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Application of near infrared hyperspectral imaging for identifying and quantifying red clover contained in experimental poultry refusals

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A R T I C L E I N F O

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A B S T R A C T

For laying hens, free-range systems are promoted to improve both animal behavior and egg quality. In this context, estimation of feed intake, and particularly the proportion of herbage in the diet, has become a hot topic in poultry feeding management. During experiments, animals are housed in individual cages to measure daily ingestion, calculated by the difference between offered feed and refusals (non-ingested feed). Due to their natural behavior, laying hens tend to put offered feed on the ground, mixing grains and herbage with impurities (wood-chip litter and droppings) which leads to actual ingestion being underestimated. Manual sorting of mixed refusals is tedious. The aim of this study was thus to propose a procedure to identify herbage, red clover in this case, among impurities and to estimate the weight of red clover in entire mixed refusals by combining near infrared reflectance (NIR) imaging systems. A discriminant analysis (based on 8074 spectra) allowed each pixel corresponding to red clover to be identified. The results showed that more than 90 % of pixels were correctly classified. On the basis of the number of pixels predicted as red clover, a linear regression was built to convert pixels into red clover weight in order to recalculate the actual feed intake. A determination coefficient (R^2) of 0.95 was achieved. Model validation was based on 12 composite refusals, and resulted in a determination coefficient of validation (R^2_v) equal to 0.83 and a root mean square error of prediction (RMSEP) equivalent to 3.4 g of dry matter (DM). The equation was considered satisfactory considering the possible bias in the manual sorting method. The main advantage of this procedure is the reduction in the time taken by the procedure by a factor of 4, while maintaining a high level of accuracy.

Abbreviations: DM, dry matter; NIR, near infrared reflectance; NIRS, near infrared reflectance spectroscopy; NIR-HIS, near infrared reflectance hyperspectral imaging system; PLS-DA, partial least squares discriminant analysis; R^2, determination coefficient; R^2_v, determination coefficient of validation; RGB, picture picture in red-green-blue system; RMSEP, root mean square error of prediction.

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1. Introduction

Forage vegetation intake by laying hens is a hot topic due to the promotion of outdoor free-range systems. These systems provide more available area to the animal, which allows natural exploring behaviors such as scratching (Weeks and Nicol, 2006; Singh and Cowieson, 2013; Jurjanz et al., 2015), and also has a positive impact on egg yolk composition, such as high omega-3 fatty acid levels (Holt et al., 2011; Rossi et al., 2013). Experiments aiming to quantify individual feed intake have to be carried out on an individual scale. Precise collection of refusals is made impossible by the natural behavior of laying hens, which tend to put feed components on the soil before eating them. This leads to the mixing of refusals with droppings (poultry excreta) and litter, as a result of which the estimated intake may be underestimated. One solution is to manually sort mixed refusals by separating feed components from impurities. The actual refusal can be weighed, and the precise forage vegetation intake can then be estimated. A possible alternative could be the development of a fast and non-destructive method using infrared spectroscopy technology. Near infrared reflectance spectroscopy (NIRS) measures the vibration of chemical bonds in organic compounds. As these vibrations are very specific to chemical groups, NIRS is an appropriate method to characterize products (Dale et al., 2013a; Fernández Pierna et al., 2014). Near infrared reflectance hyperspectral imaging systems (NIR-HIS) therefore combine NIRS (giving a spectral signature of the products to be analyzed) and imaging technologies (allowing their spatial distribution to be identified). Each spectrum from a pixel corresponds to absorption at different wavelengths, providing a complete hypercube of data to be studied with appropriate chemometric tools (Eylenbosch et al., 2017). NIR-HIS combined with chemometrics has proven to be a powerful combination for analyzing entire samples and is at the same time easy to implement and accurate (Eylenbosch et al., 2018; Vermeulen et al., 2018). It is used in a number of agronomic applications for both quantitative and qualitative analyses. Shorten et al. (2019) assessed the chemical composition of ryegrass by estimating 13 attributes such as protein, sugar and energy content. The results showed a good prediction for percentage of ash and nitrogen, achieving a determination coefficient of validation (R^2_v) of 0.55 and 0.70 and a root mean square error of prediction (RMSEP) equal to 0.77 and 0.35 respectively. Metabolizable energy was less well predicted (R^2_v = 0.33, RMSEP = 0.23). Using an airborne hyperspectral system in heterogeneous pastures, Pullanagari et al. (2018) studied metabolizable energy and protein crude content and achieved an R^2_v of 0.61 and 0.63 and an RMSEP equal to 0.86 and 2.20 respectively. Caporaso et al. (2018) performed estimates for single wheat kernels of protein content (R^2_v = 0.79; RMSEP = 0.94) and kernel weight (R^2_v = 0.62 and RMSEP = 7.4). Classification of grassland species was tested by Dale et al. (2013b). This study discriminated poaceae (R^2_v = 0.91) from other species (R^2_v = 0.90), but plants from the fabaceae family were less well differentiated (R^2_v = 0.60).

