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# The Application of Near Infrared Spectroscopy in Wheat Quality Control

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## 1. Introduction

The application of near infrared spectroscopic technique for the quantitative analysis of food products and commodities is nowadays widely accepted. However, 160 years passed from the discover of near infrared part of the spectrum to its first analytical application which is related to the work of Karl Norris who firstly demonstrated the potential of the NIRS in quantitative analysis particularly for prediction of moisture and protein content in wheat. The intense development of this technique during the last 50 years has been challenged by the development of powerful computers, softwares and chemometric tools, since the NIRS data processing is quite demanding task. The near infrared spectroscopy is an instrumental technique based on measuring the intensity of reflectance or intensity of transmission of radiation from the near infrared region of the electromagnetic spectrum (800-2500 nm) by the test sample. The intensity of the reflection and transmission depends on the rate of absorption of radiation by the sample, which leads to excitation of hydrogen bonds (CH, NH, OH). As the tested samples are very complex in composition, it happens that on the same wavelength, several organic bonds involving hydrogen vibrate producing overlapped spectral bands. Therefore, the resulting NIR spectrum looks like a slightly wavy line with no clearly defined features, with very broad and overlapped molecular overtone and combination bands, which complicate to assign them to specific chemical constituent and make impossible to determine the direct relationship between the concentration of ingredients of interest and the absorbed radiation energy (Fig. 1).

Due to the significant overlapping of NIR bands, the prerequisite for the NIRS application is the development of the calibration model which relates the concentration of certain analyte found in a sample to the spectral data collected from that sample. Calibration model development process implies the extraction of useful information from the NIR spectra by applying chemometrics methods. Multivariate calibration techniques (e.g. principal components analysis, partial least squares, or artificial neural networks) are often employed to extract the desired chemical information from the spectral data. Calibration model allows relating the NIR optical data with the compound (or property) of interest that is used to define the quality of the sample:

$$[C] = [A][\beta] + [\varepsilon] \quad (1)$$



Fig. 1. NIR reflectance spectrum of wheat flour (1100-2500 nm) recorded by FOSS's NIRSystem 6500

Also, the application of NIRS technique has been extended to determination of certain functional properties of tested samples which do not represent the unique chemical entities which manifest absorption in NIRS spectral region, but manifest relationship with certain constituents that can be used as a basis for calibration model development. So far, a number of applications of the NIRS technique have been demonstrated having commercial and/or scientific significance that was developed on the basis on different NIR spectral ranges, different ways of recording and processing of spectra, different sample presentations, various chemometrics techniques used for calibration development and different use of validation statistics.

## 2. Near infrared spectroscopy: A rapid analytical tool in cereal quality control

In the 60s, the initial application of the NIRS technique in routine analysis was demonstrated and it was related to the determination of moisture and protein in cereals. The first stage of commercial application of the NIRS technique, during the 1970s, was predominantly related to the prediction of protein, moisture and oil content in cereals. In the second stage, during the 1980s, the NIRS prediction of more complex constituents such as complex carbohydrates was enabled. The third stage of NIRS application in cereal technology has been related to the prediction of functional properties of grains. The success of this technique lays in numerous advantages it offers over wet chemical analysis such as:

- Significant reduction of testing time,
- No requirement for the use of chemicals and their preparation,
- No requirement for the sample preparation
- No requirement for additional technical expertise to carry out examination

However, the application of NIR spectroscopy is characterized by the relevant restrictions, which are primarily related to the nature of NIRS as an indirect method (Dowell et al., 2006; Jirsa et al., 2008; Miralbés, 2008; Pierce et al., 1996; Pojić et al. 2002; Pojić et al., 2003). To

facilitate the application of this method, it is necessary to develop the calibration model by which the obtained spectral data are translated into the required result – the content of the selected compound of interest, and it is often necessary to check and update the calibration models due to changes in the sample matrix. However, this restriction has been significantly overcome by development and application of calibration models based on artificial neural networks that handle very large data set and proved to be very accurate, stable, transferable and therefore globally applicable (Büchman et al, 2001).

The potential of wheat to be processed in wide range of different final products gives it the significance of upmost grain of commerce (Williams, 2002). Application of the NIRS technique in wheat quality control has been characterized by rapid development from prediction of major constituents in wheat grains (moisture, protein, oil starch, cellulose) to prediction of functional properties of wheat that define its capability to meet the requirements of the intended purposes (production of bread, pastry, cookies, pasta). Since the functionality of the commodity is strongly affected by its physico-chemical properties which do not manifest characteristics absorption in NIR spectral region, the use of NIRS to predict functionality is based on the relationship between physico-chemical properties and certain constituent having absorptions in NIR region (protein, oil, starch etc) (Williams, 2007). The value of wheat grain is dependent on its composition, functionality and safety, that all have an equal importance in wheat breeding, trade and processing (Fig. 2) (Williams, 2002).

