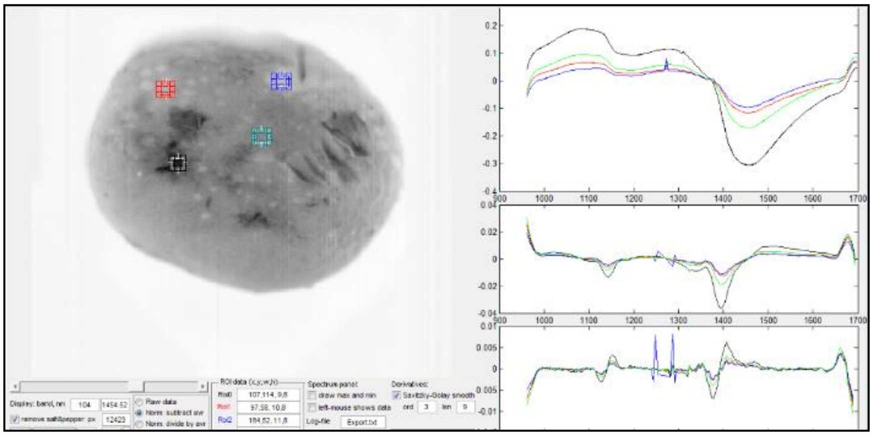

Figure 2: Image of a sample treated with Natamycin in CuBrowser at 1454 nm with rawl (above) and normalized (bottom) values

Classification was successful using the SVM method, the results are shown in Table 1.

Table 1: Results of SVM classification

Untreated		
	real	
predicted	***Cladobotryum***	**control**
Cladobotryum	**100,00%**	0,00%
control	0,00%	**100,00%**
Correctly classified	**100%**	
Prokloráz-Mn		
	real	
predicted	***Cladobotryum***	**control**
Cladobotryum	**95,42%**	16,96%
control	4,58%	**83,04%**
Correctly classified	**89,71%**	
Natamycin		
	real	
predicted	***Cladobotryum***	**control**
Cladobotryum	**60,13%**	5,60%
control	39,87%	**94,40%**
Correctly classified	**80,78%**	
Bacillus subtilis		
	real	
predicted	***Cladobotryum***	**control**
Cladobotryum	**88,89%**	4,24%
control	11,11%	**95,76%**
Correctly classified	**93,16%**	

After the MCCV validation, this ratio decreased slightly: untreated: 98.5%, natamycin: 78%, prochlorase-Mn: 88%, *Bacillus subtilis*: 91%.

Classification of white button mushrooms treated with fungicides

Classification of *Cladobotryum*-infected samples by the LDA method was successful. The natamycin- and *Bacillus subtilis*-treated groups showed overlap. 93.13% of the prochlorase-Mn-treated group and 100% of the untreated group was correctly classified by the method.

During the SVM classification, 74.58% of the spectra were correct for *Cladobotryum*-infected samples and 66.67% for control samples. In the case of infected samples, the classification efficiency was around 80-90% in the untreated and prochlorase-Mn-treated groups, and was lower in the other groups. In the case of control samples, the ratio of correctly classified spectra was 89.76% in the untreated group, 71.61% in the *Bacillus subtilis*-treated group, 53.88% in the natamycin-treated class, and 14.29% in the prochlorase-Mn- treated class.

Study of the development of of cobweb disease and green mold on champignon caps

I performed the model building using the intensity values measured at the wavelengths selected on the basis of the PCA loading values. Using the SVM model, the ratio of the correctly classified samples was around 75%, and this rate increased slightly during validation. The model created with the LDA method led definitely to a better result. The proportion of correctly classified samples reached 87.5% and decreased to 86.4% during validation. The best result was obtained in the classification of the control group, followed by the group infected with *Trichoderma* and then the group infected with *Cladobotryum*. Discriminant analysis based on PCA-selected wavelength data was suitable for distinguishing between infected and control samples already in the early phase of infections.

