

Near-Infrared Spectroscopy for Estimation of Ergosterol Content in Barley: A Comparison Between Reflectance and Transmittance Techniques

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ABSTRACT

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The fungal-specific lipid ergosterol correlates with fungal biomass and often also with the degree of mycotoxin contamination of cereals. We compared the ability of a near-infrared reflectance (NIR) instrument with a broad wavelength range (400–2500 nm) and a near-infrared transmittance (NIT) instrument with a narrower wavelength range (850–1050 nm) to predict the ergosterol content of naturally infected barley samples. The two instruments were equally good at predicting ergosterol content in Swedish samples ($r^2 = 0.81$ and 0.83 for NIT and NIR, respectively). The NIT instrument was then used for samples from three countries (Sweden,

Ireland, UK). This model had about the same root mean-squared error (≈ 5 mg of ergosterol/kg, db, of grain) as the dataset with only Swedish samples, although the r^2 value was lower (0.58). The investigation has shown that it is possible to predict ergosterol content in whole barley samples using NIR or NIT instrumentation, and acceptable models can be obtained using different barley cultivars and samples from different countries and harvest years. This should make it possible to routinely predict the fungal biomass at grain terminals.

Mold growth is one of the primary causes of deterioration of stored cereals, causing general spoilage, formation of allergenic spores, and hazardous mycotoxins (Sinha 1979; Sauer et al 1992). The presence of different mycotoxins in grain has received considerable legislative interest. A maximum level for ochratoxin A has been set to 5 $\mu\text{g}/\text{kg}$ of raw cereals within the European Community (EC Commission Regulation 472/2002) and a maximum limit for deoxynivalenol (DON, 1,250 $\mu\text{g}/\text{kg}$, db) and zearalenon (100 $\mu\text{g}/\text{kg}$, db) has recently been established (EC Commission Regulation 856/2005). For the grain trade, the most important analytical challenge regarding mold infestation is the investigation of large numbers of grain shipments for ochratoxin A and *Fusarium* toxins. It is probable that a rather high percentage of delivered grains, at least during certain years, can exceed the EU limit for ochratoxin A (Jørgensen et al 1996).

Mycotoxins constitute a large and heterogeneous group of compounds, the toxicology of many of which have never been thoroughly examined (Müller and Lehn 1988). The importance of nondetectable mycotoxins can be considerable (Trenholm et al 1994). They found more severe signs of intoxication among animals fed a diet based on cereals with naturally occurring DON, compared with a diet in which DON had been added to grain free from mold infestation. Unknown fungal toxins in the naturally infested grain were suggested to account for this discrepancy.

Considering the impossible task of monitoring all mycotoxins present, the total load of fungi should be considered as a risk assessment for encountering detrimental levels of different mycotoxins. There is also an interest in classifying grain according to general fungal load. Determining the numbers of colony forming units (CFU) or percentage of infected kernels are the most common procedures at present. The ergosterol content could be an alternative to these measures. At least for *Fusarium*, the percentage of infected kernels is not well correlated with the presence of mycotoxins (Homdork et al 2000). Ergosterol is better correlated with fungal biomass than CFU (Schnürer 1993) and is also more sensitive than chitin in detecting the early stages of fungal growth (Matcham et al 1985).

The ratio between ergosterol and biomass varies depending on species (Birmingham et al 1995; Djajakirana et al 1996) and even isolates (Richardson and Logendra 1997). On the other hand, the age of the culture, with the exception of the first few days, has not been reported to influence the ratio extensively (Matcham et al 1985; Johnson and McGill 1990; Richardson and Logendra 1997). Ergosterol content in grains does not change considerably under proper storage conditions (L. Seitz, USDA, Manhattan, KS, USA, *personal communication*, E-mail: larry.seitz@gmprc.ksu.edu) and could be considered a more stable index of mold growth than viable counts that can change drastically over time (Abramson et al 1984; Birzele et al 2000). Despite the possible limitation in that the ergosterol-to-biomass ratio differs somewhat between species, there are many reports on the strong correlation between grain deterioration and ergosterol contents (Seitz et al 1977; Müller and Lehn 1988; Schnürer and Jonsson 1992; Tothill et al 1992). Schnürer and Jonsson (1992) also proposed a tentative quality grading of grains based on ergosterol contents, leading to recommendations for grain to be graded either as food, as animal feed, or to be discarded.