The objective of the present study was to propose a complete procedure for identifying and quantifying red clover in entire mixed refusals originating from laying hens' experiments using NIR-HIS combined with chemometrics.

2. Materials and methods

2.1. Sample collection

Dried mixed refusals were used in an experiment conducted on 15 laying hens at the Walloon Agricultural Research Center in Gembloux (Belgium) with the approval (number 14–1778) of the Animal Ethics Committee of the University of Liège (Liège, Belgium). The study focused on the quantification of red clover (*Trifolium pratense* L.) intake by laying hens and the impact of red clover intake on egg composition.

The experiment was carried out indoors, in individual metal mesh cages (0.95 × 1.95 × 0.75 m), providing a large available space. Each cage contained one drinking trough, one nest, wood-chip litter and two feeders, one for crushed grain mixture and the second for fresh red clover (Fig. 1). The crushed grain mixture was composed of 470 g/kg wheat, 200 g/kg peas, 110 g/kg flaxseed meal, 90 g/kg sunflower and 130 g/kg vitamins and mineral supplement.

Every morning, crushed grain mixture and red clover were weighed and distributed individually. Once the feeders were attached to the cages, the hens systematically put the red clover on the floor, scratched and spread it out before eating. The next morning, all blades of herbage were carefully picked up from the floor; these red clover refusals were contaminated with impurities such as droppings, crushed grains and wood-chip litter (Fig. 2). These mixed refusals were dried at 60 °C until constant weight, vacuumized with a

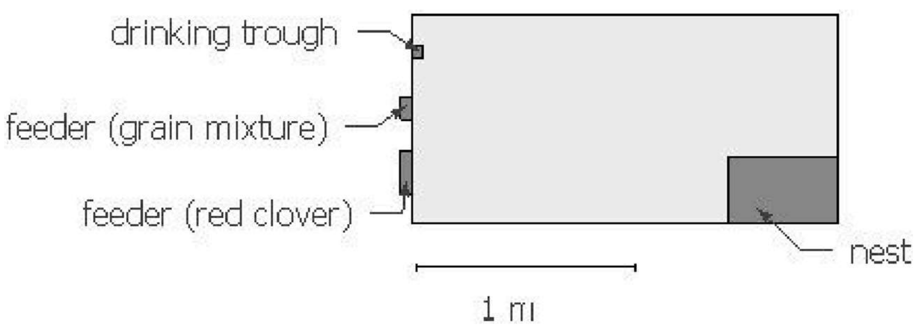

Fig. 1. Individual laying hen cage including two feeders (one for crushed grain mixture and the one for fresh red clover), a drinking trough and a nest. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 2. Example of a dried mixed refusal collected during the trial. Visible components are: dropping, red clover and litter. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

vacuum packaging machine (model UNICA GAS, Lavezzini, Fiorenzuola, Italy) and stored at room temperature. The experiment was continued for 30 days, and mixed refusals were collected every day. In total, about 450 dried mixed refusals were obtained, with an average weight of 10.6 g of dry matter (DM), ranging from 0.1 to 43.6 g of DM.

2.2. NIR hyperspectral imaging and image acquisition

The NIR-HIS used in this work was described in detail by Vermeulen et al. (2012). The push-broom imaging system is an NIR hyperspectral line scan using a SWIR XEVA CL 2.5 320 TE4 camera (Burgermetrics SIA, Riga, Latvia). The analysis surface measured 28 cm in length and 10 cm in width, in the direction of the conveyor belt. The pixel was a square with a side of 875 µm. Spectra were collected in the wavelength range from 1118 nm to 2424 nm, with a spectral resolution of 6.3 nm (i.e. 209 channels). The exposure time was 20 ms and the speed of the conveyor belt was fixed at 100 mm/s. Samples had to be carefully spread without overlapping of constituents. Illumination was provided by two halogen lamps, each with a power of 120 W.