Prediction of quality parameters on watermelon

Prediction of pH value, based on the HSI data was not successful. In this case, too, the loading plots in the regions indicating water absorption showed a relationship for the estimation. The spectra of the samples or their differences may be small if there is no significant difference between the samples in terms of acid content, and the differences do not necessarily lead to a detectable spectral difference.

The loading plots of the prediction of the distance from the center also show a maximum around 1450 nm. The water content of the tested matrix is very high and its effect is also visible spectrally. Based on this, the results mainly reflect the differences in the distribution of the water content of the sample. During the analysis of the cumulated data of samples, more than 98% of the variance could be described with 2 latent variables, and the standard error of the prediction was below 0.3.

Prediction of the distribution of SSC was successful, the individual study of the samples also allowed an acceptable estimation. The data already showed a successful fitting in case of the use of 3 latent variables (R^2 = 0.64), although in case of 10 latent variables the fit was noticeably better (R2 = 0.77). When analyzing the cumulated data of the samples, the lowest squared error of the estimation occurred for 10 latent variables RMSEV = 0.8, where the value of the coefficient of determination was R^2 = 0.74.

Prediction of toxin content in Fusarium-infected maize samples

PLSR regression was performed by randomly selecting samples from the groups 10-times, and then increasing the number of latent variables by 5 and repeating the random selection and regression with validation.

<u>Prediction of cumulated toxin content</u>

The best RMSEV result was obtained in the case of 25 latent variables, the average of the standard error of validation was 13.48 mg/kg, the highest value was obtained for 15.12 mg/kg out of ten repetitions, the coefficient of determination was 0.974.

<u>Prediction of deoxynivalenol toxin content</u>

The production of deoxynivalenol is characteristic of *Fusarium culmorum* and *Fusarium graminearum* species, therefore I used data of these groups for this prediction. The lowest mean RMSEV was obtained in the case of using 20 latent variables, it was 11.95 mg/kg, while the mean RMSEC was 2.5 mg/kg. The value of the coefficient of determination in the case of 20 latent variables was 0.985 based on the average of 10 repetitions.

<u>Prediction of of fumonisin B1 and B2 toxin content</u>

For the prediction of fumonisins, I used data of samples artificially infected with *Fusarium verticilloides,* and non-infected groups. Since there were noticeably higher RMSEC and RMSEV values for 5 and 20 latent variables, I performed only 5 repetitions in these cases. The best results were obtained for 15 latent variables where the RMSEV was 13.26 mg/kg and the RMSEC was below 1 mg. The value of the coefficient of determination in the case of 15 latent variables was 0.986 based on the average of 10 repetitions. According to the 2006/576/EC recommendation of the European Commission, the guidance value for fumonisin B1 + B2 toxin content of maize and maize poducts as feed material is 60 mg/kg. By further developing the applied PLSR model, it may be possible to achieve a practically applicable model in the case of feedingstuff.

Classification of Fusarium-infected maize samples by level of contamination, Fusarium species and maize hybrid

Classification of groups by level of contamination

Samples were classified into three categories based on total mycotoxin contamination (DON and fumonisins): high: highly contaminated (above 30 mg/kg), medium: moderately contaminated (between 15 mg/kg and 30 mg/kg), and low contaminated (below 15 mg/kg)).

PLS-DA modeling and validation was performed 10 times with different numbers of latent variables with randomly selected samples in a given ratio per group of fungal infection. During validation, the best result was obtained for 15 latent variables, where the average of the ratio of correctly classified samples based on results of the10 repetitions reached 98.8%. According to the ratio of correctly classified samples, the lowest value was obtained for the moderately contaminated group, followed by the high and then the low contaminated groups (Table 2).