Ergosterol has also been correlated with the presence of the mycotoxins DON (Abramson et al 1998; Lamper et al 2000) and ochratoxin A (Saxena et al 2001). Abramson et al (1999) found that an increase in ergosterol contents in moist barley preceded that of the toxins sterigmatocystin, ochratoxin A, and citrinin. In a study by Homdork et al (2000), ergosterol content was followed under different environmental conditions and different levels of *Fusarium* infection. During warm and humid conditions, and at certain minimum level of *Fusarium* infection, ergosterol, nivalenol, and zearalenone contents increased simultaneously. Pietri et al (2004) collected maize samples from storage bins and found a good correlation between ergosterol contents and different *Fusarium* toxins. They concluded that ergosterol seems to be a good index of the toxicological quality of maize.

Today, the analysis of ergosterol is done roughly as outlined by Seitz et al (1977): extraction, high-performance liquid chromatography (HPLC), and detection with a UV detector. The procedure has been developed further in France, and a standard method has been established (AFNOR 1991). The Nordic Committee on Food Analysis has established a slightly updated method (NMKL 2002). These methods require advanced laboratory equipment and can only be performed in centralized units.

Near-infrared spectroscopy (NIRS) is used routinely for analyzing moisture and protein contents in grains. The technique is rapid and is frequently used on delivery of grains at elevator terminals, where results are obtained within minutes. Many different constituents and properties can be analyzed at the same time, and it is possible to connect different instruments in networks,

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thus supporting control and calibrations of individual instruments (Fleurat-Lessard 1997).

The near-infrared region range of the electromagnetic spectrum is 700–2500 nm. In this region, signals mainly consist of combination bands and overtones from the mid-infrared region. The origin is mainly from bonds in organic molecules and water (C-O, C=O, C-H, N-H, and O-H) (Deaville and Flinn 2000). Due to highly overlapping absorption bands from these overtones and combination bands, direct quantitative estimates from NIR spectra are impossible (Wetzel 1983). A known reference dataset is always needed for calibration of prediction models. Statistics such as partial least squares (PLS) regression are commonly used (Martens and Martens 1986) for this multivariate.

NIRS was used successfully earlier to estimate mold growth in cereals. Roberts et al (1991) found that NIRS data could be used to predict the fungal chitin content in barley. The chitin content correlated with a visual estimation of the mold growth in incubated samples.

TABLE I
Content of Ergosterol, Moisture, Odor at Unloading, and Harvest Year for Barley Samples of Dataset 1

Ergosterol ^a	MC (%) ^a	Odor Type	Strength	Harvest Year
14.8	13.0	Musty	Strong	1993
17.2	13.9	Moldy	Pronounced	1994
8.9	13.8	Moldy	Pronounced	1994
42.5	19.8	Acid	Weak	1995
32.0	17.3	Acid	Weak	1995
8.9	15.1	Moldy	Weak	1995
46.5	18.0	Moldy	Pronounced	1995
36.5	16.8	Moldy	Weak	1995
12.7	14.4	Moldy	Weak	1995
15.8	15.9	Sour	Pronounced	1995
12.4	11.9	Moldy	Pronounced	1995
14.1	12.4	Moldy	Pronounced	1995
11.5	13.9	Moldy	Pronounced	1995
7.5	14.3	Sour	Pronounced	1995
9.9	14.3	Moldy	Pronounced	1995
29.2	15.9	Sour	Pronounced	1995
12.7	14.7	Moldy	Pronounced	1995
13.0	13.8	Musty	Pronounced	1995
6.8	13.3	Moldy	Pronounced	1995
57.7	16.7	Moldy	Strong	1995
60.7	9.8	Moldy	Strong	1995
13.0	16.8	Moldy	Strong	1996
11.8	16.6	Moldy	Pronounced	1996
11.8	17.3	Musty	Pronounced	1996
6.4	10.3	Normal		1996
19.5	7.9	Normal		1996
9.4	11.4	Normal		1996
4.6	10.4	Normal		1996
3.3	10.1	Normal		1996
2.9	10.1	Normal		1996
4.6	9.9	Normal		1996
4.1	9.6	Normal		1996
4.5	9.6	Normal		1996
29.6	17.2	Moldy	Strong	1996
10.6	15.4	Sour	Pronounced	1996
16.4	16.5	Moldy	Strong	1996
7.5	14.5	Moldy	Strong	1996