Data acquisition was performed using Burgermetrics SIA (2010). Before measurements, a system calibration was performed with two references: a white one (white ceramic) and a dark one (achieved by blocking the entrance of reflected light). Bad pixels – pixels with aberrant detection (Burgermetrics, 2009) – were detected and removed at the same time. As the stability of imaging system was checked with one standard sample, every sample was measured once (no duplicate analysis). The image recording was continuous with an average of 100 images per sample, depending on the sample size.

2.3. Procedure development

2.3.1. Sample selection

The use of 57 dried mixed refusals was necessary to perform this study, randomly selected from all samples collected in the experiment on laying hens. The robustness of the manual sorting method was tested with five dried mixed refusals. The development of the procedure required the use of 52 dried mixed refusals.

2.3.2. Manual sorting method

The manual sorting method was designed in the lab and consisted of visual screening and manual sorting of red clover to determine its dry weight. A dried mixed refusal was spread on a tray and red clover parts (leaves, stem) were manually separated from impurities such as droppings, wood-chip litter or crushed grains. Once the entire mixed refusal had been screened, the red clover portion was weighed and the impurities were discarded.

The robustness of the manual sorting method was estimated. Five samples were randomly selected, and each one was manually sorted by three different operators. The weight of red clover and the time needed to sort entire samples were taken into account for each operator and each sample. The presence of an operator effect on the weight of red clover and the time required to sort samples was determined using analysis of variance, considering a P-value below 0.05 as significant.

2.3.3. Methodology and data treatment

The strategy for identifying and quantifying red clover in dried mixed refusals was based on a two-step procedure. The first step aimed to identify red clover among the impurities in dried mixed refusals (qualitative determination) while the second step estimated the weight of red clover (quantitative determination). For the first step, five samples were randomly selected and manually sorted. Each type of component identified in dried mixed refusals (red clover, droppings, crushed grains and wood-chip litter) was separately analyzed with NIR-HIS, recording NIR images and thereby creating a spectral library with spectra of pure components. This library was used to build partial least squares discriminant analysis (PLS-DA), a multivariate analysis defining latent variables used to classify new observations in a predefined set during calibration (Chevallier et al., 2006; Lenhardt et al., 2015; Eylenbosch et al., 2017). Calibration and cross-validation of PLS-DA were performed using the PLS Toolbox software of Eigenvector Research (2007) working with Matlab (The Math Works, 2015). Furthermore, an external check was performed consisting of a comparison between RGB (red-green-blue system) picture and the prediction of the NIR hyperspectral images of dried mixed refusals, by identifying if red clover parts were differentiated from other impurities. External validation was carried out on three randomly selected dried mixed refusals. The result of this first step was the number of pixels classified as red clover in dried mixed refusals. The second step aimed to estimate the weight of red clover expressed in g of DM in dried mixed refusals, by using a linear regression between the number of pixels classified as red clover and the weight of red clover. Thirty-two dried mixed refusals were randomly selected and manually sorted by a single operator, and the red clover portion was analyzed with NIR-HIS and weighed with an analytical balance in order to obtain reference data. Linear regression was performed with Rstudio (R Core Team, 2019) and a regression line was obtained, enabling the number of pixels predicted as red clover to be converted into dry weight of red clover. The linear regression was regarded as significant with a P-value below 0.05.

To validate the entire procedure, the repeatability of the NIR-HIS procedure was calculated and an external validation was performed. To evaluate the repeatability of the NIR-HIS measurement, the variation coefficient of repeated analyses (ten times) of a dried mixed refusal was calculated. For the external validation, 12 dried mixed refusals were randomly selected and fully analyzed with NIR-HIS to predict the quantity of red clover (g of DM). These samples were then manually sorted and the red clover part was weighed, as described in the manual sorting method section. The model's prediction error was evaluated with the root mean square error of prediction (RMSEP) as follows:

$$\mathrm{RMSEP} = \sqrt{\frac{\sum_{i=1}^{n}(\hat{y}_i - y_i)^2}{n}}$$

with $\hat{y}$ being the predicted dry weight with the NIR-HIS procedure, y being the dry weight obtained with the manual sorting method and n being the number of observations. Moreover, the time required to estimate the weight of red clover in dried mixed refusals with the manual sorting method and with NIR-HIS analysis was recorded for comparison.