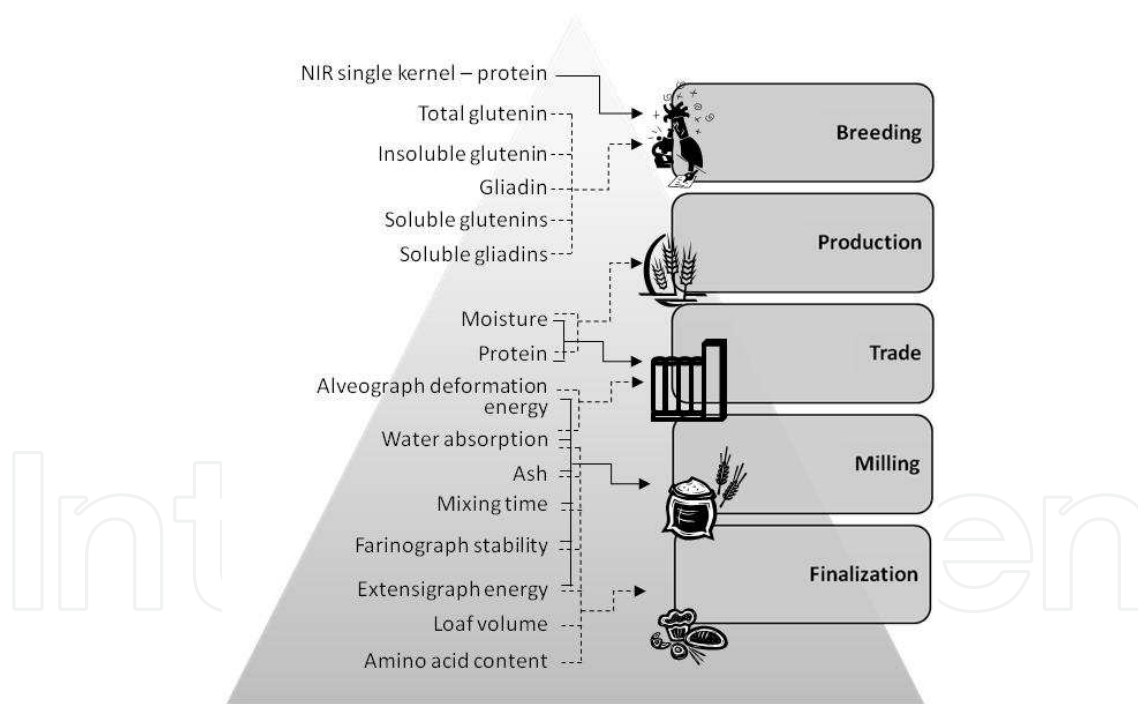


Fig. 2. The relationship between different NIRS applications in wheat quality control

### 2.1 The role of NIRS in cereal breeding

The breeding purposes require the knowledge on both composition and functional properties of grain, whilst the functionality of wheat grain has always been an issue of a great concern for wheat breeders. Functionality in wheat includes prediction of milling yield, kernel texture, rheological parameters of dough, loaf volume, product appearance,

etc. Most of these parameters are dependent on the protein-proteinase complex of wheat grain and the condition of carbohydrate complex as well (Williams, 2007).

First attempt to determine the functional properties of wheat was made by Rubenthaler and Pomeranz (1987) by development of NIRS model for prediction of water absorption, mixing time and loaf volume. The following work presented by Williams et al. (1988) showed the potential of NIRS to predict Farinograph stability, Extensigraph energy and Alveograph deformation energy. Further research reported by Pawlinsky and Williams (1998), Hrušková et al. (2001), Hrušková and Šmejda (2003), Miralbés (2003, 2004), Dowell et al. (2006) and Vázquez et al. (2007) were carried out by using analyzers of higher generation – scanning monochromators. The efficiency of the models developed were affected by the form of the samples used (whole grain, ground grain or flour), by the composition of the sample sets which were insufficiently variable and by the inherent variability of the reference rheological methods.

One of the first attempts to develop NIRS calibration model for prediction of protein composition was made by Delwiche et al. (1998). It was possible due to the fact that the main fractions of gluten - glutenin and gliadin, exhibit some differences in their NIR spectra which enable them to be determined in mixtures with starch (Wesley et al., 1999) Total glutenin, insoluble glutenin and gliadin contents can also be measured in whole wheat kernel by NIRS against HPLC as a reference method (Delwiche et al., 1998; Dowel et al., 2006; Wesley et al., 1999; Seabourn et al., 1998; Wesley, 2001) with sufficient accuracy for screening purposes in breeding programs. Although some authors has recommended the use of instrument with a monochromator in reflectance mode over the range of 2000 to 2300 nm for these application (Wesley, 2001), it has been proved that use of instruments in transmittance mode with narrower spectral range below 2000 nm could also be applicable (Dowell et al., 2006; Scholz et al., 2007). Concerning the carbohydrate complex of grain, the NIR spectra of amylose and amylopectin are very similar since they consist of the same glucose unit. Therefore, very little progress has been made in estimating the quality of carbohydrate components in wheat. Scanning visible-NIR spectrophotometers are often applicable for research and development purposes, since this application requires wider spectral ranges such as 400-2500 nm, 400-1700 nm, 1100-2500 or 1000-2600 nm. Also, since breeders commonly face with insufficient quantity of samples, the development of NIRS single kernel characterization systems has been initiated.

## 2.2 The role of NIRS in cereal trade

Methods based on near infrared spectroscopy are accepted worldwide for cereal quality control in trade, especially according to the fact that it is capable of generating results for several quality parameters rapidly and in a non-destructive way. Although different countries established their own systems for classifying wheat on the basis of different quality parameters, wheat grading systems is commonly based on the wheat protein content (Williams, 2007; Hulasare et al., 2003; Váradi et al., 1999). The price of wheat is dependent on the protein content, often with substantial price increments between grades. Measuring protein content in wheat and wheat flour has been demonstrated as successful NIRS application due to its strong and broad absorption bands in the NIR region which affect easy calibration model development. Therefore, the segregation or blending grain prior to delivery is inconceivable without the use of NIR technology. When using NIR analysis for

above mentioned purposes, special attention must be paid to demonstrate that it generates technically valid results satisfying the regulatory requirements for analytical procedures. Stand-alone bench type instruments, designed for testing whole grains by measuring the intensity of transmission of NIR radiation from the spectral range 850-1050 nm are the most suitable for cereal trade purposes. Moreover, the measurement infrastructure comprised of the network of NIRS instrument significantly improved the cereal testing in trade.

