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ORIGINAL PAPER

Validation and transferability study of a method based on near-infrared hyperspectral imaging for the detection and quantification of ergot bodies in cereals

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Abstract In recent years, near-infrared (NIR) hyperspectral imaging has proved its suitability for quality and safety control in the cereal sector by allowing spectroscopic images to be collected at single-kernel level, which is of great interest to cereal control laboratories. Contaminants in cereals include, *inter alia*, impurities such as straw, grains from other crops, and insects, as well as undesirable substances such as ergot (sclerotium of *Claviceps purpurea*). For the cereal sector, the presence of ergot creates a high toxicity risk for animals and humans because of its alkaloid content. A study was undertaken, in which a complete procedure for detecting ergot bodies in cereals was developed, based on their NIR spectral characteristics. These were used to build relevant decision rules based on chemometric tools and on the morphological information obtained from the NIR images. The study sought to transfer this procedure from a pilot online NIR hyperspectral imaging system at laboratory level to a NIR hyperspectral imaging system at industrial level and to validate the latter. All the analyses performed showed that the results obtained using both NIR hyperspectral imaging cameras were quite stable and repeatable. In addition, a correlation higher than 0.94 was obtained between the predicted values obtained by NIR hyperspectral imaging and those supplied by the stereomicroscopic method which is the reference method. The validation of the transferred protocol on blind samples showed that the method could identify and quantify ergot contamination, demonstrating the transferability of the method. These results were obtained on samples with an ergot concentration of 0.02 % which is less than the EC limit for cereals (intervention grains) destined for humans fixed at 0.05 %.

Keywords Ergot · Contaminant · Cereal · NIR hyperspectral imaging · Transferability · Validation

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Introduction

Ergot alkaloids are mycotoxins produced by fungi in the *Claviceps* genus, mainly *Claviceps purpurea*. The fungi may infect cereals and wild grasses, where they produce sclerotia that contain ergot alkaloids. These substances cause serious disorders in cattle, sheep, and horses [1], whereas there is historic evidence of human intoxications (ergotism) due to ergot alkaloids [2]. The alkaloid composition of the sclerotia depends on their maturity and other factors, such as fungal strain, host plant, geographical origin, and prevailing weather conditions. The danger posed by ergot alkaloids has been well known for centuries [3]. For the farmer, the main danger is yield reduction. For the feed and food sectors, the presence of ergot creates a high toxicity risk for animals and humans because of its alkaloid content. To reduce the risk of poisoning, European Commission (EC) directive 2002/32/EC on undesirable substances in animal feed fixed a limit of 0.1 % for ergot in all

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feedingstuffs containing unground cereals [4]. EC regulation 689/92, which sets out the procedure and inspecting cereal conditions for intervention agencies in charge of governing the cereals European market, trade with third countries, and rules regarding competition, restricted the concentration of ergot bodies in cereals for food to 0.05 % [5]. A recent EC recommendation [6] established that Member States should analyze food and feed samples (cereals, cereal products, pasture/forage grasses, compound feed, and food) for the presence of the six most prevalent ergot alkaloids (ergocristine, ergotamine, ergocryptine, ergometrine, ergosine, and ergocornine) and determine simultaneously the ergot body content. Thus, a relationship between the amount of ergot bodies and presence of ergot alkaloids could be established.

Until a few years ago, chromatographic and (to a lesser extend) immunochemical techniques were used mainly to develop methods for directly monitoring the presence of ergot alkaloids in food and feed commodities [7–9]. More recently, multiplex dipstick assays have been developed to detect several ergot alkaloids at the same time [10]. Near-infrared (NIR) spectroscopy has also been investigated for detecting the total alkaloid content of forage grasses [11]. None of the current analytical methods for ergot alkaloids has been formally validated through inter-laboratory studies, which seems partly due to the fact that legal limits for individual ergot alkaloids in food and feed are not yet fixed in the European Union [12]. Unlike the methods mentioned here, focusing on alkaloid detection per se, the current official method focuses on the detection of ergot bodies in the food and feed chain. EC regulation 687/2008 [13] states that the official method for detecting ergot bodies in cereals is the quantification of ergot bodies by the manual extraction of the ergot sclerotia in the sample and the weight of this fraction. The International Association of Feedingstuff Analysis has used light microscopy and color reaction to identify the presence of ergot bodies [14]. More recently, spectroscopy-based techniques, i.e., NIR hyperspectral imaging, combined with chemometric tools, have been proposed for detecting contaminants in food and feed [15–21].