^a Ergosterol measured in mg/kg, db. Moisture content (MC).

Dowell et al (1999) were able to predict the ergosterol content in wheat using NIR spectra from single kernels and NIR spectra have also been related to humus ergosterol (Pietkäinen and Fritze 1995). NIR has also been used to successfully predict fumonisin B1 and ergosterol in maize (Berardo et al 2005).

These examples indicate a possibility of predicting the ergosterol content in bulk samples of grain collected at elevators. One aspect that has not been previously addressed is the possible variability in output using different NIRS techniques such as the feasibility of using near-infrared transmittance (NIT) instead of near-infrared reflection (NIR). NIT should be more sensitive than NIR because light in the former case is passed through the sample, while in the latter case, the light is diffusively reflected. NIT is thus potentially more adequate for detection of ergosterol that is found at very low levels, and NIT instruments are commonly used at grain terminals. NIT models for detection of individual mycotoxins have also been investigated. For DON, it might be possible to use an NIT model directly (Kos et al 2002; Pettersson and Åberg 2003) but this is hardly possible for ochratoxin A because acceptable levels are very low.

The objective of this research was to investigate the possibility of predicting the ergosterol content of whole bulk samples of naturally infected barley using NIRS and comparing transmittance and reflectance measurements. Samples were collected in three different countries (Ireland, UK, and Sweden), representing, in total, at least 23 different barley cultivars and five different harvest years.

MATERIALS AND METHODS

Grain Samples

In the first experiment in which transmittance and reflectance instruments were compared, 37 Swedish barley samples collected at grain terminals were used (Dataset 1). Among these samples, 28 were graded as off-odorous at unloading and thus would be expected to be infected by fungi and hence have high ergosterol contents (Table I). The samples, harvested between 1993 and 1996, were more extensively described previously (Olsson et al 2002).

In the second experiment, 82 barley samples were collected (Dataset 2). Forty-nine of these were collected in Sweden, 22 in Ireland, and 11 in the UK (Table II). All samples in Dataset 2 were harvested in 1998. Mean values were about the same in the datasets but within Dataset 2, the Irish samples had higher ergosterol contents than the others (Table II). Swedish samples were delivered from farmers for routine analyses, while the Irish and British samples were procured from field trials.

The most commonly used cultivars from the different countries were represented; in total, at least 23 cultivars. Cultivars represented in the Swedish collection were different from those from Ireland and UK. However, information on cultivar was not available for every sample.

NIRS Instrumentation and Analyzing Procedure

The NIR analyses with a scanning monochromatic instrument (NIRSystems 6500, Foss, Silver Spring, MD) equipped with a sample cell with a spinning sample cup was used. The instrument collects 1.8 scans/sec and spans 400–2500 nm.

TABLE II
Summarizing Statistics for Ergosterol Values in Datasets 1 and 2^a

Dataset	Max	Min	Median	Mean	SD	Skewness
1	60.66	2.88	12.37	17.06	14.60	1.68
2, All samples	48.29	6.82	16.56	17.63	8.15	1.11
2, Swedish	33.3	6.9	15.4	15.92	5.93	0.75
2, Irish	48.3	6.8	23.25	24.47	9.54	0.37
2, British	19.2	7.9	10.2	11.59	3.71	1.04

^a Data for samples with different origins are given separately.

