



A feasibility study of non-targeted adulterant screening based on NIRM spectral library of soybean meal to guarantee quality: The example of non-protein nitrogen



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ABSTRACT

The quality and safety of soybean meal is a key matter for the livestock breeding and food industries, since it is one of the most important and widely used protein feed raw materials. As driven by commercial interests, new illegal adulterants which are unknown to consumers and regulators emerge constantly. In order to make up for the inadequacy of traditional detection methods, a novel non-targeted adulterant screening method based on a near-infrared microscopy spectral library of soybean meal is proposed. This study focused on the feasibility of non-targeted screening methods for the detection of adulteration in soybean meal. Six types of non-protein nitrogen were taken as examples and partial least squares discriminant analysis was employed to verify the feasibility of this novel method. The results showed that the non-targeted screening method could screen out adulterations in soybean meal with satisfactory results.

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1. Introduction

Soybean meal, a by-product of soybean oil extraction, with abundant protein, carbohydrate, dietary fibre, vitamins and minerals, is used as high-protein animal feed in many countries (Fernández Pierna et al., 2014). As driven by commercial interests, many types of illegal ingredients have been found in soybean meal with the purpose of increasing the nitrogen content (Bisaz & Kummer, 1983; Dorne et al., 2013). The standard protein determination method (i.e. Kjeldahl) cannot differentiate between protein nitrogen and non-protein nitrogen. Melamine (2,4,6-triamino-1,3,5-triazine), an adulterant used to increase the nitrogen content of foods and feeds, has caused illness and death of human infants and pets (cats and dogs), due to kidney damage (Administration, 2007; Organization, 2009; Reimschuessel & Puschner, 2010). In order to ensure food and protein feed safety, many platforms have been used to detect melamine, such as enzyme-linked immunosorbent assays (ELISA) (Yin et al., 2010), gas-chromatography mass-spectrometry (GC-MS) (Hong et al., 2009), high-performance liquid chromatography (HPLC) (Venkatasami & Sowa, 2010), near-infrared reflectance spectroscopy (NIRS) (Haughey, Graham, Cancouët, & Elliott, 2013), NIR hyperspectral imaging (Fu et al.,

2014), NIR microscopy (NIRM) (Yang, Wang, Han, Li, & Liu, 2014) and so on.

The detection modes of all the methods mentioned above are targeted to detect the known and reported illegal compounds in food and feed. Although those methods for the detection of known adulterants are efficient, they cannot be used for screening an emerging illegal adulterant. There is an urgent need to investigate ways to avoid future melamine-type crises. Without knowing the emerging illegal adulterants we propose a non-targeted screening method, which could detect abnormal components of food or feed caused by adulteration. Taking the feed itself as the fidelity target would make feed safety control proactive, rather than being one step behind the adulterators (Lachenmeier et al., 2009).

Near-infrared spectroscopy (NIRS) is a convenient analytical method that has been applied in a wide range of studies involving quality control and adulteration detection (Haughey et al., 2013; Teye et al., 2015). NIRS has become an important tool of analysis due to its speed, non-destructiveness and reproducibility. NIRM which combines NIRS and digital images together to characterise samples in micro scale could provide more detailed information (Yang et al., 2014). NIRM spectral data are three-dimensional: $x \times y \times \lambda$, of which x and y are the spatial information and λ represents the spectral information. Each spectrum represents the sample information within each pixel (Gendrin, Roggo, & Collet, 2007; Huang, Tian, Min, Xiong, & Du, 2015; Kamruzzaman, ElMasry, Sun, & Allen, 2011). NIRM technology could collect hundreds or

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thousands of spectra (including tens or hundreds of variables) for each sample, instead of unique average spectra. NIRM technology almost does not damage or consume sample in the process of analysis and requires minimal sample preparation (Huang, Min, Duan, Wu, & Li, 2014; Pierna et al., 2012). Based on these advantages, NIRM is now applied as an advanced analytical method in many fields such as pharmaceuticals (Amigo et al., 2008; Cruz, Bautista, Amigo, & Blanco, 2009; Gendrin, Roggo, & Collet, 2008), meat quality evaluation (He & Sun, 2015), and food and feed safety control (Jiang, Yang, & Han, 2014; Pierna et al., 2012).