3. Results

3.1. Manual sorting method

Table shows the weight of red clover (g of DM) obtained and the time required (in minutes) for the manual sorting of five dried mixed refusals by three independent operators. For the weight of red clover, the standard deviation ranges from 0.1 to 0.6 g of DM and the P-value is equal to 0.027. The average time to sort dried mixed refusals is about 18 min with a standard deviation from 3 to 8 min and a P-value of 0.180.

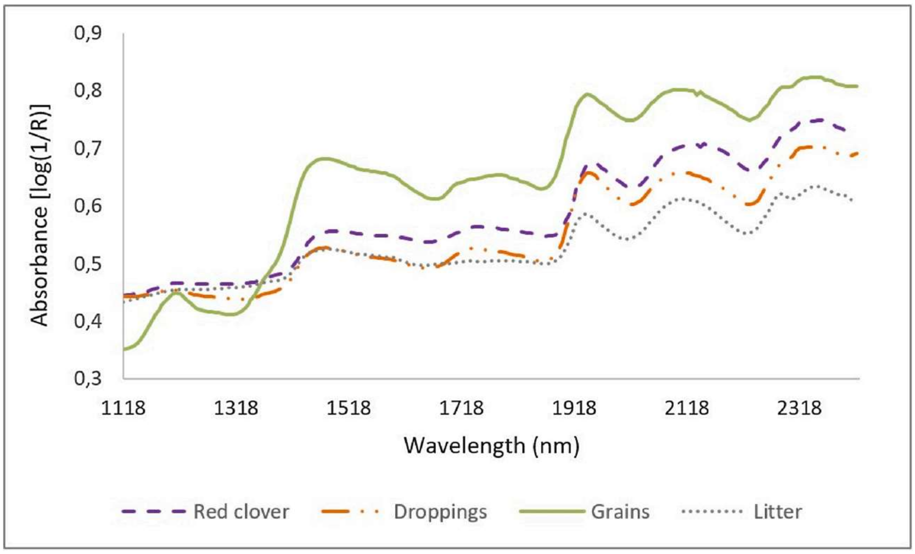

Fig. 3. Mean NIR-HIS (near infrared reflectance hyperspectral imaging system) raw spectra of pure components (i. e. red clover, droppings, crushed grains and wood-chip litter) contained in dried mixed refusals. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. Qualitative determination

In order to build the PLS-DA models, a spectral library had to be created with spectra of pure components. Based on NIR-HIS analysis of pure components from five dried mixed refusals, a total of 8074 spectra were selected, distributed as follows: red clover (2829 spectra), droppings (1050 spectra), crushed grains (672 spectra), wood-chip litter (2644 spectra) and background (879 spectra). Mean spectra of pure components are shown in Fig. 3. Absorption bands were observed in the characteristic wavelength regions of crude protein (1484 nm) and of moisture (1947 nm) (Clark and Lamb, 1991). Regions of wavelengths associated with fibers (2148 nm; 2348 nm) also showed a maximum of absorbance (Andrés et al., 2005). Regarding these features, the mean spectrum of red clover corresponds to typical spectra of forage, and the mean spectrum of crushed grains fits with typical spectra of cereals.

To identify red clover among other impurities based on NIR-HIS images of dried mixed refusals, two successive PLS-DA models were built using the library composed of pure component spectra. The results of calibration and cross-validation are shown in Table 1. The first PLS-DA model made it possible to discriminate between spectra extracted from pixels of dried mixed refusals and from the background (conveyor belt). The number of latent variables used to build this PLS-DA model was equal to height, minimizing over-fitting. For the calibration, 98.0 % of spectra extracted from pixels of mixed refusals were correctly classified and 98.2 % of those from the background. The results were similar to those in the cross-validation with 98.0 and 97.6 % of pixels from spectra of dried mixed refusals and background respectively correctly assigned. The second PLS-DA model, based on five latent variables, made it possible to discriminate between red clover and impurities (i.e. droppings, crushed grains, wood-chip litter). The results, also presented in Table 2, are substantially identical for calibration and cross-validation. Ninety percent of red clover pixels were correctly classified, and 93.0 % of pixels from impurities were properly assessed. Percentages of correctly classified pixels were high (> 90 %) for both PLS-DA models, and the results for calibration and cross-validation were similar.