Table 2: Confusion matrix of validation of PLS-DA classification by level of contamination with 15 latent variables based on an average of 10 repetitions

	Actual class		
Predicted class	**High**	**Medium**	**Low**
High	99%	1%	0%
Medium	0%	98%	0%
Low	1%	1%	100%

Classification of groups by Fusarium species

For the classification of different Fusarium species, I examined the medium and highly contaminated groups within the artificially infected groups. With 25 latent variables, the ratio of correctly classified samples exceeded 99% during validation, with only the *F. graminearum* group having any misclassification (Table 3).

Table 3: Confusion matrix for validation of PLS-DA classification by Fusarium infection with 15 latent variables based on the average of 10 repetitions

	Actual class		
Predicted class	*F. culmorum* (C)	*F. graminearum*	*F.verticilloides* (V)
F. culmorum (C)	100%	1%	0%
F. graminearum (G)	0%	99%	0%
F.verticilloides (V)	0%	0%	100%

Classification of groups according to maize hybrid

PLS-DA classification according to maize hybrids was also successful, using 10 latent variables, the efficiency is shown by the confusion matrix of validation based on the average of 10 repetitions (Table 4).

Table 4: Confusion matrix for validation of PLS-DA classification according to maize hybrid with 10 latent variables based on average of 10 replicates

	Actual class	
Predicted class	CRC	SBL
CRC	98%	0%
SBL	2%	100%

NEW SCIENTIFIC RESULTS

1. I found that based on the spectra recorded using the hyperspectral method with the normalization of the intensity values measured at 1454 nm and 1200 nm, the appearance of *Cladobotryum dendroides* infection on *Agaricus bisporus* caps can be detected in the early stage before the of symptoms are observable and it can be distinguished from mechanical damage. The significant wavelengths I have identified allow the method to be applied under industrial conditions using a multispectral system.

2. I detected successfully the presence of *Cladobotryum dendroides* infection on *Agaricus bisporus* caps treated with different antifungal agents (natamycin, Proclorase-Mn, *Bacillus subtilis* extract) applying SVM method on the spectral data collected using hyperspectral imaging over 11 days (ratio of correctly classified samples based on MCCV, 20% test sample, untreated group: 98.5%, natamycin: 78%, Proclorase-Mn: 88%, *Bacillus subtilis*: 91%).

3. Based on the intensity values at 980 nm, 994 nm, 1014 nm, 1397 nm and 1459 nm, selected based on the results of PCA on the data collected using hyperspectral imaging method, I successfully distinguished between sound, *Cladobotryum dendroides* infected and *Trichoderma aggressivum* infected *Agaricus bisporus* mushroom samples by SVM method (ratio of correctly classified samples based on CV - control: 74%, *Cladobotryum*: 76%, *Trichoderma*: 79%) and by LDA method (proportion of correctly classified samples based on CV - control: 96%, *Cladobotryum*: 78%, *Trichoderma*: 85%).

4. I found that the soluble solids content (SSC) on the cut surface of watermelon can be successfully predicted using PLSR method on the SNV transformed average spectra calculated from the data recorded with hyperspectral imaging method (LV = 10, R^2 = 0.74, RMSEV = 0.8).

5. I predicted successfully the total amount of fumonisins (FB_1 and FB_2) and deoxynivalenol toxin of ground maize samples artificially infected with *Fusarium culmorum*, *Fusarium graminearum* and *Fusarium verticilloides*, and artificially not infected samples, using PLSR method on the SNV transformed average spectra calculated from the data recorded with hyperspectral imaging method (RMSEC = 4.22 mg / kg, RMSEV = 13.48 mg / kg, R^2 = 0.974).

6. I predicted successfully the amount of deoxynivalenol toxin of ground maize samples artificially infected with *Fusarium culmorum* and *Fusarium graminearum* using PLSR method on the SNV transformed average spectra calculated from the data recorded with hyperspectral imaging method (RMSEC = 2.5 mg / kg, RMSE 0.985).