In this paper we developed a non-targeted screening method for the detection of adulterations in soybean meal. Six types of non-protein nitrogen compound (melamine, cyanuric acid, urea, biuret, mono-ammonium phosphate (MAP) and di-ammonium phosphate (DAP)) were selected as adulterants. Global H (GH) value discrimination method based on the spectral library of soybean meal was employed to select spectra of non-protein nitrogen sources in the mixtures. Partial least squares discriminant analysis (PLSDA), which is a supervised classification method successfully used for discriminant analysis in many fields (Cozzolino, Chree, Scaife, & Murray, 2005; de Almeida, Correa, Rocha, Scafi, & Poppi, 2013; Pierna et al., 2014), was used to verify the feasibility of the non-targeted screening method based on GH value.

2. Materials and methods

2.1. Experimental strategy

A novel non-targeted adulterant screening method based on an NIRM spectral library of soybean meal was proposed for abnormal spectra selection in artificially mixed samples. For verifying the

feasibility of the novel method the PLSDA, which has been successfully used for the detection of melamine and cyanuric acid in feed, was employed (Fernandez Pierna et al., 2014). The flowchart of the experimental strategy for this study is shown in Fig. 1.

2.2. Sample collection and preparation

Soybean meal samples ($n = 88$), which were collected from Argentina, Italy, Brazil, France and China, were used in this study and ground to pass through a 0.5-mm square mesh using a Retsch mill (Ultra centrifugal Mill ZM 100; Retsch GmbH, Haan, Germany). Melamine, cyanuric acid, urea, and di-ammonium phosphate (DAP) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Biuret was bought from Tianjin Fuchen chemical reagents factory (Tianjin, China). Mono-ammonium phosphate (MAP) was purchased from Beijing chemical reagent company (Beijing, China).

Exploratory principal components analysis was applied to the 88 average spectra of soybean meal samples. Different types of soybean meal samples were separated into different groups. One sample from each group was selected randomly and prepared for adulteration analysis in this study. A complete strategy and sampling is detailed in Section 3.1 of this paper.

Set 1 and set 2 were prepared for adulteration analysis in this paper as shown in Table A 1. All the mixtures were prepared in the laboratory using a mixer (REAX 20/8; Heidolph, Schwabach, Germany). In order to reach a homogeneous distribution of adulterants in the soybean meal, a stepwise dilution procedure was applied to ensure that in each dilution step the ratio of the two materials to be mixed did not exceed a factor of 3 (Gizzi, von