### 2.2.1 The role of NIRS networks

A significant advance in the application of the NIRS technique in cereal trade has been achieved by the development of global ANN calibration models, and by the establishment of measurement infrastructure composed of multiple NIRS devices interconnected in the network. Operation of the NIRS instruments through the network significantly improved the routine application of NIRS method, eliminated specified shortcomings and significantly facilitated the application of the NIRS method for the end-users. Hence, the independent measurements of protein content that are internationally equivalent have triggered off the establishment the NIRS networks in many countries around the world (Büchman, 1996; Pojić & Mastilović, 2006).

NIRS networks are formed in order to:

- ensure metrological traceability of results of measurement of grain quality
- define the common interests of entities involved in the network system
- distribute costs and improve network operation domain

Establishing a network of NIRS devices allows achieving of the same level of accuracy of determining the protein content regardless of location of devices. In addition, the NIRS networks ensure reliability and uniformity of quality control of grain crops as well as simplification of procedures for calibration model monitoring and their improvement.

NIRS network consists of two to several hundred or even thousands of NIRS devices that are controlled and configured from the central so-called master device. The initial idea of operation of NIRS instruments through the network came from FOSS Analytical AB, and currently the most impressive networks worldwide consist of FOSS's instruments - scanning monochromator Infratec 1241 Grain Analyzer. The success of such measurement infrastructure is highly dependent on the network organization and procedures and tasks proposed and divided between:

- reference laboratory that analyze samples using the reference (wet chemical) tests and monitor performance of the used calibrations
- administrative center, which manages databases, communication between devices in the network and performs the standardization of network devices
- calibration center that develops new calibration models
- network management body for the decision-making on the organization of network and calibrations updates.

Procedures that enable undisturbed functioning of the NIRS network and confirm compliance of results obtained by wet chemical tests on the one hand and consistency of results of individual devices with the central (master) device on the other hand is achieved through the following activities:



- monitoring of the accuracy of measurements of the central (master) device,
- standardization of individual NIRS devices and
- monitoring of the stability of measurements of individual NIRS device.

### 2.3 The role of NIRS in cereal processing

Analytical methods based on near infrared spectroscopy have the potential to significantly improve the quality of final cereal products by testing the products through the entire production process in processing industry (raw materials, intermediate and final products). For these purposes, on-line instruments as well as stand-alone bench type instruments designed for testing whole grains by measuring the intensity of transmission of NIR radiation from the spectral range 850-1050 nm or the intensity of diffuse reflection of NIR radiation from the spectral range 1000-1400 nm are the most suitable. For example, the determination of ash by NIRS is particularly useful for process control in the wheat milling industry to monitor the consistency of milling and the compliance with flour specifications. Although inorganic substances do not absorb energy in the NIR spectral region, some authors demonstrated that the NIRS method can be used for reliable prediction of the ash content (Dowell et al., 2006; Deaville & Flinn, 2000; Osborne, 2007; Pérez-Marín et al., 2004; Armstrong et al., 2006; Mentink et al., 2006; Pojić et al., 2010). Since ash content cannot be directly measured by NIRS, it is assumed that it is predicted by correlation with the total amount of organic compounds and water present, because of the large number of wavelengths used in the process of calibration development that give significant information (Osborne, 2007; Clark et al., 1987; Garnsworthy et al., 2000; Frankhuizen, 2008).

### 3. Validation protocol for the NIRS method

Demonstration that the NIRS method is fit for intended purpose has the great importance for the compliance with regulations, process control, making regulatory decisions, support national and international trade, support research etc.

When we talk about the validation of the NIRS as an analytical method we must be aware of the duality of the term „validation“ used within the analytical chemists utilizing NIRS. The most common use of the term „validation“ implies using an independent sample set to test the accuracy of the calibration model developed (Mark & Workman, 2007). That process results in certain statistical indicator explained later in the text. This process must be distinguished from the process of validation of NIR spectroscopic method aimed at confirmation, through the provision of objective evidence, that the requirements for a specific intended use of application are fulfilled (Lauwaars & Anklam, 2004; Dybkaer, 2011). Hence, the objective of the validation of the NIRS method is to demonstrate that its characteristics are suitable for its intended purpose. When we talk about the application of the NIRS method in wheat quality control, the validation experiment should include the testing of characteristics of NIRS method and demonstration its fitness for purpose.