Based on this technique, a complete methodology was developed for detecting and quantifying ergot bodies in cereals contaminated at a level of up to 1 % [22]. NIR hyperspectral imaging was proposed as a fast method for the online detection and quantification of ergot bodies in cereals. The main advantage of this NIR method is the higher volume of samples that can be analyzed by unit of time, as well as its ability to detect ergot bodies (entire or broken) and differentiate them from other visually similar plant structures. The method addresses the sampling issue properly, as it can be used to analyze primary samples from the first step of the procedure of sampling a large lot of cereals [23]. It does not require doing secondary or tertiary sampling before analysis and therefore drastically reduces the total sampling error associated with all analytical methods.

The purpose of this paper is to report on a validation and transfer study of a hyperspectral NIR-based method for ergot detection and quantification. The study involved transferring the method from the laboratory that developed the methodology to another laboratory and included the use of a commercial NIR hyperspectral imaging system and its application in the control laboratory of a large feed factory.

Material and methods

Samples

The first set of samples consisted of wheat and rye, contaminated or not with ergot. These samples were collected by the NUTRECO company in 2009 and 2010. Seven samples of 50 g (set A: S1 to S7) were analyzed to assess the protocol and perform the transfer of the methodology. In set A, only samples S5, S6, and S7 were contaminated by six, four, and seven ergot bodies, respectively. To validate the transfer of the NIR hyperspectral imaging method, a second set of six samples (set B: S8 to S13) of 0.5–1 kg was collected in 2011 by the same feed producer. These samples included rye, organic rye, organic triticale, oats, and black oats. Only samples S8 and S9 were contaminated by three and eight ergot bodies, respectively. The presence or absence of ergot bodies was assessed using the reference method described in the next section.

Apart from ergot, the samples, in particular, the organic rye and triticale samples, were also contaminated with other impurities. Tables 1, 2, and 3 show, for each sample, the cereal species (wheat, rye, triticale, oat, and black oat) and the quantity of impurities determined by visual observation.

In addition to these samples, a set of reference materials was prepared to be used in building the discrimination model, cereals versus contaminants. This set included crop cereals such as wheat, rye, and barley and some impurities such as rape seed, straw pieces, and ergot bodies. Each reference material was put, row by row, into a metallic holder, as shown in Fig. 1c.

Reference method

The official method [13, 14] is based on a visual identification of the suspicious ergot particles in a sample of 50–100 g after sifting (Fig. 1a). The collected material is spread on a plate and checked with a stereo-microscope (magnification

Table 1 Analytical results of samples in set A from two instruments

Sample code	Cereal	Impurities presence	Ergot									
			Reference value		Predicted value							
					Without object identification				With object identification			
					BurgerMetrics		SisuCHEMA		BurgerMetrics		SisuCHEMA	
Set A			No.	%	No.	%	No.	%	No.	%	No.	%
S1	Wheat	Few	0	0.00	9	0.38	8	0.27	0	0.00	0	0.00
S2	Wheat	Few	0	0.00	3	0.34	4	0.24	0	0.00	0	0.00
S3	Wheat	Few	0	0.00	4	0.11	6	0.21	0	0.00	0	0.00
S4	Wheat	Few	0	0.00	7	0.13	6	0.16	0	0.00	0	0.00
S5	Wheat	Few	6	0.39	6	0.35	11	0.57	6	0.35	6	0.41
S6	Rye	Few	4	0.33	3	0.25	8	0.61	3	0.25	4	0.34
S7	Rye	A lot	7	0.52	14	1.01	32	1.11	7	0.64	10	0.59

of ×10–40) to identify all particles selected as ergot (Fig. 1b). The identification is performed by using ergot reference material and draws on the knowledge and expertise of the operator. After this process, the particles identified as ergot are weighed to an accuracy of 0.01 g, and the percentage of ergot is calculated, with two decimals, as follows:

$$\%\,\text{ergot bodies} = \text{particles identified as ergot bodies(g)}/\text{total weight of sample(g)} \times 100$$

This official method was applied to the two sets of samples (sets A and B) in order to quantify the ergot bodies.