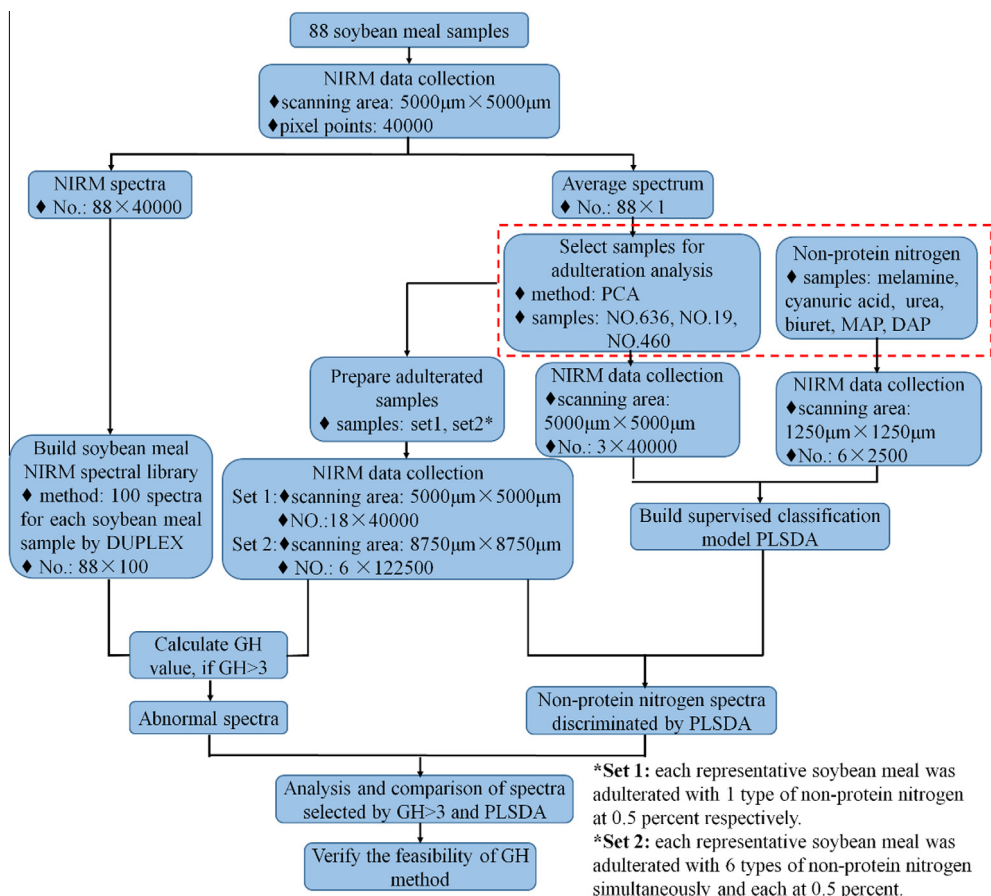


Fig. 1. Experimental flowchart for this study.

Holst, Baeten, Berben, & van Raamsdonk, 2004). The steps of artificial mixture are shown in Table A 2.

Set 1: each representative soybean meal was adulterated with 1 type of non-protein nitrogen at 0.5%.

Set 2: each representative soybean meal was adulterated with 6 types of non-protein nitrogen simultaneously, each at 0.5%.

2.3. Apparatus and data collection

NIRM spectra were obtained using a Fourier transform near infrared (FT-NIR) imaging system (Spectrum400 + Spotlight 400 FT-NIR Microscope; PerkinElmer, Waltham, MA). The instrument was equipped with a mercury cadmium telluride (MCT) line array detector, allowing the collection of spectra from arbitrary spatial regions with a high spectral resolution. Scanning areas of pure soybean meal samples, pure non-protein nitrogen, mixtures of set 1 and set 2 were $5000\ \mu\text{m} \times 5000\ \mu\text{m}$, $1250\ \mu\text{m} \times 1250\ \mu\text{m}$, $5000\ \mu\text{m} \times 5000\ \mu\text{m}$ and $8750\ \mu\text{m} \times 8750\ \mu\text{m}$, respectively. The spatial resolution was $25\ \mu\text{m} \times 25\ \mu\text{m}$ per pixel, and each spectrum was computed at $32\ \text{cm}^{-1}$ resolution across the wavelength range $7800\text{--}4000\ \text{cm}^{-1}$ by combining 8 scans.

2.4. Soybean meal spectral library

Using the scanning parameters mentioned in Section 2.3, 40,000 spectra could be acquired from one sample. It is a challenge to handle, extract, and exploit such a large amount of data. There was a great amount of redundant information in NIRM spectra and a DUPLEX algorithm could effectively remove it and reduce computational cost accordingly. So 100 spectra per sample were selected through the DUPLEX algorithm (Pierna et al., 2011; Snee, 1977) and 8800 spectra were selected and used to build a spectral library. The algorithm proceeds as follows: (1) principal component analysis (PCA) was used for reducing the dimensions of the data matrix by compressing the information into few new variables known as principal components (PCs), (2) the Euclidean distance between all possible pairs of spectra was calculated, (3) the two spectra with largest Euclidean distance were assigned to the training set, (4) the two spectra in the remaining list with largest distance were put in the test set, and (5) steps (3) and (4) were repeated until one had obtained as many spectra as predefined. The 8800 selected spectra were used as the representative spectra for the spectral library building in this work.