An external check was performed in order to visually ensure the correct identification of red clover among impurities on NIR-HIS images (Fig. 4). The RGB picture (Fig. 4a) shows a part of a mixed refusal containing red clover (green portions) and impurities (beige components) placed on the conveyor belt. On the hyperspectral image at 1740 nm wavelength (Fig. 4b), mixed refusal components can be observed, without discriminating between red clover and impurities, and the background is grey. The discrimination of background pixels using the first PLS-DA model is presented on Fig. 4c with black pixels. Classification based on the second PLS-DA model is presented on the next two figures: in white, Fig. 4d shows the pixels classified as red clover and Fig. 4e the pixels identified as impurities. Visual comparison between Figs. 4a, 4d and 4e shows that the red clover was correctly discriminated from impurities and background.

3.3. Quantitative determination

The second step of the procedure was to predict the weight of red clover (g of DM) contained in dried mixed refusals. The linear regression between the number of pixels classified as red clover by PLS-DA models and the weight of red clover obtained with the manual sorting method is shown on Fig. 5. The regression showed a determination coefficient of 0.95 and a standard error of 1.5 g of DM. Statistical analyses revealed that the model is significant (p-val < 0.001).

3.4. Procedure validation

The repeatability of NIR-HIS measurements was estimated by analyzing the same mixed refusals 10 times. The average predicted weight of red clover, measured with NIR-HIS, was equal to 0.13 g of DM and a variation coefficient of these repeated measurements was equal to 2.1 %.

An external validation was performed by analyzing 12 dried mixed refusals with NIR-HIS and with the manual sorting method. Weights obtained with the two methods are compared on Fig. 6. The determination coefficient (R^2_V) was equal to 0.83. The relationship between manual sorted and predicted weight is linear, with a slope of 0.85. The global error of the procedure, including both the pixel prediction error and the weight estimation error, was estimated with RMSEP as 3.4 g of DM. The time required to sort these refusals was around 1 h with the NIR-HIS method and more than 4 h for the manual sorting method.

Table 1
Robustness of the manual sorting method, used to estimate the weight of red clover contained in dried mixed refusals. Five samples were selected and each sample was sorted by three different operators. In total, there were three replicates per sample, for which the weight of red clover in dried mixed refusals (g of DM) and the required sorting time (in minutes) were measured.

Sample	Weight (g of DM)				Time (min)			
	Min	Max	Mean	SD	Min	Max	Mean	SD
1	16.7	18.1	17.5	0.6	13	21	17	4
2	9.0	10.0	9.4	0.4	13	25	19	6
3	3.9	4.2	4.0	0.1	13	20	16	3
4	6.1	6.4	6.3	0.1	10	16	13	3
5	6.6	7.0	6.9	0.2	14	31	22	8

Mean, mean of the three operators; SD, standard deviation of the three operators; DM, dry matter.

Table 2
Results of the first PLS-DA model discriminating pixels of dried mixed refusals from pixels of the background (conveyor belt); and of the second PLS-DA model identifying red clover pixels among impurities in dried mixed refusals (droppings, crushed wheat, wood-chip litter). The number and the percentage of pixels correctly classified and incorrect are shown for calibration and cross-validation.

	Calibration				Cross-validation			
	Number of pixels		Percent of pixels		Number of pixels		Percent of pixels	
	Correctly classified	Error	Correctly classified [%]	Error [%]	Correctly classified	Error	Correctly classified [%]	Error [%]
PLS-DA model 1								
Components of mixed refusals	7051	144	98.0	2.0	7049	146	98.0	2.0
Background	863	16	98.2	1.8	858	21	97.6	2.4
PLS-DA model 2								
Red clover	2558	271	90.4	9.6	2555	274	90.3	9.7
Impurities	4083	283	93.5	6.5	4081	285	93.5	6.5

PLS-DA, partial least squares discriminant analysis.