7. I predicted successfully the amount of the amount of fumonisins (FB_1 and FB_2) of artificially not infected and with *Fusarium verticilloides* artificially infected maize samples using PLSR method on the SNV transformed average spectra calculated from

the data recorded with hyperspectral imaging method (RMSEC = 0.11 mg/kg, RMSEV= 13.26 mg/kg, R^2 = 0.986).

8. I classified successfully the artificially not infected and with *Fusarium culmorum, Fusarium graminearum* and *Fusarium verticilloides* artificially infected ground maize samples into contamination level categories (sum of DON and fumonisin B1 and B2 below 15 mg/kg: low, between 15 mg/kg and 30 mg/kg: medium, above 30 mg/kg: high), using PLS-DA method on the SNV transformed average spectra calculated from the data recorded with hyperspectral imaging method.

9. I classified successfully the with *Fusarium culmorum*, *Fusarium graminearum* and *Fusarium verticilloides* artificially infected ground maize samples (ratio of correctly classified samples in validation: 99.6%), as well as the artificially not infected and with *Fusarium culmorum*, *Fusarium graminearum* and *Fusarium verticilloides* artificially infected ground samples of a maize hybrid more susceptible to fusarium infection (source: Cereal Researcher, Szeged) and maize hybrid more resistant to fusarium infection (source: Staatbau Linz) (ratio of correctly classified samples in validation: 99.1%), using PLS-DA method on the SNV transformed average spectra calculated from the data recorded with hyperspectral imaging method.

CONCLUSIONS AND SUGGESTIONS

As it can be seen in the literature (LORENTE ET AL., 2011; ESQUERRE, GOWEN, DOWNEY, O'DONNELL, 2012), I also applied successfully statistical models based on the intensity values measured at a few selected wavelength to detect different mould infections. Taking into account the results in the literature and the aspects of industrial applicability, I suggest the development of a multispectral system for practical use. Using this system the pieces of not conforming quality can be sorted out, and with the detection of the infection, its spread can be prevented by removing the infected pieces. Further quality attributes could be examined with the proper design of the system, as well as the detection of the most common fungal pathogens, as *Pseudomonas tolaasii* could be enabled, even using image processing (VÍZHÁNYÓ and FELFÖLDI, 2000). Deterioration due to the appearance of moulds is a serious economic problem in the case of many crops, so similarly monitoring the quality of other crops is a goal that can have direct benefits in the short term, such as in the case of citrus fruits. The technical development of special sensors, as well as the decrease in price and the increase in the computing capacity of the available computer equipment, are expected in the near future to result in the rapid spread of advanced monitoring systems in more and more segments of the food industry.

In the case of watermelon samples, building a more efficient model to estimate pH and soluble solids content can be achieved by increasing data variability. This can be accomplished by using a larger number of samples as well as measuring crops at different ripening stages, in a similar way as SUGIYAMA, TSUTA and SUN, (2010) predicted sugar content in melon samples.

Based on the results of the study on Fusarium-infected maize samples, I concluded that although the presence and metabolic activity of microscopic fungi cause a change in the composition that can be examined by spectral methods, the technique used in my work was not suitable for direct, particularly specific detection of mycotoxins.

Since mycotoxins pose an extremely serious food safety risk, I suggest the use of more specific methods in this regard. However, by using the method in addition to the commonly used analytical methods, feedstuff can be monitored in practically unlimited quantities, which can greatly contribute to the improvement of food safety in this area. This direction of the development of the method has great potential, therefore I suggest extending the research to other micromycetes potentially present in the feed, as well as the study of further mycotoxins. In the case of maize, a more accurate, robust and comprehensive model could be built in a subsequent study by measuring a larger number of samples, applying reference measurements of further regulated mycotoxins (zearalenone) in maize, and sampling at different times after the infection. In the case of the present study, the majority of the samples were contaminated far above the permissible mycotoxin content of foods suitable for human consumption, therefore with the measurement of a larger number of samples with lower mycotoxin concentrations can be expected to enable the development of a better performing model for this range.

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