The validation protocol that was performed in our laboratory in order to provide the evidence that the NIRS method as being applied in wheat quality control for protein content prediction fitted for purpose included: accuracy, repeatability, reproducibility, intermediate precision, linearity, robustness and transferability (Fig. 3). The NIRS instrument used was a

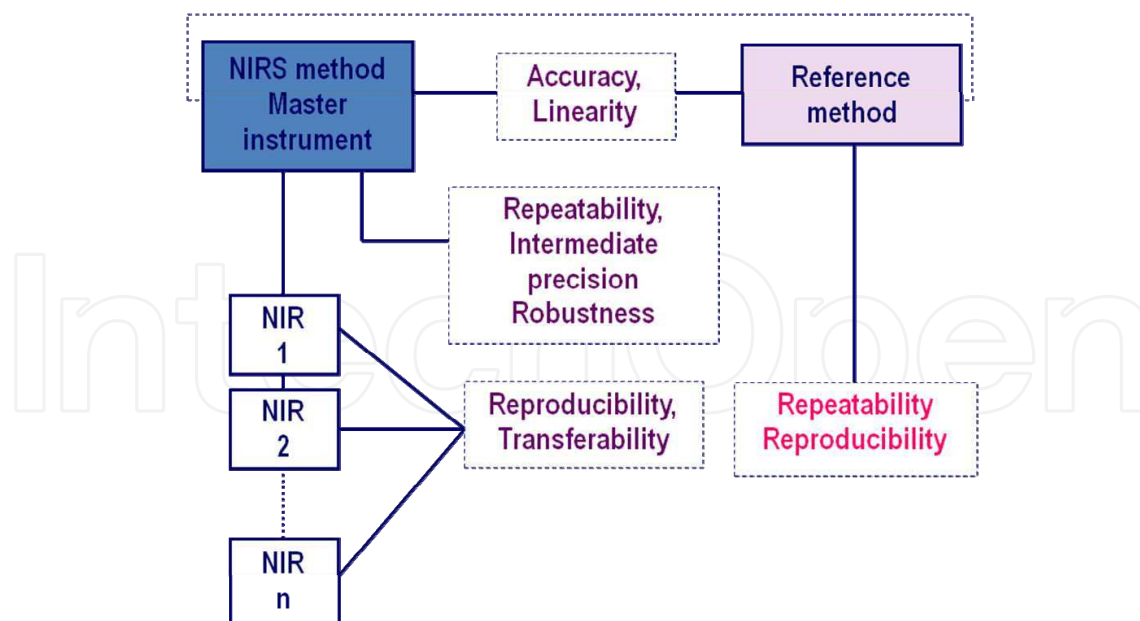


Fig. 3. The validation protocol of the NIRS method as being applied in wheat quality control scanning monochromator Infratec 1241 Grain Analyzer with the ANN calibration model for protein content used in the transmittance mode (FOSS Analytical, Denmark).

### 3.1 Accuracy

Accuracy, defined as the closeness of agreement between a measured value and a true value of a measurand, in the case of the NIRS method expresses a measure of how well NIRS predicted value match a given reference value obtained by a reference (wet chemistry) method. The accuracy of the NIRS method is commonly described by statistical terms such as SEC (standard error of calibration), SECV (standard error of cross validation),  $R^2$  (coefficient of determination), explained variance (1-VR), residual predictive deviation (RPD), standard error of prediction (SEP) etc. (Konieczka & Namieśnik, 2009). These values describe the agreement between the predicted NIRS values and the reference method values from the same sample (Ritchie et al., 2002; Moffat, 2004). SEC, SECV,  $R^2$  and 1-VR values are calculated on the basis of samples used to develop the calibration model itself, whilst the SEP value is calculated on the basis of independent sample set not included in the calibration model development procedure:

$$SEP = \sqrt{\frac{\sum_{i=1}^N (y_r - y_p)^2}{N - 1}} \quad (2)$$

where  $y_r$  is the reference value of  $i$  samples,  $y_p$  is the NIRS predicted value of  $i$  sample,  $N$  the number of samples.

The selection of suitable statistical term to express the accuracy of the NIRS method depends on the availability of the samples covering the whole range of component concentration with its even distribution. The accuracy of the NIRS method to a large extent is influenced by nonhomogeneity of sample, laboratory error, physical and chemical variation in sample



with time, population sampling error, different nature of spectroscopic and wet chemical measurements, instrument noise, sample presentation, calibration modeling, calibration transfer etc. (Workman, Jr., 2008). Statistical term used to express a systematic difference between the two sets of results obtained by the reference method and the NIRS method is bias (Workman, Jr., 2008; Shenk et al., 2008):

$$\text{Bias} = \frac{\sum_{i=1}^N (y_r - y_p)}{N} \quad (3)$$

where  $y_r$  is the reference value of  $i$  samples,  $y_p$  is the NIRS predicted value of  $i$  sample,  $N$  the number of samples.

The accuracy can be improved by a bias and slope adjustment, but it requires precaution due to the fact that the bias only fixes the problem on a temporary basis.

Subsamples	1. validation set			2. validation set		
	BIAS, %	RMSEP, %	SEP, %	BIAS, %	RMSEP, %	SEP, %
5	-0.32 <sup>a</sup>	0.4887 <sup>a</sup>	0.3851 <sup>a</sup>	0.03 <sup>a</sup>	0.2461 <sup>a</sup>	0.2494 <sup>a</sup>
7	-0.30 <sup>a</sup>	0.4926 <sup>a</sup>	0.4028 <sup>a</sup>	0.02 <sup>a</sup>	0.2385 <sup>a</sup>	0.2444 <sup>a</sup>
10	-0.32 <sup>a</sup>	0.5096 <sup>a</sup>	0.4088 <sup>a</sup>	0.04 <sup>a</sup>	0.2474 <sup>a</sup>	0.2517 <sup>a</sup>

Values in the same column marked with the same letters are no significantly different ( $P < 0.05$ , LSD test)