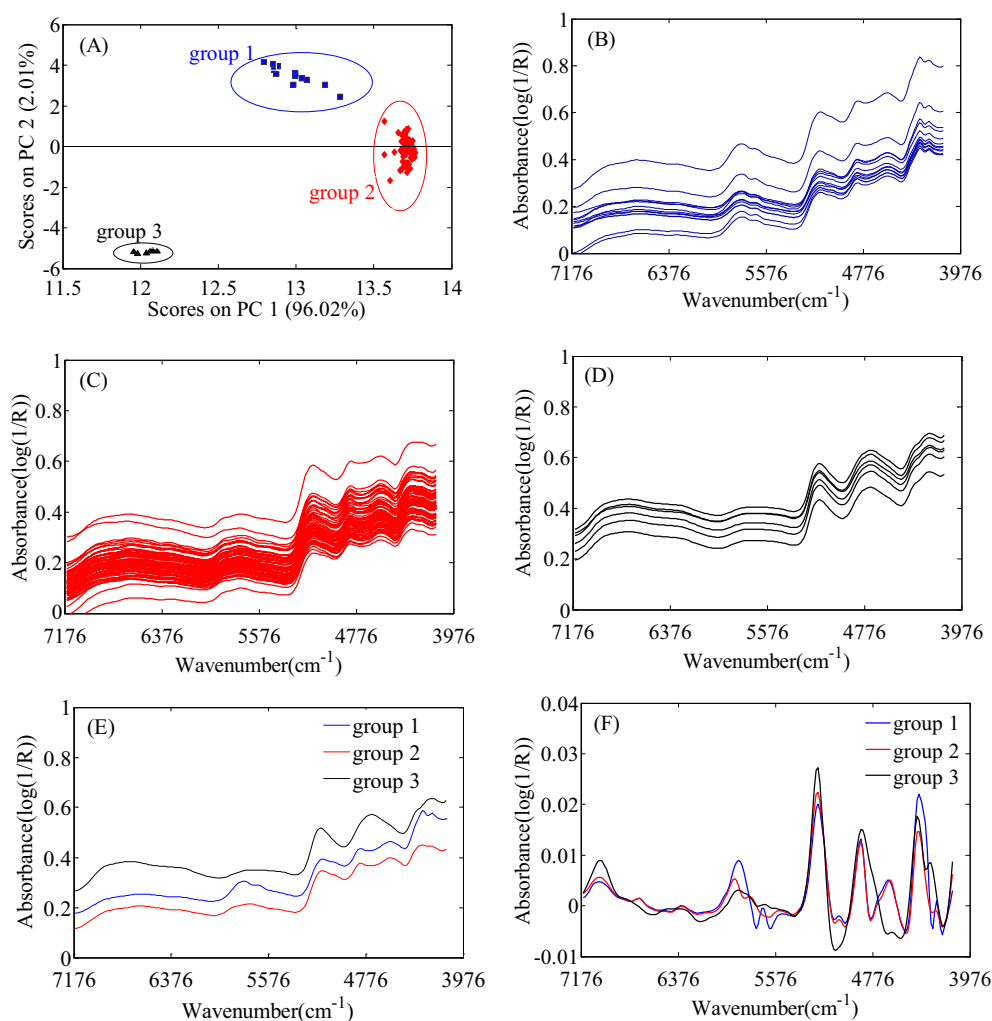


Fig. 2. (A) The PCA scores plot for 88 soybean meal samples, (B) the mean spectra per sample from group 1, (C) the mean spectra per sample from group 2, (D) the mean spectra per sample from group 3, (E) the raw mean spectra of all the samples from each group clustered by PCA analysis, (F) the first-derivative mean spectra of all the samples from each group clustered by PCA analysis.

2.5. Data processing

2.5.1. GH value

In order to remove unwanted spectral variations and scattering effects, all the spectra used in this study were pre-treated with 1st derivative Savitzky-Golay and standard normal variate (SNV). The GH value based on the spectral library of soybean meal could provide information about the distances between each spectrum of adulterated feed sample and the average spectra of the soybean meal spectral library in the principal components or latent variables space. It was a modification of the Mahalanobis distance (H) of each sample from the average spectrum of the spectral library. GH was calculated according to the equation used by Guthrie in his paper, the Eqs. (1) and (2) as follows (Guthrie, 2005; Whitfield, Gerger, & Sharp, 1987):

$$GH = \frac{H^2}{f} \quad (1)$$

$$H = (M_i - \bar{S}) * V^{-1} * (M_i - \bar{S})' \quad (2)$$

Here S is the $n \times f$ matrix of the spectral library scores, n is the number of the spectra and f is the number of dimensions for the spectral library. V is the covariance matrix of the matrix S . M is the spectral matrix need to be detected given in the $m \times f$ matrix, m is the number of spectra and f is the number of dimensions for the detected sample. GH follows the χ -square distribution, of which a value greater than 3 will indicate a 0.01 or less probability of belonging to that group. So “3” was assigned as the threshold value.

2.5.2. PLSDA

PLSDA is a variation of the Partial Least Squares (PLS) regression algorithm for discriminant analysis (Wold, Sjöström, & Eriksson, 2001). As a supervised clustering method, it requires a prior knowledge of the data (Bonifazi, Di Maio, Potenza, & Serranti, 2014). In this study, the spectra collected separately from soybean meal and 6 types of non-protein nitrogen were used to make calibration models. The PLSDA models were used to seek a direct relationship

between the X ($N \times J$) block (spectral data) and the Y ($N \times M$) matrix (categorical variables), where N is the number of samples, J the variable number and M the number of category. When there were two classes to be classified, the matrix Y was arranged in a single column where 1 was used for interest class and 0 for without interest. For more than two classes, the PLS2 algorithm was applied by constructing a matrix Y ($N \times M$), where each column represents a class (de Almeida et al., 2013; Mazivila et al., 2015).