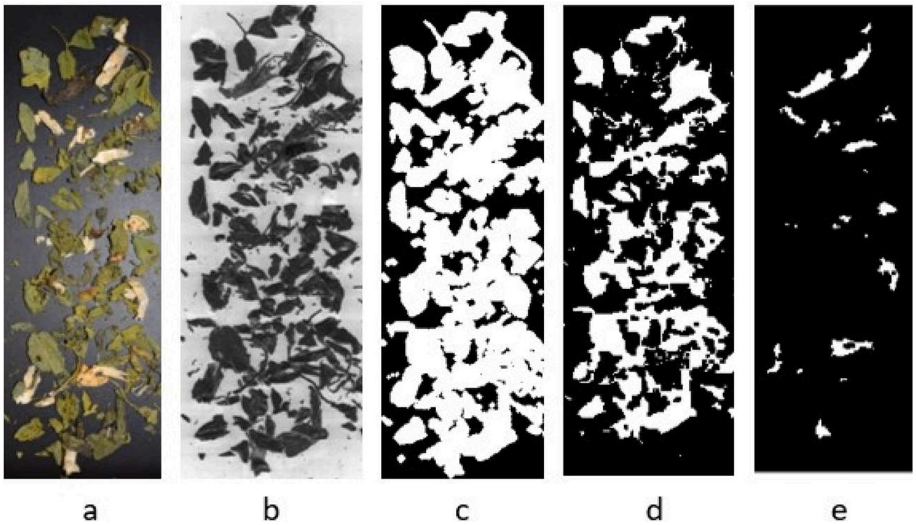

Fig. 4. Illustration of external check of correct red clover identification. (a) RGB image (picture in red-green-blue system) of a part of a mixed refusal placed on the conveyor belt (in black); green blades are red clover and beige components correspond to wood-chip litter. (b) NIR-HIS (near infrared reflectance hyperspectral imaging system) image (at 1740 nm wavelength) with dried mixed refusal components in dark and background in grey. (c) Result of the first PLS-DA (partial least squares discriminant analysis) model; pixels classified as dried mixed refusal (red clover and impurities) are in white and pixels classified as background in black. (d, e) Result of the second PLS-DA model; pixels in white are classified as red clover (d) or impurities (e). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

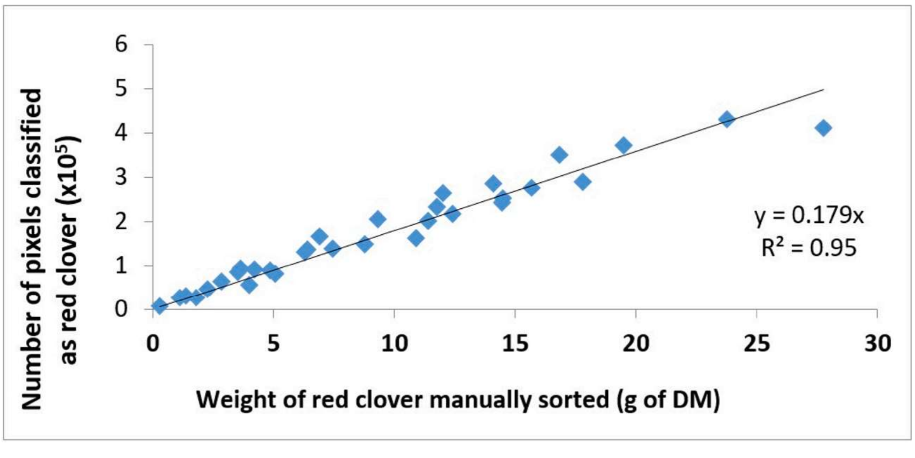

Fig. 5. Linear regression between pixels classified as red clover by PLS-DA (partial least squares discriminant analysis) models and weight of red clover manually sorted (g of DM, dry matter) in dried mixed refusals. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

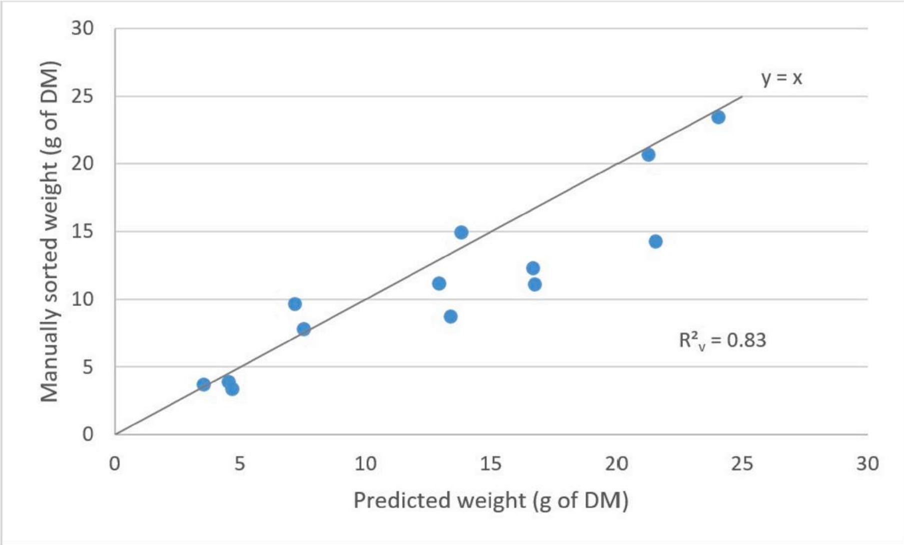

Fig. 6. Relationship between predicted and manually sorted weight of red clover contained in dried mixed refusals (g of DM, dry matter). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