NIR hyperspectral line scan imaging system

To develop the method for detecting and quantifying ergot bodies in cereals, hyperspectral images were collected using an NIR hyperspectral line scan camera, combined with a conveyor belt and allowing online prediction analysis (BurgerMetrics SIA, Riga, Latvia). This instrument, described in detail by Vermeulen [22], was not intended to be moved from one laboratory to another. To perform a complete transfer of the NIR hyperspectral imaging protocol, another commercial instrument (Specim–SisuCHEMA SWIR XL [Specim Ltd, Oulu, Finland]) was used and installed at the first stage at CRA-W and then at NUTRECO (see Fig. 1d for a picture of the instrument).

Table 2 Analytical results of samples in set A from the SisuCHEMA NIR hyperspectral camera at two locations and over two time periods

Sample code	Cereal	Impurities presence	Ergot									
			Reference value		Predicted value							
					With object identification							
					SisuCHEMA							
					Location 1				Location 2			
					Period 1		Period 2		Period 1		Period 2	
Set A			No.	%	No.	%	No.	%	No.	%	No.	%
S1	Wheat	Few	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00
S2	Wheat	Few	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00
S3	Wheat	Few	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00
S4	Wheat	Few	0	0.00	0	0.00	0	0.00	0	0.00	0	0.00
S5	Wheat	Few	6	0.39	6	0.43	6	0.37	5	0.42	6	0.41
S6	Rye	Few	4	0.33	3	0.29	4	0.34	4	0.42	4	0.33
S7	Rye	A lot	7	0.52	11	0.58	9	0.58	10	0.58	11	0.61

Table 3 Analytical results on samples in set B from the SisuCHEMA NIR hyperspectral camera

Sample code	Cereal	Impurities presence	Ergot					
			Reference value		Predicted value			
					Without object identification		With object identification	
Set B			No.	%	No.	%	No.	%
S8	Rye	Few	3	0.02	12	0.03	4	0.02
S9	Organic rye	A lot	8	0.04	21	0.12	7	0.04
S10	Organic triticale	A lot	0	0.00	23	0.04	2	0.00
S11	Oat	Few	0	0.00	3	0.00	0	0.00
S12	Black oat	A lot	0	0.00	20	0.04	0	0.00
S13	Black oat	few	0	0.00	1	0.00	0	0.00

The Specim instrument includes a NIR hyperspectral imaging system similar to that used to develop the method and a tray as a sample holder instead of a conveyor belt. The acquisition was performed with ChemaDAQ spectral imaging software (Specim Ltd, Oulu, Finland). All images consisted of 480 lines of 320 pixels which were acquired from 256 wavelength channels (970–2500 nm with 6.3 nm spectral intervals). A 15 mm lens was used to analyze the total width of the plate. The individual pixel size was 600 μm^2. A total of 153,600 spectra (320 pixels/line × 480 lines) from an area of 600 cm^2, corresponding to about 100 g of grain, were acquired in 5 s.

Experimentation plan

Using the online prediction NIR hyperspectral camera, the first measurements were performed at CRA-W on samples in set A. These measurements were then followed at CRA-W with the SisuCHEMA NIR hyperspectral instrument. The SisuCHEMA instrument was subsequently assessed over two time periods under laboratory (CRA-W) and industrial (NUTRECO) conditions. The samples in set A were analyzed at the two locations and over two time periods in order to assess the reproducibility of the method. The samples in set B were analyzed only at the feed company with the aim of validating the transfer of the methodology on the SisuCHEMA NIR hyperspectral instrument. The metallic holder with reference materials was analyzed twice each day (morning and afternoon) in order to check the stability of the instruments and assess the repeatability of the measurements.

Development of discrimination models

For the online prediction NIR hyperspectral camera, the data treatment was performed using HyperProVB v1.31 software