Pure soybean meal spectra and pure non-protein nitrogen spectra were used as the training set of PLSDA models. These groups were assigned in matrix Y ($N \times M$), where each column represented a class. PLSDA models were generated with 10-fold cross validation and the number of latent variables was chosen using the plot RMSECV (Root Mean Squared Error of Cross-Calibration) versus the number of latent variables.

PLSDA is a good method for qualitative identification (Fernandez Pierna et al., 2014), and will be used to verify the feasibility of the non-target screening method based on GH value.

All data were processed using Matlab version 7.14 (The Mathworks, Inc., Natick, MA) with the PLS_Toolbox version 7.1 (Eigenvector Research, Wenatchee, WA).

3. Results and discussion

3.1. Principal component analysis (PCA)

Using NIRM, 40,000 spectra of per pure soybean meal sample were scanned. Then the mean spectra per sample was calculated and 88 average spectra were collected. The exploratory PCA analysis was applied to the 88 average spectra. Fig. 2(A) shows the PCA scores plot for soybean meal samples using 1st derivative Savitzky-Golay and SNV of raw data generated in a satisfactory cluster trend. The first two principal components (PCs), explain the majority of the variation with PC1 explaining 96.02% and PC2 explaining 2.01% of the spectral data. PCA as an unsupervised pattern recognition method could group similar samples. All the soybean meal samples used in this experiment were separated into three major groups and their spectra are displayed in Fig. 2(B) (group 1 of blue

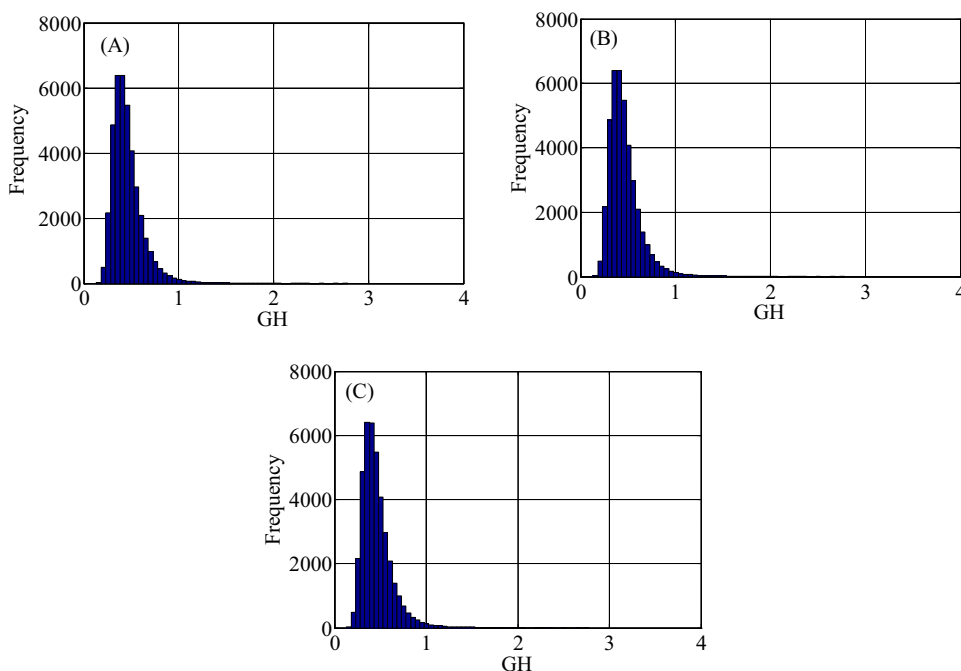


Fig. 3. Histograms of GH values for spectra of (A) NO. 19, (B) NO. 460 and (C) NO. 636. X-axis represents GH value and Y-axis represents the number of spectra.

square), Fig. 2(C) (group 2 of red rhombus) and Fig. 2(D) (group 3 of black triangle).