Two detector types are used: silicon detectors for the 400–1100 nm interval and lead sulfide detectors for the 1100–2500 nm interval. The spectral resolution was 10 nm and measurements were recorded at 2-nm intervals. The sample size was ≈ 100 g and the final spectra were the average of 38 scans collected from different sectors of the spinning sample cup.

NIT analyses used a monochromatic transmittance instrument (Infracore 1241 Analyzer, Foss, Höganäs, Sweden), measuring the range 850–1050 nm. Data was recorded at 2-nm intervals using a silicon detector with a spectral resolution of 7 nm. The sample size was 150 g; 10 subsamples were analyzed automatically by the instrument. Both instruments were used for analyzing the samples of Dataset 1, while only the Infracore 1241 Analyzer was used for Dataset 2.

Chemical Analyses

Water content was assessed at the same time as the spectra used for prediction of ergosterol content were collected. The standard NIT calibration used in the instrument was used for assessing the water content of the samples. The calibration is used for barley only and has a SEP compared with gravimetric water content of 0.19 (Henrik Josefsson, Foss, Höganäs, Sweden, *personal communication*, E-mail: hjs@foss.dk).

Ergosterol analysis was done according to the NMKL (2002), with some small deviations because this work was conducted before the NMKL method was definitely established. During hydrolysis and saponification, 10 g of barley that had been ground to pass a 0.5-mm sieve was weighed into a round-bottomed flask. Ethanol (80 mL), 10 mL of water, and 10 mL of 50% KOH solution was added. Hydrolysis or saponification was performed in a 90°C water bath during reflux boiling for 30 min. Hydrolysate (20 mL) was then purified by adding it onto a ChemElute (Varian) extraction column and after standing for 20 min, the column was extracted with 100 mL of hexane. The flow rate was 6.5 mL/min or slower.

The eluate was evaporated and dissolved in 5 mL of heptane. The sample was then stored at -20°C until analysis. The parameters for the liquid chromatography were: column VA 250/4.6 (length 250 mm, i.d., 4.6 mm) Nucleosil 50-5 (Machery-Nagel); mobile phase 2% 2-propanol in hexane. The chromatography was run at room temperature with a flow rate of 1.5 mL/min. The detection of ergosterol was done with a UV detector at 282 nm and the retention time was ≈ 6 min.

Data Evaluation

Partial least squares (PLS) was used for the calibrations with the software Unscrambler 7.8 (Camo ASA, Oslo, Norway). PLS is a multivariate linear regression technique that produces projections in a few dimensions of a data matrix with many variables (Wold 1989). Thus, a large number of variables are reduced to a few latent variables (components). The PLS models were validated using full cross-validation (leave-one-out) (Martens and Næs 1989) and by using one data set as validation set by predicting it with the calibration of the other. Before calibrations, NIT and NIR spectra were transformed by the standard normal variate (SNV) to have a standard deviation of 1 of each spectrum to reduce light-scatter effects caused by particle size differences and de-trended (DT) to remove linear and quadratic curvature of each spectrum (Barnes et al 1993). Spectra were also smoothed before analysis by fitting each recorded wavelength to a third-order polynomial using the four adjacent data points on each side (Savitzky and Golay 1964). Thus, the resulting PLS-calibrations were of the form

$$Y = \beta_0 + \beta_{\lambda_1}A_{\lambda_1} + \dots + \beta_{\lambda_i}A_{\lambda_i}$$

Here Y is the dependent variable (ergosterol); β_0 is the intercept; β_{λ_i} are the regression coefficients for the transformed and smoothed absorbance A_{λ_i} at wavelength λ_i .

To evaluate the performance of the calibrations, r^2 values, root mean-squared error of cross-validation (RMSECV) or prediction of the validation sets (RMSEP) were calculated on the relationship between measured and predicted ergosterol in the validation samples

$$RMSE = \sqrt{\frac{1}{I} \sum_{i=1}^I (y_i - \hat{y}_i)^2}$$

Here RMSE denotes RMSECV and RMSEP alike; y is the measured value and \hat{y} is the predicted value of sample i ; and I is the number of samples. RMSE indicates the average deviation between measured and predicted values in absolute values.