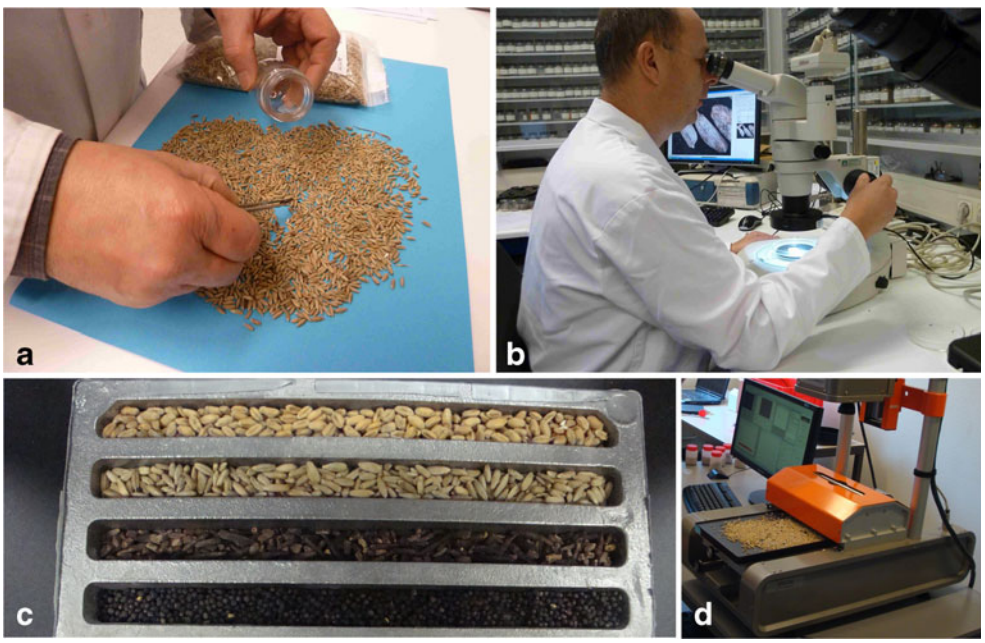

Fig. 1 Pictures showing **a** the manual removal of ergot bodies and **b** the observation by the stereo-microscopic method (official method); **c** the metallic holder with the reference material, and **d** the NIR hyperspectral SisuCHEMA instrument

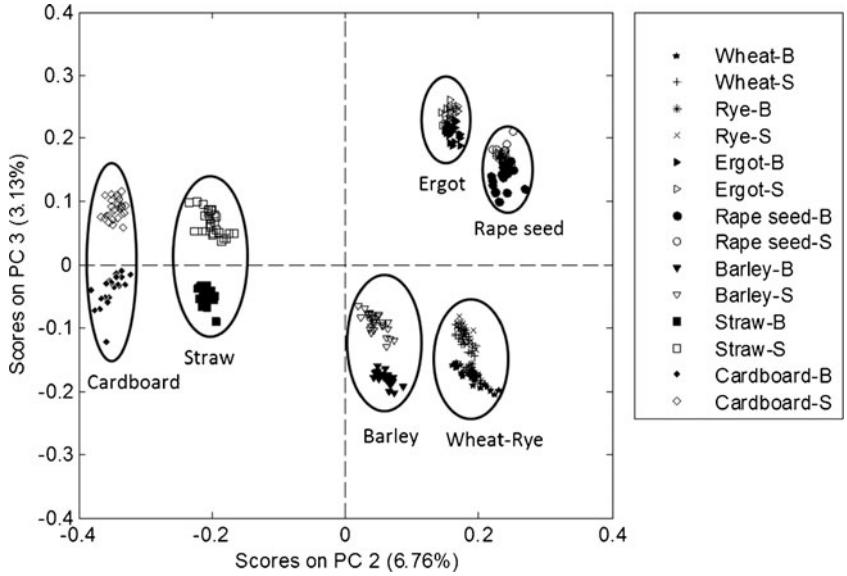

Fig. 2 PCA (PC2 versus PC3) on the seven reference materials measured on two NIR hyperspectral instruments: *B*= BurgerMetrics, *S*= SisuCHEMA

(BurgerMetrics, Latvia). The libraries for ergot bodies, cereals, and background were built by selecting pixels on the images of the reference materials, and models were developed using partial least squares discriminant analysis (PLSDA) [24] without any preprocessing being applied to the data.

For the SisuCHEMA NIR hyperspectral camera, the data treatment was performed using Evince software (Umbio, Umea, Sweden). The libraries were built by selecting spectra from the principal component analysis (PCA) scores calculated on the images of the reference materials [24]. Discrimination models were then developed using Soft Independent Method of Class Analogy (SIMCA) as a multivariate chemometric tool [24]. In this case, the preprocessing standard normal variate (SNV) and mean centering were applied to the spectral data. The wavelength range used was limited to the 1,573 2,399 nm region.