The manual sorting method, designed in the lab, shows a significant operator effect. For four of the five samples, one operator obtained the highest weight and another the lowest weight. Robustness seems to be insufficient. Moreover, operators reported that the method is time-consuming and tedious. Impurities were observed, mostly in dried mixed refusals with a high weight. In view of operators' observations, bias can be assumed to be present in the manual sorting method, due to the difficulty of reliably identifying droppings. When components of dried mixed refusals were manually sorted and a supposed dropping was observed, the operator was not able to identify if it was red clover refusal mixed with the droppings of laying hens, or if it was a dropping containing an entire blade of clover. After experimental laying hens had undergone euthanasia, entire parts of red clover were observed in their large intestine, which means that they can be excreted with droppings.

As regards the NIR-HIS procedure, qualitative determination indicates that red clover was successfully identified on NIR-HIS images of dried mixed refusals. The share of correctly classified spectra extracted from pixels of dried mixed refusals was higher than 90 %, for both PLS-DA models. Furthermore, the results for calibration and cross-validation were similar, indicating good robustness. The result of quantitative determination shows a strong linear relationship between the number of pixels classified as red clover and the weight of red clover in dried mixed refusals.

To validate the procedure, the repeatability of the NIR-HIS method was estimated and found to be good, with a variation coefficient for repeated measurements equal to 2.1 %. Comparison between the two methods using linear regression indicated a slight over-estimation of the predicted weight of red clover with the NIR-HIS method compared to the manual sorting method. The NIR-HIS method is considerably less time-consuming (by a factor of 4) and the work is less tedious compared to the manual sorting method. The global error of the procedure, estimated with RMSEP, was equal to 3.4 g of DM, a satisfactory level. This error parameter is based on the difference between predicted and manually sorted weights. However, as explained previously, the accuracy of the manual sorting method shows limitations due to the correct identification of droppings. Visual observation of droppings cannot discriminate between a refusal composed of red clover mixed with droppings of laying hens and droppings containing entire blades of red clover. As NIR-HIS gives information about the nature of components, the spectra of red clover blades mixed with droppings should be different from those of blades which have passed through the gastrointestinal tract. To check this hypothesis, intestinal contents with entire red clover blades should be analyzed with the NIR-HIS procedure and their spectra added to the spectral library and PLS-DA. This method could then be able to discriminate droppings better than the manual sorting method. Finally, the error of prediction could be explained by the fact that the hyperspectral imaging-based procedure used a two-dimensional unit (pixel) to estimate a three-dimensional unit (weight), as the weight depends on the surface and density of components.

This procedure could be applied to different cultivars of red clover, since the cultivar does not change the spectra of the forage species. Other forage species could be identified by adding their spectra in the spectral library, and quantified by including their data in the linear regression.

5. Conclusions

This study demonstrates that NIR-HIS combined with chemometric tools allows red clover to be identified and quantified in mixed refusals. PLS-DA models discriminated spectra from pixels predicted as red clover from impurities, with 90.4 % and 93.5 % respectively of the pixels correctly classified. A linear regression was then able to convert the number of pixels classified as red clover into the weight of red clover contained in mixed refusals. The determination coefficient was equal to 0.98, which is considered satisfactory.

This process could replace manual sorting, which is tedious and time-consuming. It could be adapted to many other samples, such as the presence or absence of certain ingredients in the case of specification in differentiated quality products.

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CRediT authorship contribution statement

V. Tosar: Conceptualization, Formal analysis, Writing - original draft. **J.A. Fernández Pierna:** Methodology, Software. **V. Decruyenaere:** Validation, Writing - review & editing. **Y. Larondelle:** Writing - review & editing. **V. Baeten:** Conceptualization, Investigation, Resources, Writing - review & editing. **E. Froidmont:** Writing - review & editing, Resources, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare no conflict of interest.

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