Table 1. The predictive ability of the NIRS model for protein content prediction depending on the number of subsamples in a single measurement

The accuracy of the NIRS method was evaluated by calculating bias, RMSEP and SEP in three different cases, using five, seven and ten subsamples in a single NIRS measurement. To determine the accuracy of protein content prediction, two independent validation sample sets were used where the first validation set was used before, and the second one after the bias correction. The parameters of predictive ability of protein content (RMSEP and SEP) were not influenced by variable number of subsamples. Bias adjustment affected better predictive ability for protein content expressed by lower RMSEP and SEP values for the second validation set (Table 1). By the assessment of comparable views of protein content obtained by the reference and NIRS method from measurements of 5, 7 and 10 subsamples, before and after bias adjustment, the negligible difference between the reference and NIRS method could be noticed after the bias adjustment regardless the number of subsamples in a single measurement (Fig. 4).

### 3.2 Precision (repeatability, reproducibility)

Precision is more important NIRS method characteristics than accuracy since it cannot be changed by a simple adjustment. It is affected by the instrument, the calibration and the operator. Precision can be defined as the closeness of agreement between measured values obtained by replicate measurements on the same or similar objects under specified conditions. It is commonly associated with random errors and represents a measure of

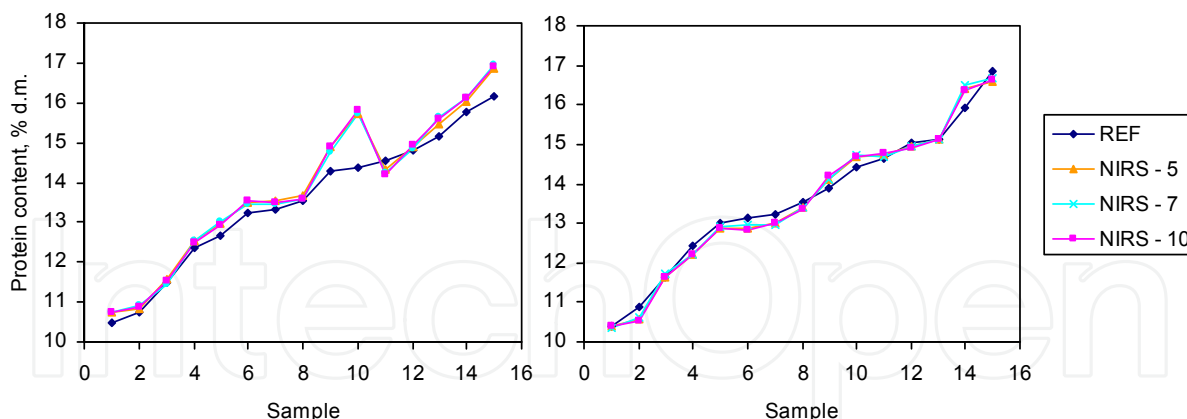


Fig. 4. Comparable view of protein content obtained by the NIRS and reference method, derived from 5, 7 and 10 subsamples, before and after bias adjustment

dispersion of results around the mean value. The precision can be considered by monitoring of repeatability, intermediate precision and reproducibility. All of them can be quantified on the basis of standard deviation, relative standard deviation, or the coefficient of variation (Konieczka & Namieśnik, 2009).

### 3.2.1 Repeatability

Repeatability is a method characteristic that indicates the measure of dispersion of results obtained under the same measurement conditions (a given laboratory, analyst, measuring instrument, reagents, etc.). Since the recommendation for repeatability determination implies measurements on samples characterized with different analyte concentrations and different matrix composition, repeatability of the NIRS methods for protein content prediction included eight consecutive measurements under the repeatability condition using a set of 15 samples.

To assess the acceptability of the repeatability, a modified Horwitz's equation can be used:

$$RSD_r = 2^{(1-0,5\log C)} * 0,67 \quad (4)$$

where the acceptable repeatability is determined on the basis of comparison of actual relative standard deviation ( $RSD_{r,i}$ ) calculated from measured values and predicted relative standard deviation ( $RSD_r$ ) calculated from the Horwitz's equation:

$$RSD_{r,i} < RSD_r \quad (5)$$

The results showed in Table 2 indicates that the repeatability of the NIRS method is better than that of the reference methods expressed by lower  $SD_{r,i}$ ,  $RSD_{r,i}$  and  $r_i$ .

The results shown in Fig. 5 show that repeatability relative standard deviation calculated from actual NIRS results for protein content ( $RSD_{r,i}$ ) are lower than Horwitz's relative standard deviation ( $RSD_r$ ). Thereby, the criterion of repeatability of NIRS method for protein content prediction is fulfilled.

	REF method			NIRS method		
	$SD_{r,i}$ %	$RSD_{r,i}$ %	$r_i$	$SD_{r,i}$ %	$RSD_{r,i}$ %	$r_i$
Mean	0,0858	0,6301	0,2401	0,0755	0,5736	0,2113
Min	0,0503	0,3540	0,1408	0,0041	0,0280	0,0115
Max	0,2484	1,7407	0,6955	0,1173	1,0867	0,3284

Table 2. Results that define the repeatability of the reference and NIRS methods for protein content determination