A morphological study was also performed in order to extract all the relevant spatial information from the samples and to combine it with the NIR spectral discrimination information. For the online predicted images, a visual analysis was performed based on the size of the groups of pixels detected as ergot bodies. For the batch-processed images, an object identification tool included in the Evince software was applied in order to remove objects corresponding to isolated false-positive pixels or objects detected as ergot bodies but showing an unusual shape. Most of the ergot bodies covered an area of between 20 and 140 pixels. The roundness of the bodies was generally less than 50 %. These thresholds were used to identify the ergot bodies. In comparison, a wheat kernel usually has an area of about 40 pixels, and the roundness of a rape seed is higher than 80 %.

Quantitative determination based on the discrimination results was also applied by taking into account both the number and the percentage of ergot bodies in the cereals. The number of ergot bodies was provided either by the number of groups of pixels counted visually or by the number of objects provided by the morphological study. The percentage of ergot bodies was provided by the proportion of pixels detected and identified as ergot bodies in the full sample. This percentage was corrected by a factor equal to 0.8, to take account of the difference in specific weight between the ergot bodies and the grains [22].

Results and discussion

Reference material

To control the stability of the two instruments over location and time, the metallic holder with reference materials was analyzed twice a day. Overall, during the study, this samples holder was analyzed 24 times on the BurgerMetrics online prediction NIR hyperspectral instrument and 29 times on the SisuCHEMA NIR hyperspectral instrument. Each spectrum was the average value calculated for each material in the metallic holder from individual images taken over time. In order to evaluate variability in the data, PCA was performed on data preprocessed by SNV and first derivative Savitzky–Golay (window, 5; polynomial, 2). The first principal component explained 87 % of the variation, which is related mainly to water content, discriminating material with high cellulose content (straw, cardboard) from seeds and ergot bodies. Figure 2 shows the second versus third principal components (PC2 versus PC3) plot, which clearly indicates that each reference material can be discriminated from each other (except wheat and rye), based on lipid, protein, and water content. It should be noted that the cereals (wheat, rye,

and barley) have similar spectral profiles; straw and cardboard also have similar spectral profiles, as do ergot bodies and rape seeds. It should also be noted that, within each class of reference material, the instruments can be discriminated and retain similar variability. This difference between the instruments is linked to the small shift in the wavelengths defined for each instrument.

Detection of ergot bodies using the reference method

The official method was applied to the two sets of samples (sets A and B) in order to quantify the ergot bodies. Tables 1, 2, and 3 show, for each sample, the number and percentage of ergot bodies determined by the reference method. Set A had four blank samples and three samples with high ergot concentration (0.33–0.67 %). Set B had four blank samples and four samples with ergot bodies (0.02–0.04 %), lower than the limits of 0.05 % and 0.1 % authorized by the EC in food and feed, respectively [4, 5].

Detection of ergot bodies using the BurgerMetrics online prediction NIR hyperspectral instrument

The PLSDA model based on three classes (background, wheat, ergot) [22] was applied to five wheat samples in set A (S1 to S5). A second PLSDA model based on three classes (background, rye, ergot), developed from libraries and built from different images taken of reference materials, was applied to the two rye samples in set 1 (S6 and S7). An overview of the results is presented in Table 1.

Between 3 and 14 objects, corresponding to 0.11–1.01 % of pixels, respectively, were detected as belonging to the ergot class. False-positives, due mainly to the presence of weed seeds and/or rape seeds, were removed, based on their smaller size, observed visually on the predicted images, in comparison to the ergot bodies. After applying this rule, no object was detected in samples S1 to S4. Six, three, and seven objects corresponding to 0.35, 0.25, and 0.64 % of ergot pixels were detected in samples S5, S6, and S7, respectively. Figure 3 shows the correlation between the ergot percent calculated using the reference method and the ergot percent calculated using the NIR hyperspectral imaging method (R^2=0.94).

Detection of ergot bodies using the SisuCHEMA NIR hyperspectral instrument

The SIMCA model (ergot-cereals) was applied to the seven samples in set A, analyzed on the SisuCHEMA NIR hyperspectral instrument at two locations and at two different times.

Table 1 (SisuCHEMA) shows the averaged results over the four measurements. Between 4 and 32 objects, corresponding to 0.16–1.11 % of pixels, were detected as belonging to the ergot class. The false-positive pixels came mainly from rape seeds, weed seed, and wheat germs classified as ergot. These false-positive pixels were removed using the object identification tool based on the size of the objects. Only objects greater than 20 pixels were selected as ergot bodies. When applying this rule, no object was detected in samples S1 to S4. Six, four, and ten objects, corresponding to 0.41, 0.34, and 0.59 % of ergot pixels, respectively, were detected on average in the four measurements in samples S5, S6, and S7, respectively. These results are comparable with those obtained using the online prediction NIR hyperspectral camera (Table 1, BurgerMetrics).