RESULTS

When constructing models based on NIT data from Dataset 1, four samples were excluded due to NIT spectra that strongly deviated from the other samples. The four samples were clearly different from the others in having high leverages and high residuals in X -space. With nine PLS components, the ergosterol content in cross-validation samples could be predicted with acceptable accuracy (Fig. 1A). Samples from different harvest years did not aggregate. Important wavelengths were distributed over several distinct peaks over the entire spectrum (Fig. 1D). The models based on NIR data gave slightly better results, with only three outlying samples excluded due to strongly deviating spectra (Fig. 1B). The model thus calibrated was able to give acceptable estimates of the ergosterol content with an 8 PLS-components calibration. Only the wavelengths of 1100–2500 nm were used, as this gave a better prediction compared with using the full range. In NIR calibration, the important wavelengths were also distributed over the whole wavelengths range (Fig. 1E).

The calibrations based on NIT data from Dataset 2 did not appear to be as good as those from Dataset 1. The r^2 was much lower compared with those from Dataset 1 (Fig. 1C). The calibrations improved only slightly if they were restricted to the samples coming from Sweden (Table III). Especially evident was a tendency for underprediction of samples with a higher ergosterol content than 20 mg/kg, db (Fig. 1C). However, the RMSECV was only slightly higher in Dataset 2. This apparent contradiction is explained by the smaller total variation in Dataset 2 (Table II). Three samples were excluded as outliers due to strongly deviating spectra. There was no strong tendency of aggregation of samples coming from the same country.

Although some similarities are suggested when comparing the most influential wavelength regions in the Swedish and mixed NIT calibrations (Fig. 1D and F), the PLS coefficients do not correlate well (data not shown).

The water content correlated weakly with the ergosterol content ($r^2 = 0.26$) for all samples in the two datasets. The water content was also used in the same way as NIT data to predict the ergosterol content using the Unscrambler software; that is, the water content was used as a single variable and cross-validation was performed as above. In both datasets, the performance of these models was inferior to models based on NIT data (Table III). The NIT data that were important for predicting ergosterol (Fig. 1D and F) also differed from the typical bands used for predicting water content in grains. Although the information on moisture is also distributed over the entire spectra, one important region is at ≈ 964 nm (Williams and Norris 2001) that was not conspicuous in the ergosterol spectra.

The NIT data from both datasets were also combined. The performance of this calibration was slightly poorer compared with the model based on Dataset 2 only (Table III). When combining the sets, it was obvious that those samples with high ergosterol contents were underpredicted and that the data points were very scarce at high ergosterol levels.