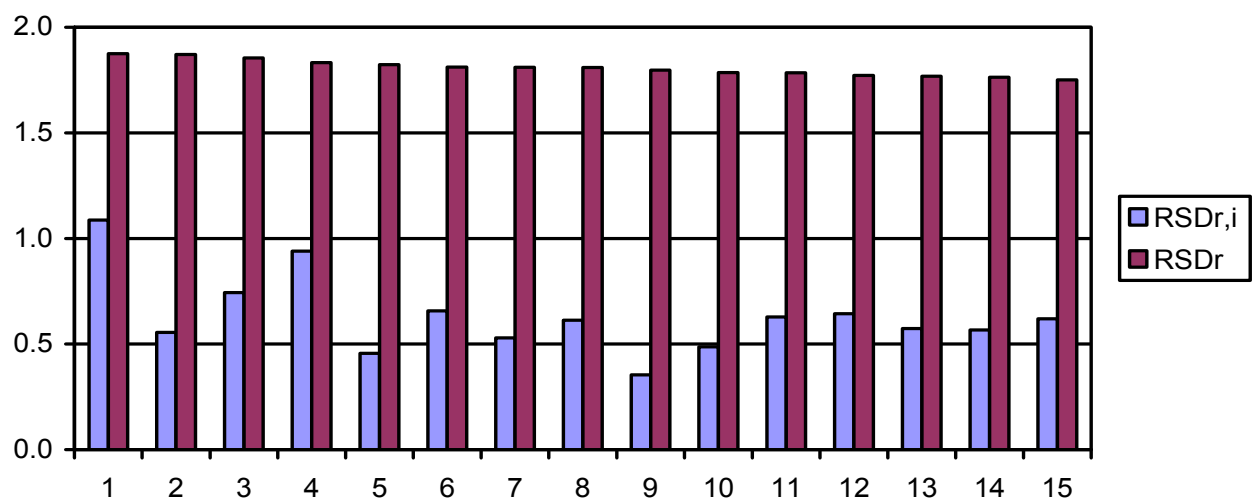


Fig. 5. Comparable view of actual  $RSD_{r,i}$  and Horwitz's  $RSD_r$  values for determining protein content by the NIRS method

### 3.2.2 Reproducibility

Reproducibility is a method characteristic that indicates the measure of dispersion of results obtained in different laboratories using a given measurement method. To assess the reproducibility of NIRS determination of protein in wheat with the Infratec 1241, set of 15 samples was measured with the 5 available NIRS analyzers Infratec 1241. As a criterion for the acceptability of the reproducibility the HORRAT value was used, which was calculated by dividing the actual value of  $RSD_{R,i}$  and  $RSD_R$  calculated from the Horwitz equation:

$$\text{HORRAT}_R = RSD_{R,i} / RSD_R \quad (6)$$

Values lower than 2.0 are considered acceptable for among-laboratory precision, expressed as a  $\text{HORRAT}_R$  value (Fig. 6).

The results showed in Table 3 indicates that the reproducibility of the NIRS method is better than that of the reference methods expressed by lower  $SD_{R,i}$ ,  $RSD_{R,i}$  and  $R_i$ .

The results shown in Fig. 6 indicates excellent reproducibility of the NIRS method as being applied for protein content prediction by the chosen NIRS instrument (Infratec 1241, FOSS Analytical AB) since all HORRAT values were less than 2.

	REF method			NIRS method		
	$SD_{R,i}$ %	$RSD_{R,i}$ %	$R_i$	$SD_{R,i}$ %	$RSD_{R,i}$ %	$R_i$
Mean	0,1743	1,400	0,4872	0,0712	0,5449	0,1993
Min	0,1231	0,9613	0,3443	0,0350	0,2178	0,0980
Max	0,2622	1,9544	0,7320	0,1825	1,6357	0,5110

Table 3. Results that define the reproducibility of the reference and NIRS methods for protein content determination

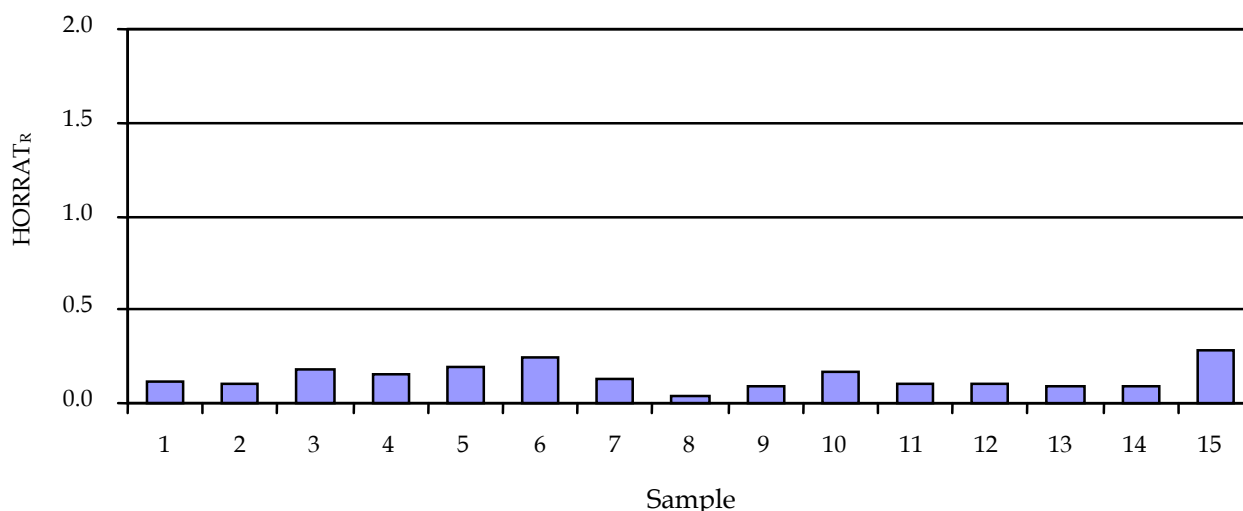


Fig. 6. HORRAT values for assessing the reproducibility of NIRS method for determining protein content