Table 2 shows the number of ergot bodies and the percentage of ergot pixels identified for the four measurements performed at two locations and over two time periods. For sample S5, six ergots were detected three times. For the fourth measurement, the sixth ergot covered 12 pixels, lower than the fixed limit of 20 pixels. For sample S6, four ergots were detected three times. For the fourth measurement, the fourth ergot also covered 12 pixels, lower than the fixed limit. For sample S7, between 9 and 11 ergots were detected for the four measurements. To explain the false-positive results of sample S7, other parameters need to be taken into account, such as the roundness of the object. These false-positives had an area of less than 30 pixels and/or roundness higher than 50 %. Some rape seeds and weed seeds complied with these criteria. The change of the area limits from 20 to 30 pixels, however, could lead to non-detection of some broken ergot bodies. Figure 4 shows the correlation between the ergot percent calculated using the reference method and the ergot percent (size >20 pixels) calculated using the SisuCHEMA NIR hyperspectral imaging method (R^2=0.99). The results show the good reproducibility of the measurements over the time periods and locations using the same model on the same instrument.

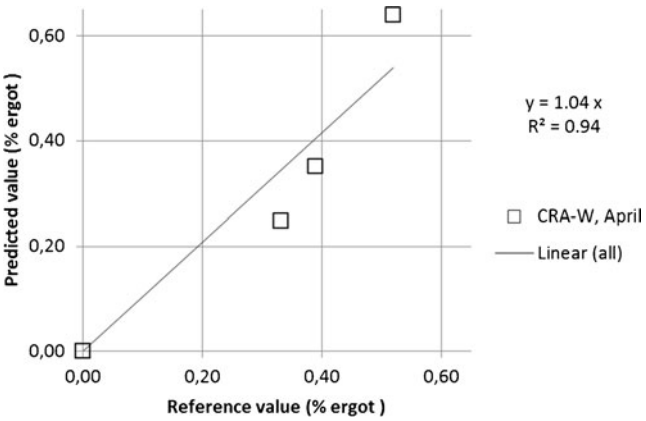

Fig. 3 Results of the analysis of samples in set A. Correlation between the reference values determined by the official method and the predicted values using the BurgerMetrics NIR hyperspectral instrument

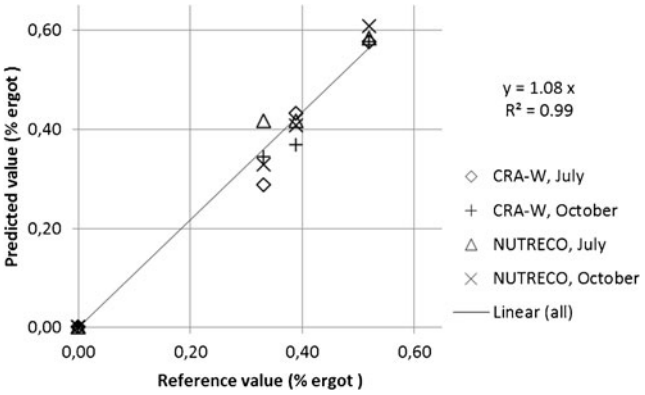

Fig. 4 Results of the analysis of samples in set A. Comparison of the analysis performed at two locations and over two time periods. Correlation between the reference values determined by the official method and the predicted values using the SisuCHEMA NIR hyperspectral instrument

To validate the transfer of the NIR hyperspectral imaging method, the same SIMCA model (ergot-cereals) and the same object identification rules were applied to the six samples (S8 to S13) in set B collected during the harvest in 2011 by the feed company and analyzed on-site, using the SisuCHEMA NIR hyperspectral instrument.