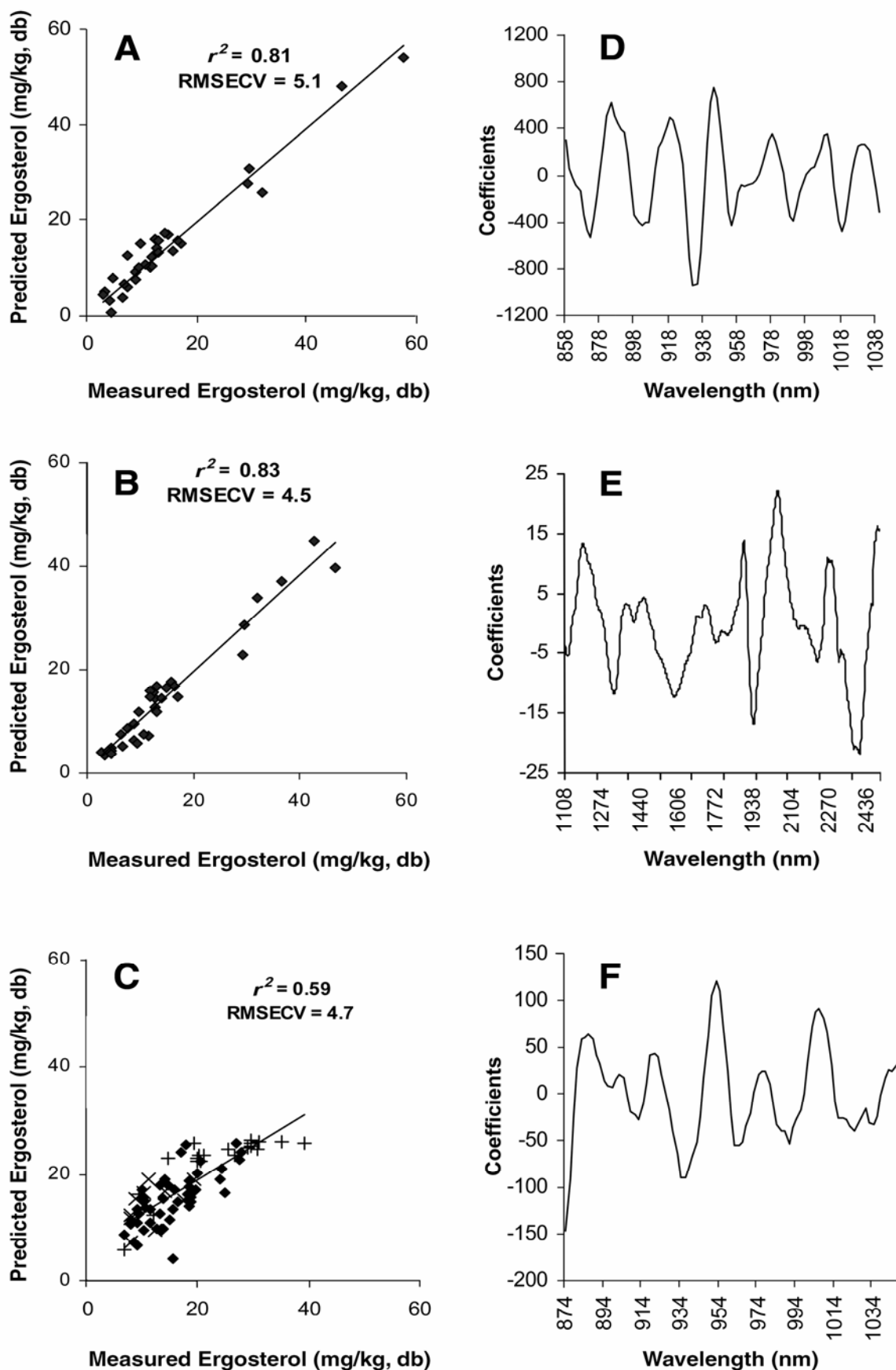


Fig. 1. Ergosterol content in barley samples predicted by full cross-validation of PLS models based on SNV-DT transformed and smoothed NIT and NIR data (A–C) and PLS coefficients for individual wavelengths used in calibrations (D–F). A and D are based on data from Dataset 1 and analyzed with NIT. B and E on Dataset 1 analyzed with NIR. C and F on NIT data collected from Dataset 2. (In C samples: ■ = Swedish, + = Irish, and X = British).

TABLE III
Comparison of Performance of Different PLS-Models for Predicting Ergosterol Content in Datasets 1 and 2 and a Combination of Datasets

Dataset	Validation	Data Type	r^2	RMSE ^a	RMSEP ^a	Sample Origins	n	Outliers
1	Cross-validation	NIT	0.81	5.1		Sweden	33	4
1	Cross-validation	NIR	0.83	4.5		Sweden	34	3
2	Cross-validation	NIT	0.59	4.7		Mixed	79	3
2	Cross-validation	NIT	0.58	3.6		Sweden	48	1
1	Cross-validation	Moisture content	0.42	7.6		Sweden	33	4
2	Cross-validation	Moisture content	0.34	6.1		Sweden	48	3
Both	Cross-validation	NIT	0.50	6.3		Mixed	110	9
Both	DS 1 = Validation set	NIT	0.47		8.6	Mixed	34	3
Both	DS 2 = Validation set	NIT	0.42		10.1	Mixed	77	6

^a Root mean-squared error of cross-validation (RMSECV) or prediction of the validation sets (RMSEP) measured in mg/kg, db.