Fig. 2(E) and (F) showed the raw and first-derivative average spectra of all the samples from each group clustered by PCA analysis. First-derivative spectra had greater amplitude than the raw spectra, which could separate out peaks of overlapping bands with satisfactory effect. It could be clearly seen that the spectra of group 3 had obvious differences with group 1 and group 2. For the spectra of group 3, absorption bands were observed around 6711 cm^{-1} (O–H stretch first overtone), 5618 cm^{-1} (C–H stretch first overtone), 4762 cm^{-1} (O–H bend and C–O stretch combination) and 4252 cm^{-1} (CH_2 bend second overtone), which were all associated with carbohydrate. The spectra of group 1 and group 2 presented similar absorption bands around 6667 cm^{-1} (N–H stretch first overtone), 4850 cm^{-1} (N–H bend second overtone or N–H bend and stretch combination) and 4587 cm^{-1} (C–H stretch and C=O stretch combination), associated with protein. The difference between group 1 and group 2 could be found in the region characteristic of fat absorption (5787 cm^{-1} and 4261 cm^{-1}). By analysing the spectra, group 3 with rich carbohydrate is soybean hull, and group 1 is full-fat soybean containing the absorption bands of fat (Burns & Ciurczak, 2007; Del Rivero et al., 2013). In this study, 3 types of soybean meal samples (one sample randomly chosen from each group) were selected and prepared for the detection of adulterations: No. 636 from group 1, No. 19 came from group 2 and No. 460 from group 3.

3.2. Selection of non-protein nitrogen sources spectra by GH and PLSDA

The GH value based on the spectral library of soybean meal was used as a criterion for the non-target screening method of adulterants in soybean meal samples. Using Eqs. (1) and (2), spectra with GH value >3 would be selected as the suspicious non-protein nitrogen sources spectra. The GH values of spectra of 3 soybean meal samples were calculated and displayed by frequency histograms (Fig. 3). If more spectra had the same GH, the bar for that frequency value would be higher. From the histograms, the graph fitted a Gaussian distribution, offering a reliable qualitative parameter for the spectra distribution. In Table 1, it can be seen that there were no spectra with the GH value >3 in pure soybean meal samples. Besides, the GH values of spectra of 6 types of pure non-protein nitrogen were also calculated and the GH values of all the spectra were >3 . The results indicated that the spectral library of soybean meal has good representativeness and can be used to recognise pure soybean meal and pure non-protein nitrogen with satisfactory performance.

Three soybean meal samples adulterated with single non-protein nitrogen (sample set 1) and 6 types of non-protein nitrogen simultaneously (sample set 2) were analysed. The non-target screening method using GH values and PLSDA was used for the detection of non-protein nitrogen adulterants. Using GH = 3 as a criterion, the distance between each spectrum of the samples

Table 1
The results of adulteration analysis by two methods of GH >3 and PLSDA.

Sample	Total number of spectra	Number of spectra		Interaction ^c
		GH >3 ^a	PLSDA ^b	
A (NO. 19)	40,000	0	–	–
B (NO. 460)	40,000	0	–	–
C (NO. 636)	40,000	0	–	–
Biuret	2500	2500	–	–
Cyanuric acid	2500	2500	–	–
DAP	2500	2500	–	–
MAP	2500	2500	–	–
Melamine	2500	2500	–	–
Urea	2500	2500	–	–
A + 0.5% biuret	40,000	181	120	120
A + 0.5% cyanuric acid	40,000	117	29	29
A + 0.5% DAP	40,000	82	63	63
A + 0.5% MAP	40,000	87	60	60
A + 0.5% melamine	40,000	98	64	64
A + 0.5% urea	40,000	165	85	85
B + 0.5% biuret	40,000	216	125	125
B + 0.5% cyanuric acid	40,000	20	9	9
B + 0.5% DAP	40,000	193	5	5
B + 0.5% MAP	40,000	119	58	58
B + 0.5% melamine	40,000	190	151	151
B + 0.5% urea	40,000	110	84	84
C + 0.5% biuret	40,000	105	36	36
C + 0.5% cyanuric acid	40,000	123	100	100
C + 0.5% DAP	40,000	132	72	72
C + 0.5% MAP	40,000	171	43	43
C + 0.5% melamine	40,000	341	270	269
C + 0.5% urea	40,000	126	100	100
A + 6 kinds of non-protein (0.5% respectively)	122,500	1549	1264(59/520/205/17/37/427) ^d	829
B + 6 kinds of non-protein (0.5% respectively)	122,500	1181	1803(195/908/292/121/278/9) ^d	907
C + 6 kinds of non-protein (0.5% respectively)	122,500	1781	814(217/169/118/217/108/85*) ^d	687

^a The number of abnormal spectra screened out by non-target screening method based on spectral library.