For each sample, Table 3 gives the number and percentage of ergot bodies using the reference method and the SisuCHEMA NIR hyperspectral imaging method, with and without object identification. It should be noted that, for the samples with no ergot bodies and few impurities, the percentage of pixels detected as ergot was very low. Samples S11 and S13 had 0.00 % of ergot pixels, and no object with more than 20 pixels detected as an ergot body was identified. For the samples with no ergot bodies but a large number of impurities, the percentage of pixels detected as ergot pixels was higher. Samples S10 and S12 showed 0.04 % of ergot. For sample S12, no object with more than 20 pixels detected as ergot bodies was identified. For sample S10, an organic triticale sample with a large number of impurities, two objects of 24 and 30 pixels were identified as false-positives. In addition to being ≤30 pixels, these two objects had a roundness higher than 50 % and were visually identified as weed seeds. Samples S8 and S9 with three and eight ergots had 0.03 % and 0.12 % of ergot bodies, respectively. It should be noted that sample S9, a sample of organic rye, also had a lot of impurities. Using the identification rules, samples S8 and S9 had four and seven objects with an area of 26 to 99 pixels, respectively, detected as ergot bodies. For sample S8, one object identified as an ergot body was a false-positive identification characterized by a size of 29 pixels and a roundness of 60 %. For sample S9, one object was not detected as ergot, characterized by a size of 17 pixels, lower than the fixed limit.

Figure 5 shows the correlation between the ergot percent calculated using the reference method and the ergot percent calculated using the object identification rules after applying the NIR hyperspectral imaging method (R^2=0.98). It should be noted that these results were obtained on the samples with an ergot concentration of less than 0.05 %, which is the fixed EC limit for cereals (intervention grains) destined for humans.

Conclusion

The conclusions drawn from the analysis of real samples at both laboratory and industrial level showed that NIR hyperspectral imaging combined with chemometric tools could be used as a control method to assess and quantify the presence of contaminants such as ergot bodies in cereals. The transfer of the concept of detecting and quantifying the ergot bodies in cereals by NIR hyperspectral imaging needed the use of ownership software for spectra acquisition and data treatment. The two configurations using PLSDA or SIMCA discrimination models, combined with an object identification tool, through an online prediction NIR hyperspectral imaging pilot system or a batch processed prediction NIR hyperspectral imaging commercial system, gave good results. The analyses performed on the reference samples using both instruments demonstrated the stability of the instruments and the repeatability of the measurements.

The discrimination models between cereals and ergot were built from spectral profiles with spectra from samples that had different fat and starch contents. Cereal kernels are characterized by high starch content and a low fat content, whereas ergot bodies, weed seeds, and wheat germs have high lipid content. The rules built for the object identification tool were based on the larger size and more elongated shape (roundness) of an ergot body compared with a cereal kernel. Based on the discrimination models, no false-

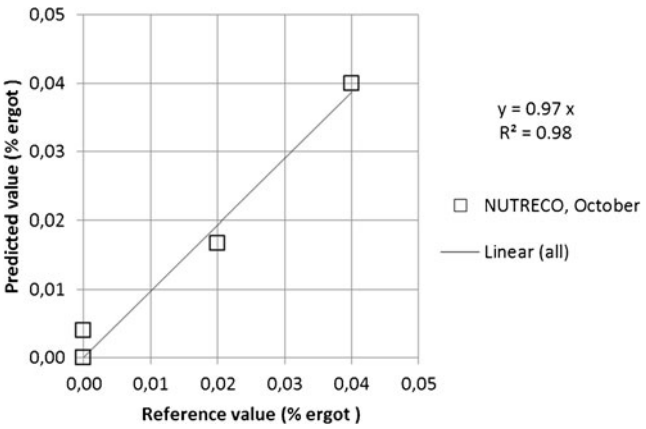

Fig. 5 Results of the analysis of samples in set B. Correlation between the reference values determined by the official method and the predicted values using the SisuCHEMA NIR hyperspectral instrument

negative ergot bodies were detected. Depending on the limits fixed for the size of ergot bodies (determined by an area of a fixed number of pixels), however, some pieces of broken ergot bodies could not be correctly classified using the identification tool. They could be confused with small weed seeds. The limits of size and roundness should be adapted, depending of the type of impurities in a cereal sample.

The successful transfer of the protocol from a pilot system used at laboratory level to a commercial system tested at industrial level showed that the NIR hyperspectral imaging could be a powerful tool within the framework of official controls. Such a method could be useful as an analytical screening tool in an automatic cereal control scheme, where the positive samples could be analyzed using official methods. In addition and with reference to EC recommendations [6], this NIR hyperspectral imaging method, combined with chromatographic methods [7–9] or multiplex dipstick assays [10], could help to improving knowledge about the relationship between the content of ergot bodies in cereals and the levels of individual ergot alkaloids.

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