### 3.2.3 Intermediate precision

Intermediate precision is a method characteristic that indicates the measure of dispersion of results obtained in a given laboratory over a long-term process of measuring defining the long-term stability or variability of a measurement process. In this way, the influence of various random effects in the measurement process (e.g. personal effects, instrumental effects, environmental effects etc.) can be monitored and quantified. Due to that, this characteristic has a wider scope than repeatability. The intermediate precision for protein content prediction was monitored over 62 days by using control (Shewart) chart (Fig. 7), having a key significance for raising wheat quality control system to the highest level. Even in cases where the measurement process is under control, control chart is a valuable tool for detection the disturbances in the measuring process. Also, this type of control represents a contribution to the accreditation of the NIRS method based on the requirements of ISO/IEC 17025.

The results shown in Fig. 7 indicates excellent stability of the NIRS method as being applied for protein content prediction by the chosen NIRS instrument (Infratec 1241, FOSS Analytical AB) since all measured values are within control limits.

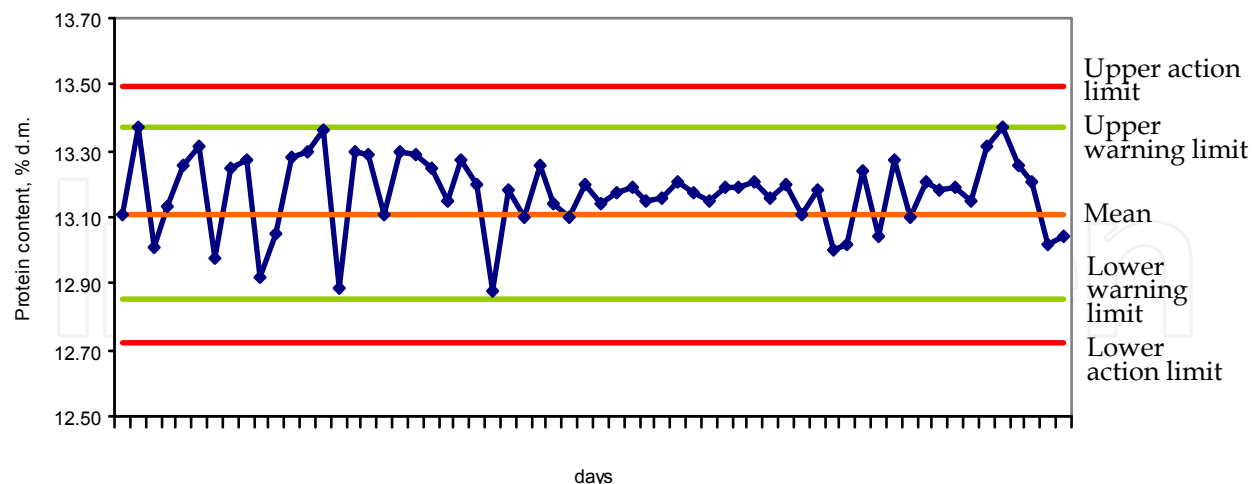


Fig. 7. Shewart (control) chart for determining protein content by the NIRS method

### 3.3 Robustness

The robustness can be defined as the resistance of a method to small deliberate changes in the experimental conditions that provides an indication of its reliability during routine use (Vander Heyden et al., 2001; Goupy, 2005; Dejaegher & Vander Heyden, 2007). The robustness test of the NIRS method for its application in analysing wheat samples examines the potential sources of variability in responses (analytical and spectral). The factors that can cause variability in the NIRS responses refer to the operational and environmental conditions. The robustness of a method is commonly examined in an experimental design, in the intervals that slightly exceed the variation that can be expected in a routine use of the method (Vander Heyden et al., 2001). Experimental design used to determine the robustness of an applied analytical method can be based on univariate (one-variable-at-a-time, OVAT) or multivariate approaches (multi-variate-at-a-time, MVAT) (Dejaegher et al., 2007; Pojić et al., 2012).

To check the robustness of the NIRS method, the OVAT experimental design included deliberate changes of number of subsamples to be measured in single NIRS measurement, environmental and sample temperature, environmental air humidity, instrument voltage and lamp aging in order to determine how tightly controlled the experimental factors should be (Table 4). The obtained results indicated that the NIRS method for determination of protein content appeared to be robust for its application in wheat quality control regardless the deliberate changes in operational conditions (Table 4) (Pojić et al., 2012).

Fig. 8 shows the average SNV second derivative spectra of wheat samples in spectral range 850-1050 nm obtained within the OVAT experimental design. It could be noticeable that the spectral differences were very small. The largest spectral variations were observed in the spectral region around 950 nm associated with OH band for water and around 968 nm, 982 nm and 1014 nm associated with overtone bands (Infrasoft International, 2000; Pojić et al., 2012).