^b The number of non-protein nitrogen spectra selected by PLSDA.

^c The number of the same spectra selected by GH >3 and PLSDA simultaneously.

^d The order of non-protein nitrogen spectra classified by PLSDA in the sample adulterated with 6 types of non-protein nitrogen is biuret, cyanuric acid, DAP, MAP, melamine, and urea.

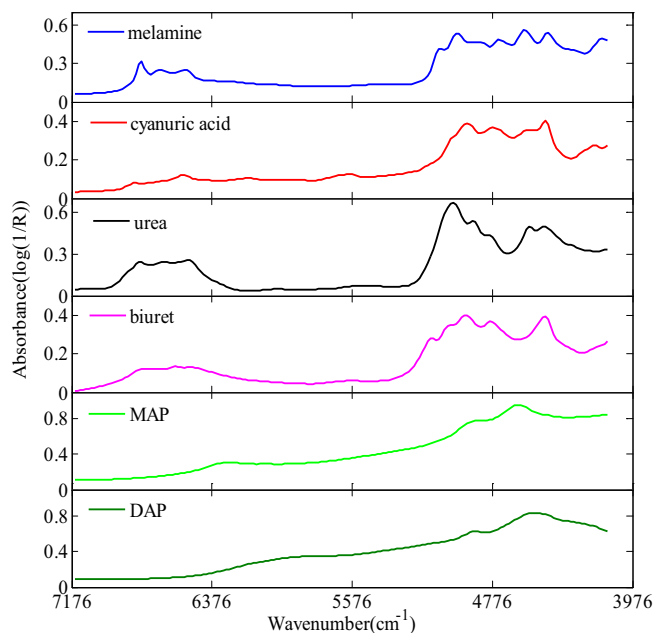


Fig. 4. The spectra of melamine, cyanuric acid, urea, biuret, MAP and DAP.

analysed and the centre of the soybean meal spectral library were calculated and the number of the spectra with $GH > 3$ were recorded. All the results are displayed in Table 1; abnormal spectra could be screened out in all artificial mixed samples by the non-targeted screening method, when 0.5% single or 6 types of non-protein (0.5% respectively) were added to soybean meal.

The PLSDA model was built and showed both 100% sensitivity (number of samples predicted as in the class divided by number actually in the class) and 100% specificity (number of samples predicted as not in the class divided by actual number not in the class) for all the classes (soybean meal, biuret, cyanuric acid, DAP, MAP, melamine, and urea). PLSDA model which had been used for discriminant analysis in many fields was used as comparison method to verify the feasibility of the non-target screening method based on GH value in this paper. Different numbers of non-protein nitrogen spectra could also be detected in all artificial mixed samples by PLSDA, and the results are also displayed in Table 1. Comparison results of the spectra selected by the non-target screening method and PLSDA showed that almost all the non-protein nitrogen spectra separated by PLSDA in each soybean meal adulterated with 0.5% non-protein nitrogen were included in the abnormal spectra with $GH > 3$, which indicated that the non-targeted screening method using GH values based on spectral library could screen

out abnormal spectra more strictly and sensitively compared to PLSDA method.

3.3. Analysis of abnormal spectra

Fig. 4 shows the spectra of 6 types of pure non-protein nitrogen melamine, cyanuric acid, urea, biuret, MAP and DAP. Each non-protein nitrogen sample had its own near infrared characteristic absorbing peaks and could be easily distinguished from soybean meal spectra. In order to verify the performance of the non-targeted screening method, the abnormal spectra screened out by $GH > 3$ and PLSDA methods were analysed. Fig. A 1 showed the abnormal spectra which were screened out by $GH > 3$ (the left side) and PLSDA (the right side). Through macroscopic observation and analysis, it can be found out that all the non-protein nitrogen spectra classified by PLSDA method are very similar to pure non-protein nitrogen spectra besides almost all of their $GH > 3$ except one spectra in NO. 636 + 0.5% melamine ($GH = 2.93$). The abnormal spectra, which were selected by $GH > 3$ but not by PLSDA, had a 0.01 or less probability of belonging to that pure soybean meal spectra group. This part of spectra with weak signal could not be recognized by the supervised clustering method PLSDA. As could be seen in Table 1 (NO. 460 + 0.5% DAP) and Fig. 5, 193 spectra were selected by $GH > 3$ but only 5 spectra were regarded as DAP by PLSDA. The reason might be that DAP exposed to the air would gradually lose ammonia and be converted into MAP, which made it impossible for PLSDA model to detect it in this situation. The peak shape of the abnormal spectra separated in NO. 460 + 0.5% DAP looks like MAP. The GH method screened out all the suspicious spectra in NO. 460 + 0.5% DAP, in spite DAP converting into MAP. The advantage of GH method is that it does not need to know which kind of illegal ingredient has been added to the soybean meal in advance, while the PLSDA model could not be built and used in this situation.