A common way of solving such problems with uneven distributions is to use the logarithm of the reference data (Eriksson et al 2001) and thus the curvature could be eliminated. However, using log transformation of ergosterol values did not give better models for the combined dataset.

To test the generality of the two data sets, we also predicted the ergosterol contents of Datasets 1 and 2, respectively, using one data set as calibration set and the other as validation set. These attempts were not generally very successful. The best results were obtained predicting the ergosterol content in Dataset 1 with the calibration of Dataset 2 (Table III).

DISCUSSION

This study clearly shows that NIR and NIT are equally apt to estimate the ergosterol content of barley. Apparently NIR bands are absorbed in sufficiently deep layers of the grain to give sufficient information related to the ergosterol content. Also, the relatively narrow wavelength range of 850–1050 nm in the NIT spectra was sufficient. The large number of well-distributed spectral bands influencing the NIT and NIR calibrations suggest that a highly complex combination of information from the spectra are used to estimate the ergosterol content.

The PLS coefficients of the best-performing calibration of the two NIT data sets were not well correlated, and predictions across the two data sets were poor. This indicates that other changes in the grains as a result of mold growth influence the calibrations apart from the possible influence from ergosterol itself. The low ergosterol content in grain, compared with that of constituents normally detected with this technique, also suggests that there is an indirect relationship between ergosterol and features actually active in the NIRS spectra. Frankenhuizen and Kisko (1997) found that spectral regions used for the detection of proteins and carbohydrates change during mold growth. Similarly, Dowell et al (1999) state that important wavelengths to determine ergosterol, scab, and DON were found all over the range 500–1700 nm. This strongly suggests that there is a combination of differences in fungal biomass and other changes occurring during fungal growth that are detected. Many of the peaking PLS coefficient regions coincide with absorption bands assigned to such structures as CH₃, CH₂, CONH₂, amides, starch, and cellulose (Shenk and Workman 2001). The performance and generality of calibrations for estimation of the ergosterol content is thus limited by the stability of indirect relationships between the ergosterol content and what can be extracted from the spectra.

The water content was correlated with the ergosterol content but the investigation showed that a prediction of ergosterol using near-infrared data was more accurate than using the water content of the samples to predict the ergosterol content. The NIT data used in the models were also different from the data used when predicting water content in the same samples.

It was possible to construct models for predicting ergosterol content in barley that could be acceptable for routine practices,

although fully satisfactory models could only be constructed using one of the two datasets tested. A relatively simple NIT instrument was equal to a more advanced NIR instrument with a much wider spectral range. The encouraging results obtained for making ergosterol predictions with a routine NIT instrument provides opportunities for screening grain samples for mold contamination much more effectively than what is done today. This makes it possible to improve the quality of grains reaching the consumers, human or animal alike. In the future, an index based on predicted ergosterol content could be used for a first classification of mold status of grains.

Further studies would give more information on the extent to which a calibration can assist in avoiding grain loads with unacceptable high contents of mycotoxins. These studies should also shed light on the possible influence of water content in samples and the effect of storage on ergosterol contents and NIRS spectra.

CONCLUSIONS

The results indicate that NIRS instrumentation can be used as a quick estimation of the content of fungal biomass in bulk grain samples. Thus, it would be possible to estimate fungal load of grain samples routinely at grain terminals. The relatively narrow wavelength range used in the NIT instruments (850–1050 nm) seems to be sufficient. It also seems to be possible to include the samples from different harvest years, different cultivars, and samples from different countries in the same calibration. The latter needs to be elucidated further because the two datasets used in this investigation were not fully compatible. This indicates that the calibration is influenced by other changes in the grain kernels apart from the ergosterol molecule. However, the water content of the samples did not influence the calibration substantially.

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