Experimental factors		Protein, % d.m.
Number of subsamples (NS)	5	13.79 <sup>a</sup>
	10	13.79 <sup>a</sup>
	15	13.83 <sup>a</sup>
Sample temperature (ST)	5°C	13.90 <sup>a</sup>
	20°C	13.83 <sup>a</sup>
	35°C	13.84 <sup>a</sup>
Ambient temperature (AT)	10°C	13.83 <sup>a</sup>
	20°C	13.83 <sup>a</sup>
	30°C	13.81 <sup>a</sup>
Environmental humidity (EH)	40%	13.81 <sup>a</sup>
	60%	13.83 <sup>a</sup>
	80%	13.86 <sup>a</sup>
Instrument voltage (IV)	200V	13.80 <sup>a</sup>
	220V	13.83 <sup>a</sup>
	240V	13.84 <sup>a</sup>
Lamp aging (L)	Old	13.83 <sup>a</sup>
	New	13.85 <sup>a</sup>

Table 4. Values in the same column marked with the same letters are no significantly different ( $P < 0.05$ , LSD test) (Pojić et al., 2012).

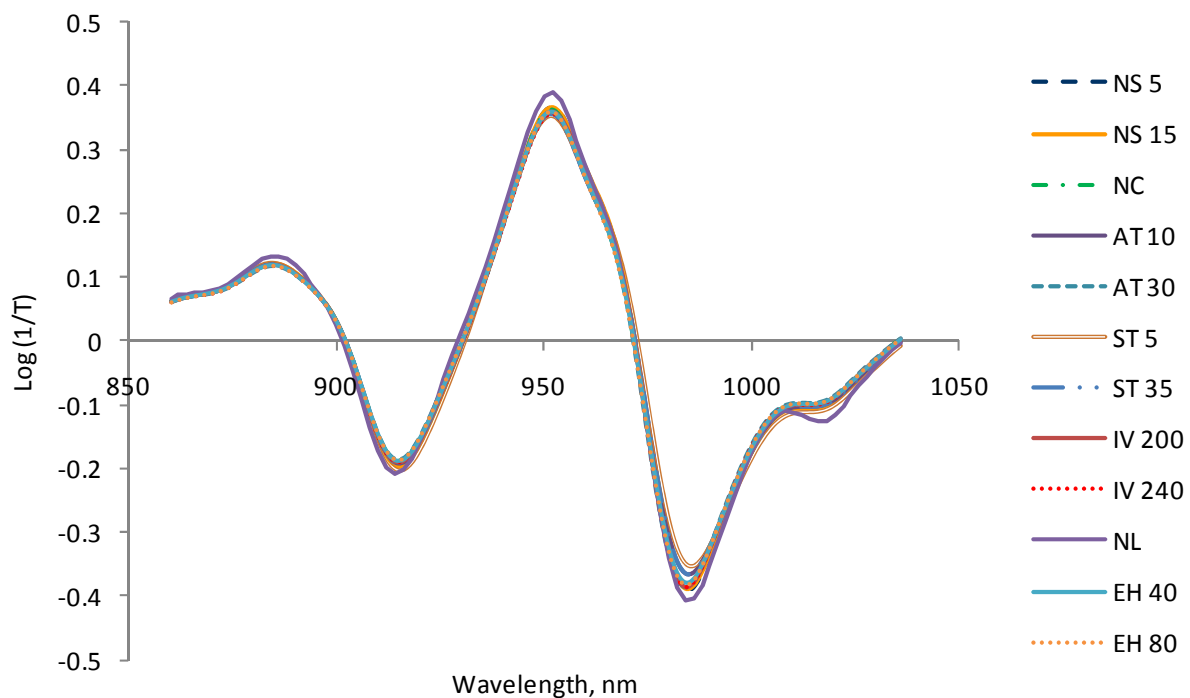


Fig. 8. The average second derivative spectra of wheat samples obtained at different experimental conditions within OVAT experimental design (NS 5, NS 15 – Five and fifteen subsamples in NIRS measurement; NC – Nominal conditions; AT 10, AT 30 – Ambient temperature of 10° and 30°C; ST 5, ST 35 – Sample temperature of 5° and 35°C; IV 200, IV 240 – Instrument voltage of 200 and 240V; NL – New lamp; EH 40, EH 80 – Environmental humidity of 40% and 80 %) (Pojić et al., 2012).

#### 4. Conclusion

Characteristics of the NIRS method as being applied for protein content determination tested within the validation experiment expressed superior quality over the wet chemical method for protein content determination. Due to that, it is not surprising that the NIRS method has been accepted as the standard method by the ISO, AACC, AOAC and ICC, which confirms its applicability for routine use. Before its adoption as an official analytical method, special attention had to be paid to the regulatory requirements for analytical procedures especially due to the fact that the NIRS technique heavily relies on the use of chemometrics calibration and statistical analysis of data. For this reason, it is of utmost importance to verify the characteristics of the NIRS method to demonstrate its fitness for intended purpose. The validation protocol for the NIRS method presented represents an important contribution to the accreditation of the NIRS method based on the requirements of ISO/IEC 17025.

#### 5. Acknowledgment

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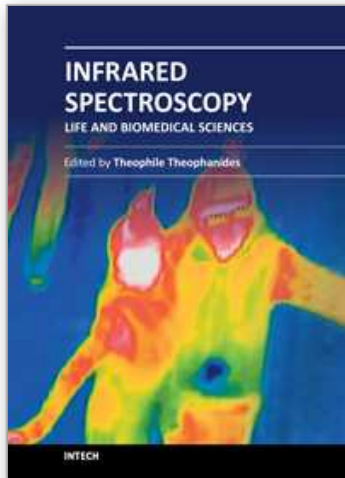


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