When the soybean meal was adulterated with 6 types of non-protein nitrogen simultaneously (0.5% respectively) the number of the selected abnormal spectra showed great disparities between these two methods. The unweighted pair-group method using centroid approach (UPGMC) was used to classify and cluster the spectra with $GH > 3$. The results of cluster analysis is shown in Fig. A 2, the abnormal spectra with $GH > 3$ in each soybean meal were grouped into 7 or 8 classes. Compared with the spectra of pure soybean meal and non-protein nitrogen, apparent variation could be seen in almost all the groups of spectra which contained the information of soybean meal and non-protein nitrogen at the same time and were regarded as suspicious spectra. When the spectra were collected under the FT-NIR imaging system, one or more different types of non-protein nitrogen might co-exist with soybean meal in one pixel when 6 types of non-protein nitrogen were added to

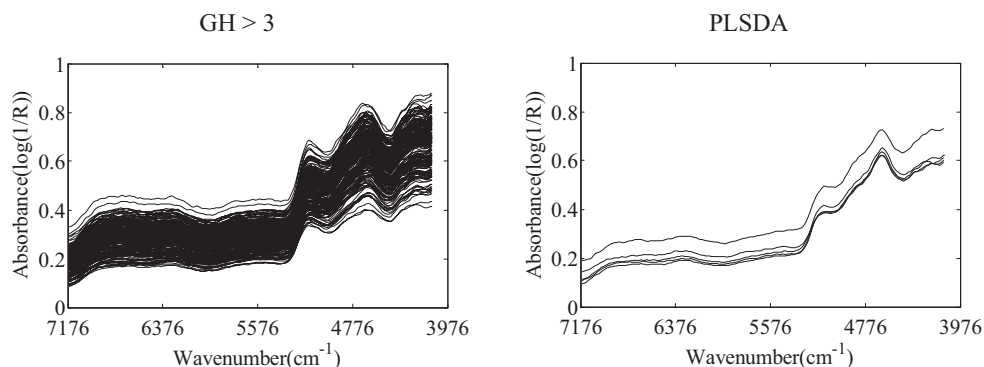


Fig. 5. The abnormal spectra screened out by $GH > 3$ and PLSDA in NO. 460 + 0.5% DAP.

soybean meal simultaneously. The spectra had very large variability due to the different content of each component. Both of these two methods screened out a lot of abnormal spectra and had a number of spectra in common.

By analysing the results showed in Table 1, Figs. A 1 and A 2, it can be seen that the performance of the GH method and PLS-DA for the detection of 0.5% single and multiple non-protein nitrogen adulteration in soybean meal is acceptable. The non-target screening method using GH values based on soybean meal spectral library could screen out more abnormal spectra and had higher sensitivity compared with PLS-DA.

4. Conclusion

The results presented in this study showed the feasibility of the non-targeted screening method for the adulteration detection of single non-protein nitrogen at 0.5%/6 types of non-protein nitrogen simultaneously each at 0.5%, which is already a low concentration added in commercial feed and could be reliably detected using PLS-DA combined with NIR imaging spectroscopy (Fernández Pierna et al., 2014). The non-targeted screening can be extended to detect other types of contaminants.

PLS-DA as a supervised classification method could only be used when the adulterants were known to the quality inspectors. However, the non-target screening method using $GH > 3$ as a criterion could screen out all spectra with 0.01 or less probability of belonging to the pure soybean meal spectra group without knowing which adulterant had been mixed into soybean meal in advance. The non-targeted screening method which took feed itself as the fidelity target showed greater potential for guaranteeing the quality and safety of soybean meal. It is hoped to change the passive situation of food and feed safety control even if this is only an exploratory attempt. Meanwhile, more research such as the quantitative analysis and the limit of detection are needed to verify the effectiveness of the non-targeted screening method and to push food and feed safety control in a better and more rigorous direction.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.foodchem.2016.04.101>.

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